UNIVERSITY OF LONDON THESIS

Degree MD  Year 2007  Name of Author ANDREW CLAYTON

COPYRIGHT
This is a thesis accepted for a Higher Degree of the University of London. It is an unpublished typescript and the copyright is held by the author. All persons consulting the thesis must read and abide by the Copyright Declaration below.

COPYRIGHT DECLARATION
I recognise that the copyright of the above-described thesis rests with the author and that no quotation from it or information derived from it may be published without the prior written consent of the author.

LOAN
Theses may not be lent to individuals, but the University Library may lend a copy to approved libraries within the United Kingdom, for consultation solely on the premises of those libraries. Application should be made to: The Theses Section, University of London Library, Senate House, Malet Street, London WC1E 7HU.

REPRODUCTION
University of London theses may not be reproduced without explicit written permission from the University of London Library. Enquiries should be addressed to the Theses Section of the Library. Regulations concerning reproduction vary according to the date of acceptance of the thesis and are listed below as guidelines.

A. Before 1962. Permission granted only upon the prior written consent of the author. (The University Library will provide addresses where possible).

B. 1962 - 1974. In many cases the author has agreed to permit copying upon completion of a Copyright Declaration.

C. 1975 - 1988. Most theses may be copied upon completion of a Copyright Declaration.

D. 1989 onwards. Most theses may be copied.

This thesis comes within category D.

☐ This copy has been deposited in the Library of UCL

☐ This copy has been deposited in the University of London Library, Senate House, Malet Street, London WC1E 7HU.
Elastic Scattering Spectroscopy
in the Management of Breast Cancer

ANDREW CLAYTON LEE
MB, ChB, AFRCS(Ed)

A thesis presented to the University of London for the degree of
DOCTOR OF MEDICINE

2006
To my parents, my wife and my brothers

To my mentors and those who made this project possible
LIST OF CONTENTS

DECLARATION 1
ACKNOWLEDGEMENTS 2
PUBLICATIONS 3
ABSTRACT 6
ABBREVIATIONS 7
LIST OF FIGURES 9
LIST OF TABLES 12

CHAPTER 1
Diagnosis of Breast Cancer and Recent Advances

1.1 Introduction 14
1.2 Triple Assessment 15
1.3 Clinical Examination 16
1.4 Imaging 17
1.4.1 Mammography 17
1.4.2 Recent advances in mammography 20
1.4.3 Ultrasonography and recent advances 24
1.4.4 Magnetic resonance imaging and recent advances 25
1.4.5 Nuclear medicine imaging 29
1.4.5.1 Scintimammography 29
1.4.5.2 Positron emission tomography 31
1.4.6 Other Imaging Modalities 32
1.5 Pathological evaluation 32
1.5.1 Excision breast biopsy 33
CHAPTER 2
Surgical Management of Breast Cancer and Recent Advances

2.1 Introduction 53
2.2 Surgical Management of Breast Cancer 53
2.3 Mastectomy 54
2.4 Breast Conservation Surgery 55
2.5 Surgical Management of the Axilla 59
2.5.1 The role of axillary surgery 59
2.5.2 Axillary lymph node dissection 61
2.5.3 Axillary lymph node sampling 62
2.6 Sentinel Lymph Node Biopsy 63
2.6.1 The sentinel lymph node concept 63
2.6.2 Validation and current status of the SLNB 65
2.6.3 The technique of SLNB 68
2.6.4 The SLNB protocol at UCLH 69
2.6.5 Pathological aspects 73
2.6.6 Intra-operative assessment 76
2.6.7 Pathological protocol at UCLH 78
2.7 Discussion 79
# CHAPTER 5

The Effects of Ambient Lighting, Tissue Handling and Formaldehyde Fixation on Elastic Scattering Spectroscopy

## 5.1 Introduction

## 5.2 The Effect of Ambient Lighting on ESS

### 5.2.1 Introduction

### 5.2.2 Methods

### 5.2.3 Results

### 5.2.4 Discussion

### 5.2.5 Conclusion

## 5.3 The Effect of Exposure in Room Condition on ESS

### 5.3.1 Introduction

### 5.3.2 Methods

### 5.3.3 Results

### 5.3.4 Discussion

### 5.3.5 Conclusion

## 5.4 The Effect of Submersion in Saline at Room Temperature on ESS

### 5.4.1 Introduction

### 5.4.2 Methods

### 5.4.3 Results

### 5.4.4 Discussion

### 5.4.5 Conclusion

## 5.5 The Effect of Tissue Chilling in Ice on ESS

### 5.5.1 Introduction

### 5.5.2 Methods

### 5.5.3 Results

### 5.5.4 Discussion

### 5.5.5 Conclusion
CHAPTER 6

Elastic Scattering Spectroscopy: An emerging technology for the detection of axillary lymph node metastasis in breast cancer

6.1 Introduction 174

6.2 Study to improve the sensitivity and specificity of ESS using MBA in determining the axillary lymph nodes status in breast cancer 176

6.2.1 Introduction 176

6.2.2 Methods 177

6.2.3 Results and Discussion 178

6.2.4 Discussion 186

6.2.5 Conclusion 188

6.3 A comparative study of ESS and TIC in assessing SLN status in breast cancer 188

6.3.1 Introduction 188

6.3.2 Methods 189

6.3.3 Results 191

6.3.4 Discussion 192

6.3.5 Conclusion 198
## CHAPTER 7

Elastic Scattering Spectroscopy for the detection of Primary Breast Cancer

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.1</td>
<td>Introduction</td>
<td>201</td>
</tr>
<tr>
<td>7.2</td>
<td>Methods</td>
<td>202</td>
</tr>
<tr>
<td>7.2.1</td>
<td>Patient selection and specimen handling</td>
<td>202</td>
</tr>
<tr>
<td>7.2.2</td>
<td>Spectral acquisition</td>
<td>202</td>
</tr>
<tr>
<td>7.2.3</td>
<td>Histological correlation</td>
<td>203</td>
</tr>
<tr>
<td>7.2.4</td>
<td>Spectral analysis</td>
<td>204</td>
</tr>
<tr>
<td>7.3</td>
<td>Analyses and Results</td>
<td>204</td>
</tr>
<tr>
<td>7.4</td>
<td>Discussion</td>
<td>213</td>
</tr>
<tr>
<td>7.5</td>
<td>Conclusion</td>
<td>220</td>
</tr>
</tbody>
</table>

## CHAPTER 8

Summary and Future Developments

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.1</td>
<td>Summary</td>
<td>222</td>
</tr>
<tr>
<td>8.2</td>
<td>Future Developments</td>
<td>224</td>
</tr>
</tbody>
</table>

## APPENDICES

<table>
<thead>
<tr>
<th>Letter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Optical Biopsy System: Operating Manual</td>
<td>226</td>
</tr>
<tr>
<td>B</td>
<td>Patient information and Consent form</td>
<td>233</td>
</tr>
</tbody>
</table>
DECLARATION

The research described in this thesis was performed whilst I held the post of Clinical Research Fellow in the National Medical Laser Centre (Department of Surgery) and Institute of Nuclear Medicine, University College London. No portion of the work referred to in this thesis has been submitted in support of an application of another degree.

I declare that I am the sole author of this thesis. The organisation and data collection of all the studies were planned and carried out by myself except for the computation of spectral analyses, which was performed by Dr D. Pickard, Dr K. Johnson and Dr B. Clark. Where assistance has been obtained from others such help has been freely acknowledged.
ACKNOWLEDGEMENTS

I owe a debt of gratitude to my three supervisors: Mr M. Keshtgar, Professor S.G. Bown and Professor P.J. Ell for their unlimited support, encouragement, guidance and supervision. My gratitude extends to Dr D. Pickard, Dr K. Johnson and Dr B. Clark, who are the physicists at the National Medical Laser Centre, for their invaluable scientific and statistical advises and for their help in performing the spectral analyses. I am also grateful to Dr M. Falzon and Dr G. Kocjan for their support and active involvement in histology and imprint cytology, to Ms W. Waddington for her expertise and help in nuclear medicine, to Mr R. Sainsbury for his support and patient referral and to Dr A. Mosse for making the grid.

I would like to acknowledge the staff at the Institute of Nuclear Medicine, National Medical Laser Centre, Department of Histopathology, the Breast Clinic and Operating theatres at Middlesex Hospital for their help, cooperation and support.

Finally, I would like to thank my family and friends especially my wife, May, for their understanding and encouragement throughout this project.
PUBLICATIONS

Peer reviewed publications

KS Johnson, DW Chicken, CDO Pickard, AC Lee, G Briggs, M Falzon, IJ Bigio, MR Keshtgar, SG Bown
Elastic Scattering Spectroscopy for Intraoperative determination of sentinel lymph node status in the breast

AC Lee, MRS Keshtgar, WA Waddington, PJ Ell
The Role of Dynamic Imaging in Sentinel Node Biopsy in Breast Cancer

Publications in preparation

DW Chicken, G Kocjan, M Falzon, B Almeida, AC Lee, JRC Sainsbury, M Douek, MRS Keshtgar
Implementation of Touch Imprint Cytology for Intra-operative Detection of Sentinel Lymph Node Metastases in Breast Cancer – Practice and Implications
Submitted to British Journal of Surgery

Elastic scattering spectroscopy in surgical oncology

Elastic scattering spectroscopy for diagnosis of primary breast cancer

Elastic scattering spectroscopy for detection of lymph node metastasis in breast cancer

Other publications and conference proceedings

Optical Biopsy for Real-Time Diagnosis, Staging and Prognostication in Breast Cancer

JJ Scarisbrick, CDO Pickard, AC Lee, GM Briggs, K Johnson, SG Bown, M Novelli, MRS Keshtgar, IJ Bigio, and R Yu
Elastic scattering spectroscopy in the diagnosis of pigmented lesions: comparison with clinical and histopathological diagnosis
Proceedings of SPIE Volume 5141, Diagnostic Optical Spectroscopy in Biomedicine II, GA Wagnieres, Editor, October 2003, pp. 147-156
SG Bown, CDO Pickard, AC Lee, GM Briggs, MRS Keshtgar, JRC Sainsbury, M Falzon, IG Bigio
Optical biopsy for immediate assessment of sentinel nodes during breast cancer surgery

MRS Keshtgar, AC Lee, WA Waddington
The Sentinel Node in Breast Carcinoma –Present Controversies

CDO Pickard, IJ Bigio, SG Bown, GM Briggs, AC Lee, PM Ripley, S Lakhani
Optical Biopsy for the Diagnosis of Breast Tumours

Published abstracts

DW Chicken, KS Johnson, MR Falzon, AC Lee, G Briggs, D Pickard, IJ Bigio, SG Bown, MRS Keshtgar
Optical biopsy utilising elastic scattering spectroscopy for intra-operative diagnosis of sentinel lymph node metastases
Journal of Clinical Oncology 2004, 22 (Supplement S): 841

DW Chicken, AC Lee, GM Briggs, MRS Keshtgar, KS Johnson, CDO Pickard, IJ Bigio, SG Bown
Optical biopsy - a novel intraoperative diagnostic tool to determine sentinel lymph node status instantly in breast cancer (Co-author)
Breast Cancer Research and Treatment 2003, 82(Supplement 1):S172

AC Lee, CDO Pickard, GM Briggs, JR Sainsbury, M Falzon, G Kocjan, I Bigio, PJ Ell, SG Bown, MRS Keshtgar
Intra-operative assessment by optical biopsy for sentinel lymph node metastasis in breast cancer: update & comparison with imprint cytology
British Journal of Surgery 2003, 90(5): 625-6

AC Lee, DW Chicken, CDO Pickard, GM Briggs, G Kocjan, M Falzon, I Bigio, PJ Ell, JR Sainsbury, MRS Keshtgar, SG Bown
Comparative study of elastic scattering spectroscopy and touch imprint cytology in assessing sentinel lymph node status in breast cancer
British Journal of Surgery 2003, 90(Supplement 1): 112

DW Chicken, AC Lee, CDO Pickard, M Falzon, SG Bown, RC Yu, J Scarisbrook, I Bigio, MRS Keshtgar
Optical biopsy – a novel perioperative diagnostic tool in surgical oncology
British Journal of Surgery 2003, 90(Supplement 1): 35
GM Briggs, AC Lee, CDO Pickard, JR Sainsbury, MR Falzon, I Bigio, PJ Ell, SG Bown, MRS Keshtgar
Optical biopsy: the technique and experience in determining lymph node status in breast cancer
European Journal of Surgical Oncology 2002, 28(7): 772-3

J Scarisbrick, CDO Pickard, AC Lee, GM Briggs, SG Bown, MRS Keshtgar, RC Yu
British Journal of Dermatology 2002, 147 (Supplement 62): 7
Optical biopsies in the diagnosis of melanocytic lesions: comparison with clinical and histopathological diagnosis

AC Lee, CDO Pickard, MRS Keshtgar, GM Briggs, M Falzon, S Lakhani, PJ Ell, I Bigio, SG Bown
Elastic Scattering Spectroscopy for the Diagnosis of Breast Cancer
British Journal of Surgery 2002, 89(Supplement1): 74

AC Lee, CDO Pickard, MRS Keshtgar, GM Briggs, M Falzon, S Lakhani, PJ Ell, I Bigio, SG Bown
Intraoperative assessment by optical biopsy for lymph node metastasis in breast cancer
British Journal of Surgery 2002, 89: 640-642

AC Lee, CDO Pickard, MRS Keshtgar, GM Briggs, M Falzon, S Lakhani, PJ Ell, IJ Bigio, SG Bown
Elastic Scattering Spectroscopy for Intraoperative Detection of Sentinel Lymph Node Metastasis

AC Lee, MRS Keshtgar, WA Waddington, PJ Ell
The Role of Dynamic Imaging in Sentinel Node Biopsy in Breast Cancer
European Journal of Cancer 2001, 37(Supplement 5): 17

AC Lee, CDO Pickard, MRS Keshtgar, SG Bown, G Briggs, S Lakhani, IJ Bigio, PJ Ell
Intra-operative assessment by optical biopsy for sentinel lymph node metastasis in breast cancer.
British Journal of Cancer 2001, 85(Supplement 1): 27

MRS Keshtgar, A Lee, I Taylor, T Davidson, PJ Ell, D Ralphs, G Kocjan
The role of intraoperative imprint cytology in determining the histological status of the sentinel lymph node.
ABSTRACT

ELASTIC SCATTERING SPECTROSCOPY IN THE MANAGEMENT OF BREAST CANCER

Elastic scattering spectroscopy (ESS) is an emerging technology capable of detecting malignant changes in tissue using elastic light scattering and light absorption. Pulses of broadband light are delivered to and subsequently collected from tissue to provide a spectrum. Automated spectral analysis using model based analysis (MBA) is under development. Potential clinical applications include ESS guided breast core biopsy, intra-operative assessment of SLN and tumour excision margin. This thesis explores various technical aspects of ESS and factors concerning spectral analysis in relation to the above potential clinical applications.

Chapter 5 studied the effects of ambient lighting, tissue handling (i.e. exposure in open laboratory conditions, submersion in saline, chilling in ice) and formaldehyde fixation over time on ESS. The effect of ambient light on the ESS spectra is minimal at intensities below 1000lux (i.e. in indoor or laboratory), but becomes significant at higher light intensities (i.e. beneath an operating lamp). As for tissue handling and formaldehyde fixation, the changes observed in ESS spectra within the first hour following excision were related to the changes in oxygenation status of haemoglobin. Otherwise, no other changes occurred during this period. However, prolonged formaldehyde fixation (beyond 4 hours) resulted in significant changes.

The first part of Chapter 6 investigated the factors influencing the sensitivity and specificity of spectral analysis in differentiating ESS spectra of normal and metastatic axillary lymph nodes. By improving the correlation between ESS spectra and histology, sensitivity and specificity increased to 88-90% and 91-96% respectively from the previously reported sensitivity of 57% and specificity of 85%. The second part of Chapter 6 is a comparative study of ESS and touch imprint cytology (TIC). Based on 89 sentinel lymph nodes, both ESS and TIC identified 6 out of 8 nodes with metastasis giving equal sensitivities of 75%. Specificity was 93% for ESS and 100% for TIC.

In Chapter 7, a grid system was used to correlate ESS spectra to the precise histology with the percentage of malignant replacement in breast tissue. Spectral analyses using MBA were performed with different training sets, and showed increasing sensitivity to differentiate normal and malignant breast ESS spectra with increases in the percentage of malignant breast tissue (specificity 0.74):

<table>
<thead>
<tr>
<th>% malignant replacement</th>
<th>1-20%</th>
<th>21-40%</th>
<th>41-60%</th>
<th>61-80%</th>
<th>81-100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>0.56</td>
<td>0.72</td>
<td>0.60</td>
<td>0.90</td>
<td>0.87</td>
</tr>
</tbody>
</table>

In conclusion, ex-vivo ESS spectra are relatively stable and reproducible within 1 hour of excision irrespective of different tissue handling techniques. Sensitivity and specificity of spectral analysis is dependent on accurate correlation between ESS spectra and histology, and the amount of replacement by malignant tissues.
ABBREVIATIONS

ALND  Axillary Lymph Node Dissection
ALNS  Axillary Lymph Node Sampling
ANN   Artificial Neural Network
BIRADS Breast Imaging Reporting and Data System
CAD   Computer-Aided Detection
CADD  Computer-Aided Detection and Diagnosis
CNB   Core Needle Biopsy
EBB   Excision Breast Biopsy
ESS   Elastic Scattering Spectroscopy
FDG   Fluorine-18 Fluorodeoxyglucose
FFS   Fresh Frozen Section
FNAC  Fine Needle Aspiration Cytology
H&E   Haematoxylin and Eosin
HCA   Hierarchical Cluster Analysis
IHC   Immunohistochemistry
ITC   Isolated Tumour Cells
LDA   Linear Discriminant Analysis
MBA   Model Based Analysis
MGG   May-Grünwald-Giemsa Stain
MRI   Magnetic Resonance Imaging
MRS   Magnetic Resonance Spectroscopy
NHSBSP National Health Service Breast Screening Programme
NMLC  National Medical Laser Centre
<table>
<thead>
<tr>
<th>NSABP</th>
<th>National Surgical Adjuvant Breast and Bowel Project</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAP</td>
<td>Papanicolaou Stain</td>
</tr>
<tr>
<td>PBB</td>
<td>Percutaneous Breast Biopsy</td>
</tr>
<tr>
<td>PCA</td>
<td>Principle Component Analysis</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
</tr>
<tr>
<td>RT</td>
<td>Radiotherapy</td>
</tr>
<tr>
<td>SLN</td>
<td>Sentinel Lymph Node</td>
</tr>
<tr>
<td>SLNB</td>
<td>Sentinel Lymph Node Biopsy</td>
</tr>
<tr>
<td>TA</td>
<td>Triple Assessment</td>
</tr>
<tr>
<td>TIC</td>
<td>Touch Imprint Cytology</td>
</tr>
<tr>
<td>UCLH</td>
<td>University College London Hospital</td>
</tr>
<tr>
<td>US</td>
<td>Ultrasonography</td>
</tr>
<tr>
<td>VACB</td>
<td>Vacuum Assisted Core Biopsy</td>
</tr>
<tr>
<td>Xe-PPS</td>
<td>Xenon lamp Pulses Per Spectrum</td>
</tr>
</tbody>
</table>
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Typical MRS spectra from different types of breast tissue</td>
<td>28</td>
</tr>
<tr>
<td>2.1</td>
<td>Intradermal injection of $^{99m}$Tc-nanocolloid</td>
<td>70</td>
</tr>
<tr>
<td>2.2</td>
<td>Dynamic and static images</td>
<td>71</td>
</tr>
<tr>
<td>2.3</td>
<td>A blue lymphatic tract after patent blue dye injection</td>
<td>72</td>
</tr>
<tr>
<td>2.4</td>
<td>SLN imprints</td>
<td>77</td>
</tr>
<tr>
<td>3.1</td>
<td>The reflection, propagation and transmission of light</td>
<td>85</td>
</tr>
<tr>
<td>3.2</td>
<td>The visible spectrum</td>
<td>87</td>
</tr>
<tr>
<td>3.3</td>
<td>Molar extinction coefficient for haemoglobin in water</td>
<td>89</td>
</tr>
<tr>
<td>3.4</td>
<td>Absorption coefficient of fat corrected for scattering contribution</td>
<td>90</td>
</tr>
<tr>
<td>3.5</td>
<td>Schematic diagram of the ESS system</td>
<td>92</td>
</tr>
<tr>
<td>3.6</td>
<td>Spectral characteristics of xenon arc lamp against a white reflectance standard</td>
<td>94</td>
</tr>
<tr>
<td>3.7</td>
<td>Optical probe</td>
<td>95</td>
</tr>
<tr>
<td>3.8</td>
<td>Typical optical spectra taken from normal breast, breast fibroadenoma and breast cancer</td>
<td>98</td>
</tr>
<tr>
<td>5.1</td>
<td>Illustration to demonstrate potential contamination of ESS by ambient light</td>
<td>110</td>
</tr>
<tr>
<td>5.2</td>
<td>ESS spectra acquired at various ambient light intensities</td>
<td>112</td>
</tr>
<tr>
<td>5.3</td>
<td>ESS spectra acquired at 1lux and 20000lux ambient light intensities in separate intensity scales</td>
<td>115</td>
</tr>
<tr>
<td>5.4</td>
<td>Examples of discarded spectra acquired at 20,000 lux</td>
<td>116</td>
</tr>
<tr>
<td>5.5</td>
<td>Normalised spectra of lymph nodes 1, 2 and 3 at various ambient light intensities</td>
<td>118</td>
</tr>
<tr>
<td>5.6</td>
<td>ESS spectra of Lymph Nodes 1 to 6 acquired between 0-10 minutes and between 10-60 minutes following excision</td>
<td>126</td>
</tr>
</tbody>
</table>
5.7 Normalised ESS Spectra of Lymph Nodes 1 to 6 acquired between 0-10 minutes and between 10-60 minutes following excision

5.8 (a) ESS spectra demonstrating the observed changes seen in Lymph Nodes 2, 3 and 4
(b) Inverse molar extinction coefficient for oxygenated and de-oxygenated haemoglobin

5.9 (a) Normalised spectra of Lymph Node 2
(b) Normalised absorption spectra of de-oxygenated haemoglobin normalised to oxygenated haemoglobin

5.10 Illustrations to show (a) the fixed position of optical probe onto tissue and (b) submersion of tissue in normal saline

5.11 ESS spectra of Lymph Nodes 1 and 2 acquired between 0-10 minutes and 10-60 minutes following submersion in normal saline

5.12 Normalised ESS spectra of Lymph Nodes 1 and 2 acquired between 1-10 minutes and between 10-60 minutes following submersion in normal saline

5.13 ESS spectra of Lymph Nodes 1, 2 and 3 acquired between 0-10 minutes and 10-60 minutes following chilling in ice

5.14 Normalised ESS spectra for Lymph Nodes 1, 2 and 3 between 0-10 minutes and 0-60 minutes following chilling in ice

5.15 Normalised spectra of Lymph Nodes (a) 2 and (b) 3 between 10 and 30 minutes

5.16 Normalised spectra of Lymph Node 2 after 60 min and inverse molar extinction coefficient of oxygenated Hb normalised to deoxygenated Hb

5.17 Oxygen dissociation curve of haemoglobin illustrating a “left” shift with decreased temperature

5.18 ESS spectra of Lymph Nodes 1-4 plotted at 0-10 minutes and 10-60 minutes following submersion in formaldehyde solution

5.19 Normalised spectra of Lymph Nodes 1-4 plotted at 0-10 minutes and 10-60 minutes following submersion in formaldehyde solution

5.20 ESS spectra of Lymph Nodes 5 and 6 plotted at 0-1 hour, 1-4 hours and beyond 4 hours following submersion in formaldehyde solution
5.21 Normalised ESS spectra for Lymph Nodes 5 and 6 at 0-1 hours, 1-4 hours and beyond 4 hours 166

5.22 Absorption spectra of methaemoglobin in the visible range 170

6.1 Potential pitfalls in TIC 197

7.1 Diagram of grid 203

7.2 Distribution of percentage cancer overall and within tumour grids 2, 3, 4, 5 and 6 206

7.3 Study of variance to determine the area within ESS spectrum influenced by the actual tissue specimen 209

7.4 Illustration to demonstrate the influence of the site of tumour in relation to the path of light 216
LIST OF TABLES

1.1 Advantages and disadvantages of non-operative diagnosis of breast cancer compared to excision breast biopsy 15
1.2 Breast Imaging Reporting and Data System (BIRADS) categories 18
1.3 Performance of NHSBSP between 1999 and 2000 19
1.4 Comparison of Papanicolaou and May-Grünwald-Giemsa stains 34
1.5 Diagnostic categories for breast FNAC reporting 35
1.6 General diagnostic criteria for the recognition of benign and malignant conditions 37
1.7 The suggested threshold for FNAC performance in breast screening in the UK 38
1.8 Performance of imaging guided FNAC for impalpable breast lesions 39
1.9 Diagnostic categories for breast CNB reporting 41
1.10 Suggested threshold for CNB performance in breast screening in the UK 42
1.11 Calcification retrieval, ADH and DCIS underestimates at stereotactic breast biopsy using CNB and VACB 44
1.12 Positive excision margins using PBB 45
1.13 Sensitivity and specificity of the existing diagnostic modalities for breast cancer 46
2.1 Types of mastectomy 55
2.2 Complications following ALND 62
2.3 Summary of ongoing clinical trials evaluating the role of SLNB in breast cancer 67
2.4 Successful localisation rate, sensitivity, specificity of SLNB using blue dye, radioisotope and combination of tracers 69
3.1 Categories of optical biopsy technologies 86
3.2 Key features of ESS 93
3.3 Published clinical results of optical biopsy

5.1 Number of Xe-PPS of Lymph Nodes 1-3 at various ambient light intensities

6.1 Key features of ESS

6.2 Sensitivities and specificities of ESS in determining normal and metastatic axillary lymph nodes using ANN, HCA and MBA

6.3 Results of Analysis 1

6.4 Results of Analysis 2: Spectra from lymph nodes with >80% replacement with cancer

6.5 Results of Analysis 3

6.6 Results of Analysis 4

6.7 Comparison of TIC results with histopathology

6.8 Comparison of ESS results with histopathology

6.9 Published studies of TIC in determining SLN status in breast cancer

6.10 Published studies of FFS in determining SLN status in breast cancer

7.1 Breakdown of grid data

7.2 Results of Analysis 1

7.3 Results of Analysis 2

7.4 Results of Analysis 2 with 510-610nm range of spectra excluded

7.5 Results of Analysis 3 (a) without and (b) with 510-610nm range of spectra excluded

7.6 Comparison of sensitivity and specificity of various modalities for breast cancer diagnosis
Chapter 1  Diagnosis of Breast Cancer and Recent Advances

1.1 Introduction

In 2002, 1,151,298 women worldwide were diagnosed with breast cancer accounting for 23% of all female cancers excluding non-melanoma skin malignancies\(^1\). Over this period, 410,712 deaths were attributed to breast cancer\(^1\). Survival in the western world has improved since the early 1990s. This was primarily due to diagnosis at earlier stages and better treatment.

A definitive diagnosis of breast cancer requires direct histological evidence of breast cancer cells from within the lesion concerned. Historically, excision breast biopsy (EBB) has been the diagnostic procedure of choice. However, EBB is not a definitive surgical procedure for breast cancer because it does not necessarily achieve complete excision margins and it does not provide the axillary lymph node status. Therefore, further surgery is required.

The current strategy in the diagnosis of breast cancer is to use non-operative methods. The advantage of such methods is to enable clinicians and patients to plan surgical management as a single definitive operation. Additional advantages and disadvantages are outlined in Table 1.1.

In the United Kingdom, patients with breast cancer present either with symptoms, such as a breast lump, to the breast clinic or without symptoms through the National Health Service Breast Screening Programme (NHSBSP). In both cases, non-operative
diagnosis of breast cancer is based on triple assessment (TA) consisting of clinical examination, imaging and pathological evaluations.

**Table 1.1: Advantages and disadvantages of non-operative diagnosis of breast cancer compared to excision breast biopsy**

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diagnosis with simple tests</td>
<td>Error in diagnosis may lead to overtreatment or delay in making the correct diagnosis</td>
</tr>
<tr>
<td>Cheap compared with EBB</td>
<td>Occasional complications</td>
</tr>
<tr>
<td>Avoids excision breast biopsy in some cases and allows treatment of cancers at a planned operation</td>
<td>Requirement of skilled personnel with additional cost of training</td>
</tr>
<tr>
<td>Performed as an outpatient procedure</td>
<td></td>
</tr>
<tr>
<td>Avoidance of frozen section</td>
<td></td>
</tr>
<tr>
<td>Reduction of patient uncertainty and anxiety</td>
<td></td>
</tr>
<tr>
<td>Low complication rate compared with EBB</td>
<td></td>
</tr>
</tbody>
</table>

(Modified from Table 1: Non-operative diagnostic subgroup of the National Coordinating Group for Breast Screening Pathology: Guidelines for non-operative diagnostic procedures and reporting in breast cancer screening. NHSBSP Publication, Sheffield, 2001)

1.2 Triple Assessment

TA is the current diagnostic procedure of choice for breast diseases. For symptomatic patients, TA consists of clinical examination followed by mammography and finally pathological evaluation. This is to ensure that any vascular injuries and haematomas
do not compromise the interpretation of mammography. For asymptomatic patients, TA begins with mammography, and proceeds once an abnormality is found.

TA is positive if any one of its 3 components is positive and negative if all 3 components are negative. In women over the age of 35 years, the diagnostic accuracy of TA for breast cancer is over 95%\textsuperscript{3,6}.

1.3 Clinical Examination

Despite advances in imaging technologies, clinical examination plays an important role in the diagnosis of breast cancer. It is comprised of a detailed visual inspection for skin and nipple changes, asymmetry and dimpling, followed by a systematic palpation of the breasts, axillae and supraclavicular fossae for discrete lumps. In women presenting with nipple discharge, the subareolar area should be massaged to provoke secretion.

The main goal of clinical examination is to identify palpable lesions and to differentiate normal physiological nodularity from discrete lumps. The latter require mandatory evaluation to exclude breast cancer. Clinical examination requires trained staff and is subject to inter-observer variation. Sensitivity ranges between 48\% and 69\% and specificity ranges between 85\% and 99\%\textsuperscript{7}. Clinical examination can be unreliable in women below the age of 35 with a sensitivity of 37\% in predicting breast cancer\textsuperscript{8}. The uses of oral contraceptives and hormone replacement therapy can increase breast nodularity thereby affecting clinical interpretation.
1.4 Imaging

The principal imaging modality used in breast cancer diagnosis is mammography. The role of ultrasonography (US) is increasing particularly in cases where mammography is equivocal. Other imaging modalities include magnetic resonance imaging (MRI) and nuclear medicine imaging.

1.4.1 Mammography

Since the 1960's, mammography has played an important role in the diagnosis of breast cancer. Mammography can be used for both screening and diagnosis. Screening mammography aims at detecting breast cancer at the asymptomatic stage. Diagnostic mammography provides diagnostic evaluation of a specific area within the breast when a clinical abnormality such as a lump or a screening abnormality has been found. Both screening and diagnostic mammograms are usually taken in 2 perpendicular views, lateral and craniocaudal. Additional views at different angles can be taken for more accurate images of a suspicious area.

Mammography is based on the principle that various types of breast tissue have different x-ray densities, which can be utilised to produce a grey-scale image of the breast. High-density tissues such as calcifications and dense glandular tissues appear white whilst low-density tissues such as fatty tissue appear black. To achieve optimal quality, adequate compression of breasts is required to separate overlapping structures, to decrease the amount of radiation and to obtain sharp images.

During interpretation of mammograms, radiologists identify specific features, which are associated with breast cancer. These include the shapes, margins and density of
lesions and microcalcifications. Mammograms are usually reported with a description of findings and a diagnostic category, such as the Breast Imaging Reporting and Data System (BIRADS) (Table 1.2)\textsuperscript{10}.

\textit{Table 1.2: Breast Imaging Reporting and Data System (BIRADS) categories}

<table>
<thead>
<tr>
<th>Categories</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Inconclusive</td>
<td>Additional mammography views required</td>
</tr>
<tr>
<td>1 Negative</td>
<td>No mammographic abnormality present</td>
</tr>
<tr>
<td>2 Benign</td>
<td>Benign findings, no short-interval follow-up required</td>
</tr>
<tr>
<td>3 Probable benign finding</td>
<td>Findings with high probability of being benign, biopsy recommended</td>
</tr>
<tr>
<td>4 Suspicious finding</td>
<td>Findings not characteristic of malignancy but requires biopsy</td>
</tr>
<tr>
<td>5 High suggestive of malignancy</td>
<td>High probability of breast cancer</td>
</tr>
</tbody>
</table>

In general, the sensitivity and specificity of mammography for diagnosing breast cancer in symptomatic patients ranges between 72\% and 94\% and between 75\% and 94\% respectively\textsuperscript{11}. For breast screening, performance is measured by the rates of invasive and in-situ cancers detected, the benign excision breast biopsy per 1000 women screened (false positive), and recall for assessment. The overall false negative rate for screening mammography is approximately 10\%. The performance of NHSBSP in the UK between 1999 and 2000 are illustrated in Table 1.3\textsuperscript{12}.
Table 1.3: Performance of NHSBSP between 1999 and 2000

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Standard*</th>
<th>Achieved at 1st screen**</th>
<th>Achieved***</th>
</tr>
</thead>
<tbody>
<tr>
<td>Invasive cancers per 1000 women</td>
<td>&gt;3.6 (&gt;4% for subsequent screen)</td>
<td>5.0</td>
<td>4.4</td>
</tr>
<tr>
<td>In-situ cancers per 1000 women</td>
<td>≤0.4 to ≤1.0</td>
<td>1.7</td>
<td>1.14</td>
</tr>
<tr>
<td>Benign biopsies per 1000 women</td>
<td>&lt;3.6 (&lt;4 for subsequent screen)</td>
<td>2.7</td>
<td>0.7</td>
</tr>
<tr>
<td>Recall rate (%)</td>
<td>&lt;7%</td>
<td>8.3</td>
<td>3.9</td>
</tr>
</tbody>
</table>


*Standard set by NHSBSP, **Results achieved by NHSBSP after one round of screening mammography per woman screened, ***Overall result achieved including repeat mammography and additional views)

In general, the diagnostic accuracy of mammography for breast cancer depends on several factors: (1) operator skills of the radiographer in positioning the breast, (2) interpretation skills of the radiologist, (3) high breast density, (4) scarring secondary to previous surgery, radiotherapy and chemotherapy to breast and (5) breast implants. High breast density is the main patient factor for reducing the diagnostic accuracy of mammography. This is because non-calcified breast cancers have a similar x-ray density to normal fibroglandular breast tissue\textsuperscript{13}. Hence, mammography is not as effective in younger pre-menopausal women, those who are pregnant or lactating, and those on hormone replacement therapy.
In patients with a discrete palpable breast lesion, mammography contributes little to the diagnosis, which should be pathological. However, the role of mammography in this situation is to determine the extent of the cancer and to detect occult synchronous cancers within the ipsilateral or contralateral breast. Additional uses of mammography include stereotactic localisation of impalpable tumours or lesions prior to surgery and regular post-operative follow-up of breast cancer patients.

1.4.2 Recent advances in mammography

Recent advances in mammography include digital mammography, Computer-aided detection and diagnosis (CADD), Digital Tomosynthesis and Contrast Digital Mammography.

Digital mammography is one of the most important advances in mammography. Similar to standard mammography, x-rays are used to generate images of the breast. Instead of a film, a 50mm by 50mm phosphor screen is used to detect the x-rays leaving the breast and to convert them into light. The latter is transferred through a fibre optic reducer to a charged coupled device (CCD) detector. A typical CCD measures 25mm by 25mm with a 1024 by 1024 pixel matrix giving a 0.05mm resolution per pixel. The CCD converts the incident light into a digitised signal, which can be displayed onto a computer monitor or stored.

The main advantage of digital mammography is that contrast and density can be adjusted after image acquisition, thus allowing visualisation of subtle details such as spiculations at the margin of small breast cancers and granular calcifications in low grade ductal carcinoma in-situ (DCIS). So far, digital mammography has shown
equivalent spatial resolution to conventional film mammography. Therefore, digital mammography has the potential to improve breast cancer detection whilst reducing additional exposures due to under or over-exposure of films.

In the largest published study to date, Lewin et al. compared the performance of digital mammography and conventional film mammography in a screening setting\textsuperscript{14}. Screening mammograms were performed both on conventional and digital mammography systems. 42 cancers were detected in 6736 paired examinations. 9 cancers were detected solely on digital mammography, 15 solely on conventional mammography and 18 were detected on both. The difference was not statistically different partly due to the small patient numbers and the relatively low numbers of cancers detected through breast screening. However, there were significant reductions in recall and biopsy rates with digital mammography (799 vs 1007).

Currently, the Digital Mammographic Imaging Screening Trial is on going. It has 49,528 women enrolled. Each participant had both digital and conventional mammography at the time of enrolment and a follow-up mammogram one year later. Closed to further enrolment since 2003, the results are awaited.

Additional advantages of digital mammography include (1) improved contrast between dense and non-dense breast tissue, (2) faster image acquisition (<1 minute), (3) easier image storage through picture archiving and communication systems, (4) transmission of images over internet i.e. teleradiology and (5) the provision of a platform for further advances such as CADD, tomosynthesis and contrast medium mammography.
The disadvantages of digital mammography are the cost of the system and the lack of convincing evidence that it improves breast cancer detection. The costs and benefits involved will decide whether this technology becomes part of routine practice.

CADD is a recent development in mammography. The objectives of CADD are to identify radiological features associated with breast cancer through computer vision and to systematically analyse these features using computer algorithms such as artificial intelligence to provide an output, thereby improving the diagnostic accuracy and performance of mammography. To achieve computer vision, all mammographic images must first be in a digital format either by digitisation of conventional mammographic films or acquired directly by digital mammography. This is followed by a processing stage, which enhances important image features whilst suppressing those of little interest. Individual images are subsequently divided into various areas by the process of segmentation, which are ready for analysis.

Currently, only two systems, R2 ("Image Checker", R2 Technology, Los Altos, California, USA) and CADx ("Second Look", CADx Medical Systems, Quebec, Canada) are commercially available for computer-aided detection (CAD). In a recent prospective study, 12,860 screening mammograms were interpreted with the assistance of a CAD system\textsuperscript{15}. Each mammogram was initially interpreted without CAD assistance and was immediately followed by a re-evaluation of areas marked by the CAD system. The study showed an increase in recall rate from 6.5\%-7.7\%, no change in the positive predictive value for biopsy at 38\%, a 19.5\% increase in number of cancer detected and an increase in the proportion of early stage (0 and 1) malignancies detected from 73\% to 78\% when CAD was used. However, the
experience and skill of the reporting radiologist had a significant influence in this study, and this was the main limitation. At present, the ability of CAD to improve diagnostic accuracy and performance is unclear from the limited number of published studies\cite{16,17}.

Digital tomosynthesis is a technique through which 3-dimensional tomograms can be generated from a series of low dose digital mammograms at different levels within the breast\cite{18}. This is performed by moving the x-ray source to various positions above the breast\cite{19}. This technique has the potential to reduce the visibility of normal breast tissue and the effects of superimposed tissue, thus improving the visibility of breast cancers and providing better demonstration of lesion morphology, especially in women with radiographically dense breasts\cite{19,20}.

Contrast digital mammography involves the use of an intravenous iodine based contrast agent and the acquisition of a series of pre and post contrast digital images\cite{20}. Images are subsequently processed by digital subtraction of the pre-contrast image from post-contrast image thereby enhancing visibility of contrast agent. A series of images over time is obtained with time-series subtraction. Contrast digital mammography enables tumour visualisation based on the increased vascularity of malignant tissue over normal breast tissue\cite{18}, and can potentially visualise smaller lesions than those currently seen with screening mammography\cite{20}. Both digital tomosynthesis and contrast digital mammography are emerging technologies, currently being evaluated by clinical studies.
1.4.3 Ultrasonography and recent advances

Ultrasonography or ultrasound (US) uses high-frequency sound waves, which are reflected from different tissue structures to generate images of internal structures. As it does not involve ionised radiation, it is considered safe for women of any age, as well as those who are pregnant. A typical examination takes 5 to 15 minutes and is totally painless.

The main diagnostic role of US is to differentiate solid lesions (e.g. breast cancer, fibroadenomas and prominent breast tissue) from fluid filled lesions (e.g. breast cysts) based on the differences in their echogenic pattern. Other indications for US include (1) the initial diagnostic imaging for palpable lumps in young women due to the intrinsic high density of breasts, (2) the evaluation of masses not completely evaluated with mammography, (3) in inflammatory breast conditions, (4) as guidance for intervention procedures, (5) the evaluation of mammographic asymmetry and (6) the evaluation following breast augmentation and breast conservation surgery.\(^\text{21}\)

US has a sensitivity range between 49-100% and a specificity range between 29-100% for breast cancer diagnosis in a recent systematic review.\(^\text{21}\) Although US is not part of TA, it is often used as a supplement to mammography. In a study of 2020 patients, US detected 8 extra malignancies whilst correctly downgraded 332 cases from a positive to a negative diagnosis (i.e. from a suggested malignancy to no malignancy) following clinical examination and mammography\(^\text{22}\). The systematic application of breast US improved the overall diagnostic yield especially in patients with palpable breast lumps or with abnormal screening mammography\(^\text{22}\).
Recent advances in breast US include 3-dimensional US and tissue harmonic imaging. 3-dimensional US provides additional images and characteristics of the breast lesions. Whether this provides additional value in differentiating breast masses is controversial\(^{23,24}\).

Tissue harmonic imaging is a new US technology. It produces images from US frequencies that are generated as the incident US wave propagates through tissue. Harmonic frequencies are multiples of the fundamental beam frequency. Few prospective clinical studies have reported reductions in image artefacts, improvements in lesion contrast and margin evaluation\(^{25-27}\).

### 1.4.4 Magnetic resonance imaging and recent advances

MRI generates images of breast structures using signals emitted from tissues following excitation by radiofrequency waves in a strong magnetic field. Excellent imaging of breast lesions can be achieved with intravenous contrast such as dimeglumine gadopentate (Magnevist\(^{®}\)) and by placing the breast within a special magnetic coil to ensure a high magnetic field. Various imaging techniques such as dynamic imaging, fat suppression and subtraction imaging are available, but are beyond the scope of this thesis.

Contrast-enhanced breast MRI has demonstrated a sensitivity of 94%-100% in the detection of breast cancer. The main limitation of MRI in breast cancer diagnosis is its specificity which is lower and more variable, ranging between 37% and 97\(^{28}\). This is partly because benign lesions such as fibroadenomas, proliferative benign disorders, areas of inflammation and active glandular tissue can all show enhancements. The
other contributing factor is the lack of standard interpretation criteria for image evaluation. The two major approaches to image interpretation are (a) evaluation of enhancement kinetics following contrast agent administration and (b) evaluation of lesion morphology.

The advantages of MRI over mammography are that it involves no ionising radiation, it can identify lesions down to 2-3mm, it has a near 100% sensitivity in detecting breast cancer using contrast enhancement, its images are not affected by breast density and breast implants, it gives a good definition of the local extent of cancer, and it is sensitive to lobular carcinoma. However, the drawbacks of MRI include a low and variable specificity, the cost of equipment and maintenance, poor reliability in showing microcalcifications, failure of breast lesions to selectively take up contrast agent and time taken for acquisition and interpretation of multiple MRI images.

In the United Kingdom, MRI is not a standard imaging procedure for breast cancer diagnosis. However, MRI has diagnostic roles in (1) diagnosing multi-centric or bilateral diseases (MRI sensitivity of 89-100% versus Mammography sensitivity of 0-66%\textsuperscript{29}), (2) evaluating difficult diagnostic problems and (3) identifying occult tumours in patients with axillary metastasis and negative clinical examination, mammography and US\textsuperscript{30}. This is particularly the case in patients with invasive lobular carcinoma because of its variable presentation on clinical examination and mammography. Other roles include (1) cancer staging such as assessment of lesion size and disease extent to nipple, skin and chest wall, and axillary lymph node involvement\textsuperscript{29}, (2) imaging used to evaluate silicone implants and (3) monitoring changes following breast conservation surgery, chemotherapy and interstitial laser
photocoagulation. MRI has a limited role in breast screening due to its cost, its time consuming nature and more importantly, its inability to detect microcalcifications. However, MRI appears to have a role in screening high risk women younger than age 40 due to the poorer sensitivity and specificity of mammography.

Recent advances in MRI include developments in functional MRI, magnetic resonance spectroscopy (MRS), 3-dimensional imaging, and magnetic resonance elastography.

Functional MRI is a relatively new technology that uses T2 weighted imaging technique to measure metabolic changes in tissues during disease process. It differs from traditional MRI by combining anatomical changes with physiological changes resulted from disease process. There are several types of functional MRI such as perfusion MRI, diffusion-weighted MRI and Blood-Oxygen-Level-Dependent MRI. However, these are beyond the scope of this thesis.

MRS is a type of functional MRI, which is capable of measuring certain metabolites. In breast cancer cells, there is an accumulation of choline-containing compounds, which can be detected by MRS. Figure 2.1 demonstrates typical MRS spectra of different breast tissue types. Malignant breast tissues are characterised by a “choline” peak at 3.3ppm.
Although individual MRS spectrum can be analysed by visual inspection, such strategy is not optimal when there is a large volume of data. A potential solution is to devise a classification strategy, which was developed by Mountford et al.32. Their technique involves a three-stage process. Firstly, a genetic algorithm-based programme was used to pre-process the MRS spectra to define the maximally discriminatory subregions in the spectra. The second stage involves the use of these discriminatory subregions to develop a linear discriminant analysis (LDA) classifier. Approximately half of the MRS spectra were randomly selected to train the LDA classifier. The LDA classifier was then used to validate on the remaining MRA spectra. If classification is problematic, the third stage, computerised consensus diagnosis, was used. Using this technique, MRS has an overall sensitivity of 92% and specificity of
93% in determining malignant and benign fine needle aspirate. This compares to a sensitivity of 85% and specificity of 82% with visual inspection of MRS spectra.

A typical MRS examination takes 20 to 30 minutes to perform. Both in-vivo and ex-vivo tissues can be studied. Potential applications of MRS include (1) incorporation of MRS into MRI to improve its low specificity and (2) to monitor and predict response to chemotherapy.

1.4.5 Nuclear Medicine Imaging

The nuclear medicine imaging modalities for breast cancer diagnosis are scintimammography and positron emission tomography (PET). These depend on the physiological distribution of a radiolabelled tracer (radiopharmaceutical), which consists of a radioactive isotope such as $^{99m}$Technetium ($^{99m}$Tc) and $^{18}$Fluorine ($^{18}$F) attached to a carrier molecule. The latter can be a metabolite or antibodies to oestrogen receptors. Radiopharmaceuticals are usually administered intravenously and then accumulate preferentially in breast cancer cells, which can be detected as an area of focal uptake by appropriate radiation imaging.

1.4.5.1 Scintimammography

Scintimammography is a physiological imaging technique used to visualise primary breast cancer using a radiopharmaceutical. Although various radiopharmaceuticals are available, $^{99m}$Tc sestamibi ($^{99m}$Tc MIBI) is the agent of choice because of its higher sensitivity and specificity. In addition, $^{99m}$Tc sestamibi is the only agent with Food and Drug Administration (FDA) approval for this purpose. Although the precise mechanism of uptake in breast cancer cells is unknown, it appears that $^{99m}$Tc
sestamibi is taken up by active transport mechanisms and is accumulated in mitochondria. Uptake appears to be influenced by the degree of tumour differentiation but not by tumour size or histological type. A review of 21 studies using $^{99}$Tc sestamibi totalling 2682 patients with at least 1541 palpable and 939 impalpable lesions showed sensitivity range of 50% to 95% and specificity range between 58 to 100%. Of these studies, the largest was a prospective multi-centre trial of 673 women. The blinded readers’ diagnostic accuracy was 78% to 81% with an inter-reader agreement between 95% and 100%. Sensitivity was higher for tumours $\geq 1$ cm than for tumours <1 cm (74.2% and 48.2% respectively).

When compared to other imaging modalities, sensitivity and specificity of $^{99}$Tc sestamibi was shown to be 84% and 80% respectively compared to values of 68% and 65% for US in 76 patients undergoing excision breast biopsy or FNAC. A separate study of 56 patients comparing scintimammography, mammography and MRI reported sensitivities of 60%, 60% and 100% respectively and specificities of 75%, 25% and 50% respectively.

The advantage of scintimammography is that the diagnostic accuracy is not affected by breast density and nodularity, the presence of breast implants, and scarring from previous surgery or radiotherapy. Therefore, scintimammography has a specific diagnostic role in these clinical situations.
1.4.5.2 Positron emission tomography

PET is a computed tomographic imaging technique using positron emitting radioisotopes (fluorine-18 and carbon-11) which are labelled to biologically active molecules such as metabolites, hormones and antibodies. The majority of PET studies in oncology have concentrated on fluorine-18 fluorodeoxyglucose (FDG). As a glucose analogue, FDG is taken by cells particularly those in high metabolic state such as malignant cells, and is subsequently converted into FDG-6-phosphate by phosphorylation. FDG-6-phosphate does not undergo glycolysis and therefore accumulates within the cells. The rationale behind using FDG is based on the observations by Warburg that glucose metabolism is increased in malignant tissue. Therefore, interpretation and diagnostic criteria of FDG-PET is based on the intensity of tracer uptake, which is expected to be more pronounced in breast cancer.

Several studies of the use of FDG-PET in diagnostic imaging for primary breast tumour have reported sensitivity and specificity ranging from 80% to 100%. However, these studies were based on a pre-selected patient population with a bias for breast cancer and a small number of benign lesions. Hence, specificity and negative predictive value have not been fully determined.

In the largest study to date of 144 patients with 132 histologically confirmed breast cancer and 53 benign lesions, Avril et al observed an overall sensitivity of 64.4% using conventional imaging reading (i.e. regarding only focal tracer accumulation as to represent malignancy) and 80.3% using sensitive image reading (i.e. including probable and definite malignant lesions). Detection rate is significantly lower in pT1 (<2 cm) tumours (68.2%) than in pT2 (>2 cm – 5cm) tumours (91.9%) with a higher...
false negativity for invasive lobular carcinoma (65.2%) compared with invasive ductal carcinoma (23.7%). As for multi-focal tumours, only 50% of multi-focal tumours were identified \(^4^1\). In a separate study by the same group, FDG uptake by breast cancer has a positive correlation with histological tumour type, microscopic tumour growth pattern and tumour cell proliferation. However, other factors such as tumour microenvironment may have significant effects on metabolism \(^4^2\). In view of limited sensitivity, FDG-PET currently has no defined clinical role in the diagnosis of breast cancer \(^4^3\).

**1.4.6 Other Imaging Modalities**

There are several other imaging technologies currently under development for the screening and diagnosis of breast cancer. These include thermography, optical imaging, electrical impedance imaging, thermoacoustic computed tomography and magnetic/ultrasonic elastography. Unlike MRI, Scintimammography, and PET, these technologies are not widely available. In addition, there is a lack of published clinical data to merit further discussion.

**1.5 Pathological Evaluation**

Pathological evaluation provides the definitive diagnosis of breast cancer with microscopic evidence of malignant cells from within the lesion concerned. Various techniques are available to obtain cellular or tissue specimens. These include EBB, fine needle aspiration cytology (FNAC) and core needle biopsy (CNB). More recently, vacuum assisted core biopsy (VACB) and percutaneous breast biopsy (PBB) have been developed.
1.5.1 **Excision breast biopsy**

EBB is historically the “gold standard” for pathological diagnosis\(^4\). It is an operative procedure performed under local or general anaesthesia. Skin incisions are usually made immediately over the palpable lump, but circum-areolar incisions can be used in lesions close to nipple for better cosmesis. Lesions are removed with or without a rim of normal breast tissue. Excised specimens are routinely sent for histology, but fresh frozen section (FFS) has been used for intra-operative diagnosis.

For impalpable lesions detected by mammography, EBB can be performed with a preoperative localisation. This involves the insertion of a needle (under local anaesthetic) using imaging guidance (usually mammography) to locate the lesion. The lesion is excised with the needle in situ, and is subsequently x-rayed to confirm complete removal\(^4\). Needle localisation EBB is the gold-standard procedure for impalpable breast lesion because of its diagnostic accuracy (between 99% and 100%)\(^4\).

The main complications of EBB are wound infection and post-operative haematoma. The latter may require re-operation for evacuation of haematoma or to arrest on-going haemorrhage\(^4\). Clinical disadvantages of EBB as a primary pathological diagnostic procedure are (1) the requirement of an invasive operation, which may not be necessary in benign diseases, (2) inability to offer preoperative patient counselling in cases turning out to be malignant, (3) inability for clinicians and patients to plan a single definitive operation for malignant lesions, (4) potential post-operative cosmetic defects and (5) potential dissemination of cancer if excision line goes through part of the tumour\(^2,4\). Hence, EBB is not the pathological diagnostic procedure of choice, and is usually reserved for equivocal cases after TA.
1.5.2 Fine needle aspiration cytology

FNAC is the simplest, cheapest and least invasive of the non-operative diagnostic techniques. It requires a venepuncture needle, a 5- or 10-ml syringe and microscope slides. The needle is guided through the breast lesion. With suction applied, a few forward and backward passes of the needle are made within the lesion. Suction is relieved before withdrawing. The content in the hub of the needle is smeared onto microscope slides. The slides are either air-dried or fixed prior to staining. For palpable lesions, FNAC can be performed “freehand” by clinicians whilst for impalpable lesions, FNAC can be performed under ultrasound imaging by allowing real time demonstration of needle traversing the lesion47.

The main staining methods for breast FNAC are Papanicolaou (PAP), and May-Grünwald-Giemsa (MGG or Diff-Quik™), and their essential differences are outlined in Table 1.4.

Table 1.4: Comparison of Papanicolaou and May-Grünwald-Giemsa stains

<table>
<thead>
<tr>
<th>Papanicolaou Stain</th>
<th>May-Grünwald-Giemsa Stain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Requires wet fixed specimens</td>
<td>Requires air-dried specimens</td>
</tr>
<tr>
<td>Cells remained 3-dimensional</td>
<td>Cells flatten out and enlarge</td>
</tr>
<tr>
<td>Cells within clusters seen on focusing</td>
<td>Cells within clusters difficult to see</td>
</tr>
<tr>
<td>Cytoplasm –pale blue &amp; translucent</td>
<td>Cytoplasm –blue/pale purple &amp; dense</td>
</tr>
<tr>
<td>Nucleus –bluish purple &amp; sharp margins</td>
<td>Nucleus –purple &amp; margins not as sharp</td>
</tr>
<tr>
<td>Chromatin pattern clearly visible</td>
<td>Chromatin pattern not as well seen</td>
</tr>
<tr>
<td>Nucleoli –blue or red</td>
<td>Nucleoli seen as pale areas</td>
</tr>
</tbody>
</table>
PAP is the method of choice because of its superior quality. For example, cells remain 3-dimensional enabling close examination of all cells and architectural patterns within the clusters. Subtle variations in chromatin patterns and clear nucleoli provide additional features for interpretation. However, PAP is time consuming and laborious. MGG is used if immediate reporting is required. Other rapid staining techniques include toluidine blue and the Rapid Papanicolaou methods. Immunocytochemistry is also possible and requires wet fixation. In general, the choice of staining is dependent on the individual preference of the cytologist or pathologist and on whether immediate reporting is required\textsuperscript{44,47}.

In the UK, FNAC results should be reported in the following categories used by the NHS Breast Screening Programme\textsuperscript{48} (Table 1.5), which is similar to the BIRADS system for mammography\textsuperscript{10}.

\textbf{Table 1.5: Diagnostic categories for breast FNAC reporting}

<table>
<thead>
<tr>
<th>Categories</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>Inadequate specimen for diagnosis</td>
</tr>
<tr>
<td>C2</td>
<td>Benign cytology</td>
</tr>
<tr>
<td>C3</td>
<td>Atypia probably benign</td>
</tr>
<tr>
<td>C4</td>
<td>Suspicious of malignancy</td>
</tr>
<tr>
<td>C5</td>
<td>Malignant cytology</td>
</tr>
</tbody>
</table>
The success of FNAC depends on a sample that is adequate and representative of the lesion, a suitable processing and staining without artifact and an accurate interpretation of cytological material with a clear report conveyed to the rest of the clinical team\(^2\). FNAC is operator dependent with a superior success rate and lower rate of technically inadequate specimens found in experienced aspirators\(^49\).

Interpretation of FNAC requires careful examination of several cytological features, which are outlined in Table 1.6. However, it is important to appreciate that cancers, particularly low-grade carcinomas, do not always exhibit all features of malignancy. Hence, interpretation and reporting of FNAC requires special cytological training and experience.

The accuracy of FNAC is dependent on the operator skill and experience during the aspiration process, and the interpretation skill and experience of the reporting pathologist. Other significant factors affecting the success rate of FNAC include poor tumour palpability, small tumour size, low tumour grade, scattered cellular distribution of cancer cells and benign-like ultrasound findings\(^50\). Other sources of errors in diagnosis include (1) the dilution of aspirate by blood or fluid, (2) blood clotting of aspirate, (3) fibrosis masking tumour cells, (4) presence of nuclear pleomorphism of some benign tumours, and (5) lack of clinical information for reporting cytologist/pathologist\(^51\). Therefore, FNAC results should be interpreted in the light of clinical history and imaging findings. It is not possible to provide the absolute sensitivity and specificity of FNAC in the diagnosis of breast cancer. However, the maximum attainable performance of individual breast services can be described through auditing. The suggested thresholds for FNAC performance in breast screening in the UK are illustrated in Table 1.7\(^52\).
Table 1.6: General diagnostic criteria for the recognition of benign and malignant conditions

(From Table 3: Non-operative diagnostic subgroup of the National Coordinating Group for Breast Screening Pathology: Chapter 3 FNAC Reporting Guideline, Guidelines for non-operative diagnostic procedures and reporting in breast cancer screening. NHSBSP Publication, Sheffield, 2001 p.22.48)

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Benign</th>
<th>Malignant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellularity*</td>
<td>Usually poor or moderate</td>
<td>Usually high</td>
</tr>
<tr>
<td>Cell to cell adhesion*</td>
<td>Good with large defined clusters of cells</td>
<td>Poor with cell separation resulting in dissociated cells with cytoplasm and small groups of intact cells</td>
</tr>
<tr>
<td>Cell arrangement</td>
<td>Even, usually in flat sheets (monolayers)</td>
<td>Irregular with overlapping and 3-dimensional arrangement</td>
</tr>
<tr>
<td>Cell types</td>
<td>Mixture of epithelial, myoepithelial and other cells with fragments of stroma</td>
<td>Usually uniform cell population</td>
</tr>
<tr>
<td>Bipolar (elliptical) bare nuclei</td>
<td>Present, often in high numbers</td>
<td>Not conspicuous</td>
</tr>
<tr>
<td>Background</td>
<td>General clean except in inflammatory conditions</td>
<td>Occasionally with necrotic debris and sometimes inflammatory cells including macrophages</td>
</tr>
<tr>
<td><strong>Nuclear characteristics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Size (in relation to red blood cell diameter)*</td>
<td>Small</td>
<td>Variable, often large, depending on tumour size</td>
</tr>
<tr>
<td>Pleomorphism*</td>
<td>Rare</td>
<td>Common</td>
</tr>
<tr>
<td>Nuclear membrane (PAP stain)*</td>
<td>Smooth</td>
<td>Irregular with indentations</td>
</tr>
<tr>
<td>Nucleoli (PAP stain)*</td>
<td>Indistinct or small and single</td>
<td>Variable but may be prominent, large and multiple</td>
</tr>
<tr>
<td>Chromatin (PAP stain)*</td>
<td>Smooth or fine</td>
<td>Clumped and may be irregular</td>
</tr>
<tr>
<td>Additional features</td>
<td>Apocrine metaplasia, foamy macrophages</td>
<td>Mucin, intracytoplasmic lumina</td>
</tr>
</tbody>
</table>

* indicates major cytological feature
Table 1.7: Suggested threshold for FNAC performance in breast screening in the UK

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Minimum (%)</th>
<th>Preferred (%)</th>
<th>Current Median (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute sensitivity*</td>
<td>&gt;60</td>
<td>&gt;70</td>
<td>57.1</td>
</tr>
<tr>
<td>Complete sensitivity*</td>
<td>&gt;80</td>
<td>&gt;90</td>
<td>81.5</td>
</tr>
<tr>
<td>Specificity</td>
<td>&gt;55</td>
<td>&gt;65</td>
<td>58.4</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>&gt;98</td>
<td>&gt;99</td>
<td>99.6</td>
</tr>
<tr>
<td>False positive rate</td>
<td>&lt;1</td>
<td>&lt;0.5</td>
<td>0.2</td>
</tr>
<tr>
<td>False negative rate</td>
<td>&lt;6</td>
<td>&lt;4</td>
<td>6.3</td>
</tr>
<tr>
<td>Inadequate rate*</td>
<td>&lt;25</td>
<td>&lt;15</td>
<td>23.4</td>
</tr>
<tr>
<td>Inadequate rate for cancer</td>
<td>&lt;10</td>
<td>&lt;5</td>
<td>9.8</td>
</tr>
<tr>
<td>Suspicious rate</td>
<td>&lt;20</td>
<td>&lt;15</td>
<td>15.8</td>
</tr>
</tbody>
</table>

* Absolute sensitivity = \( \frac{\text{number of breast cancers with C5 on FNAC}}{\text{total number of carcinomas undergoing FNAC}} \times 100\% 

Complete sensitivity = \( \frac{\text{number of breast cancers with C3, 4 and 5 on FNAC}}{\text{total number of carcinomas undergoing FNAC}} \times 100\% 

Inadequate rate = \( \frac{\text{number of inadequate specimen}}{\text{total number of cases undergoing FNAC}} \times 100\% 

(From Table 9: Non-operative diagnostic subgroup of the National Coordinating Group for Breast Screening Pathology: Chapter 6 Quality Assurance, Guidelines for non-operative diagnostic procedures and reporting in breast cancer screening. NHSBSP Publication, Sheffield, 2001 p.50.)

For impalpable lesions, the performance of imaging guided FNAC from a review of 16 studies by Britton is illustrated in Table 1.8.3.
Table 1.8: Performance of imaging guided FNAC for impalpable breast lesions

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Stereotactic FNAC (%)</th>
<th>US FNAC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of studies (No. of FNAC)</td>
<td>12 (5895)</td>
<td>4 (2673)</td>
</tr>
<tr>
<td>Absolute sensitivity</td>
<td>62.4</td>
<td>83.1</td>
</tr>
<tr>
<td>Complete sensitivity</td>
<td>83.1</td>
<td>95.1</td>
</tr>
<tr>
<td>Specificity</td>
<td>86.9</td>
<td>84</td>
</tr>
<tr>
<td>Positive predictive value of C5</td>
<td>99.3</td>
<td>98.3</td>
</tr>
<tr>
<td>False negative rate</td>
<td>0.5</td>
<td>1.4</td>
</tr>
<tr>
<td>Inadequate rate</td>
<td>6.4</td>
<td>12.8</td>
</tr>
<tr>
<td>Inadequate rate in cancers</td>
<td>5.0</td>
<td>2.1</td>
</tr>
</tbody>
</table>

The main advantages of FNAC are that it is simple and accurate in expert hands. Immediately reporting is possible. Other advantages of FNAC are that it is quick (usually takes 1-2 minutes for aspiration), well tolerated by patients and cost effective. In addition, FNAC has the least complications of all biopsy techniques. These include pain during aspiration, slight haematoma and pneumothorax. The latter is rare and occurs usually in women with small breasts or medial lesions. There have been reports of epithelial displacement and tumour seeding, which are more common with CNB.

1.5.3 Core needle biopsy

CNB is a percutaneous procedure that involves removing a core of breast tissue approximately 1-2cm in length and 1-3mm in width using a cutting needle. The needle consists of an inner trocar with a special notch for trapping a sliver of breast tissue and an outer hollow needle. The needle is larger than that used in FNAC, and local anaesthetics are required. A variety of automated devices such as Bard Biopsy
gun and Manan gun (Manan Medical Products) are available in addition to the original manual needles (Trucut, Surecut).

Automated devices are preferred because of higher sampling success rate, greater diagnostic sensitivity, better specimen quality and ease of use. Upon loading and releasing the trigger, the inner trocar is propelled first into the lesion and is immediately followed by the hollow needle coring the breast tissue. The CNB needle is subsequently removed from the patient and reloaded to retrieve the specimen.

For palpable lesions, CNB can be performed “freehand”, and a minimum of five cores is recommended to ensure adequate sampling. For impalpable lesions, CNB is usually performed with stereotactic guidance. This can be carried out with the patient in prone, lateral recumbent or upright position on a specially designed stereotactic mammography system. The patient’s breast is slightly compressed and held in position throughout the procedure. A pair of x-ray images is acquired, and computer analysis provides precise settings, enabling correct placement of the CNB needle by radiologists. Alternately, US can be used to guide the biopsy needle precisely to the correct location under continuous imaging.

The core specimens are fixed in formaldehyde and sent for standard histopathology. The standard histopathology is a 3-step process involving fixation, embedding and staining. Fixation preserves the physical structure of tissue using a buffered isotonic solution of 4% formaldehyde. Fixation typically takes 12 hours, and the minimum time for CNB specimens is 6 hours. Embedding involves dehydrating the tissue specimen by bathing it in a graded series of ethanol and water mixtures, and clearing,
in which ethanol is cleared by xylene to allow impregnation with paraffin. Embedding takes between 8 to 30 hours. The paraffin-embedded tissue is sectioned longitudinally by a microtome to a thickness of 1-10μm, and sections are transferred onto glass slides for staining\textsuperscript{57}. The standard stain for histopathology is haematoxylin and eosin (H&E) and the staining process takes between 15 to 30 minutes. For mass lesions, H&E stained sections from one level are usually sufficient, but for microcalcifications, cores should have a minimum of 3 levels examined. Further levels and immunohistochemistry can be performed\textsuperscript{56}. Alternately, core specimens can be examined using fresh frozen section (FFS) for immediate reporting. However, this is associated with higher false positive diagnosis\textsuperscript{58}.

CNB yields a larger sample for histological assessment, thus enabling a definitive diagnosis with histological type and interpretation by any pathologist without the need of cytological expertise. In addition, CNB provides further prognostic information such as histological grade and hormone receptor status\textsuperscript{59}. Like FNAC, CNB results should be reported in the following categories (Table 1.9) used by the NHSBSP\textsuperscript{56}.

\textbf{Table 1.9: Diagnostic categories for breast CNB reporting}

<table>
<thead>
<tr>
<th>Categories</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>Normal tissue</td>
</tr>
<tr>
<td>B2</td>
<td>Benign lesion</td>
</tr>
<tr>
<td>B3</td>
<td>Lesion of uncertain malignant potential</td>
</tr>
<tr>
<td>B4</td>
<td>Suspicious of malignancy</td>
</tr>
<tr>
<td>B5</td>
<td>Malignant</td>
</tr>
</tbody>
</table>
The accuracy of CNB is dependent on the operator skill and experience, tumour palpability and tumour size. For palpable lesions, freehand CNB has the maximum attainable sensitivity of 95% and specificity of 100% from published data. Like FNAC, sensitivity and specificity vary between individual centres. For breast screening, the current suggested thresholds for CNB in UK are outlined in Table 1.10.

**Table 1.10: Suggested thresholds for CNB performance in breast screening in the UK**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Minimum (%)</th>
<th>Preferred (%)</th>
<th>Current Median (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute sensitivity</td>
<td>&gt;70</td>
<td>&gt;80</td>
<td>&gt;76.4</td>
</tr>
<tr>
<td>Complete sensitivity</td>
<td>&gt;80</td>
<td>&gt;90</td>
<td>84.5</td>
</tr>
<tr>
<td>Specificity</td>
<td>&gt;75</td>
<td>&gt;85</td>
<td>81.2</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>&gt;99</td>
<td>&gt;99.5</td>
<td>100</td>
</tr>
<tr>
<td>False positive rate</td>
<td>&lt;0.5</td>
<td>&lt;0.1</td>
<td>0</td>
</tr>
<tr>
<td>False negative rate</td>
<td>&lt;15</td>
<td>&lt;10</td>
<td>15.1</td>
</tr>
<tr>
<td>Miss rate from cancer</td>
<td>&lt;15</td>
<td>&lt;10</td>
<td>15.1</td>
</tr>
<tr>
<td>Suspicious rate</td>
<td>&lt;10</td>
<td>&lt;5</td>
<td>4.8</td>
</tr>
</tbody>
</table>

(From Table 10: Non-operative diagnostic subgroup of the National Coordinating Group for Breast Screening Pathology: Chapter 6 Quality Assurance, Guidelines for non-operative diagnostic procedures and reporting in breast cancer screening. NHSBSP Publication, Sheffield, 2001 p.50.)
CNB allows a more accurate assessment with larger samples and also histological assessment. However, CNB is more invasive and can be uncomfortable. CNB is more time consuming, and has a higher risk of haematoma. CNB is limited by the histological processing, and therefore cannot provide an immediate diagnosis. Some published studies reported the superiority of CNB over FNAC in both freehand and imaging guided setting\textsuperscript{60,61}, whilst others reports claimed higher accuracy with FNAC\textsuperscript{62}. It is important to appreciate that both techniques are highly operator dependent.

**1.5.4 Vacuum assisted core biopsy**

VACB (Mammotome, Ethicon Endo-Surgery, Cincinnatti, USA, and Minimally Invasive Breast Biopsy, United States Surgical, Norwalk, USA) provides breast cores for histological evaluation similar to CNB, but differs fundamentally in its design and mechanism of action. VACB uses biopsy probes with a sampling chamber that has a vacuum channel incorporated. The latter applies a gentle negative pressure drawing breast tissue into the probe's sampling chamber before a rotating cutting cylinder is passed down within the probe separating the core of tissue. Specimens are retrieved using the negative pressure without the need of removing the probe. Further specimens can be obtained from the same insertion. A simple rotational mechanism is incorporated to orientate the aperture of the sampling chamber for sampling outer areas. A small metal clip can be accurately deposited to localise the biopsy area should surgical excision be required\textsuperscript{63}.

Because no firing mechanism is involved, image-guided VACB of impalpable breast lesions has shown to be superior to CNB in (1) calcification retrieval, (2) ADH underestimates and (3) DCIS underestimates (Table 1.11)\textsuperscript{64}.
**Table 1.11: Calcification retrieval, ADH and DCIS underestimates at stereotactic breast biopsy using CNB and VACB**

<table>
<thead>
<tr>
<th></th>
<th>Number of Studies</th>
<th>Number of Lesions using CNB</th>
<th>Number of Lesions using 14-/11-gauge VACB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcification retrieval</td>
<td>7</td>
<td>1474/1591 (93%)</td>
<td>2241/2269 (99%)</td>
</tr>
<tr>
<td>ADH Underestimates</td>
<td>9</td>
<td>90/194 (46%)</td>
<td>36/177 (20%)</td>
</tr>
<tr>
<td>DCIS Underestimates</td>
<td>7</td>
<td>118/579 (20%)</td>
<td>107/985 (11%)</td>
</tr>
</tbody>
</table>

Other advantages of VACB include less internal scarring and bleeding because the probe remains in situ during the entire sampling procedure. The vacuum mechanism can be used to remove bleeding during and at the end of the procedure. Epithelial displacement mimicking stromal invasion is less in VACB\textsuperscript{65}. The disadvantage of VACB over CNB is the cost of expensive equipment, whilst the learning curves of both techniques are similar\textsuperscript{66}.

### 1.5.5 Percutaneous breast biopsy

PBB (Advanced Breast Biopsy Instrumentation, United States Surgical, Norwalk, USA) is designed to provide a precise total removal of mammographic lesions using a large bore cutting cylinder with digital stereotactic imaging as an alternative to needle localisation breast biopsy. PBB is also referred as large volume breast biopsy.

PBB is performed with the patient lying on a prone biopsy table. Under local anaesthesia, a wire is stereotactically guided into the breast lesion, and is used as a guide for the insertion of cutting cylinder. A variety of sizes up to 2cm are available.
The specimen produced is in the form of a core, and is removed with the wire guide. Post-procedure mammography and specimen radiology can be performed to confirm complete excision.

PBB is more invasive than CNB and VACB, requiring a 15-20mm skin incision for the insertion of the biopsy probe. Clinical studies showed that ABBI is a reliable stereotactic breast biopsy technique and a safe alternative to needle localisation breast biopsy, with considerable cost savings. In a large series of 1785 lesions during a 7-year period, imaging-histologic discordance occurred in 3.1% and is significantly higher in first 2 years. In this series, there was a high prevalence (24.4%) of breast cancer found.

As a primary procedure for breast cancer, PBB has a high percentage of positive excision margins requiring further open surgical re-excision (Table 1.12). However, if restricted to less than 10mm, tumour-free excision margins can be achieved in 55% with no residual disease found at re-excision in 89%. Therefore, there is no clear advantage of PBB over stereotactic CNB at present. The average operating time using PBB is over 60 minutes. This is significantly greater than EBB.

Table 1.12: Positive excision margins using PBB

<table>
<thead>
<tr>
<th>Study</th>
<th>Number of Lesions</th>
<th>Number of cancer</th>
<th>Positive margins (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liberman</td>
<td>54</td>
<td>7</td>
<td>6 (86%)</td>
</tr>
<tr>
<td>Rebner</td>
<td>Unspecified</td>
<td>11</td>
<td>7 (64%)</td>
</tr>
<tr>
<td>Smathers</td>
<td>101</td>
<td>27</td>
<td>23 (85%)</td>
</tr>
<tr>
<td>Lifrange</td>
<td>Unspecified</td>
<td>53</td>
<td>11 (79%)</td>
</tr>
</tbody>
</table>
1.6 Discussion

An accurate and reliable system for breast cancer diagnosis is of paramount importance. Misdiagnosis and delays at any stage of the diagnostic process may result in considerable anxiety and distress for the patient. TA is the current standard diagnostic system in the UK, because of its simplicity and accuracy. Despite this, false negative and equivocal cases continue to occur resulting in diagnostic delays and benign breast biopsies. This is partly due to the inherent limitations of the existing technologies such as the reduced sensitivity of mammography in dense breast and human errors, such as misinterpretation of mammograms. These in turn have been the driving force behind the efforts to develop and refine existing and new technologies. Table 1.13 summaries the sensitivity and specificity of the existing diagnostic techniques for breast cancer quoted in this chapter.

Table 1.13: Sensitivity and specificity of the existing diagnostic techniques for breast cancer

<table>
<thead>
<tr>
<th>Diagnostic modality</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical examination(^{75})</td>
<td>48-69%</td>
<td>85-99%</td>
</tr>
<tr>
<td>Mammography(^{11})</td>
<td>72-94%</td>
<td>75-94%</td>
</tr>
<tr>
<td>US(^{21})</td>
<td>49-100%</td>
<td>29-100%</td>
</tr>
<tr>
<td>MRI(^{76})</td>
<td>94-100%</td>
<td>37-97%</td>
</tr>
<tr>
<td>Scintimammography(^{34})</td>
<td>50-95%</td>
<td>58-100%</td>
</tr>
<tr>
<td>PET(^{77})</td>
<td>80-100%</td>
<td>-</td>
</tr>
<tr>
<td>FNAC(^{52})</td>
<td>57%</td>
<td>58%</td>
</tr>
<tr>
<td>CNB(^{52})</td>
<td>76%</td>
<td>81%</td>
</tr>
<tr>
<td>VACB</td>
<td>Not available</td>
<td>Not available</td>
</tr>
<tr>
<td>PBB</td>
<td>Not available</td>
<td>Not available</td>
</tr>
</tbody>
</table>
It is important to appreciate that none of the above techniques are 100% sensitive and 100% specific in diagnosing breast cancer. However, each one of them has specific roles.

Advances in the diagnostic technologies for breast cancer can be classified into 3 groups: (1) improvements in existing technology such as digital mammography, 3-dimensional ultrasound, VACB and PBB, (2) reduction human error such as CADD and (3) developments using other modalities such as MRI, PET, scintimammography and EIS. The first group is essentially a technological up-grade to provide higher quality using existing techniques, whilst the third group explores the differences between normal and malignant breast tissue in other physical parameters such as tumour vascularity, biochemical, electrical and optical. Although such advances are welcomed, it is important that these technologies provide additional value without additional complexity. The latter can only result in unnecessary anxiety and delays in patients.

Reference List


48. Non-operative Diagnostic Subgroup of the National Coordinating Group for Breast Screening Pathology. FNAC Reporting Guidelines. *Guidelines for Non-


76. Goscin CP, Berman CG, Clark RA. Magnetic resonance imaging of the breast. *Cancer Control* 2001; 8: 399-406.

Chapter 2 Surgical Management of Breast Cancer and Recent Advances

2.1 Introduction
Breast cancer is widely recognised by clinicians as a systemic disease originating in the breast with local and systemic manifestations. This reflects the evolution of breast cancer management over the past century from radical surgery alone to multimodal therapy guided by individual patient factors and disease stage. Although recent advances in breast cancer management have centered on systemic treatments such as chemotherapy, immunotherapy and hormonal therapy, the role of surgery remains important for local disease control and disease staging. The aim of this chapter is to review the surgical management of the breast cancer and in particular sentinel lymph node biopsy (SLNB).

2.2 Surgical Management of Breast Cancer
The aims of surgical management of breast cancer are:

- To cure patients where the disease is confined to the breast
- To control disease locally, preventing progression to ulceration, bleeding and lymphoedema
- To determine tumour characteristics such as tumour grade, hormonal receptor status and other prognostic markers
- To determine lymph node involvement
Surgery consists of either mastectomy or breast conservation surgery together with axillary surgery. Reconstructive surgery to rebuild the contour of the breast is also an important option for patients undergoing mastectomy. However, this is beyond the context of this thesis and will not be discussed further.

At present, surgery remains an integral part of the breast cancer management. Most patients undergo surgery prior to systemic therapy. Occasionally, some patients undergo systemic therapy before surgery to reduce the size of the tumour or in the case of inflammatory breast cancer. Surgery may be contraindicated in patients with extensive local disease, in those who are unfit for anaesthesia or in those who are too old. In these situations, disease control can be provided by hormonal therapy such as tamoxifen and anastrozole.

2.3 Mastectomy

Mastectomy is the surgical removal of a breast. There are several types of mastectomy (Table 2.1). Simple mastectomy and modified radical (Patey) mastectomy are the most commonly used. Classical radical (Halsted) mastectomy is rarely performed because of its radical nature. Subcutaneous mastectomy spares the skin overlying the breast, which is used for breast reconstruction.

The advantages of mastectomy are (1) post-operative radiotherapy is not required, and (2) the risk of ipsilateral metachronous breast cancer is abolished. The main disadvantage of mastectomy is disfigurement following the removal of the breast. Common complications after mastectomy include wound complications such as haematoma, infection, delayed healing, and shoulder weakness or stiffness.
### Table 2.1: Types of mastectomy

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Tissues removed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple or Total Mastectomy</td>
<td>Removal of the entire breast with skin, nipple and areola, but no lymph nodes</td>
</tr>
<tr>
<td>Modified radical (Patey) mastectomy</td>
<td>As simple mastectomy with pectoralis minor muscle</td>
</tr>
<tr>
<td>Classical radical (Halsted) mastectomy</td>
<td>Removal of the entire breast, nipple, areolar, pectoralis major and minor muscles and lymph nodes</td>
</tr>
<tr>
<td>Subcutaneous mastectomy</td>
<td>Removal of entire breast only leaving skin</td>
</tr>
</tbody>
</table>

The indications for mastectomy are:

- Large tumour and/or extensive in-situ disease
- Multifocal disease
- Patient preference e.g. fear of recurrence and ease of follow-up
- High risk e.g. young patients with known genetic predisposition
- Cosmetic outcome e.g. small breasts

Although breast conservation surgery is the procedure of choice for the primary tumour, mastectomy continues to be an important and useful operation for many patients and constitutes around a third of all operations for primary breast cancer.

#### 2.4 Breast Conservation Surgery

Breast conservation surgery involves the removal of the tumour whilst preserving the remainder of the breast. This is done either by quadrantectomy in which an entire of quadrant of the breast is removed or by wide local excision in which the cancer is
excised with a margin of normal breast tissue. Breast conservation surgery for small tumours (T1 and T2) is favored because the breast is preserved without the need for prosthesis. More important is the similar patient survival in both breast conservation and mastectomy as demonstrated by numerous studies. The most important are the three randomized trials with more than 20 years follow-up.

The National Surgical Adjuvant Breast and Bowel Project (NSABP) B-06 trial started in 1976 to determine whether breast conservation with or without radiotherapy was as effective as mastectomy. 1851 women were randomized into three treatment groups: mastectomy, lumpectomy alone and lumpectomy with breast irradiation. The trial showed no significant differences among the three groups with respect to disease-free survival, distant-disease-free survival and overall survival.

The European Institute of Oncology trial recorded similar findings as NSABP B-06. Between 1973 and 1980, 701 women with breast cancers less than 2cm were randomized to radical mastectomy (349 women) and quadrantectomy (352 women) followed by radiotherapy to ipsilateral breast. After 1976, patients in both groups with positive lymph nodes also received adjuvant chemotherapy. The 20 year death rate from breast cancer was 24.3% in the mastectomy group and 26.1% in the breast conservation group. However, 30 women in the breast conservation group developed local recurrence and 8 women in the mastectomy group over the 20 year period giving a crude cumulative incidence of 8.8% and 2.3% respectively. Similar findings were recorded by the National Cancer Institute trial, which had 247 patients enrolled and had a median follow-up of 18 years.
The risk of ipsilateral recurrence of tumour is a major concern for women undergoing breast conservation surgery. The NSABP B-06 trial showed a significant ipsilateral recurrence rate of 39.2% when lumpectomy was performed without post-operative breast radiotherapy compared with a recurrence rate of 14.3% when lumpectomy was accompanied with breast radiotherapy\textsuperscript{1}. This compares with a recurrence rate of 14.3% when lumpectomy was accompanied with breast radiotherapy. Therefore, breast conservation surgery is commonly followed by at least six weeks of radiotherapy.

Another important factor for local recurrence is the resection margins. In a recent study by Leong et al.\textsuperscript{4}, 452 women with pathologically node-negative breast cancer underwent breast conservation surgery and radiotherapy. 352 (77.9%) had a negative margin, 42 (9.3%) with positive (invasive and/or in situ) margin and 58 (12.8%) with indeterminate margin. With a median follow-up of 80 months, the 5-year ipsilateral recurrence rates were 3.1%, 11.9% and 6.9% respectively. Similar findings were observed by Smitt et al. in their study of 535 patients undergoing breast conservation surgery with radiotherapy\textsuperscript{5}. With a median follow-up of 6 years, local recurrence occurred in 14% of margin-positive patients and 3% of margin-negative patients.

Although it is widely accepted that resection should aim at complete macroscopic removal of tumour with clear margins, there is no consensus in the definition of a negative margin and the subsequent management of a positive margin. By the NSABP definition, a negative margin is defined as the absence of tumour cells at the specimen surface on microscopy\textsuperscript{1}, whilst the European Institute of Oncology trial had relatively wide margins because of quadrantectomy\textsuperscript{2}. In the National Cancer Institute study,
complete macroscopic removal of tumour was mandated with no requirement of microscopically clear margins. In a recent review of the significance of resection margins, Gennaro et al. concluded that negative margins do not guarantee complete removal of the disease and positive margins are not necessarily associated with residual disease⁶. In the case of negative margins, the risk of leaving residual tumour is low but not negligible. The risk of local recurrence is high if margin involvement is extensive or more than one margin is involved. The group proposed to divide marginal involvement into five distinct categories: absent, focal, minimal, moderate and extensive. If more than one margin is involved or there is extensive involvement of one margin, further excision or mastectomy should be considered. For others, re-operation may not be necessary if adjuvant therapy is given.

Breast conservation surgery is suitable when there is no evidence of advanced local disease (i.e. tumour ≤4cm and no extensive nodal involvement), multifocal disease, or distant metastasis. Post-operative complications after breast conservation surgery are similar to those of mastectomy, which includes haematoma, wound infection, delayed healing, and seroma formation. Long-term complications include breast fibrosis, pneumonitis and pulmonary fibrosis, fat necrosis, rib fracture and arm oedema, which are secondary to radiotherapy.

The disadvantages of breast conservation surgery are (1) the need for post-operative radiotherapy, which currently consists of daily treatments for 6 weeks, and (2) the risk of local recurrence, which requires further surgery in approximately 1% of patients per year and also requires regular long term follow up with mammogram surveillance. Breast conservation surgery is not feasible where facilities for radiotherapy and follow-up are not available locally or are too expensive.
2.5 Surgical Management of the Axilla

2.5.1 The role of axillary surgery

Lymphatic metastasis to axillary lymph nodes was the first route through which systemic dissemination of breast cancer was recognised. Its importance has been appreciated for more than 200 years. Axillary surgery in the form of axillary lymph node dissection (ALND), also known as axillary lymph node clearance, was developed in the 19th century to remove axillary lymph nodes with an intention to improve survival. Since then, axillary surgery has become an integral part of the surgical management of breast cancer.

ALND was initially thought to improve survival by removing all potentially involved lymph nodes. This belief no longer holds, as many studies have demonstrated no survival advantage with ALND. However, the axillary lymph node status remains a major prognostic indicator and a principal determinant in adjuvant chemotherapy. Hence, the role of ALND has evolved over the past 30 years to that of disease staging and local disease control.

At present, the axillary lymph node status is determined by histological examination of axillary lymph nodes. ALND is the standard procedure used to obtain these lymph nodes. Other methods such as physical examination, lymphoscintigraphy, US, computed tomography, MRI, 99mTc-sestamibi scanning, and PET have been evaluated, but have failed to achieve equivalent sensitivity and specificity to histology. The limitation of imaging techniques is their inability to detect small metastatic lesions within a macroscopically normal lymph node. It seems that histology will remain the standard method for axillary nodal staging, using axillary
surgery to harvest the axillary lymph nodes. The controversy over axillary surgery is the extent of the dissection. ALND is an extensive procedure with significant morbidity. As a result, less radical procedures such as axillary lymph node sampling (ALNS) and sentinel lymph node biopsy (SLNB) were developed.

Axillary lymph node status plays an important role as an indicator for chemotherapy. However, indications for chemotherapy have widened over the years and have become less reliant on axillary lymph node status. Other factors such as tumour features may provide adequate information for evaluating the risk of metastasis and the decision on chemotherapy.

The role of ALND in local disease control in the axilla is well recognised. Local failure after complete axillary dissection is rare and in the range of 0-2%. However, in terms of local disease control, axillary radiotherapy offers the same efficacy. A recently published 15 year update on a prospective randomized trial comparing ALND and axillary radiotherapy in 658 patients with a breast tumour less than 3 cm in diameter and clinically uninvolved lymph nodes, found no difference in long term survival in both groups (73.8% versus 75.5% at 15 years). Axillary recurrence was less in ALND group (1% versus 3%), but there were no difference in recurrence rates in the breast or supraclavicular and distant metastases.

In summary, axillary surgery in the management of breast cancer has evolved from playing a therapeutic role to playing a role in disease staging. In the absence of a reliable test for axillary lymph node staging, axillary surgery forms an important part of breast cancer management. The controversy in axillary surgery is over the extent of the axillary dissection.
2.5.2 Axillary lymph node dissection

ALND is the surgical removal of the axillary lymph nodes. The extent of ALND can be defined by the level from which the lymph nodes are removed. The axillary lymph nodes are grouped into 3 levels in relation to the pectoralis minor muscle. Level 1 consists of axillary lymph nodes inferolateral to pectoralis minor, level 2 posterior to pectoralis minor and level 3 superomedial to pectoralis minor.

ALND is usually limited to level 1 and 2, because the risks of complications are higher with the more extensive dissection. Complications of ALND are outlined in Table 2.2. With the exception of damage to the nerves to serratus anterior and latissimus dorsi, which is rare and avoidable, early complications are treatable and settle with time.

Late complications are more troublesome. Paraesthesia in the axilla and the medial aspect of the arm is one of the most common post-operative problems resulting from the surgical division of the intercostal brachial nerve. This can be avoided by identifying and preserving the nerve in the dissection as it emerges from the medial aspect of the axilla. Restriction of shoulder mobility may improve with physiotherapy. The most troublesome complication is lymphoedema secondary to the disruption or obstruction of lymphatics, because it is often resistant to treatment and causes significant morbidity. The incidence of lymphoedema following ALND ranges from 5.5% to 80%, and may be underestimated because milder cases might readily be overlooked. The extent of breast surgery and subsequent axillary irradiation are the main risk factors for lymphoedema.
Table 2.2: Complications following ALND

<table>
<thead>
<tr>
<th>Early complications</th>
<th>Late complications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemorrhage &amp; Haematoma</td>
<td>Lymphoedema</td>
</tr>
<tr>
<td>Damage to the nerves to serratus anterior and latissimus dorsi</td>
<td>Reduced upper limb mobility</td>
</tr>
<tr>
<td>Wound infection</td>
<td>Axillary recurrence</td>
</tr>
<tr>
<td>Seroma formation</td>
<td>Paraesthesia in upper arm and axilla</td>
</tr>
</tbody>
</table>

At present, ALND is the standard axillary procedure for breast cancer. However, its role is controversial because of its complications and the increasing diagnosis of breast cancer at early stages with decreased likelihoods of nodal metastasis. ALNS and SLNB provide alternative, less invasive procedures when there is no clinical evidence for nodal involvement.

2.5.3 Axillary lymph node sampling

Numerous methods of ALNS have been described, but the technique most widely used in the United Kingdom is the sampling of the firmest or largest four nodes within the axilla. A recently published randomized clinical trial using this technique reported no increase in axillary recurrence or mortality rate compared with ALND\textsuperscript{25}. In this study, 466 patients aged under 70 years with unilateral invasive breast tumours less than 4cm and no evidence of metastatic disease were randomized to ALND (232 patients) and ALNS (234 patients) with axillary irradiation given selectively. There were no differences in the rates of ipsilateral breast (ALND 14 versus ALNS 15), axillary (8 versus 7) or distant (29 versus 29) recurrences, and no statistically significant differences in 5-year survival rates (82.1% in ALND group versus 88.6 in...
ALNS group)\(^{25}\). Morbidity in general was least in ALNS group without radiotherapy. In terms of arm swelling, ALND had an early mean increase in arm volume of 4%, which remained constant over the next 2.5 years whilst ALNS had an increase of 2% which improved with time\(^{25}\). This result was supported by the similar findings from an earlier randomized trial of 417 patients by the same unit in Edinburgh\(^{26}\) and another similar randomized trial of 100 patients by a Swedish group\(^{27}\).

The main argument against ALNS is the risk of axillary recurrence. In the NSABP B-04 trial, the local failure rate for patients who had mastectomy and six or less axillary nodes removed was 12% compared to a 0.3% local failure rate in patients with more than 10 nodes removed\(^{21}\). However, a re-analysis of this data by Harris and Osteen showed that these figures have no statistical power because of a small patient group\(^{28}\). A more recent study from Denmark consisting of 3128 patients showed the 5-year probability for axillary recurrence was 19% in patients with no ALND, 10% with 1-2 removed and negative nodes, 5% with 3-4 nodes removed, 3% with 5-10 nodes removed and 3% with more than 10 removed and negative nodes\(^{22}\). From the above studies, it appears that the optimal number of nodes that need to be harvested for ALNS is between 4 to 5 nodes. In practice, the removal of an extra lymph node does not result in a statistically significant difference in outcome.

2.6 Sentinel Lymph Node Biopsy

2.6.1 The sentinel lymph node concept

Sentinel lymph node (SLN) is a lymph node that receives direct lymphatic drainage from the primary tumour. Over the recent years, there have been significant interests
in SLNB as an alternative and less invasive procedure for lymphatic staging of breast
cancer. This is because a large number of clinical studies have demonstrated the high
sensitivity and specificity of SLN in predicting the axillary lymph node status.
Therefore patients with a negative SLN can be spared from unnecessary ALND.

The SLN concept is based fundamentally on two principles. First, the pattern of
lymphatic drainage to a specific regional lymph node i.e. the SLN is orderly and
predictable. Secondly, the SLN functions as an effective filter for tumour cells.
Therefore, metastatic breast cancer cells would disseminate to the SLN first where
they form metastatic deposits before progressing onto other lymph nodes. The SLN
concept follows the lymphatic permeation theory as the mechanism of breast cancer
metastasis, which was central to the ALND. The aim of SLNB is to identify and
retrieve the first “temporary barrier” in patients with clinically node-negative early
stage breast cancer. In these patients, the risk of metastasis is relatively low, and
ALND could be avoided if the SLN is free of metastasis.

Opponents to the SLN concept view tumour dissemination as a random process via
both the blood and lymphatic system. This is determined by the biological nature of
the tumour and the immunocompetence of the host. In the case of lymphatic
dissemination, the process is unpredictable and lymph nodes do not act as barriers as
was once suggested29. However, it is important to bear in mind that haematological
dissemination is now widely regarded as an independent mechanism for tumour
dissemination. As for lymphatic dissemination, there is still no consensus as to the
precise mechanism. The validation of the SLN concept would provide further
evidence to support the lymphatic permeation theory as the mechanism for tumour dissemination within the lymphatic system.

2.6.2 Validation and current status of the SLNB

Since the first publications on SLNB in breast cancer\(^{30-32}\), there has been immense research and clinical interest in SLNB because of its potential clinical benefits. This resulted in a large volume of published research evaluating SLNB. Much of these studies centred on (1) whether the SLN can be accurately identified, (2) whether SLN status predicts status of the axilla, (3) the optimal technique for SLN biopsy, and (4) identifying the appropriate population for SLN. Many of these studies shared similar protocols, which involve the SLNB using various techniques followed by ALND. The SLN status is then compared with the axillary lymph node status.

A meta-analysis of 69 studies between 1994 and 2000 involving 8059 patients found an overall SLN identification rate of 96% (7765 patients) and an overall false negative rate of 8.4% (range: 0-29%)\(^{33}\). In 28 (44.8%) studies, successful SLN mapping was >90%, and these studies were also associated with lower false negative rates (6.3% versus 11.1%)\(^{33}\). 11 trials reported zero false negative rate whilst 26 trials had rates >10%\(^{33}\). Other studies identified factors influencing successful SLN identification and false negativity. These include patient factors such as age and body mass index, and surgeons' experience\(^{32,34,35}\). These studies have shown that SLN can be identified and that SLN can predict the axillary lymph node status with acceptable accuracy.

The potential role of SLNB is to identify patients with negative SLN, who do not require ALND thereby avoiding the risk of axillary morbidity. The current phase of
research addresses the various clinical aspects of SLNB such the safety of implementation of SLNB in the management of breast cancer, the establishment of optimal SLN localisation technique, and the management of the axilla following a positive SLNB.

To date, there is only one published randomised clinical trial comparing SLNB with routine ALND. In this study (March 1998 to December 1999), 516 patients with primary breast tumours (≤2 cm in diameter) were randomly assigned either to SLNB and immediate ALND (the ALND group, 257 patients) or to SLNB followed by ALND only if the SLN contained metastases (the SLN group, 259 patients). 83 patients in ALND group (32.3%) had metastasis, and the overall accuracy of SLNB in this group was 96.9% (sensitivity 91.2%, specificity 100%). 92 patients in the SLNB group (35.5%) had positive SLN and had ALND. The 167 patients with negative SLN in the SLNB group had less pain and better arm mobility and had no cases of overt axillary metastasis during follow-up. Other trials are currently underway and are summarised in Table 2.3.

SLNB is rapidly becoming standard practice for axillary lymph node staging in breast cancer. In the case of a negative SLN, ALND is not indicated. In the case of a positive SLN, ALND is currently recommended because of the risk of residual disease in the axilla. However, this is controversial as there are also alternatives such as axillary irradiation and observation. These are currently evaluated through randomised trials (Table 2.3). As the results of these randomised trials are unlikely to be available in the immediate future, a number of consensus statements have been issued to guide the clinical management of SLNB performed out with clinical trial settings. In the Consensus Statement on Guidelines for Performance of Sentinel Lymphadenectomy...
for Breast Cancer (third revision, October 2003) from the American Society of Breast Surgeons, a level I or II ALND is recommended in the event of a positive SLNB\textsuperscript{37}.

\textbf{Table 2.3: Summary of ongoing clinical trials evaluating the role of SLNB in breast cancer (from Goyal A. et al.\textsuperscript{38})}

<table>
<thead>
<tr>
<th>Study</th>
<th>Start date</th>
<th>Sample size</th>
<th>Design</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axillary Lymphatic Mapping Against Nodal Axillary Clearance (ALMANAC)</td>
<td>2000</td>
<td>1260</td>
<td>Randomise to: ALND or ALNS vs. SLNB</td>
</tr>
<tr>
<td>After Mapping of the Axilla: Radiotherapy or Surgery (AMAROS)</td>
<td>2001</td>
<td>3485</td>
<td>If SLN positive randomise to: ALND vs. Radiotherapy</td>
</tr>
<tr>
<td>German Clinical Interdisciplinary Sentinel Study (KiSS)</td>
<td>2000</td>
<td>1912</td>
<td>If SLN negative randomise to: ALND vs. no ALND</td>
</tr>
<tr>
<td>French Randomised Sentinel Node Study (Fransenod)</td>
<td>NA\textsuperscript{*}</td>
<td>446</td>
<td>SLNB patients randomised to peritumoural injection vs. periareolar injection</td>
</tr>
<tr>
<td>International Breast Cancer Study Group Trial (IBCSG 23-01)</td>
<td>2001</td>
<td>1960</td>
<td>If SLN positive for micrometastases(IHC), randomise to: ALND vs. no ALND</td>
</tr>
<tr>
<td>American College of Surgeons Oncology Group (ACOSOG-Z0010)</td>
<td>1999</td>
<td>5300</td>
<td>IHC positive SLN patients(H&amp;E negative) observed to determine prognostic significance; bone marrow also assayed for micrometastasis to determine incidence and significance</td>
</tr>
<tr>
<td>American College of Surgeons Oncology Group (ACOSOG-Z0011)</td>
<td>1999</td>
<td>1900</td>
<td>If SLN positive(H&amp;E) randomise to completion ALND vs. observation</td>
</tr>
<tr>
<td>National Surgical Adjuvant Breast and Bowel Project (NSABP-B-32)</td>
<td>1999</td>
<td>4500</td>
<td>If SLN negative randomise to: completion ALND vs. no additional axillary treatment</td>
</tr>
<tr>
<td>Royal Australasian College of Surgeons Sentinel lymph Node biopsy versus Axillary Clearance (RACS SNAC)</td>
<td>2001</td>
<td>1000</td>
<td>SLN negative patients randomised to completion ALND vs. no additional axillary treatment</td>
</tr>
</tbody>
</table>

IHC, Immunohistochemical staining; H&E, Haematoxylin and eosin. *Not available
2.6.3 The technique of SLNB

SLNB is a two-staged procedure involving first the localisation of SLN followed by the surgical biopsy. Current techniques for SLN localisation use a vital blue dye (such as Patent Bleu V and Isosulphan bleu) and/or $^{99m}$Tc Technetium ($^{99m}$Tc) labelled radioactive colloid tracers. The commonly used colloids are $^{99m}$Tc sulfur colloid, $^{99m}$Tc human serum albumin nanocolloid and $^{99m}$Tc antimony trisulfide, which vary in particle size and range.

The tracers are injected into or in close proximity to the primary breast tumour. The rationale is that the tracer particles will enter the lymphatic system and be transported to the SLN by the lymphatic flow. The tracers are retained by the SLN, which is identifiable by the accumulation of blue dye and/or by the radioactivity using a handheld gamma probe.

Despite the consensus over the principles underlying SLNB, there are significant variations in the actual techniques used such as the choice of tracers, the site of injection and pre-operative imaging. In a meta-analysis of 40 studies, Liberman et al demonstrated 91% localisation rate using radioisotope only and combination techniques compared to 80% using blue-dye technique (Table 2.4). With a narrower range of localisation rate, the combination technique appeared to be more consistent and reliable over radioisotope technique.

Operative issues such as the surgical definition of SLN, multiple SLNs, removal of additional non-SLN and credentialing have also contributed to the significant variations in SLN biopsy between institutions. However, these are beyond the scope of this thesis.
Table 2.4: Successful localisation rate, sensitivity and specificity of SLNB using blue dye, radioisotope and combination of tracers

<table>
<thead>
<tr>
<th>Tracer (no. of studies)</th>
<th>Number of SLN Procedures</th>
<th>Successful localisation rate</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue Dye (12)</td>
<td>993</td>
<td>80% (69-99%)</td>
<td>91%</td>
<td>100%</td>
</tr>
<tr>
<td>Radioisotope (16)</td>
<td>1934</td>
<td>91% (66-98%)</td>
<td>93%</td>
<td>100%</td>
</tr>
<tr>
<td>Combination (12)</td>
<td>873</td>
<td>91% (81-100%)</td>
<td>95%</td>
<td>100%</td>
</tr>
<tr>
<td>Total (40)</td>
<td>3800</td>
<td>88%</td>
<td>93%</td>
<td>100%</td>
</tr>
</tbody>
</table>

(Adapted from Table 27.2 Validation studies of SLN biopsy in breast cancer: Hirman S Cody III: Sentinel Lymph Node Biopsy. Dunitz, London, 2002)

2.6.4 The SLNB Protocol at UCLH

At UCLH, patients, who were diagnosed with breast cancer by TA or following EBB, were recruited for SLNB following informed consent. Exclusion criteria were pregnancy, lactation, previous axillary surgery or irradiation, multifocal breast cancers and clinically positive axilla.

SLN localisation uses both $^{99m}$Tc-nanocolloid (Nycomed Amersham, UK) and Patent V blue dye. $^{99m}$Tc-nanocolloid is characterised by a very small size of albumin particles with typically more than 95% of the labelled colloid below 80nm. 15MBq of $^{99m}$Tc-nanocolloid in 0.2ml volume is injected intradermally overlying the palpable tumour or the site of previous excision breast biopsy (Figure 2.1). In the case of
impalpable tumour, the site of injection is at the areolar border of the corresponding quadrant. The injection takes place approximately 18 to 24 hours before surgery.

**Figure 2.1: Intradermal injection of $^{99m}$Tc-nanocolloid**

(Illustration from Figure 5b: M.R.S. Keshtgar, W.A. Waddington, S.R. Lakhani and P.J. Ell: Chapter 5 Injection techniques, The sentinel node in surgical oncology. Springer-Verlag, 1999 p.53)

Following injection, anterolateral dynamic imaging is acquired for a period of 10 minutes followed by static imaging with anterior and lateral views each for a period of 5 minutes using a low energy high-resolution collimator (400XC/T Single-headed large field-of-view, GE Medical Systems, USA). Dynamic imaging visualises the passage of the radio-colloid along the lymphatics (Figure 2.2a), whilst static imaging confirms the number of SLN localised using radio-colloid (Figure 2.2b).
Figure 2.2: Dynamic and static images. (a) Dynamic images taken at different time frames illustrating the passage of radioisotope (dotted arrow) and uptake of colloid by SLN (arrow) and (b) anterior-posterior (left) and lateral (right) static images confirming successful localisation of SLN.
At the time of surgery, 2ml of Patent V blue dye is injected in the same fashion as the $^{99m}$Tc-nanocolloid (Figure 2.3). Using the gamma probe detector (Neoprobe 1500), the site of the maximal radioactivity is determined and marked as the site of incision. Following incision, the gamma probe is used to guide the dissection towards the SLN. SLN was defined as any lymph node which is radioactive and/or blue in colour. SLN is removed and ex-vivo radioactivity is measured to verify the excision of SLN. The residual radioactivity within the cavity is measured to confirm completion of SLNB. Biopsy of internal mammary SLN was not performed. All patients had ALND unless they refused.

*Figure 2.3: A blue lymphatic tract after patent blue dye injection*

(Illustration from Figure 5a: M.R.S. Keshtgar, W.A. Waddington, S.R. Lakhani and P.J. Ell: Chapter 7 Surgical techniques, The sentinel node in surgical oncology. Springer-Verlag, 1999 p.82)
The SLNB protocol at UCLH was evaluated by Mr Mohammed Keshtgar in 2 phases that is in learning and recruitment. In the learning phase, a total of 30 patients were recruited, in which SLN localisation was successful in 29 (97%). Three patients consented to SLNB only. Two patients had SLN in the internal mammary chain, but also had ALND. In the 24 patients, who had both SLNB and ALND, 7 patients had axillary nodal metastasis. SLN status was positive in 5 out of 7 patients (sensitivity of 71%) and there were 2 false negatives (specificity 89%). In total, SLN correctly predicted the axillary status in 22 patients (91%).

In the recruitment phase, a total of 51 patients were recruited. One patient underwent bilateral procedures. SLN localisation was successful in all 52 procedures (100%). 15 patients had axillary metastasis (28.8%), and all had positive SLN (sensitivity 100%). In 4 of 15 patients, the SLN was the only involved lymph node. There was no false positive (specificity 100%). Following the recruitment phase study, the combined intra-dermal injection technique was adopted for SLN localisation at UCLH.

2.6.5 Pathological aspects

The role of pathology is crucial to the success of SLNB as an alternative to ALND. An accurate pathological assessment of SLN is necessary in order for the SLN to predict axillary lymph node status.

In a standard level III ALND, 20 or more lymph nodes are usually removed for pathological analysis. Conventionally, each lymph node is longitudinally bisected through its hilum and is examined with H&E staining at the level of the bisection.
However, this had been shown to underestimate lymph node metastases in several studies\textsuperscript{41}. This is particularly so for smaller metastatic lesions such as micrometastases, which were first defined by Huvos et al. as tumour deposits smaller than 2mm\textsuperscript{42}. In the Ludwig trial consisting of 921 patients, micrometastases were missed in 9\% of node-negative patients, which were associated with significantly worse disease-free survival and overall survival at 5 and 10 years\textsuperscript{43,44}.

With an average yield of 1 to 3 SLN, SLNB provides an opportunity for a detailed examination of nodal metastasis by making step sectioning and immunohistochemistry practical. Previous studies showed that the detection rate of metastasis increases by 9 to 47\% using these techniques\textsuperscript{45}. However, these techniques are too labour intensive and expensive for routine use in ALND specimens. In the Ludwig study\textsuperscript{43}, it took 12 sections per tissue block and 12 tissue blocks per patient to identify micrometastasis in 9\% of node-negative patients or 1600 slides to identify one additional node-positive patient. In a separate study, Wilkinson et al. found that an addition of 1449 slides was required to identify each additional node-positive patient\textsuperscript{46}.

Many studies have confirmed the upstaging of breast cancers on the basis of a more detailed pathological work-up for SLNs\textsuperscript{45}. However, there are considerable discrepancies in practice and varying opinions on the optimal histological protocol\textsuperscript{47}. These include the number of step sections, the step size, the roles of immunohistochemistry and molecular techniques, and intra-operative assessment. The main factor for these controversies is the significance of micrometastasis.
Micrometastasis is an arbitrary term first defined in the 1970s to describe any metastatic lesions less than 2mm\textsuperscript{42}. This ranges from a single cell or a small group of cells to tumour deposits. The advent of SLNB has led to more specific definitions. In the 6\textsuperscript{th} edition of the American Joint Committee on Cancer Staging Manual\textsuperscript{48}, micrometastasis is defined as a tumour deposit between 0.2 and 2 mm, which is usually seen on H&E staining. Such lymph nodes are designated as pN1mi, which is distinguishable from the traditional macrometastasis pN1. As for single cells or small groups of cells, the term "isolated tumour cells" (ITC) applies with the pN0(i+) designation. ITC are usually detected by immunohistochemistry but can be confirmed by H&E staining. The nature of ITC is controversial and some authors regard ITC as merely dislodged cells during surgery that are biologically meaningless. Although the Ludwig and other studies have demonstrated a poorer prognosis associated with micrometastasis, whether the same applies to ITC is uncertain. Prospective trials (IBCSG23-01, ACOSOG-Z0010 and NSABP-B32) are currently underway to determine the significance of micrometastasis and ITC. However, in a recently published case-control study, the axillary lymph nodes of 48 patients with stage 1 breast cancer who subsequently developed metastasis after 15 years of follow-up were re-examined and compared with the axillary lymph nodes from an age- and stage-matched group of patients, who did not develop metastasis. It was concluded that micrometastasis >0.2mm leads to a worse prognosis whilst the significance of ITC remained unclear\textsuperscript{49}. If ITC is found to be clinically significant, then a vigorous protocol for pathological examination may be required.
2.6.6 Intra-operative assessment

SLNB is a significant advancement in the management of breast cancer. At present, patients undergoing SLNB would require ALND if SLN is found to be positive for metastasis. This occurs at a rate between 25% and 40% of SLN biopsies\textsuperscript{45}. Because of the histological processing of the SLN, ALND has to be delayed as a second operation. This is inconvenient for patients, and may increase patients' anxiety whilst waiting for the outcome of SLN histology. In addition, readmission for ALND incurs additional resources on the health service, which may limit the widespread clinical application of SLN biopsy. Intra-operative assessment of SLN is a potential solution as it enables clinical decisions to be made on ALND at the time of SLNB.

Touch imprint cytology (TIC) and fresh frozen section (FFS) are the two main techniques that have been widely evaluated for this purpose. Other techniques such as scrape cytology and intra-operative serial sectioning have also been described.

TIC is an established cytological technique. It has been used for intra-operative assessment of lymph node for metastasis in a variety of malignant diseases and intra-operative assessment of excision margins of breast lesions. At UCLH\textsuperscript{50}, the technique for assessing SLN involves bisecting the SLN along its longitudinal axis and through the hilum and gently touching the sectioned surface onto glass slides. These can be separately stained with rapid cytological stain such as RAL555 (Cell Path, UK), MGG and anticytokeratin immunocytochemistry. Slides can then be examined by an experienced cytologist under light microscope (Figure 2.4).
TIC is simple and quick to perform. TIC provides clear cytological details for evaluation and avoids the consumption of tissue. However, potential pitfalls are the presence of large aggregates of lymphoid cells, which may mimic metastatic lesions, the presence of single malignant cell, which can be missed, and immunocytochemistry staining of follicular dendritic cells. 

Fresh frozen section is also an established pathological technique. FFS involves the rapid freezing of specimen followed by sectioning and staining. As with TIC, the SLN is initially bisected so that the sectioned surface is examined. Unlike TIC, the tissue removed for FFS is consumed and cannot be re-used for definitive histopathology. The limitations of FFS include freezing artefact with loss of good tissue architecture and cytological detail, difficulty in obtaining a good complete section and difficulty in identifying small foci of tumour especially in lobular carcinoma.

The drawbacks for intra-operative assessment of SLN include (1) variation in sensitivity of TIC and FFS, (2) delays whilst waiting for the results, (3) the need for an experienced pathologist, (4) pressure for the pathologist to provide a definitive report within a limited time frame and (5) patient anxiety resulting from false results.

2.6.7 Pathological protocol at UCLH

The protocol for pathological assessment of SLN at UCLH is:

1. Bisection of SLN along its longitudinal axis and through the hilum
2. TIC obtained from the sectioned surface: 3 slides with 3 imprints per slide. Staining using RAL555 (Cell Path, UK), MGG stain and anticytokeratin immunocytochemistry
3. SLN individually labelled and fixed in formalin
4. Standard H&E sections at 3 levels
5. IHC performed if the H&E sections show no evidence of metastasis.
2.7 Summary

Surgical management of breast cancer has evolved significantly over the past few decades. For the primary tumour, radical surgery alone has given way to breast conservation with radiotherapy in clinically appropriate cases. For the axilla, the trend is towards less radical axillary dissection such as ALNS and SLNB. The overall aim is to minimize the extent of surgery thereby reducing the morbidity and disfigurement without compromising clinical outcome. With the exception of SLNB, landmark randomized clinical trials with long-term follow-up have supported such advances.

To facilitate widespread adoption, such advances must have a positive impact on resource utilization as well as clinical and patient benefits. For breast conservation surgery, post-operative radiotherapy is required, but further demands on resources are made by positive resection margins, which may require readmission for re-excision of margins. Likewise for SLNB, additional resources such as radioisotopes, gamma-probe detectors and nuclear medicine facilities are required. Readmission may be required for the completion of ALND or axillary radiotherapy in the event of a positive SLN. In these situations, readmissions increase the demands on resources and create inconveniences and anxiety for the patients. If re-operation is to be avoided, intra-operative margin and SLN assessments are necessary. This can be provided by TIC and FFS, but this requires additional specialist histopathology support.

Reference List


Chapter 3 Elastic Scattering Spectroscopy

3.1 Introduction

Elastic scattering spectroscopy (ESS) provides information on the elastic light scattering and light absorption properties of matter in the form of a spectrum. Recently, there has been significant interest in its potential applications in biomedicine. These include the detection and diagnosis of tissue pathologies, non-invasive drug concentration monitoring, monitoring tissue-engineered constructs and early repair in collagenous tissues. This is because optical properties of tissue can change along with structural changes to tissues brought about by disease processes, trauma and healing. Such changes are often predictable and detectable by ESS.

ESS belongs to a group of technologies known as optical biopsy. Optical biopsy exploits the various interactions between light and tissue (1) to study the mechanical or biochemical structures of tissue, (2) to detect and to diagnose tissue pathology, and/or (3) to image tissue structures. There are numerous types of optical biopsy such as Raman spectroscopy, fluorescence spectroscopy or imaging, and optical coherence tomography, which have been evaluated clinically. However, these are beyond the scope of this thesis. The aim of this chapter is to discuss the physical basis of ESS, the design principles of ESS system, spectral analysis and results of clinical studies.
3.2 Physical Basis of ESS

Several optical processes take place when light interacts with biological tissues. In combination, these interactions enable biological tissues to have different physical appearances to the human eye. At the basic level, reflection, propagation and transmission occur (Figure 3.1). When a beam of light is incident upon the tissue surface, some of the light is reflected. The remainder enters and propagates through the tissue medium. If enough light propagates through to the back surface, light can be transmitted through or be reflected by the back surface.

Figure 3.1: The reflection, propagation and transmission of light

![Diagram showing reflection, propagation, and transmission of light](image)

1 Reflection
2 Propagation
3 Transmission

It is during the propagation phase that most of the optical interactions take place. The principal interactions are elastic scattering and absorption. Other interactions such as Raman scattering and fluorescence also occur but these effects are considerably weaker in comparison. Optical biopsy technologies can be classified according to the type of optical interaction (Table 3.1).
Table 3.1: Categories of optical biopsy technologies

<table>
<thead>
<tr>
<th>Optical Interaction</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescence</td>
<td>Steady-state fluorescence spectroscopy</td>
</tr>
<tr>
<td></td>
<td>Time gated fluorescence spectroscopy</td>
</tr>
<tr>
<td></td>
<td>Auto-fluorescence imaging</td>
</tr>
<tr>
<td></td>
<td>Enhanced-Fluorescence imaging/Photodynamic diagnosis</td>
</tr>
<tr>
<td>Raman scattering</td>
<td>Raman spectroscopy</td>
</tr>
<tr>
<td>Elastic scattering</td>
<td>Elastic scattering/Diffuse reflectance spectroscopy</td>
</tr>
<tr>
<td></td>
<td>Polarised light scattering spectroscopy</td>
</tr>
<tr>
<td>Optical coherence</td>
<td>Optical coherence tomography</td>
</tr>
</tbody>
</table>

3.2.1 The nature of light

Light is a form of electromagnetic radiation characterized by its dual nature as a wave and a particle. Electromagnetic radiation transfers energy in discrete ‘packs’, known as photons. Each photon has a specific frequency of oscillation ($\nu$) and a specific wavelength ($\lambda$). The energy ($E$) of a photon is proportional to its frequency ($\nu$) by the equation $E = \nu \cdot h$, where $h$ is Plank’s constant.

The electromagnetic spectrum classifies photons according to their frequency. Electromagnetic radiation between the wavelength ranges of 400 to 700 nm is visible to the human eye as light. This is often referred as the visible spectrum and ranges from infrared to ultraviolet (Figure 3.2).
3.2.2 Light absorption

Absorption is a major optical process, which determines the colour and transparency of biological tissues. It occurs at atomic and molecular levels, where light is captured and converted into internal energy by an absorber (chromophore). The process involves the transition of the chromophore from a lower energy state to a higher energy state, which is quantized. Absorption is governed by the Beer-Lambert Law, which states a linear relationship between absorbance and the concentration of the chromophore.

Each chromophore has a specific absorption profile (extinction coefficient), which describes the level of absorption at different wavelengths. The major chromophores in
biological tissue are haemoglobin, melanin, deoxyribonucleic acid (DNA), cytochrome c oxidase and β-carotene.

Water is an absorber of electromagnetic radiation below 200nm and above 900nm wavelengths. Therefore, it has very little effect on the visible spectrum thus allowing the propagation of light through biological tissue.

Haemoglobin is one of the most important chromophores in human tissue because of its omnipresence and its high absorptive properties. In the human body, haemoglobin exists in two main forms: deoxygenated (Hb) and oxygenated (HbO_2). Each has a characteristic extinction coefficient (Figure 3.3). The absorptive properties derive from the haem protein, which has two characteristic bands. The main band is in the blue region (390-450nm) of the visible spectrum (black arrow), and is known as the Soret band. The other lies in the 450 to 700 nm range and is called the "visible" or "alpha" and "beta" band(s) (white arrow). The Soret band is typically 5 to 10 times more intense than the visible bands. The Soret band of HbO_2 appears at 415 nm (black arrow, red spectra) and the alpha and beta bands appear at 577 nm and 542 nm respectively (white arrow, red spectra). For Hb, the Soret band is a red shifted to 431nm (black arrow, blue spectra), and a single broad band at 555nm replaces the alpha and beta bands (white arrow, blue spectra). In addition, Hb has a much weaker but characteristic absorption peak at 760 nm (dotted arrow).
DNA absorbs in the ultraviolet range between 280 and 320nm, which can induce structural changes in DNA resulting in carcinogenesis e.g. malignant melanoma and other skin cancers. The design of ESS systems must take this into account. In addition, DNA has scattering properties in the visible spectrum.

Other chromophores such as melanin and β-carotene are present in specific parts of the body such as skin and fat (Figure 3.4) respectively. These must be taken into account in the analysis of ESS data.
3.2.3 Elastic scattering

Scattering is an optical process, which causes light to change its direction of propagation. There are several different scattering processes such as refraction, Rayleigh scattering, Raman scattering and Elastic scattering. These are different processes occurring at different levels e.g. subatomic, molecular and cellular levels. Not all scattering processes take place in biological tissue. The scattering processes in biological tissues are elastic scattering and Raman scattering.

Elastic scattering is the predominant scattering process in tissue. It is termed elastic because the frequencies of the scattered photons are the same as the incident photons. Elastic scattering occurs when light interacts with spherical bodies such as cells and
An important characteristic of elastic scattering is the angular distribution of the scattered light also known as the angular effect. Differences in the light scattering properties of normal and malignant cells are attributed to a change in the average size of the scattering centres.

The angular distribution can be described by Mie theory, which consists of a series of complex formulae. The angular distribution is dependent on the wavelength of the photon and size of scattering particle. In general, a tissue component whose size is small compared to the wavelength will scatter light more isotropically than larger components, and in biological tissue, elastic scattering is highly anisotropic. The physics of light scattering in biological tissue is complex and is beyond the context of this thesis.

When light propagates through biological tissue, it is subjected to multiple scattering events. Therefore, the data acquired do not provide information as to the individual scattering events but rather indicate the overall scattering properties of the tissue, which is the effective scattering coefficient. This coefficient is proportional to the density of the scattering particles.

Raman scattering is a complex phenomenon occurring at molecular level. It is an inelastic scattering process, because the scattered photons have different frequencies from the incident photons. Several Raman scattering systems are currently under evaluation for their abilities to diagnose tissue pathology including breast cancer.
3.3 The Elastic Scattering Spectroscopy System

3.3.1 Key features

The ESS system at NMLC consists of a pulsed xenon lamp, an optical probe, a charged couple device (CCD)-spectrometer and a laptop personal computer for system control and spectral record. Figure 3.5 illustrates the schematic diagram of the ESS system. The design of the ESS system reflects on the four basic processes of optical biopsy: (1) light delivery to tissue, (2) light-tissue interaction, (3) light collection from tissue and (4) display of collected data.

Figure 3.5: Schematic diagram of the ESS system
The operating manual of ESS system is enclosed in Appendix 1 of this thesis. Spectral acquisition is full automated upon activation. It initiates by acquiring a “dark spectrum” without triggering the xenon lamp. This is followed by repeated pulses of light from the xenon lamp until a threshold of light intensity is reached at the spectrometer. The “dark spectrum” is subtracted from the spectrum recorded to provide an ESS spectrograph. The entire process takes approximately one second. Table 3.2 illustrates the key features of the ESS system.

**Table 3.2: Key features of ESS**

<table>
<thead>
<tr>
<th>Key Features of ESS</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESS system is portable and is easy to set up</td>
</tr>
<tr>
<td>Components are widely available and relatively inexpensive</td>
</tr>
<tr>
<td>Spectral acquisition takes approximately one second</td>
</tr>
<tr>
<td>ESS system is safe and does not involve ionised radiation or laser (see Light Source)</td>
</tr>
<tr>
<td>Optical probes can be supplied sterilised for intra-operative use (see Optical Probe)</td>
</tr>
<tr>
<td>ESS permits in-vivo and ex-vivo spectral acquisition</td>
</tr>
<tr>
<td>Software is currently under development to analyse spectrographs. No expert interpretation is needed (See Spectral Analysis)</td>
</tr>
</tbody>
</table>

### 3.3.2 Light source

The light source is a pulsed xenon arc lamp (Perkin Elmer, Inc.). It produces short pulses (approximately 1μs) of broad spectrum light. Because of potential health hazard, ultraviolet B (280-315nm) and C (100-280nm) light have been filtered and the
light output of system ranges between 320-920nm. The spectral output taken against a white reflectance standard (Spectralon™, Labsphere, Inc., North Sutton, New Hampshire) is illustrated in Figure 3.6.

Figure 3.6: Spectral characteristics of xenon arc lamp against a white reflectance standard

Prior to operation, the ESS system is referenced against Spectralon™, which is spectrally flat between 250-1000nm, thereby allowing spectral variations in the light source, spectrometer, fibre transmission and fibre coupling to be accounted for.

The main considerations regarding the light source of the ESS system are: (1) the type of light source and (2) the safety aspects. Many types of light sources such as light emitting diodes, fluorescent bulbs and filament lamps are commercially available and can be used for optical biopsy. The choice of a pulsed xenon arc lamp for our ESS system is because of the requirement for the production of multiple pulses of stable broadband light in millisecond duration at high repetition rates.
As regards to safety, the health hazard associated with ultraviolet B (280-315nm) and C (100-280nm) light and the thermal effect of light must be appreciated. In our ESS system, both ultraviolet B and C are filtered. In our experience, there has been no discomfort or pain felt by subjects during in-vivo use and no histological evidence of tissue damage.

3.3.3 Optical probe

The optical probe (Figure 3.7a) consists of two parallel flexible optical fibres, the illuminator fibre and collection fibre, which are housed in a metal casing for the ease of handling. The illuminator fibre is connected to the pulsed xenon lamp and delivers light to the tissue specimen upon activation of the ESS system. Following optical interactions, a portion of scattered light enters the collection fibre, and is delivered to the CCD-spectrometer. For ESS acquisition, the optical probe has to be in direct physical contact with the tissue specimen. Figure 3.7b is a schematic illustration of this process.

Figure 3.7(a & b): Optical probe
Connections of the proximal ends of the optical fibres to the pulsed xenon lamp and CCD-spectrometer are by SMA connectors. The illuminator fibre is 400\(\mu\)m in diameter, and the collection fibre is 200\(\mu\)m in diameter. The centre-to-centre separation distance between the two fibres is 350\(\mu\)m. For this probe geometry, the volume of tissue investigated is approximately 500 \(\mu\)m long, 300\(\mu\)m wide and 300\(\mu\)m deep. This was determined using Monte-Carlo simulation with Mie theory incorporated for the details of the scattering events\(^25\).

The separation of the fibres has a profound bearing on the character of the received signal\(^26\). For narrow separation, the ESS spectrum depends mainly on the scattering properties of tissue although some absorptive features also influence the signal\(^26\). With a wider separation distance, the ESS spectrum is predominantly influenced by absorption. The 350\(\mu\)m separation distance is an engineering compromise for optimal performance\(^26\).

### 3.3.4 Tissue specimen

The advantage of ESS is that ESS acquisitions can be made from in-vivo and ex-vivo tissues by simply placing the optical probe directly onto the tissue and without the need of tissue preparation i.e. fixation, staining and freezing. The optical probe can be sterilized by ethylene oxide for on-table use during surgical operations. Hence, the potential applications of ESS are vast.

As an emerging technology, technical information concerning the effects of tissue handling on ESS is not available. However, this may have potential significance, for
example, in situations where ex-vivo ESS data are used for in-vivo applications and visa versa. Other variables such as time between surgical excision and ESS acquisition, the effects of temperature, tissue hydration and tissue fixation may have significant impact on the optical properties of tissues. At present, there are only a few published studies into the effects of tissue handling on optical biopsy. These include studies on non-human tissues and other optical biopsy technologies apart from ESS\textsuperscript{13,27-30}.

For example, fluorescence intensity in fresh mouse skeletal tissue increases over a period of days with tissue dehydration and with formaldehyde fixation\textsuperscript{30}. Raman spectroscopy of human bronchial tissues is significantly affected by tissue dehydration and formaldehyde fixation\textsuperscript{27}. In contrast, formaldehyde fixation does not seem to induce significant changes in Raman spectra of human breast tissue\textsuperscript{13}.

### 3.3.5 CCD-Spectrometer

CCD-spectrometer (S2000 Ocean Optics) produces a spectrograph of the light entering the collection fibre from tissue following elastic scattering. A CCD-spectrometer is used because of its high sensitivity for measurement at low light intensities and its short data collection time. The alternative is a photomultiplier tube, which is more sensitive at low-intensity but has a longer data collection time. CCD is most commonly used in optical biopsy. The information is transferred to laptop computer, where it is displayed in the form of a spectrograph.
3.4 Elastic Scattering Spectrograph

Figure 3.8 illustrates typical ESS spectra of normal breast tissue, breast fibroadenoma and breast cancer acquired using the ESS system. The X-axis is wavelength of light from 320nm to 820nm. The Y-axis is the light intensity. Each spectrograph contains 1801 data points. Individually, each data point and its absolute intensity value are of no significance. It is the overall patterns generated by the data points that are of significance.

An ESS spectrograph is characterised by troughs and linear gradients. The troughs are absorption bands of chromophores. The most prominent are those of haemoglobin (Soret, alpha and beta bands), which are clearly demonstrated in the tumour spectrum (red) above. The other feature of spectrographs is the linear gradients such as those at 345-375nm and 620-820nm, which are attributed to elastic light scattering.

Figure 3.8: Typical optical spectra taken from normal breast, breast fibroadenoma and breast cancer
3.5  Spectral Analysis

The ESS spectrographs illustrated in Figure 3.8 are typical examples. However, there are wide variations in the spectrographic patterns within different breast tissue types. Visual inspection of ESS spectra is sub-optimal for spectral interpretation and also time consuming especially when a large number of ESS spectra is involved. A system for spectral analysis would be advantageous for the following reasons:

- Improving speed of spectral analysis,
- No requirements for training to interpret ESS spectra
- No human factor/error involved in misinterpretation

The technique used for ESS spectral analysis at NMLC is Model Based Analysis (MBA), which was developed in-house as there are no specifically designed and commercially available software. In addition to MBA, hierarchical cluster analysis and artificial neural networks were also studied. MBA is a two-staged process consisting of Pre-processing and Linear Discriminant Analysis (LDA).

An ESS spectrum consists of 1801 data points, which together with its absolute intensity value are of no significance. It is the overall patterns generated by the data points that are significant. The role of pre-process is to reduce the dimensionality into various coefficients (or eigenvalues) suitable LDA.

First, ESS spectra underwent “smoothing”, in which each data point was replaced by the average of the 20 neighbouring data points thereby reducing 1801 data points to 180 intensity values. Each “smoothed” spectrum was subsequently standardised by
subtracting to the mean intensity of the spectrum (i.e. the average intensity over the full spectral range) from each data point and then divided by the standard deviation of the smoothed spectrum. This gave all the spectra a mean intensity of zero and a standard deviation equal to 1. The standardisation process enabled each spectrum to have relative intensities rather than absolute values allowing spectral comparison and further processing. Once standardised, principal component analysis (PCA) was applied to the spectra to reduce the data to only those regions with large variability, which are referred as principal components. This was carried out using the statistical package, Systat 9.0 (Systat Software Inc., Richmond, California, USA).

The second stage, LDA, used all the principal components to develop an algorithm for discrimination between tissue pathologies (also using Systat 9.0). This was done by calculating a set of weights that specify a hyperplane that splits the data into groups such as cancerous and normal breast tissues and selecting a set of 6-10 principal components to form a LDA algorithm.

As spectral analysis is under development, clinical studies in this thesis were validated using 2 analysis techniques: bootstrapping and jackknife. In the bootstrap analysis, 50% of ESS spectra with corresponding pathology were randomly selected as the “training set” to train and develop a LDA algorithm. The LDA algorithm was then used to test the remaining spectra (the testing set) and the results were compared with the corresponding reported pathology. This process was repeated 1000 times with re-sampling of training and testing sets each time. The average sensitivity and specificity of spectral analysis is reported with standard variance. Bootstrapping provides a reliable test of our spectral analysis technique: it reduces the potential
effects of a stochastic selection of training and testing sets, which would favour either exceptionally good or poor sensitivity and specificity.

A jackknife analysis is essentially a “leave one out” analysis, in which one spectrum was used for testing whilst the remainder were used for training. Analysis is repeated until each spectrum has been tested. Therefore, the sensitivity and specificity of the spectral analysis are absolute.

Other techniques for spectral analysis are available and have been evaluated by other institutions on different tissue types. These include mathematic models to calculate specific parameters such as haemoglobin concentration and saturation, scattering parameters\textsuperscript{16}, fluorescence indices\textsuperscript{9,33} and nuclear sizes\textsuperscript{9,33}. Spectral analysis in general is in the early stages of development. Many of the systems have been developed in-house and therefore not available for multi-institutional scrutiny. In addition, there is no consensus on the optimal techniques or comparative studies on different techniques.

3.6 Review of Clinical Data

Currently, only a limited number of clinical studies on optical biopsy have been published. These are illustrated in Table 3.3 together with their respective sensitivity and specificity. It is important to appreciate that most of these studies are only “proof of concept” as they are not prospective studies comparing against established standards such as histology, cytology and frozen section\textsuperscript{4}. The sensitivities and specificities provide a general guide and reference demonstrating the potential of these emerging technologies. Direct comparisons between these studies should be
avoided because of the differences in hardware, spectral analysis, different organ and diseases being studied, specimen handling and protocol in histological correlation. However, these sensitivities and specificities are acceptable and promising. Further developments and evaluations of optical biopsy technologies are merited.

Table 3.3: Published clinical results of optical biopsy

<table>
<thead>
<tr>
<th>Study</th>
<th>Type of optical biopsy</th>
<th>Organ studied</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Patient Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bigio$^{31}$</td>
<td>ESS</td>
<td>Breast cancer*</td>
<td>69%</td>
<td>85%</td>
<td>31 (72 tissue sites)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sentinel nodes (Breast)*</td>
<td>58%</td>
<td>93%</td>
<td>31 (54 tissue sites)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Breast cancer**</td>
<td>67%</td>
<td>79%</td>
<td>31 (72 tissue sites)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sentinel Node (Breast)**</td>
<td>91%</td>
<td>77%</td>
<td>31 (54 tissue sites)</td>
</tr>
<tr>
<td>Jerjes$^2$</td>
<td>ESS</td>
<td>Oral cancer (Excision margins)</td>
<td>87%</td>
<td>80%</td>
<td>21</td>
</tr>
<tr>
<td>Jerjes$^1$</td>
<td>ESS</td>
<td>(Lymph nodes)</td>
<td>98%</td>
<td>68%</td>
<td>13 (130 lymph nodes)</td>
</tr>
<tr>
<td>Johnson$^3$</td>
<td>ESS</td>
<td>Sentinel Node (Breast)</td>
<td>84%</td>
<td>91%</td>
<td>68 (169 lymph nodes)</td>
</tr>
<tr>
<td>Lovat$^4$</td>
<td>ESS</td>
<td>Barrett’s oesophagus</td>
<td>75%</td>
<td>80%</td>
<td>41 patients (71 tissue sites)</td>
</tr>
<tr>
<td>Mourant$^5$</td>
<td>ESS</td>
<td>Bladder cancer</td>
<td>100%</td>
<td>97%</td>
<td>10</td>
</tr>
<tr>
<td>Muller$^{34}$</td>
<td>F, RS and ESS</td>
<td>Oral cancer</td>
<td>96%</td>
<td>96%</td>
<td>15 (91 tissue sites)</td>
</tr>
<tr>
<td>Panjehpour$^{16}$</td>
<td>F</td>
<td>Barrett’s oesophagus</td>
<td>90%</td>
<td>96%</td>
<td>36</td>
</tr>
<tr>
<td>Scarisbrick$^6$</td>
<td>ESS</td>
<td>Malignant melanoma</td>
<td>100%</td>
<td>77%</td>
<td>77 (100 lesions)</td>
</tr>
<tr>
<td>Stone$^{14}$</td>
<td>Raman</td>
<td>Epithelial cancer</td>
<td>&gt;90%</td>
<td>&gt;90%</td>
<td>Not stated</td>
</tr>
<tr>
<td>Utzinger$^7$</td>
<td>RS</td>
<td>Ovarian tissue (Malignant)</td>
<td>86%</td>
<td>79%</td>
<td>16 (72 tissue sites)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ovarian tissue (Benign)</td>
<td>86%</td>
<td>80%</td>
<td></td>
</tr>
<tr>
<td>Wallace$^8$</td>
<td>ESS</td>
<td>Barrett’s oesophagus</td>
<td>90%</td>
<td>90%</td>
<td>13 (76 tissue sites)</td>
</tr>
<tr>
<td>Yamasaki$^{15}$</td>
<td>Raman</td>
<td>Lung cancer</td>
<td>91%</td>
<td>97%</td>
<td>Not stated (210 tissue sites)</td>
</tr>
</tbody>
</table>

ESS: Elastic Scattering Spectroscopy; F: Florescence; RS: Reflectance Spectroscopy
* using Artificial Neural Network, ** using Hierarchical Cluster Analysis
3.7 Potential Applications of ESS in the Management of Breast Cancer

ESS is an emerging technology with potential widespread clinical applications because of its ability to detect and diagnose tissue pathology and its key features (Table 3.2). Its eventual success will depend on the adoption of this technology by clinicians and acceptance by patients. Therefore, it is important for ESS to complement and improve existing practices without complexity. Chapters 1 and 2 reviewed the current diagnostic techniques and surgical management of breast cancer, and provided certain clinical situations where ESS can be applied. These include percutaneous diagnosis of breast cancer, ESS guided breast core biopsy, intra-operative assessment of SLN and tumour excision margin.

3.8 Summary

ESS and other optical biopsy technologies are exciting developments in biomedicine with potential clinical and scientific applications. They exploit the interactions between light and tissue, and the change in optical properties of tissue with disease processes. Various systems are specifically designed to suit the particular optical interaction studied. As an emerging technology, technical information such as the effect of tissue handling is not available. These issues have to be considered.

The ESS system at NMLC is designed as a portable system for in-vivo and ex-vivo detection and diagnosis of tissue pathologies in particular malignant and pre-malignant conditions. The system involves broad spectrum light with harmful ultraviolet B and C light filtered, and is considered safe. Other advantages include the relatively low cost compared with existing technologies such as US, MRI and PET.
The ultimate goal at NMLC is to produce an expert system, which is capable of analysing ESS spectra in real-time without the need of human interpretation. The technique used for spectral analysis is MBA, which involves the pre-processing of data and subsequent LDA, and is currently under development.

Reference List


Chapter 4  Aims of Thesis

Elastic scattering spectroscopy (ESS) is an emerging technology with potential widespread clinical applications. In the management of breast cancer, potential applications include percutaneous diagnosis of breast cancer, ESS-guided breast biopsy, intra-operative assessment of sentinel lymph nodes (SLN) for metastasis and tumour resection margins. The ultimate goal at the National Medical Laser Centre is to produce an expert system, which is capable of analysing ESS spectra in real-time without the need of human interpretation. This is considered as one of the critical success factors for its eventual success. The current stage of development is centered on the development of the spectral analysis system, which has direct influence on the sensitivity and specificity of ESS. The thesis of Dr Gavin Briggs identified Model Based Analysis as a suitable technique for spectral analysis. However, further validation and improvements are necessary. Other aspects, such as different proportions of normal and malignant breast tissue within the field of study, may have significant influence on the sensitivity and specificity of ESS, but have yet to be determined. In addition, other technical issues, such as the effects of ambient lighting, tissue handling and formaldehyde fixation, may have important but unknown effects.

The aims of thesis are:

1. To investigate the effects of ambient lighting, tissue handling and formaldehyde fixation on ESS
2. To improve the sensitivity and specificity of ESS in determining the axillary lymph node status in breast cancer
3. To undertake a comparative study of ESS and touch imprint cytology in assessing SLN status in breast cancer

4. To determine the effect of different proportions of normal and malignant breast tissue within the field of study on the sensitivity and specificity of ESS, and to evaluate the impact of this, if any.
Chapter 5: The Effects of Ambient Lighting, Tissue Handling and Formaldehyde Fixation on Elastic Scattering Spectroscopy

5.1 Introduction
ESS is capable of detecting pre-malignant and malignant changes in human tissues. The ultimate goal is to develop an expert system, which would analyse the spectra in real time. Therefore, it is necessary to acquire a large volume of spectra both for the development and the testing of MBA. Although all ESS spectra used in this thesis were acquired from freshly excised specimens, this may not always be possible, and spectral acquisition may take place after a significant period of time. Therefore tissue specimens need to be handled appropriately to prevent autolysis. Current standard practice for handling tissue specimens includes immediate fixation in 4% formaldehyde solution, storage in chilled conditions with ice and submersion in normal saline. The effects of these handling techniques on ESS are unknown. However, a few publications have demonstrated that tissue handling can have effects on Raman, fluorescence and diffuse reflectance spectroscopy. The aim of this chapter is to investigate these effects on ESS in axillary lymph nodes. In addition, this chapter will evaluate the effect of ambient lighting on ESS. Axillary lymph nodes were specifically chosen because a major theme of this thesis is the clinical application of ESS in detecting lymph node metastases in breast cancer (Chapter 6).

5.2 The Effects of Ambient Lighting on ESS

5.2.1 Introduction
The ESS system at NMLC is a portable system, which can be used in various settings. These include surgical operating theatres, laboratories, clinics and even the outdoors.
The ambient light intensities of these environments vary significantly from approximately 700lux in a normal room to 20,000lux under a standard operating lamp. As spectral acquisition involves the collection of scattered light from the tissue, ambient light could theoretically enter the tissue, undergo scattering and absorption, and be collected by the ESS system (Figure 5.1). This may contaminate ESS spectra and ultimately affect spectral analysis. The aim of this study is to investigate the effect of ambient lighting on ESS spectra.

Figure 5.1: Illustration to demonstrate potential contamination of ESS by ambient light

Collection fibre
Illuminator fibre

Light from xenon lamp
Ambient light
5.2.2 Methods

Axillary lymph nodes were harvested from surgical specimens of patients with proven breast cancer undergoing axillary surgery following informed consent. The lymph nodes were bisected along their longitudinal axes. The optical probe was applied to the sectioned surface and was fixed in position using a clamp during the study. Two ESS spectra were acquired at different ambient light intensities from 1lux (total darkness) to 20,000lux. All lymph nodes were fixed in 4% formaldehyde solution and sent for pathology at the end of the study.

ESS spectra were visually inspected. For comparison, ESS spectra at various ambient light intensities were normalised to the spectra taken in total darkness by division. The number of xenon lamp pulses per spectrum (Xe-PPS) was also recorded.

5.2.3 Results

In total, 3 sets of measurements were made from 3 normal axillary lymph nodes. A selection of ESS spectra from all 3 lymph nodes is illustrated in 3 ambient light intensity ranges: 1-100lux, 100-1000lux and 1000-20000lux in Figure 5.2.
Figure 5.2: ESS spectra acquired at various ambient light intensities (a) between 1-100 lux, (b) between 100-1000 lux and (c) between 1000-20000 lux

(a) Lymph Node 1 (1-100 lux)

(b) Lymph Node 2 (1-100 lux)

(c) Lymph Node 3 (1-100 lux)
On visual inspection, there are minor changes in the spectra of all 3 lymph nodes between 100 and 1000 lux. These are mainly differences in specific intensities within the
On visual inspection, there are minor changes in the spectra of all 3 lymph node between 1 and 1000lux. These are mainly differences in spectral intensities whilst the
overall spectral profiles (shape) remain unchanged. As the ambient light intensity increases from 1000lux, there is a decreasing trend in the overall spectral intensities (i.e. downward shift of spectra) in all 3 lymph nodes. The spectral profiles also appear to flatten particularly in the Soret, alpha and beta bands, which are the strong absorption bands of oxygenated haemoglobin at 415nm, 577nm and 542nm wavelengths respectively (See Section 3.2.2). However if the scale of spectral intensity is adjusted (Figure 5.3), the spectral profiles at 20000lux remained similar to those at 1lux between 320 and 620nm.

Figure 5.3: ESS spectra acquired at 1lux and 20000lux ambient light intensities in separate intensity scales
From the practical point of view, difficulties in acquiring satisfactory spectra were encountered at 20,000 lux. Several spectra had to be discarded (Figure 5.4).

**Figure 5.4:** *Examples of discarded spectra acquired at 20,000 lux*

Further comparisons between two given spectra can be made by normalising one against the other. This is carried out by dividing the spectral intensities of one spectrum by the corresponding spectral intensities of the other. An increase in spectral intensity at a particular wavelength in the former would result in a normalised...
intensity value greater than 1, and visa versa. A normalised intensity value of 1 indicates no change. Figure 5.5 illustrates normalised spectra of Lymph Nodes 1, 2 and 3 at various ambient light intensities, which were normalised to their respective spectrum at 1lux.

In all 3 lymph nodes, a sequence of changes can be seen in the Soret, alpha and beta bands even at low ambient light intensities. The sequence begins with an increase in normalised intensity in the Soret band followed by increases in normalised intensities in the alpha and beta bands. The sequence proceeds with a gradual resolution of the raised normalised intensities. The sequence occurs at different ambient light intensities in the 3 lymph nodes.

Otherwise, there are no significant changes in spectral profile between 620 and 920nm. It is remarkable that the normalised spectra of all 3 lymph nodes at 10,000 and 20,000 lux have a relatively horizontal trend between 620 and 920nm.
Figure 5.5: Normalised spectra of Lymph Nodes 1, 2 and 3 at various ambient light intensities (a) between 10-100lux, (b) between 100-1000lux and (c) between 1000-20000lux normalised to their respective spectrum at 1lux

(a)

Lymph Node 1 (10-100 lux)

Lymph Node 2 (10-100 lux)

Lymph Node 3 (10-100 lux)
Lymph Node 1 (1000-20000 lux)

![Graph showing normalized intensity vs wavelength for different lux levels for Lymph Node 1.](c)

Lymph Node 2 (1000-20000 lux)

![Graph showing normalized intensity vs wavelength for different lux levels for Lymph Node 2.](c)

Lymph Node 3 (1000-20000 lux)

![Graph showing normalized intensity vs wavelength for different lux levels for Lymph Node 3.](c)
The number of Xe-PPS at different ambient light intensities is illustrated in Table 5.1. This is determined by the threshold of light intensity being reached at the CCD-spectrometer during spectral acquisition, which in turn deactivates the xenon lamp (see Section 3.3.1). A reduction in the number of Xe-PPS suggests either more photons from the Xenon lamp or photons from an external source, i.e. ambient light, are being collected by the collecting fibre of the optical probe and delivered to the CCD-spectrometer. In general, the number of Xe-PPS remained the same at lower ambient light intensity ($\leq 1000$ lux), and reduced in all 3 lymph nodes with increases in ambient light intensity.

Table 5.1: Number of Xe-PPS of Lymph Nodes 1-3 at various ambient light intensities

<table>
<thead>
<tr>
<th>Ambient light intensity (lux)</th>
<th>Lymph node 1</th>
<th>Lymph node 2</th>
<th>Lymph node 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17</td>
<td>41</td>
<td>20</td>
</tr>
<tr>
<td>100</td>
<td>17</td>
<td>41</td>
<td>20</td>
</tr>
<tr>
<td>500</td>
<td>17</td>
<td>41</td>
<td>20</td>
</tr>
<tr>
<td>1000</td>
<td>17</td>
<td>41</td>
<td>20</td>
</tr>
<tr>
<td>2000</td>
<td>17</td>
<td>31</td>
<td>20</td>
</tr>
<tr>
<td>3000</td>
<td>17</td>
<td>31</td>
<td>15</td>
</tr>
<tr>
<td>4000</td>
<td>17</td>
<td>31</td>
<td>15</td>
</tr>
<tr>
<td>5000</td>
<td>17</td>
<td>23</td>
<td>15</td>
</tr>
<tr>
<td>8000</td>
<td>13</td>
<td>23</td>
<td>11</td>
</tr>
<tr>
<td>10000</td>
<td>9</td>
<td>15</td>
<td>11</td>
</tr>
<tr>
<td>20000</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
</tbody>
</table>
5.2.4 Discussion

The findings of this study are as follow: (1) the overall spectral profile remained stable with minor changes at lower ambient light intensities (≤1000lux), (2) there was a gradual reduction of overall spectral intensity and flattening of spectral profile as the ambient light intensity increased above 1000lux, (3) spectral profiles at higher ambient light intensities were similar to those acquired in darkness after re-scaling, (4) normalised spectra demonstrated a sequence of changes that occurred in the Soret, alpha and beta bands, and (5) Xe-PPS reduced as the ambient light intensity increased.

In order to analyse these findings, it is important to appreciate the process of spectral acquisition. Spectral acquisition initiates with a background measurement from the tissue without triggering the xenon lamp. This is followed immediately by ESS measurement with the pulses of light from the xenon lamp until a threshold of light intensity is reached at the spectrometer. ESS spectrum is generated by subtracting the background measurement from the ESS measurement.

As the xenon lamp is controlled by the spectrometer, reductions in Xe-PPS at high ambient light intensities indicate that ambient photons entered the detector fibre of the optical probe via the lymph node, and were recorded by the spectrometer. Subsequently, less photons from the xenon lamp were needed to reach the light intensity threshold of the spectrometer; hence the reduction in Xe-PPS (Observation 5). However, the level of ambient light intensity, at which Xe-PPS decreases, differed in all 3 lymph nodes, likewise the degree of Xe-PPS reduction. These may reflect the individual differences in optical properties of the lymph nodes in particular the surface of the lymph node.
At lower ambient light intensities ($\leq 1000$ lux), Xe-PPS was unchanged in all 3 lymph nodes. In addition, spectral profiles only showed minor changes. These suggest that the ambient lighting has little effect on ESS spectra at intensities below 1000 lux, which is typical of normal indoor conditions such as laboratories and clinics.

ESS spectra are generated by subtracting the background measurement from the ESS measurement. The difference between the background and ESS measurements is greatest when ESS spectra are acquired in total darkness. As the ambient light intensity increases, the difference decreases due to the contribution of ambient photons to the background measurement and the reduction of Xe-PPS. This explains the gradual reduction of overall spectral intensity and the flattening of spectral profile as ambient light intensities increase (Observation 2).

By subtracting the background measurement from the ESS measurement, the ambient light is accounted for in the ESS spectra. The essential difference between ESS spectra acquired at low and high ambient light intensities is that more photons from the xenon lamp were used in the former. This provides an explanation for the similarities in spectral profiles of the spectra taken at 1 and 20,000 lux after re-scaling (Observation 3).

Normalised spectra provide comparisons between ESS spectra at various ambient light intensities to that at 1 lux. The most obvious changes in the normalised spectra occurred in the Soret, alpha and beta bands, which are the absorption bands of haemoglobin (Observation 4). As absorption is governed by the Beer-Lambert law,
which states that there is a linear relationship between absorbance and the concentration of the chromophore, the changes in the normalised spectra cannot be explained by the changes in the ambient light intensity since the concentration of haemoglobin remained constant. Possible explanation would be the change in oxygenation status of haemoglobin during this study (see Section 5.3).

Apart from the above changes, the normalised spectra were relatively horizontal indicating that the only difference between ESS spectra at various ambient light intensities is in spectral intensity. This is resolved by standardisation during the pre-processing stage of spectral analysis (See Section 3.5). The spectral profile, from which the principal components are derived for analysis, remained relatively unaffected. Theoretically, ambient light intensity should not influence Principle Component Analysis (PCA) and subsequent spectral analysis despite the observed changes in ESS spectra. Further investigations with larger specimen numbers is needed to establish the effect of ambient light intensity on spectral analysis.

5.2.5 Conclusion

In conclusion, ambient light can enter the ESS system via tissue. Increases in ambient light intensity can result in decrease in spectral intensity, flattening of ESS spectra and reduction in Xe-PPS. These changes are explicable by the process of spectral acquisition, and can be resolved potentially by standardisation during the pre-processing stage of spectral analysis. Underlying changes in spectral profile can be seen in the Soret, alpha and beta bands, which are not related to the changes in ambient light intensity. No other changes were seen in the spectral profile. Therefore, increases in ambient light intensity are not expected to affect spectral analysis.
5.3 The Effect of Exposure in Room Condition on ESS

5.3.1 Introduction

ESS is an emerging technology with potential in-vivo and ex-vivo clinical applications. For ex-vivo applications such as intra-operative assessment of resection margins of excised tumours and SLN for metastasis, the time between excision and spectral acquisition may vary. During this time, significant changes in the ESS spectra may occur. The aim of this study is to investigate the effect of time on ESS spectra of axillary lymph nodes in room conditions following excision.

5.3.2 Methods

Axillary lymph nodes were harvested from surgical specimens of patients with proven breast cancer undergoing axillary surgery following informed consent. The lymph nodes were bisected along their longitudinal axes. The optical probe was applied to the sectioned surface and was fixed in position using a clamp during the study. The initial ESS spectrum was taken at time zero. Subsequent ESS spectra were taken every 30 seconds for the first 15 minutes, then every 60 seconds for the next 15 minutes and finally every 5 minutes for the next 30 minutes. At the end of the study, the lymph nodes were fixed in 4% formaldehyde solution and sent for pathology. ESS spectra were visually inspected. For comparison, ESS spectra at various time intervals were normalised to the spectra taken at zero minute by division.

5.3.3 Results

In total, 6 sets of measurements were made from 5 normal lymph nodes and 1 metastatic lymph node (Lymph node 2). A selection of ESS spectra from all 6 lymph nodes is illustrated over 2 time intervals: 0-10 min and 10-60 min in Figure 5.6.
Figure 5.6: ESS spectra of Lymph Nodes 1 to 6 acquired between 0-10 minutes and between 10-60 minutes following excision.
Figure 5: Spectral changes in Lymph Nodes 5 and 6 for different time periods. The intensity changes were observed in the spectral range from 320 to 920 nm.

Lymph Node 5 (10-60 minutes)

- 10 min
- 30 min
- 60 min

Wavelength (nm)

Lymph Node 6 (0-10 minutes)

- Start
- 1 min
- 3 min
- 5 min
- 10 min

Wavelength (nm)

Lymph Node 6 (10-60 minutes)

- 10 min
- 30 min
- 60 min

Wavelength (nm)

No significant changes in spectral patterns were observed after 2 hours in Lymph Nodes 3 and 4.
On visual inspection, a sequence of changes can be seen in Lymph Nodes 2, 3 and 4. This is characterised by (1) a right shift of the Soret band trough from approximately 415nm to 431nm, (2) a gradual replacement of the alpha and beta bands by a single broad band at 555nm and (3) flattening of peak at 390nm. These changes occurred after 2 minutes for Lymph Node 2, after 50 minutes for Lymph Node 3, and after 4 minutes for Lymph Node 4. No other spectral changes were observed in these lymph nodes. For lymph nodes 1, 5 and 6, no significant spectral changes were observed throughout the 60 minute period.

Figure 5.7 illustrates the corresponding normalised spectra of the 6 lymph nodes. This is done by dividing individual ESS spectra by the ESS spectrum at zero minutes. As in Section 5.2, an increase in spectral intensity at a particular wavelength results in a normalised intensity value greater than 1. Conversely, a decrease in spectral intensity results in a normalised intensity value less than 1. The peaks and troughs in the normalised spectra signify changes in spectral profiles with increasing and decreasing spectral intensities respectively over that bandwidth. A horizontal line represents no spectral change.

In all 6 lymph nodes, normalised spectra demonstrated changes in the Soret, alpha and beta bands. These are more prominent in Lymph Nodes 2, 3 and 4, and correspond to the spectral changes observed by visible inspection. The same pattern of changes can also be seen in the normalised spectra of Lymph Nodes 1, 5 and 6 but were less in magnitude. The remaining band areas have a horizontal profile in all 6 lymph nodes indicating the stability of ESS spectra over the duration of this study.
Figure 5.7: Normalised ESS spectra of Lymph Nodes 1 to 6 acquired between 0-10 minutes and between 10-60 minutes following excision.
Lymph Node 2 (10-60 minutes)

Lymph Node 3 (0-10 minutes)

Lymph Node 3 (10-60 minutes)
5.3.4 Discussion

The spectral changes in Lymph Nodes 2, 3 and 4 are illustrated in Figure 5.8a. In this illustration, the blue spectrum is the start spectrum at 0 minutes, whilst the red spectrum is acquired at 10 minutes. The right shift of the Soret band trough (black arrow), the gradual replacement of the alpha and beta bands by a single broad band at 555nm (white arrow) and the flattening of peak at 390nm (brown arrow) correspond to the differences in the inverse molar extinction coefficient of oxygenated and deoxygenated haemoglobin in Figure 5.8b.

Figure 5.8: (a) ESS spectra demonstrating the observed changes seen in Lymph Nodes 2, 3 and 4 and (b) Inverse molar extinction coefficient for oxygenated and deoxygenated haemoglobin

(a)
The similarities between the two indicate that de-oxygenation of haemoglobin is the process responsible for the observed spectral changes in Lymph Nodes 2, 3 and 4. These can be confirmed by comparing their corresponding normalised spectra (Figures 5.9a and b), which are very similar with peaks and troughs at almost identical wavelengths.

**Figure 5.9:** (a) Normalised spectra of Lymph Node 2 and (b) Normalised absorption spectra of de-oxygenated haemoglobin normalised to oxygenated haemoglobin.

Apart from the changes associated with de-oxygenation of haemoglobin, PWS spectra remain stable over time. However, further studies with larger numbers may reveal a small possibility of malignant cells are concluded to have a
The reason for the de-oxygenation of haemoglobin is likely to be the continual oxygen demand of viable cells within the lymph nodes. This process was evident in the normalised spectra of all 6 lymph nodes even though only Lymph Nodes 2, 3 and 4 had corresponding changes in the ESS spectra. The timing and pace of de-oxygenation cannot be determined by this study. However, further studies with larger numbers may reveal a trend especially if malignant cells are considered to have a higher metabolic rate.

Apart from the changes associated with de-oxygenation of haemoglobin, ESS spectra remained stable over 60 minutes. Individual spectra within each series can be superimposed upon each other, and the corresponding normalised spectra have a horizontal profile. In addition to the stability of ESS spectra over time, this study also demonstrated the reproducibility of ESS spectra from a fixed anatomical point.

The limitation of this study is that spectral acquisition did not progress beyond 60 minutes. Certainly, it would be of interest to investigate beyond the 60 minutes with serial spectral acquisitions once the process of tissue degradation is established.
5.3.5 Conclusion
De-oxygenation of haemoglobin occurs following surgical excision, which is detectable by ESS. The timing and pace of this process vary between different specimens. Apart from this, ESS spectra remain stable for at least 60 minutes following surgical excision in a normal in-door environment. This study also demonstrated the reproducibility of ESS spectra from a constant anatomical point. The implication of this study is that ex-vivo spectral acquisitions can take place within 60 minutes following surgical excision with no significant effects other than de-oxygenation of haemoglobin.

5.4 The Effect of Submersion in Saline at Room Temperature on ESS

5.4.1 Introduction
Tissue hydration is important when freshly excised tissue specimens such as lymph node and renal biopsies are required for pathological processing. Drying must be avoided. Usually, specimens are wrapped in paper or pads moistened with normal saline and transported to laboratories immediately. If there will be any delay in transportation, the specimen should be placed in normal saline solution. Specimens may be transported at room temperature for up to two hours. For delays of 2-24 hours, specimens should be stored at 4°C or cooled on wet ice but not allowed to freeze. The effect of submersing specimens in normal saline on ESS is unknown. The aim of this section is to investigate this effect on ESS of axillary lymph nodes at room temperature.
5.4.2 Methods

Axillary lymph nodes were harvested from surgical specimens of patients with proven breast cancer undergoing axillary surgery following informed consent. The lymph nodes were bisected along their longitudinal axes and were submerged in 0.9% saline solution. The optical probe was applied to the sectioned surface and was fixed in position using a clamp during the study (Figure 5.10). The initial ESS spectrum was taken at zero minutes. Subsequent ESS spectra were taken every 30 seconds for the first 15 minutes, then every 60 seconds for the next 15 minutes and finally every 5 minutes for the next 30 minutes. At the end of the study, all lymph nodes were fixed in 4% formaldehyde solution and sent for pathology.

ESS spectra were visually inspected. For comparison, ESS spectra at various time intervals were normalised to the spectra taken at zero minute by division.

Figure 5.10: Illustrations to show (a) the fixed position of the optical probe onto tissue and (b) submersion of tissue in normal saline
5.4.3 Results

In total, 2 sets of measurements were made from 1 metastatic lymph node (Lymph Node 1) and 1 normal lymph node (Lymph node 2). A selection of ESS spectra from both lymph nodes is illustrated over 2 time intervals: 0-10 minutes and 10-60 minutes in Figure 5.11. The corresponding normalised spectra are illustrated in Figure 5.12. As in Sections 5.2 and 5.3, normalisation was carried out by dividing individual ESS spectra by the ESS spectrum at zero minutes.

Figure 5.11: ESS spectra of Lymph Nodes 1 and 2 acquired between 0-10 minutes and 10-60 minutes following submersion in normal saline

![Figure 5.11: ESS spectra of Lymph Nodes 1 and 2 acquired between 0-10 minutes and 10-60 minutes following submersion in normal saline](image-url)
On visual inspection, de-oxygenation of haemoglobin can be observed in Lymph Node 1 with no other spectral changes. The corresponding normalised spectra closely resemble to those of Lymph Nodes 2, 3 and 4 in Section 5.3.

As for Lymph Node 2, spectral profiles show that haemoglobin was already in the de-oxygenated form. No significant changes in spectral profile were observed throughout the 60 minute period. In the normalised spectra, there are changes at the Soret, alpha and beta band areas as well as in the ultra-violet region.
Figure 5.12: Normalised ESS spectra of Lymph Nodes 1 and 2 acquired between 1-10 minutes and between 10-60 minutes following submersion in normal saline.
5.4.4 Discussion

The findings in Lymph Node 1 are consistent with the findings in the previous study demonstrating the haemoglobin de-oxygenation. The late changes seen in normalised spectra of Lymph Node 2 are not characteristic of the de-oxygenation of haemoglobin. However, the corresponding ESS spectral profile of Lymph Node 2 showed only slight changes in the band regions concerned. Therefore, its significance is doubtful. However, this cannot be verified because only 2 lymph nodes were studied in this series. Further studies may reveal more information.

Tissue hydration is an important consideration in the handling of tissue specimens for optical biopsy. It is known to affect Raman spectroscopy in biological tissue. However, its effect on ESS is unknown. An inadvertent variable in Section 5.3 is the potential desiccation of the lymph nodes by evaporation. The similar findings in both studies suggest that this is not the case. However, these two studies were not designed to investigate the effect of tissue hydration on ESS specifically.
5.4.5 Conclusion

This study demonstrated that submersion of tissue specimens in normal saline has no effect on ESS for up to 60 minutes although de-oxygenation of haemoglobin can occur during this period.

5.5 The Effect of Tissue Chilling on Ice on ESS

5.5.1 Introduction

Storage in ice is standard practice for handling freshly excised tissue specimen during transportation to the laboratory. The effect of this practice on ESS is unknown. However, the process of freezing and thawing was found to affect fluorescence spectroscopy. The aim of this study is to investigate the effect of tissue chilling on ESS.

5.5.2 Methods

Axillary lymph nodes were harvested from surgical specimens of patients with proven breast cancer undergoing axillary surgery following informed consent. The lymph nodes were bisected along their longitudinal axes. The optical probe was applied to the sectioned surface and was fixed in position using a clamp for the duration of the study. The lymph node was chilled using a bag of ice. Initial ESS spectra were taken at zero minutes. Subsequent ESS spectra were taken every 30 seconds for the first 15 minutes, then every 60 seconds for the next 15 minutes and finally every 5 minutes for the next 30 minutes. At the end of the study, all lymph nodes were fixed in 4% formaldehyde solution and sent for pathology. ESS spectra were visually inspected. For comparison, ESS spectra at various time intervals were normalised to the spectra taken at zero minutes by division.
5.5.3 Results

In total, 3 sets of measurements were made from 1 metastatic lymph node (Lymph Node 1) and 2 normal lymph nodes (Lymph Node 2 and 3). A selection of ESS spectra from both lymph nodes is illustrated over 2 time intervals: 0-10 minutes and 10-60 minutes in Figure 5.13. The corresponding normalised spectra are illustrated in Figure 5.14. As in previous studies, normalisation was carried out by dividing individual ESS spectra by the ESS spectrum at zero minute.

*Figure 5.13: ESS spectra of lymph nodes 1, 2 and 3 acquired between 0-10 minutes and 10-60 minutes following chilling in ice*
On visual inspection, spectral profiles at zero minutes demonstrated that haemoglobin was in a de-oxygenated state in Lymph Nodes 1 and 2, and in an oxygenated state in Lymph Node 3. For Lymph Node 1, this and the overall spectral profile remained unchanged throughout the 60 minute period. In the normalised spectra, only minor changes occurred in the Soret bandwidth.

For Lymph Node 2, the ESS spectral profile remained unchanged for the initial 10 minutes. However, the normalised spectra over this period are identical in profile as those of Lymph Node 2, 3 and 4 in Section 5.3. Between 20 and 30 minutes, alpha and beta bands became evident together with a left shift of the Soret band. These changes are indicative of the oxygenation of haemoglobin and were associated with changes in the profiles of corresponding normalised spectra. No other significant changes were observed in ESS spectra and normalised spectra.

For Lymph Node 3, de-oxygenation of haemoglobin occurred in the same manner as the Lymph Nodes 2, 3 and 4 in Section 5.3 during the first 10 minutes of this study. No other changes were observed in ESS spectra and normalised spectra.
Figure 5.14: Normalised ESS spectra for Lymph Nodes 1, 2 and 3 between 0-10 minutes and 10-60 minutes following chilling in ice.
Lymph Node 2 (10-60 minutes)

Wavelength (nm)

Lymph Node 3 (1-10 minutes)

Wavelength (nm)

Lymph Node 3 (10-60 minutes)

Wavelength (nm)
5.5.4 Discussion

The most interesting observation in this study is of the oxygenation of haemoglobin in Lymph Node 2. The initial ESS spectral profile indicated that haemoglobin was in a de-oxygenated state, which is characterised by a single broad band at 555nm. The spectral profile remained unchanged for 10 minutes. However, the corresponding normalised spectra showed that further de-oxygenation of haemoglobin was in progress. Between 10 and 30 minutes, a single broad band at 555nm was replaced by the alpha and beta bands of oxygenated haemoglobin with accompanied changes in normalised spectra. Figure 5.15a demonstrates the changes in the normalised spectra of Lymph Node 2 during this period. This is different from that of Lymph Node 3 (Figure 5.15b) where de-oxygenation was in slow progress.

Figure 5.15: Normalised spectra of Lymph Nodes (a) 2 and (b) 3 between 10 and 30 minutes
The process observed in Lymph Node 2 is the oxygenation of haemoglobin. This can be confirmed by comparing the normalised spectra at 60 minutes (Figure 5.16a) and the inversed normalised molar extinction coefficient of the oxygenation of haemoglobin (Figure 5.16b). The profiles of Figures 5.14a and b appear similar with corresponding peaks.

**Figure 5.16:** (a) Normalised spectra of Lymph Node 2 after 60 min and (b) Inverse molar extinction coefficient of oxygenated Hb normalised to deoxygenated Hb
The likely explanation for the oxygenation of haemoglobin after initial de-oxygenation is the change in haemoglobin’s affinity to oxygen with temperature. At lower temperatures, the stability of the bond between oxygen and haemoglobin increases resulting in greater affinity of haemoglobin to oxygen and a “left shift” of the oxygen dissociation curve. This is demonstrated by the oxygen dissociation curve of haemoglobin in Figure 5.17. Otherwise, no other changes were observed in the ESS spectra and normalised spectra of Lymph Node 2.

For Lymph Node 1, only minor changes were noted in the normalised spectra between 320 and 420nm, whilst Lymph Node 3 exhibited de-oxygenation of haemoglobin in the same fashion as described in Sections 5.3 and 5.4.
5.5.5 Conclusion

Oxygenation and de-oxygenation of haemoglobin can occur in tissue during storage in chilled conditions following excision and can be detected by ESS. Otherwise, ESS spectra of tissue specimens remain stable and reproducible when stored in ice for at least 60 minutes following surgical excision. Therefore, storage of tissue specimens in ice up to 60 minutes is acceptable for subsequent ESS studies.
5.6 The Effect of Formaldehyde Fixation at Room Temperature on ESS

5.6.1 Introduction
Formaldehyde fixation is an established technique for the storage of tissue specimens prior to pathological processing. Formaldehyde prevents tissue degradation by forming covalent bonds between two components of a protein or between two different proteins. Formaldehyde fixation has been shown to significantly affect near-infrared Raman spectroscopy of normal and cancerous human bronchial tissues\(^1\), whilst it has only a weak effect on the fluorescence spectroscopy\(^4\). However, the effect of formaldehyde fixation on ESS is unknown. The aim of this section is to investigate the effects of formaldehyde fixation over two time frames: up to 60 minutes in fixation (Study A) and overnight in fixation (Study B).

5.6.2 Methods
Axillary lymph nodes were harvested from surgical specimens of patients with proven breast cancer undergoing axillary surgery following informed consent. The lymph nodes were bisected along their longitudinal axes and were submerged in 4% formaldehyde solution. The optical probe was applied to the sectioned surface and was fixed in position using a clamp during the study. Initial ESS spectra were taken at zero minutes. For Study A, subsequent ESS spectra were taken every 30 seconds for the first 15 minutes, then every 60 seconds for the next 15 minutes and finally every 5 minutes for the next 30 minutes. For Study B, ESS spectra were taken at various time points up to 24 hours. At the end of the study, all lymph nodes were sent for pathology.
ESS spectra were visually inspected. For comparison, ESS spectra at various time intervals were normalised to the spectra taken at zero minutes by division.

5.6.3 Results

Study A

In total, 4 sets of measurements were made from 4 normal lymph nodes (Lymph Nodes 1-4). A selection of ESS spectra from both lymph nodes is illustrated over 2 time intervals: 0-10 minutes and 10-60 minutes in Figure 5.18. The corresponding normalised spectra are illustrated in Figure 5.19. As in Sections 5.2, 5.3 and 5.4, normalisation was carried out by dividing individual ESS spectra by the ESS spectrum at zero minute.

*Figure 5.18: ESS spectra of Lymph Nodes 1-4 plotted at 0-10 minutes and 10-60 minutes following submersion in formaldehyde solution*
On visual inspection, initial spectral profiles demonstrated that haemoglobin was in the oxygenated state in Lymph Nodes 1, 3 and 4, and in the de-oxygenated state in Lymph Node 2.

For Lymph Node 1, there was a right shift in the Soret band in the initial 10 minutes, which was accompanied by minor changes in the alpha and beta bands. The corresponding normalised spectra were consistent with progressive de-oxygenation of haemoglobin as seen in previous studies. Beyond 10 minutes, there was a reversal of the changes so that by 60 minutes the Soret band returned to its initial position within the ESS spectrum with increased prominence of the alpha and beta bands. The normalised spectrum at 60 minutes resembled that of Lymph Node 2 in Section 5.5 and was suggestive of oxygenation of haemoglobin. No changes were noted in other bandwidths during the 60 minutes period.

For Lymph Node 2, there were no changes in the ESS spectra in the first 10 minutes, but the corresponding normalised spectra suggested a small degree of de-oxygenation...
of haemoglobin. In the 10-60 minute period, alpha and beta bands became evident together with a left shift of the Soret band. Normalised spectrum at 60 minutes was similar to that of Lymph Node 2 in Section 5.5 indicating that oxygenation of haemoglobin had occurred. Otherwise, no other changes were observed.

For Lymph Node 3, minor changes occurred in both ESS and normalised spectra over the 60 minute period. As for Lymph Node 4, there were no changes in ESS spectra in the initial 10 minutes, but normalised spectra during this period showed evidence of de-oxygenation. In the subsequent 10-60 minutes, a slight left shift and increased prominence of alpha and beta bands occurred with associated changes in the normalised spectra identical to those observed in Lymph Node 1.

Figure 5.19: Normalised spectra of Lymph Nodes 1-4 plotted at 0-10 minutes and 10-60 minutes following submersion in formaldehyde solution
Lymph Node 1 (10-60 minutes)

Lymph Node 2 (1-10 minutes)

Lymph Node 2 (10-60 minutes)
Lymph Node 3 (1-10 minutes)

Lymph Node 3 (10-60 minutes)

Lymph Node 4 (1-10 minutes)
Study B

In total, 2 sets of measurements were made from 1 metastatic lymph node (Lymph Node 5) and 1 normal lymph node (Lymph Node 6). A selection of ESS spectra from both lymph nodes is illustrated over 3 time intervals: 0-1 hour, 1-4 hours and beyond 4 hours in Figure 5.20. The corresponding normalised spectra are illustrated in Figure 5.21. As in previous studies, normalisation was carried out by dividing individual ESS spectra by the ESS spectrum at zero minutes. Because of the exceptionally high value of the normalised intensity of Lymph Node 6 between 400 and 450nm band width, the normalised intensity axis of Lymph Node 6 was restricted to 0-5 range in order to reveal the details in other areas.
Figure 5.20: ESS spectra of Lymph Nodes 5 and 6 plotted at 0-1 hour, 1-4 hours and beyond 4 hours following submersion in formaldehyde solution.
Lymph Node 6 (0-1 hour)

Wavelength (nm)

Intensity

0 min
15 min
30 min
1 hr

Lymph Node 6 (1-4 hours)

Wavelength (nm)

Intensity

1 hr
2 hr
3 hr
4 hr

Lymph Node 6 (4-24 hours)

Wavelength (nm)

Intensity

4 hr
5 hr
6 hr
24 hr
On visual inspection, minor changes were observed in the spectral profiles of both lymph nodes in the first hour. During this period, the normalised spectral profiles of both lymph nodes were identical to those of Lymph Nodes 1, 2 and 4 in Study A and therefore indicative of haemoglobin oxygenation.

Between 1 and 4 hours, the most notable change is in the gradient of spectra between 720 and 920nm in Lymph Node 6. Minor changes were also noted in the ESS spectra, but the overall profile of both lymph nodes remained similar. Over this period, the normalised spectra of both lymph nodes resembled those in the earlier period and those of Lymph Nodes 1, 2 and 4 in Study A. However, as time progressed, there was a gradual change in the normalised spectra in Lymph Node 6 between 500-620nm where the triple peaks were gradually replaced by a single broad peak at 560nm. This change also occurred in Lymph Node 5 in the 4-17 hour period.

Beyond 4 hours, ESS spectra of both lymph nodes changed beyond recognition from their initial spectra at zero minute. Both the alpha and beta bands were obliterated, and changes in the spectral gradient between 720-920nm also occurred. The spectral changes were accompanied by the change in the profile of the normalised spectra.
**Figure 5.21:** Normalised ESS spectra for Lymph Nodes 5 and 6 at 0-1 hours, 1-4 hours and beyond 4 hours.
Lymph Node 6 (0-1 hour)

Lymph Node 6 (1-4 hours)

Lymph Node 6 (4-24 hours)
5.6.4 Discussion

Formaldehyde fixation is a standard technique for tissue handling. Due to its chemical interaction with biological tissue, it is important to determine its effect on ESS. If the effect is significant, ESS spectral acquisition should take place prior to formaldehyde fixation. Also, ESS spectra taken from formaldehyde fixed specimens cannot be used for spectral analysis of in-vivo or unfixed ex-vivo tissues, and visa versa.

The findings of Studies A and B are as follows: (1) changes in ESS spectra within 60 minutes of formaldehyde fixation occurred within the bandwidths of haemoglobin absorption, (2) beyond the initial 60 minutes, there were changes in the gradient of spectra between 620 and 920nm, (3) beyond 4 hours, ESS spectra did not bear any resemblance to the initial spectra at zero minutes, and (4) oxygenation of haemoglobin was evident in 5 out of 6 lymph nodes (Lymph Nodes 1, 2, 4, 5 and 6) within 60 minutes of formaldehyde fixation.

The changes in ESS spectra within the first 60 minutes occurred within the bandwidths of haemoglobin absorption with no other changes in the remaining bandwidths. Therefore, it appears that ESS spectra acquired from formaldehyde fixed tissue specimens is acceptable for subsequent in-vivo or fresh ex-vivo studies if spectral acquisition takes place within one hour of fixation and haemoglobin effects are accounted for during spectral analysis. However, beyond this period, changes in ESS spectra also involved bandwidths independent of haemoglobin absorption. The resultant spectra bore less resemblance to the initial spectra, and therefore, cannot be used in in-vivo and fresh ex-vivo applications. Further studies with larger numbers would be useful (1) to confirm the findings of these studies, (2) to establish whether
there is a predictable trend of changes induced by formaldehyde and (3) to investigate
whether ESS spectra acquired within the first 60 minutes of formaldehyde fixation
affects subsequent spectral analysis.

Although Lymph Node 2 was the only lymph node to demonstrate the re-oxygenation
of de-oxygenated haemoglobin in the ESS spectra, normalised spectra of Lymph
Nodes 1, 4, 5 and 6 in the first 60 minutes had similar profiles to that of Lymph Node
2 thus suggesting that further oxygenation of haemoglobin took place, which is an
interesting finding. One possible explanation is that formaldehyde increased the
oxygen affinity of human haemoglobin. This interaction may be due to combination
with sulphhydryl groups of the protein, but nitrogenous groups are probably also
involved5.

However, it has been suggested that formaldehyde causes the formation of
methaemoglobin6. Figure 5.22 is the light absorption spectra of methaemoglobin
which is characterised by peak absorptions at 500nm and 632nm (arrows)7. If
formation of methaemoglobin was to be the case, two troughs would be expected to
develop at 500nm and 632nm in the ESS spectra and normalised intensity at 500nm
and 632nm bandwidths would less than 1. This was not observed in Study B, and
hence, the formation of methaemoglobin was unlikely to be responsible for the
observed changes.
Figure 5.22: Absorption spectra of methaemoglobin (Curve 1, solid) in the visible range

The extended duration of Study B provides a glimpse of the changes in ESS induced by formaldehyde. Unfortunately, ESS spectra of Lymph Nodes 5 and 6 were acquired at different times and therefore are not comparable. However, Study B achieved its aim by demonstrating significant changes in ESS following formaldehyde fixation.

Two published studies from NMLC investigating the ability of ESS to detect cervical intranodal metastasis and to assess bony resection margins in oral cancer used
formaldehyde fixed tissues\textsuperscript{6,8}. In the lymph node study, specimens were fixed between 24 and 72 hours, and spectral analysis involved analysis of spectral intensities at 360, 450, 630 and 690 nm providing a sensitivity of 98% and a specificity of 68% for detecting cancer\textsuperscript{6}. As for the bony resection study, specimens were fixed for up to 10 years, and spectral analysis involved analysis of spectral intensity at 400nm and spectral gradient between 570 and 680nm\textsuperscript{8}. Sensitivity and specificity of ESS in this study was 87% and 80% respectively for detecting cancer. Formaldehyde fixation appears to provide a much simpler discrimination between normal and malignant tissue when compared to detecting metastasis in freshly excised unfixed axillary lymph nodes in breast cancer. A possible hypothesis is that the elastic scattering and absorption properties of normal and malignant tissues change differentially during formaldehyde fixation.

5.6.5 Conclusion

In conclusion, the changes in ESS spectra within the first hour of formaldehyde fixation are attributable to oxygenation of haemoglobin, which is probably secondary to the increased oxygen affinity of haemoglobin by formaldehyde. If this is accounted for in subsequent spectral analysis, formaldehyde fixation appears to be suitable for tissue storage for up to 60 minutes prior to ESS spectral acquisitions. Beyond 60 minutes, significant changes occurred. Therefore, ESS spectral acquired after 60 minutes of formaldehyde fixation should not be used for clinical applications unless future studies can allow for formaldehyde induced changes in the analysis.
5.7 Summary and Conclusion of Chapter

The studies in this chapter have provided much needed technical information on the effects of ambient light and tissue handling on ESS. It is important to establish these effects prior to large scale data acquisition and clinical studies so that these effects can be accounted for in subsequent spectral analysis if necessary. Failure to do so may result in the corruption of the database ultimately affecting sensitivity and specificity.

Section 5.2 demonstrated that ambient light could enter the ESS system via tissue. At higher ambient light intensities (>1000lux), effects on ESS spectra become more prominent. Theoretically, these effects may be abolished by the processes of spectral acquisition and analysis. However, further studies involving spectral analysis of a large number of spectra would establish whether this is the case.

The studies into the effects of tissue handling have established that the changes that occurred in ESS spectra within the initial 60 minutes following excision when tissue specimens were left in various conditions were attributed to the change in oxygenation status of haemoglobin. In general, de-oxygenation of haemoglobin occurred over time, but oxygenation of haemoglobin was also observed during chilling in ice and formaldehyde fixation. Otherwise, ESS spectra remain stable and reproducible within this time period thus confirming that the various methods of tissue handling are suitable for subsequent spectral acquisition within 60 minutes. More important is the reproducibility of ESS spectra from a given anatomical point, which is fundamental to ESS.
Study B in Section 5.6 demonstrated significant changes to ESS spectra following prolonged formaldehyde fixation. Therefore, formaldehyde fixation should not be used for tissue handling prior to spectral acquisition if the subsequent ESS spectra database is to have a clinical application unless some way can be found to correlate the ESS spectra before and after formaldehyde fixation.

Reference List


Chapter 6 Elastic Scattering Spectroscopy: An emerging technology for the detection of axillary lymph node metastasis in breast cancer

6.1 Introduction

ESS is an emerging technology, which is capable of detecting changes in tissue structures by studying the elastic scattering and absorption of light in tissues. The key features of ESS system at NMLC are listed in Table 6.1.

Table 6.1: Key features of ESS

<table>
<thead>
<tr>
<th>Key Features of ESS</th>
</tr>
</thead>
<tbody>
<tr>
<td>• ESS system is portable and is easy to use</td>
</tr>
<tr>
<td>• Components are widely available and relatively inexpensive</td>
</tr>
<tr>
<td>• Spectral acquisition takes approximately one second</td>
</tr>
<tr>
<td>• ESS system is safe and does not involve ionised radiation or laser</td>
</tr>
<tr>
<td>• Optical probes can be supplied sterilised for intra-operative use</td>
</tr>
<tr>
<td>• ESS permits in-vivo and ex-vivo spectral acquisition</td>
</tr>
<tr>
<td>• Software for spectral analysis is currently under development</td>
</tr>
</tbody>
</table>

A potential application of ESS is the intra-operative detection of SLN metastasis in breast cancer. The purpose of intra-operative assessment is to identify the patients with SLN metastases so that ALND can be carried out at the time of SLNB. Since 24-31% of patients with T1 (<2cm) tumour and 44-49% of patients with T2 (2-5cm) tumour have axillary metastases\(^1\), a reliable intra-operative technique to assess the
SLN would reduce readmissions for ALND thereby conserving resources and popularizing SLNB in breast cancer. An ideal technique for intra-operative assessment of SLN should be reliable, rapid, simple, cheap and non-operator dependent – “an expert system”. The ability for “in-theatre” or “on-table” assessment would be desirable.

At present, TIC and FFS are established techniques for intra-operative assessment for tissues, and have been evaluated for this purpose. Many studies have demonstrated that a high degree of sensitivity and specificity can be achieved by both (Tables 6.9 and 6.10). Although rapid in the actual processing of specimen, both TIC and FFS can be time consuming considering the time taken for the delivery of SLN to laboratories and for detailed examination. In addition, both techniques are operator dependent requiring highly trained and experienced pathologists for interpretations. Therefore, intra-operative SLN assessment by TIC and FFS is not widely practiced.

Studies by Gavin Briggs at NMLC have demonstrated the ability of ESS to detect metastases in excised axillary lymph nodes in patients with breast cancer. In his thesis, Briggs recruited 34 patients. 11 patients underwent SLNB, 2 patients had both SLNB and ALND, and the remaining 21 patients had ALND. 62 axillary lymph nodes were harvested, and 14 were found to have metastases. A total of 117 ESS spectra were acquired. Spectral analysis was performed using Artificial Neural Network (ANN), Hierarchical Cluster Analysis (HCA) and Model Based Analysis (MBA). The sensitivities and specificities are illustrated in Table 6.2.
Since this study, MBA has become the analysis technique of choice at NMLC for ESS. However, the sensitivity and specificity achieved using MBA were significantly lower than the 94% sensitivity and 92% specificity in differentiating normal and malignant breast tissue using identical spectral analysis technique.

The aims of this chapter are (1) to improve the sensitivity and specificity of ESS in determining the axillary lymph nodes status in breast cancer and (2) to undertake a comparative study of ESS and TIC in assessing SLN status in breast cancer.

### 6.2 Study to improve the sensitivity and specificity of ESS using MBA in determining the axillary lymph nodes status in breast cancer

#### 6.2.1 Introduction

In Dr Brigg’s thesis, the sensitivity and specificity of ESS in detecting axillary lymph node metastases using MBA were 57% and 85%, which were significantly lower than the 94% sensitivity and 92% specificity he achieved in differentiating normal and metastatic axillary lymph nodes using ANN, HCA and MBA (from Gavin Briggs).

<table>
<thead>
<tr>
<th></th>
<th>ANN</th>
<th>HCA</th>
<th>MBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>58%</td>
<td>91%</td>
<td>57%</td>
</tr>
<tr>
<td>Specificity</td>
<td>93%</td>
<td>76%</td>
<td>85%</td>
</tr>
</tbody>
</table>
malignant breast tissues using an identical spectral analysis technique\textsuperscript{2}. One possible factor was the small number of ESS spectra and lymph nodes in this study. Our initial approach was to increase the size of the data. Following further recruitment, a total of 2804 ESS spectra from 327 lymph nodes were obtained. ESS spectra were correlated to the overall histology of the lymph node. Using identical spectral analysis technique, a similar sensitivity of 69\% and specificity of 85\% were achieved.

Subsequent scrutiny of our methodology found 4 areas of weakness. Firstly, ESS spectra were acquired randomly from the sectioned surface of the lymph nodes. With an average of 8.6 spectra acquired per lymph node, spectral acquisition did not cover the entire sectioned surface of the lymph nodes. Secondly, ESS spectra were correlated with the overall histology of the lymph nodes. These 2 factors resulted in a lack of precise correlation between ESS spectra and the histology of the area within the lymph nodes, where ESS spectra were acquired. This becomes problematic in lymph nodes with minimal or partial metastases because spectra taken from a normal area would be classified as metastatic. Thirdly, the use of spectra from the same lymph node in both training and testing datasets could potentially influence spectral analysis, and finally, there was a lack of discrimination between different types of breast cancer in spectral analysis. The aim of this study is to explore these issues by running different spectral analyses using MBA with different criteria for selecting datasets for training and testing.

6.2.2 Methods

All the lymph nodes used in this study were selected from the 327 lymph nodes in the preliminary study. These lymph nodes were harvested from female patients
undergoing ALND and SLNB for breast cancer following informed and written consent (Appendix B). Immediately following surgical excision, each lymph node was bisected along its longitudinal axis. Multiple ESS spectra were acquired from the sectioned surfaces at random. Each lymph node was labeled individually and was fixed with 4% formaldehyde in separate containers.

Histological sections of the sectioned surface were made individually and were stained with H&E. For SLN, 2 further H&E sections were made at different levels, and IHC was performed if the SLN was negative on H&E. Histopathological reporting was performed by Dr Mary Falzon, Consultant breast pathologist. The lymph nodes were classified as negative or positive for metastasis. For the latter, the amount of metastatic replacement was assessed as a percentage. The histopathological report was correlated with the ESS spectra.

Spectral analyses using MBA was performed with different criteria for datasets selected for training. Only negative lymph nodes and those with more than 80% metastatic replacement were selected for analysis in order to minimise the effect of minimal and partial metastases.

6.2.3 Results and Discussion

In total, there were 28 lymph nodes with more than 80% metastatic replacement. 19 of 28 lymph nodes (non-SLN) were from invasive ductal carcinoma. 4 lymph nodes were completely replaced by invasive ducto-lobular carcinoma whilst 3 were replaced by invasive lobular carcinoma. The remainder consisted of lymph nodes from
invasive ductal carcinoma with extensive necrosis (1 node) and extensive microcalcification (1 node).

In presenting the statistical results, sensitivity, specificity and accuracy are presented in the standard way:

\[
\text{Sensitivity} = \frac{\text{True Positive}}{\text{True Positive} + \text{False Negative}}
\]

\[
\text{Specificity} = \frac{\text{True Negative}}{\text{True Negative} + \text{False Positive}}
\]

\[
\text{Accuracy} = \frac{\text{True Positive} + \text{True Negative}}{\text{Total number}}
\]

**Analysis 1: Sensitivity and specificity of ESS in determining axillary lymph node status in invasive ductal carcinoma**

This analysis used the spectra of the 19 lymph nodes which had invasive ductal carcinoma metastasis and an equal number of spectra taken from normal lymph nodes. In total, there were 291 spectra, of which 194 were used in the training set and the remaining 97 were used in the testing set.

The results are illustrated in Table 6.3. Of the 97 spectra used in the testing set, 50 were taken from metastatic lymph nodes, and 47 from normal lymph nodes. ESS correctly identified 44 of the 50 positive spectra as positive with 6 false negatives giving a sensitivity of 88%. ESS correctly identified 45 out of 47 negative spectra as negative with 2 false positives giving a specificity of 96%. ESS correctly classified 89 spectra giving an accuracy of 92%. The Kappa value for this analysis is 0.835.
In this analysis, MBA achieved a sensitivity of 88\% and specificity of 96\% in determining the metastatic status of ESS spectra taken from lymph nodes in patients with invasive ductal carcinoma. Such sensitivity and specificity are comparable with the reported sensitivities and specificities of TIC and FFS in determining lymph node status in breast cancer thus demonstrating the potential of ESS in this application.

The improved sensitivity and specificity demonstrates the importance of the correct correlation of the ESS spectra and histology. In both of our preliminary studies, all ESS spectra taken from metastatic lymph nodes were classed as positive even if they were acquired from a normal area. If these spectra were subsequently used for training, this would result in the spectral analysis recognising spectra from normal lymph nodes as metastatic thereby resulting in false positives. On the other hand if these spectra were used for testing, spectral analysis would have recognised these spectra as negative, but these would be classed as false negatives because the histological correlation was metastatic. By selecting lymph nodes with >80\% metastasis, this analysis reduced the error in spectra-histology correlation. Whether the sensitivity and specificity could be improved further by selecting lymph nodes with complete
metastatic replacement cannot be determined in this study because only 6 lymph nodes were completely replaced.

Additionally, this analysis has demonstrated the suitability of our spectral analysis technique. MBA has been evaluated for similar tasks in other areas. One interesting study is computer gender classification from facial images. In this study, the dimensionality of the face images was reduced into 8 coefficients by preprocessing. This was followed by analysis using other algorithms as well as LDA. The study found LDA to be the preferred algorithm because of its performance (92% accuracy) and also it was considerably less expensive to compute.

*Analysis 2: A jackknife analysis of ESS spectra of axillary lymph nodes with metastatic invasive ductal carcinoma*

In this analysis, the testing set consisted of spectra from 1 of the 19 lymph nodes with >80% metastatic replacement by invasive ductal carcinoma. The spectra of the remaining 18 metastatic lymph nodes were used as the training set along with an equal number of spectra from normal axillary lymph nodes. The tested lymph node was classified positive if 1 or more of spectra tested positive. The process was repeated for each of the 19 lymph nodes.

The result is illustrated in Table 6.4. Spectral analysis correctly classified 17 out of 19 positive lymph nodes giving a “per node” sensitivity of 89.5%. In total, 129 spectra were tested, and MBA was correct in classifying 105 ESS spectra as positive giving a “per spectrum” sensitivity of 81.4%.
Table 6.4: Results of Analysis 2: Spectra from lymph nodes with >80% replacement with cancer

<table>
<thead>
<tr>
<th>Node</th>
<th>Number of Spectra taken</th>
<th>Number of spectra classified as positive</th>
<th>Number of spectra classified as negative</th>
<th>Correct</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0%</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0%</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>75%</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>100%</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>8</td>
<td>0</td>
<td>100%</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>100%</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>8</td>
<td>0</td>
<td>100%</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>8</td>
<td>0</td>
<td>100%</td>
</tr>
<tr>
<td>9</td>
<td>8</td>
<td>8</td>
<td>0</td>
<td>100%</td>
</tr>
<tr>
<td>10</td>
<td>8</td>
<td>8</td>
<td>0</td>
<td>100%</td>
</tr>
<tr>
<td>11</td>
<td>8</td>
<td>8</td>
<td>0</td>
<td>100%</td>
</tr>
<tr>
<td>12</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td>50%</td>
</tr>
<tr>
<td>13</td>
<td>8</td>
<td>6</td>
<td>2</td>
<td>75%</td>
</tr>
<tr>
<td>14</td>
<td>8</td>
<td>8</td>
<td>0</td>
<td>100%</td>
</tr>
<tr>
<td>15</td>
<td>8</td>
<td>2</td>
<td>6</td>
<td>25%</td>
</tr>
<tr>
<td>16</td>
<td>8</td>
<td>8</td>
<td>0</td>
<td>100%</td>
</tr>
<tr>
<td>17</td>
<td>12</td>
<td>12</td>
<td>0</td>
<td>100%</td>
</tr>
<tr>
<td>18</td>
<td>8</td>
<td>1</td>
<td>7</td>
<td>13%</td>
</tr>
<tr>
<td>19</td>
<td>7</td>
<td>7</td>
<td>0</td>
<td>100%</td>
</tr>
<tr>
<td>Total</td>
<td>129</td>
<td>105</td>
<td>24</td>
<td>81%</td>
</tr>
</tbody>
</table>
The “leave one node out” approach of jackknife analysis provides a robust assessment of MBA. This is because all the spectra were used for training except for the one used for testing, and all metastatic lymph nodes were individually tested. Therefore the reported sensitivity is absolute. Two additional advantages of jackknife analysis are: (1) the potential influence of using spectra of the same lymph node for training and testing is abolished, and (2) jackknife analysis simulates a prospective analysis. Unfortunately, normal lymph nodes were not included in the testing, and therefore specificity could not be determined.

Of the 17 correctly classified lymph nodes, spectral analysis was correct in classifying all the spectra in 12 lymph nodes. In the other 5 lymph nodes, the percentage of spectra correctly classified ranged from 13% to 75%. As lymph nodes with >80% metastatic involvement were selected, one possible reason for the false negatives is that these ESS spectra were taken from a normal area of the lymph node. This would account for the false negatives in Lymph Node 3, 12 and 13. However, there may be other factors involved in Lymph Nodes 15 and 18. Without direct correlation of ESS spectra to actual histology, this cannot be investigated further. As for Lymph Nodes 1 and 2, only 2 ESS spectra were taken from each, and this may not have been representative of the lymph nodes.

**Analysis 3: Jackknife analysis using spectra of metastatic lymph nodes of different breast tumour types as testing set**

In this analysis, the training set consisted of spectra acquired from lymph nodes with more than 80% metastatic replacement by invasive ductal carcinoma and equal
number of spectra from negative nodes. The testing set consisted of spectra taken from lymph nodes with more than 80% metastatic replacement by other breast tumour types or by invasive ductal carcinoma with unusual histology.

The results of this analysis are illustrated in Table 6.5. ESS correctly classified 35 out of 49 ESS spectra giving a “per spectra” sensitivity of 71%. However, 8 out of 9 lymph nodes were correctly classified giving a “per node” sensitivity of 89%.

**Table 6.5: Results of Analysis 3**

<table>
<thead>
<tr>
<th>Node</th>
<th>Histology</th>
<th>Number of Spectra</th>
<th>Number of spectra classified positive</th>
<th>Number of spectra classified negative</th>
<th>Correct</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lobular carcinoma</td>
<td>6</td>
<td>0</td>
<td>6</td>
<td>0%</td>
</tr>
<tr>
<td>2</td>
<td>Lobular carcinoma</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>100%</td>
</tr>
<tr>
<td>3</td>
<td>Ducto-lobular carcinoma</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>100%</td>
</tr>
<tr>
<td>4</td>
<td>Ducto-lobular carcinoma</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>75%</td>
</tr>
<tr>
<td>5</td>
<td>Ducto-lobular carcinoma</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>75%</td>
</tr>
<tr>
<td>6</td>
<td>Ducto-lobular carcinoma</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>50%</td>
</tr>
<tr>
<td>7</td>
<td>Lobular carcinoma</td>
<td>7</td>
<td>5</td>
<td>2</td>
<td>71%</td>
</tr>
<tr>
<td>8</td>
<td>Ductal cancer with 80% necrosis</td>
<td>8</td>
<td>6</td>
<td>2</td>
<td>75%</td>
</tr>
<tr>
<td>9</td>
<td>Ductal carcinoma with extensive microcalcification</td>
<td>8</td>
<td>8</td>
<td>0</td>
<td>100%</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>49</td>
<td>35</td>
<td>14</td>
<td>71%</td>
</tr>
</tbody>
</table>
The purpose of this analysis is to determine whether spectra from negative and metastatic invasive ductal carcinoma lymph nodes alone are sufficient as training sets for testing of spectra from metastatic lymph nodes of other breast tumour types or with unusual pathology. It appears that spectral analysis using the training set consisting only of spectra from normal and metastatic invasive ductal carcinoma lymph nodes can determine lymph node metastasis from other tumour types. Despite correctly classifying 8 out of 9 as metastatic lymph nodes, the “per spectrum” sensitivity is less at 71%. This may be due to (1) the small numbers in this analysis, (2) the incorrect histological correlation of the testing spectra as discussed in Analysis 1 and (3) the different tissue types. The implication of the latter is that the addition of spectra from metastatic lymph nodes of other tumour types is needed to the existing training set. The obvious limitation of this analysis is the small number of lymph nodes and spectra, and further studies with larger numbers would provide clarification.

**Analysis 4: The effect of adding spectra of other tumour types to the training set consisting of normal and metastatic invasive ductal carcinoma lymph nodes**

The aim of this analysis is to determine whether the addition of spectra of other tumour types to the training set influences the sensitivity and specificity of spectral analysis. All the spectra taken from lymph nodes with more than 80% metastatic replacement by all tumour types and equal number of spectra from normal lymph nodes are used. Two-thirds were randomly selected for the training set. Once a LDA algorithm was established, the remaining one-third was used for testing.
In total, there were 555 spectra, of which 370 were randomly chosen as the training set and the remaining 185 were used in the testing set. Of the 185 ESS spectra used for testing, 91 spectra were histologically positive. ESS correctly classified 82 spectra with 9 false negatives giving a sensitivity of 90% (Table 6.6). 94 spectra were histologically negative, and 86 were correctly classified with 8 false positives giving a specificity of 91%. Spectral analysis was correct in classifying 168 spectra giving an overall accuracy of 91%. The Kappa value in this study is 0.816136.

**Table 6.6: Results of Analysis 4**

<table>
<thead>
<tr>
<th></th>
<th>Histology</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>ESS</td>
<td>Positive</td>
<td>82</td>
<td>8</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>9</td>
<td>86</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>91</td>
<td>94</td>
<td>185</td>
</tr>
</tbody>
</table>

The sensitivity of 90%, specificity of 91% and accuracy of 91% achieved in this analysis is comparable to those of Analysis 1 (88%, 96% and 91% respectively) suggesting that the addition of spectra from metastatic lymph nodes of other breast cancer types has little impact on the performance of spectral analysis.

### 6.2.4 Discussion

Spectral analysis is an essential component of our ESS system. This study demonstrated (1) that current spectral analysis using MBA is suitable for analyzing
ESS spectra and (2) the importance of correct spectral classification of the training datasets. By refining the training and testing sets, significant improvements in sensitivity and specificity (88% and 96% from Analysis 1) were achieved. For the purpose of this thesis, spectra from lymph nodes with partial metastasis and micrometastasis have to be discounted because it was not possible to correlate the ESS spectra to the precise histology.

The sensitivities and specificities achieved in Analyses 1 and 4 are comparable with the reported sensitivities and specificities of TIC and FFS in determining SLN metastasis indicating the potential of ESS technology in this application. In the clinical situation, a lymph node would be deemed metastatic if one or more ESS spectra are classified as positive as in the case of Analyses 2 and 3. Combining Analyses 2 and 3, spectral analysis correctly classified 25 of the 28 metastatic lymph nodes even though 10 of these lymph nodes had some of spectra classified as negative. Whether these negative spectra were taken from a normal area of the lymph node or were misclassified by spectral analysis is uncertain. However, the effect of misclassifying spectrum/spectra can be compensated by a correct classification of a positive spectrum as demonstrated. Clinically, there is a clear requirement for a thorough spectral acquisition from the entire sectioned surface of the lymph node.

The reporting of sensitivity and specificity of ESS in this chapter can be confusing. On one hand, Analyses 1 and 4 reported sensitivity and specificity per spectrum whilst Analyses 2 and 3 were presented per lymph node. Therefore, it is important for clarification when presenting the results to avoid confusion and to allow direct comparisons between different optical biopsy technologies. In addition, sensitivity
and specificity per spectrum are more relevant for those with interests in the development of spectral analysis whilst for the surgeon, the sensitivity and specificity per node are more relevant as these would determine whether or not to proceed with ALND during SLNB.

The limitation of this study is that only normal and metastatic lymph nodes with more than 80% replacement were studied. Although spectral acquisitions were also made from lymph nodes with partial and micro-metastases, it was not possible to correlate the spectra to the precise histology. This raises the question of whether ESS can detect small or micro-metastases where the area of spectral acquisition may be partially involved with tumour infiltration. This is to be determined in Chapter 7.

6.2.5 Conclusion

Spectral analysis using MBA has “per spectrum” sensitivity of up to 90% and specificity of up to 96% in differentiating ESS spectra of normal and metastatic lymph nodes in breast cancer. This study confirms the suitability of MBA for spectral analysis in ESS, and also demonstrated the importance of correct histological correlation of ESS spectra.

6.3 A comparative study of ESS and TIC in assessing SLN status in breast cancer

6.3.1 Introduction

Touch imprint cytology (TIC) is an established cytological technique first described by Dudgeon and Patrick in 1927. TIC has proven useful as an intra-operative
diagnostic tool for the detection of lymph node metastasis in a variety of malignant diseases⁵-⁷. In the management of breast cancer, TIC has been used to determine excision margins of breast lesions intra-operatively⁸,⁹.

TIC is simple and quick to perform. It provides clear cytological details for evaluation without artifacts. In addition to standard staining, it is also possible to perform immunocytochemistry. Furthermore, TIC avoids the consumption of tissue. For these reasons, TIC is more favored than FFS for intra-operative SLN assessment. The aim of this study is to compare TIC and ESS in assessing SLN status of patients with breast cancer.

6.3.2 Methods

Women with invasive breast cancer (< 5 cm in size) proven by triple assessment and clinically axillary node negative (T1/T2, N0) were recruited following informed consent. Exclusion criteria included (1) pregnant and lactating women and (2) previous axillary surgery. SLNB was performed using the combined blue dye and radioisotope technique.

On the day prior to surgery, each patient received 0.2ml of 15MBq⁹⁹mTc-nanocolloid (Nycomed Amersham, UK) via a single intra-dermal injection to the skin overlying the tumour if palpable or at the areolar border of the corresponding quadrant if tumour was impalpable. The injection site was then massaged for 1 minute to encourage lymphatic drainage. The uptake of radiotracer was confirmed by pre-operative gamma camera imaging.
At the time of surgery, intra-dermal injection of 2ml of patent blue dye was administered in the same manner as the radioisotope injection 5 minutes prior to the surgical incision. SLNB was performed using gamma probe detection. Biopsy for internal mammary SLN was not performed. A SLN was defined as a lymph node, which was radioactive or blue-stained or both.

Once harvested, the SLN was bisected along its longitudinal axis and through the hilum. The sectioned surface was gently touched onto glass slides, and if possible, 3 imprints were made per slide. In total, 3 slides per SLN were made. This was followed by ESS spectral acquisition. The SLN were labeled and fixed separately in formaldehyde. TIC slides and SLN were sent to the Department of Histopathology.

For TIC, one slide was stained with rapid cytological stain (RAL555, Cell Path, UK), one with standard Giemsa stain and one with ICC staining (anticytokeratin). Slides were examined by Dr Gabrijela Kocjan, consultant cyto-pathologist, under light microscope but not in real time. The TIC results were compared with histology.

Histological sections of the cut surface of the SLN were made and were stained with H&E. In addition, 2 further H&E sections were made at different levels. Immunohistochemistry was performed if there were no metastases seen on H&E sections. Histo-pathological reporting was performed by Dr Mary Falzon. The lymph nodes were classified as negative or positive for metastases.

ESS spectra were correlated to histology. MBA was carried out, and two-thirds of the ESS spectra were randomly selected to establish the LDA algorithm. Once this has was done, the remaining one-third of the ESS spectra were used for testing.
6.3.3 Results

A total of 89 SLN were harvested from 53 patients. Of these 89 SLN, 8 were found to contain metastases. TIC correctly identified 6 of the 8 SLN with metastases and all 81 negative SLN giving sensitivity of 75% and specificity of 100% (Table 6.7). Of the 2 false negatives for TIC, one SLN had metastatic deposits on the sectioned surface which were seen on histological section but not on TIC despite a detailed review. The second false negative had a small focus of metastasis in deeper sections detected by immunohistochemistry.

**Table 6.7: Comparison of TIC results with histopathology**

<table>
<thead>
<tr>
<th></th>
<th>Histology</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>TIC</td>
<td>6</td>
<td>0</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>2</td>
<td>81</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>81</td>
<td>89</td>
<td></td>
</tr>
</tbody>
</table>

ESS correctly identified 6 of the 8 SLN with metastases and 75 of the 81 negative SLN. This gives ESS sensitivity of 75% and specificity of 93% (Table 6.8). Of the 2 false negatives for ESS, one SLN was the same false negative as TIC which had a small metastasis deposit in deeper sections detected by immunohistochemistry. The other is a different SLN which had metastatic deposits on the sectioned surface seen on histological section. A Student’s t-test on overall accuracy and Kappa value for TIC and ESS indicated equivalence at the 95% confidence level.
Table 6.8: Comparison of ESS results with histopathology

<table>
<thead>
<tr>
<th></th>
<th>Histology</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>ESS</td>
<td>Positive</td>
<td>6</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>2</td>
<td>75</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>8</td>
<td>81</td>
<td>89</td>
</tr>
</tbody>
</table>

6.3.4 Discussion

This study showed that TIC and ESS have similar sensitivity and specificity in determining SLN status when compared to standard histopathology with IHC. For ESS, the spectra were not tested prospectively in that two-thirds of the spectra were used for training whilst the remaining one-third was used for testing. Although this is not a direct comparison between ESS and TIC, the sensitivity of 75% and specificity of 93% achieved by ESS are comparable with most published reports on TIC and FFS in determining SLN status in breast cancer (Tables 6.9 and 6.10), and provide further evidence to support the potential role of ESS in assessing SLN status in breast cancer.

Unlike TIC, ESS assesses the SLN at individual points. To obtain the best performance, spectral acquisition has to be thorough, covering the entire sectioned surface. This may be responsible for one of the false negative where a metastasis was present in the sectioned surface. The other false negative had a small metastasis deposit in deeper sections detected by immunohistochemistry and was also missed by TIC. One possible solution to this problem is to have multiple sectioning. Further
development should aim to produce an ESS system capable of prospective spectral analysis so that a true comparison can be made between TIC and ESS.

There are numerous advantages of ESS in assessing SLN status. For example, individual spectral acquisition requires just over 1 second, and for an average size SLN, probably 15 to 20 spectral acquisitions are required. With the eventual development of real time spectral analysis, ESS would not require specialist interpretation. The ESS system is portable and optical probes can be sterilised. So SLN assessment can be performed by the operating surgeon in sterile conditions following excision. Furthermore, ESS systems are relatively cheap to produce.

The sensitivity and specificity of TIC in this series were 75% and 100% respectively, which is comparable with most published reports on TIC and FFS in determining SLN status in breast cancer (Tables 6.9 and 6.10). However, in an earlier series at our institute, Keshtgar et al. reported a higher sensitivity and specificity\textsuperscript{26}. The main advantages of TIC include: (1) TIC is a simple and quick technique, (2) TIC does not consume tissues unlike FFS. However the chief drawback is the requirement of an experienced cyto-pathologist, who is happy to interpret and to report the TIC in real time.
Table 6.9: Published studies of TIC in determining SLN status in breast cancer

<table>
<thead>
<tr>
<th>Study</th>
<th>Number of procedures</th>
<th>Gold Standard</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barranger</td>
<td>185</td>
<td>IHC</td>
<td>33.3</td>
<td>78.4</td>
</tr>
<tr>
<td>Bochner</td>
<td>79</td>
<td>IHC</td>
<td>37</td>
<td>100</td>
</tr>
<tr>
<td>Deo</td>
<td>76</td>
<td>Not stated</td>
<td>96.9</td>
<td>100</td>
</tr>
<tr>
<td>Ravichandran</td>
<td>132</td>
<td>H&amp;E</td>
<td>70</td>
<td>97</td>
</tr>
<tr>
<td>Aihara</td>
<td>118</td>
<td>H&amp;E</td>
<td>84</td>
<td>97.4</td>
</tr>
<tr>
<td>Bochner</td>
<td>53</td>
<td>IHC</td>
<td>53</td>
<td>100</td>
</tr>
<tr>
<td>Leidenius</td>
<td>139</td>
<td>IHC</td>
<td>68</td>
<td>98.5</td>
</tr>
<tr>
<td>Nagashima</td>
<td>124</td>
<td>Not stated</td>
<td>70.3</td>
<td>99.6</td>
</tr>
<tr>
<td>Creager</td>
<td>678</td>
<td>IHC</td>
<td>53</td>
<td>98</td>
</tr>
<tr>
<td>Baitchev</td>
<td>87</td>
<td>IHC</td>
<td>83.3</td>
<td>100</td>
</tr>
<tr>
<td>Yu</td>
<td>78</td>
<td>H&amp;E</td>
<td>97.4</td>
<td>100</td>
</tr>
<tr>
<td>Cserni</td>
<td>60</td>
<td>IHC</td>
<td>59</td>
<td>100</td>
</tr>
<tr>
<td>Motomura</td>
<td>101</td>
<td>IHC</td>
<td>90.9</td>
<td>98.5</td>
</tr>
<tr>
<td>Usman</td>
<td>55</td>
<td>H&amp;E</td>
<td>100</td>
<td>99.2</td>
</tr>
<tr>
<td>Van Diest</td>
<td>54</td>
<td>IHC</td>
<td>63</td>
<td>100</td>
</tr>
<tr>
<td>Total</td>
<td>2019</td>
<td></td>
<td>Average 70.6</td>
<td>Average 97.8</td>
</tr>
</tbody>
</table>

(Modified from Table 3: Implementation of Touch Imprint Cytology for Intra-operative Detection of sentinel lymph node metastases in breast cancer – Practice and implications. DW Chicken et al.25)
Table 6.10: Published studies of FFS in determining SLN status in breast cancer

<table>
<thead>
<tr>
<th>Study</th>
<th>Number of procedures</th>
<th>Gold Standard</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leidenius16</td>
<td>375</td>
<td>IHC</td>
<td>83</td>
<td>98.3</td>
</tr>
<tr>
<td>Nagashima17</td>
<td>124</td>
<td>Not stated</td>
<td>83.8</td>
<td>100</td>
</tr>
<tr>
<td>Motomura27</td>
<td>101</td>
<td>H&amp;E</td>
<td>52</td>
<td>100</td>
</tr>
<tr>
<td>Van Diest24</td>
<td>54</td>
<td>IHC</td>
<td>91</td>
<td>100</td>
</tr>
<tr>
<td>Cao28</td>
<td>234</td>
<td>IHC</td>
<td>85.7</td>
<td>100</td>
</tr>
<tr>
<td>Wada29</td>
<td>569</td>
<td>H&amp;E</td>
<td>84</td>
<td>100</td>
</tr>
<tr>
<td>Khalifa30</td>
<td>96</td>
<td>Not stated</td>
<td>86</td>
<td>100</td>
</tr>
<tr>
<td>Aihara14</td>
<td>42</td>
<td>Not stated</td>
<td>92</td>
<td>100</td>
</tr>
<tr>
<td>Van der Loo31</td>
<td>275</td>
<td>IHC</td>
<td>83</td>
<td>100</td>
</tr>
<tr>
<td>Gulec32</td>
<td>157</td>
<td>IHC</td>
<td>44</td>
<td>100</td>
</tr>
<tr>
<td>Veronesi33</td>
<td>295</td>
<td>H&amp;E</td>
<td>95.4</td>
<td>100</td>
</tr>
<tr>
<td>Tanis34</td>
<td>262</td>
<td>IHC</td>
<td>74</td>
<td>99</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>2584</strong></td>
<td></td>
<td><strong>Average 79.5</strong></td>
<td><strong>Average 99.8</strong></td>
</tr>
</tbody>
</table>

(Modified from Table 4: Implementation of Touch Imprint Cytology for Intra-operative Detection of sentinel lymph node metastases in breast cancer – Practice and implications. DW Chicken et al.25)

It is important to note that there are significant variations in the TIC technique. These include the number of sections made per SLN, longitudinal versus transverse sectioning, touching versus scraping of sections, the choice of stain and the inclusion of ICC, which may all have significant implications35. Apart from a comparative study on 635 axillary lymph nodes by Anastasiadis et al to show greater accuracy for MGG stain over H&E stain for TIC36, there are no large studies available to compare
the various variations specifically and to establish the optimal protocol. With multiple sectioning, this could prevent one of the false negatives (the one with a small metastasis deposit in deeper sections detected by immunohistochemistry) in this study. However, extra intra-operative time would be needed for additional processing and interpretation, which may not be desirable in a busy operating schedule. Also multiple sectioning can be difficult in small SLN.

As for the other false negative of TIC, tumour cells failed to be imprinted onto the glass slides therefore highlighting a technical limitation of TIC. However, it is important to stress that care and attention are required throughout the preparation process even though TIC is a “quick and simple” technique. Although there were no false positives for TIC in this study, potential pitfalls can occur. For example, large aggregates of lymphoid cells (Figure 6.1a) may have a deceptive epithelial appearance\textsuperscript{37}. Non-lymphoid cells such as follicular dendritic cell (Figure 6.b) can be confused with metastasis on immunocytochemistry\textsuperscript{37}.

A general limitation of this study is that both TIC and spectral analysis were not carried out in real time. However, a study is currently undertaken to compare the effect of real time reporting of TIC at our institution\textsuperscript{25}.
Figure 6.1: Potential pitfalls in TIC (a) Large aggregates of lymphoid cells and (b) Follicular dendritic cell

6.3.5 Conclusion

ESS has sensitivity and specificity comparable to TIC and FFS in assessing SLN status in breast cancer, but further developments and evaluations are necessary. TIC in our experience is a good technique for assessing SLN status

Reference List


Chapter 7  Elastic Scattering Spectroscopy for the detection of
Primary Breast Cancer

7.1  Introduction

A number of studies investigating the various optical biopsy technologies have so far indicated acceptable sensitivities and specificities in distinguishing normal, pre-malignant and malignant tissues in a variety of organs (Table 3.3). The reported sensitivities and specificities can be considered as the "headline" values giving an overall performance. There are numerous factors that can affect sensitivity and specificity. The underlying change in the optical properties of the tissue and the point where this becomes detectable are especially important.

From our experience in Chapter 6, a metastatic axillary lymph node can be replaced by cancerous tissue completely, partially or as micrometastasis. In the same manner, the focus where an ESS spectrum was acquired may contain a variable proportion of normal and malignant tissues. As both normal and abnormal tissues contribute to the overall optical properties of the tissue, the proportion of normal and abnormal tissues is likely to have significant influence on the sensitivity and specificity.

The aims of this chapter are (1) to determine whether the proportion of normal and cancerous breast tissues within the field of optical biopsy influences the sensitivity and specificity of ESS, (2) to determine the sensitivities and specificities of ESS in detecting breast cancer at specific percentage field change and (3) to evaluate the impact, if any, on the potential clinical application of ESS in the management of breast cancer.
7.2 Methods

7.2.1 Patient selection and specimen handling

Female patients with proven invasive breast carcinoma by triple assessment undergoing mastectomy were recruited following informed and written consent (Appendix B). Following mastectomy, the surgical specimen was placed in a sterile plastic bag, which was subsequently sealed. The bag containing the specimen was then placed in a plastic container of ice. The specimen was taken immediately to the Department of Pathology where the specimen was processed.

The breast specimen was first coated with a blue-black colored dye and was then sliced using a knife. Each individual slice measured 1cm in thickness. An appropriate slice of breast tissue containing the breast tumour was selected for this study.

7.2.2 Spectral acquisition

The ESS system was set up as described in Appendix A. An area on the breast slice, which contained both the breast tumour and normal breast tissue, was selected for the study. A plastic grid with an 8 by 8 array of 2mm diameter holes (Figure 7.1) was placed over the selected area and was secured to the breast tissue with the application of pins into the tissue through the additional holes at the corner of the grid (yellow holes). ESS spectra were acquired systematically starting from the top left corner hole then across the top row then moving down to the next lower row. A maximum of 64 spectra per grid were acquired. Following this, the specimen area was cut out into a separate tissue block using a scalpel and the slots in the plastic grid. The sides of the
tissue block were colored using a different colored dye to allow histological correlation later. The different colours used for the side of the tissue block were recorded.

**Figure 7.1:** Diagram of grid

7.2.3 Histological correlation

The breast tissue block underwent standard histological processing. The surface where ESS spectra were obtained was sectioned, stained with H&E and mounted onto a glass slide. A transparent sheet with an 8 by 8-printed grid to scale was placed over the slide. The slide was orientated according to the colour coding of the original breast tissue block. Individual grid cells were reviewed by Dr Mary Falzon, consultant pathologist. Estimated percentages of tumour, fat and normal breast tissue per grid cell were recorded and correlated to the corresponding ESS spectra.
7.2.4 Spectral analysis

Spectral analysis using MBA as described in Section 3.5 was performed with different criteria for the selection of training and testing datasets (Analysis 2 and 3) in order to determine the sensitivities and specificities of ESS in detecting breast cancer at different percentages of field change. Because of the use of a grid system, there is a potential that spectra from a specific grid may have grid-specific characteristics. This could result in spectra being classified as normal or malignant breast tissue spectra based on the grid-specific characteristics rather than tumour-specific characteristics. The aim of Analysis 1 is to evaluate this possibility.

7.3 Analyses and Results

Data were obtained from 8 tissue blocks, which will be referred to as “Grids” for the purposes of simplicity. Each grid cell within each grid will be referred to as a “cell”. The paired ESS spectrum and corresponding histology of a cell will be referred to as a “dataset”.

Table 7.1 shows the breakdown of data. Some of the Grids have less than 64 datasets because some of the cells particularly at the edges of the grids were destroyed during histological processing. Therefore, ESS spectra with no corresponding histology were discarded. Grid 3 was taken from a smaller piece of tissue, and therefore only has 28 datasets. Grids 5 and 6 were taken from the same patient, and Grids 7 and 8 were also taken from the same but a different patient. The number of categories describes the number of groups of datasets with different percentages of cancer replacement. Grid 8 was taken from macroscopically normal breast tissue with no malignant tissue seen on histology and hence has only 1 category in Table 7.1.
Table 7.1: Breakdown of grid data

<table>
<thead>
<tr>
<th>Grid</th>
<th>Number of Datasets</th>
<th>Number of Categories</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>60</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>64</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>28</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>64</td>
<td>12</td>
</tr>
<tr>
<td>5</td>
<td>58</td>
<td>14</td>
</tr>
<tr>
<td>6</td>
<td>57</td>
<td>13</td>
</tr>
<tr>
<td>7</td>
<td>64</td>
<td>4</td>
</tr>
<tr>
<td>8</td>
<td>64</td>
<td>1</td>
</tr>
</tbody>
</table>

The overall distribution of cells with different percentages of cancer is illustrated in Figure 7.2. The overall distribution is skewed as the majority of cells were normal without cancer (i.e. 0%). The distribution of other categories is not even. Within each grid, the distribution was also uneven (Figure 7.2 lower panel). For example, Grids 2, 5 and 6 have significantly more cells with no cancer whilst Grid 4 has majority of cells with high percentages of cancer.
Figure 7.2: Distribution of percentage cancer overall and within tumour grids 2, 3, 4, 5 and 6.

% Cancer across all tumours

(Tumours 1, 7 and 8 are omitted because they only have 2, 4 and 1 categories respectively.)
Analysis 1: Analysis to determine the influence of grid-specific characteristics on spectral classification

Because of the variations among the grids in the distribution of cells with different percentages of cancer, there is a potential that this may influence subsequent analyses. The purpose for this analysis is to investigate the influence of patient grid-specific characteristics on spectral classification by determining whether spectral analysis can identify the correct patients' grids from which spectra were acquired.

Standard principle components were calculated. Spectra were paired to the grid they were acquired (i.e. Grid 1) irrespective of the underlying histology. 60% of all the datasets were randomly selected as training set and the remaining 40% were used for testing. The analysis was repeated several times in a bootstrap manner to provide an average result, which is illustrated in Table 7.2. Sensitivity and specificity of a particular grid are defined as the sensitivity and specificity of spectra analysis in distinguishing spectra of that particular grid from all the rest in the testing set. For example, the sensitivity of 0.84 in Grid 8 means that the spectral analysis correctly classified 84% of Grid 8 spectra in the testing set as coming from Grid 8. Specificity of 0.97 means that spectra analysis correctly classified 97% of non-Grid 8 spectra in the testing set as non-Grid 8. The analysis showed that it is possible to classify spectra from Grids 1, 2, 3 and 4 according to their grid with good accuracy. However, the analysis found difficulty in discriminating the spectra between Grids 5 and 6, and between 7 and 8. In view that Grid 5 and 6 were specimens from one patient, and Grids 7 and 8 were another, this suggests that certain factors within the spectra that are specific to the particular surgical specimen or patient.
In view of these findings, an investigation was carried out to identify the parts of the ESS spectra that are characteristic of the actual tissue specimen of the grids (i.e. individual patient) rather than to the underlying histology (i.e. cancer or normal). A mathematical model was created which calculated the variance within the spectra that were due to the actual tissue specimen (i.e. the patient grid) and the residual variance at individual wavelengths. The ratio of the residual variance and variance of the tissue specimen were plotted in Figure 7.3 (solid line). If the ratio is below 1 at a particular wavelength, this suggests that this part of the spectrum is influenced by the actual tissue specimen. This found that the part of ESS spectra between 510 and 660nm (the grey shaded area in Figure 7.3) is influenced mainly by the actual tissue specimen irrespective of the actual underlying histology.

<table>
<thead>
<tr>
<th>Grid</th>
<th>Number of Datasets</th>
<th>Sensitivity</th>
<th>Standard error</th>
<th>Specificity</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>60</td>
<td>1.00</td>
<td>0.00</td>
<td>0.99</td>
<td>0.00</td>
</tr>
<tr>
<td>2</td>
<td>64</td>
<td>0.99</td>
<td>0.00</td>
<td>0.99</td>
<td>0.00</td>
</tr>
<tr>
<td>3</td>
<td>28</td>
<td>0.96</td>
<td>0.01</td>
<td>1.00</td>
<td>0.00</td>
</tr>
<tr>
<td>4</td>
<td>64</td>
<td>1.00</td>
<td>0.00</td>
<td>1.00</td>
<td>0.00</td>
</tr>
<tr>
<td>5</td>
<td>58</td>
<td>0.86</td>
<td>0.01</td>
<td>0.99</td>
<td>0.00</td>
</tr>
<tr>
<td>6</td>
<td>57</td>
<td>0.83</td>
<td>0.01</td>
<td>0.98</td>
<td>0.00</td>
</tr>
<tr>
<td>7</td>
<td>64</td>
<td>0.81</td>
<td>0.01</td>
<td>0.97</td>
<td>0.00</td>
</tr>
<tr>
<td>8</td>
<td>64</td>
<td>0.84</td>
<td>0.01</td>
<td>0.97</td>
<td>0.00</td>
</tr>
</tbody>
</table>
Figure 7.3: Study of variance to determine the area within ESS spectrum influenced by the actual tissue specimen

Analysis 2: To determine the sensitivity of ESS in discriminating spectra taken from cells containing between 1-80% cancer tissue

For this analysis, datasets from Grids 2, 3, 4, 5 and 6 were used because these grids contain a variety of cells with different percentages of cancer. Datasets were grouped into the following: 0% (i.e. no cancer), 1-20%, 21-40%, 41-60%, 61-80 and 81-100% according to the percentage of cancer detected by histology. MBA was performed using all 0% (51 datasets) and 80-100% (42 datasets) datasets for training. Once a LDA algorithm was established, the remaining datasets (1-20%, 21-40%, 41-60%, 61-80%) were used for testing. The results are illustrated in Table 7.3.
**Table 7.3: Results of Analysis 2**

<table>
<thead>
<tr>
<th>Percentage cancer</th>
<th>Number of spectra tested</th>
<th>% correctly classified as cancerous</th>
<th>% incorrectly classified as normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-20%</td>
<td>39</td>
<td>69%</td>
<td>39%</td>
</tr>
<tr>
<td>21-40%</td>
<td>28</td>
<td>91%</td>
<td>9%</td>
</tr>
<tr>
<td>41-60%</td>
<td>36</td>
<td>67%</td>
<td>33%</td>
</tr>
<tr>
<td>61-80%</td>
<td>39</td>
<td>96%</td>
<td>4%</td>
</tr>
</tbody>
</table>

This showed that sensitivity in detecting cancer is higher when the cell has a higher percentage of cancer. It is worth noting that there is no specificity in this analysis as all 0% (normal spectra) were used for training. A second analysis was also carried out using the same criteria except that the ESS spectra between 510 and 660nm (as identified in Figure 7.3) were excluded. The result is illustrated in Table 7.4 showing a slight improvement.

**Table 7.4: Results of Analysis 2 with 510-610nm range of spectra excluded**

<table>
<thead>
<tr>
<th>Percentage cancer</th>
<th>Percentage correctly classified as cancerous</th>
<th>Percentage incorrectly classified as normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-20%</td>
<td>72%</td>
<td>28%</td>
</tr>
<tr>
<td>21-40%</td>
<td>91%</td>
<td>9%</td>
</tr>
<tr>
<td>41-60%</td>
<td>78%</td>
<td>22%</td>
</tr>
<tr>
<td>61-80%</td>
<td>96%</td>
<td>4%</td>
</tr>
</tbody>
</table>
Analysis 3: MBA using 60% of datasets randomly selected for training to determine the sensitivity and specificity of ESS in discriminating between the remaining 40% of datasets

For this analysis, datasets from Grids 2, 3, 4, 5 and 6 were used. Datasets were grouped into the following: 0% (i.e. no cancer), 1-20%, 21-40%, 41-60%, 61-80 and 81-100% according to the percentage of cancer detected by histology. 60% of datasets from all groups were randomly selected for MBA. Once a LDA algorithm was established, the remaining 40% of datasets were used for testing. This analysis was repeated 100 times (“bootstrapping”) to give an average sensitivity and specificity for each group. The results are illustrated in Table 7.5a. As the figures are averages, the positive and negatives do not necessarily add up to 1. This shows that the average specificity is 74% (i.e. 0% datasets classified as negative). Between 1-20%, average sensitivity is 56% indicating that spectral analysis is poor at discrimination. However the average sensitivity increases as the amount of cancer increases. Table 7.5b showed a slight improvement when the ESS spectra between 510 and 660nm were excluded from analysis.
Table 7.5: Results of Analysis 3 (a) without and (b) with 510-610nm range of spectra excluded

(a)

<table>
<thead>
<tr>
<th>Group</th>
<th>Positive (SD)</th>
<th>Negative (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>0.28 (0.02)</td>
<td>0.74 (0.02)</td>
</tr>
<tr>
<td>1-20%</td>
<td>0.56 (0.02)</td>
<td>0.44 (0.02)</td>
</tr>
<tr>
<td>21-40%</td>
<td>0.72 (0.02)</td>
<td>0.28 (0.02)</td>
</tr>
<tr>
<td>41-60%</td>
<td>0.60 (0.01)</td>
<td>0.40 (0.01)</td>
</tr>
<tr>
<td>61-80%</td>
<td>0.90 (0.01)</td>
<td>0.10 (0.01)</td>
</tr>
<tr>
<td>81-100%</td>
<td>0.87 (0.04)</td>
<td>0.17 (0.01)</td>
</tr>
</tbody>
</table>

(b)

<table>
<thead>
<tr>
<th>Group</th>
<th>Positive (SD)</th>
<th>Negative (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>0.26 (0.02)</td>
<td>0.76 (0.02)</td>
</tr>
<tr>
<td>1-20%</td>
<td>0.60 (0.02)</td>
<td>0.40 (0.02)</td>
</tr>
<tr>
<td>21-40%</td>
<td>0.78 (0.02)</td>
<td>0.22 (0.02)</td>
</tr>
<tr>
<td>41-60%</td>
<td>0.69 (0.01)</td>
<td>0.31 (0.01)</td>
</tr>
<tr>
<td>61-80%</td>
<td>0.95 (0.00)</td>
<td>0.05 (0.00)</td>
</tr>
<tr>
<td>81-100%</td>
<td>0.90 (0.04)</td>
<td>0.14 (0.01)</td>
</tr>
</tbody>
</table>
7.4 Discussion

The original intentions for the grid system were to increase our collection of ESS spectra on breast tissues and to improve the correlation between ESS spectra and histology. Subsequently, this evolved to become a study to determine the minimal percentage of breast cancer present within the tissue that could be detected by ESS. This study has therefore provided further information and understanding into ESS in addition to the original objectives.

With the exception of Grid 8, the variety in the distribution of cells with different percentages of cancer among the grids was not intended. However, with Grids 2, 5, and 6 having more normal datasets and Grid 4 having more datasets with higher percentages of cancer replacement, this creates an interesting situation. Could this influence spectral classification i.e. can an ESS spectrum be classified as normal or cancerous based on which grid it came from? Do ESS spectra from a particular grid have some aspects in common and specific to that particular grid? Analysis 1 showed that this appears to be the case, and was particularly good at distinguishing the spectra of Grids 1, 2, 3 and 4 from each other. It was less accurate in distinguishing spectra between Grids 5 and 6, and between Grids 7 and 8. Indeed, Grids 5 and 6 were taken from the same breast specimen of one patient, and Grids 7 and 8 were also taken from the same breast specimen but from a different patient. This suggests that certain aspects of an ESS spectrum are specific to the tissue specimen rather than to the actual histology. These may be due to (1) patient factors (i.e. ESS spectra with characters specific to the individual patient or tumour), (2) technical factors (i.e. the set-up and functioning of the ESS system on the day), (3) specimen factors (i.e. specimen handling and timing of spectral acquisition) and (4) environmental factors.
Subsequent analysis found that ESS spectra between 510 and 660nm showed patient grid-specific characteristics. As this spectra region is dominated by the absorption effects of haemoglobin, the oxygenation status of haemoglobin in the tissue may be responsible for the findings. However, this does not exclude patient, technical and environmental factors, and further investigations may reveal more information.

The limitation of the axillary lymph node study in Chapter 6 was that spectral analyses were performed using normal lymph nodes and lymph nodes with more than 80% metastatic tumour replacement. Therefore, the 88% sensitivity and 96% specificity achieved was only for discriminating ESS spectra taken from an area of the lymph node with no or extensive metastatic cancer. Because it was not possible to correlate ESS spectra taken from areas with lesser metastatic tumour replacement with the underlying histology, it was not possible to assess sensitivity and specificity of ESS with partial metastatic replacement. An attempt to use the grid system on lymph nodes failed because histological processing of lymph nodes altered the shape of the lymph node significantly and it was not possible to correlate ESS spectra to the precise histology. However it was possible for primary breast tissue as in this chapter.

Analysis 2 is an extension of the lymph node study in Chapter 6. As in the lymph node study, only the datasets from normal breast tissue and tissue with extensive invasive ductal carcinoma were used for training. The testing set consisted of ESS spectra taken from tissues with variable amount of breast cancer. The result showed that ESS is able to detect cancer in spectra taken from areas with 1-20% tumour. The sensitivity and specificity improve where there is a higher proportion of cancer.
The datasets used for training are as important as the actual process of spectral analysis. In Analysis 2, only datasets from normal breast tissue and tissue with extensive invasive ductal carcinoma were used showing good sensitivities. The purpose for Analysis 3 was to determine whether sensitivity and specificity can be improved with additional datasets from tissues with less extensive invasive ductal carcinoma. Unlike Analysis 2, datasets were randomly selected for training and testing with a 60/40 split, and analysis was bootstrapped (i.e. carried out 100 times with different random selections of training and testing datasets) (1) to provide an average sensitivity and specificity and (2) to negate the effect of a randomly selected training and testing sets with particular bias. The results showed reduced sensitivity for the detection of cancer by ESS in tissue which has between 1-20% cancer. However as with Analysis 2, sensitivity increases with more cancer within the field of ESS spectra.

The second parts of Analyses 2 and 3 attempted to determine the impact of the “grid-specific” part of ESS spectra. This was done by removing the part of the spectra between 510- 660nm from the analyses. In both cases, there were slight improvements in the sensitivities thus suggesting a minor effect.

The low ESS sensitivity in the 1-20% cancer group can be explained by the small amount of tumour resulting in no or insignificant changes in the elastic scattering and light absorption properties of tissue. However, another possibility is the position of the tumour tissue in relation to the optical fibres of the optical probe. Given that light has to propagate through the tissue from the delivery fibre to the receiving fibre of the optical probe, if the tumour is situated in this pathway, it is more likely to cause...
alterations in ESS spectra (Figure 7.4a) then otherwise (Figure 7.4b). If this is a contributory factor for the low sensitivity, then multiple receiving fibres surrounding the delivery fibre may improve sensitivity.

**Figure 7.4:** Illustration to demonstrate the influence of the site of tumour in relation to the path of light (blue arrows)

The results of Analyses 2 and 3 indicate that ESS has acceptable sensitivity to detect primary breast cancer when the field of ESS interrogation contains 21% or more of breast cancer. This would influence the potential applications of ESS in the management of breast cancer. Whether ESS has equivalent sensitivity in detecting
metastatic breast cancer in lymph nodes needs further investigation. Assuming that this is possible, ESS has a potential role to play in the diagnosis and surgical management of breast cancer. These could involve (1) the optical diagnosis of breast cancer, (2) optical guided breast core biopsy, (3) intra-operative assessment of resection margins of breast cancer and (4) intra-operative assessment of SLN in breast cancer.

Table 7.7 illustrates the range of sensitivity and specificity of the various modalities in the diagnosis of breast cancer. Although this is a crude comparison of sensitivities and specificities, ESS appears to be comparable with other modalities. It is important that optical diagnosis should complement existing modalities without additional complexity, which can only result in unnecessary anxiety, excision biopsy and delay in breast cancer diagnosis. Therefore situations where optical diagnosis may be of benefit include remote areas where there are no radiological and pathological services. In this situation, the optical probe can be inserted into a breast through a hollow needle. ESS spectra can be taken and immediately analysed to provide the diagnosis or alternatively give an indication whether the lump contains suspected malignancy so that appropriate management can be arranged. Otherwise, optical diagnosis can be incorporated into other technologies such as mammary duct endoscopy. Optical probes can be miniaturized to fit into channels of the endoscope for this application. Obviously, the role of optical diagnosis in this situation requires further evaluation.
Table 7.6  Comparison of sensitivity and specificity of various modalities for breast cancer diagnosis

<table>
<thead>
<tr>
<th>Modality</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mammography(^1)</td>
<td>72-94%</td>
<td>75-94%</td>
</tr>
<tr>
<td>Ultrasound(^2)</td>
<td>86%</td>
<td>90%</td>
</tr>
<tr>
<td>MRI(^3)</td>
<td>Near 100%</td>
<td>Low</td>
</tr>
<tr>
<td>Scintimammography(^4)</td>
<td>50-95%</td>
<td>58-100%</td>
</tr>
<tr>
<td>PET(^5)</td>
<td>80-100%</td>
<td>80-100%</td>
</tr>
<tr>
<td>FNAC(^6)</td>
<td>Absolute 57.1%*</td>
<td>58.4%</td>
</tr>
<tr>
<td>Core biopsy(^6)</td>
<td>Absolute &gt;76.4%*</td>
<td>81.2%</td>
</tr>
<tr>
<td>ESS</td>
<td>65-95%</td>
<td>76%</td>
</tr>
</tbody>
</table>

* Absolute sensitivity = \( \frac{\text{number of breast cancers with C/B 5 on FNAC/CNB}}{\text{total number of carcinomas undergoing FNAC/CNB}} \) x 100%

Complete sensitivity = \( \frac{\text{number of breast cancers with C/B 3, 4 and 5 on FNAC/CNB}}{\text{total number of carcinomas undergoing FNAC/CNB}} \) x 100%

ESS technology can also be incorporated into existing diagnostic modalities such as breast core biopsy. The biopsy needles can be manufactured with incorporated optical fibres. In this context, ESS could confirm the correct position of the biopsy needle. The principle advantage is to minimize false negative core biopsies. In our institution, ESS has been applied in gastro-intestinal endoscopy, although it is not yet firmly established.
Resection margins in breast conservation surgery continue to be an important issue in the surgical management of breast cancer. Although TIC has been shown to be useful for intra-operative assessment of resection margins, it has not been widely practiced because of practicalities. At present, resection margins are assessed with histology. The limitations are (1) only a small area is actually assessed and (2) if the resection margin is positive, further resection would have to be carried out as a separate operation. ESS has a potential role in assessing resection margins in real time without the need of tissue preparation and pathological interpretation. Further margin resection can be undertaken to achieve complete resection at the same time if necessary. Although ESS sensitivity is poor when there is small amount (<20%) of cancer present, this should not be a significant issue as further resection is indicated when there is a moderate to extensive presence of cancer. For focal or minimally involved margins, post-operative radiotherapy is adequate in the management. This was discussed in Chapter 2.

The value of intra-operative assessment of SLN was already discussed in Chapter 2 and 6. If the sensitivity of ESS in detecting metastatic breast cancer in lymph nodes is equivalent to that in detecting primary breast cancer as demonstrated in this study, ESS has a definitive role in the intra-operative detection of SLN metastasis. Again the low ESS sensitivity when only 1-20% of cancer is present means that ESS is unlikely to detect micrometastasis. However, the optimal management of the axilla with micrometastatic disease has not yet been established and randomised clinical trials are currently in progress to evaluate surveillance and axillary radiotherapy in place of ALND. As for larger SLN metastases where ALND is indicated, ESS is expected to be able to detect these lesions.
At present, spectral analysis is empirical as it is not completely known which histological and cytological features correspond to specific features of the ESS spectra. With better understanding of how ESS spectra are generated, more accurate spectral analysis can be produced.

7.5 Conclusion

This study has demonstrated:

- ESS spectra reflect both the characteristics of the underlying histology and of the individual tumour
- The individual tumour characteristics are in the spectral region between 520-660nm, which is dominated by the absorption properties of haemoglobin
- Sensitivity of ESS is greater when there is a higher percentage of cancer within the field of spectral acquisition
- ESS is able to detect breast cancer from areas with >20% tumour replacement with acceptable sensitivity
- ESS has potential clinical roles in the diagnosis and surgical management of breast cancer

Reference List


Chapter 8  Summary and Future Developments

8.1  Summary

The concept of ESS is relatively simple. Light entering tissue undergoes elastic scattering or absorption, which is dependent on the optical properties of the tissue. ESS is sensitive to those changes brought on by the malignancy. However, the eventual success and widespread clinical applications of ESS depend on various factors. These include scientific and technical development, validation of technology, intellectual rights, funding of start-ups, regulatory approval, physician adoption and social acceptance. This thesis has addressed a few of these aspects within the clinical setting of breast cancer.

Chapters 1 and 2 reviewed the current status in the diagnosis and surgical management of breast cancer. The sensitivities and specificities of various modalities for the diagnosis and detection of breast cancer were stated providing the basis for comparison with ESS. Chapter 3 provided a review of ESS which was pitched for clinicians. It also identified potential clinical applications of ESS in the management of breast cancer. These include ESS guided percutaneous breast core biopsy, intra-operative assessment of tumour excision margin and SLN for metastasis. Subsequent chapters were designed with these potential applications in mind.

Chapter 5 addressed the various technical issues involving the effects of ambient light intensities, tissue handling and formaldehyde fixation on ESS, which have not been investigated before. It found that ambient light can enter the ESS system. At ambient light intensities ≤1000lux (i.e. indoor or laboratory light intensity), the effect on the
ESS spectra is unremarkable. At higher light intensities (i.e. beneath an operating lamp), the effects on ESS spectra becomes significant. However, the processes of spectral acquisition and standardisation can potentially resolve this effect as discussed in Section 5.2.4. Until this is fully investigated, spectra acquisition under strong ambient lighting (i.e. under an operating lamp or direct sunlight) should be avoided.

As for the effects of tissue handling, the changes observed in ESS spectra within the first hour following excision were related to the changes in oxygenation status of haemoglobin. Otherwise, there were no other changes to the ESS spectra irrespective of leaving the tissue specimen exposed in open laboratory conditions, chilled in ice or submersed in saline and formaldehyde during this time frame. However, significant changes in ESS spectra were observed gradually with formaldehyde fixation. Further studies are needed to clarify the effects of formaldehyde fixation in the first hour. Therefore, spectra acquisition from tissue specimens which have been submerged in formaldehyde is not recommended if spectra are intended for clinical use. In addition to technical issues, these studies demonstrated the reproducibility of ESS spectra, which is fundamental to the ESS concept.

Chapters 6 and 7 provided technical validation of the ESS concept and spectral analysis using MBA. In Chapter 6, ESS was applied to the detection of breast cancer metastasis in axillary lymph nodes. The “per spectrum” sensitivity and specificity achieved were between 88-90% and 91-96% respectively. The overall “per node” sensitivity was 89%. The “per spectrum” results are significant improvements from those achieved by Dr Briggs. This is primarily because of the improved correlation between spectra and histology, and the restriction in using only normal and metastatic
lymph nodes with >80% metastasis. This chapter highlighted the importance of accurate histological correlation of the training and testing datasets. It also identified the importance of partial replacement by cancer within the area of spectral acquisition, which subsequently aided the development of the grid system in Chapter 7.

The comparative study of ESS and TIC in the second part of Chapter 6 was the first clinical study comparing ESS to other established clinical techniques. Although it was not prospective and has a relatively small numbers, it demonstrated that both techniques have similar sensitivity and specificity, and provides support for further studies.

In addition to a more accurate histological correlation, the grid system used in Chapter 7 enabled the reporting of sensitivities at different percentage replacement by cancer within the area of spectral acquisition, which in turn allowed the evaluation of this issue. This found that the sensitivity of ESS increases (up to 96%) as the percentage of cancer replacement increases (Tables 7.3 and 7.5). The use of bootstrap and jackknife analyses provided more robust spectral analyses and in turn further technical validation to the spectral analysis. This provides a platform for further developments of spectral analysis. Furthermore, the reporting of sensitivities at different percentages of cancer replacement should be adopted as the standard format for reporting of performance of optical biopsy technologies.

8.2 Future Developments

ESS is an exciting technology. As an emerging technology, further developments are essential in order to further the understanding of ESS and to improve its performance.
Further scientific and technical developments such as statistical/mathematical aspects of spectral analysis and optical probe technology are necessary. However, these should be balanced with clinically orientated studies in order to address the clinical issues since the barrier for new technologies is often the end-users i.e. the clinicians. Several areas have already been identified and discussed such as the effect of strong ambient light intensities on spectral analysis and further formaldehyde studies. The development of spectral analysis systems for real time prospective studies is the most important of all for the ultimate clinical validation of ESS, to allow prospective comparisons with other modalities and to gain regulatory approval. Therefore, the expansion of datasets with accurate histological correlation for use as training data remains a top priority.

Societal acceptance of ESS technology is essential and the need for this should not be underestimated. Despite the advantages and safety of ESS, careful consideration is required when considering the potential clinical applications of ESS. It is important to appreciate that ESS should compliment or enhance the existing technologies and clinical practices without adding complexity or patient anxiety. In addition to assessing tumour excision margins and metastases, potential roles for ESS include guiding biopsies during endoscopy and surveillance of pigmented skin lesions.
Optical Biopsy System

Operating Manual

Prepared by Andrew C Lee
Before setting off

Before setting off from the NMLC, it is advised to check that all equipment are complete in order to avoid unnecessary trips to collect forgotten items. The following is the check list:

In the computer bag: Computer, Power cable for computer, Foot switch, Calibration pot, Optical probe

(Figure 1)

In the travel case: OBS, power cable for OBS

*Figure 1: Equipment as packed in computer bag*

It is also important to check that the optical probe is functional. This is done by pointing the tip of the probe to a light source and checking that light is present at the connection point with good lamination (Figure 2). The optical probe is designed to have 2 optical fibres of different diameter.

*Figure 2: Checking the optical probe*
The OBS should be hand carried to avoid lose connection between the components within the OBS as the result of bumpy pavements.

**Setting up the OBS**

The OBS should be set up within reach of power point. To avoid unintentional damage to the optical fibre by treading on or bending it, the OBS system and the fibre should not cause any obstruction. The OBS should be set up in the following steps:

1. Place the computer on top of the OBS
2. Connect the connection cable of the OBS to the interface card of the computer situated on the right hand side of the computer (Figure 3). Ensure that the white dot of the connection cable corresponds to the white dot on the interface card (Figure 4).

*Figure 3: Interface card at side of computer (Note the white dot (dotted circle))*
3. Connect the OBS and computer to the power mains. If only one power socket is available, use it for the OBS. The computer can operate with its own power supply.

4. Connect the optical probe to the OBS. Ensure that the lamp fibre is connected to the “Lamp” socket on the front of the OBS and the detector fibre to the “Detector” socket (Figure 5). The lamp fibre has a larger diameter which is obvious when looking at the connection end of the optical probe whilst pointing the tip of the probe to a light source.

Figure 5: Connection sockets for optical probe

5. Switch the computer on
Setting up the software

Once the Microsoft Windows 98 is loaded, please follow the following steps:

1. Select the “Optical biopsy” icon
2. Select Bms2000
3. Select Bmsspec2.exe and a separate window opens
4. Under “LANL”, select “Use Standard Run Configuration”
5. Place the probe tip in the calibration pot with the lid on and keep the tip approximately 1mm away from the Spectralon

**Figure 6:** Calibration in progress

6. Press “F5” on the keyboard to trigger the lamp to fire and a spectrum would appear.
7. Examine the overall pattern of the spectrum (Figure 7), which can be altered by dirt/blood at the tip of the probe.

**Figure 7:** Typical optical spectrum from spectralon
8. Use alcohol wipes to remove any blood/dirt.
9. Once a satisfactory spectrum is obtained, press the button with the yellow light bulb in the tool bar and followed by the button with the blue R.
10. Note the “np” number at the bottom right of the screen. This is required for spectral analysis latter on.
11. To save optical spectra, select “LANL” and “File save option”
12. A separate window appears with two boxes. Enter the unique study number for your patient e.g. “L299”. In the second box, enter 11. Press return to exit. The first spectrum will be saved as L29911.
13. To use the foot pedal, connect the pedal (Figure 8)

Figure 8: Connecting the foot pedal

14. To enable auto save, select “Setup” option and then click on “Auto save on foot pedal”.
15. The OBS is now ready for use.

Acquiring spectral data

To acquire spectral data, the tip of the probe must be in direct physical contact with the tissue concerned. Care should be taken not to apply too high a pressure onto tissue. Spectral measurement without saving is executed by pressing “F5” key or the foot pedal if the latter is not set up to auto save. Spectral measurement with auto save is executed by pressing “F6” key or the foot pedal if it is activated for auto save. The spectral number illustrated in the bottom of the screen indicates the spectral number of the next spectrum to be saved. It is generally advised to document the spectral number and the tissue specimen to ensure accurate correlation between spectrum and tissue histology. After the acquisition of spectra from
After spectral acquisition, go back to BMS200 folder. Click on Data folder. Inside this folder are the saved spectra. In this folder create a folder and name it with your patient’s study number. Transfer all the spectra of this study into this folder so that it can be exported for analysis.

**Packing up**

To exit the Bmsspec2, simply close the window. Turn computer off. Disconnect the OBS. Do not eject the computer interface card especially if the computer is still on. Ensure that all components are clean. Remove any blood/tissue fragments using alcohol wipes. To avoid any damage to optical probes, coil all fibres and place them in container bags. Pack all components into their appropriate carriers. Make sure nothing is left behind.

**Probe care**

Optical probes are very delicate and expensive. After use, please clean the entire length of the probe and the probe tip and connection ends with alcohol wipes. Do not use any abrasive materials to clean the ends.

Probes can be cleaned using the endoscopy wash cycle. The connection end must be outside the cleaning fluid. Staff at the endoscopy unit are happy to run the wash cycle for you.

For sterile probe, ethylene oxide is used. This is done by a commercial company in Scotland. The probe must be cleaned from physical dirt/blood/tissue, and placed in the instrument bags, which need to be sealed and sent via courier. The process takes 1 week minimum. Please consult Dr Sandy Mosse.

If using the probe under sterile condition, the calibration pot must also be sterilized. This can be done by autoclave.

**Further queries**

If you have further queries, you can contact me (Andrew Lee) on 0788 755 0608 or via email: aclee@doctors.org.uk
APPENDIX B

CONFIDENTIAL
PATIENT INFORMATION SHEET

OPTICAL BIOPSY FOR DIAGNOSIS OF LESIONS IN THE BREAST

Mr A Lee, Mr M Keshtgar, Dr M Hall-Craggs, Prof S G Bown, Mr R Sainsbury, Dr S Lakhani

We are sorry you have developed an area in your breast that needs investigation. The normal way to do this is to insert a thin needle into the suspicious area under local anaesthetic and remove a tiny piece of tissue to examine under the microscope. Sometimes this is just done in the outpatient clinic and on other occasions some form of imaging (usually an ultrasound scanner or a magnetic resonance (MR) scanner) is used to be sure the needle is in the right place. The tissue specimen is then processed and examined under a microscope. We are assessing a new technique known as "optical biopsy" which in due course, may be able to give an immediate answer, but at present we need to study how best to use it. We should like to ask you if we could take optical biopsy measurements on your breast at the same time as a conventional biopsy so the results from the two can be compared.

The optical biopsy system is very simple in concept. A thin optical fibre (about the thickness of a piece of thread) is put down the needle used for the conventional biopsy and a short burst of white light passed down the fibre. The diagnostic information is obtained by analysing the light that is reflected back up the fibre. The measurement only takes a few seconds and the power of the light used is so low that it will not affect you in any way. Thus the optical biopsy will only increase the time of your routine biopsy procedure by a few minutes and will not cause any additional discomfort.

If it is decided that you need an operation on your breast or your axilla (the area under your arm next to the breast), we should also like to take optical biopsy measurements during surgery on the tissues seen during the operation that are going to be removed, and then compare the results with microscopic examination of the tissues in the laboratory. One of our research team will explain to you which areas we would like to measure in your case.

You do not have to take part in this study if you do not want to. Please discuss it with anyone you wish before making a decision. If you decide to take part, you may still withdraw at any time without giving a reason. Your decision whether to take part will not affect the rest of your care and management in any way. If you agree to take part, we will ask you to sign a consent form. All proposals for research using human subjects are reviewed by an ethics committee before they can proceed. This proposal was reviewed by the joint UCL/UCLH committees on the ethics of Human Research.
Supplementary information sheet

Principal investigator: Prof SG Bown.
Other investigators: Mr A Lee, Mr M Keshtgar, Dr M Hall-Craggs, Mr R Sainsbury, Dr S Lakhani

As this research programme is being funded by the United States Army Medical Research and Materiel Command (USAMRMC) as part of a major research programme on the diagnosis and treatment of breast cancer, we are asked to provide further specific information to you before you agree to participate in the study.

Naturally, you will not be asked to pay any costs in relation to this study. We are not able to pay you for your participation, but we will reimburse you for any out of pocket travel expenses incurred if you make additional visits to this hospital solely related to your participation in this study. Should you be injured as a direct result of participating in this research project, you will be provided medical care, at no cost to you, for that injury. You will not receive any injury compensation, only medical care. You should also understand that this is not a waiver of your legal rights. You should discuss this issue thoroughly with the principal investigator before you enroll in this study. If there is any possibility that you might be pregnant, you should not volunteer for this study. A urine pregnancy test will be done prior to treatment, and you will be excluded if the results are positive.

Representatives from the US Army Medical Research and Materiel Command or the Food and Drug Administration may inspect the records of the research in their duty to protect human subjects in research. By enrolling in this study, you should understand that the United States Army Medical Research and Materiel Command (USAMRMC) will collect certain information about you, including your name, address, study name and dates. The purpose is, first, to readily answer an individual's questions about their participation in research sponsored the USAMRMC; and second, to ensure that the USAMRMC can exercise its obligation to ensure research volunteers are adequately warned of risks and to provide new information as it becomes available. The information will be retained in this database for a minimum of 75 years. All information obtained in this database is protected under the Privacy Act of 1974. Personal identifying information may not and will not be released unless the subject (or legal guardian) provides written approval of such disclosure. Each subject on whom data are collected, upon written request to Human Subjects Protection Division, Office of the Deputy Chief of Staff for Regulatory Compliance and Quality, USAMRMC may have access to their record, and only their record, contained in the database.

In the event of a research related injury, you should contact any of the investigators at the National Medical Laser Centre, Institute of Surgical Studies, 67-73 Riding House Street, London W1W 7EJ.

Any questions on subjects rights should be addressed to UCL/UCLH Committee on the Ethics of Human Research, Chairman - Professor A Maclean, UCLH NHS Trust, 1st Floor Vezey Strong Building, 112 Hampstead Road, London NW1 2LT.
CONFIDENTIAL
PATIENT CONSENT FORM

OPTICAL BIOPSY FOR DIAGNOSIS OF LESIONS IN THE BREAST

Mr A Lee, Mr M Keshtgar, Dr M Hall-Craggs, Prof S G Bown, Mr R Sainsbury,
Dr S Lakhani

Have you read the information sheet about this study? YES/NO

Have you had the opportunity to ask questions and discuss this study? YES/NO

Have you received satisfactory answers to all your questions? YES/NO

Have you received enough information about this study? YES/NO

My questions concerning the study has been answered by

.............................................................................................................(Doctor's name)

I understand that I am free to withdraw from the study at any time without giving a reason
and withdrawal from the study will not affect my routine care and management. I agree to
take part in this study.

Patient
signature:........................................................................Date................................................................................................

Please print name and
Address ........................................................................................................
........................................................................................................

Witnessed: ..........................................................Date.............................................................

Please print name
(doctor)........................................................................................................

Investigator: ..........................................................Date.............................................................