

CRANFIELD UNIVERSITY

LAURENCE MAXIMILIAN ALMOND

**TOWARDS OBJECTIVE ENDOSCOPIC
DIAGNOSIS OF BARRETT'S ASSOCIATED
EARLY NEOPLASIA USING
FIBRE-OPTIC RAMAN SPECTROSCOPY**

CRANFIELD HEALTH

DM THESIS

Supervisors: Dr C. Kendall, Dr J. Hutchings, Dr S. Morgan,
Professor H. Barr

August 2012

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Academic Year 2012

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This thesis is submitted in partial fulfilment of the requirements for
the degree of Doctorate of Medicine.

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Abstract

This translational research evaluates a novel, custom-built Raman probe for potential application as an in vivo diagnostic tool in the oesophagus. Raman spectroscopy (RS) is a well established analytical technique which is capable of probing the biochemical changes associated with neoplastic progression in oesophageal tissue. RS relies on measurement of subtle inelastic scattering signals following monochromatic laser excitation.

Clinical utilisation of RS within hollow organs requires accurate collection and transmission of signal through fibre-optic cables. This research aims to evaluate the ability of a custom built fibre-optic Raman probe, in conjunction with multivariate classification models, to differentiate between benign and neoplastic oesophageal pathologies ex vivo. The need for spectral stability and reproducibility are addressed, as are difficulties associated with multi-system reliability. In addition, the biochemical basis of spectral classification is evaluated and discussed.

Seven hundred and ninety-eight 1 s Raman probe spectra were acquired from 673 ex vivo oesophageal tissue samples from 62 patients. 5 s and 0.1 s spectra were also recorded for comparison. Principal component fed linear discriminant analysis was used to calculate probe accuracy by reference to a consensus histopathological ‘gold standard’ diagnosis. All results were statistically cross validated.

Based on characteristic spectral signatures, high-grade dysplasia and adenocarcinoma could be discriminated from Barrett’s oesophagus, low-grade dysplasia and normal squamous oesophagus with a sensitivity of 86% and a specificity of 88%. The ability to detect early superficial mucosal disease, including discrimination between low-grade and high-grade dysplasia, was also demonstrated despite short, clinically applicable (1 s) spectral acquisition times. Background subtraction and green glass correction algorithms were not shown to enhance diagnostic performance. However, improved diagnostic accuracy was demonstrated using longer acquisition times.

A single probe was shown to measure consistent spectra over a 20 month period. In addition, a potential algorithm for multi-system spectral classification was defined, and consistent measurements were recorded by two independent operators using two identically built

probes. The potential for bimodal diagnosis using a combination of RS and narrow band imaging was also demonstrated. No single incident of thermal tissue injury was reported.

The ability for rapid, accurate, objective diagnosis of a range of oesophageal pathologies using a novel endoscopic Raman probe has been demonstrated. RS has the potential to aid targeting of high-grade dysplasia and intramucosal cancer during endoscopic resection in order to maximise R0 resection rates. A potential barrier to clinical application of RS will be the transferability and repeatability of spectral measurements acquired with different Raman systems. This will require further evaluation following the construction of multiple probes.

Acknowledgements

I would like to thank Ian and May McIntyre for their kind and generous sponsorship of the Three Counties Cancer Network Oesophageal Research Fellowship. Their significant donation supported the first year of this work and enabled me to generate some preliminary results to support applications for further funding. I am also grateful to the Royal College of Surgeons of England for awarding me a Research Training Fellowship to support my second year of research.

I would like to thank my academic supervisors Dr Jo Hutchings and Dr Catherine Kendall for their incredible support and kindness. I would also like to express my thanks to Dr Gavin Lloyd for being so generous with his time and willing to engage in helpful discussions. His programming support in Matlab was also hugely appreciated. In addition, I would like to express my gratitude to Professor Nick Stone for giving me the opportunity to undertake important clinical research within the internationally recognised Biophotonics Unit.

Finally, I would like to thank Professor Hugh Barr. There can be no more thoughtful or selfless supervisor. His incredible enthusiasm and motivation stem from his willingness for others, including myself, to succeed. He has been an inspiration and role model, and has become a good friend.

Contents

Abstract.....	i
Acknowledgements.....	iii
List of Figures.....	XII
List of Tables.....	XVII
List of Abbreviations.....	XiX
1 INTRODUCTION: RESEARCH CONTEXT AND OBJECTIVES	1
2 LITERATURE REVIEW: BARRETT’S OESOPHAGUS	4
2.1 Oesophageal cancer – Epidemiology and Clinical Significance	4
2.2 Barrett’s Oesophagus – definition, epidemiology, significance	5
2.2.1 Definition:	5
2.2.2 Epidemiology.....	Error! Bookmark not defined.
2.2.3 Risk Factors for Developing Barrett’s Oesophagus.....	Error! Bookmark not defined.
2.2.4 Pathogenesis and Natural History of Barrett’s Oesophagus and Adenocarcinoma Error! Bookmark not defined.	
2.2.5 Barrett’s Neoplasia.....	10
2.3 Histological Diagnosis and Classification	10
2.3.1 Histological Appearances of Barrett’s Oesophagus	12
2.3.2 Histological Grading of Dysplasia / Cancer	13
2.3.3 Histological Diagnosis and Staging of Oesophageal Cancer.....	15
2.3.4 Histological T-staging of Oesophageal Carcinoma and Clinical Significance.....	16
2.3.5 Buried Glands	18
2.4 Risk of cancer and mortality in Barrett’s oesophagus	19
2.5 Natural History of Dysplasia in Barrett’s Oesophagus	21
2.5.1 Low-grade dysplasia	22
2.5.2 High-grade dysplasia	23

2.6	Endoscopic Recognition and Assessment of Barrett's Oesophagus	24
2.7	Barrett's Oesophagus Surveillance	26
2.8	Screening for Barrett's Oesophagus	28
2.9	Management of Barrett's Neoplasia.....	29
2.9.1	Endotherapies.....	29
2.9.2	Management of LGD	Error! Bookmark not defined.
2.9.3	Management of HGD.....	Error! Bookmark not defined.
2.9.4	Management of Early Oesophageal Cancer.....	Error! Bookmark not defined.
2.10	Summary: Clinical Need for a Novel Diagnostic Tool.....	40
3	LITERATURE REVIEW: RAMAN SPECTROSCOPY	43
3.1	Raman Scattering – Principles and Basic Science	43
3.1.1	Molecular Vibration and Polarisation.....	45
3.2	The Raman Spectrum.....	47
3.3	Raman Instrumentation	50
3.4	Application of Raman spectroscopy to medical diagnostics	52
3.4.1	Fluorescence	54
3.4.2	Summary	55
3.5	Raman Spectroscopy in the assessment of oesophageal pathology (in vitro).....	55
3.6	Fibre-optic Raman Spectroscopy	60
3.7	Analysis of Raman Spectral Signatures Using Multivariate Statistics	65
3.7.1	Data Pre-Processing.....	Error! Bookmark not defined.
3.7.2	Principal Component Analysis	Error! Bookmark not defined.
3.7.3	Linear Discriminant Analysis	67
3.7.4	Validation Using Test and Training Datasets	68
3.7.5	Leave-one-out Cross Validation	69
3.7.6	Diagnostic Outcome Measures	69

3.8 Competing Advanced Imaging Techniques.....	70
3.8.1 Wide-Field Detection Techniques	71
3.8.2 Point Measurement Techniques	74
3.8.3 Novel Imaging Techniques: Summary	75
4 METHODS.....	78
4.1 Raman Probe-Spectrometer Interface.....	78
4.1.1 Probe Design.....	79
4.2 Laser Safety	80
4.3 System Calibration.....	81
4.4 Evaluation of Probe Stability.....	82
4.5 Ex Vivo Tissue Measurements.....	83
4.5.1 Ethical Approval and Patient Consent	83
4.5.2 Sample preparation and spectral acquisition.....	83
4.6 Spectral Reproducibility Testing Using Two Probe Prototypes.....	86
4.6.1 Multi-Operator Probe Comparison Using PTFE Standard	87
4.6.2 Two Probe Comparison Using Ex Vivo Tissue	87
4.7 Transferability between Spectrometers: Two Spectrometer Comparison.....	88
4.8 Raman probe performance in the presence of endoscopic white light and narrow band imaging	89
4.9 Statistical Methods.....	90
4.10 Additional Projects	92
4.10.1 Correlation of Raman spectral signatures with molecular markers of progression in Barrett's Oesophagus	92
4.10.2 Oesophageal Spectral Cytopathology	93
5 EVALUATION OF DIAGNOSTIC PERFORMANCE.....	94

5.1 Summary of Measurements	94
5.2 Spectral Exclusion.....	96
5.3 Unprocessed Spectral Data	97
5.4 Diagnostic Classification Models	99
5.4.1 Three Group Models	100
5.4.2 Two Group Models	101
5.4.3 Detection of Epithelial Disease.....	104
5.4.4 Distinction of Barrett's Oesophagus from Gastric Mucosa.....	107
5.4.5 Non-Homogenous Tissue.....	107
5.5 Assessing the Value of Background Subtraction Techniques.....	109
5.5.1 Green Glass Correction.....	110
5.5.2 Air Background Subtraction	111
5.5.3 Background Subtraction / Correction: Impact on Classification Performance.....	111
5.6 Evaluation of the 5 s Spectral Dataset.....	112
5.7 Evaluation of the 0.1 s spectral dataset.....	114
5.8 Assigning error bars to the classification using repeated cross validation.....	116
5.9 Leave One Patient Out Cross Validation.....	117
5.10 Principal Component Optimisation	119
5.11 Influence of Technique of Tissue Collection.....	120
5.12 Influence of Consensus Histopathology:	123
5.13 Oesophageal Mapping	125
5.14 Summary.....	127
6 UNDERSTANDING THE BASIS OF SPECTRAL CLASSIFICATION.....	129
6.1 Mean Tissue Spectra.....	129

6.2	Difference Spectra.....	130
6.3	Background Subtraction Algorithms.....	132
6.4	Elucidation of Subtle Biochemical Features Using Multivariate Analysis	133
6.5	Exclusion of Early Principal Components.....	138
6.5.1	Assessment of Classification Performance	138
6.5.2	Reconstruction of the Dataset Following Exclusion of PC1	139
6.6	Evaluation of Principal Component Contributions to Spectral Classification	140
6.7	Spectral Truncation	143
6.8	Biochemical Transition from Barrett’s to Adenocarcinoma	144
6.9	Summary.....	146
7	CONSISTENCY, REPRODUCIBILITY AND TRANSFERABILITY	148
7.1	Spectral Consistency across the duration of the study period.....	149
7.1.1	Temporal Consistency of Air Background and PTFE Measurements.....	149
7.2	Influence of the Date of Measurement on the Spectral Classification	151
7.2.1	Summary	153
7.3	Probe Comparison	153
7.3.1	Two Operator, Two Probe Comparison using a PTFE Standard.....	153
7.4	Transferability between Spectrometers.....	157
7.4.1	Optimising a Calibration Correction.....	157
7.4.2	Diagnostic Classification Performance.....	162
7.5	Evaluation of the Potential for Compatibility with Narrow Band Imaging.....	165
7.6	Prediction of Ability to Classify Pathology In Conjunction with NBI Based on Truncated Dataset Analysis	168
7.7	Is Classification Possible Based on Spectral interference from white light endoscopy or NBI?.....	169

7.8 Summary.....	170
8 SUMMATIVE DISCUSSION AND CONCLUSIONS.....	171
8.1 Clinical Perspective.....	171
8.1.1 Potential for Multimodality Imaging	177
8.1.2 Optimising the Classification Model for Purpose.....	179
8.1.3 Potential Role During Oesophagectomy or Oesophageal Cancer Staging	182
8.2 Limitations.....	182
8.3 Future Work.....	184
8.3.1 Translation to an In Vivo Tool.....	185
8.3.2 Alternative Potential Future Projects Utilising Oesophageal RS	187
8.4 Conclusions.....	188
REFERENCES.....	193
APPENDICES	222

List of Figures

Figure 2-1. Oesophageal adenocarcinoma incidence ratio relative to 1975..	4
Figure 2-2. The metaplasia–dysplasia–adenocarcinoma sequence.....	9
Figure 2-3. Depiction of layers of the oesophageal wall.	10
Figure 2-4. Oesophageal wall (H&E).	11
Figure 2-5. Histology of Barrett’s oesophagus.....	12
Figure 2-6. Barrett’s oesophagus with high-grade dysplasia.....	14
Figure 2-7. Barrett’s associated cancer.....	15
Figure 2-8. The T-stage classification of T1 oesophageal adenocarcinoma is illustrated.	17
Figure 2-9. Illustration of T-staging in normal (squamous) oesophageal mucosa.	17
Figure 2-10. A: Oesophageal biopsy following previous photodynamic therapy (PDT). Neosquamous epithelium is situated on top of residual buried Barrett’s glands, which may retain some malignant potential. Note that goblet cells are clearly visible. B: Buried high grade dysplasia and intramucosal adenocarcinoma in a Barrett’s patient who had previously received PDT.	18
Figure 2-11. Nodular multifocal HGD in Barrett’s oesophagus. Clinicians must have a very high level of suspicion for the presence of invasive malignancy.	26
Figure 2-12. Treatment Depths. T-stage classification is illustrated. Mucosa (M), submucosa (SM), muscularis propria (MP), adventitia (ADV).....	30
Figure 2-13. Endoscopic resection of focal HGD with clear margins (A), and an early invasive cancer (B).	32
Figure 2-15. Current clinical practice. Clinicians are advised to take multiple, random oesophageal biopsies. This policy samples <5% of the mucosa.....	41
Figure 3-1. Changes in energy states during elastic (Rayleigh) and inelastic (Raman) scattering.	44
Figure 3-2. Three modes of vibration are possible for a CO ₂ molecule: symmetrical stretch, a bending mode and an asymmetrical stretch. In triatomic molecules, symmetrical stretch causes large polarisation changes and therefore strong Raman signals, whereas bending and asymmetric stretch cause a dipole change and little polarisation change and therefore a weak Raman signal.....	46
Figure 3-3. A Raman spectrum acquired from normal squamous oesophageal mucosa.	47
Figure 3-4 . Examples of spectra from a variety of human molecular constituents. Characteristic peaks can be seen for each substance at reproducible positions.....	49

Figure 3-5. Standard apparatus within a laboratory Raman spectrometer.....	51
Figure 3-6. A biochemical distribution plot. Reproduced from Stone <i>et. al.</i>	57
Figure 3-7. A high quality Raman map is able to identify 4 distinct areas which have been discriminated through differences in their biochemical composition. Each has a characteristic spectral fingerprint which can be distinguished using PCA.	58
Figure 3-8. A rapid Raman map (right) acquired from a region of clinical interest (left).....	59
Figure 3-9. Progress towards in vivo Raman spectroscopy in the upper gastrointestinal tract.	65
Figure 3-10. (A) Illustration of the two principal components, PC1 and PC2, for the two variables, X_1 and X_2 . (B) Datapoints have been referred to the principal component axes.	67
Figure 4-1. The probe-spectrometer interface. Reproduced from Almond <i>et. al.</i> [10] The photograph shows the internal components of the Renishaw system 100 spectrometer.	78
Figure 4-2. Optical components of the custom-built Raman probe. The confocal design facilitates an extremely shallow depth of field and ensures the excitation volume matches the sampling volume.....	80
Figure 4-3. Photograph taken prior to tissue measurements after placement of the perspex mapping grid. The distal oesophagus and proximal stomach have been opened and the grid has been pinned in place so that it overlies the distal oesophagus and gastro-oesophageal junction.	84
Figure 4-4. Schematic depicting methods of spectral measurement from bulk tissue and biopsies.	85
Figure 4-5. The components of ‘spectrometer 2’ - a portable spectrometer which was custom-built by Renishaw (Renishaw Plc., Wotton-under-Edge, Gloucestershire, UK)..	88
Figure 4-6. Schematic of PC-fed LDA followed by leave-one-spectrum-out cross validation. The cycle was repeated n times using 1 spectrum in the test dataset and n-1 spectra in training dataset until all spectra had been included as a test.	91
Figure 5-1. A collection of spectra measured from a single patient using a 5 s acquisition time. Note that a single spectrum (light blue) has a significant fluorescence background making tissue classification impossible.	96
Figure 5-2. (A) Ex vivo tissue spectra measured using 5s, 1s and 0.1s acquisition times. These unprocessed spectra are presented individually in (B), (C) and (D) to allow comparison of SNR.	98
Figure 5-3. Unprocessed spectra measured from a single patient.	99
Figure 5-4. One second, three group cross-validated LD scores plot 3 group (20 PCs).	100

Figure 5-5. Two group histograms demonstrating the classification performance of (A) the two group model: HGD / cancer versus NDB; and (B) normal squamous oesophagus versus HGD / cancer.	103
Figure 5-6. LD scores histogram illustrating the performance of a 2 group model at distinguishing LGD from HGD and adenocarcinoma.	105
Figure 5-7. A green glass correction function has been calculated by dividing the green glass spectrum measured using the probe system by the green glass standard.	110
Figure 5-8. The classification performance of a 3 group (left) and 2 group model (right) is illustrated following PC-fed LDA and LOSOCV.....	113
Figure 5-9. LD scores plot of the standard 3 group model. 0.1 s spectra (circles) have been projected onto the 1 s model (crosses) to see how they classify.....	115
Figure 5-10. Performance of training and test datasets following 2 group PC-fed LDA and repeated (1000 times) cross validation leaving out one third of the dataset. The sensitivity and specificity for separating HGD/cancer from LGD/Barrett's are illustrated.....	117
Figure 5-11. Comparison of (a) PC6 and (b) PC20. It is clear that significant biochemical information is contained within PC6 whereas PC20 appears to contain mostly noise and is unlikely to aid spectral classification.....	119
Figure 5-12. Influence of number of PCs on sensitivity / specificity performance.	120
Figure 5-13. standard 3 group LD scores prediction scatter plot identifying samples which have been measured from fresh (stars) and from fresh frozen (circles) tissue samples.	121
Figure 5-14. The pathology of the distal oesophagus has been mapped out.	126
Figure 6-1. Mean 1 s tissue spectra for 3 pathologies.....	129
Figure 6-2. Difference spectra for non-dysplastic Barrett's (NDB) and adenocarcinoma spectra (top); adenocarcinoma and normal squamous (NSq) spectra (middle); and NDB and NSq spectra (bottom).	131
Figure 6-3. Mean 1 s spectra from three pathology classes following air background subtraction (left) and green glass correction (right).....	133
Figure 6-4. Raman spectra from (A) histone; and (B) glycogen acquired on a laboratory based system using 10 s acquisition time.....	134
Figure 6-5. A selection of PC loadings are illustrated following PCA of the 1 s spectral dataset measured from normal squamous; LGD/NDB; and HGD/cancer tissue. Peak labels have been tentatively assigned to prominent features within the loadings.	136
Figure 6-6. Linear discriminant function plots 1 (A) and 2 (B).....	137

Figure 6-7. (A) Normalised raw spectral data. (B) Normalised data which has been reconstructed after exclusion of PC1.	139
Figure 6-8. LDF plots following exclusion of PC1.	140
Figure 6-9. A three group classification predictions scatter plot (left); and LD loads showing the individual contribution of different PCs to spectral classification.....	141
Figure 6-10. PC 8 and PC 9 loadings following PCA on spectra measured from three pathology groups.....	142
Figure 6-11. PC 6 and PC 11 loadings following PCA on spectra measured from three pathology groups.....	142
Figure 6-12. An LD scores histogram showing spectral predictions following PC-fed LDA for a two group model (NDB/LGD and HGD/cancer)..	145
Figure 7-1. Mean 1 s air spectra measured on randomly selected days demonstrating spectral consistency over time.....	149
Figure 7-2. Visual illustration of variation in air background spectra compared to day 1 (Air 4). 95% and 99% confidence intervals are illustrated.....	150
Figure 7-3. Eight randomly selected 5 s PTFE spectra.....	151
Figure 7-4. An LD scores scatter plot illustrating spectral predictions for a three group (NSq; NDB/LGD; HGD/cancer) classification model. Three randomly selected sets of spectra, measured on different days, have been highlighted.....	152
Figure 7-5. Mean PTFE spectra measured in 5 s using probe 1 and probe 2.	154
Figure 7-6. Mean PTFE spectra taken by each operator.....	154
Figure 7-7. PTFE spectra measured by both operators with each probe.	155
Figure 7-8 A, B&C: 5 s air background spectra; and D, a 1 s silica spectrum. All have been measured with the custom built probe in conjunction with one of the two spectrometers as indicated.....	158
Figure 7-9. Normalised 5 s PTFE spectra measured using the two systems. A silicon offset correction has been applied to the spectrum measured on Spectrometer 2.	159
Figure 7-10. Normalised 5 s PTFE spectra measured using the two systems. A linear data stretch has been applied to the data measured using Spectrometer 2.	160
Figure 7-11 (A) Green glass spectra measured on the two experimental systems, and at the National Physical Laboratory; and (B) 5 s PTFE spectra measured on each system. A silicon offset correction, normalisation and green glass correction has been applied to the spectrum acquired on Spectrometer 1. A linear data stretch, normalisation and green glass correction has been applied to the spectrum acquired on Spectrometer 2.	161

Figure 7-12. Air background spectra have been subtracted from the two PTFE spectra presented. Subsequently the spectra have been normalised and a linear data stretch has been applied.....	162
Figure 7-13. Unprocessed 1 s ex vivo tissue spectra measured using the Raman probe in conjunction with Spectrometer 2.	163
Figure 7-14. One second tissue spectra measured using Spectrometer 2 following air background subtraction.	163
Figure 7-15. Air background spectra measured in conjunction with WLE and NBI, and following exclusion of ambient light (control).	166
Figure 7-16. Five second PTFE spectra (A); and mean tissue spectra from 13 oesophageal biopsies (B); measured in conjunction with WLE and NBI, and following exclusion of ambient light (control).	167
Figure 7-17. Variation in tissue spectra acquired in the presence of endoscopic light and NBI compared to those acquired after exclusion of all ambient light. Confidence intervals for the ‘dark’ control are illustrated. The figure on the right represents a truncated dataset (400:1465); the figure on the left includes the whole spectral range.....	168
Figure 8-1. ROC curves showing sensitivity / specificity pairings for the discrimination of HGD and adenocarcinoma from (A) LGD and non-dysplastic Barrett’s; and from (B) all other pathologies. Note that buried neoplasia has been excluded from these models.	180
Figure 8-2. (A) An LD scores histogram illustrating probe classification of glandular neoplasia from all other benign tissue. (B) An ROC curve for the same cross validated classification model is also illustrated..	180

List of Tables

Table 2-1. Two large recent studies have reported the incidence of oesophageal adenocarcinoma in Barrett's oesophagus.....	20
Table 2-2. Studies reporting the incidence of adenocarcinoma in resected specimens following oesophagectomy for high-grade dysplasia.	24
Table 3-1. List of common biochemical components found in oesophageal tissue with their corresponding peak position.	50
Table 3-2. Accuracy of Raman spectroscopic diagnosis of 8 pathological groups in the oesophagus.....	56
Table 3-3. <i>In vivo</i> Raman spectroscopy in the upper GI tract..	63
Table 3-4. Advanced endoscopic imaging modalities being investigated for use in Barrett's oesophagus surveillance programmes and for facilitation of targeted endoscopic resection. .	77
Table 4-1. Vienna Classification of gastrointestinal epithelial neoplasia.....	86
Table 5-1. Summary of spectra measured from tissue where a corresponding consensus expert histological diagnosis is available. Spectra recorded using alternative probe / spectrometer systems have been excluded.....	94
Table 5-2. Summary of histological diagnoses.....	95
Table 5-3. A 1 s three group PC-fed LDA classification model which has been tested using LOSOCV. Probe sensitivities and specificities have been calculated.	101
Table 5-4. The 3 group PC-fed LDA classification model has been tested using LOSOCV. Probe sensitivities and specificities are displayed.	101
Table 5-5. The cross validated results of a series of 2 group classification models are displayed.	102
Table 5-6. Performance of a two group classification model (LGD / NDB) and HGD / cancer) after the inclusion and exclusion of buried adenocarcinoma.....	108
Table 5-7. The effect of air background subtraction and green glass correction on the classification performance of a 3 group model tested using LOSOCV.....	111
Table 5-8. A 5 s three group PC-fed LDA classification model which has been tested using LOOCV. Probe sensitivities and specificities have been calculated.	113
Table 5-9. A 5 s two group PC-fed LDA classification model which has been tested using LOOCV. Probe sensitivities and specificities have been calculated.	114

Table 5-10. Performance of the 0.1 s spectra after projection onto a 1s 3 group classification model.....	115
Table 5-11. The standard 3 group model has been tested using leave one spectrum out cross validation (LOSOCV) and leave one patient out cross validation (LOPOCV).	118
Table 5-12. Proportions of spectra measured from fresh and snap frozen tissue from each of the main pathology categories.	121
Table 5-13. Three group classification performance inclusion and exclusion of fresh and snap frozen samples.	122
Table 5-14. Performance of a two and three group model using pathology data reported by each expert pathologist and by a consensus of the two.	124
Table 6-1. Three group classification performance is illustrated following exclusion or inclusion of early PCs from the analysis.....	138
Table 6-2. Classification performance of the 1 s three group classification model is illustrated following truncation of the lower wavenumber range.	144
Table 7-1. The probability that there is no difference in PTFE peak heights when measured by the two probes, or the two operators, or by any combination of the two (interaction column), is illustrated following 2-way ANOVA.....	156
Table 7-2. Prior to two-way ANOVA, air background subtraction and green glass correction were applied to the raw data. The probability that there is no difference in PTFE peak heights when measured by the two probes, or the two operators, or by any combination of the two (interaction column), is illustrated.	157
Table 7-3. Comparison of diagnostic classification performance when including the full spectral range (400-1850 cm ⁻¹); and truncated spectra (400-1465cm ⁻¹).	169
Table 7-4. Classification of spectra acquired in the dark, and in the presence of endoscopic white light and NBI.....	170
Table 8-1. The diagnostic performance of the Raman probe is indicated..	174

List of Abbreviations

AFI – Autofluorescence imaging
ANOVA – Analysis of variance
APC – Argon plasma coagulation
AUC – Area under curve
BSG – British Society of Gastroenterology
BOSS – Barrett’s oesophagus surveillance study
CCD – Charge coupled device
CLO – Columnar lined oesophagus
CM – Cardiac mucosa
CM – Confocal microscopy
DNA – Deoxyribonucleic acid
ER – Endoscopic resection
ESD – Endoscopic submucosal dissection
ESS – Elastic scattering spectroscopy
EUS – Endoscopic ultrasound
FM – Fundic mucosa
GI – Gastrointestinal
GORD – Gastroesophageal reflux disease
GRIN – Graded index
H&E – Haematoxylin and eosin
HGD – High grade dysplasia
HRE – High resolution endoscopy
IM – intestinal metaplasia
IMC – Intramucosal cancer
LD (A) – Linear discriminant (analysis)
LGD – Low grade dysplasia
LOPOCV – Leave one patient out cross validation
LOSOCV – Leave one spectrum out cross validation
NBI – Narrow band imaging
NDB – Non-dysplastic Barrett’s oesophagus
NICE – National Institute of Clinical Excellence

NIR – Near infra-red
NSq – Normal squamous
OCT – Optical coherence tomography
OGD - Oesophagogastroduodenoscopy
OGJ – Oesophagogastric junction
PC (A) – Principal component (analysis)
PDT – Photodynamic therapy
ROC – Receiver operator curve
RFA – Radiofrequency ablation
RS – Raman spectroscopy
SCC – Squamous cell carcinoma
SNR – Signal to noise ratio
TSG – Tumour suppressor gene
WLE – White light endoscopy

CHAPTER 1

1 Introduction: Research Context and Objectives

Barrett's oesophagus is known to progress through a series of histologically identifiable dysplastic changes to oesophageal adenocarcinoma and confers an increased risk of malignancy. However, endoscopic detection of high grade dysplasia (HGD) (the premalignant lesion) and early intramucosal cancer (T1a) within Barrett's segments is unreliable, even for experienced endoscopists.[1-6] British Society of Gastroenterology guidelines therefore recommend *random* quadrantic biopsies every 2cm of Barrett's oesophagus.[7] This gold standard biopsy regimen samples <5% of the oesophageal mucosa and may fail to diagnose up to 57% of dysplastic lesions.[8] In addition, patients with confirmed HGD have a significant risk of occult invasive malignancy (around 35%) which may be missed by random biopsy.[9-26]

The early detection of premalignant breast, colon and cervical neoplasia has facilitated timely intervention in patients with these tumours leading to decreased cancer-related mortality.[27] However, despite the recognition of Barrett's oesophagus as a significant risk factor for oesophageal adenocarcinoma, and a variety of novel techniques to resect and ablate early neoplastic lesions, five year survival rates remain dismal (8-15%).[28-30]

The relative lack of progress in terms of mortality outcomes stems partly from difficulties in predicting the small cohort of patients with non-dysplastic Barrett's oesophagus who will eventually progress to cancer. It also reflects difficulties in identifying neoplastic lesions endoscopically and the absence of a non-endoscopic screening test.

Recently improved therapeutic strategies for treating HGD / T1a disease, including endoscopic resection and radiofrequency ablation, have created a profound clinical need for a safe and accurate test to endoscopically diagnose and grade early Barrett's-associated neoplasia. Since patients with Barrett's oesophagus are at an increased risk of malignant progression, this cohort are currently kept under close endoscopic surveillance. However,

improved endoscopic diagnostic techniques are required to: target areas of dysplasia for point biopsy (minimising missed neoplasia and the risks of bleeding/perforation); assess resection margins intra-operatively; and ultimately enable instant diagnosis of dysplasia and intramucosal cancer in order to facilitate early targeted endoscopic resection with meticulous endoscopic margin assessment.

Raman spectroscopy is an optical diagnostic technique which has shown considerable potential for early diagnosis of a variety of malignant disease states.[31-51] Raman spectroscopy measures the molecular-specific, inelastic scattering of laser light within tissue, producing a molecular ‘fingerprint’ unique to that tissue. Normal and abnormal tissue has differing biochemical cellular components leading to characteristic spectral differences which can be analysed. Laboratory based Raman spectrometers have previously been shown to be capable of discriminating *eight* pathological tissue types in the distal oesophagus (including Barrett’s metaplasia, HGD and adenocarcinoma) with high sensitivities and specificities.[49]

The objective of this Doctorate is to facilitate the translation of this laboratory technique into a clinical tool which can be used endoscopically in patients with Barrett’s oesophagus. Raman spectroscopy has clear potential as a medical diagnostic tool, it is exquisitely sensitive to minute biochemical changes; objective; rapid; safe; suitable for analysis of fresh unprepared tissue; and feasible for use in conjunction with specialised fibre-optics to enable hollow organ assessment.

Following a multi-unit collaboration a novel fibre-optic Raman probe has been designed and built at the Analysis Interface Centre at Bristol University. The probe, which has been specifically designed to fit through the instrument channel of a gastroscope, is novel in its design which aims to facilitate interrogation of the epithelial surface so potentially enabling detection of early pre-malignant neoplasia. In addition, the probe has been engineered for direct contact with oesophageal epithelium.

This Doctorate aims to evaluate the potential of this novel Raman probe to discriminate clinically relevant oesophageal pathologies. The probe has been coupled to a near-infrared monochromatic laser source and a Renishaw system 100 spectrometer (Renishaw Plc., Wotton-under-Edge, Gloucestershire, UK). Initial proof of concept experiments have been conducted using laboratory standards and porcine tissue phantoms. Further evaluation has

used fresh human oesophageal tissue to evaluate the diagnostic potential of this technique with the aim of supporting a future application for an in vivo clinical trial. Probe performance has been evaluated from a translational standpoint, ensuring clinically applicable laser power at the probe tip and short spectral acquisition times which will be feasible during routine oesophagoscopy. Besides diagnostic accuracy, other translational aspects have also been addressed including inter-user variability, consistency of spectral acquisition over a prolonged (18 month) time period, compatibility with narrow band imaging, variation of accuracy with spectral acquisition times, and reproducibility and transferability of data measured using independent (optically identical) probes and using different spectrometers.

This is a translational research project with huge clinical significance. All data has been collected safely and meticulously using rigorous scientific standards in order to generate robust, reproducible and clinically relevant results. The data has been carefully and thoroughly evaluated so as to present an accurate, rather than over-optimistic, description of probe performance in order to truly establish potential for future clinical use.

CHAPTER 2

2 Literature Review: Barrett's Oesophagus

2.1 Oesophageal cancer – Epidemiology and Clinical Significance

Over the past 40 years the incidence of oesophageal adenocarcinoma has increased more rapidly than any other solid tumour in the Western world (Figure 2-1). Oesophageal cancer has become the ninth most prevalent cancer in the UK[52] and now represents a major public health problem.

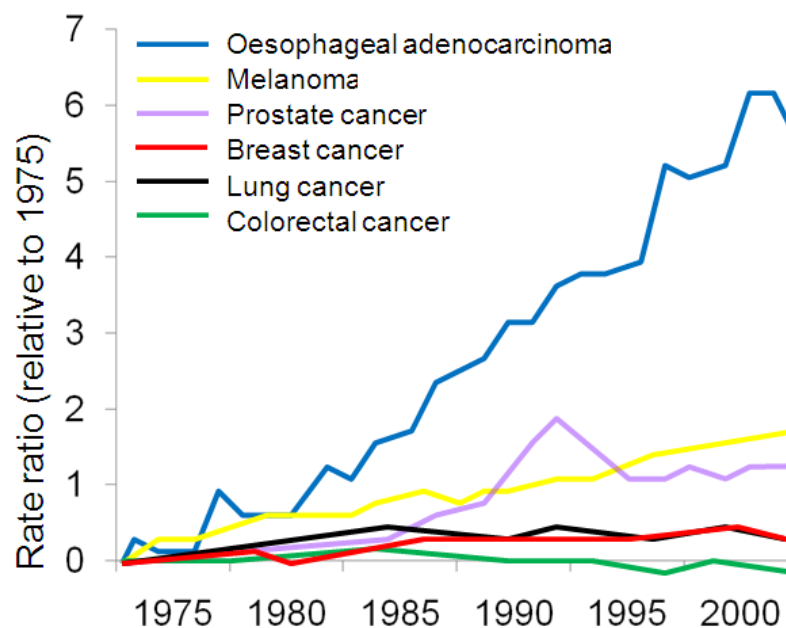


Figure 2-1. Oesophageal adenocarcinoma incidence ratio relative to 1975. Reproduced from the National Cancer Institute's Surveillance, Epidemiology, and End Results database.[30]

The other common pathological type of oesophageal cancer, squamous cell carcinoma (SCC), has by contrast declined by around 2% per year over the past decade, and adenocarcinoma now accounts for more than 50% of cases.[52][28] This trend is likely to result from the changing pattern of risk factors which influence the development of the two diseases. Rates

of smoking - a major risk factor for SCC - are declining, and rates of gastro-oesophageal reflux, the principal risk factor for adenocarcinoma, have increased dramatically.

The rapid increase in incidence of oesophageal adenocarcinoma is given a greater significance when survival figures are considered. Between 1993 and 1997 pooled data from the United States illustrated a one year survival of 44% with a 5 year survival of just 13%.[53] Figures from the Office for National Statistics quoted UK survival rates from 2001 as just 28% and 8% respectively.[29] More recently the National Oesophagogastric Cancer Audit reported a 5 year age-adjusted survival rate of 10%.[28] In addition the audit reported a 1 year survival of just 76% in those patients who underwent oncological and surgical therapy with curative intent.[28] Five year survival rates following surgery with curative intent remain between 25% and 40%.

It is clear that oesophageal cancer outcomes have lagged hugely behind those for other common malignancies including bowel, breast, and cervical cancer and there is now a pressing clinical need to diagnose oesophageal cancer earlier, and to identify and treat dysplastic changes in the oesophagus in order to prevent progression to malignancy.

2.2 Barrett's Oesophagus – definition, epidemiology, significance

2.2.1 Definition:

Barrett's oesophagus is a well known risk factor for the development of oesophageal adenocarcinoma.[54; 55] Barrett's oesophagus is defined by the American College of Gastroenterology as **“a change in the distal oesophageal epithelium of any length that can be recognised as columnar type mucosa at endoscopy and is confirmed to have intestinal metaplasia by biopsy of the tubular oesophagus”**.[56] The need for recognition of intestinal metaplasia (IM), as opposed to two other histologically distinct types of columnar metaplasia: cardiac (CM) and fundic (FM), reflects the fact that it is IM which has been shown to have significant malignant potential.[54; 57-61] IM can be easily recognised histologically by the presence of goblet cells occurring in association with columnar epithelium (Figure 2-5).

However, both British and Japanese guidelines on Barrett's oesophagus exclude the need for goblet cells in their definitions. The British Society of Gastroenterology prefers the term 'columnar-lined oesophagus' (CLO) and defines Barrett's oesophagus as **“an oesophagus in which any portion of the normal squamous lining has been replaced by a metaplastic columnar epithelium which is visible macroscopically. In order to make a positive diagnosis of “Barrett's oesophagus”, a segment of columnar metaplasia of any length must be visible endoscopically above the oesophago-gastric junction and confirmed or corroborated histologically.”**[7] The explanation for the difference in definitions is that UK pathologists consider that goblet cells can always be identified within a Barrett's segment provided sufficient biopsies are taken over an adequate time-scale.[7]

In the UK, the definition of Barrett's oesophagus is therefore regarded as a clinicopathological construct from both endoscopic and histopathological findings.

2.2.2 Epidemiology

The exact population prevalence of Barrett's oesophagus is unclear. Reports from endoscopic series range from 0.9% to 5.6% depending on the precise definition used and the type of study.[62; 63] A well designed population based study recruited a representative sample of 1000 people from two communities in Northern Sweden to undergo upper endoscopy and confirmed the presence of intestinal metaplasia in 1.6% of the population studied.[64]

When extrapolated to the UK and US populations, conservative estimates of prevalence are approximately 1 million and 4 million affected individuals respectively.[26; 65]

There is also evidence that the incidence of Barrett's oesophagus is increasing by around 2% per year.[63; 64; 66; 67] Although this may be due in part to improved endoscopic recognition, it is likely that there has been some true increase in incidence - data from Holland reported an increase in the number of cases of Barrett's oesophagus despite a decrease in the number of endoscopies being performed over the same period, suggesting a true increase in incidence.[26; 67]

The prevalence of Barrett's oesophagus increases with age, the mean age at diagnosis being approximately 62 years for men and 68 years for women. The condition predominantly affects Caucasians[68] with a male to female ratio of approximately 1.7:1.[69]

2.2.3 Risk Factors for Developing Barrett's Oesophagus

The chief risk factor for the development of Barrett's oesophagus is chronic gastro-oesophageal reflux disease (GORD). Between 8% and 30% of all adults are affected by GORD and 5% to 12% of these may subsequently develop Barrett's oesophagus.[64; 70-72] Evidence suggests that symptom frequency and symptom chronicity are better predictors of the presence of Barrett's oesophagus than symptom severity at any given time.[64; 70; 73; 74]

In addition to GORD, other causes of oesophageal inflammation have also been linked to Barrett's oesophagus formation including viral infections, chemotherapy and non-steroidal anti-inflammatory drugs. There is also evidence that smoking, high alcohol intake, high BMI and centripetal obesity are linked to Barrett's and an increased risk of progression to adenocarcinoma (although smoking is much more commonly associated with squamous cell carcinoma).[75] [76; 77]

Eradication of gastric *H. Pylori* has also been shown to predispose to the development of Barrett's oesophagus.[78] *H. pylori* bacteria produce the enzyme urease which catalyses the breakdown of urea, releasing ammonia which neutralises acid produced by gastric parietal cells. Gastritis caused by *H. pylori* infection also causes destruction of parietal cells, further decreasing gastric acid production.[78] The presence of *H. pylori* has been shown to diminish the prevalence of complications secondary to GORD, including the development of Barrett's oesophagus.

2.2.4 Pathogenesis and Natural History of Barrett's Oesophagus and Adenocarcinoma

Understanding the pathogenesis and natural history of Barrett's oesophagus is vital to understanding and identifying which patients will progress to oesophageal adenocarcinoma.

Barrett's oesophagus develops as an adaptive response to increased cell loss as a result of chronic inflammation, secondary to the synergistic effect of longstanding pulsatile reflux of acid and bile.[79; 80] Metaplasia appears to originate from stem cells located in the oesophageal gland squamous ducts which undergo point mutations in tumour suppressor genes (TSG), typically p16 and p53, in response to chronic reflux.[81]

Although traditionally thought of as an acquired condition, inherited genetic factors may have an influence in a subset of patients who develop Barrett's oesophagus. Family and twin studies suggest that certain individuals with a strong familial history of Barrett's oesophagus are at an increased risk.[82; 83] For example, a family in the UK has been identified with a male index case with oesophageal adenocarcinoma, three siblings with neoplastic metaplasia, and six children with non-dysplastic metaplasia.[26; 84]

Although Barrett's oesophagus and oesophageal adenocarcinoma appear to be polygenic conditions, it is hoped that results from on-going genome-wide association studies, and whole genome sequencing studies, will direct further investigation towards particular loci of interest.[85]

Following the initial trigger, commonly a point mutation, competing populations of distinct metaplastic clones colonise the Barrett's segment. Subsequently, in order for neoplasia to develop the metaplastic oesophageal epithelial cells must undergo a series of molecular changes which classically give rise to cancer via the metaplasia-LGD-HGD-adenocarcinoma sequence.[86]

Several of these molecular changes have been defined (mostly those necessary for the development of Barrett's oesophagus and early changes in the pathogenesis of dysplasia) but most of the key steps that enable cells to become cancerous remain unclear. It appears that mutations in genes responsible for DNA repair, chemical processing and the inflammatory response may all have a role.[87-89]

p53 abnormalities have been detected in up to 95% of Barrett's associated cancers and studies have shown that Barrett's patients with p53 mutations are at a far higher risk of malignant progression than those patients with Barrett's oesophagus that lack the mutation. Another tumour suppressor gene p16 has also been shown to be important in the development of Barrett's cancers. Like p53, p16 prevents progression of abnormal cells from G1 to the S

phase. p16 mutations are the earliest detected genetic alterations in patients with Barrett's oesophagus and are detectable prior to aneuploidy, p53 abnormalities, increased tetraploidy, and HGD. More than 85% of Barrett's cells have been shown to possess at least one form of p16 mutation, and in fact, the number of p16 abnormalities present increases with increasing length of Barrett's segment. This has led to the hypothesis that Barrett's starts out as a single clone that spreads out as subclones develop with greater numbers of genetic abnormalities.

Figure 2-2 illustrates the metaplasia–dysplasia–adenocarcinoma sequence. It appears there are multiple genetic variants of cancer which arise through a series of differing mutations along the pathway of carcinogenesis. Some of the most well described early genetic and epigenetic changes, which appear to be necessary for cancer development, are indicated. A more thorough review of the molecular biology of Barrett's oesophagus is not within the scope of this thesis.

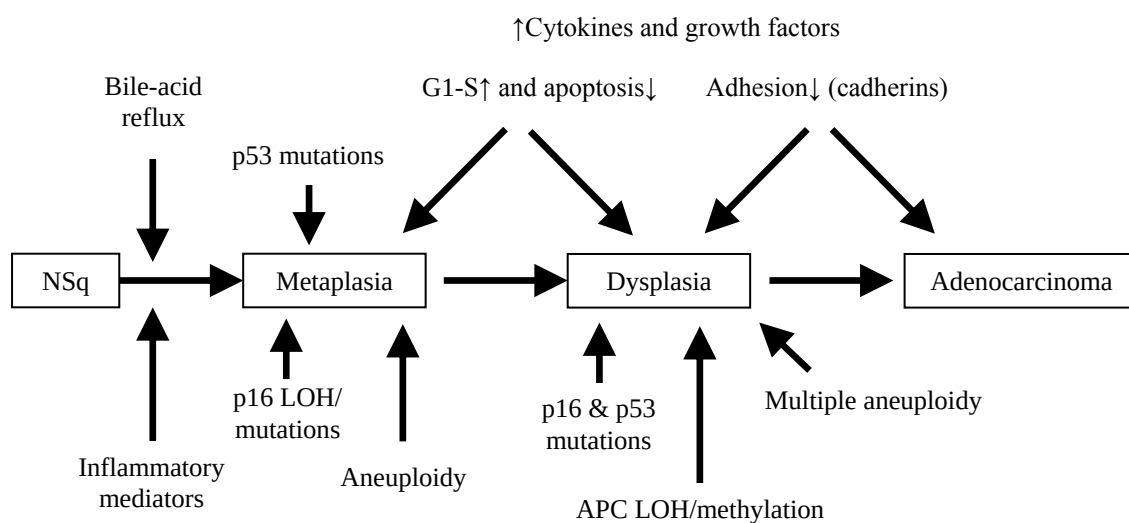


Figure 2-2. The metaplasia–dysplasia–adenocarcinoma sequence. Histological stages of progression are initiated by genetic, epigenetic and environmental factors which drive cell cycle and cell adhesion sequelae. LOH: loss of heterozygosity. APC: adenomatous polyposis coli gene. Figure adapted from Almond et. al..[26]

2.2.5 Barrett's Neoplasia

Dysplasia is defined as “**an unequivocal neoplastic alteration of epithelium which has the potential to progress to invasive malignancy but remains confined within the basement membrane of the epithelium within which it arose.**”[90; 91] Dysplasia is classified as either low grade (LGD), or high grade (HGD), based on its histological appearances. HGD has a higher malignant potential than LGD and malignant transformation classically occurs through a stepwise progression of pathology as described above. The diagnosis, classification and clinical significance of dysplasia and early adenocarcinoma will be discussed further below.

2.3 Histological Diagnosis and Classification

When diagnosing dysplasia and adenocarcinoma it is essential to first understand and recognise normal oesophageal histology.

Figure 2-3 depicts the layers of the normal oesophagus. Most of these layers can be seen in the biopsy specimen in Figure 2-4 which has been stained with Haematoxylin and Eosin.

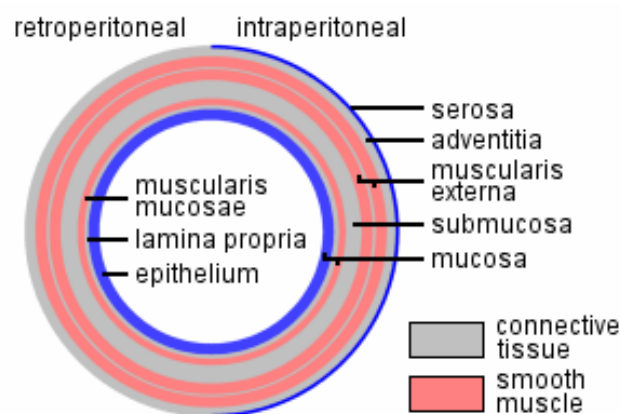


Figure 2-3. Depiction of layers of the oesophageal wall.[92]

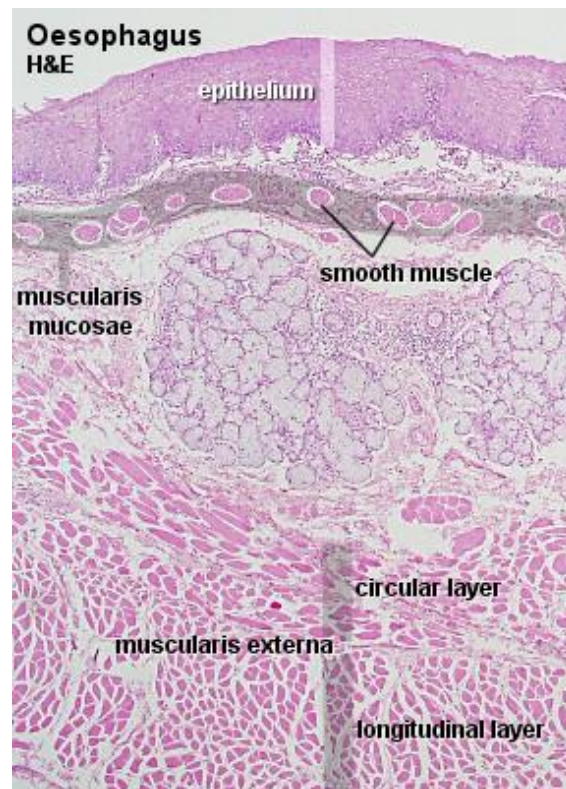


Figure 2-4. Oesophageal wall (H&E).[92]

The innermost layer of the oesophagus is a thick protective stratified squamous epithelium (not keratinised). This is supported on a basement membrane and overlies the lamina propria which contains scattered lymphoid aggregations, lymphatics, nerves and blood vessels. It also contains some mucous glands named ‘cardiac glands’ because of their resemblance to the cardiac glands of the stomach. Deep to the lamina propria is the muscularis mucosae, containing mucosal smooth muscle. These layers together form the mucosa. At the oesophagogastric junction the mucosa changes abruptly to a tightly packed glandular secretory mucosa.

The mucosa sits on a submucosa which is a relatively loose layer of areolar connective tissue which enables some sideways movement of the mucosa on the muscularis propria (as occurs as the oesophagus distends during passage of a food bolus). The submucosa is highly vascular and also contains lymphatics and parasympathetic ganglion cells (Meissner's submucosal plexus). In addition, it contains some small mucous glands (predominantly in its lower third) which aid lubrication of the mucosa.

The muscularis propria is a thick muscular layer consisting of distinctly separate inner circular muscle and outer longitudinal muscle. The muscle of the upper third of the oesophagus is skeletal, the middle third consists of both smooth and skeletal muscle, and the lower third consists of only smooth muscle. The circular and longitudinal muscular layers are separated by loose connective tissue containing Auerbach's myenteric plexus.

The outermost layer of the oesophagus is a connective tissue adventitia which merges with connective tissue surrounding adjacent structures such as the trachea. Below the diaphragm the oesophagus is surrounded by serosa.

2.3.1 Histological Appearances of Barrett's Oesophagus

As previously discussed, Barrett's oesophagus is a metaplastic change from normal stratified squamous epithelium to a single-layered columnar epithelium. The presence of Goblet cells is necessary to demonstrate intestinalised metaplasia (IM) (Figure 2-5).

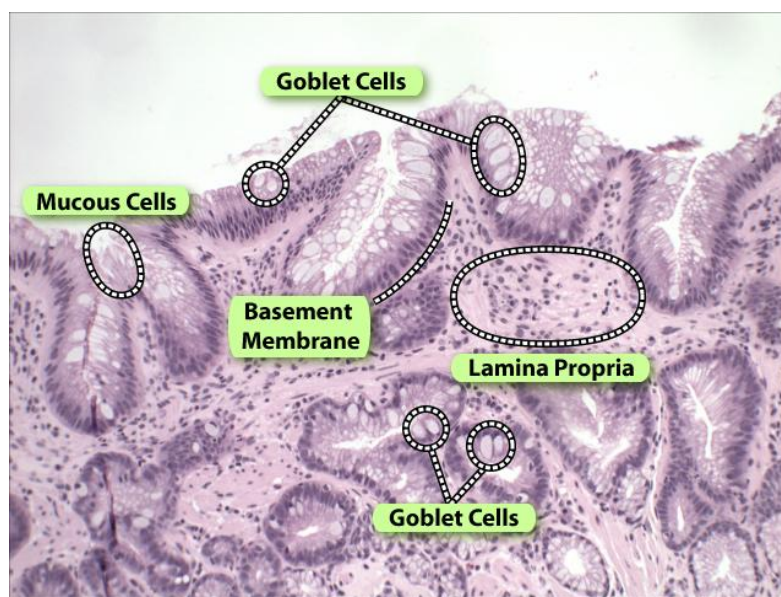


Figure 2-5. Histology of Barrett's oesophagus.[93] Goblet cells are clearly visible confirming the diagnosis of IM.

‘Gold-standard’ biopsy regimens only demonstrate histological confirmation of IM in 35-45% of patients who have endoscopic appearances of Barrett’s oesophagus.[105] IM is more readily identified in longer Barrett’s segments: 80% if >3cm, but only 30% if <3cm.[12; 106; 107] In addition, repeat biopsies in patients with shorter Barrett’s segments may increase the likelihood of histologically identifiable IM by around 20%.[97] These findings explain the refusal of the BSG to include IM in the definition of Barrett’s oesophagus but also indicate the difficulties in making a diagnosis based on microscopic appearances alone.

Intestinalised metaplasia may also be found at the gastric cardia or fundus and biopsies from these sites can therefore be histologically indistinguishable from oesophageal IM. The distinction relies entirely on correct endoscopic recognition of the site from which the biopsy was taken.

Occasionally oesophageal biopsies can be histologically diagnostic for Barrett’s oesophagus where a native oesophageal gland or a duct from these glands can be seen in close juxtaposition to metaplastic mucosa.[26] This is however an uncommon finding due to the superficial nature of most biopsies.

2.3.2 Histological Grading of Dysplasia / Cancer

Dysplasia is an atypical change in the nuclei and cytoplasm of the cells of the epithelium. In normal Barrett’s tissue the pattern of cellular growth is very even and the nuclei are of a consistent size and shape (Figure 2-5).

Differentiating normal Barrett’s mucosa from low-grade dysplasia can be difficult, particularly in the presence of inflammation and/or hyperplasia (see below). In these circumstances pathologists may report the specimen as ‘indefinite for dysplasia’.

Low-grade dysplasia is characterised by atypical cellular changes in association with normal architectural appearances of the glands and the epithelium. In LGD, some (but less than 50%) of the nuclei are large, often with prominent nucleoli. Some cells may be seen to be dividing – indicating an increased mitotic rate. In HGD most cells display atypical changes and the architecture of the glands has been disrupted. Most cells have prominent nuclei containing

large nucleoli and reduced cytoplasm. In addition, many may be seen to be dividing. There is also typically a reduced number of goblet cells (Figure 2-6).

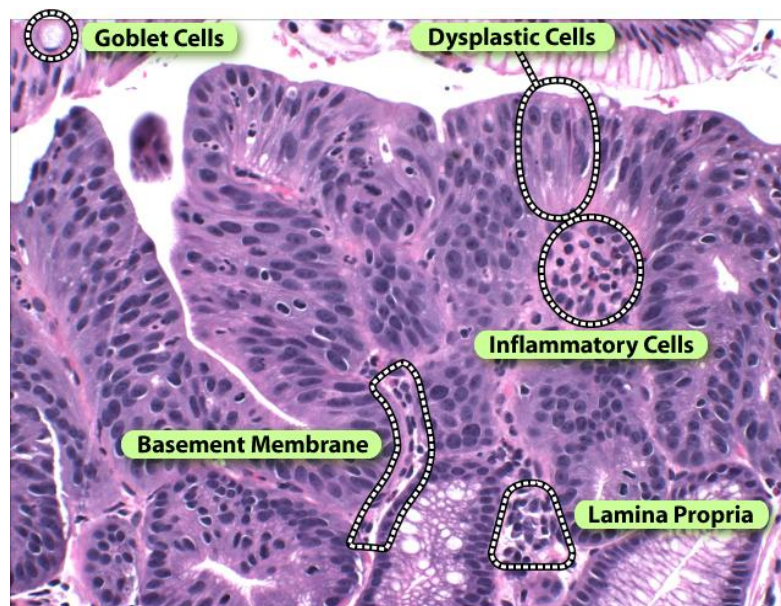


Figure 2-6. Barrett's oesophagus with high-grade dysplasia. [93] The epithelium is no longer arranged in neat, ordered rows and the glands have become distorted. The nuclear to cytoplasmic ratio has increased. However, there is no invasion through the basement membrane and this is therefore not cancer. Some inflammatory cells are seen within the lamina propria as are normal stromal cells.

HGD may be occasionally reported as 'carcinoma in situ'. By definition this describes cells that appear cancerous but are confined to the basement membrane and have therefore not invaded the lamina propria. This term has now been abandoned.

In malignant disease the atypical cells have breached the basement membrane and invaded into the lamina propria or beyond (Figure 2-7).

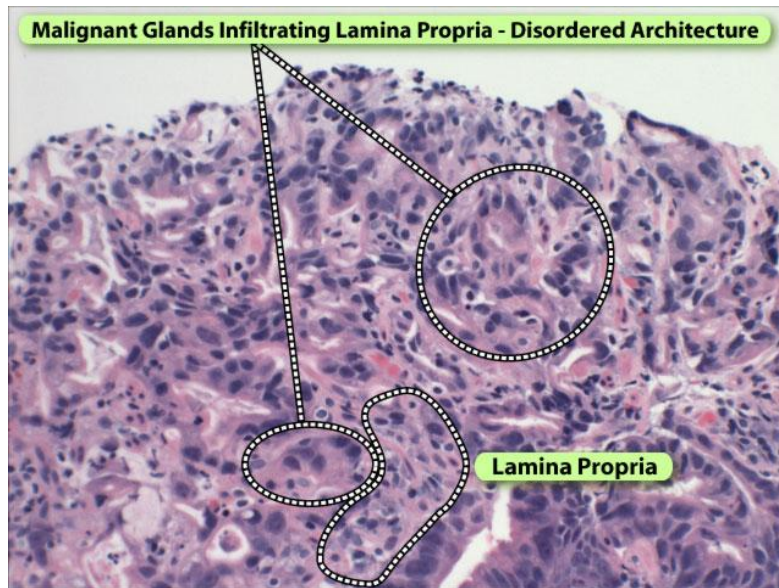


Figure 2-7. Barrett's associated cancer. This is a biopsy of the lamina propria from a patient with Barrett's oesophagus. Cancer cells are evident scattered throughout the lamina propria having invaded through the basement membrane.[93]

The identification and grading of dysplasia within an area of Barrett's oesophagus is notoriously difficult. Agreement between pathologists has often been shown to be poor to moderate ($K=0.2-0.6$) and only minimally improved by education.[85] Histological assessment however remains the gold-standard technique for distinguishing between grades of dysplasia, and also permits assessment of the integrity of the basement membrane. It is hoped that development of novel technology (such as Raman spectroscopy) may act as an aid to histological diagnosis in the future, and help to minimise subjectivity. The implications of inter-observer disagreement in grading dysplasia are discussed further in later chapters.

2.3.3 Histological Diagnosis and Staging of Oesophageal Cancer

Achieving an accurate histological diagnosis is an essential step in assigning treatment strategies for early Barrett's malignancies. Importantly though, histopathologists can also have difficulty differentiating between HGD and early invasive (T1) carcinoma based on point biopsy appearances alone. In recent years endoscopic resection (ER) has become recognised as not only a potentially curative therapy, but also a vital diagnostic technique.

This role has expanded as pathologists have realised the clinical relevance of accurate assessment and grading of early (T1) tumours.

The current BSG guidelines from 2005 do not recognise the diagnostic role of EMR. However, new guidelines are currently being formulated which will stress the need for accurate assessment of the presence and depth of invasive cancer and will recommend confirmation using ER.

2.3.4 Histological T-staging of Oesophageal Carcinoma and Clinical Significance

A complete description of pathological TNM staging of oesophageal cancer is included in Appendix I.

Recent studies have demonstrated the importance of accurate staging of early (T1) tumours (Figures 2-8 and 2-9). Intramucosal cancers (T1a lesions confined to the mucosa) have a very low incidence of lymphatic invasion (<5%) whereas, invasion into the submucosa is associated with lymphatic spread in 20-45% of cases. New evidence has suggested that the distinction between T1sm₁ and T1sm₂ (i.e. between the upper 1/3 and lower 2/3 of the submucosa) may be particularly significant as the risk of lymphatic spread appears to significantly increase in T1sm₂ tumours. This data is important as surgical oesophagectomy and lymphadenectomy provide the only chance of cure for patients with lymphatic spread, whereas endoscopic therapy is potentially curative in those without lymphatic invasion.

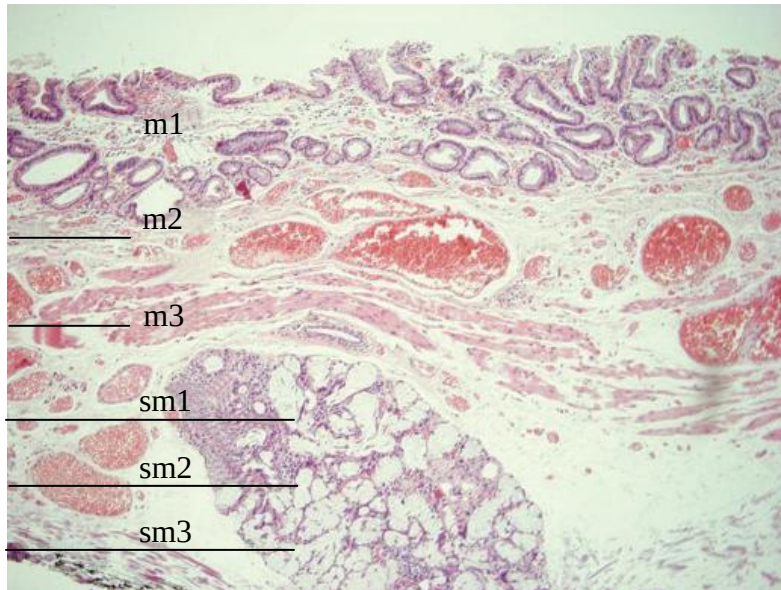


Figure 2-8. The T-stage classification of T1 oesophageal adenocarcinoma is illustrated.
Figure from Gut, 2007. [3]

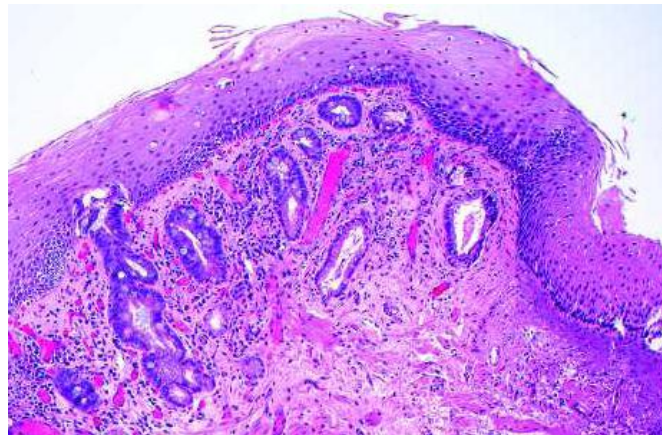


Figure 2-9. Illustration of T-staging in normal (squamous) oesophageal mucosa. Figure reproduced from Pech et. al.. Gut, 2007. [3]

2.3.5 Buried Glands

In some cases following ablative therapy for Barrett's oesophagus, a normal squamous epithelium may re-grow over a portion of Barrett's tissue (Figure 2-10). Endoscopically this appears normal, but these buried Barrett's glands can retain malignant potential.[99] This potential for buried Barrett's has implications for post-endotherapy surveillance.

A:



B:

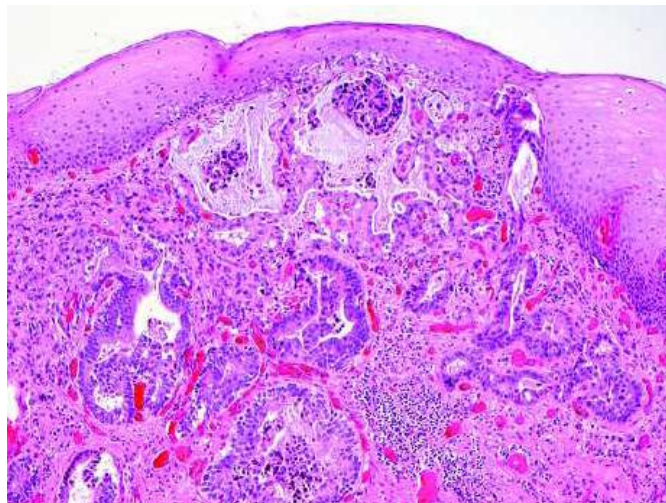


Figure 2-10. A: Oesophageal biopsy following previous photodynamic therapy (PDT). Neosquamous epithelium is situated on top of residual buried Barrett's glands, which may retain some malignant potential. Note that goblet cells are clearly visible. B: Buried high grade dysplasia and intramucosal adenocarcinoma in a Barrett's patient who had previously received PDT. Images reproduced from Odze et. al..[99]

2.4 Risk of cancer and mortality in Barrett's oesophagus

The presence of Barrett's metaplasia is accepted as a significant risk factor for the development of adenocarcinoma. However, the overall risk of progression to adenocarcinoma and the risk of disease specific mortality is low.

A large number of studies have estimated the risk of adenocarcinoma arising from Barrett's oesophagus, with very variable results.[100-110] Many of the early studies were likely subject to publication bias, with results only being published if they demonstrated a high incidence of cancer, leading to an overestimate of risk.[111] A meta-analysis from 2012 by Desai et. al. which investigated the incidence of adenocarcinoma in non-dysplastic Barrett's included 57 studies and a total of 58 547 patients-years of follow-up.[112] The pooled annual incidence of adenocarcinoma was 0.33% (95% CI 0.28% to 0.38%) and a subgroup analysis showed an even lower rate of 0.19% in patients with short segment Barrett's (<3 cm) on index endoscopy. However, as is the case with meta-analyses, this report was limited by selection bias affecting each of the included studies.

Recently two large population based cohort studies reported significantly lower annual rates of progression to adenocarcinoma (0.12-0.13% per year) than previously reported.[100; 101] (Both of these studies were published just prior to publication of the meta-analysis reported by Desai et. al., but neither were included in this analysis).

Reference	Patients	Mean follow-up (years)	Total follow-up (patient years)	Annual risk of progression to cancer
Bhat et. al.. (2011)	8,522 3,179* 3917^	7.0 - -	59,784 23,417* 28,323^	0.13% 0.04%* 0.23%^
Hvid-Jensen et. al.. (2011)	11,028	5.2 (median)	67,105	0.12%

Table 2-1. Two large recent studies have reported the incidence of oesophageal adenocarcinoma in Barrett's oesophagus. Unless stated figures represent the annual risk of malignant progression from non-dysplastic intestinal metaplasia. Figures from Bhat et. al.. Are divided into *patients where IM was absent following index biopsy, and ^patients who had IM identified on index biopsy. (The presence or absence of IM was unknown for 1426 patients). Table reproduced from Almond et. al..[26]

It is important to note that these figures exclude carcinomas of the gastric cardia and do not reflect progression to HGD. In addition, the two studies used slightly different mechanisms for identifying patients with Barrett's oesophagus.

Hvid-Jensen et. al.. conducted a population-based case control study including 11,028 patients who were followed for a median of 5.2 years. Patients with Barrett's oesophagus were shown to have an annual risk of malignant progression of just 0.12%, and a relative risk of 11.3 for developing adenocarcinoma compared to the general population. Only 7.6% of the total oesophageal adenocarcinomas diagnosed nationwide over the study period had a previous diagnosis of Barrett's oesophagus. However, this study used the Danish National Pathology Registry without corroboration with endoscopic findings to identify patients with a previous diagnosis of IM. It is therefore possible that patients with cardiac IM rather than oesophageal IM were included leading to a potential under-estimate of the risk of disease progression.

Bhat et. al. included patients with CLO at endoscopy who had Barrett's metaplasia corroborated histologically. However, the validated Prague system for recording the Barrett's segment was not used and it was unclear whether there may have been an overestimate of CLO due to the presence of hiatal hernia.

Interestingly, the data reported by Bhat et. al. demonstrated an increased risk of neoplastic progression in patients who had a histological diagnosis of IM at index endoscopy, compared to those with CLO without confirmed IM. This finding supports evidence from smaller studies demonstrating a higher risk of disease progression in patients with confirmed IM.[7; 113; 114]

It is also important to note that previous studies have suggested a significant geographical variation in the incidence of carcinoma arising in Barrett's oesophagus in Western countries. Previously rates of malignant progression in the UK have been shown to be almost double those in the US.[115] For this reason variations in population demographics are important to consider when interpreting population based studies such as the Danish study by Hvid et. al. discussed above. For example, compared to Denmark, rates of obesity, a risk factor for disease progression, are significantly higher in the UK. Likewise, a greater proportion of males, who are also at a higher risk of malignant progression, develop Barrett's oesophagus in the UK.

It is essential to appreciate that whilst patients with Barrett's oesophagus have an increased relative risk of developing adenocarcinoma, the majority will die from other causes. A study from the UK has demonstrated an increase in overall mortality and oesophageal cancer mortality in Barrett's patients compared with an age- and sex-matched population without Barrett's. Interestingly, there were more cancer deaths from other cancers (18%) than from oesophageal cancer (10%) and by far the greatest cause of mortality was cardiorespiratory disease (49%).[116]

2.5 Natural History of Dysplasia in Barrett's Oesophagus

The true natural history of dysplastic Barrett's oesophagus has been difficult to ascertain. Studies have suffered from inadequate power, insufficient follow-up durations, sampling errors at endoscopy and considerable diagnostic variability due to histological subjectivity.

2.5.1 Low-grade dysplasia

Reported rates of regression / progression from LGD vary considerably reflecting the difficulties discussed above.

A recent prospective cohort study of 713 Barrett's patients, including 111 with LGD, reported that LGD was a significant risk factor for progression to HGD or adenocarcinoma compared to non-dysplastic disease (RR 9.7; 95% CI 4.4-21.5).[117] Similarly, Hvid-Jensen et. al.. reported that the relative risk of oesophageal cancer among those who had LGD at baseline endoscopy was 4.8 (95% CI 2.6-8.8). The annual risk of progression to HGD or cancer was found to be 1.27% for those with LGD at baseline. Bhat et. al. reported a hazard ratio of 5.67% (95% CI 3.77 to 8.33) for patients with LGD compared to no dysplasia.[100] However, a recent study by Wani et. al.. which followed up 210 patients with Barrett's oesophagus with or without LGD for a mean of 6.2 years found no associations between presence of prevalent, incident or persistent LGD, or the extent of LGD, with progression rates.[26; 100]

Bergman et. al. recently reported considerable over-diagnosis of LGD in non-specialist hospitals and concluded that the true significance of the diagnosis has probably been underestimated by most reported series.[118] 1198 Barrett's patients underwent surveillance at six non-specialist hospitals identifying 147 (12.5%) patients with LGD. However, following a review of the histology by two external expert pathologists only 8 (0.7%) patients were deemed to have a diagnosis of LGD and the majority of samples were reclassified as non-dysplastic Barrett's oesophagus. In this study 42% of patients with LGD diagnosed by consensus expert pathologists demonstrated progression to either carcinoma or HGD, and 2.2% regressed to non-dysplastic Barrett's oesophagus, over a mean follow-up period of 51 months.[118]

In summary, although the true natural history of LGD has been difficult to ascertain it appears clear that the majority of patients with LGD diagnosed without consensus pathology reporting will either remain stable or regress to non-dysplastic disease or squamous mucosa. The majority of evidence from large recent trials reports that the presence of LGD at baseline endoscopy increases the relative risk of malignant progression (RR 4.8-9.7). However, it is possible that widespread misclassification of non-dysplastic Barrett's oesophagus or

oesophagitis as LGD may have led to a widespread under-recognition of the true risk of LGD.[26]

2.5.2 High-grade dysplasia

The natural history of HGD is also unclear although this diagnosis is known to confer a significant risk of progression to adenocarcinoma. Reid et. al. reported that 59% (45/76) patients with HGD who were followed for 5 years developed adenocarcinoma. Another study reported that 3 of 24 patients (13%) with focal HGD and 17 of 42 patients (40%) with diffuse HGD developed carcinoma after a mean follow-up of 41 and 23 months respectively.

It is important to consider what proportion of HGD patients who undergo oesophagectomy have an occult cancer detected in the resected specimen. Reported rates vary widely (0-73%) but the average appears to be around 40% (Table 2-2).[9-26] Those with visible, nodular HGD, appear to have a significantly greater risk of harbouring coexisting cancer.[119; 120] This emphasizes the importance of using strict biopsy protocols in patients with Barrett's oesophagus.

Given that intramucosal cancers (T1a) may be managed by endoscopic therapy, it may be more pertinent to ask the prevalence of submucosal invasive cancer at oesophagectomy for HGD. Following a retrospective assessment of 41 patients with preoperative HGD and 19 with preoperative IMC who underwent oesophagectomy, Wang et. al. demonstrated rates of invasive cancer of 5% in patients with pre-operative HGD, and 11% in patients with pre-operative IMC.[24]

Reference	Patients with high-grade dysplasia	Invasive cancer at postoperative histology	Percentage
Altorki et. al. (1991)[23]	8	4	50
Pera et. al. (1992)[24]	18	9	50
Streitz et. al. (1993)[25]	9	2	22
Levine et. al. (1993)[26]	7	0	0
Peters et. al. (1994)[28]	9	5	56
Edwards et. al. (1996)[27]	11	8	73
Rice et. al. (1997)[29]	16	6	38
Collard et. al. (1997)[30]	12	4	33
Ferguson and Naunheim (1997)[31]	15	8	53
Cameron and Carpenter (1997)[32]	19	2	11
Falk et. al. (1999)[33]	28	10	36
Headrick et. al. (2002)[34]	54	19	35
Tseng et. al. (2003)[35]	60	18	30
Sujendran et. al. (2005)[36]	17	11	65
Read et. al. (2005)[37]	49	18	37
Wang VS et. al. (2009)[38]	41	16 (14 IMC; 2 inv. ca.)	39% (34% IMC, 5% inv. ca.)
Nasr and Schoen (2011)[39]	68	12 (4 IMC; 8 inv. ca.)	17.6 (5.9 IMC; 11.7 inv. ca.)
Total	441	152	34

Table 2-2. Studies reporting the incidence of adenocarcinoma in resected specimens following oesophagectomy for high-grade dysplasia. IMC – intramucosal cancer; inv. ca. – invasive cancer (denotes invasion into submucosa or beyond). Reproduced from Almond et. al.[26]

2.6 Endoscopic Recognition and Assessment of Barrett's Oesophagus

In the majority of cases, Barrett's oesophagus is identified incidentally in patients who are undergoing an oesophagogastroduodenoscopy (OGD) for investigation of reflux symptoms. The National Institute for Health and Clinical Excellence (NICE) now recommends that all patients with long-term symptomatic reflux (greater than 5-10 years) should be referred for a screening endoscopy to check for Barrett's oesophagus.[116; 121; 122]

Barrett's oesophagus has a classical appearance on OGD. There is proximal displacement of the squamocolumnar junction which in normal circumstances lies at the proximal limit of the linear gastric mucosal folds. 'Salmon pink' columnar mucosa is seen in the distal oesophagus arising from the oesophago-gastric junction (OGJ), often with characteristic 'tongues' extending proximally. Occasionally there may also be readily identifiable islands of columnar mucosa. Following endoscopic recognition, the extent of proximal extension above the OGJ should be measured and documented (taking care to accurately identify any sliding hiatus hernia which may confuse this measurement). The diagnosis must then be confirmed / corroborated histologically by multiple pinch biopsies of the affected segment. When biopsies are obtained it is crucial that they originate from the oesophagus and that their site is recorded as accurately as possible.

The 'Prague C and M criteria', defined by an International Working Group on Barrett's oesophagus, offers a validated method of classifying Barrett's based on its endoscopic appearance.[123] The extent of circumferential ('C') involvement in centimetres from the OGJ should be recorded, as should the maximum ('M') length of the Barrett's segment (including tongues of Barrett's but excluding isolated 'islands').

Although the risk of malignant progression is greater in long Barrett's segments (>8cm), it is now recognised that shorter lengths, even below 3cm have malignant potential.[124-127] However, short Barrett's segments can cause considerable diagnostic difficulties - what appears endoscopically to be a short segment of metaplasia in the distal oesophagus or an irregular z-line may in fact represent intestinal metaplasia of the gastric cardia known as cardia intestinal metaplasia (CIM).[7] This can lead to misclassification of CIM as Barrett's oesophagus. For this reason the endoscopist has a crucial role in defining the exact position from which biopsies are taken.

It is important that clinicians make a thorough and systematic inspection of the mucosa in order to identify any evidence of early macroscopic neoplastic disease. Water or 1% acetylcysteine should be used to remove blood, saliva and refluxate from the oesophagus and insufflation must be carefully optimized to allow clear visualization of any mucosal abnormalities whilst still permitting recognition of the mucosal folds at the OGJ. Clinicians should be aware that the majority of early neoplastic lesions arise between 6 o'clock and 12 o'clock in the endoscopists view.[128]

Despite meticulous inspection using white light endoscopy (WLE), recognition of early neoplastic lesions is difficult and subjective, even for expert endoscopists. Guidelines therefore recommend that quadrantic biopsies are taken every 2cm of Barrett's oesophagus with additional targeted biopsies from any visible areas of mucosal abnormality.[7] Currently, the routine use of jumbo biopsy forceps is not recommended.

Rigorous biopsy protocols, which are commonly poorly adhered to outside of specialist centres, sample less than 5% of the mucosa and may miss up to 57% of dysplasia.[129][8] Several advanced endoscopic imaging techniques have been developed which may aid targeted biopsies from high-risk areas, improving diagnostic yield (see section 3.14 on advanced endoscopic imaging).[130-134]

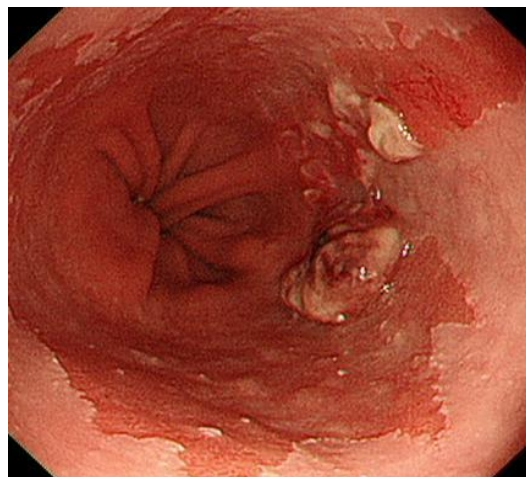


Figure 2-11. Nodular multifocal HGD in Barrett's oesophagus. Clinicians must have a very high level of suspicion for the presence of invasive malignancy.

2.7 Barrett's Oesophagus Surveillance

Patients with Barrett's oesophagus are typically kept under endoscopic surveillance to monitor for dysplastic and malignant change. The surveillance interval depends predominantly on the presence and degree of dysplasia identified, but also to a lesser extent on patient age, comorbidity and preference.

Several small retrospective studies have demonstrated a survival benefit for patients with cancers detected by surveillance endoscopy rather than following symptom investigation.[135-139] However, others have failed to support this finding.[140] It is likely that these small studies have been subject to both selection bias and length bias and doubt therefore remains as to the true benefits of a surveillance-for-all strategy. The on-going randomised multicentre Barrett's Oesophagus Surveillance Study (BOSS) aims to define the objective value of endoscopic surveillance and the most appropriate surveillance protocol. BOSS randomises patients with at least 1 cm of circumferential or 2cm non-circumferential Barrett's oesophagus to either protocol endoscopic surveillance with protocol biopsy or endoscopy at the time of need, the latter group being discharged unless they develop new symptoms or alarm symptoms.[26]

Despite the current lack of high level evidence, most clinicians elect to follow up patients with Barrett's oesophagus provided the pros and cons of surveillance have been fully discussed with the patient and that they subsequently wish to proceed with surveillance endoscopy. Clearly, this approach is not suitable for all-comers, and management should therefore be individualised appropriately. For example, long-term follow-up of elderly patients who are unfit for intervention is not usually recommended.

Patients should also understand the disadvantages and limitations of surveillance programmes. In addition to a physical and psychological burden, it must be remembered and communicated to patients at enrolment, that early neoplasia may be missed by surveillance due to sampling error and limitations of current endoscopic imaging techniques. Similarly, curative intervention will not necessarily be available for all surveillance detected cancers.

Where surveillance is deemed appropriate current BSG guidelines suggest that individuals with Barrett's oesophagus without dysplasia should undergo surveillance endoscopy every 2 years. The current recommendation for quadrantic biopsies every 2cm of Barrett's oesophagus is based on a mathematical model which assumes the risk of progression to adenocarcinoma in Barrett's oesophagus to be approximately 1% per annum.[26] However, the recent evidence suggesting significantly lower rates of malignant progression questions the rationale for surveillance of patients with non-dysplastic disease. Outcomes of the BOSS trial are awaited, however it is possible that future recommendations for surveillance will require additional risk factors such as segment length >3cm, a history of dysplasia, or

molecular markers of increased risk. This may enable categorization, or even individualization of surveillance protocols reflecting patient and disease factors. Surveillance of dysplastic disease will be discussed further below.

2.8 Screening for Barrett's Oesophagus

There are now successful screening programmes in the United Kingdom for colorectal, breast and cervical cancer. Screening programmes aim to identify curable premalignant or early malignant disease, however despite the recognition of Barrett's dysplasia as a risk factor for the development of adenocarcinoma, no oesophageal screening test has been developed.

In light of the recent developments in endotherapy for HGD and intramucosal cancer there is a significant clinical need for a screening test to facilitate identification and treatment of early oesophageal neoplasia. The proximity of the distal oesophagus lends itself to safe and quick assessment with an endoscope. However, widespread population screening using OGD would be expensive, impractical, and not without associated morbidity. A more favourable technique would be screening of a targeted group of individuals who are regarded to be at a higher risk of oesophageal neoplasia than the general population.

Patients with symptomatic chronic GORD have a 5-15% chance of developing Barrett's oesophagus, with males over fifty having the highest risk of progression to adenocarcinoma.[71; 141; 142] This data has prompted a call for selective screening of this group. However, the absolute risk of adenocarcinoma in patients with GORD is low, in the region of 1 in 1000 per annum[90] and it is also important to note that up to 40% of patients who develop adenocarcinoma have no history of symptomatic reflux. In addition, a recent study has shown that 44% of patients with Barrett's oesophagus are asymptomatic for at least 3 months prior to their diagnosis. A policy of screening based on reflux symptoms alone would therefore seem inadequate and endoscopic screening of individuals with chronic reflux symptoms to detect either Barrett's or cancer is therefore not currently recommended in either the UK or US.[7; 56]

Several attempts have been made to develop a scoring system using symptoms and patient demographics to predict a diagnosis of Barrett's oesophagus for screening purposes.[143;

144] However, recently interest in these risk prediction strategies has waned due to inability to generate sufficient accuracy of diagnostic predictions.

In the future it is hoped that non-invasive screening tests could be developed to detect patients with susceptible phenotypes using spectroscopic or molecular techniques. Several clinical trials are underway including the Chemoprevention of Premalignant Intestinal Neoplasia (ChOPIN) trial, and the Barrett's Oesophagus Screening Trial (BEST2) trial which aim to explore non-invasive methods of screening for Barrett's oesophagus and molecular predictors of malignant progression. ChOPIN aims to detect a panel of predictive serum biomarkers, whereas BEST2 aims to investigate the potential of a non-endoscopic immunocytological device (Cytosponge).[145; 146] This trial requires patients to swallow a small capsule which dissolves into a 3cm sponge within the stomach and is then withdrawn through the oesophagus in order to collect cells. The cells are assessed for a range of predictive biomarkers including TFF3 positivity (the principle endpoint) as well as ploidy, Mcm2, cyclin A, TP53, and methylation. Cost data and the impact of screening on psychosocial well-being are also being evaluated. It is hoped that non-invasive screening tests such as these could enable safe, accurate and cost-effective population based screening in the future.[12] Currently, identifying patients with early neoplasia and determining which of those will progress to adenocarcinoma, remains a significant and as yet unsolved challenge.

2.9 Management of Barrett's Neoplasia

All patients with Barrett's oesophagus, with or without neoplasia, should be commenced on high dose acid suppression therapy unless contraindicated. Subsequent management is largely dependent on patient comorbidity, patient preference, and the presence, grade and extent of dysplasia (see Appendix II).

2.9.1 Endotherapies

There are two main forms of endoscopic therapy available to treat HGD and intramucosal tumours – endoscopic resection and endoscopic ablation. These techniques aim to destroy the lining of the oesophagus and promote regenerative re-growth of normal squamous mucosa. In

order for this to occur, (as opposed to columnar re-growth) some of the superficial squamous lined ducts must survive the process. Techniques for mucosal ablation include photodynamic therapy, thermal ablation, radiofrequency ablation and argon plasma coagulation.

Endotherapy is indicated in selected patients with HGD, intramucosal cancer (T1a) and early submucosal cancers (T1b). It offers an attractive alternative to radical surgery in terms of reduced mortality and morbidity, with excellent short and medium term results, but long-term efficacy remains unclear.

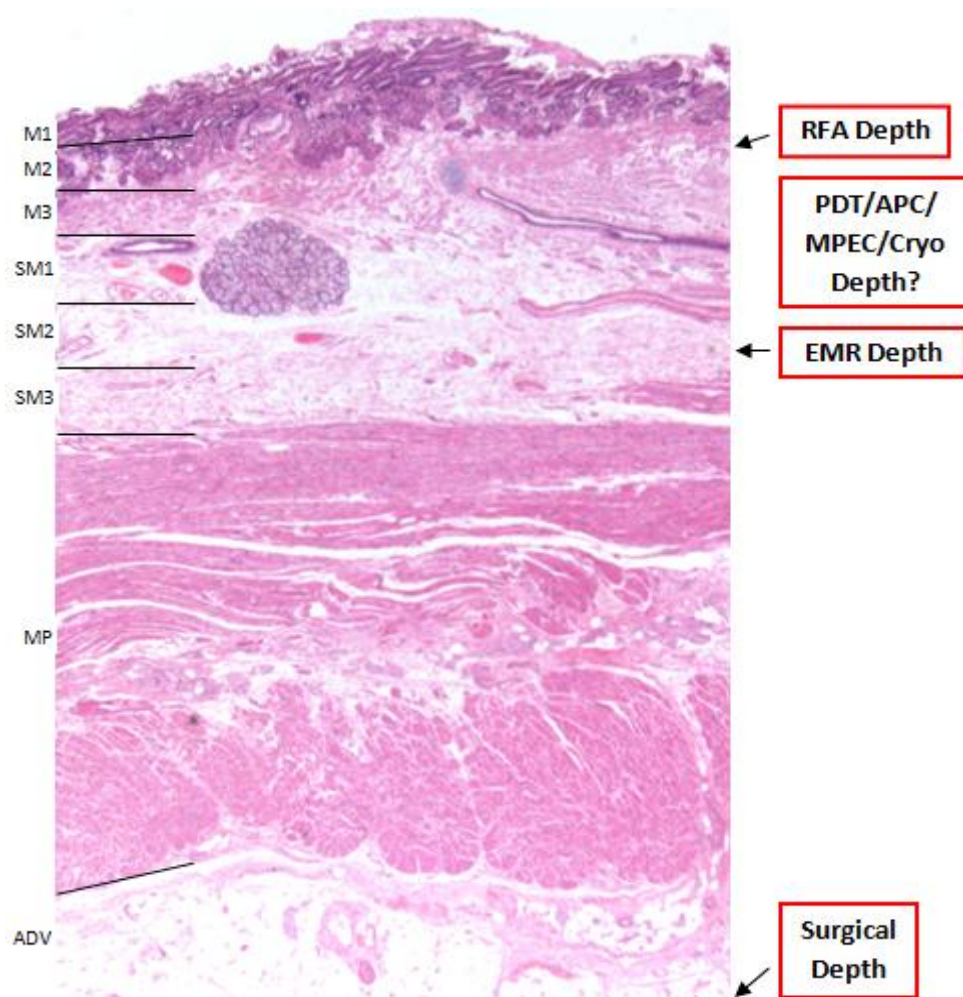


Figure 2-12. Treatment Depths. T-stage classification is illustrated. Mucosa (M), submucosa (SM), muscularis propria (MP), adventitia (ADV).

2.9.1.1 Endoscopic Resection

Endoscopic mucosal resection aims to remove the mucosa and submucosa down to the muscularis. For this reason the term endoscopic resection (ER) is now preferred. The

technique involves raising an area of mucosa using suction or by submucosal injection, and then snaring it off in a similar manner to a colonic polyp. It is a useful technique for removing focal areas of HGD or early cancer, and as well as being therapeutic it provides important diagnostic information.

ER preserves tissue architecture, obtains specimens that are easy to orientate, and enables accurate assessment of the depth of invasion (Figure 2-13).[99] A retrospective study of 150 ER specimens (focal lesions) found that initial diagnoses based on point mucosal biopsies were changed in 49% of cases following assessment of ER specimens. A change in management plan was demonstrated in 30% of patients.[147] Similarly, Mino-Kenudson et al. recently demonstrated that interobserver reporting agreement between pathologists was improved when reporting ER specimens rather than point biopsies, particularly when differentiating between intramucosal and submucosal carcinoma, a key distinction when planning a treatment strategy.[148] In addition, ER specimens also enable improved assessment of other important prognostic factors such as the grade of cellular differentiation and the presence of lymphovascular invasion. ER has been shown to be the best technique for assessment of visible mucosal abnormalities within Barrett's oesophagus.

ER is indicated for removal of focal HGD. Piecemeal resection is required for lesions greater than 2cm with meticulous care being taken to ensure completeness of excision. Complications are uncommon - bleeding (3%) and perforation (0.1-5%) - and most can be managed endoscopically.[149]

ER has been shown to achieve remission in 82.5% to 95% of patients with HGD, but may be associated with metachronous lesions or disease recurrence in up to 14% of patients within 12 months, and 21.5% of patients over 5 years.[150-153] Factors associated with recurrence include piecemeal resection, long-segment Barrett's oesophagus (>5 cm), delayed treatment of HGD >10 months, multifocal disease and omission of adjuvant ablative therapy.[150] Recurrent disease necessitates re-treatment which can be successful and provide long-term disease control but which may have higher complication rates.

Several trials have reported circumferential ER for removal of widespread multifocal disease however this practice has led to high rates of post-treatment stricture formation (17-26%) and

higher rates of perforation (3%) and is therefore not widely recommended.[154; 155] In addition, significant recurrence rates have been reported using this strategy. Also, in up to 25% of cases, complete resection of the Barrett's segment may be impossible despite multiple staged attempts at treatment.[98]

As discussed previously, oesophageal lesions that invade into the lamina propria but are confined to it (do not invade the submucosa), T1a lesions, have a 5% chance or less of nodal involvement. Recent data have suggested that shallow submucosal invasion T1sm1 also has a significantly lower risk of nodal metastases than deeper invasion.[156; 157][158] In early cancers with a low risk of lymphatic spread, ER therefore offers a potentially curative, minimally invasive, treatment option, which may be particularly appropriate in older patients at higher risk of operative morbidity / mortality. Average 3-year survival rates of more than 80% have been reported for T1a adenocarcinomas treated by ER.

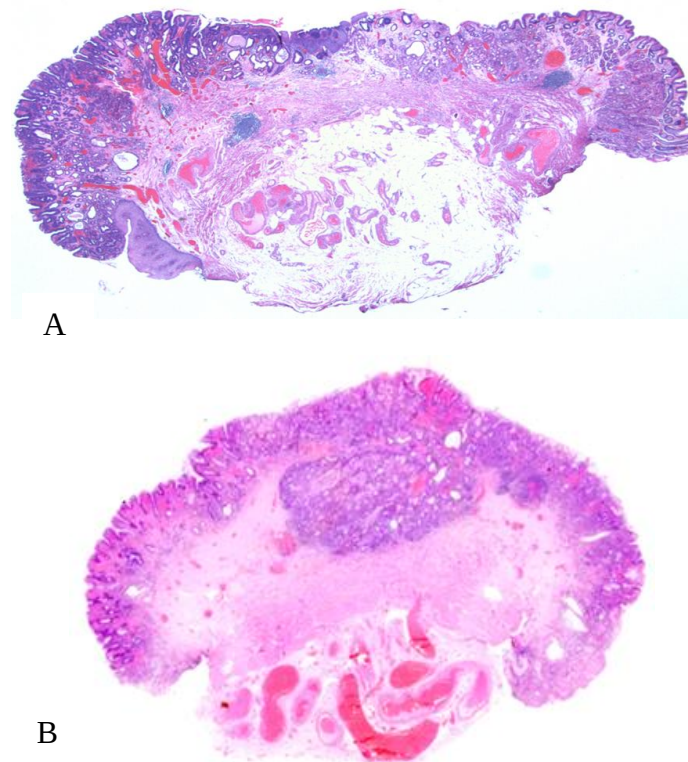


Figure 2-13. Endoscopic resection of focal HGD with clear margins (A), and an early invasive cancer (B).

2.9.1.2 Mucosal ablation therapy

Endoscopic ablation techniques aim to destroy the lining of the oesophagus and promote regenerative growth of normal squamous mucosa. Techniques for mucosal ablation include argon-beam plasma photocoagulation (APC), multipolar electrocautery (MPEC), laser therapy and cryotherapy; chemical methods, such as photodynamic therapy (PDT); and radio-frequency (RFA) ablation. The ablation depths achieved by the respective therapies are illustrated in Figure 2-12 and evidence supporting each technique is discussed below.

2.9.1.3 Radiofrequency Ablation

RFA is a relatively new technique which can be used to ablate circumferential (HALO³⁶⁰) or focal (HALO⁹⁰) Barrett's oesophagus. Circumferential ablation is performed using a balloon to apply radiofrequency energy evenly to the lining of the oesophagus. The procedure is described below.

The length of the Barrett's segment is first measured endoscopically. N-acetylcysteine is then used to wash saliva and gastric juice from the oesophagus and a guidewire is placed into the gastric antrum. The endoscope is removed and a sizing balloon is inserted over the guide wire and inflated once in the distal oesophagus. In long segment Barrett's oesophagus several measurements are taken and subsequently, an appropriately sized ablation catheter is selected (based on the smallest oesophageal diameter measurement). The catheter is then passed over the guide wire and positioned at the proximal extent of the Barrett's segment. The endoscope is re-passed to ensure correct positioning of the catheter and the balloon is then inflated and a standardised dose of energy is delivered (which has a power density sufficient to ablate down to the muscularis mucosae, 700-1000µm deep). After a short period of treatment (<5s) the catheter is passed distally to the next portion of the oesophagus to be treated, trying to minimise overlap between zones by endoscopic visualisation. Once the entire Barrett's segment has been ablated the catheter is removed and the endoscope is reinserted in order to debride the ablated mucosa. The procedure is then repeated so that the whole Barrett's segment receives two treatments.

Complications are rare but include significant bleeding (1-2%), stricture formation (6%) and perforation (very rare).[159] Repeat OGD is recommended after 2 – 3 months and any residual focal Barrett's oesophagus can then be treated using HALO⁹⁰ RFA.

A large randomised trial of RFA by Shaheen *et. al.* demonstrated successful resolution of dysplastic Barrett's oesophagus following treatment with RFA.[159] Complete eradication of LGD was seen in 90.5% (ablation group) compared to 22.7% (control group) ($P<0.001$). Complete eradication of HGD occurred in 81.0% (ablation group) versus 19.0% (control group) ($P<0.001$). RFA also decreased the likelihood of disease progression (3.6% vs. 16.3%, $P=0.03$) and cancer (1.2% vs. 9.3%, $P=0.045$).

Recently encouraging longer term follow-up results have been reported. A randomised trial of 127 patients with dysplastic Barrett's oesophagus demonstrated eradication of dysplasia in 55 of 56 subjects after 3 years. The rate of oesophageal adenocarcinoma was calculated at 0.55% per patient-years over the study period.[169]

2.9.1.4 Photodynamic Therapy

Photodynamic therapy (PDT) involves systemic (intravenous) administration of a photosensitising agent (porfimer sodium) which is retained selectively by dysplastic cells. After 48 hours the patient undergoes an upper endoscopy and a laser is used to excite a cytotoxic reaction in dysplastic Barrett's cells, leading to their destruction. There is now strong evidence that PDT can prevent the progression of disease in patients with Barrett's HGD. [170] A five year randomised multicentre trial by Overholt *et. al.* demonstrated that PDT was significantly more effective at eradicating HGD than omeprazole only (odds ratio 2 ± 0.7). It also significantly lengthened the time taken to progress to malignancy and reduced the overall risk of malignant progression by half.[26; 161]

Side-effects of PDT include nausea and chest pain in the first day or two after treatment. In the longer term, oesophageal strictures may occur in up to a quarter of patients. Oesophageal perforation has also been described (very rarely). In addition, due to the photosensitising affect of porfimer sodium, patients are required to minimise light exposure to their skin for up to 4-6 weeks after the treatment.

Several trials in Europe have used 5-ALA as the photosensitising agent in an effort to reduce skin sensitivity and oesophageal strictures. However, additional blood pressure and cardiac complications have been reported with 5-ALA and further work is therefore required to clarify the most effective drug with the least side-effects.

2.9.1.5 Argon Plasma Coagulation

APC is the most widely used form of thermal ablation. A jet of ionized argon (plasma) is directed through an endoscopic probe to ablate short segments of Barrett's oesophagus or areas of persistent disease following other ablative treatment. A trial by Pech *et. al.* retrospectively assessed disease recurrence in patients treated by ER with or without subsequent APC ablation of the residual Barrett's segment. Rates of recurrence fell from 33.3% to 17.6% with the inclusion of APC ablation. Other studies support these results and report low complication rates.[26; 162-164]

2.9.1.6 Multipolar Electrocoagulation

MPEC is widely used as a method of endoscopic therapy for acute gastrointestinal haemorrhage, however it also has a well recognised role in thermal ablation of Barrett's oesophagus. A RCT comparing MPEC with APC followed 35 patients over a two year period. Complete reversal of Barrett's oesophagus was demonstrated in 75% of patients treated by MPEC and 63% treated by APC. One patient treated by MPEC developed an oesophageal stricture but no major complications (bleeding / perforation) were reported.[165] Another trial of MPEC by Sampliner *et. al.* showed that 78% (45/58) patients had complete reversal of Barrett's oesophagus following a mean number of 3.5 treatments.[26]

2.9.1.7 Cryotherapy

Cryotherapy has recently been approved for use in the United States in patients with dysplastic Barrett's oesophagus. The technique involves the non-contact delivery of liquid nitrogen to Barrett's epithelium via an endoscopic catheter. A retrospective multicentre study

of endoscopic cryotherapy by Shaheen *et. al.* included sixty patients who underwent a median of 3.4 treatments. 97% had complete eradication of HGD, 87% had eradication of all dysplasia with persistent non-dysplastic intestinal metaplasia, and 57% had complete eradication of all intestinal metaplasia.[166] However, patients were followed for a median time of just 10.5 months. Evidence of ‘buried’ metaplastic cells were found in 2 patients (3%). Strictures were described in 3 patients, bleeding (requiring admission) in 1 patient and severe chest pain in 2 patients. No perforations have been reported following cryotherapy.[26]

2.9.1.8 Lasers

A variety of lasers (KTP, Nd:YAG, CO₂, diode) have been used for ablative therapy in Barrett’s oesophagus with mixed results. Barham *et. al.* used KTP laser ablation in 16 patients with non-dysplastic Barrett’s oesophagus.[167] 11/13 patients were found to have persistent Barrett’s despite a mean of three treatments per patient. Biddlestone *et. al.* demonstrated similarly unconvincing results – 11/24 biopsy samples from 10 patients treated using KTP showed evidence of residual Barrett’s mucosa.[168] In 2011 Polese *et. al.* published results of long-term follow-up of Barrett’s oesophagus treated by diode laser ablation.[169] Twenty patients with IM (including four with LGD) were treated with a mean of eight treatments over 2 years. Complete remission was demonstrated in 13 patients (11/12 with BO ≤ 3 cm and 2/8 with BO >3 cm). Similar results have been obtained previously by Weston *et. al.* using Nd:YAG laser.[170]

Laser therapy (whichever modality is chosen) is safe and may have a role in ablating short Barrett’s segments. However, as with APC and MPEC, its most appropriate use may be in targeted ablation of small persistent areas of Barrett’s oesophagus following earlier RFA or PDT ablation.[26]

2.9.2 Management of LGD

The detection of LGD should prompt a course of high-dose acid suppression with a PPI followed by repeat endoscopy with extensive biopsies at 2-3 months. If LGD persists then surveillance endoscopy should be repeated at 6-monthly intervals and the patient should

remain on a PPI. If regression to metaplasia without dysplasia occurs on two consecutive examinations then the surveillance interval may return to 2-yearly.[7]

Endoscopic treatment of LGD is controversial and is not supported by current UK guidelines based on a number-needed-to-treat analysis. However, simple surveillance of LGD is not universally supported and there is growing backing for early intervention.

The reported significant over-diagnosis of LGD in routine clinical practice may account for low reported rates of disease progression and supports the widely recommended policy of close surveillance, without endoscopic therapy. However, an alternative option might be for referral of all histology slides showing suspected LGD for specialist consensus reporting, with subsequent endoscopic treatment for confirmed cases of LGD. This approach would have considerable economic and practical implications and is not currently feasible in the UK.

2.9.3 Management of HGD

The detection of HGD has serious implications for the patient and should be considered analogous to a malignant lesion. The diagnosis should always be confirmed by a second expert pathologist within the setting of a multidisciplinary meeting. The diagnosis of HGD is usually an indication to end surveillance and patients should be fully informed of the significance of the diagnosis and of the pros and cons of endoscopic and surgical treatment.

Falk et. al. demonstrated that 38% of cancers were missed when taking quadrantic biopsies every 2 cm from patients with HGD. Jumbo biopsy forceps made little difference to detection rates (67% versus 62%).[19] Similarly, Cameron and Carpenter found 2/19 (10.5%) unsuspected adenocarcinomas following quadrantic 2 cm biopsies in patients who subsequently underwent oesophagectomy.[171] Reid et. al. compared a quadrantic 2 cm biopsy protocol to biopsies taken at 1 cm intervals in 45 patients diagnosed with HGD who subsequently developed cancer.[172] The 2 cm protocol missed 50% of the cancers that were detected by the 1 cm protocol in Barrett's segments 2 cm or more without visible lesions.

Following referral to specialist centres patients with HGD should therefore be re-biopsied with quadrantic biopsies every 1 cm with further targeted biopsies from suspicious areas. Where indicated a large number of samples should be taken - up to 84 biopsies from a single patient has been reported.[12] However, it is important to consider that Barrett's adenocarcinomas may still be missed in up to 29% of cases.[173] Patients should be carefully staged, including use of diagnostic ER, and patients with nodular disease should be considered at particular risk of harbouring occult invasive disease.[119; 120]

The choice of intervention in patients with HGD remains a controversial subject. Traditionally, individuals who were fit enough were recommended oesophagectomy while endoscopic techniques were reserved for those unfit for resection. However, with increasing evidence of therapeutic benefit, endoscopic therapy is now generally considered to be first line treatment ahead of oesophagectomy in fit patients provided they have been adequately staged.

Most units favour a policy of focal lesion resection using ER followed by ablation of the entire Barrett's segment using RFA to destroy the neoplastic field change in adjacent metaplasia. ER improves histological assessment and aids detection of occult adenocarcinoma[174; 175] but the need for subsequent ablation of the surrounding non-dysplastic Barrett's oesophagus is controversial. There are no RCTs addressing this directly, however there is some evidence to suggest lower recurrence rates if ER is used in conjunction with whole segment ablation.[19; 113]

Patients must be informed of the potential need for lifelong endoscopic surveillance (including biopsy) following endotherapy, even in cases of complete response, to ensure the absence of long-term recurrence and the identification of buried glandular elements which may retain malignant potential. Surveillance after oesophagectomy is not routine as recurrence rates are low, although Barrett's associated adenocarcinoma above the gastric conduit has been described.

The choice of ablative modality is also open to debate. RFA and PDT deliver an even distribution of treatment over a consistent therapeutic depth and can be readily applied to large areas of circumferential disease. Both have been shown to be highly efficacious at eradicating dysplastic Barrett's oesophagus,[159-162; 168; 176-181] however RFA is now

widely regarded as the first line therapy due to its relative ease of administration, more favourable side-effect profile, and low rates of recurrent dysplasia.[180]

There is RCT evidence supporting the efficacy of both MPEC and APC for treatment of dysplastic Barrett's although, compared to RFA, these techniques are less user-friendly for treating large Barrett's segments and treatment depths are less consistent. In addition, they may be more likely to be complicated by strictures and the development of buried glandular mucosa beneath neosquamous epithelium.[165] Currently these techniques are favoured for 'touch-up' therapy in patients with small patches of persistent metaplasia following previous RFA treatments. A RCT comparing the two thermal techniques, MPEC and APC, found no significant advantage with either technique.[165]

The decision between endotherapy and oesophagectomy is controversial and dependent on patient comorbidity and the nature of the disease: HGD versus intramucosal cancer; unifocal versus multifocal; long versus short segment Barrett's; presence or absence of lymphovascular invasion; and grade of differentiation. Endotherapy can effectively eradicate HGD with low rates of disease recurrence in the medium term (long-term data awaited).[159; 160; 182] In addition, any recurrent disease can usually be managed endoscopically, or if necessary surgically, without excess mortality.[98; 183] However, advocates of surgery state the risk of occult adenocarcinoma, particularly in nodular disease, and the possible risk of under-staging of disease using a non-operative approach. In addition, there is evidence that prophylactic oesophagectomy in HGD is associated with a lower risk of operative mortality than routine oesophagectomy as patients are typically younger with fewer comorbidities, and have not undergone neoadjuvant therapy.[21] There are no randomised trials comparing surgery and endotherapy in the management of HGD and these studies should be undertaken as a matter of urgency. However, it is certainly clear that endotherapy is a viable treatment option for patients with HGD, with close surveillance, early endoscopic treatment of recurrence, and progression to oesophagectomy if indicated. The Surveillance Epidemiology and End Results database of the US National Cancer Institute found no difference in survival between patients with HGD or stage 1 (T1N0M0) tumours treated by endoscopic therapy compared to radical surgery.[184; 185] However, most of the current studies directly comparing radical surgery and endoscopic therapy have been severely limited by selection bias.[156; 184; 185] A recent Cochrane review was unable to recommend either surgery or endotherapy as first line treatment in HGD due to the paucity of high level evidence and the

multitude of contributing factors, and made strong recommendations for the initiation of well designed multi-centre randomised trials.[186][26]

2.9.4 Management of Early Oesophageal Cancer

Patients with confirmed oesophageal adenocarcinoma should undergo complete tumour staging to establish the presence or absence of distant or locoregional metastases. Surgery should be regarded as the treatment of choice in young patients with multifocal nodular IMC provided they are fit enough to tolerate oesophagectomy. Fit patients with T1sm1 disease should also be offered surgery as a first-line therapy due to the significant risk of lymph node involvement.

Patients with a small focus of IMC should be considered for dual modality endotherapy, with surgical resection reserved for salvage. EUS to assess nodal status could be considered in these patients, however, it is generally thought to be poor at distinguishing between T1a and T1b tumours (33-85% accuracy) and importantly under-diagnosis of T1b is common.[4; 187] EUS is therefore not usually undertaken in early (T1) disease.

Older patients and those with significant co-morbidities should be treated by endotherapy even in the case of T1b disease as not all will have lymph node involvement, particularly those with disease limited to the upper third of the submucosa (T1sm1).

2.10 Summary: Clinical Need for a Novel Diagnostic Tool

For more than a hundred years clinicians have relied on the histological appearance of cells in order to establish the diagnosis of most malignant and inflammatory conditions. Although a trusted technique, histological assessment of tissue is time consuming, expensive, and necessitates tissue biopsy which carries with it the risks of bleeding and visceral perforation. Furthermore, as previously discussed, histological diagnosis can be extremely subjective. Kerkhof *et. al.* demonstrated a poor level of interobserver agreement between expert histopathologists (K=0.58) in the grading of low-grade (LGD) and high-grade (HGD) oesophageal dysplasia.[188] However, this distinction has vital consequences for patient management as LGD can be monitored by serial surveillance endoscopy whereas HGD

necessitates early endoscopic therapy or even surgery, and carries a significant risk of malignant progression. Although histological assessment remains the ‘gold standard’ at present, there is a clear need for an objective diagnostic test which relies on biochemical or molecular analysis of target tissues rather than an individual’s assessment of cellular appearance.

As well as being difficult to classify histologically, dysplasia and IMC can be extremely difficult or even impossible to recognise at endoscopy even for skilled endoscopists. Progression to dysplasia or IMC in Barrett’s oesophagus is rare and the lack of familiarity of most endoscopists with the typical appearances of early neoplasia is a significant limiting factor in its detection.[100; 101; 103] Guidelines therefore advise that multiple biopsies are taken whenever Barrett’s oesophagus is identified at endoscopy.[7] Most endoscopists take four quadrant biopsies every 2 cm of Barrett’s oesophagus, or fewer.

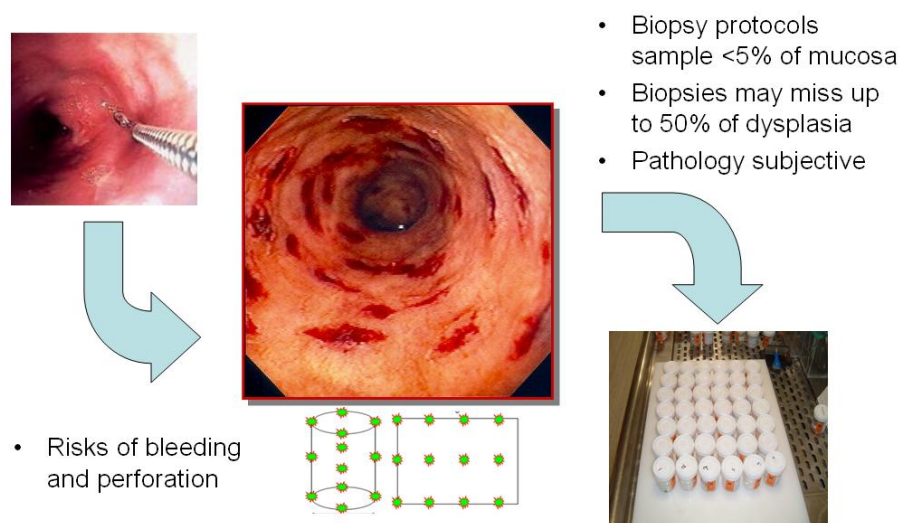


Figure 2-15. Current clinical practice. Clinicians are advised to take multiple, random oesophageal biopsies. This policy samples <5% of the mucosa.

Surveillance biopsy protocols sample less than 5% of the mucosa and may miss up to 57% of early neoplastic lesions.[8] In addition, patients with HGD are known to have a 30-40% chance of occult adenocarcinoma which may be missed by sampling error in up to 50% of patients when using a standard biopsy regimen. Several studies have compared the results of

endoscopic biopsy assessment to surgical pathology following oesophagectomy for the detection of adenocarcinoma in dysplastic Barrett's segments. Falk et. al. demonstrated that over a third of cancers (38%) were missed when quadrantic biopsies every 2 cm were taken from patients with HGD. Jumbo biopsy forceps made little difference to detection rates (67% versus 62%).[19] Cameron and Carpenter found 2/19 (10.5%) unsuspected adenocarcinomas following quadrantic 2 cm biopsies in patients who subsequently underwent oesophagectomy.[171] Similarly, Reid et. al. showed that this biopsy regimen would have missed 13/26 (50%) of cancers in their cohort. They also demonstrated that targeted biopsies of endoscopically visible suspicious lesions were only able to establish the correct diagnosis in 15 out of 45 (33%) patients who were subsequently proven to have cancer.[172]

There is a clear clinical need for advanced imaging tools that could improve endoscopic detection of HGD and IMC and enable targeted treatment.

CHAPTER 3

3 Literature Review: Raman Spectroscopy

Raman spectroscopy is a technique that measures the interaction between light and tissue in order to obtain specific biochemical information. Over recent years scientists have recognised that Raman spectroscopy has considerable potential for use in medical diagnostics. The technique is quick and non-invasive and is capable of differentiating between normal, pre-malignant, and cancerous tissue. It is hoped that with further development, Raman probes may provide accurate, non-invasive optical diagnosis in ‘real time’, without the need for biopsy.

3.1 Raman Scattering – Principles and Basic Science

When light interacts with a molecule it may be absorbed, or scattered, or may pass straight through it. For absorption to occur, the energy of the incident light must match the specific energy required to excite the molecule from its resting ‘ground state’ to an ‘excited state’. Only a particular frequency of light, with the specific energy required, can be absorbed by a particular molecule. This principle forms the basis of absorption spectroscopy.

In contrast to absorption, scattering of light does not require the energy of the incident light to correspond to the energy gap between the ground and excited states of the molecule. Scattering results when the incident light causes polarisation of the electron cloud around the molecule as the electrons move into a higher energy state. At this instant the interaction between the light and the molecule can be considered a very short-lived ‘complex’ where energy is transferred from the light wave instantaneously to the molecule. This changes the configuration of the electrons in the molecule but does not necessarily cause nuclear motion. Almost immediately the unstable ‘complex’ releases the light as scattered radiation. This scattered light characteristically has the same wavelength (i.e. the same energy) as the incident light and the process is therefore termed ‘elastic’ scattering. This effect is known as Rayleigh scattering and is by far the most intense form of scattering (Figure 3-1).

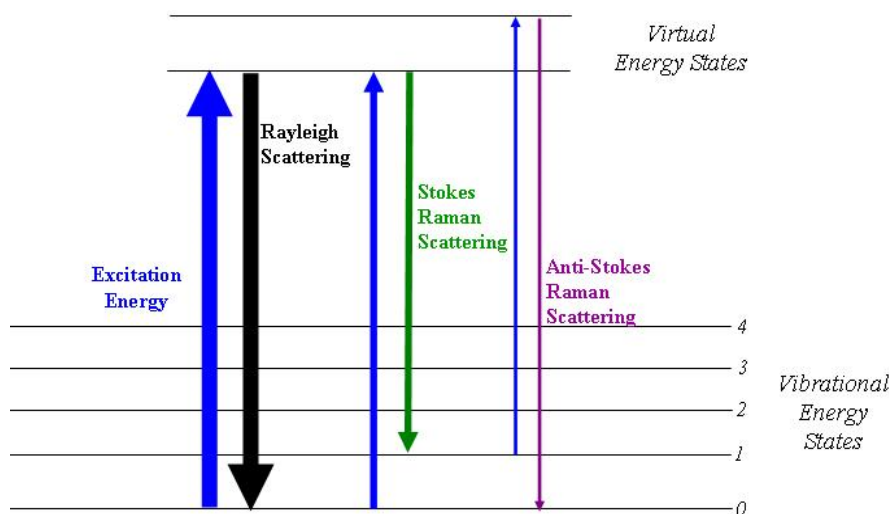


Figure 3-1. Changes in energy states during elastic (Rayleigh) and inelastic (Raman) scattering.

The other form of scattering which can be readily identified is Raman scattering. This is a much rarer event and occurs in only $1 \text{ in } 10^6 - 10^8$ of the photons scattered. Raman scattering was first described in 1928 by an Indian physicist of the same name who was later awarded the Nobel Prize. [189]

The key differentiating characteristic of Raman scattering is that there is an appreciable and measureable change in energy between the incident and the scattered light. This form of scattering is therefore termed ‘inelastic’ scattering (Figure 3-1).

Raman scattering occurs when the incident light interacts with a molecule and not only causes electron polarisation but also induces nuclear motion. In this process energy is effectively passed from the incident light to the molecule to create nuclear vibration. The scattered light is therefore of lower energy (lower frequency) than the incident light. The energy change between the incident and scattered light, which corresponds to the energy of vibration of the molecule, is known as the Raman shift. This is measured in wavenumbers and can be plotted against intensity. This plot is termed a Raman spectrum (see section 3.2).

Two types of Raman scattering have been described, dependent on the state of the molecule when the interaction with light occurs. Stokes scattering results when light interacts with a

molecule in its ground (unexcited state). Nuclear motion is initiated and the scattered light has a lower energy than the incident light. However, where the molecule exists in an already vibrationally excited state, as is seen at high temperatures, the scattered light is of a higher energy than the incident light. This is known as anti-Stokes scattering.

At room temperature, most molecules are at the ground vibrational state and therefore Stokes Raman scattering is much more prevalent than anti-Stokes scattering. It is possible to calculate the proportion of molecules in a substance that are in the ground and excited states using the ratio of intensities of Stokes and anti-Stokes scattering.

3.1.1 Molecular Vibration and Polarisation

Within a molecule there are a defined number of electrons in molecular orbits. A molecule also has a defined number of positional or vibrational modes in which these electrons can be found in relation to their nuclei. The number of vibrational modes possible for a molecule is described by the equation $3N-6$, where N is the number of atoms in the molecule. Hence, a triatomic molecule such as H_2O or CO_2 will have three modes of vibration. Importantly, the energy required to alter these vibrational modes differs for each molecule.

When a molecule vibrates the change in configuration of the nucleus alters the shape of the surrounding electron cloud. This can induce a change of the dipole moment or a change in the polarisation of the molecule. For the vibrational change in a molecule to be Raman active there must be a change in the polarisation of the molecule. That is to say that in shifting from one vibrational state to another, the electron density relative to the position of the nucleus must change. However, not all changes in vibrational states can be detected by Raman spectroscopy as in some cases the change in vibrational mode represents a change in the dipole moment of the molecule and not its polarisation. This change is not detected by Raman spectroscopy but could be detected by IR spectroscopy. For this reason IR and Raman spectroscopy are often regarded to be complimentary to one another.

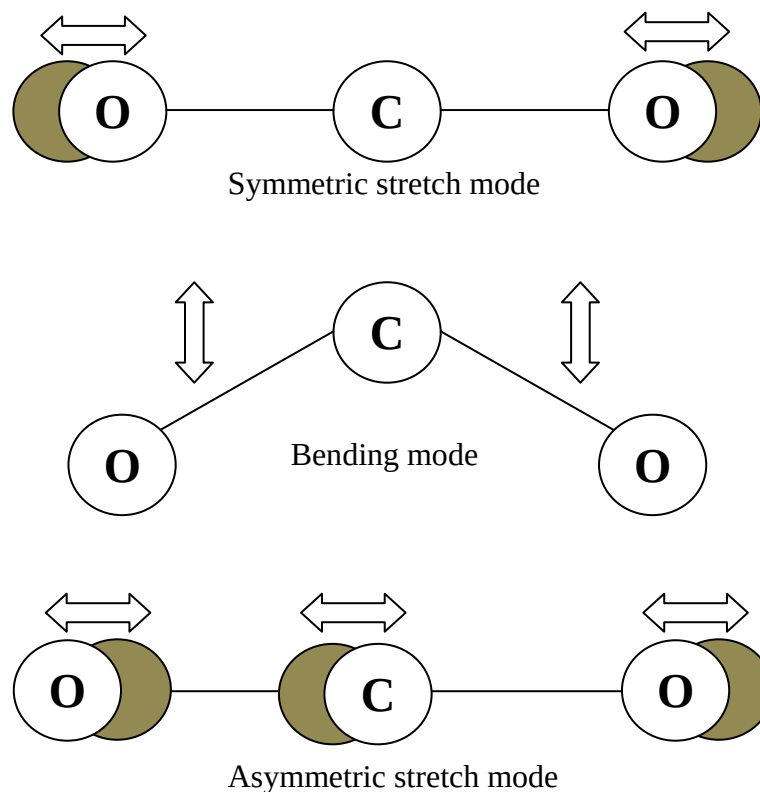


Figure 3-2. Three modes of vibration are possible for a CO₂ molecule: symmetrical stretch, a bending mode and an asymmetrical stretch.[190] In triatomic molecules, symmetrical stretch causes large polarisation changes and therefore strong Raman signals, whereas bending and asymmetric stretch cause a dipole change and little polarisation change and therefore a weak Raman signal.

Another example of the differences in vibrational modes can be appreciated when the properties of a phenyl ring are compared to an OH bond. The phenyl ring has a large electron cloud and is highly polarisable giving a strong Raman signal, whereas an OH bond is less polarisable (so gives a weak Raman signal), but a dipole change is readily induced causing a strong IR signal.

The properties of the OH bond have important consequences for the application of spectroscopy to living tissue diagnostics. As a result of the strong IR signal, all water must be excluded from tissue samples before using IR spectroscopy to prevent profound distortion of the spectra from the marked dipole effect of the OH bonds. This effect is not seen with

Raman spectroscopy and so allows this technique to be used to analyse fresh tissue. It is usually seen that any molecule of sufficient complexity will exhibit a usable Raman signal.

3.2 The Raman Spectrum

A Raman spectrum is a plot of 'Raman shift' measured in wavenumbers (on the x-axis) against intensity (on the y-axis) (Figure 3-3). The zero point on the x-axis corresponds to the energy of the incident light. Data to the right of this point therefore represents Stokes scattering, whereas data to the left represents anti-Stokes scattering.

A Raman spectrum is always relative to the wavelength of the incident light. However, the x-axis of a Raman spectrum is rarely measured in wavelength as this would vary with the incident light chosen for excitation. Instead, wavenumber is used to represent the 'Raman shift' and facilitate comparison between spectra.

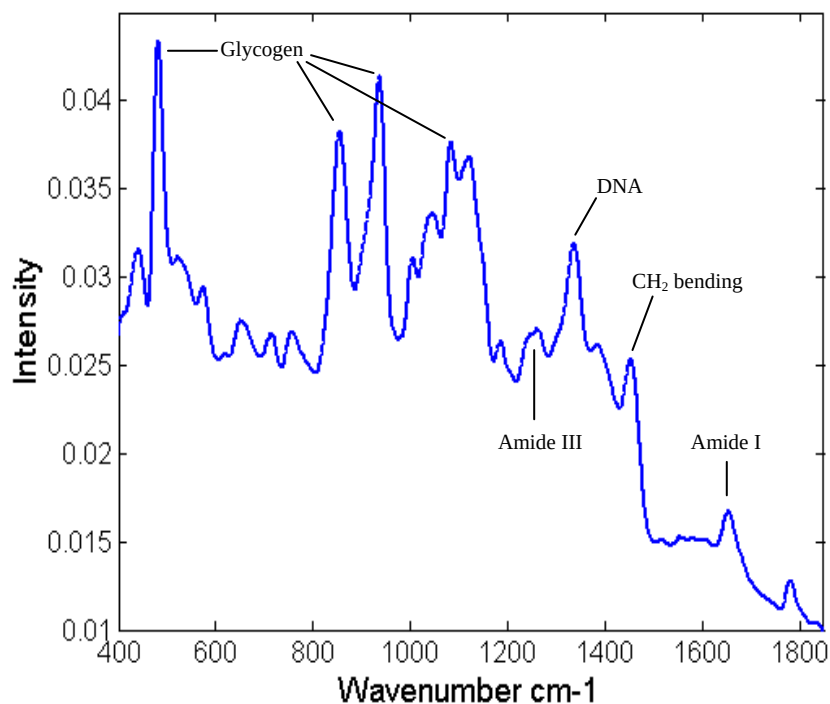


Figure 3-3. A Raman spectrum acquired from normal squamous oesophageal mucosa.

A larger Raman shift indicates that a greater energy is required to create vibrational motion in a given molecule and crucially, the ‘value’ of the Raman shift for a given compound is independent of the frequency of the excitational light. However, excitational light frequency does directly affect the intensity of the Raman signal.

Raman peaks are spectrally narrow and correspond precisely to the vibration of specific bonds within molecules. In addition, the intensity of a peak is proportional to the concentration of the specific molecular constituent that gives rise to it. This allows specific vibrations to be assigned to certain spectral peaks enabling a precise qualitative and quantitative biochemical fingerprint to be obtained.[191] However, in complex molecules peak assignment can be difficult and unreliable as the various peaks merge to form bands. It is then the shape and distribution of these bands that can be used as a chemically sensitive signature of the specimen.

Analysis of pure natural compounds such as glycogen and collagen has enabled scientists to assign peaks that are characteristic of these molecules (Figure 3-4 and Table 3-1). For example, glycogen molecules have characteristic peaks at 490, 853, 937, 1083 and 1123 cm^{-1} . [192] Although potentially obscured by signal from other molecular constituents, modern multivariate analysis techniques can be used to extract these subtle spectral features and enable conclusions to be drawn regarding the nature of a tissue specimen. These statistical techniques can help realise the potential of Raman spectroscopy to deliver objective, quantitative information which can enable precise tissue analysis and clinical diagnosis.

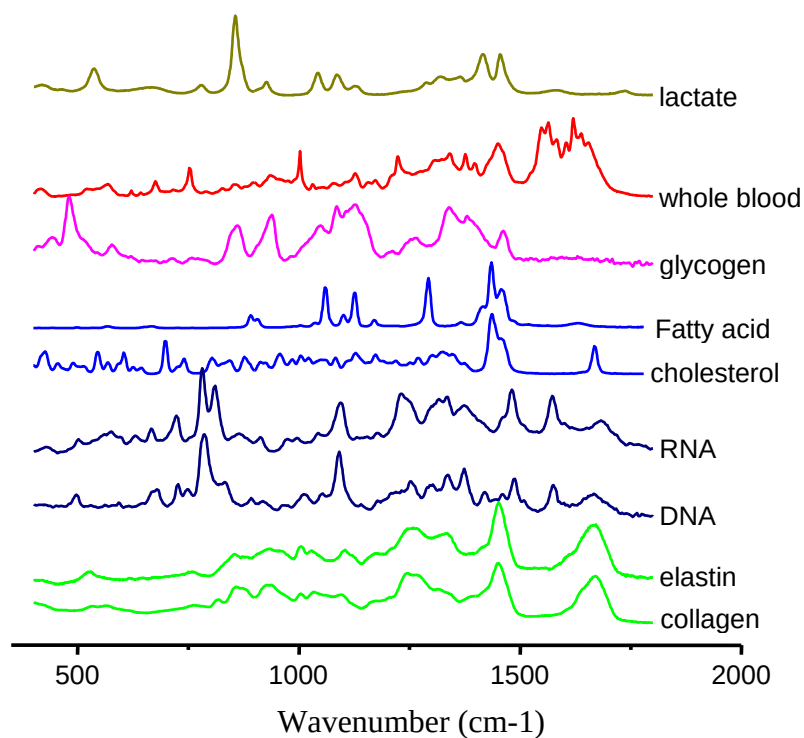


Figure 3-4 . Examples of spectra from a variety of human molecular constituents. Characteristic peaks can be seen for each substance at reproducible positions.[192]

Table 3-1 displays a list of common biochemical constituents found in oesophageal tissue with their corresponding characteristic Raman peak positions obtained from the literature.[191; 193-195] A more comprehensive list is provided for reference in Appendix III.

Peak position (cm ⁻¹)	Major assignments
490	Glycogen
524	Disulfide stretch in proteins
574	Glycogen
643	Tyrosine
669	Cysteine
720	Nucleic acids
755	Tryptophan
781	RNA/DNA
853	Glycogen
937	Glycogen
1001	Symmetric ring mode of phenylalanine
1083	Glycogen
1128	Glycogen
1262	Amide III
1313	Collagen I
1335	DNA
1446	CH ₂ bending of proteins and lipids
1575	DNA (Nuclei acids and heam protein)
1655	Amide I (Histones)

Table 3-1. List of common biochemical components found in oesophageal tissue with their corresponding peak position. Table reproduced from Stone et. al.[191]

3.3 Raman Instrumentation

Raman spectrometers must be enormously sensitive in order to detect Raman signal which is of extremely low intensity.[196] They must also be able to exclude all other signal, including elastically scattered light, to ensure the Raman signal is not completely obscured. This is achieved using a series of filters within the instrument.

The basic components of a modern Raman system are identified in Figure 3-5.

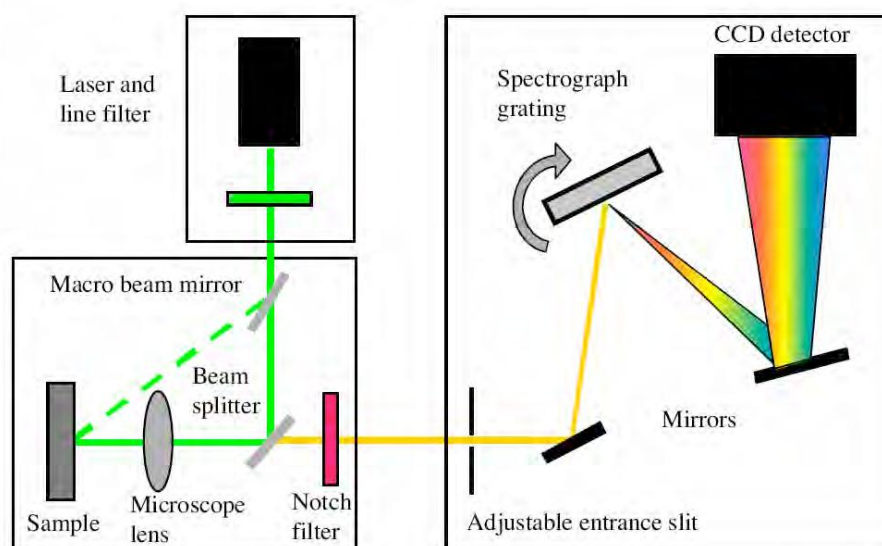


Figure 3-5. Standard apparatus within a laboratory Raman spectrometer.

As the position of Raman peaks are relative to the wavelength of incident light, a monochromatic (single wavelength) incident laser is used for excitation. When assessing biological tissue, incident laser light in the near-infrared (NIR) region is generally the preferred source for excitation. At this wavelength there is minimal tissue absorption and the risks of thermal or mutagenic effects are therefore avoided. In addition, the effect of fluorescence seen with visible and UV light is largely overcome.

A series of mirrors typically guide the laser onto the sample. Scattered light passes back into the spectrometer and is filtered to remove elastically scattered light. A lens focuses the light onto a slit, which removes any stray light. The light is then split and dispersed by the diffraction grating and then reflected onto the charge-coupled device (CCD) (Figure 3-5).

The CCD typically consists of a sectorised length of silicon. This enables different frequencies of scattered light to be collected separately, and this information can then be relayed to a computer in order to construct a spectrum. The shape of the spectrum will depend on the number of photons striking each sector of the silicon.

In this thesis a custom-built fibre-optic probe has been coupled to a Renishaw system 100 spectrometer (Renishaw Plc., Wotton-under-Edge, Gloucestershire, UK) to assess potential

for endoscopic RS in the oesophagus. Full details of the probe-spectrometer design are included in Chapter 4.

3.4 Application of Raman spectroscopy to medical diagnostics

The ability of Raman spectroscopy to accurately identify pathological changes in tissues has been proven using *ex vivo* tissue specimens from breast, cervix, larynx, bladder, prostate, lymph nodes and oesophagus.[31-51]

These studies have demonstrated the huge potential for future clinical and pathological use of Raman spectroscopy, particularly in oncological diagnosis. On-going research utilising modern fibre-optic technology now aims to develop a Raman probe for use endoscopically or laparoscopically to enable rapid tissue diagnosis. In the oesophagus, a Raman probe could identify early neoplastic changes which are often invisible using standard endoscopy. With further development Raman spectroscopy could remove the subjectivity from histological assessment and even eliminate the need for a biopsy altogether (so called ‘optical biopsy’).

Objective endoscopic diagnosis of neoplastic change in the oesophagus would enable immediate intervention to take place. HGD in Barrett’s oesophagus could be diagnosed and then treated by endoscopic resection (ER) during a single endoscopy session; or similarly intra-operatively tumour margins could be assessed to ensure an adequate surgical resection. In addition, when re-scoping a patient with biopsy-proven focal HGD or IMC, Raman spectroscopy could accurately identify the lesion so that ER (of the correct area) could be performed.

The diagnostic potential of Raman spectroscopy is significant. Laboratory results have been consistent and encouraging, and the development of fibre-optic Raman probes is another significant step towards possible *in vivo* use. However, the challenge of developing *in vivo* Raman systems is considerable as several important factors must be addressed and overcome: [191]

- Weak signal strength
 - Raman signals are inherently weak (only 1 in $10^6 - 10^8$ photons are inelastically scattered).
 - Biochemical changes in cells that have become dysplastic are very difficult to detect as they may be present in minute concentrations.
 - When used on living tissue, the intensity of the excitatory laser must be limited to prevent tissue damage. This limits the ability to generate strong scattering signals from the tissue.
- Signal interference
 - Biological tissues are inhomogeneous in composition and thus highly scattering.
 - Tissue fluorescence.
 - Spectral contamination caused by the background Raman and fluorescence signals generated in the fibre-optic materials.

Previous work has addressed many of the issues related to signal strength and signal interference. One method of circumventing this problem is to utilise highly sophisticated data analysis techniques. Previous studies have recognised that simple peak analysis comparing the intensities and wavenumbers of individual peaks, fails to provide discrimination between pathology. Instead, one must consider diagnostic information to be spread throughout the spectrum and complex chemometric approaches such as principal component analysis (PCA) and linear discriminant analysis (LDA) have been utilised to tease out biochemical differences between tissue types (see Chapter 4).

When aiming to optimise signal strength it must be considered that the intensity of a Raman peak is dependent on the polarisability of the molecule being excited, the concentration of that molecule in the tissue, and the intensity of the incident laser. In fact, the power of Raman scattering is known to increase with the fourth power of the frequency of the excitation source. In addition, signal to noise ratio (SNR) is proportional to the square of the intensity of the incident light multiplied by the acquisition time.

Therefore, increasing both the incident laser power and the acquisition time would be expected to generate a stronger Raman signal. However, in-practice this could cause injury to

human tissue and therefore does not offer a realistic solution. Moreover, prolonged acquisition times of more than a few seconds would be impractical for in vivo use.

Using excitatory light in the near-infrared (NIR) wavelength helps to minimise its absorption by human tissue and so enables relatively high laser power to be used safely. In addition, NIR light can penetrate more deeply into tissue (mm or more) which can be of particular advantage when trying to detect internal organs or deep tissues.

Another problem when using Raman spectroscopy to examine biological tissue is that of tissue fluorescence which often obscures the Raman signal.

3.4.1 Fluorescence

Fluorescence is the emission of light by a substance following absorption of light of a different wavelength. Although fluorescence is an absorptive process, its result is essentially very similar to that of Raman scattering – both transfer a molecule to a higher (or lower) energy level, and both lead to the production of photons with different frequencies from those used for excitation. The major difference is that Raman scattering occurs with any frequency of incident light, whereas for fluorescence to occur, the incident light must have a frequency specific to the molecule it is exciting. This means that Raman peaks are completely independent of the wavelength of the incident light, whereas fluorescence peaks are dependent on it. For this reason a wavelength of incident light can be chosen which aims to minimise tissue fluorescence.

In practice, fluorescence poses a major challenge to the application of Raman spectroscopy to medical diagnostics. Its effect can however be diminished considerably by using an excitation laser in the ‘near-infrared’ (NIR) wavelength which dramatically reduces the number of molecules with excited states low enough in energy to give fluorescence.

Further discussion on spectral interference caused by excessive fluorescence signal is included in Chapter 5.

3.4.2 Summary

The intensity of the Raman signal is related to:

- the power of the excitatory laser;
- the square of the polarisability of the molecule being analysed;
- the fourth power of the frequency used for excitation.

It is clear then that only one molecular property (its polarisability) affects the intensity of the Raman signal. By contrast there are two instrumentation parameters, chosen by the spectroscopist, which can influence the intensity of scattered output.

It might seem logical to maximise the frequency of the incident light in order to improve Raman sensitivity. Use of UV excitation would therefore appear beneficial, particularly as this would also help minimise tissue fluorescence. However, UV radiation is absorbed by many compounds, and as a result may cause tissue destruction through thermal injury. NIR light has been shown to provide the best balance between adequate scattering intensity, minimisation of fluorescence, and avoidance of thermal or mutagenic effects on the tissue being assessed. Similarly, increasing the power of the incident laser would increase peak intensity but may also cause thermal injury to the tissue (depending on the area of tissue being irradiated i.e. the power density). Shim *et. al.* previously demonstrated that a 250mW 830nm laser focused to a spot size of 500 μm produced no detectable rise in temperature following an exposure time of 1 minute.[197]

3.5 Raman Spectroscopy in the assessment of oesophageal pathology (in vitro)

Several groups have explored the potential of Raman spectroscopy for diagnosis of oesophageal pathology *in vitro*.

In 2003 Kendall *et. al.* analysed snap-frozen oesophageal biopsy specimens using Raman microspectroscopy.[198] Their subset analysis included normal squamous mucosa, Barrett's mucosa and adenocarcinoma, and showed a high level of agreement ($k=0.89$) between

consensus pathological opinion (the gold standard) and Raman measurements. They also showed that the level of agreement between Raman spectroscopy and consensus histological diagnosis compared favourably to that between an independent pathologist and the consensus pathology opinion for the same biopsy specimens ($k=0.76$). This illustrated the importance of gaining a consensus pathology opinion in order to reduce inter-observer variability, a finding supported by others.[199, 200]

Kendall *et. al.* reported the sensitivity and specificity of Raman microscopy for characterising eight different pathological subtypes of oesophageal tissue (Table 3-2). Visible differences in spectra were not evident and complex multivariate analysis was required to distinguish between the pathological groups. Where differences were found between Raman data and the ‘gold standard’ pathological opinion, Raman microscopy tended to overestimate the degree of dysplasia, for example, in several cases Barrett’s metaplasia was predicted by the model to be HGD.

	Normal squamous	Cardiac-type	Fundic-type	IM	HDG	Adeno-carcinoma	Squamous dysplasia	SCC
Sensitivity	94%	100%	96%	77%	88%	73%	100%	100%
Specificity	99%	100%	98%	99%	92%	99%	99%	100%

Table 3-2. Accuracy of Raman spectroscopic diagnosis of 8 pathological groups in the oesophagus. [200]

Shetty *et. al.* used Raman spectroscopy to characterise some of the early biochemical changes that occur in oesophageal mucosal cells as they progress to Barrett’s metaplasia and then to dysplasia and cancer.[43] Shetty *et. al.* found a marked variation in the distribution of a collection of biochemical constituents in normal, dysplastic and malignant cells. Biochemical constituents studied included sugars, lipids, and amino acids. Raman spectroscopy identified increased levels of DNA, actin and oleic acid in dysplastic glandular tissue compared to normal Barrett’s or squamous mucosa (Figure 3-6). Conversely, glycogen was particularly prominent in normal squamous tissue and reduced in neoplastic tissue. This study further supported the potential of Raman spectroscopy for early diagnosis of malignancy, and raised

the possibility that Raman spectroscopy could perhaps enable detection of biochemical changes in tissue before morphological changes manifest.

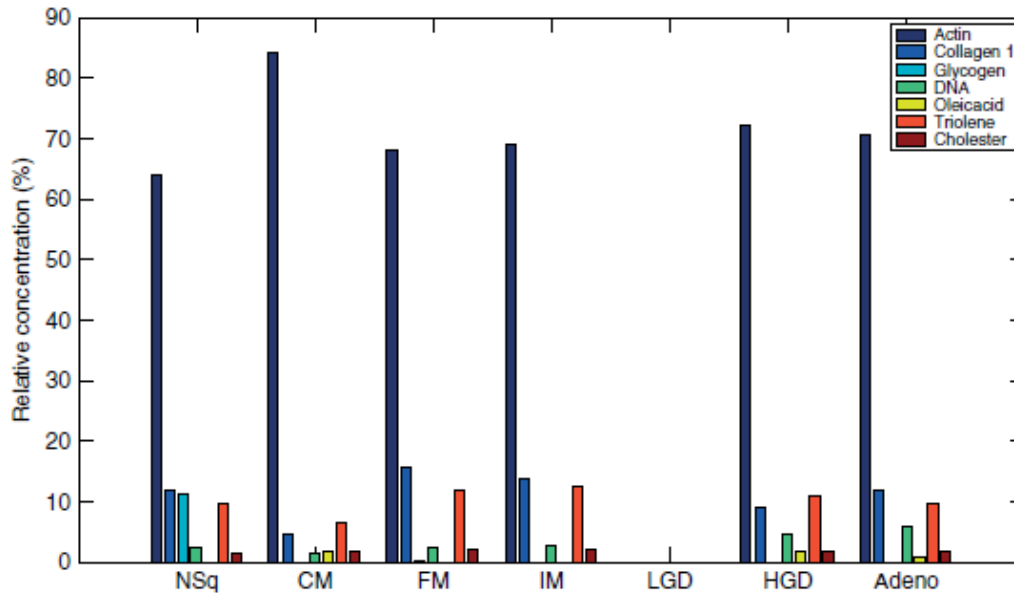


Figure 3-6. A biochemical distribution plot. Reproduced from Stone *et. al.* [8] Normal squamous (NSq), cardiac metaplasia (CM), fundic metaplasia (FM), intestinal metaplasia (IM), low-grade dysplasia (LGD), high-grade dysplasia (HGD), adenocarcinoma (adeno). Relative concentrations have been normalised to a total of 100%. Note there is no LGD as histopathological agreement was not consistent.

The proven ability of RS to analyse and classify biopsied oesophageal tissue has demonstrated potential for the technique to be used to aid histological assessment of tissue samples. The ability of RS to objectively characterise tissue based on its biochemical profile could help to overcome the inherent subjectivity associated with histopathological classification.

High quality Raman microspectrometers can be used to acquire oesophageal Raman maps based on distinction of histologically relevant biochemistry within frozen tissue sections. Figure 3-7 displays a high quality map of a sectioned biopsy from a patient with Barrett's oesophagus. This pseudocolour PC scores image was acquired in 48 hours using an x and y step size of 8.4 μm and an acquisition time of 30s per pixel.

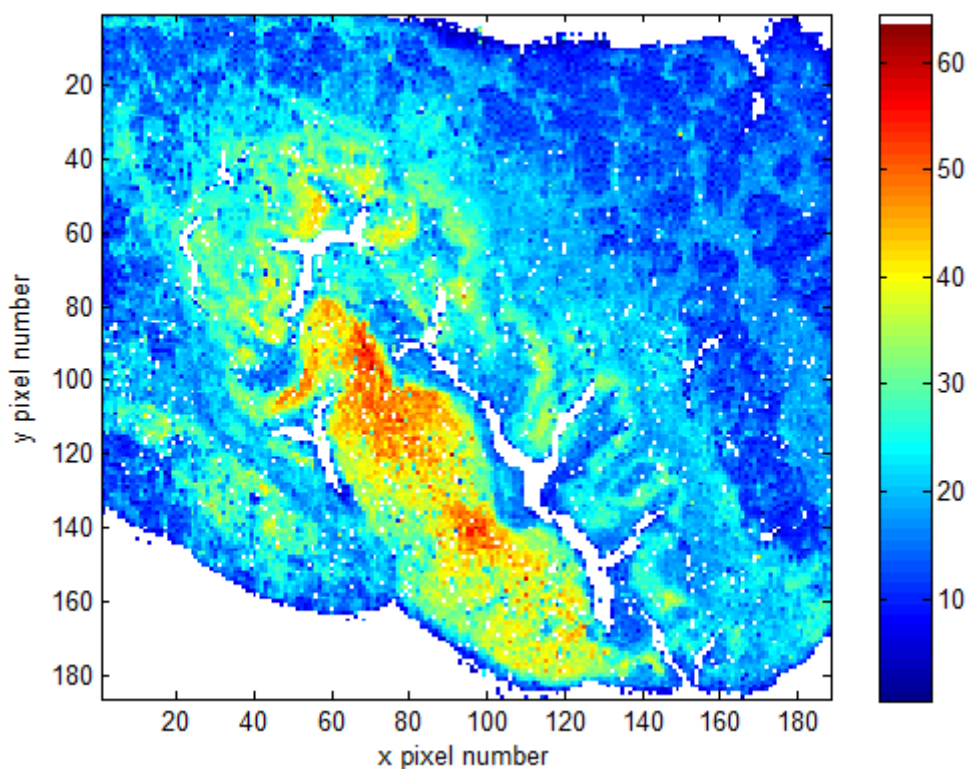


Figure 3-7. A high quality Raman map is able to identify 4 distinct areas which have been discriminated through differences in their biochemical composition. Each has a characteristic spectral fingerprint which can be distinguished using PCA.

Recently, Hutchings et. al. demonstrated that ‘rapid Raman maps’ of oesophageal tissue can be acquired in clinically practicable timescales.[38] Hutchings et. al. used a Renishaw StreamLine system (Renishaw Plc., Wotton-under-Edge, Gloucestershire, UK) coupled to a microscope to map 12 oesophageal biopsy samples which had been snap frozen and sectioned. An 830 nm diode laser was used for excitation which had a power of 70 mW at the sample.

Continuous readout of the CCD minimised time between sequential data measurements resulting in the acquisition of thousands of spectra in short timescales. Mapping of a 2mm oesophageal biopsy in 30-90 minutes (0.5 s acquisition time per 25.3 μm Raman pixels) in conjunction with principal component analysis was shown to be sufficient to generate spectral maps with clearly identifiable pathological regions.[34]

Figure 3-8 illustrates a rapid Raman map of a portion of an oesophageal tissue section. This map was acquired in 15 minutes using an x and y step of 26.4 μm and a 1 s acquisition time per pixel.

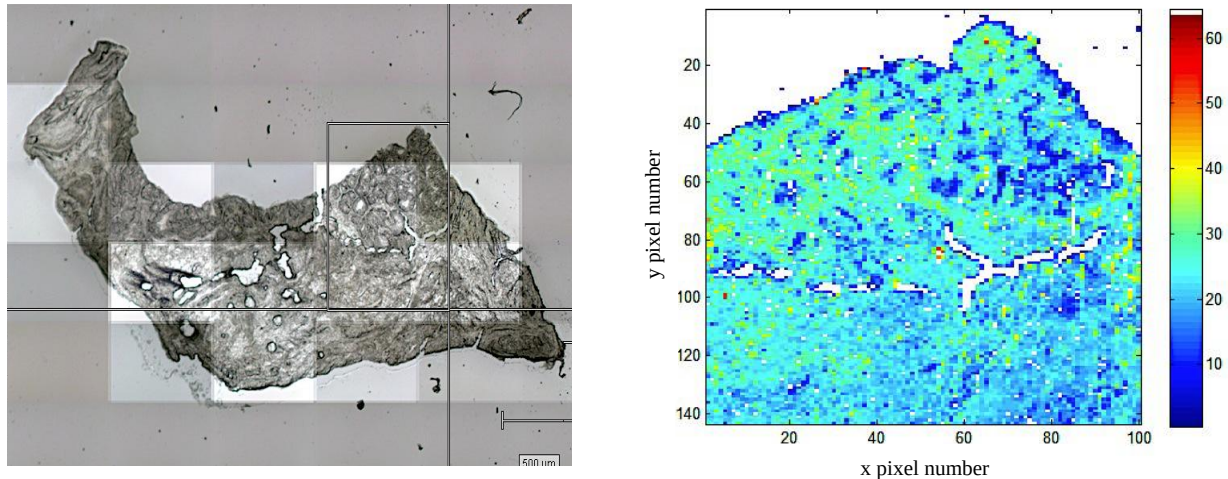


Figure 3-8. A rapid Raman map (right) acquired from a region of clinical interest (left).

Rapid Raman mapping has the potential to provide prompt objective assessment of biopsied tissue samples without the need for tissue fixation or staining (Figure 3-8). This low signal to noise mapping lends itself to automation. Furthermore, objective diagnosis can be obtained by projecting measured spectral data onto pre-established multivariate algorithms in order to obtain a diagnosis based on the biochemical profile of the tissue, thus removing the subjectivity of histological reporting.

Work is currently underway to further evaluate the diagnostic potential of rapid Raman mapping as an aid to oesophageal histopathology evaluation, particularly with regard to the grading of Barrett's-associated dysplasia.

This ability to map out pathological regions within oesophageal biopsy specimens, which can be correlated with histology appearance, provides further evidence of the ability of Raman spectroscopy to objectively assess tissue and therefore gives additional support to the proposal of developing diagnostic Raman probes for in vivo use in the oesophagus.

3.6 Fibre-optic Raman Spectroscopy

In order to develop a clinically applicable *in vivo* Raman probe for upper gastrointestinal endoscopy, there are several specific scientific and practical considerations which must be addressed and overcome. These are in addition to those discussed in section 3.4 and relate to the nature of the fibre-optic probe.

- The probe must be narrow and long enough to fit down an endoscope channel - modern endoscopes have a 2.8 mm diameter instrument port and are 90 cm in length. In practical terms this necessitates construction of a probe of at least 2 m in length.
- The design of the probe tip must not interfere with manipulation of the articulated distal end of the endoscope.
- Signal-to-noise ratio must be maximised, although acquisition times must remain clinically applicable.
- The probe and spectrometer must be coupled to a data processing unit so that short acquisition times are translated to meaningful clinical results in near 'real time'.
- Data measurement should also be close to 'real time' (short acquisition times) to enable rapid assessment of large areas of oesophagus and to minimise inaccuracy caused by scope movement or oesophageal peristalsis.
- The probe must be safe for use *in vivo* – laser wavelength and power must be carefully chosen so as to not cause tissue destruction or long-term mutagenic effects.
- The probe must function effectively in the presence of endoscopic light – laboratory Raman spectroscopy requires reduced ambient light to minimise unwanted spectral noise.
- The sampling depth and volume must be optimised to enable diagnosis of epithelial disease. Metaplasia and dysplasia are epithelial conditions and therefore the optimum depth from which to attain Raman signal is 10-200µm.[43] Sampling from large surface areas would provide more rapid assessment of clinical pathology but may decrease sensitivity and specificity for focal abnormalities.
- The probe must work in direct contact with the mucosal surface in order to facilitate endoscopic use.
- The probe must work safely and accurately in the presence of blood, saliva and gastric juice.

- The probe must be packaged so that it can be reused for multiple endoscopies and must be robust enough to withstand sterilisation procedures or must be simple and cheap enough to manufacture to enable single use.
- Measurements must be reproducible on different days, by different endoscopists and using different probes and spectrometers;
- High sensitivity and specificity must be achievable in the clinical setting – levels of diagnostic accuracy must be tested and reproduced *in vivo*.

As Raman scattering is weak and the power of the incident laser must be low (to minimise thermal injury), the design of a fibre optic Raman probe must centre on maximising collection efficiency whilst minimising intrinsic scattering and fluorescence produced by the probe.

One of the most potentially difficult problems to overcome is the propensity of probe fibres to generate their own Raman and fluorescence signals.[201; 202] In some cases, the Raman signals may be greater than those arising from the tissue being studied. Fibre signal is proportional to fibre length and to the intensity of excitatory light. It is generated in the delivery fibre due to excitation by the laser light and in the collection fibres by elastically scattered light transmitting back along the collection fibre. Notch or edge filters can be used to remove elastically scattered light from the collection fibre, and bandpass filters are commonly used to remove fibre-optic signal that has been generated in the delivery fibre.

In 1998, Mahadevan-Jansen *et. al.* published *in vivo* results using a fibre-optic Raman probe to analyse dysplastic change in cervical tissue.[203] Silica-silica fibres were shown to cause less signal disruption than sapphire and liquid guide fibres despite their tendency to generate significant background signal at low wavenumbers (below 900 cm^{-1}). The probe consisted of a single excitation fibre and 50 collection fibres and had a total diameter of 12 mm. The excitation fibre was positioned at the edge of the probe, and the laser output (at 789 nm) was reflected by a gold mirror at an angle to produce a 900 μm diameter excitation spot on the tissue surface. A bandpass filter on the distal tip of the excitation fibre blocked fluorescence and Raman scattering generated by the fibre, but transmitted the excitation light. Although demonstrating that Raman spectra could feasibly be measured *in vivo* using a fibre-optic, the dimensions of this probe and the time taken for spectral acquisition (90 s) meant this design was impractical for use in the gastrointestinal tract.

The next important challenge was to develop an effective miniature fibre-optic probe that employed state-of-the-art probe technology but which was compatible for use with a medical endoscope. An endoscope is 90 cm in length and has a 2.8 mm diameter instrument channel through which a Raman probe could be delivered. This presents a considerable technical challenge; to package a probe with all its constituent parts (filters, collimating lenses, illumination and collection channels, and external casing) into a durable device with a diameter smaller than 2.8 mm.

In 1999 Shim *et. al.* reported on utilising an endoscope compatible, fibre-optic Raman probe consisting of one central delivery fibre (400 μm diameter) surrounded by seven collection fibres each with a 300 μm diameter (Visionex, Inc, Sunnyvale, CA, USA, probe).[204] Filters were placed over the fibres approximately 2.5 cm from the probe tip and the collection fibres were part bevelled so that they predominantly collected signal from a zone in front of the delivery fibre and at a specified collection depth, determined by the bevelled angle. This design provided a means of controlling the sampling volume whilst optimising collection efficiency.

In 2000, Shim *et. al.* reported the first *in vivo* Raman measurement of gastrointestinal tissue using the Visionex probe described above (Table 2).[197] In this feasibility study the probe was passed through an endoscope into the oesophagus, stomach and colon. Twenty patients successfully underwent the endoscopic procedure following routine sedation and no complications were reported. The probe was positioned to touch an area of mucosa for 5 seconds during which time readings were taken. The probed site was then biopsied to allow comparison of Raman spectra with histopathology at that site. Subtle differences were reported in spectra from normal and diseased areas but the data acquired was not sufficient to discriminate between pathological groups. Whilst demonstrating feasibility, Shim *et. al.* concluded that more work was needed to enhance the signal-to-noise ratio (SNR), to minimise acquisition times, and to improve data interpretation before reliable results could be generated. This study illustrated that endoscopic Raman spectroscopy could be performed safely in human patients, but was not large enough to enable comparison with results generated using laboratory based Raman spectrometers.

Year	Authors	Site	Number of Patients / Spectra	Measurement Parameters	Probe Design	Accuracy / Conclusions
2000	Shim <i>et. al.</i> [197]	Oesophagus, stomach (& colon)	20 patients 400 spectra	Diode laser at 785nm Acquisition time, 5s ~500mW of excitation power.	Visionex probe, 7 bevelled collection fibres, 1 excitation fibre Silica fibres Shutter to block white light Tissue contact Collection depth ~500µm	Feasibility demonstrated Subtle difference in normal and diseased spectra not sufficient to discriminate between pathologies. More work to enhance SNR, minimise acquisition times, improve data analysis Background signal subtraction demonstrated
2005	Motz <i>et. al.</i> [205]	Rat palate		Diode laser at 830 nm Acquisition time, 1s Power output, 100mW	Single central delivery fibre, 15 collection fibres. Collection depth exceeded 500µm	Improvements in collection optics necessary
2005	Wilson <i>et. al.</i> [206]	Oesophagus	65 192 spectra	Diode laser at 785nm Acquisition time, 5s 70mW power output	Single delivery fibre, 6 bevelled collection fibres Collection depth 800µm	Sensitivity 86% and specificity 88% for dysplasia (compared to normal dysplastic oesophagus)
2007	Hattori <i>et. al.</i> [207]	Rat oesophagus and stomach		Ti:sapphire laser at 785nm 80mW power output	Single central delivery fibre, 8 collection fibres	Feasibility demonstrated Further improvements in SNR necessary
2010	Huang <i>et.al.</i> [209]	Stomach	71 patients 1102 spectra	Diode laser at 785nm Acquisition time, 0.5s 1.5 W/cm ² power density at probe tip	Single central delivery fibre, 32 collection fibres (diameter 1.8mm) System used in conjunction with NBI Collection depth 800µm	Delineation of malignant gastric ulcers (from benign gastric tissue): Sensitivity, 82.1% Specificity, 95.3%
2011	Huang <i>et. al.</i> [210]	Oesophagus	27 patients 75 spectra	Diode laser at 785nm Acquisition time, 0.5s 1.5 W/cm ² power density at probe tip	Probe as above. Multimodal imaging – NBI ⁺ plus AFI [*] Collection depth 800µm	Delineation of oesophageal cancer (from normal oesophageal mucosa): Sensitivity, 97.0% Specificity, 95.2%

Table 3-3. *In vivo* Raman spectroscopy in the upper GI tract. (Trials are in humans unless stated). All probes incorporated miniature in-built optical fibres. ⁺NBI - narrow band imaging, ^{*} AFI – autofluorescence imaging.

Motz *et. al.* used a novel probe to study dysplasia in rat palate tissue using 100 mW of laser light (at 830 nm) for excitation.[197; 205] Multivariate data analysis of Raman spectra demonstrated differences in low and high grade dysplasia, but it was concluded that further modifications to the probe were necessary to optimise collection depth as a significant spectral contribution was collected from tissue that was deeper than 500 µm. More recently a greater

emphasis has been placed on targeted illumination of the epithelial lining of the gastrointestinal tract. Precancerous dysplasia develops in epithelial cells and, by definition, has not yet invaded deeper structures. For optimum diagnostic accuracy, probes must collect signal from the epithelial layer and minimise spectral contributions from scattering occurring in deeper tissues.

In 2005, Wilson and co-workers published results of an *in vivo* trial in the upper gastrointestinal tract of humans. They used a probe consisting of a central delivery fibre surrounded by six 300 μm diameter bevelled collection fibres that achieved an estimated sampling depth of 500 μm . [206] In conjunction with a diode laser (emitting at 785 nm) and a modern CCD detector they reported an accuracy of 87% for delineating between normal oesophageal tissue and dysplasia.

Huang and co-workers recently described the use of a ball lens at the distal tip of a novel Raman probe to focus excitation light onto the epithelium and help collimate scattered light into the collection fibres. [210] In 2010, following a number of preliminary *in vitro* and *in vivo* studies in rats, Huang et. al. published a series of results from *in vivo* trials of endoscopic Raman spectroscopy for the detection of gastric neoplasia in humans. They used a 1.8 mm diameter Raman probe consisting of 32 collection fibres surrounding a single central delivery fibre. The probe had two stages of optical filters, at its proximal and distal ends, and used 785 nm excitation. Each spectrum was acquired in 0.5 s. In December 2010 they reported a diagnostic sensitivity of 82.1% and a specificity of 95.3% for delineating malignant gastric ulcers from benign tissue. [208] In 2011, the same Raman probe system was used in the oesophagus demonstrating a sensitivity of 97.0% and a specificity of 95.2% for detecting oesophageal cancer. [209]

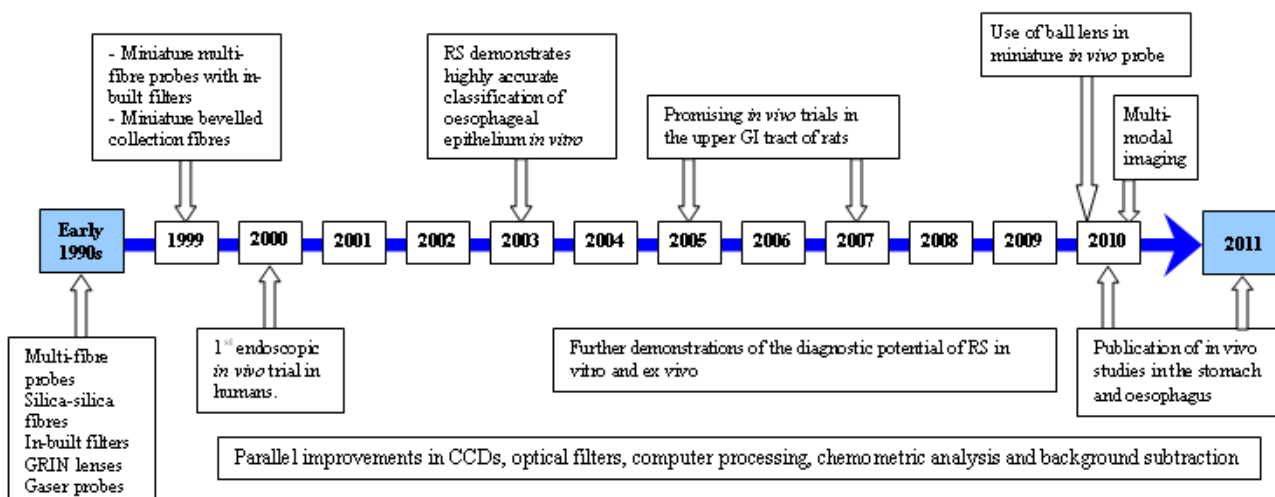


Figure 3-9. Progress towards in vivo Raman spectroscopy in the upper gastrointestinal tract.

3.7 Analysis of Raman Spectral Signatures Using Multivariate Statistics

Measured Raman spectra represent a combination of tissue autofluorescence, weak tissue Raman scattering signals and noise from background signals generated by the probe and spectrometer components. Moreover, cells undergoing neoplastic progression demonstrate simultaneous biochemical alterations that are subtle and complex. For these reasons visual comparison of spectra, or peak ratio calculations, are insufficient to extract the subtle spectral differences that correspond to distinct pathological groups. Complex multivariate statistical analysis is therefore necessary to enable accurate discrimination of varying spectral characteristics across the dataset which can be used to inform pathological classification algorithms.

A variety of different multivariate techniques are currently being investigated for use in diagnostic spectral classification.[36; 39; 211] The most commonly described algorithm for this particular application, termed linear discriminant analysis (LDA), has been widely shown to be successful.[31; 32; 38; 47; 206; 211; 212] Prior to the application of LDA, principal component analysis (PCA) is performed to reduce the dimensionality of the data. Principal component (PC) fed LDA has previously enabled Raman signatures to be ascribed to 8 distinct pathologies in the oesophagus.[49] This multivariate approach is widely used in

spectral analysis and has been chosen as the principal method of spectral characterisation in this thesis.[34]

3.7.1 Data Pre-Processing

Prior to multivariate analysis several pre-processing steps are advisable to reduce any systematic and random errors in the Raman spectra.[213] Spectral normalisation acts to eliminate significant differences in intensity, and mean centring (subtraction of the mean of the entire dataset from each individual spectrum) scales the data facilitating subsequent spectral comparison.

3.7.2 Principal Component Analysis

PCA simplifies the differences between spectra into a number of principal components which describe the variance in the sample dataset. This preserves the biochemical information that is characteristic of each pathology but does not necessarily split the data in the direction most suitable for separating the pathological groups.

Figure 3-10(A) illustrates the principle of PCA when there are only two variables. The dotted lines represent the principal components 1 and 2 which are orthogonal to each other. These can be re-plotted as shown in Figure 3-10(B). This demonstrates that PC1 accounts for the majority of the variation within the data points.

PCA can be considered a rotation of the axes in such a way that PC1 is in the direction of maximal variation whilst the angle between the axes is maintained at 90 degrees. The first principal component (PC1) accounts for most of the variation in the data set, the second (PC2), accounts for the next largest variation and is orthogonal to the first, and so on.

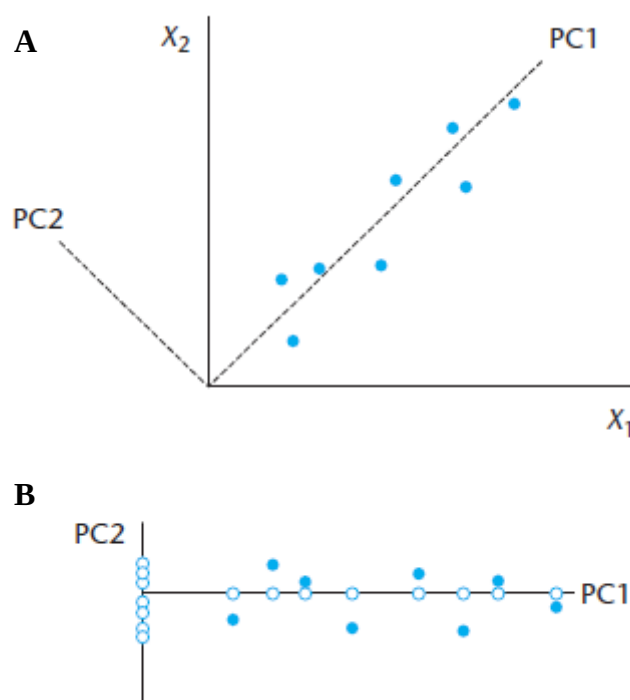


Figure 3-10. (A) Illustration of the two principal components, PC1 and PC2, for the two variables, X_1 and X_2 . (B) Datapoints have been referred to the principal component axes. ● Indicates data points, ○ indicates their projection onto the axes. Figure reproduced from Miller and Miller.[213]

PCA is essentially a mathematical method of data reduction. It does not assume that there is any particular distribution within the dataset and is an ‘unsupervised’ technique, that is to say it incorporates no prior knowledge of the expected groupings. However, in many cases PCA alone is insufficient to enable optimum classification of a group of spectra. Additional multivariate techniques which incorporate information about the nature of the samples, so called ‘supervised’ techniques must then be employed to maximise separation. LDA has been widely used for this purpose and its success is well documented.[31; 32; 38; 47; 206; 211; 212]

3.7.3 Linear Discriminant Analysis

Linear discriminant analysis (LDA) can be employed to aid pathological separation. In contrast to PCA, LDA is a supervised technique, which incorporates information about the pathological nature of the samples in order to maximise the variation between the groups. Linear discriminant functions describe the directions in spectral space that provide maximal

separation between groups whilst minimising separation within each group.[34] They can be used to construct a classification model onto which individual spectral datasets can be projected in order to statistically test and validate the model. These statistical mechanisms also allow for alterations in the classification of groups within the model so that sensitivity for detection of cancer can be maximised to the expense of specificity or vice versa. When used in a clinical setting, this could enable a Raman probe to have a programmed high level of sensitivity for the detection of cancer or dysplasia (prompting targeted tissue biopsy), in an attempt to minimise the prevalence of missed disease.

LDA essentially aims to create a classification model for allocating a new spectrum to a particular group. The linear discriminant function represents a line of maximal variation between two groups and therefore, depending on where a new datapoint sits in relation to the line, enables discrimination between two groups. This holds true for groups that have similarly shaped distributions.

LDA can be extended to the situation where there are more than two groups of objects, for example when attempting to classify three or more different oesophageal pathologies. The ability of LDA to classify correctly is assessed initially by way of a confusion matrix. This uses the LD classification model to classify each spectrum and compares this to its true classification which in this case was provided by consensus histological assessment of the particular biopsy specimen. However, an inherent problem with this method is its tendency to be over-optimistic in its ability to correctly classify. This so-called ‘overfitting’ of the training data is a consequence of the fact that the spectra being classified form part of the dataset which was originally used to derive the classification model.[214] Problems of overfitting can be overcome by using separate test and training datasets, however, this solution compromises the number of spectra which can be used to train the model. Instead a technique known as cross-validation, in particular leave-one-out cross validation, is preferred.

3.7.4 Validation Using Test and Training Datasets

The most rigorous way to test the validity and accuracy of a classification model is to project onto it a completely new spectral dataset, known as a ‘test’ dataset. In this case the classification model will have been trained using a ‘training’ dataset to define the linear

discriminant function(s). The ‘test’ spectra will then be classified based on their spectral information and this classification compared to the consensus histological diagnosis in order to calculate the level of agreement between the two. This method is ideal for comparing between spectra acquired in different timescales or using different Raman systems. Test spectra should be scaled and/or have a calibration correction applied and then projected onto the trained classification model to assess its ability to classify the pathology of the test spectra. Using these techniques enables a statistical assessment how the results of an analysis will generalise to an independent dataset.

3.7.5 Leave-one-out Cross Validation

When attempting to calculate probe accuracy the need to split the data into independent training and test datasets means that a proportion of the data is excluded from training the model. However, clearly the more data that is used to develop the model the more reflective it will be of the potential variation within the oesophagus. Therefore, in practice a technique termed leave-one-out cross validation is applied. This utilises $n-1$ of all the spectral data collected to ‘train’ the model and then projects on the single spectrum which has been excluded as the ‘test’ dataset. This is repeated n times until all the spectra have been individually excluded and projected onto the model as a test. Crucially, no sample is ever found in both training and test datasets at the same time.

This method of simulating the predictive performance of the algorithm for data not part of the training process mimics as closely as possible a real situation. For instance, in a clinical setting it would be impossible to include all potential patients in a training dataset. Therefore a classification algorithm must be trained from a sub-group of patients but be capable of making diagnostic predictions in patients that were not included in the training process.

3.7.6 Diagnostic Outcome Measures

The most commonly used measures to describe the ability of a diagnostic test to correctly predict the presence or absence of a medical condition are the sensitivity and specificity. The sensitivity of a test is the percentage of individuals with a disease who are correctly classified as having the disease. A highly sensitive test would therefore be positive for the majority of

individuals who have the disease. The specificity of a test is the percentage of individuals without the disease who are correctly classified as not having the disease. A test is therefore highly specific if it is positive for only a tiny percentage of those who do not have the disease. In the case of assigning a sensitivity and specificity to the Raman system probe the spectral prediction has been regarded as a true positive result if both the test and the consensus histopathology for a sample were in agreement.

Receiver Operating Characteristic (ROC) curves plot the true positive rate (sensitivity) against the false positive rate (1-specificity) (see Chapter 8). The area under an ROC curve (AUC) represents another measure of how well the test can distinguish between two diagnostic groups: the higher the AUC the greater the overall accuracy of the test.

An alternative measure of agreement besides sensitivity / specificity and AUC is the kappa statistic which allows for the fact that that observers will sometimes agree or disagree purely by chance (see Chapter 8). In the case of Barrett's oesophagus, the kappa statistic is often used to report the level of subjectivity between histopathological diagnoses.

3.8 Competing Advanced Imaging Techniques

In an attempt to address the need for a reliable endoscopic diagnostic tool to detect early Barrett's neoplasia, a variety of techniques at varying stages of preclinical / clinical development have been explored. Although all of these appear to have the potential to enhance endoscopic diagnosis, no one single technique has garnered universal favour with clinicians. This reflects a variety of limitations including subjectivity, poor sensitivity or specificity, excessive cost and demanding training requirements.

This section illustrates the current position with regards to advanced endoscopic diagnosis in Barrett's oesophagus. It is hoped that endoscopic RS may overcome many of the pitfalls associated with these alternative techniques. There may also be an indication to combine RS with a wide-field imaging modality, a concept which is investigated further in Chapter 7.

3.8.1 Wide-Field Detection Techniques

3.8.1.1 High Resolution Endoscopy (HRE)

Modern high resolution endoscopes which generate up to one million pixel images (compared to the 300,000 pixel images of traditional scopes) have been shown to have a higher sensitivity than standard white light endoscopy (WLE) for the detection of early Barrett's neoplasia provided they are used by expert endoscopists.[132; 215] So not to negate their effect these endoscopes should be used in conjunction with high definition television monitors to further enhance the projected image quality and prevent loss of resolution when larger images are required.

Even in the hands of experts, Kara et. al. showed that targeted biopsy using HRE was only capable of detecting 79% of dysplasia, and differentiation between HGD and LGD for the purposes of targeted treatment was difficult.[215] HRE should replace standard WLE where possible. Although to significantly improve endoscopic diagnosis of dysplasia, HRE may be best utilised in conjunction with additional endoscopic imaging technology.

3.8.1.2 Chromoendoscopy

Chromoendoscopy involves exogenous administration of stains to the oesophageal mucosa in order to improve tissue characterization during endoscopy. Absorptive stains (such as methylene blue) cross epithelial membranes selectively, whereas contrast stains (such as indigo carmine) permeate into mucosal crevices highlighting surface topography and mucosal irregularities.[216]

Studies utilising chromoendoscopy in Barrett's oesophagus have had mixed results and have highlighted problems such as difficulties in uniformly coating the oesophageal mucosa with the stain, and excessive times necessary for stain spraying.[85; 217; 218] The technique has not been shown to consistently out-perform HRE in the detection of early neoplastic lesions.[133]

Chromoendoscopy is relatively widely available and does not require any particular equipment except for a spray catheter which is easy to use and cheap to purchase. However, chromoendoscopy is often both labour-intensive and operator-dependent and is therefore unlikely to become widely utilised.

3.8.1.3 Narrow Band Imaging

Narrow band imaging (NBI) illuminates the mucosa with blue and green light in order to enhance the resolution of the mucosal surface by relying on the principal that longer wavelengths of light penetrate deeper into tissue than shorter wavelengths. Narrow band blue light displays the superficial capillary networks, while green light displays the subepithelial vessels, and a combination of the two images produces an extremely high definition image of the mucosal surface allowing visualisation of subtle mucosal irregularities and alterations in vascular patterns consistent with dysplasia and IMC.[219]

NBI is a widely available technique which avoids the need for staining or intravenous contrast agents. It has shown promise in detection of dysplastic lesions when in the hands of experienced users.[220; 221] A recent review of NBI with magnification demonstrated high accuracy for the diagnosis of HGD in Barrett's oesophagus based on recognition of irregular mucosal pit patterns and/or irregular microvascularisation.[222] However, NBI is time-consuming and results have been mixed due to high levels of inter-observer variability.[133; 223] Overall, data on the accuracy of NBI in Barrett's oesophagus are inconclusive and results of multicentre randomised controlled trials are awaited.

3.8.1.4 Autofluorescence Imaging

AFI detects fluorescence radiation following excitation of tissue using light of short wavelengths. Variation in the type and concentration of fluorophores enables differentiation between normal, metaplastic and neoplastic tissue. Several studies have suggested that AFI is sensitive for detection of HGD in Barrett's oesophagus although the technique appears limited by low specificity.[224][225; 226] Curvers et. al. demonstrated an increased detection

rate of HGD/IMC using AFI compared to WLE alone (53% Vs 90%) but this came at the expense of a high false positive rate of 81%.[227]

Further trials are necessary to demonstrate improvements in specificity through combination with other imaging techniques such as confocal microscopy.

3.8.1.5 Optical Coherence Tomography

OCT is analogous to ultrasound imaging but can produce higher resolution images as it relies on backscattering of near-infrared light (as opposed to radio waves) to generate cross-sectional images of epithelial and sub-epithelial tissues.

OCT is performed using probes passed through the instrument channel of endoscopes. It does not require the use of exogenous contrast and, unlike with ultrasonography, tissue contact is not required.

Several studies have assessed the role of OCT in the detection of dysplasia. In a study of 55 patients with Barrett's oesophagus, OCT was shown to delineate between HGD and adenocarcinoma with a sensitivity of 83% and a specificity of 75%.[228] Another study of 33 patients demonstrated a diagnostic accuracy of 78% for the identification of dysplastic Barrett's oesophagus, however considerable user discrepancy (56% to 98%) was demonstrated.[229]

Further clinical evaluation is required to assess the diagnostic performance of OCT in the oesophagus. In the future the technique may have a greater role in the staging of early oesophageal tumours rather than in the detection of dysplasia, however the considerable time required to acquire and interpret the images may become a significant limiting factor.

3.8.1.6 Labelling of Biomarkers

Visually tagged probe molecules have been engineered which selectively bind to neoplastic cells.[230; 231] Lu et. al. identified a cell surface peptide specific to adenocarcinoma which

they labelled using a fluorescein-tagged antibody delivered topically. The oesophagus was then washed to remove any unbound antibody and a fluorescence endoscope was used to visualise neoplastic disease.[232]

Similarly, the Fitzgerald group recently demonstrated that alterations in cell-surface glycans during progression to adenocarcinoma could be identified through changes in their lectin binding properties.[2] Selective binding of a candidate lectin (wheat germ agglutinin) sprayed into an ex vivo oesophagus enabled visualization of high-grade dysplastic lesions, which were not detectable by conventional endoscopy.

These highly promising molecular techniques require further work in order to identify novel molecular targets to improve sensitivity and specificity before clinical implementation can be considered.

3.8.2 Point Measurement Techniques

3.8.2.1 Confocal Microscopy

CM magnifies the mucosa 1000-fold enabling real-time visualisation of cellular structures. CM has shown considerable potential in Barrett's oesophagus with reported accuracy of up to 97.4% for the detection of dysplasia.[233] However, due to considerable inter-user variation these results have not been universally matched.[3] The technique has also been criticised for being both time-consuming and expensive as well as requiring considerable training to interpret and relying on the use of exogenous contrast to demonstrate the irregular neovascularisation that is characteristic of neoplastic tissue. Further trials are required before this technique can be recommended for widespread use.

3.8.2.2 Elastic Scattering Spectroscopy (ESS)

ESS uses a fibre-optic probe passed through the instrument port of an endoscope to generate morphological information about the nature of the Barrett's segment. White light is

elastically scattered from the mucosa and submucosa with varying signal depending on the size and shape of the cell nuclei and the degree of cellular crowding.

Spectral signal can be acquired in short acquisition times which approach 'real-time' imaging.

Dysplastic and malignant tissues have been shown to have a characteristic ESS signature however, due to signal interference from deeper structures the accuracy of ESS appears limited to around 85%.[234] Lovat et. al. measured spectra from 181 tissue sites from 81 patients which were correlated with consensus histopathology. ESS identified HGD with a sensitivity of 92% and a specificity of 60% and was able to differentiate these sites from inflammation with a sensitivity and specificity of 79%.[235] This current level of accuracy was deemed insufficient to support clinical uptake of ESS unless it can be improved through refinement of the technology or the use of a concomitant imaging modality.

Recently Qiu et. al. described a novel endoscopic polarised scanning spectroscopy (EPSS) system capable of performing rapid optical scanning of the oesophageal surface in near real-time.[131] The technique records elastically scattered light and filters signal from deeper tissue enabling epithelial interrogation. 5 patients with Barrett's oesophagus underwent EPSS. 95 biopsies were taken for comparison to spectral data which, compared to a histological gold standard, demonstrated a sensitivity of 92% and a specificity of 96% (all pathologies). Further on-going development of this technology is necessary before sound conclusions on its potential for widespread clinical use can be drawn.

3.8.3 Novel Imaging Techniques: Summary

Currently, none of these advanced endoscopic imaging techniques have yet been widely adopted into clinical practice. This reflects a variety of limitations including insufficient sensitivity or specificity, inter-observer variability, cost and technical difficulties. There is a continuing need for a novel imaging technique that could enable objective assessment of Barrett's segments in order to minimise missed disease and aid targeted ER.

The advent and now widespread use of ER has meant that accurate targeting of focal HGD/IMC has become essential. A study by Vieth et. al. showed that a complete (R0)

resection was achieved in just 26.8 % (87/326) of patients at the first attempt due to inadequate endoscopic assessment of lesion margins.[8] This improved to 74.8% (244/326) following repeat endoscopy. The study concluded that failure to achieve R0 resections at the initial endoscopy would introduce an unnecessary time delay and may increase complication rates, and the development of improved endoscopic imaging techniques was strongly advocated.

The potential ability to objectively target neoplastic lesions makes endoscopic RS an intriguing and exciting possibility and has led to the exploration of this technology within this translational research thesis.

Imaging modality	Concept	Reference
White light endoscopy		
High-resolution endoscopy (HRE)	Greater magnification and resolution than normal endoscopy allowing more detailed visualisation of the mucosa	May et. al. (2004)[18]
Chromoendoscopy	Topical application of dyes improves visualisation of mucosal surfaces. Examples: methylene blue – absorbed with different patterns into different types of mucosa; indigo carmine – accumulates in mucosal fissures accentuating surface topography	Canto et. al. (2006)[220]
Optical endoscopy		
Autofluorescence imaging (AFI)	Short-wavelength light causes excitation of endogenous biological tissues with subsequent release of longer wavelength fluorescent light.	Kara et. al. (2005)[230]
Narrow-band imaging (NBI)	Narrow-bandwidth green and blue light (with exclusion of red light) only superficially penetrates mucosa, improving visualisation of mucosal microvasculature and surface morphology	Curvers et. al. (2008)[142]
Confocal microscopy (CM)	Real-time magnification of the mucosa up to 1000-fold enables visualisation of cellular structures.	Dunbar et. al. (2010)[139]
Elastic scattering spectroscopy (ESS)	Elastic scattering of white light generates real-time morphological information about the size and shape of the cell nuclei and the degree of cellular crowding in the mucosa and submucosa.	Qiu et. al. (2010)[140]
Trimodal imaging	Incorporates HRE, AFI and NBI in a single endoscope with ability to switch between modalities during procedure.	Curvers et. al. (2008)[20; 240]
Molecular imaging	Fluorescently-tagged molecular probes bind selectively to metaplastic or dysplastic cells.	Fitzgerald et. al.(2012)[16]

Table 3-4. Advanced endoscopic imaging modalities being investigated for use in Barrett’s oesophagus surveillance programmes and for facilitation of targeted endoscopic resection. Reproduced from Almond et. al.[26]

CHAPTER 4

4 Methods

4.1 Raman Probe-Spectrometer Interface

The internal optical components of the novel Raman probe are described in detail below. For all spectral measurements, unless otherwise stated, the custom-built probe was used in conjunction with a monochromatic 830 nm Innovative Photonics Systems (IPS) laser (Princeton, New Jersey, US) and a Renishaw system 100 spectrometer (Renishaw Plc., Wotton-under-Edge, Gloucestershire, UK) which was optimised for NIR signals using a deep depletion CCD camera. Figure 4-1 illustrates the probe-spectrometer system. The photograph in Figure 4-1 displays the internal components of the Renishaw system 100 spectrometer. Laser light enters the spectrometer and is filtered and collimated before being reflected onto a dispersion grating and projected onto the CCD (see Figure 3-5).

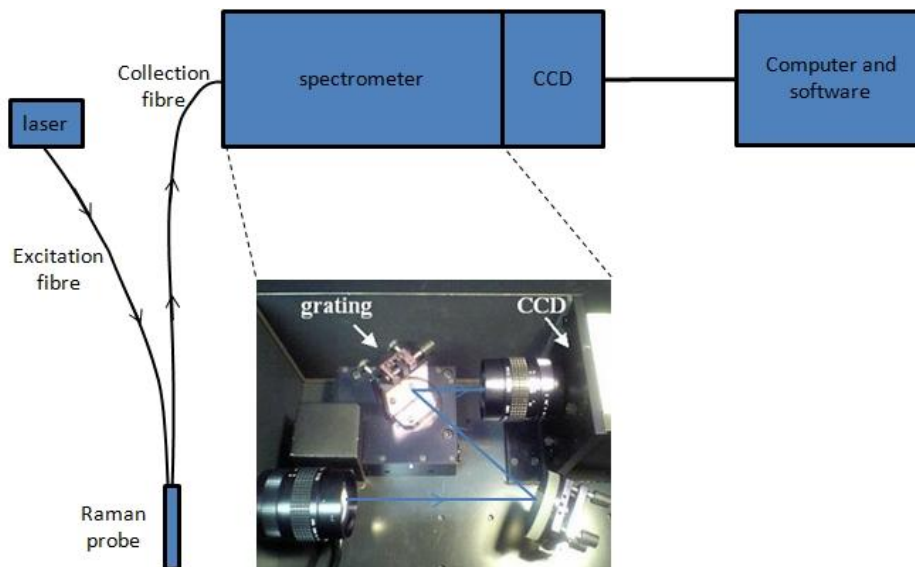


Figure 4-1. The probe-spectrometer interface. Reproduced from Almond et. al.[237]
The photograph shows the internal components of the Renishaw spectrometer.

Since Raman scattering increases with the fourth power of the frequency of the excitation source an excitation wavelength was chosen which maximised signal output whilst minimising tissue absorption and generation of unwanted fluorescence signal. Excitation using 830 nm wavelengths has previously been shown to be safe, and yielded acceptable tissue signal during preliminary experiments (Appendix IV).

4.1.1 Probe Design

This Doctorate assesses the potential for in vivo analysis of oesophageal tissue using a novel, custom-built, confocal Raman probe which has been designed specifically for compatibility with a standard medical endoscope. The Raman probe was designed and built at the Interface Analysis Centre, at the University of Bristol.

A full description of an earlier prototype probe has been published previously by Day J et. al.[37] The probe has now been modified to incorporate a graded index (GRIN) lens at its distal tip so that it may be placed in contact with a tissue sample. The probe has a fibre-optic length of 90 cm and is 2.7 mm in diameter enabling passage through the instrument channel of a standard medical endoscope. Its internal optical constituents are illustrated in Figure 4-2 which also illustrates the direction of the excitation laser (red) and collected signal (green).

The laser is transmitted through the excitation fibre, collimated by a GRIN lens, and filtered to remove Raman and elastic scattering signals generated within the fibre. The monolith reflects the light onto a dichroic filter which reflects only light at the specified excitation wavelength onto a focussing GRIN lens. After interaction with the sample, scattered light is collimated by the GRIN lens and transmitted back to the dichroic filter which reflects the elastically scattered light but allows inelastically scattered wavelengths to pass through to a second dichroic filter. The signal is then focussed onto the collection fibre by another GRIN lens and transmitted back to the spectrometer.

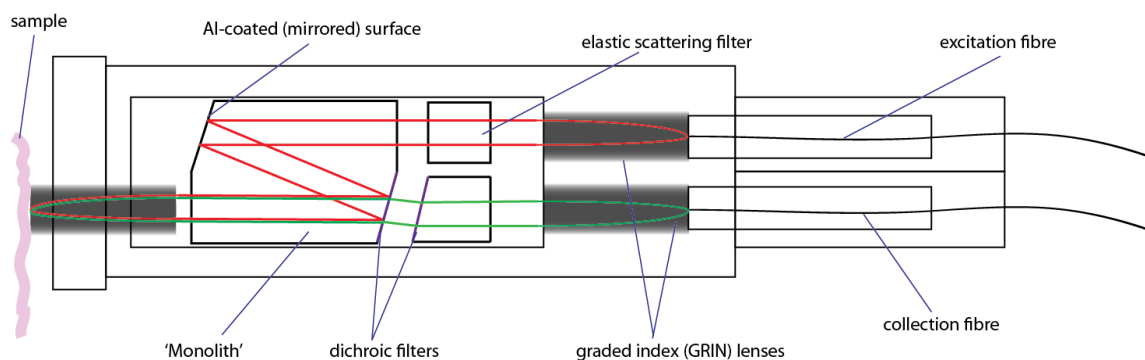


Figure 4-2. Optical components of the custom-built Raman probe. The confocal design facilitates an extremely shallow depth of field and ensures the excitation volume matches the sampling volume. Image courtesy of Oliver Stevens, Interface Analysis Centre, University of Bristol.

The probe is confocal, that is to say, the exit aperture of the excitation fibre is equal to the entrance aperture of the collection fibre.[37] It has been designed to focus to 150µm in order to sample from the oesophageal epithelium and minimise signal interference from deeper tissues. In addition, unlike previous commercial fibre-optic Raman probes it has a short tip so that endoscopic manipulation will be possible when taking probe measurements *in vivo*. It is also designed for direct tissue contact in order to facilitate *in vivo* endoscopic measurements.

4.2 Laser Safety

A strict laser safety policy was adhered to during all spectral measurements.

Prior to independent measurements training was provided on laser safety issues and safe handling of the instrument (via a laser safety course and laboratory tutorials). An assessment of laser competence was also completed.

A customised set of laser safety ‘local rules’ was developed to enable measurement of Raman spectra both within the laser laboratory, and within the endoscopy department at Gloucestershire Royal Hospital (Appendix V). Local rules were approved by the Gloucestershire NHS Trust laser protection advisor following inspection of the sites.

The local rules detailed the nature of the potential hazards when using the laser and the mandatory safety precautions including the need for protective eyewear, and for designation of a ‘controlled laser area’ with dedicated access.

A risk assessment was also completed prior to work in the laser laboratory and the endoscopy department (Appendix VI).

4.3 System Calibration

If Raman probe systems are to be used widely in clinical settings spectral datasets must be shown to be consistently reproducible. However, standardisation of spectrometer specifications and probe construction is unlikely to prevent minor spectral variation between different systems. It is therefore necessary to define a clear method for Raman system calibration.

Prior to each set of spectral measurements the system was calibrated using laboratory standards. Power output was initially measured and adjusted to 60 mW at the probe tip. The intensity of Raman signal is dependent on both excitatory laser power and laser frequency. However, excessive escalation of laser power can lead to thermal tissue injury. An excitation power of 60mW was chosen as this output is consistent with those in other published studies.[194; 197; 204; 206; 238] The novel Raman probe focuses excitation signal onto the epithelium (rather than interrogating deeper tissues as in previous studies). Therefore, a careful histological assessment for the presence of thermal injury was conducted by two independent pathologists following interrogation of tissue samples using 60 mW of 830 nm excitation for 5 s.

Calibration of the Raman system aimed to ensure that CCD read-out was reproducible so that spectral data could be compared from day to day. It also enabled comparison between Raman systems. Spectra were measured from silicon, green glass, a neon argon lamp and PTFE standards to enable both offset correction and, where necessary, correction of variation of CCD efficacy across the entire spectral range. In practice, following silicon measurement an offset correction of the main spectral peak at 520.4 cm^{-1} was performed immediately prior to

measurement of the other optical standards. The green glass standard was measured routinely to enable future correction of CCD readout variation which commonly varies slightly with wavelength, particularly in the higher wavenumber range. Green glass has a broad spectral signal so can potentially enable system calibration across a large wavenumber range through calculation of an energy transfer function correction. Multiplying a raw tissue spectrum by the transfer function allows correction and standardisation of the measured tissue spectra for spectrometer energy sensitivity over the entire wavelength range (Chapters 5 and 7).

Following silicon correction an air background signal was also measured to ensure visual consistency with previous readings and to enable air background subtraction during data analysis where indicated.

All calibration and tissue spectra, unless otherwise stated, were acquired after exclusion of ambient light.

4.4 Evaluation of Probe Stability

Prior to tissue measurement it was necessary to establish that the Raman probe could be used to record accurate and reproducible spectra from a variety of laboratory compounds with predictable spectral signatures. Spectra were acquired from PTFE, green glass and a porcine tissue phantom.

All spectra were measured by gently apposing the probe onto the surface of the material / tissue being interrogated.

The following experiments were undertaken:

- assessment of temporal variation in PTFE spectra. Serial 5s PTFE spectra were measured at 3 minute intervals for a total of 30 minutes;
- assessment of variation caused by switching the laser source on and off;
- assessment of the effect of disconnecting the laser source;

- assessment of variation due to probe re-positioning (including alteration in angle) between measurements;
- assessment of the importance of adequate probe contact;
- measurement of tissue spectra using a porcine tissue phantom in conjunction with a custom built perspex grid;
- assessment of the effect of saline and water lavage on spectral recordings;
- comparison of spectral signals acquired using an original prototype probe, and the current novel confocal probe.

Further methodological details and results of these initial probe-spectrometer evaluations are included in Appendix IV.

4.5 *Ex Vivo Tissue Measurements*

This section describes the methods of spectral collection from ex vivo oesophageal tissue. Ex vivo tissue data are presented and discussed in Chapters 5 and 6.

4.5.1 Ethical Approval and Patient Consent

Informed consent was provided by all patients permitting ex vivo spectral analysis of their oesophageal tissue. Ethical approval for the study was obtained from the Gloucestershire Local Research Ethics Committee, Gloucester, UK (Appendix VII).

All patients who participated in the study received a patient information leaflet (Appendix VIII). They were also required to read and complete a customized consent form (Appendix IX).

4.5.2 Sample preparation and spectral acquisition

Following surgical oesophagectomy with curative intent in patients with neoplastic disease, multiple spectra were measured from fixed points across the mucosal surface of the distal oesophagus using a custom built perspex grid to define measurement locations (Figure 4-3).

Following surgical resection the specimen was opened longitudinally and the mucosa gently washed using water (Sigma-Aldrich 34478) to remove any blood. The grid was then positioned to overlie the mucosa and pinned in place at each corner to prevent movement during spectral measurements. The probe was passed through specified grid positions and gently apposed to the tissue by hand, to mimic the clinical environment. The Perspex grid comprised 200 holes, each with a diameter of 3mm arranged in a 10 x 20 rectangular orientation. Each of the holes were 1mm apart. The columns and rows of the grid were labeled to enable correlation of the grid position with the Raman spectral measurement.

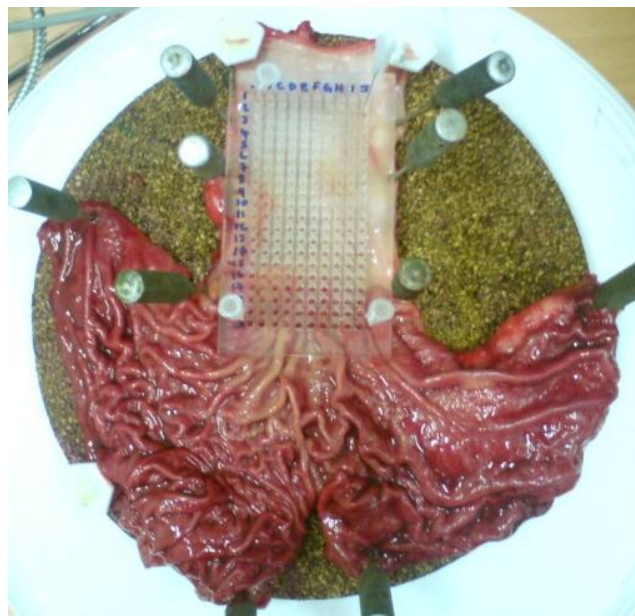


Figure 4-3. Photograph taken prior to tissue measurements after placement of the perspex mapping grid. The distal oesophagus and proximal stomach have been opened and the grid has been pinned in place so that it overlies the distal oesophagus and gastro-oesophageal junction.

Raman spectra were acquired using 1 s, 5 s and / or 0.1 s acquisition times across the wavenumber range 400 – 1850 cm^{-1} . Following spectral measurement, a biopsy was taken using standard endoscopy forceps at each of the specified grid positions and immediately fixed in formalin. All samples were paraffin embedded, microtomed and stained with H&E (Haematoxylin & Eosin) prior to expert histological reporting.

Where tumours were too bulky to enable use of the grid, biopsies were taken from the resected specimen and mounted on calcium fluoride substrates before immediate spectral measurement. Between 1 and 5 spectra were measured from different sites across the biopsy depending on its size. Following spectral measurement the tissue was immediately fixed in formalin. Samples subsequently underwent routine processing prior to expert histological analysis.

Endoscopic resection (ER) and endoscopic biopsy samples were used to augment the spectral dataset and increase the numbers of Barrett's and dysplastic samples (Figure 4-4). When collecting endoscopic samples the specimens were immediately placed on acetate and snap frozen in liquid nitrogen in order to prevent the tissue from drying out prior to spectral analysis. Samples were subsequently allowed to defrost at room temperature for 2 minutes (point biopsies) or 5 minutes (ERs) and mounted on calcium fluoride substrate with their mucosal surface facing upwards before spectral measurement. One spectrum was acquired per point biopsy specimen. Up to five spectra were acquired from different locations across ER specimens depending on their size. Biopsy samples were subsequently formalin fixed and processed as described above.

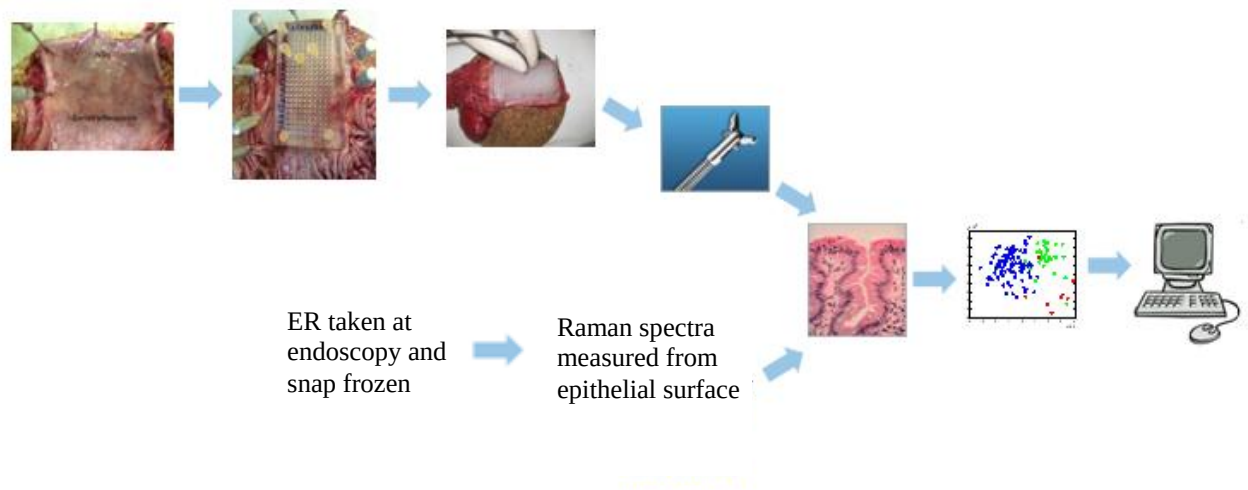


Figure 4-4. Schematic depicting methods of spectral measurement from bulk tissue and biopsies.

All tissue sections were independently reported by two expert Consultant GI Pathologists based at different hospitals in order to gain consensus diagnoses. Oesophageal neoplasia was recorded using the Vienna classification (Table 4-1).[239]

Category	Description
Category 1	Negative for neoplasia/dysplasia
Category 2	Indefinite for neoplasia/dysplasia
Category 3	(Non-invasive) low grade dysplasia
Category 4	(Non-invasive) high-grade neoplasia
Category 5	Invasive neoplasia
Category 5.1	Intramucosal cancer
Category 5.2	Submucosal carcinoma or beyond

Table 4-1. Vienna Classification of gastrointestinal epithelial neoplasia.[239]

Following an initial period of data collection and a preliminary analysis, a power calculation was performed to guide future spectral measurements (Appendix X).

4.6 Spectral Reproducibility Testing Using Two Probe Prototypes

This section corresponds to results presented and discussed in Chapter 7.

Experiments were undertaken to assess whether two Raman probes of the same design could perform consistently.

4.6.1 Multi-Operator Probe Comparison Using PTFE Standard

PTFE spectra were independently measured by two operators using two identically built Raman probe prototypes. Both operators acquired spectra over 5 seconds using each probe on three separate occasions. The sequence of measurements (which probe, which operator) was randomized.

Between each set of measurements the probe system was fully calibrated. Both probes were used in conjunction with the 830 nm IPS laser and the Renishaw system 100 spectrometer described previously.

A wavenumber representing the midpoint of each of the 5 prominent PTFE peaks was selected. Following baseline correction and baseline correction plus green glass correction, the intensity of each peak was measured at each specified wavenumber for all spectra. Two-way ANOVA (analysis of variance) was used to investigate the relationship between the spectral measurements and the two independent variables.

4.6.2 Two Probe Comparison Using Ex Vivo Tissue

Ex vivo tissue spectra were measured using a third probe. This had an earlier prototype design with a different GRIN lens at its distal tip which had not yet been modified for tissue contact. This probe was used in conjunction with the 830 nm IPS laser and the Renishaw system 100 spectrometer. Spectra were recorded in 1 and 5 seconds with the probe positioned 2-5 mm from the mucosal surface. Tissue collection, processing and histological reporting were performed as described previously. The results of these measurements are included in Appendix IV.

The second probe described in section 4.7.1 above was damaged prior to tissue measurements. No identical probes were available for further reproducibility testing due to probe construction difficulties. These are discussed further in Chapter 8.

4.7 Transferability between Spectrometers: Two Spectrometer Comparison

This section corresponds to results presented and discussed in Chapter 7.

In order to assess the reproducibility and transferability of spectra acquired using different spectrometers, a second, portable system, was evaluated (spectrometer 2). Both spectrometers were used in conjunction with the novel Raman probe and a consistent 830 nm monochromatic laser source.

Optical standards (PTFE, green glass, silicon) and ex vivo tissue were interrogated using the probe in conjunction with spectrometer 2. The internal components of spectrometer 2 are illustrated in Figure 4-5.

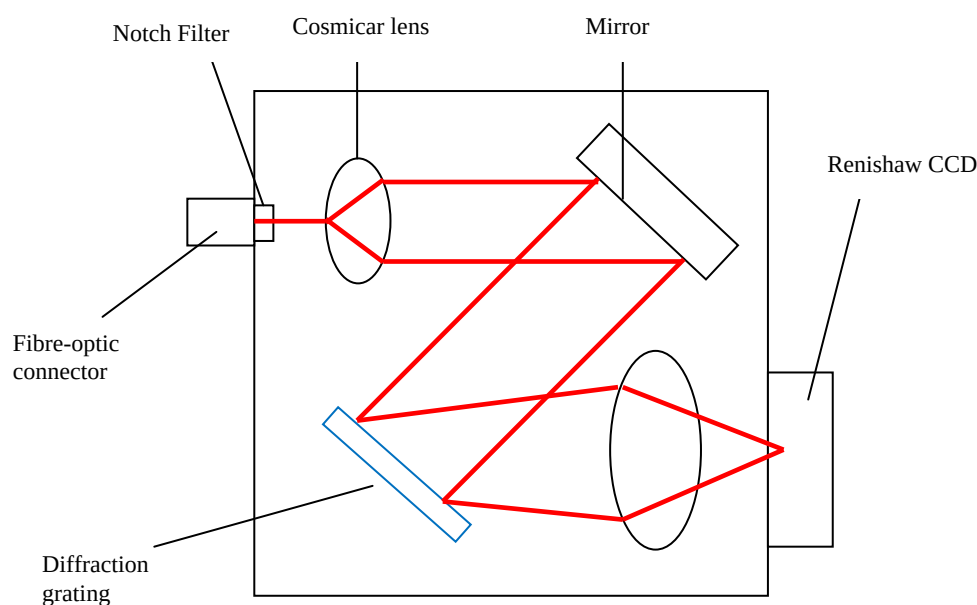


Figure 4-5. The components of ‘spectrometer 2’ - a portable spectrometer which was custom-built by Renishaw (Renishaw Plc., Wotton-under-Edge, Gloucestershire, UK). (Published with permission from Renishaw).

Spectra recorded from the optical standards were used to define a calibration correction to enable cross system comparison between measurements acquired on spectrometer 2 and those recorded on the Renishaw system 100 spectrometer (spectrometer 1).

Ex vivo tissue spectra were acquired from endoscopic biopsy samples using spectrometer 2 in conjunction with the novel probe. Tissue spectra were acquired in 5 s, 1 s and 0.1 s. Comparison was made between tissue spectra measured using the two systems.

4.8 Raman probe performance in the presence of endoscopic white light and narrow band imaging

This section also corresponds to results presented and discussed in Chapter 7.

To assess Raman probe performance in the presence of white light endoscopy (WLE) and narrow band imaging (NBI) light sources the novel probe was used in conjunction with the portable spectrometer described above (spectrometer 2).

Spectral measurements were undertaken in conjunction with a modern Olympus endoscope in the endoscopy department at Gloucestershire Royal Hospital. Full laser safety measures were implemented in line with customized local rules following a prior inspection of the endoscopy suite by the Gloucestershire NHS Trust laser safety officer (see section 4.5.1).

Ex vivo Raman spectra were measured from oesophageal biopsies collected during endoscopy. Spectra were measured from each biopsy to assess spectral interference from simultaneous: (i) white light endoscopy; (ii) narrow band imaging; and (iii) following exclusion of all ambient light (dark control measurement). Equivalent measurements were also made from PTFE as a consistency standard. All spectra were acquired in 5, 1 and 0.1 seconds. Spectral measurements, tissue processing and histological reporting were performed as described previously.

4.9 Statistical Methods

The measured Raman spectra represent a combination of tissue autofluorescence, weak tissue Raman scattering signals and noise from background signals generated by the probe and spectrometer components. Raw spectral data were normalised using standard vector normalisation, mean-centred, and interpolated to integer wavenumber spacings between 400 and 1850 cm^{-1} (see Chapter 3).

The effect of air background subtraction and energy sensitivity correction (using the green glass standard) was evaluated. When comparing between different probe-spectrometer systems a calibration algorithm was defined in order to optimize cross system spectral comparison. In addition, spectra measured using different acquisition times were scaled accordingly to enable accurate comparison.

Principal component (PC) fed linear discriminant (LD) classification models were developed for both the 5s and 1s oesophageal tissue datasets using Matlab (Mathworks, Inc.). A thorough explanation of PCA, LDA and cross validation has been included in Chapter 3.

PCA was employed to simplify the variance within the dataset into PC scores and loads. The number of PC loads chosen was optimised to maximise classification performance and prevent over-fitting due to noise. LDA was then employed which utilised pathological information in order to define functions which enabled spectral classification.

The classification performance of a variety of ‘models’ was validated using leave one spectrum out cross-validation (LOSOCV) (Figure 4-6). This involved projecting a single spectrum onto a diagnostic classification model which was created using n-1 spectra. The process was repeated n times until each spectrum had been projected as a test dataset. Following cross validation Raman probe sensitivity and specificity could be calculated.

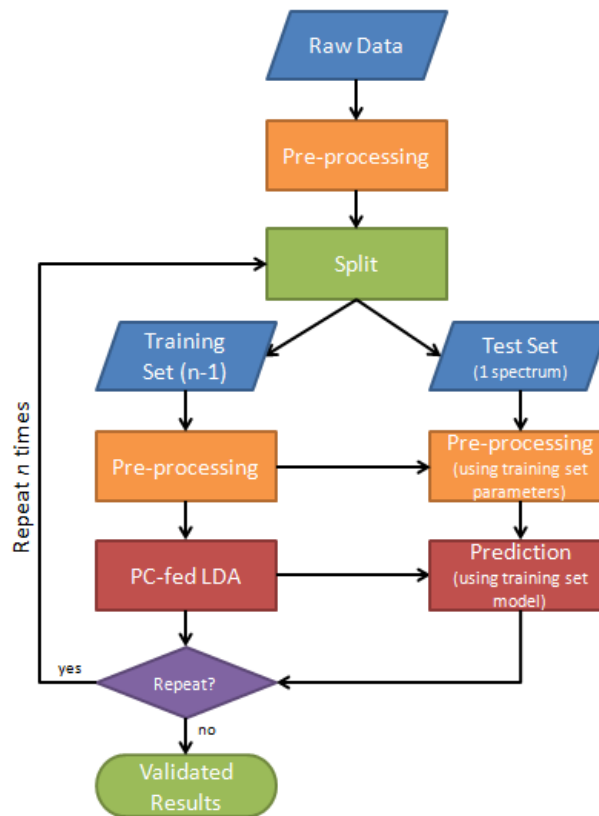


Figure 4-6. Schematic of PC-fed LDA followed by leave-one-spectrum-out cross validation. The cycle was repeated n times using 1 spectrum in the test dataset and n-1 spectra in training dataset until all spectra had been included as a test.

A variety of classification models were evaluated in order to assess the capability of the probe to distinguish a variety of oesophageal pathologies. In addition, the probe's ability to distinguish early mucosal disease (e.g. LGD and HGD); types of glandular mucosa (IM, CM or FM); and buried neoplasia was also assessed.

The effects of longer (5 s) and shorter (0.1 s) spectral acquisition times on classification performance were evaluated. Similarly, the impact on diagnostic performance following inclusion and exclusion of non-homogenous tissue specimens, and the effect of training the classification model using consensus (as opposed to single pathologist) diagnoses, were also evaluated. In addition, sub-group analyses were performed to assess differences in classification between spectra measured from snap frozen (biopsies) and fresh (surgical resections) tissue.

Independent test datasets were projected onto the 1s classification model to assess the ability of the algorithms to classify spectra acquired using different systems. This strategy was also employed to assess the classification of spectra measured in the presence of WLE and NBI light sources.

Testing the classification model using an independent test dataset required calculation of a set of PC scores for the test dataset based on the loads calculated following PCA of the training dataset, (the loads of the training set were used to calculate a set of scores for the test set). The test scores could then be classified using LD functions described following LDA on the training dataset, and were then compared to consensus histology data to calculate classification performance.

In addition to LOSOCV, the analysis was repeated using leave one patient out cross validation (LOPOCV). Repeated k fold cross validation was also employed in order to assign error bars to the classification performance.

Examination of PC loadings and identification of those loadings most important to tissue diagnosis was conducted to examine the biochemical basis spectral of spectral classification.

4.10 Additional Projects

4.10.1 Correlation of Raman spectral signatures with molecular markers of progression in Barrett's Oesophagus

I was responsible for initiating a proof of principle study aiming to correlate Raman spectral signatures with molecular markers of progression in Barrett's oesophagus. This study was undertaken by Ms Marta Losa, an MSc student at Cranfield University whom I co-supervised.

The study aimed to determine whether laboratory based Raman spectroscopy had the potential to identify histologically normal Barrett's cells which had undergone molecular changes making them more susceptible to neoplastic transformation.

Following a review of the literature, the patterns of expression of three biomarkers: cyclin A, CDX2 and hTERT were studied using immunohistochemistry, and correlated with Raman signatures acquired using a Renishaw StreamLine system (Renishaw Plc., Wotton-under-Edge, Gloucestershire, UK). Data collection was undertaken by Ms Marta Losa, myself, Dr Jo Hutchings and Dr Catherine Kendall. Appendix XI summarises the aims, methodology and results of this study.

4.10.2 Oesophageal Spectral Cytopathology

During a British Society of Gastroenterology travelling fellowship I initiated a collaboration between the Biophotonics Research Unit in Gloucester and the Department of Medical Diagnostics, Northeastern University, Boston, US. The intention of the collaboration was to investigate the potential for interrogation of oesophageal cells using spectral cytopathology (SCP). Successful results could suggest a potential role for SCP in oesophageal cancer screening.

A proof of principle study was conducted and further on-going data collection is in progress. A report of the methodology, results and conclusions of the proof of principle study is included in Appendix XII.

CHAPTER 5

5 Evaluation of Diagnostic Performance

This chapter presents and analyses the ex vivo tissue data measured using the Raman probe system. Further discussion of the results from a clinical perspective is included in Chapter 8.

5.1 Summary of Measurements

A total of 2226 spectra were measured from ex vivo tissue from 62 patients. To enable evaluation of diagnostic classification performance a histological diagnosis was obtained at the site of each spectral measurement. Table 5-1 illustrates the 1342 spectra for which a corresponding consensus histological diagnosis is available. The nature of the tissue samples (biopsies / resections) and the acquisition times used for spectral measurements is illustrated. Spectra recorded using alternative probe/spectrometer systems have been excluded from this table. Data on these will be included in Chapter 7.

	Total number of patients	Number of spectra with corresponding pathology		
		5 s	1 s	0.1 s
Oesophageal / Gastric resections	27	231	651	121
Biopsies / EMRs from endoscopy	35	74	147	18
Total	62	405	798	139

Table 5-1. Summary of spectra measured from tissue where a corresponding consensus expert histological diagnosis is available. Spectra recorded using alternative probe / spectrometer systems have been excluded.

Following initial proof of concept experiments, 1 s measurements were generally favoured over longer measurements as they are more applicable to potential clinical use in vivo and demonstrated potential for diagnostic classification.

Table 5-2 summarises the most common histological diagnoses after an independent review of all the biopsy samples by two leading upper gastrointestinal pathologists from different UK centres. Only those samples which were homogenous for a particular pathology were included here unless stated. Non-homogenous samples were classified as ‘mixed pathology’. Mixed pathology specimens e.g. mixed LGD and HGD, or mixed Barrett’s and normal squamous tissue, were included in the subsequent analyses only where indicated. Samples reported as non-homogenous by one pathologist have been recorded as ‘mixed pathology’ in order to optimise training of classification models. The impact of using consensus, rather than individual pathology reporting, to train the classification model is evaluated and discussed in section 5.12.

Pathology	Number of Biopsies		
	Pathologist 1	Pathologist 2	Consensus
Normal Squamous	253	256	252
IM	137	109	100
Non-dysplastic Barrett’s oesophagus (NDB)	142	144	114
Stomach with native structures	15	36	12
Homogenous LGD	26	85	21
Homogenous HGD +/- IMC	25	49	10
All dysplasia including mixed LGD / HGD	82	119	68
All neoplasia (LGD +/- HGD +/- cancer) ⁺	231	241	184
All adenocarcinoma ⁺	150	139	115
Benign epithelium over adenocarcinoma	49	65	74
Any benign pathology [*]	453	424	406
Connective tissue	6	4	2
Mixed pathology [*]	189	125	189
Squamous neoplasia	3	3	3

Table 5-2. Summary of histological diagnoses. *Includes non-homogenous tissue samples. Note that non-dysplastic Barrett’s (NDB) oesophagus includes all columnar lined oesophagus (CLO) samples: intestinal metaplasia, cardiac metaplasia and fundic metaplasia were classified as CLO provided the tissue sample was taken from above the mucosal folds. Glandular samples with clear native gastric features were included as ‘stomach’. This was to avoid confusion caused by misclassification of CLO as stomach or vice versa. ⁺Excludes invasive cancer beneath normal epithelium. Other diagnoses, where there was no consensus agreement, are not included.

5.2 Spectral Exclusion

Fluorescence is the emission of light by a substance following *absorption* of light of a different wavelength. It is well recognised that excessive fluorescence signal can mask subtle inelastic signals preventing Raman spectral analysis.

Despite the use of monochromatic NIR excitation and a modern dispersive spectrometer, occasional florid fluorescent spectra were recorded (Figure 5-1). In some instances the degree of fluorescence signal was significantly higher than that shown in Figure 5-1 leading to saturation of the CCD.

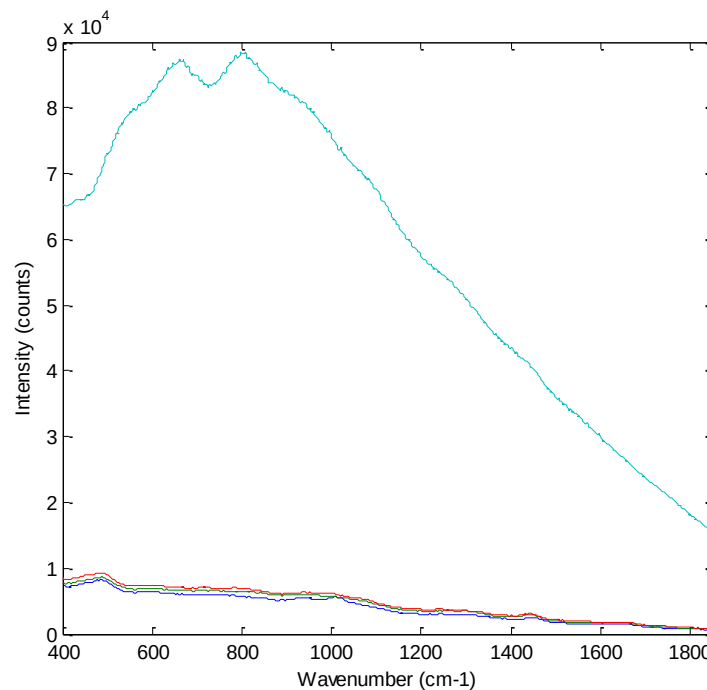


Figure 5-1. A collection of spectra measured from a single patient using a 5 s acquisition time. Note that a single spectrum (light blue) has a significant fluorescence background making tissue classification impossible.

An initial proof of concept study recorded 180 spectra from a single resected oesophagus. 18% of spectra, from a range of pathologies, displayed considerable fluorescence signal. The reason for this was thought to be excessive blood on the surface of the mucosa following dissection of the specimen - blood is known to fluoresce due to a variety of endogenous fluorophores.[240; 241] Subsequently, mucosal lavage was performed using water, prior to spectral measurement from oesophagectomy specimens.

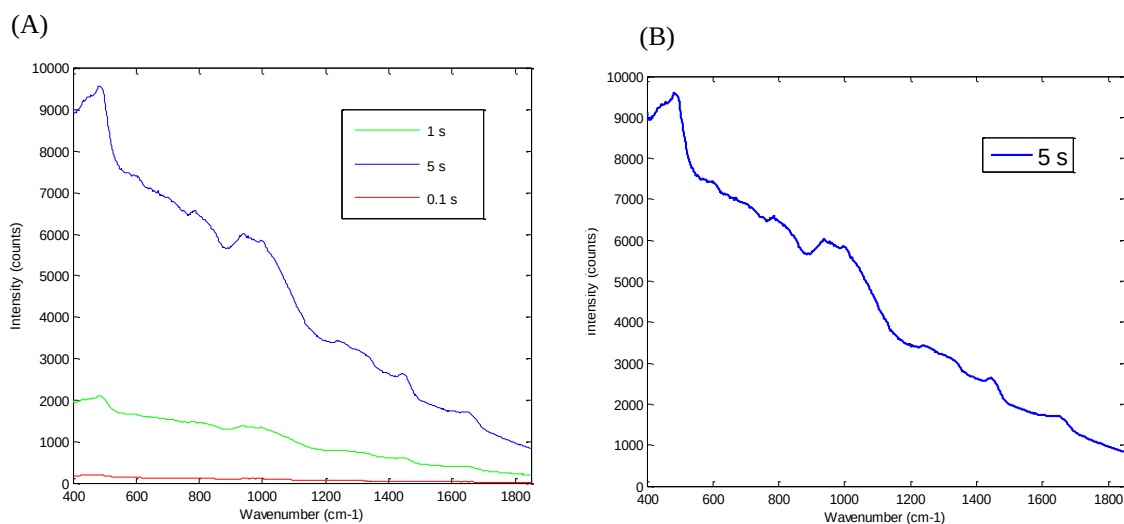
To assess the effect of mucosal lavage on unwanted fluorescence signal, 100 consecutive spectra were evaluated. Only a single spectrum displayed significant fluorescence and this was not reproduced following cleaning of the probe tip and repeated measurement at the same tissue site.

1.3% of all 1 s spectra from a variety of pathologies displayed significant fluorescence signal despite mucosal lavage. These readily identifiable outliers were excluded from the subsequent analysis. In a clinical setting these spectra could be automatically highlighted as unreliable prompting the endoscopist to perform lavage and to take a repeat measurement.

To enable a true assessment of the diagnostic performance of the probe, measurements from tissue sites for which there was no consensus histological diagnosis, and similarly those from non-homogenous tissue, were initially excluded from the multivariate models.

5.3 Unprocessed Spectral Data

Figure 5-2(A) illustrates unprocessed tissue spectra measured from a single patient using 5s, 1s and 0.1s acquisition times. These spectra are also illustrated individually (Figure 5-2B, C and D) to permit visual comparison of the signal-to-noise ratio (SNR) obtained using each acquisition time. Despite lower SNR, Figure 5-2 illustrates that tissue spectra can be acquired using short, clinically applicable, timescales. Further evaluation will demonstrate whether these spectra are capable of diagnostic classification and the impact of reducing spectral acquisition times.



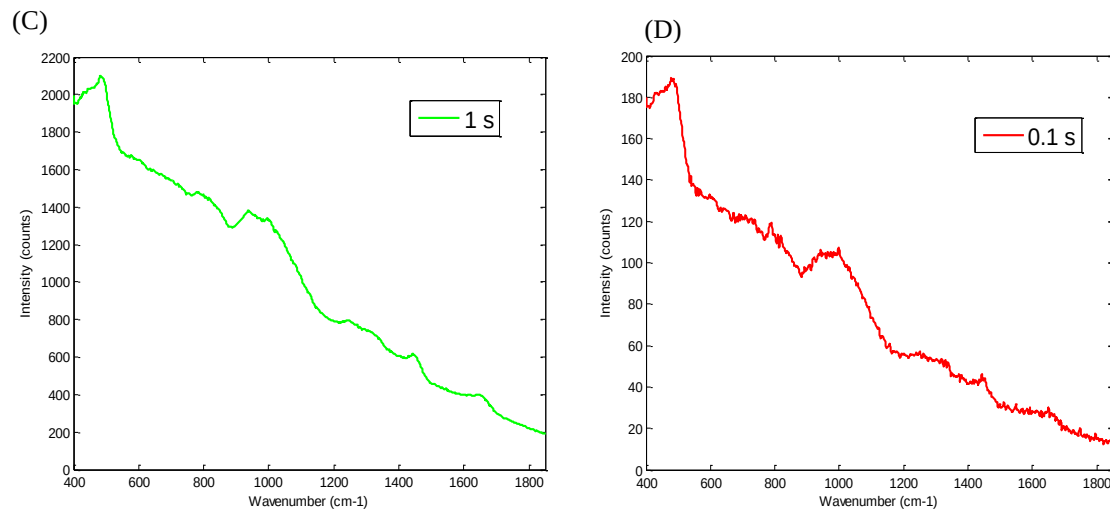


Figure 5-2. (A) Ex vivo tissue spectra measured using 5s, 1s and 0.1s acquisition times. These unprocessed spectra are presented individually in (B), (C) and (D) to allow comparison of SNR.

A series of 5 s spectra taken from multiple epithelial sites across a single resected oesophagus are shown in Figure 5-3. Normal squamous spectra are coloured in blue, cancer in red, and Barrett's in green. This figure illustrates the variation in spectral appearance and intensity that was observed following raw spectral measurements. The intensity variation may reflect slight angulation of the probe on the tissue, minor fluctuations in laser power output or alterations in the amount of pressure used to apply the probe to the tissue. Whilst power output was standardised at the start of each set of measurements, no attempt was made to directly control the other two variables. This was an intentional decision since clinical application of the probe in vivo would not permit precise regulation of probe angulation or pressure. In addition, the effects of probe angulation and pressure have previously been investigated and shown not to influence spectral classification.[197]

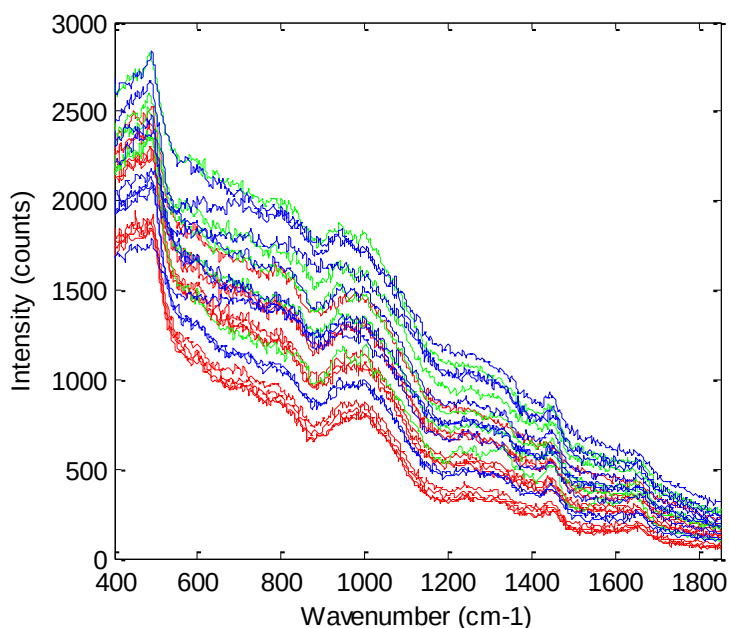


Figure 5-3. Unprocessed spectra measured from a single patient. Each spectrum was acquired over 5s. Normal squamous spectra are coloured in green, cancer in red and Barrett's in blue.

Data pre-processing (routinely normalisation and mean centring) served to minimise variations in intensity values. Additional statistical calibration measures were also trialled in an attempt to minimise background signal and will be discussed in later sections. However, the variation in intensity illustrated in Figure 1-3 did not prevent PC-fed LDA from extracting the spectral information required for diagnostic spectral classification.

5.4 Diagnostic Classification Models

The large 1 s spectral database has been used for spectral prediction unless stated. The diagnostic accuracy achieved with 5 s and 0.1 s acquisition times is also evaluated below.

Consensus pathology results have been used to inform the linear discriminant analysis (LDA) unless stated. In addition, the term ‘cancer’ is used in the tables to refer to adenocarcinoma rather than squamous cell carcinoma or other rarer cancers.

5.4.1 Three Group Models

A 1 s PC-fed LDA classification model was validated using leave-one-spectrum-out cross validation (LOSOCV). Spectra measured from three groups (HGD / adenocarcinoma; LGD / Barrett's oesophagus; and normal squamous) were included. LGD was included with non-dysplastic Barrett's (NDB) samples, and HGD with adenocarcinoma, as the division between the two groups represents the current clinical treatment line - LGD and NDB are typically observed whereas HGD and adenocarcinoma are treated with ER, ablation or surgery.

Figure 5-4 illustrates a scatter plot of cross-validated LD scores for each Raman probe prediction. The first 15 PCs were included in the classification algorithm.

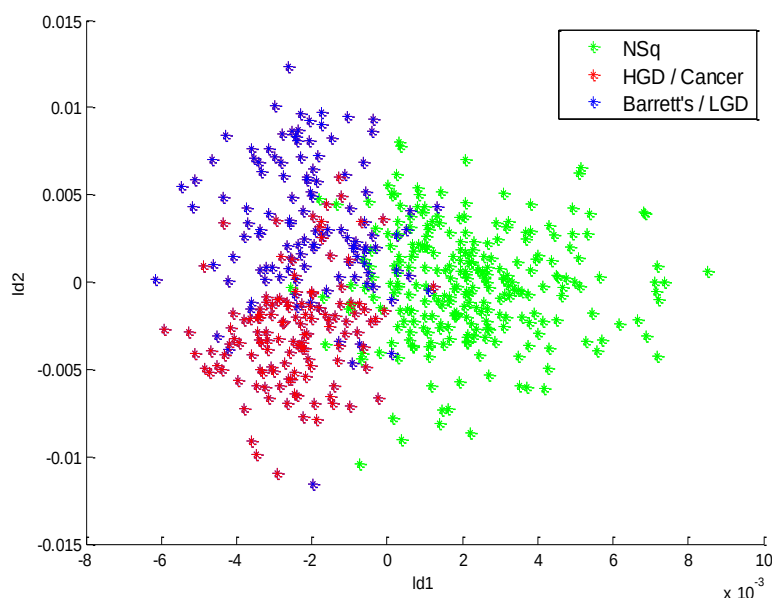


Figure 5-4. One second, three group cross-validated LD scores plot 3 group (20 PCs).

Figure 5-4 clearly illustrates the predictive capability of the model. The numerical data is displayed in Table 5-3. A sensitivity of 86% and specificity of 88% were attained for distinguishing HGD / cancer (advanced neoplasia) from the other common pathologies. For reference, the sensitivity and specificity of this model for correctly predicting advanced neoplasia prior to cross validation were 87% and 90%.

		Raman Spectroscopy		
		NSq	NDB/LGD	HGD/cancer
Pathological Assessment	NSq	215	20	16
	NDB/LGD	9	97	29
	HGD/cancer	2	15	108
Sensitivity		87%	72%	86%
Specificity		96%	91%	88%

Table 5-3. A 1 s three group PC-fed LDA classification model which has been tested using LOSOCV. Probe sensitivities and specificities have been calculated.

Table 5-4 displays the results of a cross validated 3 group Raman probe classification model for distinguishing NDB; LGD; and HGD/IMC. Distinction between these pathologies during endoscopy can be difficult or sometimes impossible but Raman probe sensitivities of 80-85% and specificities of 81-99% are demonstrated for all three groups.

The reliability of this classification performance is limited by the relatively small numbers in each group. This was due to small numbers of homogenous LGD and HGD samples, with additional sample exclusion due to lack of consensus pathology agreement. These limitations are discussed further in Chapter 8.

		Raman Spectroscopy		
		NDB	LGD	HGD/IMC
Pathological Assessment	NDB	97	15	2
	LGD	4	17	0
	HGD/IMC	2	0	10
Sensitivity		85%	81%	80%
Specificity		81%	88%	90%

Table 5-4. The 3 group PC-fed LDA classification model has been tested using LOSOCV. Probe sensitivities and specificities are displayed.

5.4.2 Two Group Models

Separating two pathology groups may be more clinically relevant than 3 or 4 group classification. Table 5-5 displays a series of one second, two group classification models.

Model	Cross-validation			
	Confusion matrix		Sensitivity	Specificity
NSq Vs. HGD/Ca	230	21	92%	93%
	19	119	93%	92%
NSq Vs. Ca	228	23	91%	94%
	7	108	94%	91%
NSq Vs. NDB	218	33	87%	89%
	12	102	89%	87%
NSq Vs. IM	223	28	89%	82%
	17	79	82%	89%
NDB Vs. Ca	104	11	90%	80%
	20	81	80%	90%
LGD+NDB Vs. HGD+Ca	111	24	82%	88%
	15	110	88%	82%
LGD Vs. HGD/IMC	21	0	100%	100%
	0	10	100%	100%
LGD Vs. HGD+Ca	19	2	90%	100%
	0	93	100%	90%
LGD Vs. HGD/IMC	21	0	100%	100%
	0	10	100%	100%
NDB Vs. LGD	99	15	87%	76%
	5	16	76%	87%
NDB Vs. Stomach	110	4	96%	92%
	1	11	92%	96%
IM Vs. CB/FB	87	9	91%	80%
	1	4	80%	91%
NSq Vs. Buried Ca	225	26	90%	78%
	17	57	78%	90%
NDB Vs. Buried Ca	90	24	79%	81%
	60	14	81%	79%
NSq Vs. HGD/ca inc buried	227	24	90%	90%
	21	180	90%	90%
NDB Vs. HGD/ca inc buried	88	26	77%	83%
	34	167	83%	77%
Benign ⁺ Vs. Neoplastic	304	102	75%	82%
	33	151	82%	75%
Benign ⁺ Vs. Neoplastic inc buried	303	103	75%	77%
	59	202	77%	75%

Table 5-5. The cross validated results of a series of 2 group classification models are displayed. All spectra used for training and testing these models were acquired in 1 s.

⁺In this analysis all benign tissues including e.g. connective tissue have been separated from dysplastic and cancerous samples. Non-homogenous benign and neoplastic samples (e.g. NSq and NDB, or LGD with cancer) have been included provided they do not contain both benign and neoplastic elements. Where there is not an exact consensus diagnosis but is agreement of benign versus neoplastic, the data has also been included.

NDB includes all columnar lined oesophagus (CLO) samples: IM, CM and FM were classified as CLO provided the tissue sample was taken from above the mucosal folds.

Normal squamous epithelium can usually be differentiated from Barrett's oesophagus at endoscopy. However, HGD and early cancer may be macroscopically indistinguishable from LGD and NDB. Therefore, a two-group classification model including NDB/LGD and HGD/cancer is of particular clinical significance (Figure 5-5A). For comparison the results of a normal squamous versus HGD/cancer algorithm is also illustrated (Figure 5-5B).

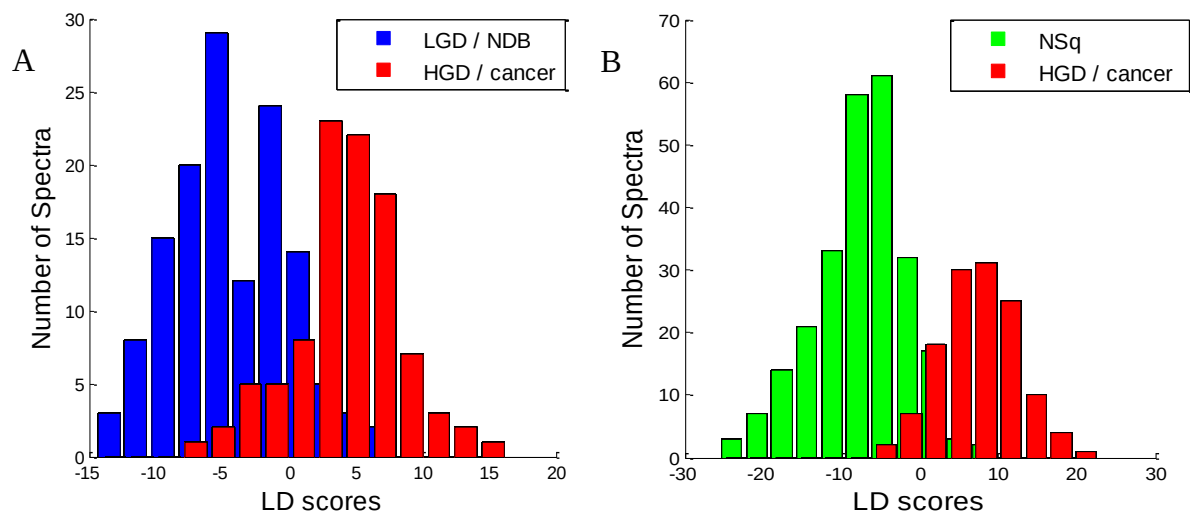


Figure 5-5. Two group histograms demonstrating the classification performance of (A) the two group model: HGD / cancer versus NDB; and (B) normal squamous oesophagus versus HGD / cancer.

Cross validated results yielded a sensitivity of 82% and a specificity of 88% for separating advanced neoplasia from LGD and NDB (Table 5). This division represents the current clinical treatment line. The positive predictive value for detecting a neoplastic lesion was 95% in this model. These data demonstrate significant potential for fibre-optic RS to be used to aid therapeutic decision making.

In addition, advanced neoplasia could be distinguished from normal squamous oesophagus with a sensitivity of 92% and a specificity of 93% (Table 5). The ability to distinguish these pathologies may be of particular use in patients with dysphagia who are found to have an oesophageal stricture. In some cases it can be difficult to distinguish benign from malignant strictures and biopsies can be inconclusive or misleading due to sampling error. Taking

several rapid Raman measurements in vivo could act to re-assure clinicians or prompt more extensive biopsying.

These analyses demonstrate that fibre-optic Raman measurements of fresh unprocessed tissue can accurately delineate between a series of pathologies in the oesophagus. Encouragingly, these results have been achieved with the novel probe system using just 1 s spectral acquisition times suggesting real potential for rapid, non-invasive, objective, in vivo diagnosis during endoscopy.

5.4.3 Detection of Epithelial Disease

Normal squamous mucosa, Barrett's oesophagus and invasive cancer can usually be readily identified using standard white light endoscopy. For RS to have a role as an in vivo diagnostic aid it is essential that it demonstrates the potential for recognition of superficial pre-malignant epithelial changes (and early intramucosal cancer (IMC) – T1a lesions). Laboratory spectrometers have been shown to detect Raman signals from the epithelium of oesophageal biopsy samples in vitro, enabling accurate classification of pre-malignant disease (see Chapter 3). However, to date, prototype probe designs have failed to address the need for specific interrogation of the epithelial surface in the oesophagus.[197; 205-209] Recently published data reported in vivo detection of cancer in the stomach and oesophagus with high degrees of diagnostic accuracy but made no mention of the capacity for diagnosis of early (HGD / IMC) disease.[209; 212; 242]

By definition dysplasia is an epithelial condition which has the potential to become an invasive malignancy but has not yet breached the epithelial basement membrane. It is essential to assess the ability of the Raman probe to detect and differentiate between grades of dysplasia and IMC since these lesions are treatable endoscopically.

Despite an array of advanced imaging modalities, differentiating LGD and HGD endoscopically is often impossible. Where possible, expert clinicians favour an approach of treating HGD with focal endoscopic resection (ER) prior to whole segment ablation.[243] By contrast LGD is kept under close surveillance. ER of extensive areas is associated with increased risks of bleeding, perforation and stricture formation. Therefore, there is a clear

clinical need for an objective diagnostic tool, such as an in vivo RS probe, which could aid lesion targeting. As well as confirming which areas should be removed, it could help with defining margins and thus potentially improve R0 resection rates.

A two group classification model including LGD and HGD/IMC has been evaluated. This demonstrates that the RS probe system is capable of distinguishing HGD/IMC from LGD with a sensitivity and specificity of 100% following LOSOCV. This particular model contains relatively small numbers of spectra due to the low numbers of consensus homogenous LGD and HGD/IMC tissue samples (Table 5-5 and Figure 5-6). However, these results are still extremely promising and demonstrate a clear ability to detect signal from the epithelium of the oesophagus. It should also be considered that these cross validated results are objective, and based on spectra which were acquired in just 1 s.

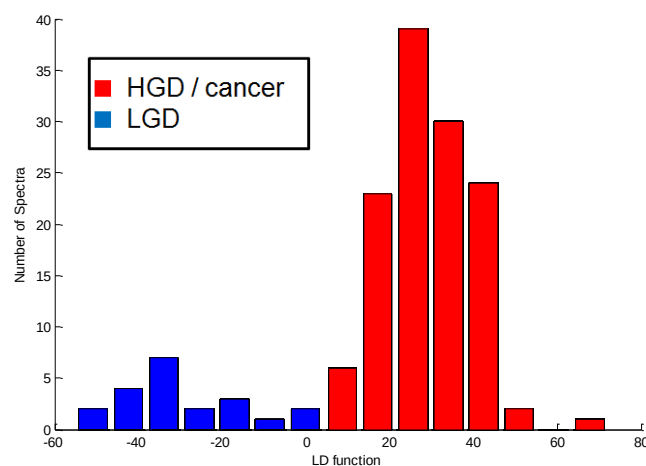


Figure 5-6. LD scores histogram illustrating the performance of a 2 group model at distinguishing LGD from HGD and adenocarcinoma.

Interestingly there is a growing body of evidence that supports early treatment of LGD provided an accurate diagnosis can be established. The premise for this is the well documented over-diagnosis of LGD by non-specialist pathologists which has tended to lead to underestimates of the risk of progression of LGD in many published series.[118] 1 s Raman probe measurements are capable of discriminating LGD from NDB with a sensitivity of 76% and specificity of 87% (Table 5-5). This potential to provide an objective diagnosis of LGD could possibly enable early targeted endoscopic treatment of these lesions. In addition, it may help prevent significant over-diagnosis and reduce unnecessary surveillance

endoscopy. However, it must be noted that the diagnostic ability to distinguish these two pathologies appears lower than for other tissue types. This may reflect the fact that only extremely subtle biochemical changes occur during the progression from Barrett's to LGD. Alternatively, since histological discrimination between Barrett's and LGD is particularly fraught with inter-observer disagreement it is possible that there has been some histological misclassification leading to incorrect training of the classification model.

Raman probe measurements were also able to discriminate intestinal metaplasia (IM) from cardiac metaplasia (CM) and fundic metaplasia (FM) with a sensitivity and specificity of 91% and 80% respectively. IM, which is characterised histologically by goblet cells, has a greater malignant potential than CM or FM, and in the United States is considered necessary to confirm a diagnosis of Barrett's oesophagus.

The analyses presented in this section demonstrate that the custom-built Raman probe is capable of detecting early epithelial changes which occur prior to the development of invasive malignancy. This could potentially help reduce the incidence of missed dysplastic disease and also facilitate early targeted therapy for these patients. In addition, it raises the intriguing prospect for potentially detecting subtle spectral differences in non-dysplastic Barrett's tissue which may be associated with an increased risk of disease progression.

Barrett's is a multiclonal condition consisting of multiple phenotypes. Certain clones are at a greater risk of progression than others due to early molecular changes which increase the risk of disease progression but do not lead to morphological change. The ability to detect detailed biochemical information from the oesophageal epithelium raises the possibility that RS could be used to detect particular 'high risk' Barrett's phenotypes. This could trigger a policy of closer surveillance in these patients. The prospect for the detection of high risk metaplasia needs to be investigated further through in vitro Raman analysis with correlation with molecular markers.

5.4.4 Distinction of Barrett's Oesophagus from Gastric Mucosa

Table 5-5 illustrates the ability of the Raman probe to distinguish between Barrett's oesophagus and gastric mucosa. The diagnosis of Barrett's oesophagus is dependent on a biopsy being taken from the oesophagus, as opposed to the cardia (the upper stomach), as the histological appearances of the two can be identical. Therefore, attempting to distinguish Barrett's oesophagus from gastric mucosa is reliant on all biopsies being correctly labelled as either 'oesophageal' or 'gastric'. Distinction between these groups can be very difficult at endoscopy, particularly in patients with hiatus hernias, and therefore considerable cross-over between these groups in the classification model was expected.

To overcome this problem the analysis presented in Table 5-5 compares glandular samples recorded as oesophageal, with stomach samples which contained native gastric features (so were definitely of gastric origin). A sensitivity of 96% and specificity of 92% were demonstrated for correctly identifying Barrett's oesophagus. However, it is important to acknowledge that these figures were based on relatively small numbers of spectra with consensus histology (see Table 5-5), and therefore may represent slightly over-optimistic classification performance.

An ability to distinguish gastric from Barrett's mucosa could be of particular use to clinicians in patients with an irregular z line who may be labelled with a diagnosis of Barrett's oesophagus. Over-diagnosis of Barrett's oesophagus can have significant psychological and economic implications (particularly in the US) and may necessitate unnecessary long-term endoscopic surveillance. On the other hand, a missed short tongue of Barrett's may have the potential to give rise to an adenocarcinoma.

5.4.5 Non-Homogenous Tissue

Only tissue that is homogenous for a particular pathology has been included in the classification models presented (unless stated). This was to ensure optimal training of the models so that the true accuracy of disease classification could be evaluated.

However, in certain circumstances tissue with mixed pathology has been included as stated. For example, samples with HGD overlying invasive cancer were included in the advanced neoplasia group. Of particular interest was the potential for the RS probe to detect cancer that was buried beneath normal squamous epithelium (Table 5-6).

		Sensitivity	Specificity
Excluding buried adenocarcinoma	LGD / Barrett's Vs.	80%	85%
	HGD / Adenocarcinoma	85%	80%
Including buried adenocarcinoma	LGD / Barrett's Vs.	81%	90%
	HGD / Adenocarcinoma	90%	81%

Table 5-6. Performance of a two group classification model (LGD / NDB) and HGD / cancer) after the inclusion and exclusion of buried adenocarcinoma.

The results displayed in Table 5-6 imply that the Raman probe can detect subepithelial, or potentially submucosal spread of cancer due to inelastic scattering of laser light transmitted beneath the epithelium. This suggests a possible role in intra-operative margin assessment during oesophagectomy. Current guidelines recommend that surgeons ensure a 10 cm margin proximal to the tumour in addition to a 5 cm distal margin.[185] However, in some cases significant margins can be difficult to achieve, and in these circumstances a tool which could provide immediate objective margin assessment would prove a significant asset.

Similarly, it is possible that the Raman probe could be used to detect buried metaplasia which may occur, and retain malignant potential, following ablative therapies. No samples containing buried metaplasia were collected and therefore this could not be evaluated further.

A second possible explanation for the ability to correctly classify buried neoplastic elements is that the overlying epithelium is in some way different biochemically to normal epithelium despite its consistent histological appearance. Evaluation of this requires further study in conjunction with molecular markers (see Chapter 8).

5.5 Assessing the Value of Background Subtraction Techniques

This section evaluates the effects of air background subtraction and green glass correction on the ability of the algorithms to classify pathology.

Probe spectra contain broad background spectral artefacts which are particularly prominent in lower wavelength regions (Figure 5-3). The propensity of probe fibres to generate their own Raman and fluorescence signals is a significant factor affecting their ability to detect the subtle spectral differences between tissue types.[201; 202] In early probe systems these signals were significantly greater than those arising from the tissue making spectral classification impossible.[197]

Fibre signal is generated in delivery fibres due to excitation by the laser light, and in the collection fibres by elastically scattered light transmitting back along the collection fibre. In-built optical filters within the probe and spectrometer help minimise these background signals (see Chapter 3) and use of 830 nm excitation aids limitation of unwanted tissue fluorescence. In addition, since unwanted fibre signal is proportional to fibre length, excess fibre length (above the 2 m required for endoscopic use) has been avoided.

However, despite these measures the Raman probe system displays a considerable degree of unwanted background signal prompting exploration of computational methods of background reduction in an effort to enhance classification performance.

Several methods of background subtraction have been described in the literature although none are used routinely due the inherent possibility of introducing systematic or non-systematic error during the data subtraction process.[244] In addition, although enhancing visible identification of biochemical tissue peaks, these techniques frequently fail to improve the ability of PCA-LDA algorithms to classify tissue spectra.[245]

Tissue spectra following air background subtraction and green glass correction are illustrated in Chapter 6 in order to illustrate some of the biochemical features important for spectral classification.

5.5.1 Green Glass Correction

Green glass standards can be used to calculate correction (transfer) functions which correct for variations in CCD readout across the entire wavenumber range.[191] Figure 5-7 illustrates a green glass spectrum measured using a laboratory spectrometer which had been calibrated using a lamp standard measured at the National Physical Laboratory; a green glass spectrum measured using the Raman probe system immediately prior to recording a set of tissue measurements; and the calculated correction function.

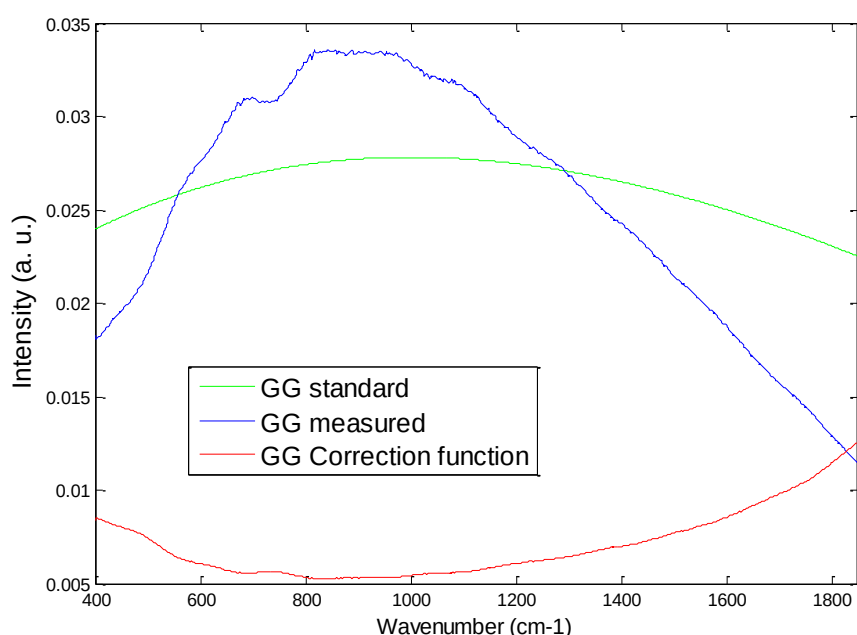


Figure 5-7. A green glass correction function has been calculated by dividing the green glass spectrum measured using the probe system by the green glass standard.

Although the data presented in this chapter were acquired using a single probe-spectrometer system, a wavenumber calibration using a green glass correction was applied to raw spectral data to account for any variability in CCD readout from day to day and to assess its impact on the accuracy of spectral classification. The results of a repeat analysis with and without green glass corrected spectra are displayed in Table 5-7 and discussed below.

5.5.2 Air Background Subtraction

Following initial system calibration, air background spectra were recorded during each set of tissue measurements to ensure visual consistency between spectra. Reproducible air spectra between measurements reassured the probe operator that there was no contamination of the probe tip, that intensity recordings were consistent, and that no systematic error had been introduced.

In some instances multiple air spectra were measured during a single set of measurements. In these cases a mean air spectrum has been used for subtraction from all tissue spectra measured on that day.

The effect of air background subtraction on diagnostic classification performance is displayed in Table 5-7 and discussed below.

5.5.3 Background Subtraction / Correction: Impact on Classification Performance

The impact of air background subtraction and green glass correction on the performance of a 3 group 1 s classification model are displayed in Table 7.

		Sensitivity (%)	Specificity (%)
No correction	NSq	87%	96%
	LGD / Barrett's	72%	91%
	HGD / Adenocarcinoma	86%	88%
Air background subtraction	NSq	83%	70%
	LGD / Barrett's	62%	98%
	HGD / Adenocarcinoma	70%	87%
Green glass correction	NSq	74%	94%
	LGD / Barrett's	81%	88%
	HGD / Adenocarcinoma	85%	86%

Table 5-7. The effect of air background subtraction and green glass correction on the classification performance of a 3 group model tested using LOSOCV.

Neither green glass correction nor air background subtraction improved the ability of the algorithm to classify pathology. It is clear that the Raman probe system obtained sufficiently reproducible spectra to enable tissue classification across the time period of the study without significant difficulties due to CCD readout variability. In addition, the capability of PCA-LDA to extract the important spectral features that enable tissue classification has been demonstrated in spite of the significant background signal.

Spectral background subtraction using iterative subtraction of the fifth order polynomial has also been described previously[45; 51; 197; 244; 246] However, this was not previously shown to improve classification performance and was thought to introduce false variance which might in fact diminish classification performance.[247] This mechanism of data subtraction is therefore not repeated here.

Despite the ability of multivariate analysis to classify probe spectra with significant background signal, engineering developments should still be implemented where possible with the aim of further boosting SNR so that more prominent biochemical peaks are identified and the accuracy of pathological classification, perhaps in even shorter timescales, is maximised.

It appears that PCA-LDA algorithms have the ability to extract the subtle spectral differences necessary for pathological separation without the need for prior background subtraction processes. It is possible that the techniques of green glass correction and air background subtraction are of greater value when attempting to compare and transfer spectral datasets acquired using different probes and / or spectrometers. More extensive discussion of the role of these background correction strategies is therefore included in Chapter 7.

5.6 Evaluation of the 5 s Spectral Dataset

Consensus pathology data is available for 405 spectra which were each acquired in 5 s using the standard Raman probe system. Although 5 s measurements would be less amenable to clinical implementation, it was interesting to assess the effect on diagnostic classification performance.

PC-fed LDA was used to generate both a 2 and 3 group classification model which were tested using LOSOCV (Figure 5-8).

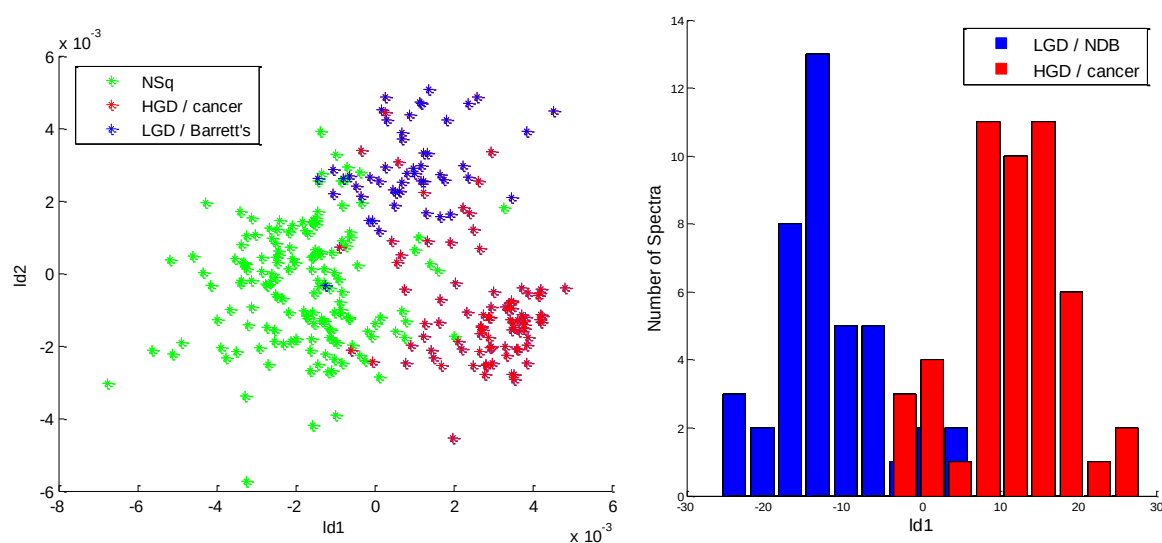


Figure 5-8. The classification performance of a 3 group (left) and 2 group model (right) is illustrated following PC-fed LDA and LOSOCV.

The corresponding data for each model is shown below in Table 5-8 and Table 5-9.

		Raman Spectroscopy		
		NSq	Barrett's /LGD	HGD/cancer
Pathological Assessment	Nsq	132	12	10
	Barrett's/LGD	1	46	2
	HGD/cancer	3	13	72
Sensitivity		86%	94%	82%
Specificity		97%	90%	94%

Table 5-8. A 5 s three group PC-fed LDA classification model which has been tested using LOOCV. Probe sensitivities and specificities have been calculated.

		Raman Spectroscopy	
		HGD/cancer	Barrett's/LGD
Pathological Assessment	HGD/cancer	166	27
	Barrett's/LGD	1	48
Sensitivity		86%	98%
Specificity		98%	86%

Table 5-9. A 5 s two group PC-fed LDA classification model which has been tested using LOOCV. Probe sensitivities and specificities have been calculated.

The prediction accuracies achieved were slightly higher for the 5 s models than for the corresponding 1 s models as expected. However, the results are perhaps less clinically applicable as 5 s measurements from precise areas might be difficult to generate in vivo due to patient movement and oesophageal peristalsis.

The fact that increases in acquisition time can enhance classification performance suggests that other mechanisms of improving signal uptake could also improve diagnostic accuracies (see Chapter 8).

5.7 Evaluation of the 0.1 s spectral dataset

139 spectra, for which consensus pathology is available, were measured from tissue from 9 patients using just a 0.1 s acquisition time. The aim of this series of measurements was to assess the effect of this much shorter acquisition time on the diagnostic performance of the model.

Projecting new data onto a predefined model provides the best method of validating classification performance. The 0.1 s spectra have therefore been projected onto the 1 s classification model as a 'test' dataset (Figure 5-9). Table 5-10 illustrates the numbers of spectra measured in 0.1 s and how they classify.

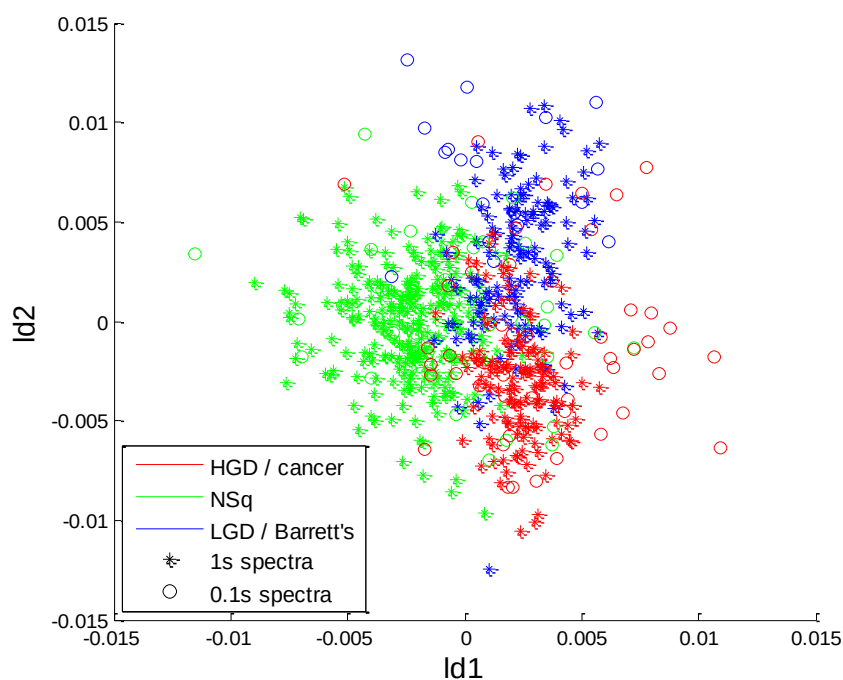


Figure 5-9. LD scores plot of the standard 3 group model. 0.1 s spectra (circles) have been projected onto the 1 s model (crosses) to see how they classify.

		Raman Spectroscopy		
		NSq	Barrett's /LGD	HGD/ cancer
Pathological Assessment	Nsq	17	9	10
	Barrett's/LGD	3	15	0
	HGD/cancer	9	13	28
Sensitivity		47%	83%	56%
Specificity		83%	74%	81%

Table 5-10. Performance of the 0.1 s spectra after projection onto a 1s 3 group classification model.

Clearly, diagnostic accuracy has been diminished considerably by using a 0.1 s spectral acquisition time. However, a degree of diagnostic classification has still been possible. This raises the significant possibility that future Raman probe analysis could approach 'real-time'.

This would potentially enable clinicians to scan across large areas of mucosa in rapid timescales until an abnormality is highlighted. Real-time imaging could be particularly advantageous in minimising the incidence of missed dysplasia in long Barrett's segments.

5.8 Assigning error bars to the classification using repeated cross validation

Probe based RS is potentially capable of providing clinicians with specific information to help inform treatment decisions. A Raman prediction of HGD would by no means be an indication to proceed straight to ER, but could act to support the clinical findings.

However, in clinical practice Raman predictions would best be presented to clinicians as probabilities, rather than as definitive diagnoses. For instance, when assessing a suspicious area of mucosa in the oesophagus a Raman system could inform a clinician that there is a 90% likelihood that it represents HGD.

In addition, when assessing the performance of the probe it is useful to have a means of assigning error bars to the classification prediction. In a clinical setting this might inform clinicians that a suspicious lesion has, for example a 90% +/- 5% likelihood of being HGD.

Repeated cross validation, as described by Lloyd et. al., has therefore been employed in order to validate diagnostic accuracy and define error bars.[34,248] Figure 5-10 displays the results of a 2 group model (Barrett's/LGD; HGD/cancer) followed by repeated cross validation. In this case cross validation has been performed by randomly selecting a third of the dataset to 'leave out' and project onto the remaining training samples. This process has been repeated 1000 times using 1000 different random combinations in the test dataset.

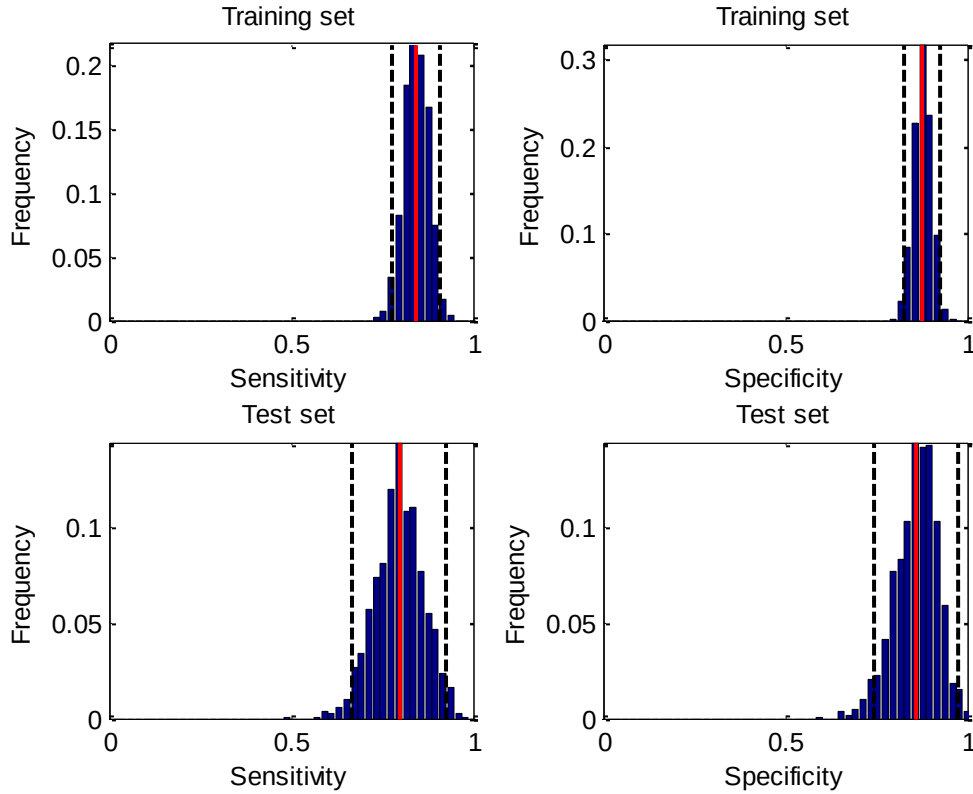


Figure 5-10. Performance of training and test datasets following 2 group PC-fed LDA and repeated (1000 times) cross validation leaving out one third of the dataset. The sensitivity and specificity for separating HGD/cancer from LGD/Barrett's are illustrated.

Figure 5-10 demonstrates that this two group model is able to separate HGD/cancer from LGD/Barrett's with a sensitivity of 82% (68% - 93%) and a specificity of 88% (75%-98%).

5.9 Leave One Patient Out Cross Validation

In this analysis the results of the 1 s three group model (normal squamous; NDB/LGD; HGD/adenocarcinoma) have been cross validated using leave one patient out cross validation (LOPOCV). This method of cross validation was employed as it infers how the model might perform for an entirely new patient attending for an endoscopy. Leave one spectrum out cross validation (LOSOCV), which has been used previously, assesses the performance of the model in classifying individual test spectra which have been 'left out'. However, using

LOSOCV includes in the ‘training’ dataset spectra measured from the same patient as the ‘test’ spectrum.

By contrast LOPOCV uses all the spectra measured from an individual patient as the ‘test’ dataset whilst all other spectra form the ‘training’ set. This overcomes the theoretical concern that using LOSOCV projects spectra onto to a model that has been trained by other spectra from that patient.

		Sensitivity	Specificity
LOSOCV	NSq	87%	96%
	LGD / Barrett’s	72%	91%
	HGD / Adenocarcinoma	86%	88%
LOPOCV	NSq	94%	78%
	LGD / Barrett’s	77%	66%
	HGD / Adenocarcinoma	86%	60%

Table 5-11. The standard 3 group model has been tested using leave one spectrum out cross validation (LOSOCV) and leave one patient out cross validation (LOPOCV).

Although accurate sensitivities have been demonstrated following LOPOCV, this is at the expense of a significant drop in specificity. The reasons for this probably reflect the fact that the patients did not all have an equal mix of pathology. That is to say, there were some patients for which no consensus could be achieved except for the normal squamous samples, and others where the samples were predominantly Barrett’s, with little or no other pathologies (this was particularly true for endoscopy, rather than oesophagectomy, patients).

In addition, in this analysis it is possible that if just a small number of homogenous spectra were measured from a particular patient and all misclassified, perhaps due to a systematic error on that day (e.g. probe lens contamination), the 0% sensitivity and specificity calculated for that patients’ spectra would significantly reduce the overall performance following LOPOCV.

In order to improve the statistical validity of LOPOCV, and thus make this analysis more representative of the classification of spectra from new patients, the analysis would ideally include a larger sample of patients with an equal distribution of pathologies from the three groups. This would also provide a better description of the variance within the patient

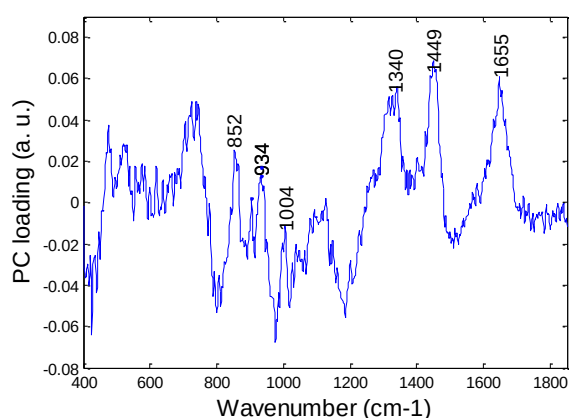
population so that future analyses are able to discriminate between patient spectral differences and biochemical spectral differences.

5.10 Principal Component Optimisation

PCA simplifies the variance within the dataset into a variable number of principal components (typically 15) which describe the major sources of independent variation between the spectra. PCA has been performed on the entire dataset without prior knowledge of which spectra were measured from which patient or from which pathology. As described previously the first principal component (PC1) reflects the direction of greatest variance between all spectra in the dataset. PC2 is in the direction of the largest residual variance and so on.

The PCs therefore contain information which is vital for spectral classification (Figure 6-5). In theory, the more PCs that are generated the better the description of the variation within the dataset. However, later PCs often only represent spectral noise and may therefore be of no benefit when attempting to classify a spectrum (Figure 5-12).

PC6:



PC20:

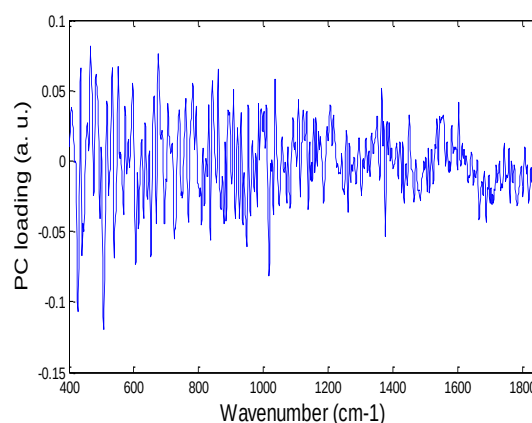


Figure 5-11. Comparison of (a) PC6 and (b) PC20. It is clear that significant biochemical information is contained within PC6 whereas PC20 appears to contain mostly noise and is unlikely to aid spectral classification.

In the example illustrated in Figure 5-12, PC-fed LDA was used to assess the probe's accuracy at delineating normal squamous tissue from adenocarcinoma. The influence of each of the principal components in achieving accurate diagnostic classification was assessed by repeating the LDA using increasing numbers of PCs. A combination of sensitivity and specificity over 85% was achieved only when utilising 11 PCs or more, and the optimum number of PCs was found to be between 11 and 16 with inclusion of later PCs adding no additional benefit to the classification performance. In this example, inclusion of higher numbers of PCs in fact led to a drop in performance due to over-fitting of noise.

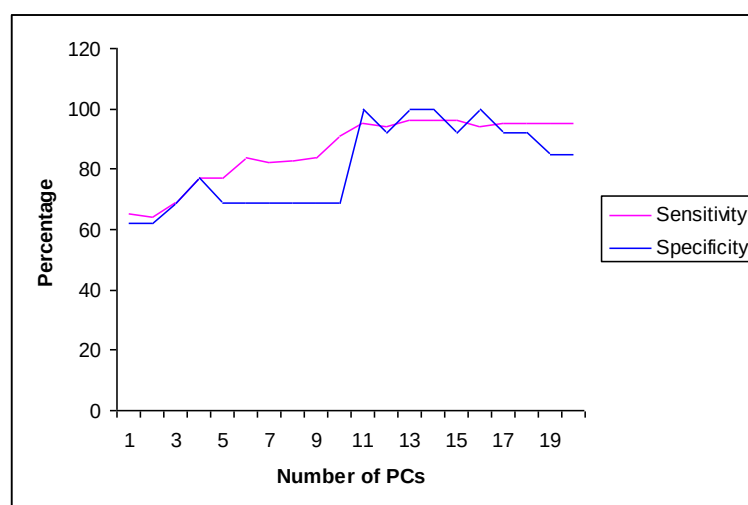


Figure 5-12. Influence of number of PCs on sensitivity / specificity performance.

5.11 Influence of Technique of Tissue Collection

A limitation of the reliability of the main 1 s dataset is that spectra were acquired using two different methods: both from fresh tissue (following oesophagectomy) and from tissue which had been snap frozen (following endoscopic biopsy or ER). This section evaluates any classification differences pertaining to variations in how the raw data were acquired.

Table 5-12 illustrates the proportion of spectra which were measured direct from a resected oesophagus compared to those from endoscopic biopsy or ER specimens for three common pathology groups.

Pathology Group	Snap frozen	Fresh (%)
Normal Squamous	26/251 (10%)	225/251 (90%)
Barrett's / LGD	55/135 (41%)	80/135 (59%)
HGD / Cancer	10/128 (8%)	118/128 (92%)
Total	91/514 (18%)	423/514 (82%)

Table 5-12. Proportions of spectra measured from fresh and snap frozen tissue from each of the main pathology categories.

To assess the impact of including both snap frozen biopsy samples and fresh surgically resected samples in the same dataset, an LD scores plot displaying spectral predictions for 3 pathology groups has been re-plotted to identify the nature of the tissue samples (Figure 5-13).

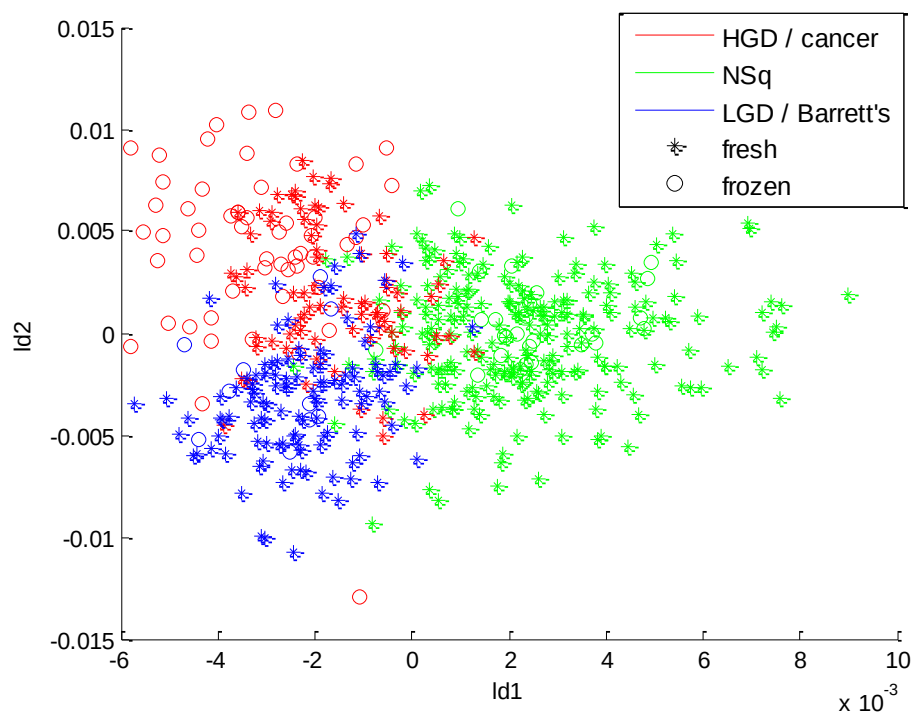


Figure 5-13. standard 3 group LD scores prediction scatter plot identifying samples which have been measured from fresh (stars) and from fresh frozen (circles) tissue samples.

In addition, 3 group PC-LDA with LOSOCV has been repeated for both a 'fresh only' dataset after exclusion of the frozen samples, and for a 'snap frozen only' dataset (Table 5-13).

		Sensitivity (%)	Specificity (%)
Both fresh and frozen samples	NSq	87%	96%
	LGD / Barrett's	72%	91%
	HGD / Adenocarcinoma	86%	88%
Fresh, surgically resected samples only	NSq	80%	96%
	LGD / Barrett's	71%	86%
	HGD / Adenocarcinoma	83%	90%
Snap frozen endoscopic samples only	NSq	96%	98%
	LGD / Barrett's	96%	98%
	HGD / Adenocarcinoma	96%	97%

Table 5-13. Three group classification performance inclusion and exclusion of fresh and snap frozen samples.

Spectra acquired from snap frozen biopsy samples appear to classify with greater accuracy than those measured directly from fresh tissue. However, this classification performance is likely to be influenced by the small number of samples in the frozen biopsy group.

It is encouraging that the process of snap freezing and thawing does not appear to alter the biochemistry of the tissue so as to prevent spectral classification. However, there is no clear reason why snap frozen samples would be expected to classify more accurately than fresh samples. A possible explanation is that the majority of snap frozen samples were measured from point biopsies which have the potential to dry out rapidly. Despite thawing only small batches of tissue samples prior to spectral analysis, it is possible that drying of some of the tinier biopsy samples may have concentrated the biochemicals responsible for inelastic Raman scattering.

Another possible explanation is due to the fact that all spectra from fresh Barrett's samples were measured from patients who had undergone oesophagectomy for either HGD or adenocarcinoma. It is therefore possible that the non-dysplastic Barrett's (NDB) from these patients had a different spectral signal to NDB from patients who did not have neoplasia elsewhere in their Barrett's segment. This raises the possibility that RS could be capable of detecting early molecular changes which increase the risk of neoplastic progression but which precede morphological changes. This could be useful in helping to predict those patients with NDB who are at a higher risk of malignant progression (see Chapter 8).

5.12 Influence of Consensus Histopathology

In order to reliably assess the performance of Raman spectroscopy as a diagnostic tool it was essential to establish the correct histological diagnosis for use as the ‘gold standard’. A reliable tissue diagnosis would enable highly accurate training of the classification model, whereas an incorrect histological diagnosis could confuse the model and potentially cause significant misclassification.

Histological diagnosis of dysplastic Barrett’s is known to suffer from considerable inter-reporter variation.[85; 188; 199; 200] In addition, diagnostic accuracy has been shown to be improved considerably when slides are reported by specialist gastrointestinal pathologists as opposed to non-specialists.

To ensure the most rigorous possible gold standard, all the tissue samples included in the analyses were independently reported by two internationally acknowledged expert gastrointestinal pathologists from separate centres within the UK. Only tissue with a consensus diagnosis was used as the ‘gold standard’ to train and test the diagnostic performance of the probe.

Cross validated three group and two group analyses were repeated using the pathology results reported by each of the independent pathologists. This enabled an evaluation of the effect of using consensus diagnoses as the gold standard.

			Sensitivity	Specificity
Three group model	Pathologist 1	NSq	84%	96%
		LGD / Barrett's	68%	86%
		HGD / Adenocarcinoma	76%	85%
	Pathologist 2	NSq	84%	95%
		LGD / Barrett's	72%	86%
		HGD / Adenocarcinoma	70%	85%
	Consensus	NSq	87%	96%
		LGD / Barrett's	72%	91%
		HGD / Adenocarcinoma	86%	88%
Two group model	Pathologist 1	LGD Vs.	81%	71%
		HGD/IMC	71%	81%
	Pathologist 2	LGD Vs.	99%	81%
		HGD/IMC	81%	99%
	Consensus	LGD Vs.	100%	100%
		HGD/IMC	100%	100%

Table 5-14. Performance of a two and three group model using pathology data reported by each expert pathologist and by a consensus of the two.

The results presented in Table 5-14 demonstrate that classification performance was improved by using consensus pathology diagnoses to train the classification models. It must be borne in mind when interpreting the results presented in this thesis, that the classification performance reflects the proportion of samples that are classified in accordance with the consensus pathology diagnosis. That is to say, the consensus pathology results, used as the gold standard, are assumed to be 100% correct. It is possible therefore that performance could have been further improved by inclusion of an additional one or more pathologists, in order to further optimise the gold standard diagnosis. If this was the case it could suggest that Raman probe measurements are in fact capable of out-performing consensus pathology when it comes to establishing an accurate diagnosis.

It must also be considered however, that current clinical management decisions are frequently based on the diagnosis of just a single pathologist, who may not be a gastrointestinal specialist.

An interesting additional analysis could compare the diagnoses of a third independent pathologist with the consensus diagnosis provided by the first two pathologists. This would enable direct comparison between Raman predictions and the diagnostic performance of the

third pathologist. However, this analysis would require re-reporting of 673 biopsy samples for a third time.

5.13 Oesophageal Mapping

A Raman probe may have a role as a mapping tool during surveillance endoscopy. Currently, clinicians are recommended to take quadrantic biopsies every 2 cm of oesophageal mucosa during Barrett's surveillance. However, this biopsy protocol can increase endoscopy times considerably; samples less than 5% of the oesophageal mucosa; produces a considerable workload for pathologists; and can miss dysplasia in up to 57% of patients.[8] An alternative strategy could utilise a Raman probe to map across the Barrett's segment to identify neoplastic areas and prompt targeted tissue biopsies. The short acquisition times required to collect reliable spectra make Raman mapping a feasible option, even at first presentation of Barrett's oesophagus. The ability to map out a Barrett's segment would also be beneficial in patients referred with known HGD without macroscopic abnormality. This would enable the lesion(s) to be identified, and targeted ER to be performed.

To investigate the potential for mapping of Barrett's segments, spectra measured from resected oesophagi in conjunction with the perspex grid were projected, as a test dataset, onto the Raman classification model and then displayed as a map. Each Raman probe prediction map is displayed adjacent to a corresponding consensus histology map that illustrates the gold standard diagnosis at each tissue site.

Figure 5-14 (top left) illustrates maps corresponding to 20 (2 by 10) Raman measurements and 20 tissue biopsies. The single tissue site diagnosed as neoplastic by consensus pathology was also identified as malignant by the Raman probe. A second site was also predicted as neoplastic by the probe – this may represent a false positive prediction. Alternatively, it is possible that the probe detected submucosal involvement which was not sampled by tissue biopsy. It is also possible that the probe detected biochemical features of residual cancer in that area which had been present prior to neoadjuvant chemotherapy, but were no longer apparent histologically.

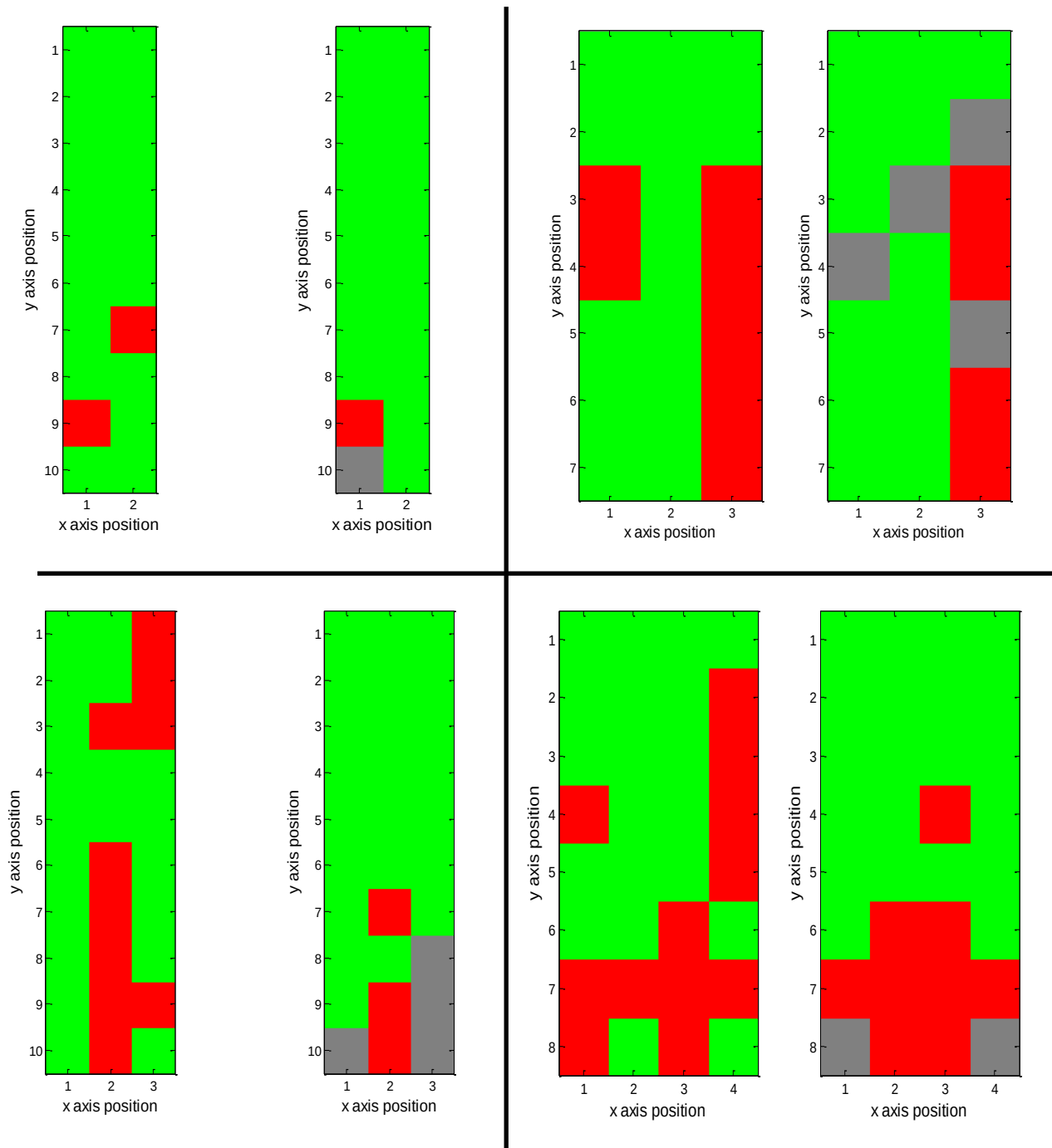


Figure 5-14. The pathology of the distal oesophagus has been mapped out (red – HGD / adenocarcinoma; green – NSq; grey – no consensus pathology agreement.) Four pairs of oesophageal maps are illustrated. The left map of each pair corresponds to Raman predictions at each grid position. The right image represents the consensus histopathological diagnosis at each grid position (gold standard).

When considering the potential for use of a Raman probe during endoscopic surveillance it must be remembered that probe spectra represent a point measurement from a specific tissue site. This is analogous to biopsying, and therefore probe-based surveillance may be liable to sampling error in the same way as standard biopsy protocols. Figure 5-14 does not illustrate the gaps between each interrogated area of tissue which could potentially harbour neoplastic disease. Although it may be feasible to take many more Raman measurements than biopsies, this advantage may be offset by the smaller area of interrogation ($<1\text{ mm}^2$) when using the probe.

However, the advantage of the probe during surveillance endoscopy may be in its ability to target biopsies to specific areas thus potentially drastically reducing the numbers of tissue samples for processing and reporting, and minimising the chance of significant post-procedural bleeding. The potential for use of the Raman probe during endoscopic surveillance is discussed further in Chapter 8.

5.14 Summary

The data presented in this chapter have demonstrated clear potential for the use of probe based Raman spectroscopy in the oesophagus. Following 1 s, 830 nm, spectral interrogation of 798 tissue sites a novel custom built Raman probe has demonstrated a sensitivity of 86% and a specificity of 88% for detecting advanced glandular neoplasia. Even higher diagnostic accuracies were achieved following sole analysis of endoscopic biopsy samples. All the data presented have been cross validated using independent test datasets, leave one spectrum out cross validation (LOSOCV) or leave one patient out cross validation (LOPOCV).

The ability of the probe system to detect mucosal changes has been demonstrated. This suggests potential for detection and targeted early excision of premalignant dysplastic lesions, analogous to the current treatment of cervical intraepithelial neoplasia. This would aim to prevent progression to invasive malignancy.

In addition, promising results have been obtained from a 0.1 s spectral dataset, raising the possibility for future ‘real-time’ imaging and detailed oesophageal mapping. The ability to map (or potentially scan across) the oesophagus in short timescales could aid rapid biopsy

targeting during Barrett's surveillance to minimise missed dysplasia. In addition, it could be used to rapidly and accurately locate areas of HGD / IMC in patients with known disease prior to targeted endoscopic resection.

No improvements in spectral classification performance were seen following air background subtraction or green glass correction. This infers that PC-fed LDA was capable of extracting the majority of the subtle spectral information required for classification despite the considerable probe background signal.

The biochemical basis of spectral classification will be discussed further in Chapter 6. In addition, the results presented here will be discussed again in Chapter 8, in the context of additional data presented in Chapters 6 and 7.

CHAPTER 6

6 Chapter 6: Understanding the Basis of Spectral Classification

It is important to evaluate and understand the basis of spectral classification so that probe design, spectral acquisition, and/or algorithm specifications can be optimised to maximise diagnostic performance. In this chapter established peak position tables have been used to correlate particular biochemicals with their corresponding spectral features.

6.1 Mean Tissue Spectra

1 s tissue spectra for normal squamous mucosa, Barrett's oesophagus, and HGD / adenocarcinoma are illustrated in Figure 6-1. Spectra have been offset for clarity and spectral peaks have been tentatively assigned following a review of the literature.[193-195]

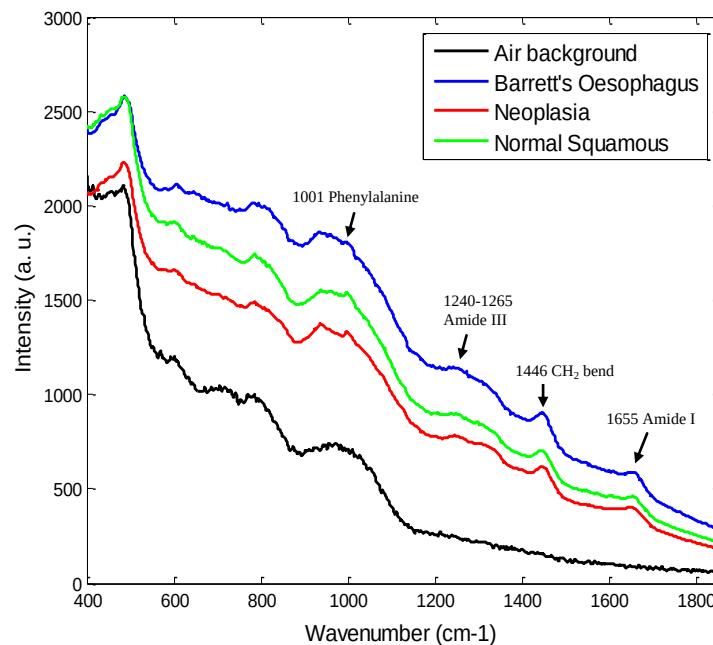
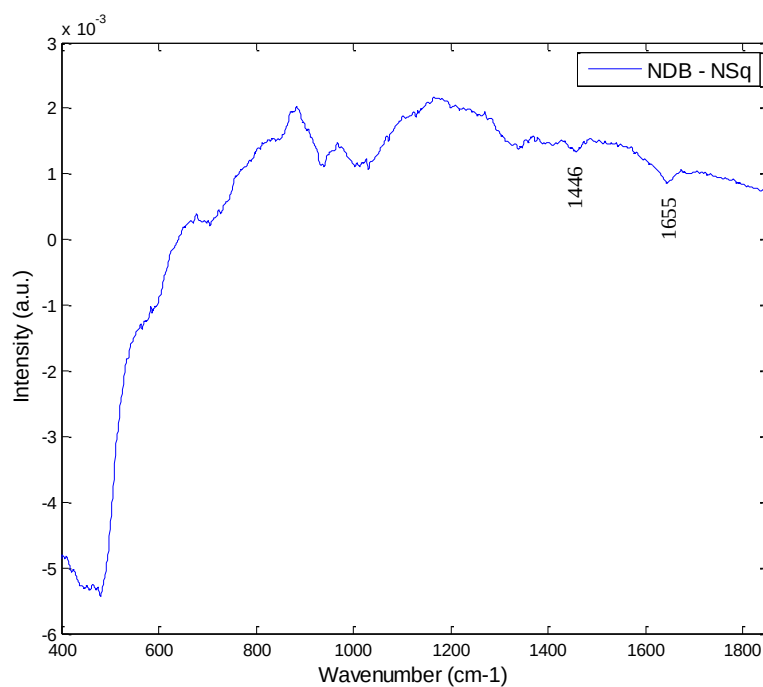


Figure 6-1. Mean 1 s tissue spectra for 3 pathologies. Spectra have been offset for clarity and spectral peaks have been tentatively assigned. ‘Neoplasia’ includes spectra from both HGD and adenocarcinoma.

Figure 3-3, included previously, shows a Raman spectrum for normal squamous oesophageal tissue measured on a laboratory based spectrometer. This demonstrates peak assignments and is referred to here to illustrate the variation in spectral quality between conventional systems and the probe based system. Probe spectra have a significantly lower signal to noise ratio (SNR) making visual classification of spectra impossible and necessitating the multivariate analysis presented in Chapter 5.

6.2 Difference Spectra

Difference spectra can be plotted to aid identification of the major differences between spectra from different pathology classes (Figure 6-2). This can potentially elucidate the peaks that are important for diagnostic classification.



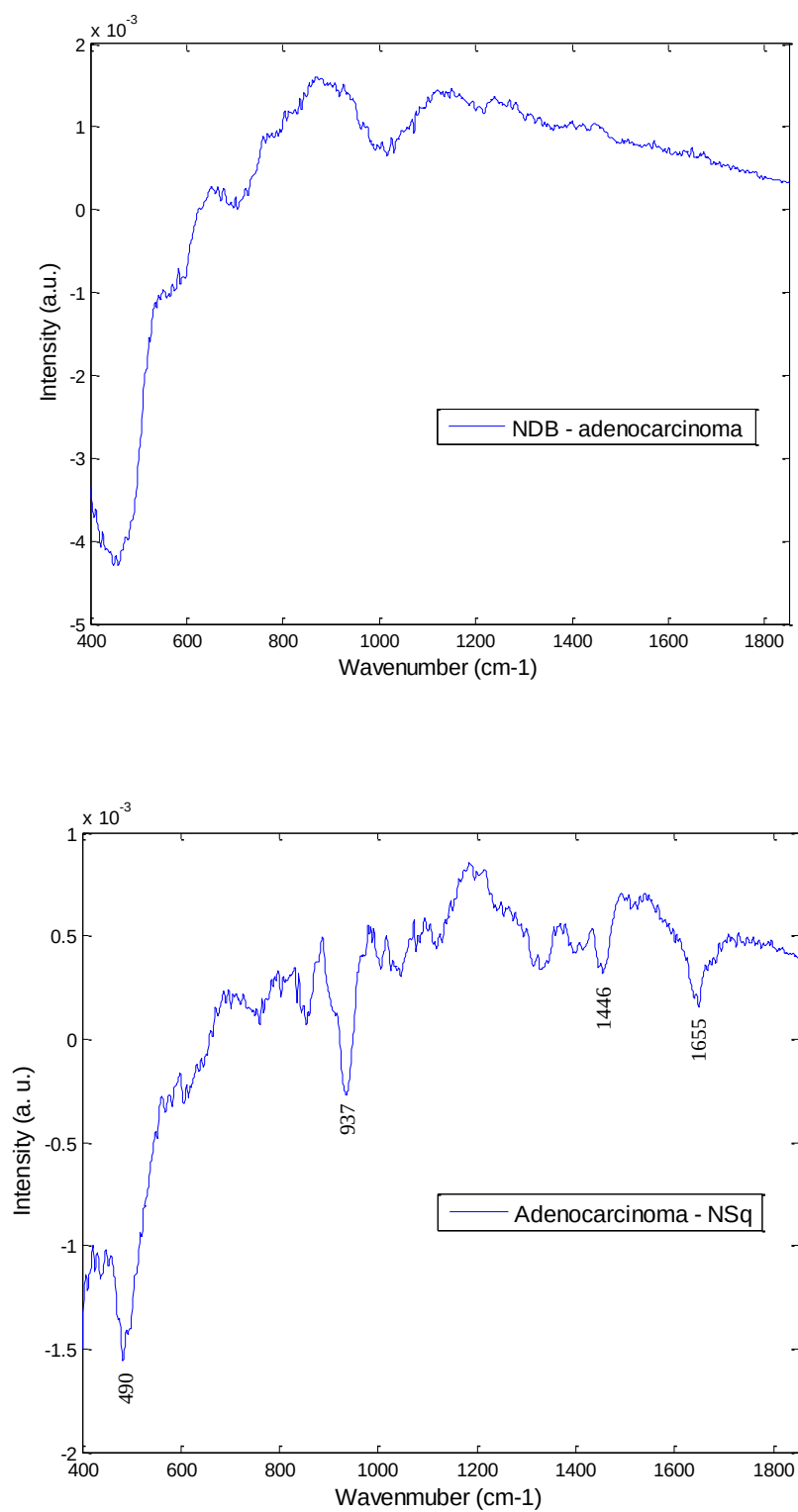


Figure 6-2. Difference spectra for non-dysplastic Barrett's (NDB) and adenocarcinoma spectra (top); adenocarcinoma and normal squamous (NSq) spectra (middle); and NDB and NSq spectra (bottom).

Figure 6-2 demonstrates that variation in the amount of fluorescent background signal accounts for the major visible spectral differences between normal squamous, Barrett's oesophagus and adenocarcinoma. This reflects the fact that background signal varies considerably from measurement to measurement (see Figure 5-3). Few biochemical peaks are reliably visible in Figure 6-2, particularly when comparing Barrett's and adenocarcinoma spectra. However, we cannot conclude from this that differences in fluorescence signal account for the ability to classify pathologies as we have previously noted no visually identifiable pattern in background appearance with respect to pathology (Figure 5-3). The significance of the biochemical peaks identified in Figure 5-3, and mechanisms to elucidate further spectral differences between pathologies are discussed extensively below.

6.3 Background Subtraction Algorithms

A variety of background correction and / or subtraction techniques can be employed to aid identification of the spectral features which may be important for classification. Mean air background subtracted and green glass corrected spectra for three pathology groups are illustrated in Figure 6-3. Comparison with Figure 6-1 demonstrates that these techniques have helped to emphasise tissue peaks. However, simple visual comparison of these spectra is still not sufficient to enable reliable diagnostic classification, which is why multivariate PC-fed LDA has been employed.

Both air background subtraction and green glass correction algorithms have been shown to fail to improve diagnostic classification performance when employed in multivariate PC-fed LDA models.

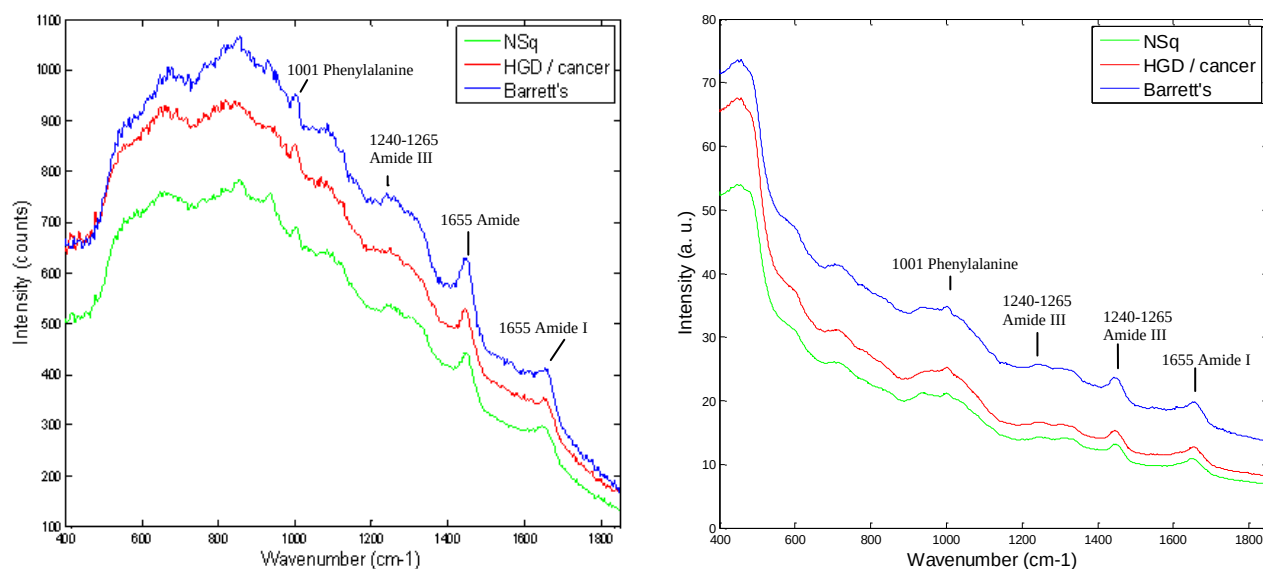


Figure 6-3. Mean 1 s spectra from three pathology classes following air background subtraction (left) and green glass correction (right). The prominent peaks have been labeled.

Peaks at 1655, 1446, 1262 and 1001 cm^{-1} are entirely consistent with previously reported oesophageal tissue measurements.[49; 206; 209; 212; 237] These peaks can be assigned to vibrational modes associated with amide I, CH_2 bending, amide III and phenyl ring structures respectively.[206].

6.4 Elucidation of Subtle Biochemical Features Using Multivariate Analysis

Previous work by Shetty *et. al.* used ordinary least squares analysis to fit the spectra of a variety of biochemical constituents to the mean spectra of each pathology. Changes in the distributions of these specific biochemical constituents were correlated with neoplastic progression. Concentrations of DNA, oleic acid (a fatty acid), collagen I and actin appeared increased in neoplastic tissue, whereas the concentration of glycogen appeared to have diminished.[43; 209; 212]

Shetty *et. al.* showed that the DNA peaks at 720 and 755 and 781 cm^{-1} , and the histone peak at 1655 cm^{-1} (a protein which packages DNA into nucleosomes), were more prominent in

neoplastic tissue (Figure 6-4A).[43] Conversely, glycogen peaks at 490, 853, 937 and 1335 cm^{-1} were less evident in oesophageal neoplasia (Figure 6-4B).

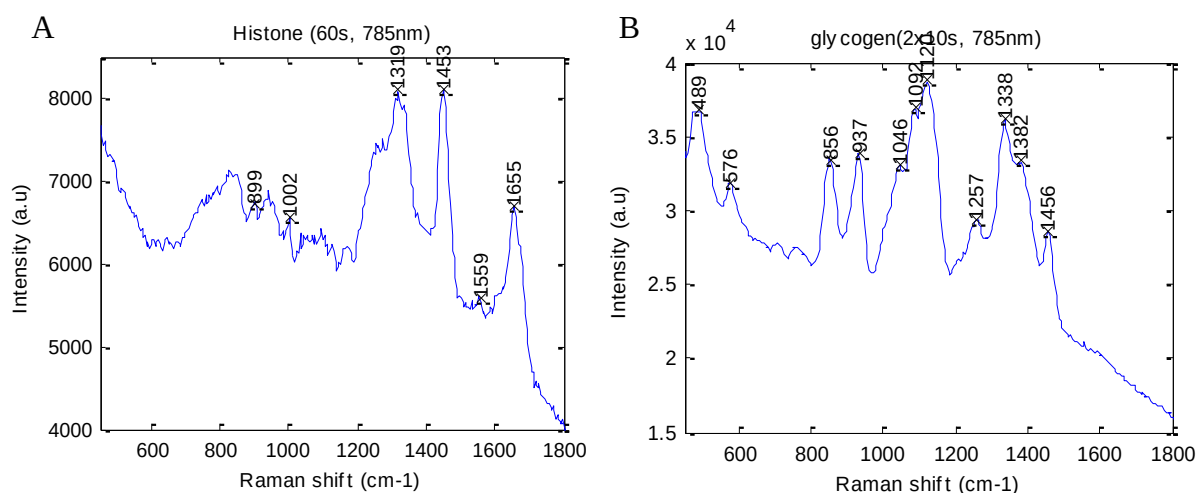


Figure 6-4. Raman spectra from (A) histone; and (B) glycogen acquired on a laboratory based system using 10 s acquisition time.[247]

This work has since been supported by Bergholt et. al..[209; 212] They measured reference Raman spectra from 35 different biomolecules associated with oesophageal tissue (including actin, albumin, pepsin, pepsinogen, RNA, DNA, myosin, hemoglobin, collagen I, collagen II, collagen V, mucin, flavins, elastin, phosphatidylcholine, cholesterol, glucose, glycogen, triolein, histones, and beta-carotene). Their subsequent analysis indicated that five biochemicals: actin, histones, collagen I, nucleic acids, and triolein (a lipid) were the most significant Raman-active biochemicals for characterising and discriminating normal squamous and cancerous oesophageal tissue. Raman peaks at 1335 and 1575 cm^{-1} were highly associated with increased DNA content, and increased intensity of the 1655 cm^{-1} amide I (C = O) band reflected a higher content of α -helical proteins (e.g. histones) in neoplastic tissue. A small decrease in collagen I concentrations in cancerous oesophageal tissue was also documented. This is in keeping with reports that invasion into underlying stromal tissue leads to a reduction of collagen content.[242; 249; 250]

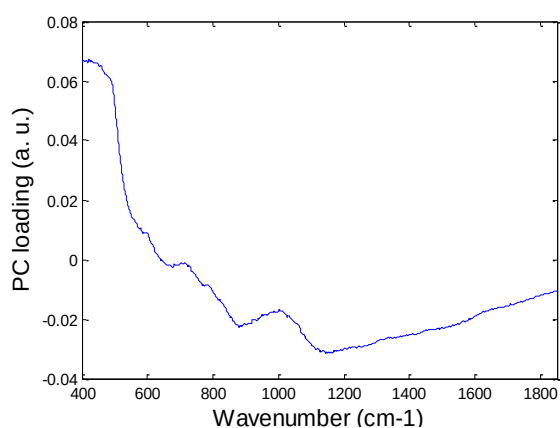
Previous studies have also demonstrated relative increases in Raman signals from a variety of proteins (e.g. at positions 1003, 1265, and 1655 cm^{-1}) which are thought to represent increased mitotic activity in neoplasia. [212; 242; 249-251] Similarly, studies on gastric

tissue have demonstrated reduction in Raman signals related to lipids (at 1078, 1302, 1440, and especially at 1745 cm^{-1}) in advanced gastric neoplasia.[242] In addition, prominent Raman bands at $1575\text{--}1618\text{ cm}^{-1}$ have been highly associated with the process of angiogenesis that occurs during neoplastic progression.[242; 249; 250]

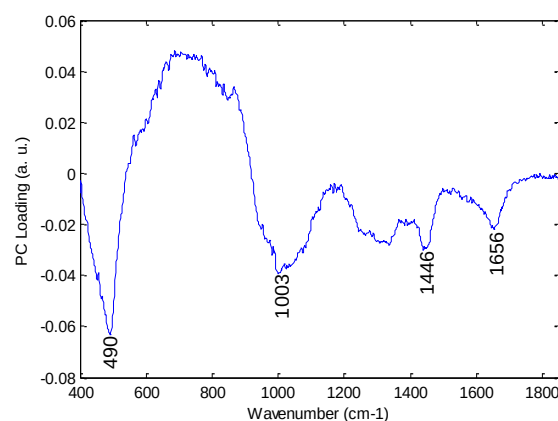
Plotting of PC loadings (Figure 6-5) and linear discriminant functions (LDF) (Figure 6-6) has been used to illustrate biochemical peaks that are important for spectral classification. Appendix XIII displays all 20 PC loadings computed for the standard three group classification model (NSq; NBD/LGD; HGD/cancer).

Evaluation of the PC loadings can enable distinction of those peaks seemingly most important for spectral classification based on their relative intensity at positions known to correlate with biochemical differences between tissue types.

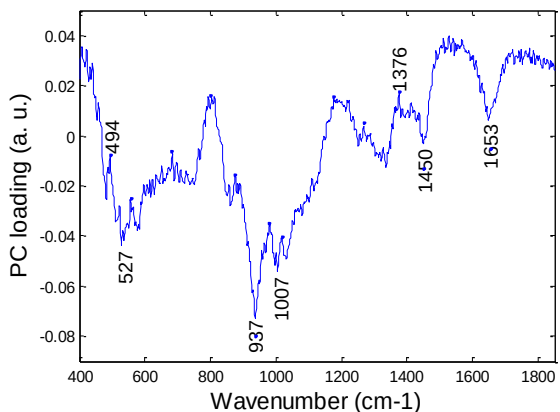
PC1:



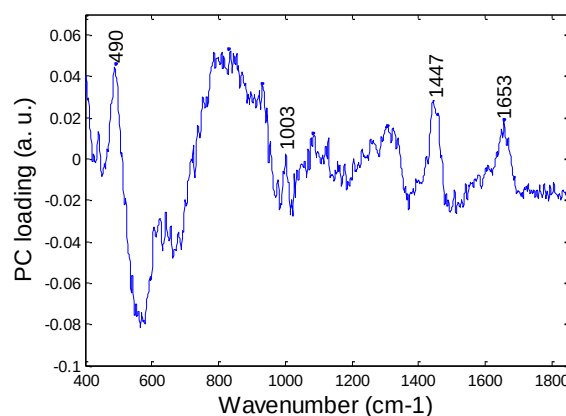
PC2:



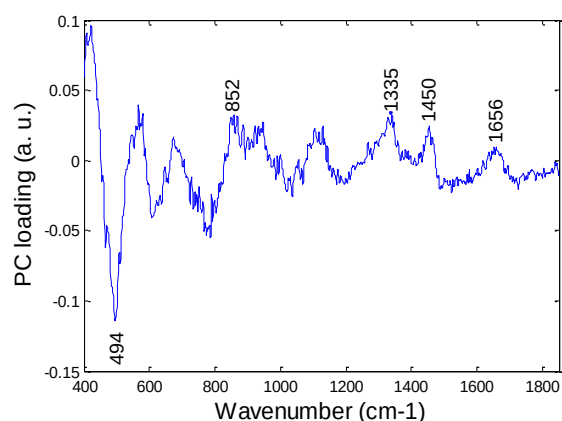
PC3:



PC4:



PC6:



PC7:

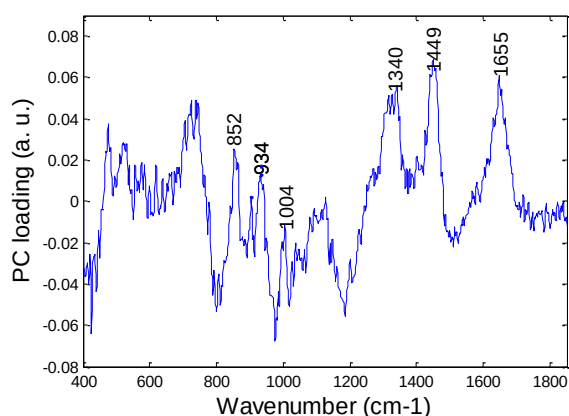


Figure 6-5. A selection of PC loadings are illustrated following PCA of the 1 s spectral dataset measured from normal squamous; LGD/NDB; and HGD/cancer tissue. Peak labels have been tentatively assigned to prominent features within the loadings.

The PC loadings plots presented in Figure 6-5 highlight peaks which are entirely consistent with the published literature. Prominent features, clearly important for spectral classification, are seen at 490, 1003, 1335, 1446 and 1655 cm⁻¹.

PC1 and to a lesser extent PC2, display a considerable degree of background fluorescence signal. This reflects the fact that variation in fluorescence signal across the dataset is significant, as was illustrated previously by the raw spectra presented in Figure 5-3 and the difference spectra in Figure 6-2.

The largest difference between the spectra is in fact the fluorescence signal variation which therefore represents the dominant feature in PC1. The ability to classify tissue spectra following the exclusion of these early PCs, is discussed further below. However, it appears that classification performance may not only depend on variations in Raman peaks, but also to some extent on differences in fluorescence signal.

Encouragingly the linear discriminant functions (LDF) for the same three group model plotted in Figure 6-6 define group separation based on recognisable biochemical differences between pathologies. In addition, in these plots there does not appear to be a significant fluorescence contribution. It is likely that the Raman bands at 487, 863, 933 and 1128 cm⁻¹ in

the LDF1 plot represent a higher glycogen concentration in the squamous epithelium and contribute significantly to the ability to separate between normal and metaplastic / neoplastic tissue.

The LDF2 plot (Figure 6-6B) illustrates prominent Raman bands at positions 498, 686 and 1571 cm^{-1} . These features can be tentatively assigned to nucleic acid bases which predominate in neoplastic tissue and correspond to increased DNA and RNA concentrations.[43]

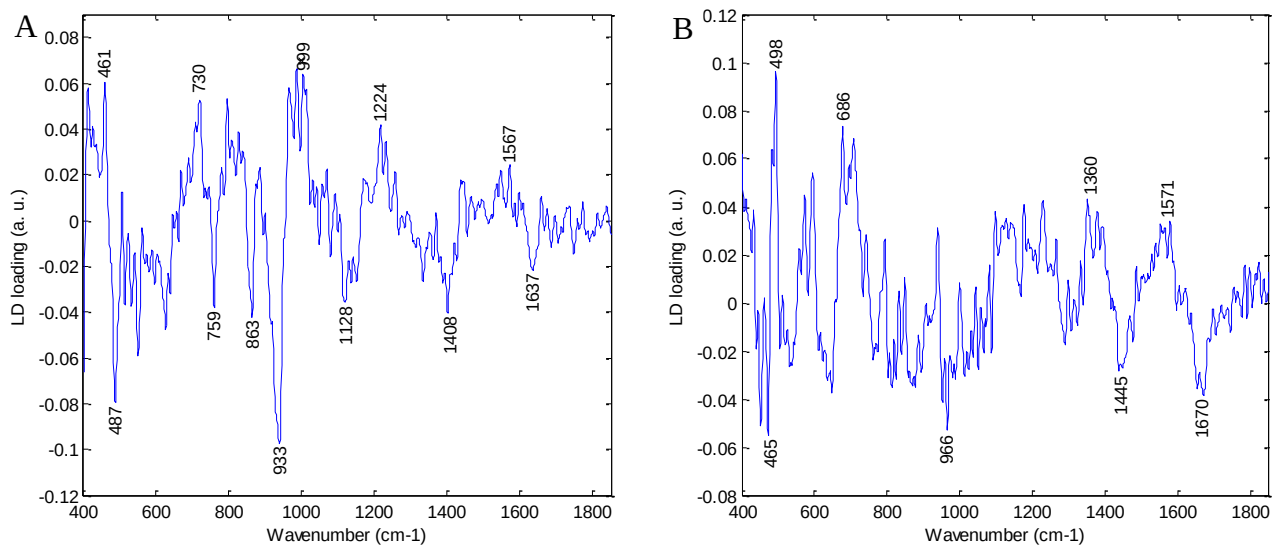


Figure 6-6. Linear discriminant function plots 1 (A) and 2 (B). Prominent peaks have been tentatively assigned.

Peak assignment of the PC loadings and LDF plots demonstrates that differences in biochemical composition detected by RS are important for spectral classification.

Visible distinction between unprocessed probe spectra measured from different pathologies is not possible. Similarly, visual identification of many of the biochemical peaks important for diagnostic classification is impossible from unprocessed spectra. However, data presented in Chapter 5 demonstrated that PC-fed LDA was capable of extracting the subtle spectral differences which can enable tissue classification. In this section the specific peaks which have enabled spectral classification have been identified.

6.5 Exclusion of Early Principal Components

6.5.1 Assessment of Classification Performance

Review of the PC loadings for the 3 group model (NSq; LGD/NDB; HGD/cancer) clearly demonstrates prominent peaks in several of the PCs at sites known to correlate with significant biochemical differences between squamous, Barrett's and neoplastic tissue (Figure 6-5 and Appendix XIII). However, the early PC loadings (particularly PC1) which represent the dominant difference between the spectra, appear to predominantly reflect differences in fluorescence signal, without apparently containing important biochemical information.

Although background fluorescence signal varies considerably between measurements (Figure 5-3) it is unclear what contribution this variation has on the ability to discriminate between pathologies. To investigate this further PC-fed LDA was repeated for this 3 group model after exclusion of PC1 and PC2. In addition, the classification performance achieved using just PC1 and PC2 was also evaluated. The predicted diagnostic accuracies achieved are displayed in Table 6-1.

		Sensitivity (%)	Specificity (%)
PC1-15 fed into LDA	NSq	87%	96%
	LGD / Barrett's	72%	91%
	HGD / Adenocarcinoma	86%	88%
Exclusion of PC1	NSq	82%	94%
	LGD / Barrett's	71%	87%
	HGD / Adenocarcinoma	82%	91%
Exclusion of PC1 and PC2	NSq	84%	90%
	LGD / Barrett's	63%	87%
	HGD / Adenocarcinoma	77%	91%
Only PC1 and PC2 fed into LDA	NSq	44%	71%
	LGD / Barrett's	67%	74%
	HGD / Adenocarcinoma	33%	76%

Table 6-1. Three group classification performance is illustrated following exclusion or inclusion of early PCs from the analysis.

Table 6-1 illustrates that exclusion of PC1 and 2 has a small effect on classification performance. This may result from loss of a degree of biochemical information which is hidden by the fluorescence signal. However, alternatively, it may reflect loss of fluorescence information which is itself playing a small role in spectral classification.

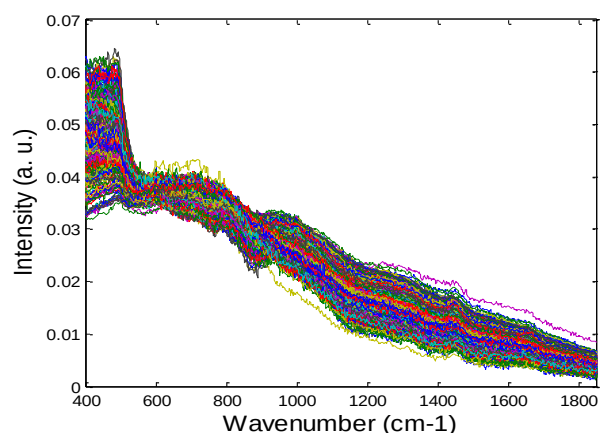
When just PC1 and 2 have been fed into LDA the model loses its ability to classify pathology. This demonstrates that the variation in fluorescence background signal alone is insufficient to enable any degree of reliable spectral classification.

6.5.2 Reconstruction of the Dataset Following Exclusion of PC1

Following exclusion of PC1 the normalised spectral data can be re-constructed by multiplying the remaining PC loadings by the PC scores. In this instance this effectively acts as an alternative technique of background subtraction and enables LD functions to be plotted without the contribution of PC1.

Normalised data prior to further analysis is shown in Figure 6-7(A). Normalised data is also illustrated in Figure 6-7(B) this time following re-construction of the dataset after exclusion of PC1.

A:



B:

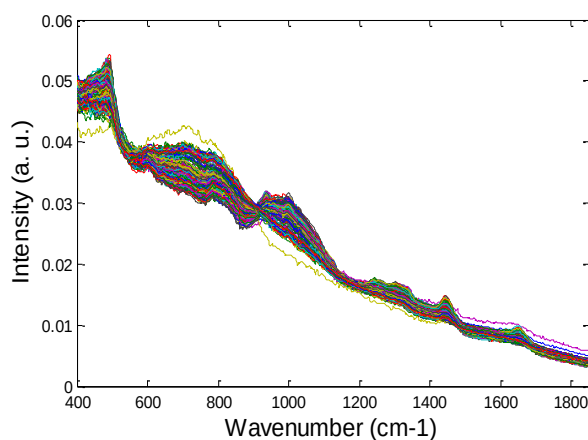


Figure 6-7. (A) Normalised raw spectral data. (B) Normalised data which has been reconstructed after exclusion of PC1.

Figure 6-7(B) demonstrates that exclusion of PC1 reduces the intensity variation within the spectral dataset. The visible peaks are still present and are in fact more pronounced following PC1 exclusion.

Figure 6-8 illustrates newly defined LDF plots following three group LDA performed on the reconstructed dataset. Encouragingly, many of the biochemical features present in Figure 6-6 are visible in Figure 6-8 providing strong evidence that they contribute significantly to diagnostic classification.

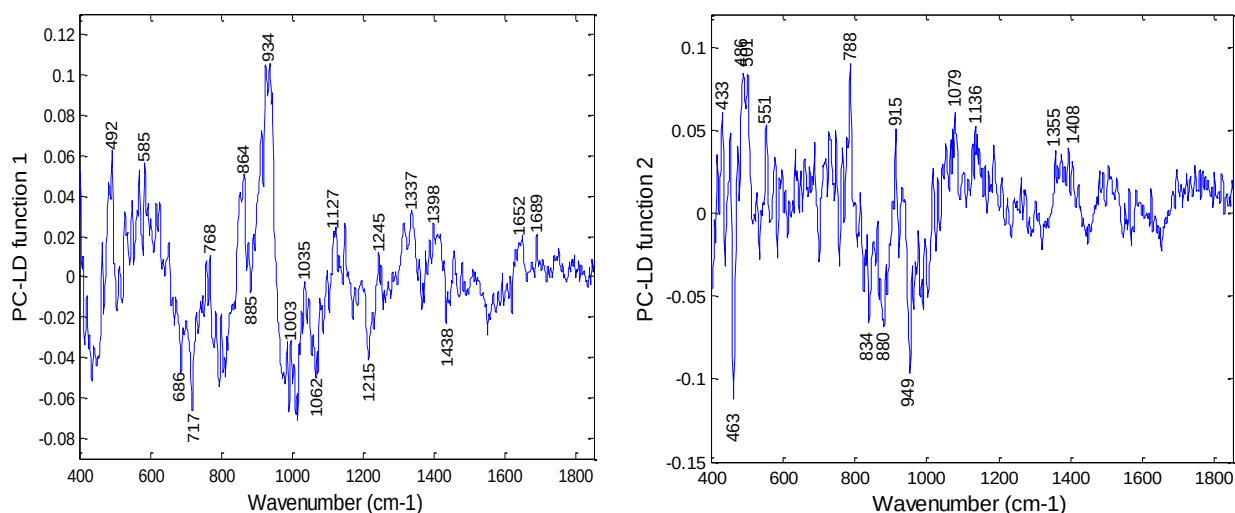


Figure 6-8. LDF plots following exclusion of PC1.

6.6 Evaluation of which Principal Components Contribute to Spectral Classification

Inspection of the PC loadings can be useful for defining biochemical peaks which account for spectral classification. However, this approach can be refined further by identifying which of the PC loadings contributes the greatest to spectral classification.

LDA generates an LD load value for each of the PCs which describes the relative contribution of each PC to spectral classification. Figure 6-9 shows a 3 group (NSq; NDB/LGD; HGD/cancer) LD scores plot following PC-fed LDA. LD load 1 accounts for the separation between normal squamous and Barrett's/neoplastic spectra, whereas LD load 2 accounts for separation between NDB/LGD and HGD/cancer spectra. Inspection of the LD loads plots reveals that PC8 and PC9 are particularly significant in separating normal squamous and Barrett's/neoplastic spectra, and PC11 and PC6 contribute most to the separation of NDB/LGD and HGD/cancer spectra.

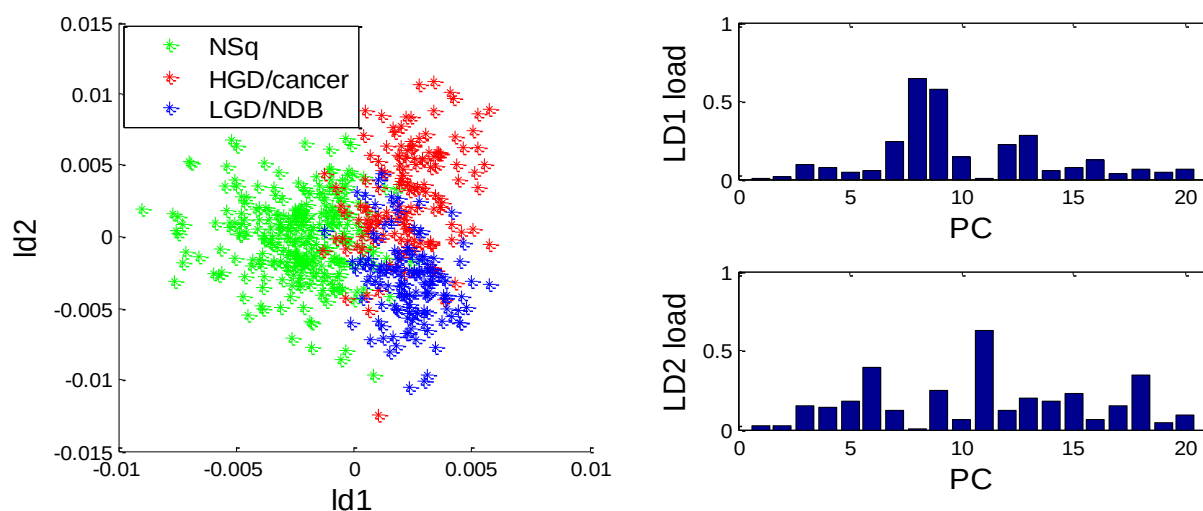
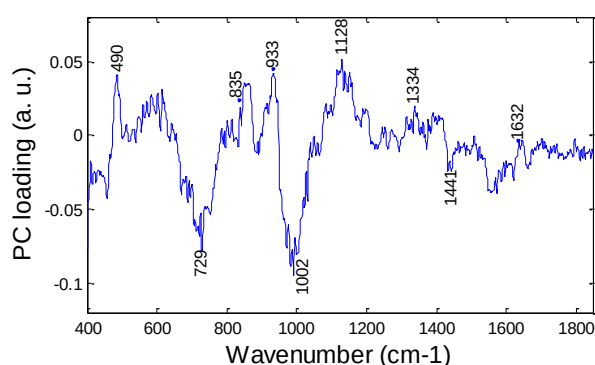


Figure 6-9. A three group classification predictions scatter plot (left); and LD loads showing the individual contribution of different PCs to spectral classification.

Examining the most significant PC loadings reveals several specific spectral features which are important for diagnostic classification. Figure 6-10 shows a plot of PC8 and PC9. Prominent peaks are again seen at 490, 933, 1086-1110 and 1128 cm^{-1} , all of which are consistent with a higher glycogen concentration in normal squamous epithelium compared to both Barrett's and neoplastic tissue. Reference to peak assignment tables (see Appendix III) allows other prominent peaks at 638, 731, 782, 885, 1021, 1095, 1334 and 1621 cm^{-1} to be tentatively assigned to nucleic acids and protein structures, including histone, which imply alterations in RNA and DNA concentrations between normal squamous and metaplastic / neoplastic tissues.

PC8:



PC9:

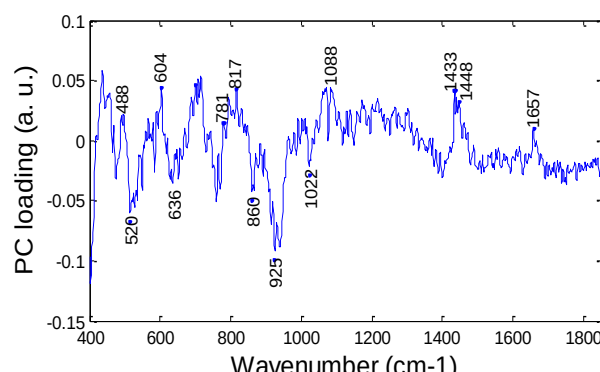
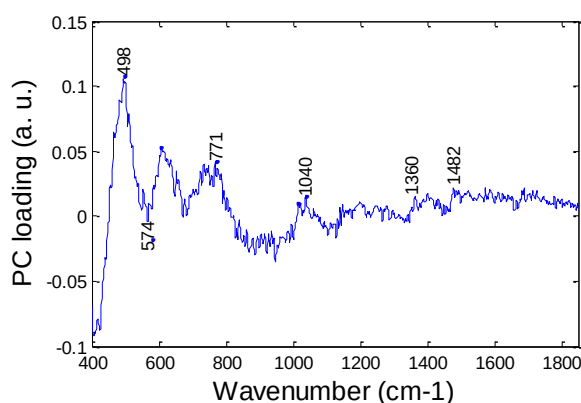


Figure 6-10. PC 8 and PC 9 loadings following PCA on spectra measured from three pathology groups.

The plot of the LD2 load in Figure 6-9 demonstrates that PCs 11 and 6 contribute particularly significantly to the differentiation of advanced neoplasia from LGD and NDB. Plotting of these PC loads (Figure 6-11) also reveals prominent glycogen peaks at 492, 574 and 937. Peaks at 771, 782, 1018, 1360 and 1511 can be tentatively assigned to nucleic acids which again reflect changes in DNA and RNA concentrations between metaplastic and malignant tissues.

PC6:



PC11:

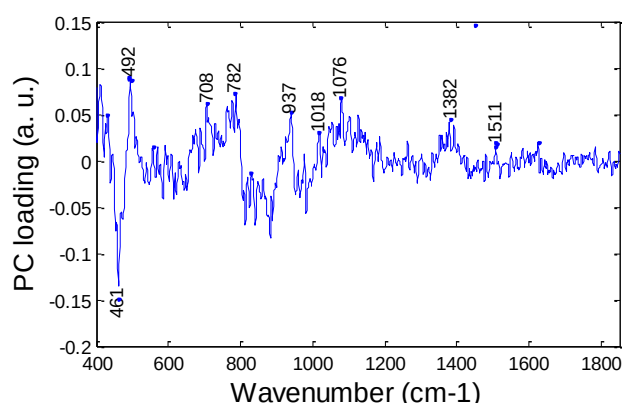


Figure 6-11. PC 6 and PC 11 loadings following PCA on spectra measured from three pathology groups.

Whilst the PCs illustrated here account for some of the ability to classify spectra, it must be remembered that a significant amount of important information is described by the other PCs.

There are therefore many other biochemical peaks, in addition to those highlighted here, which could contribute to spectral classification. Further work should continue to elucidate these significant classifying features, although this will best be studied through in vitro measurements using long acquisition times and high quality spectrometers with high SNRs.

It is important to note that PC1 and PC2 appear to contribute very little to spectral classification (Figure 6-9). This supports the earlier analysis demonstrating little change in performance after exclusion of these PCs. Since PC1, and to a lesser extent PC2, consists almost entirely of background fluorescence signal this analysis reinforces the fact that the chief contributor to spectral classification is biochemical features detected by Raman spectroscopy rather than a difference in fluorescence background in the three tissue groups.

It is also noteworthy that some particularly prominent peaks are seen at the lowest wavenumber positions in PCs 9 and 6. It is unclear whether these represent biochemical features or significant fluorescence signal. However, the latter is a possibility as there is clear visual variation in unprocessed spectral data in this range (Figure 5-2). To assess the contribution to spectral classification of features in the low wavenumber range the dataset was truncated, and classification performance was re-evaluated.

6.7 Spectral Truncation

The effect of spectral truncation was evaluated to assess the impact of exclusion of spectral regions which display significant background signal and minimal biochemical information. In the lower wavelength range there is a particularly high background contribution and spectral plots show that variation is marked in this region (Figure 5-2). Previous studies have demonstrated that exclusion of this low wavelength range may improve spectral classification.[45; 203]

Spectra were therefore truncated below 600 cm^{-1} and the classification performance was re-evaluated (Table 16).

		Sensitivity	Specificity
Whole spectral range included (400-1850)	NSq	87%	96%
	LGD / Barrett's	72%	91%
	HGD / Adenocarcinoma	84%	88%
Truncated spectral range (600-1850)	NSq	83%	96%
	LGD / Barrett's	76%	88%
	HGD / Adenocarcinoma	84%	89%

Table 6-2. Classification performance of the 1 s three group classification model is illustrated following truncation of the lower wavenumber range.

Table 16 shows that truncation of the lower wavenumber range made little difference to the ability of the model to classify. This infers that there is sufficient biochemical information retained in the remaining spectral range to permit classification and that the significant background in the early wavenumber range may act to mask some of the influence of glycogen peak variation (at 490 nm) on spectral classification.

6.8 Biochemical Transition from Barrett's to Adenocarcinoma

There is a well described histological sequence of progression from normal squamous mucosa, to Barrett's oesophagus, LGD, HGD and then adenocarcinoma. Many of the molecular changes, particularly those early in this sequence have been elucidated and are now well described. However, the *biochemical* changes that occur during each stage of the progression to adenocarcinoma are as yet less well described.

The above sections have identified and discussed many of the Raman peaks thought to be most significant for pathological classification. Encouragingly, these are consistent with previous published reports and reflect particular changes in glycogen, nucleic acids, oleic acid, collagen I, actin and histone peaks which are known to vary during progression to malignancy.

Probe based spectra have a considerably lower SNR than can be recorded using laboratory spectrometers. In addition, the spectra presented in this thesis have been acquired in short, clinically applicable timescales. These factors make a more detailed evaluation of

biochemical features difficult. However, an attempt has been made to demonstrate a biochemical progression between Barrett's and adenocarcinoma (Figure 6-12).

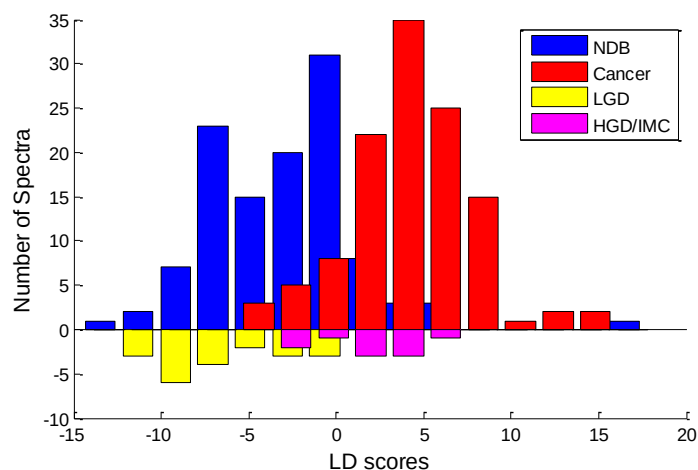


Figure 6-12. An LD scores histogram showing spectral predictions following PC-fed LDA for a two group model (NDB/LGD and HGD/cancer). Spectra from each individual pathology have been plotted separately in order to visually illustrate where and how they classify. LGD and HGD/IMC spectra have been plotted inversely on the y axis for visual clarity.

The data presented in Chapter 5 indicated that, for a variety of reasons, there was some misclassification in the NDB/LGD Vs. advanced neoplasia model. However, the majority of spectra can clearly be seen to classify correctly (Figure 6-12). Assuming a linear progression of biochemical changes from NDB to invasive adenocarcinoma, those spectra tending to misclassify could reasonably be expected to be the dysplastic group. Examination of Figure 6-12 shows that this appears to be the case for the HGD/IMC samples which predominantly occupy a central position in the histogram. However, one would expect the LGD spectra to also cluster predominantly in the middle of the plot, just to the left of the HGD/IMC spectra. This is not convincingly the case.

Despite not being able to clearly demonstrate biochemical progression here, the data certainly provokes interest and demonstrates potential for further investigation.

6.9 Summary

Understanding the basis of spectral classification is important in order to facilitate measures to improve diagnostic performance. The data presented in this chapter demonstrate that classification is predominantly due to biochemical differences between tissue types detected by Raman spectroscopy. Several characteristic biochemical differences along the pathway of progression to cancer have previously been reported including changes in glycogen, histone, DNA and RNA concentrations. In this chapter these spectral features were all shown to be important for spectral classification.

The biochemical changes detected during neoplastic progression are entirely consistent with increasing nuclear activity (e.g. a hyperchromatic state) and angiogenesis in neoplastic tissue. These are well described characteristic features of tissue carcinogenesis and the ability of Raman spectroscopy to detect these features is of considerable value for tissue diagnosis and characterisation.

In addition, the fact that these biochemical features are consistent with the literature reconfirms the potential suitability and versatility of Raman spectroscopy as an endoscopic technique for early detection and diagnosis of dysplasia and cancer based on spectral features derived from proteins, DNA, and lipids.

A greater appreciation of the spectral features which account for diagnostic classification could potentially facilitate a more targeted approach to spectral measurement. For example, measurement of significantly truncated spectra from a limited wavenumber range of interest could enable significant reductions in spectral acquisition times whilst maintaining classification performance. Alternatively, multiple repeat measurements could be made from a focussed wavenumber range in the time it takes to acquire a single set of measurements from the relatively broad spectral range recorded at present. This could increase SNR and may yield improvements in diagnostic performance. Knowledge of particular biochemical characteristics between tissue types could also potentially influence probe design, or perhaps more likely, impact on the design of statistical algorithms used for classification.

It is possible that differences in the fluorescence signal generated by different tissue types have also contributed to the ability to classify tissue. However, detailed analyses including an

evaluation of which principal components contribute to spectral classification, and spectral truncation analyses, have demonstrated that any contribution from fluorescence signal appears small. It is also important to consider that the priority aim of the custom-built fibre-optic probe evaluated in this thesis is to correctly classify oesophageal tissue in vivo. Ultimately, it is this ability to accurately classify that is important, not the need for classification to be based solely on Raman spectral signatures.

CHAPTER 7

7 Consistency, Reproducibility and Transferability

Prior to clinical use of endoscopic RS, spectrometer specifications and probe construction must be standardised in order to minimise variation in spectral acquisition. It is likely that attaining identically reproducible measurements using different, although standardised, equipment will be extremely challenging. Therefore it will be essential to ensure that mechanisms exist to correct for inevitable multi-system, multi-site and multi-operator variability enabling transferable classification models to be generated. Clinical application will necessitate the use of a pre-defined automated classification algorithm based on known spectral fingerprints correlating with specific tissue types in order to attain instantaneous diagnoses. However this requires that spectral signals measured across all Raman systems can be precisely calibrated.

In this chapter the consistency of spectral acquisition over an eighteen month period of data collection is evaluated. In addition, the performance of two probes consisting of identical optical components is compared. Both these probes are evaluated in conjunction with the same Renishaw system 100 spectrometer. A further set of measurements is also presented which were acquired using a single probe in conjunction with two different spectrometers. Mechanisms for data comparison are explored following a variety of calibration techniques and subsequently the ability to correctly classify pathology with the different systems is evaluated.

The potential for use of RS in conjunction with narrow band imaging (NBI) is also evaluated.

7.1 Spectral Consistency across the duration of the study period

7.1.1 Temporal Consistency of Air Background and PTFE Measurements

It is also essential to demonstrate that spectra can be measured consistently over time. A random selection of mean air background spectra measured on eight different days across an eighteen month period, using the standard probe-spectrometer system are illustrated in Figure 7-1.

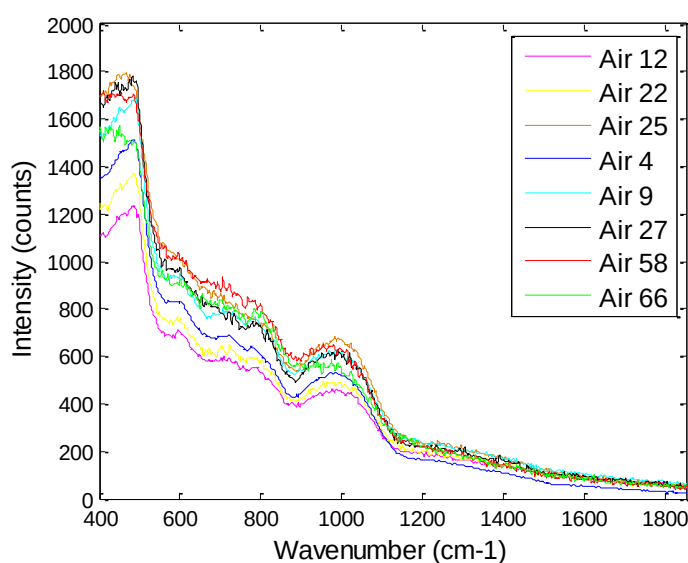


Figure 7-1. Mean 1 s air spectra measured on randomly selected days demonstrating spectral consistency over time. Air 4 was measured in November 2010; Air 66 was measured in April 2012.

PCA was performed on the air background spectra measured in November 2010 (Air 4). Figure 7-2 illustrates the variance within this dataset and plots 95% and 99% confidence intervals. Projection of individual air spectra measured on other days (randomly selected) demonstrates the levels of consistency achieved.

Projection of new spectra onto this plot allows a simple assessment of spectral consistency and reproducibility. Prior to clinical usage, this method could be applied to ensure accurate instrument calibration and exclude extraneous contamination of the probe tip.

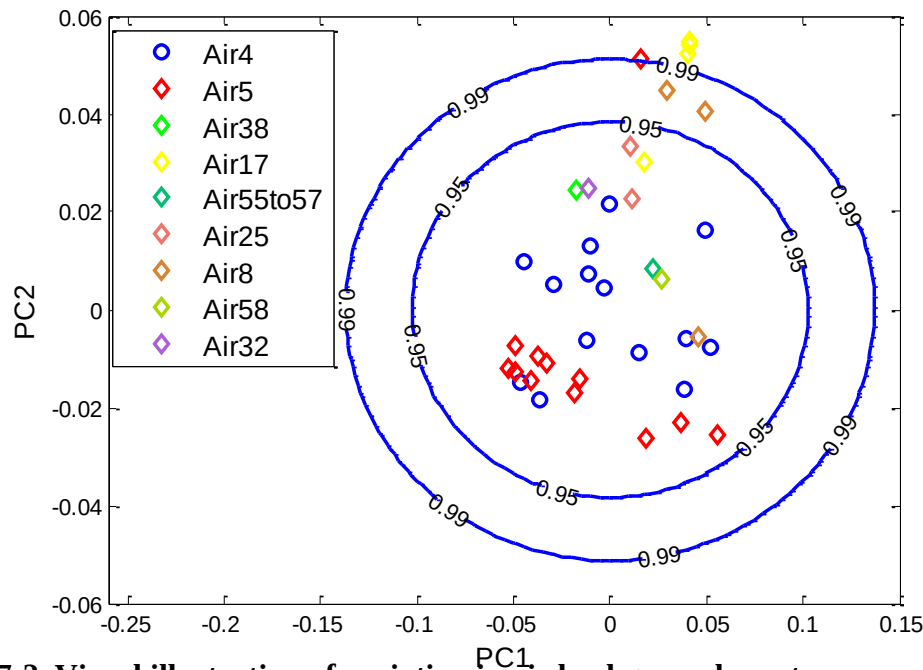


Figure 7-2. Visual illustration of variation in air background spectra compared to day 1 (Air 4). 95% and 99% confidence intervals are illustrated.

Methods analogous to this could be used to clearly define instrument tolerability. For example, let's assume a tolerance level based on a 95% confidence interval (CI) of a predefined set of calibration air measurements. In this case, a pre-endoscopy 'test' air background measurement falling within the 95% CI boundary would be considered acceptable. This would indicate that the probe-spectrometer system is functioning, using acceptable laser power, and without significant spectral contamination. However, a pre-endoscopy 'test' measurement falling outside of the pre-defined tolerance level would prompt cleaning of the probe tip, a check of laser power output, and/or replacement of the probe.

In order to define levels of equipment tolerability in this way it must be clear what levels of spectral consistency are required for tissue classification. This will necessitate measuring sufficient spectra from sufficient patients and with sufficient probes in order to account for spectral variation due to either pathology, patient, or probe-spectrometer factors.

Alternatively, it may be possible to define levels of tolerability mathematically by introducing random background to the spectral data and assessing the limits of where spectral classification becomes impossible. Automated generation of equipment tolerance levels is discussed further in later sections.

Figure 7-3 illustrates that PTFE spectra have also remained relatively consistent throughout the period of data collection despite minor variations in intensity signals as seen with the unprocessed tissue data presented in Chapter 5.

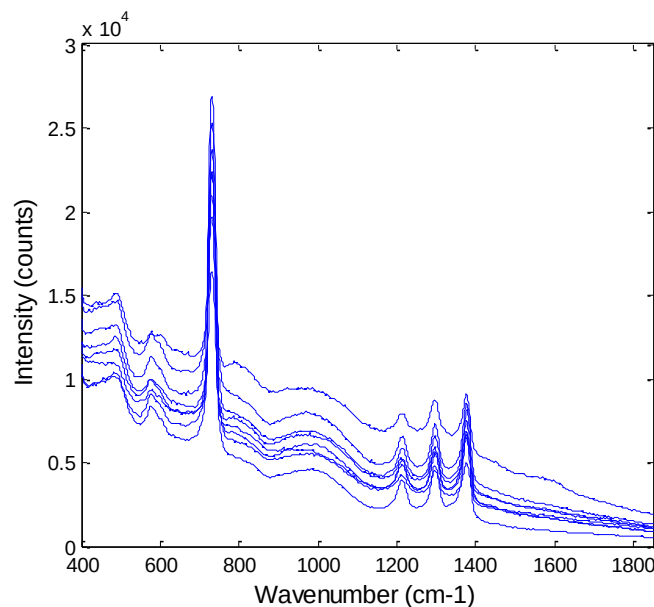


Figure 7-3. Eight randomly selected 5 s PTFE spectra.

The need for reliable and reproducible spectral acquisition will be discussed and evaluated in greater detail in later sections. However, the plots presented above have demonstrated consistent spectral measurements over the eighteen month period of data collection.

7.2 Influence of the Date of Measurement on the Spectral Classification

As discussed in Chapter 6, it is important to appreciate the factors that account for spectral variation within the groups. Figure 7-4 illustrates an LD scores plot for the standard three group classification model. To investigate any variation in tissue spectra measured on different days, three sets of spectra measured on three different days, have been highlighted in colour. This plot visually demonstrates that there is no particular clustering of these spectra.

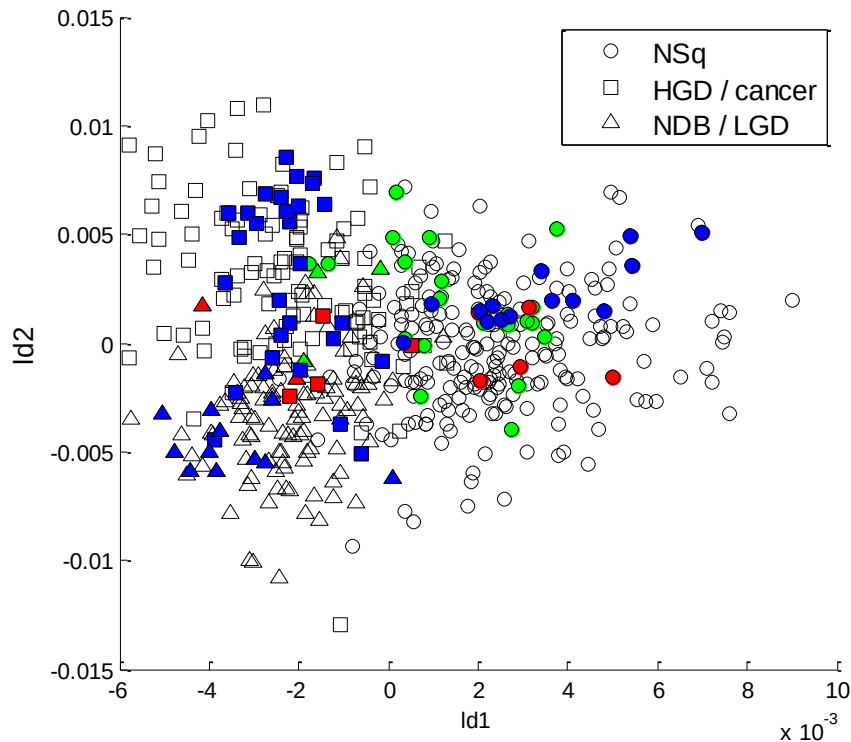


Figure 7-4. An LD scores scatter plot illustrating spectral predictions for a three group (NSq; NDB/LGD; HGD/cancer) classification model. Three randomly selected sets of spectra, measured on different days, have been highlighted.

This illustration does not solely give a visual impression of consistency of spectra measured on a particular day. It also reflects any spectral differences pertaining to specific patient factors since each set of spectra was acquired from tissue from a different patient. Confounding due to snap freezing of a portion of the dataset has been removed here by only including fresh tissue spectra measured from resected oesophagi.

This visual illustration demonstrates that the date of measurement, and unique patient factors for these three patients, appear to have little impact on the ability of the probe system to record spectral data.

7.2.1 Summary

This section has demonstrated that despite minor fluctuations in intensity which occur from measurement to measurement, spectral data has remained consistent over an 18 month period. In addition, results presented in Chapter 5 have demonstrated highly accurate classification performance for tissue spectra measured across this time period.

It is not clear at this stage whether Raman probes will be disposable, with the intention of single use, or whether they will be amenable to multi-use with sterilisation between patients. In the latter case they will be required to operate consistently over an extended period of time in a similar way to standard medical endoscopes. The data presented here has demonstrated that significant systematic error was not introduced over the course of the study period suggesting potential for reliable multi-use systems. However, it is important to note that the ability of the probe to withstand multiple sterilisation procedures remains to be evaluated.

7.3 Probe Comparison

If Raman probes are to be used as clinical diagnostic tools, probe construction must be standardised and consistent so that spectral measurements can be accurately reproduced. In this section data recorded using two identically constructed Raman probes is compared and evaluated.

7.3.1 Two Operator, Two Probe Comparison using a PTFE Standard

7.3.1.1 Unprocessed Spectra

Initial measurements aimed to demonstrate that two Raman probes of the same design could measure consistent PTFE spectra when used by two operators.

Figure 7-5 shows mean PTFE spectra measured using each probe. Probe 2 has a prominent background feature between ~900 and 1100 nm which enables easy visual differentiation between the spectra. The source of this additional background signal is unclear although it

may be due to subtle variations in one or more of the probe components or a minute difference in the alignment of the optical components within the probe.

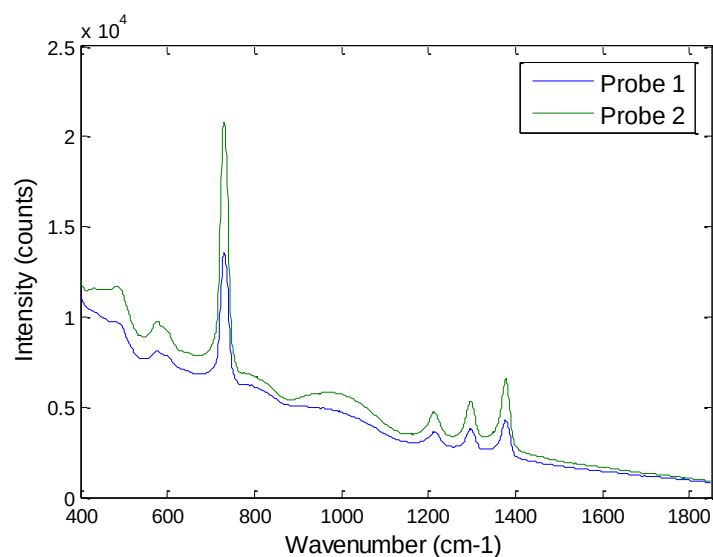


Figure 7-5. Mean PTFE spectra measured in 5 s using probe 1 and probe 2.

Figure 7-6 illustrates mean PTFE spectra measured by each operator using both probes. Visually these appear consistent except for a small variation in the background signal. This difference is accounted for by an obvious spectral outlier (see Figure 7-7 below).

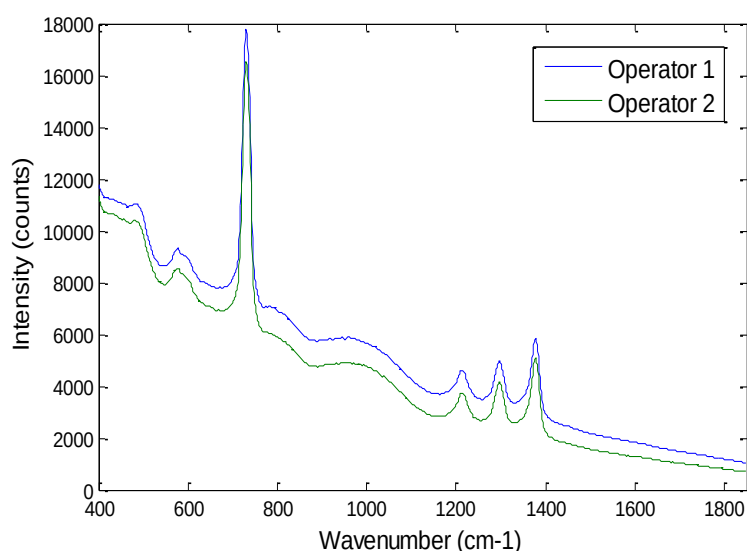


Figure 7-6. Mean PTFE spectra taken by each operator.

Figure 7-7 shows unprocessed PTFE spectra measured by two independent operators using the two probes in conjunction with the same laser source and spectrometer.

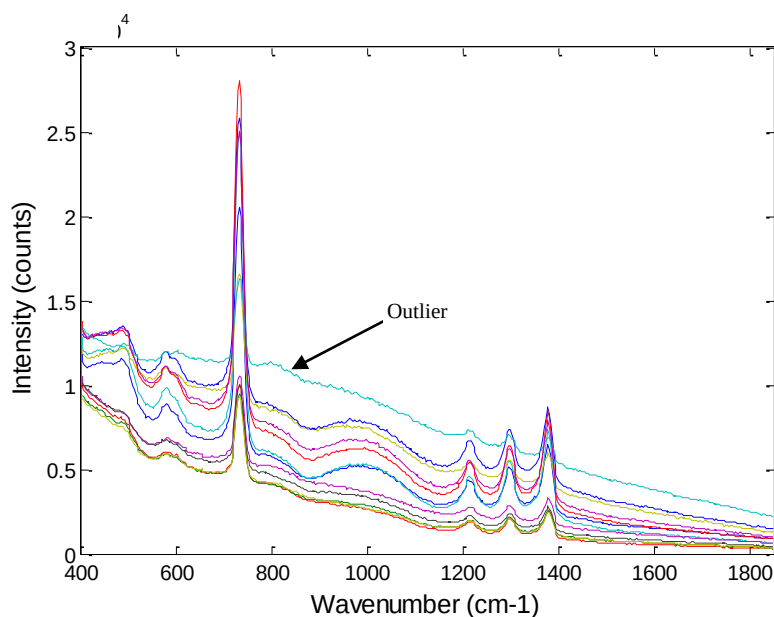


Figure 7-7. PTFE spectra measured by both operators with each probe.

One spectrum which was measured by operator 1 using probe 2 is a clear visual outlier. This spectrum, which has been highlighted, has a considerable additional background signal. The measurement was repeated following cleaning of the probe and was found to persist. It is unclear what caused the increased background signal detected in this spectrum, the most likely explanation being debris on the on the surface of the probe lens.

This spectrum was included in the subsequent analysis. However, it could be reasonably argued that visible outliers – those *unprocessed* spectra which can be easily identified – could be removed prior to statistical analysis. A more precise approach is to use pre-defined levels of performance tolerance to identify outlying measurements. This approach lends itself to automation and could be used to prompt repeat measurements where the initial spectra fail to meet the tolerance criteria.

7.3.1.2 Statistical Evaluation of Spectral Consistency

Two-way analysis of variance (ANOVA) was used to investigate the relationship between the measured spectra and the two independent variables - the operator and the probe. The analysis aimed to establish whether there was any true variation between the groups.

A wavenumber representing the midpoint of each of the 5 prominent PTFE peaks was selected. Prior to pre-processing (Table 1), and following air background correction plus green glass correction (Table 2), the intensity of each peak was measured at each specified wavenumber.

The tables illustrate the results of 2-way ANOVA calculations which demonstrate that, with one exception, there were no statistical differences between the PTFE peaks measured by the 2 probes, or by the 2 operators, or by any combination of a particular operator with a particular probe (interaction column). (A *p* value of less than 0.05 indicates that there was a statistically significant difference in peak height at a specific peak due to the effects of a particular independent variable (or combination). In this instance, the null hypothesis that there is no difference in peak height irrespective of the probe or operator, is false).

The single statistically significant peak height difference is highlighted in bold. It is unclear why the interaction of a particular operator with a particular probe at this individual peak was shown to be significant, although it is possible that inaccuracies were introduced during the data pre-processing (air background subtraction and green glass correction).

Wavenumber (cm ⁻¹)	Interaction (<i>p</i> value)	Probe (<i>p</i> value)	Operator (<i>p</i> value)
731	0.9444	0.0716	0.2908
981	0.7797	0.067	0.178
1214	0.9041	0.0685	0.3518
1297	0.9273	0.0727	0.2762
1378	0.997	0.0736	0.3645

Table 7-1. The probability that there is no difference in PTFE peak heights when measured by the two probes, or the two operators, or by any combination of the two (interaction column), is illustrated following 2-way ANOVA.

Wavenumber (cm ⁻¹)	Interaction (<i>p</i> value)	Probe (<i>p</i> value)	Operator (<i>p</i> value)
731	0.0992	0.8481	0.1472
981	0.0289	0.1624	0.116
1214	0.1027	0.7859	0.3001
1297	0.1349	0.6781	0.1744
1378	0.0722	0.8798	0.2665

Table 7-2. Prior to two-way ANOVA, air background subtraction and green glass correction were applied to the raw data. The probability that there is no difference in PTFE peak heights when measured by the two probes, or the two operators, or by any combination of the two (interaction column), is illustrated. The single statistically significant result is highlighted in bold.

It must be stressed that these analyses have evaluated the spectra at just five wavenumber positions. Variations in background signal at other positions have not been taken into account. Whilst these results demonstrate that reliable data can be measured using two optically identical probes without significant inter-user variation, further analysis of tissue spectra across the whole wavenumber range is important to evaluate the feasibility of generating transferable diagnostic algorithms (see below).

7.4 Transferability between Spectrometers

7.4.1 Optimising a Calibration Correction

Spectra have been measured using a custom built portable spectrometer (see Chapter 4), in addition to the standard Renishaw system 100 spectrometer used routinely for previous measurements. This section investigates the potential for cross system comparison of spectra measured using the two spectrometers after a variety of statistical calibration algorithms have been applied.

Background subtraction and calibration strategies have been discussed in detail previously. Neither air background subtraction nor green glass correction improved the performance of the diagnostic classification models presented in Chapter 5. However, these techniques may

have a greater importance when attempting to project new data, measured with a different system, onto a universal classification model.

Calibration aims to ensure reproducible CCD read-out so that consistent spectral data can be recorded from day to day. Calibration standards were measured, as previously described, to facilitate both offset correction and correction of variation of CCD efficacy across the entire spectral range. Both silicon and PTFE spectra were used to assess any shift in wavenumber due to their well defined and reproducible peaks. Figure 7-8 illustrates air background and silica spectra measured using a single Raman probe in conjunction using two different spectrometers. ‘Spectrometer 1’ was the Renishaw System 100 spectrometer used for all the ex vivo tissue measurements presented in Chapter 5. ‘Spectrometer 2’ was the custom-built portable spectrometer.

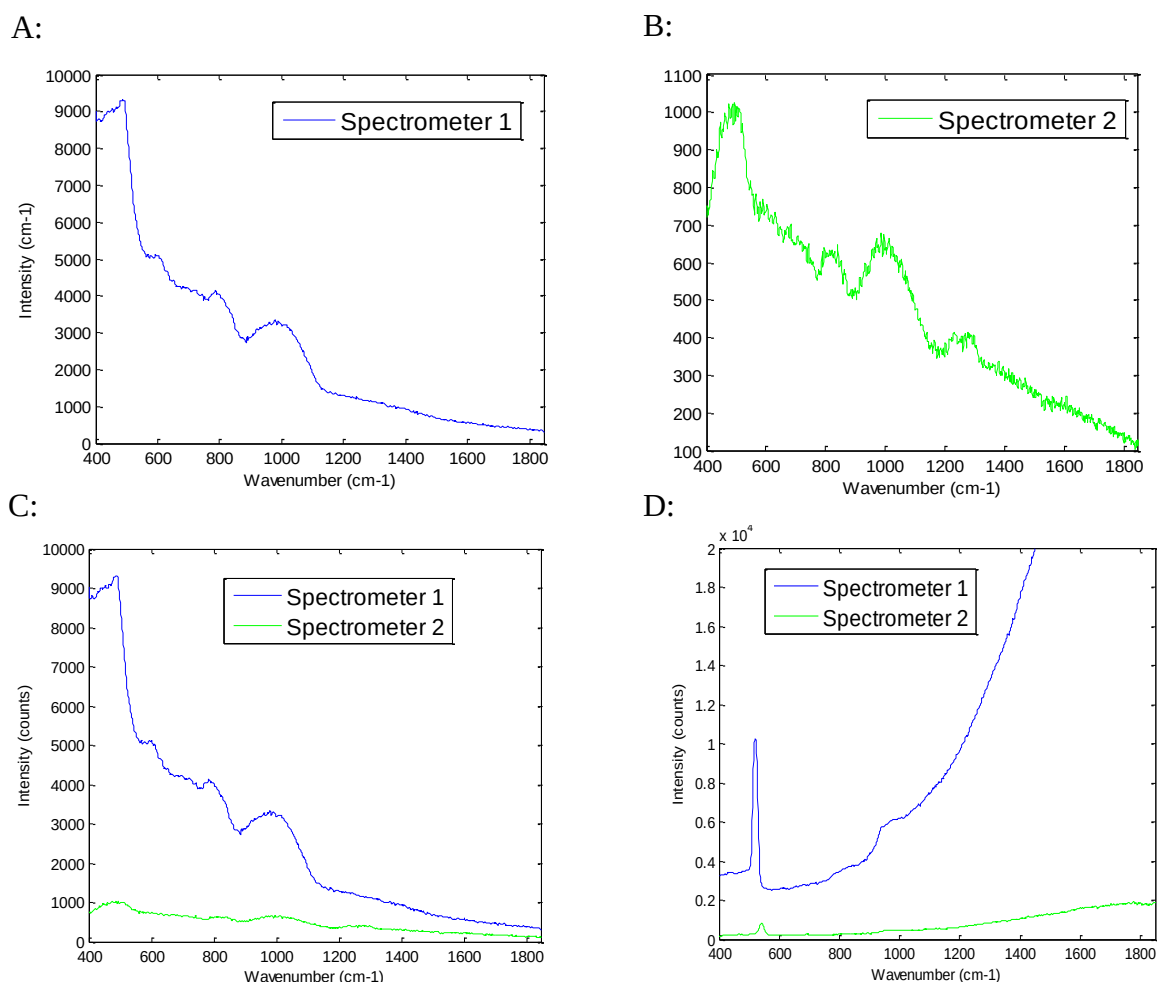


Figure 7-8 A, B&C: 5 s air background spectra; and D, a 1 s silica spectrum. All have been measured with the custom built probe in conjunction with one of the two spectrometers as indicated.

The shape of the background signal acquired with the two spectrometers was visually comparable although an additional feature was noted between 1200-1300 cm^{-1} when using spectrometer 2. However, the intensity acquired using spectrometer 2 was significantly lower than with the Renishaw System 100 spectrometer (spectrometer 1) meaning a lower SNR was achievable. The intensity difference is clearly illustrated in Figure 7-8 C&D which show air and silica spectra measured on the two systems.

The software with spectrometer 2 did not permit a silicon offset correction to be applied as was performed routinely for calibration of spectrometer 1. It can be seen from Figure 7-8 D that the silica peaks are therefore at slightly different wavenumbers. In order to correct this difference an offset correction was applied to the spectrometer 2 data in Matlab.

Figure 7-9 illustrates normalised 5 s PTFE spectra acquired using each spectrometer. A silicon offset correction has been applied to the spectrometer 2 measurement as described.

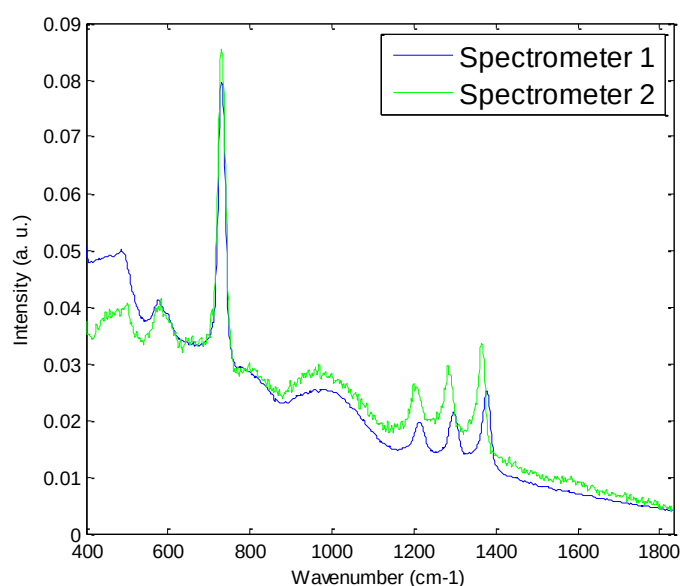


Figure 7-9. Normalised 5 s PTFE spectra measured using the two systems. A silicon offset correction has been applied to the spectrum measured on Spectrometer 2.

Inspection of Figure 7-9 demonstrates that there is a persistent peak offset in the higher wavenumber range. Therefore a linear data stretch was applied to the spectrum measured on Spectrometer 2 based on the expected peak positions of the 4 prominent PTFE peaks. Again the normalised data was compared (Figure 7-10).

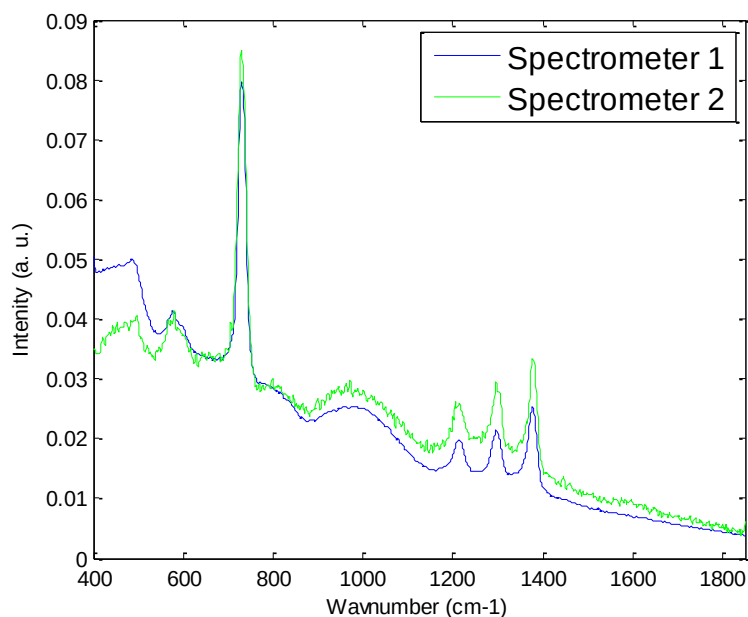


Figure 7-10. Normalised 5 s PTFE spectra measured using the two systems. A linear data stretch has been applied to the data measured using Spectrometer 2.

In an attempt to further improve spectral comparability a green glass correction was applied to both PTFE spectra (Figure 7-11). Green glass correction aimed to eliminate variations in instrument sensitivity across the spectral range. CCD readout typically varies slightly with wavelength, classically tending to fall-off at higher wavelengths. This is due to several factors including a gradual reduction in efficiency of the detector across the spectral range; and also to a lesser extent variation in CCD pixel sensitivity.[191]

Figure 7-11A shows a reference green glass spectrum measured at the National Physical Laboratory which was used as a calibration standard. Green glass measurements acquired using the two spectrometers were then used to calculate energy transfer function corrections by dividing the reference green glass spectrum by the two measured spectra. Unprocessed PTFE spectra measured on each system were then multiplied by the corresponding transfer function to calculate green glass corrected spectra (Figure 7-11B).

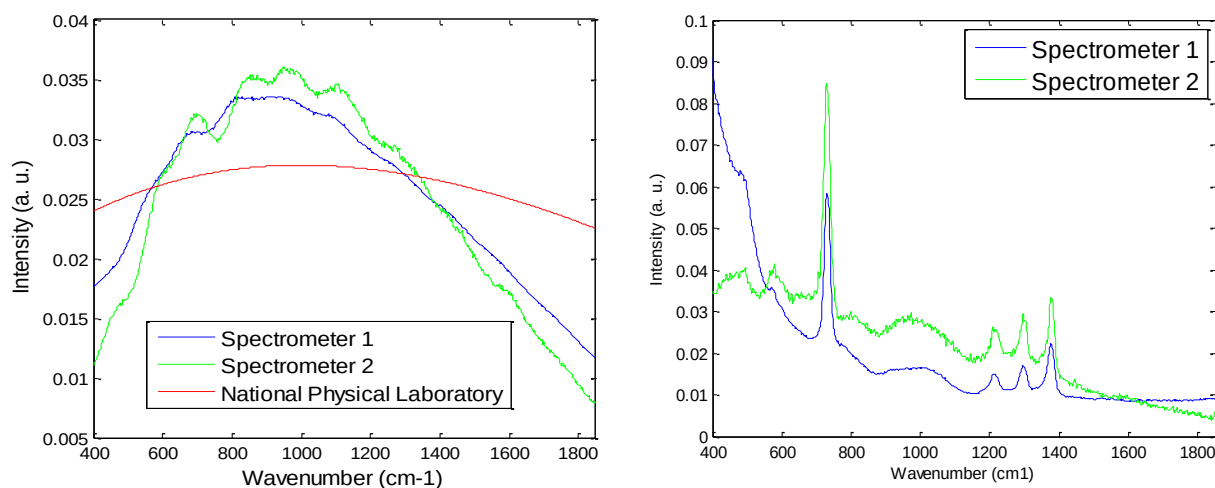


Figure 7-11 (A) Green glass spectra measured on the two experimental systems, and at the National Physical Laboratory; and (B) 5 s PTFE spectra measured on each system. A silicon offset correction, normalisation and green glass correction has been applied to the spectrum acquired on Spectrometer 1. A linear data stretch, normalisation and green glass correction has been applied to the spectrum acquired on Spectrometer 2.

Figure 7-11B shows that the use of green glass correction as an additional calibration strategy has failed to improve spectral comparability between the two systems (in comparison to that seen in Figure 7-10).

Background subtraction was also employed in an attempt to remove some of the spectral disparity, particularly in the upper wavenumber range. Corresponding air background data were subtracted from each PTFE spectrum and the spectra were then normalised and linearly stretched as before. This series of calibration corrections was shown to further improve spectral comparability (Figure 7-12). A combination of air background subtraction and green glass correction provided no additional improvement in spectral comparability.

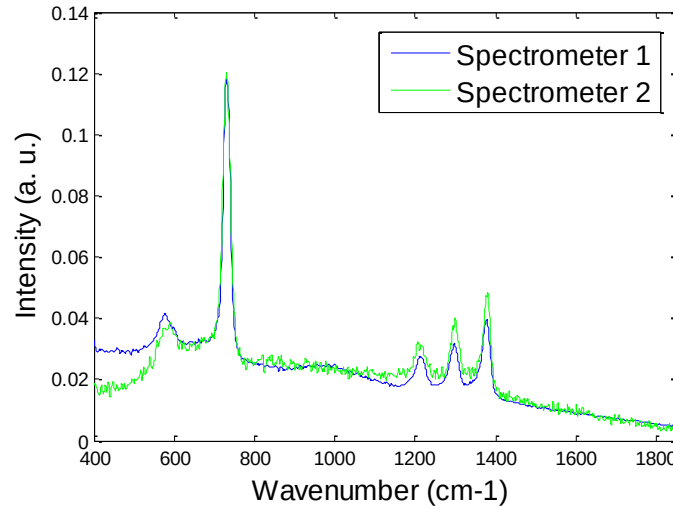


Figure 7-12. Air background spectra have been subtracted from the two PTFE spectra presented. Subsequently the spectra have been normalised and a linear data stretch has been applied.

In this model it appears that the most visually consistent spectra can be achieved through a process of normalisation, linear data stretching and air background subtraction (Figure 7-12). By defining this process for spectral calibration between the two systems it was hoped that tissue spectra, measured using Spectrometer 2, could be classified using the multivariate model generated from data acquired on Spectrometer 1.

7.4.2 Diagnostic Classification Performance

In clinical practice the intention would be to record spectra using identically built probe-spectrometer systems with clearly pre-defined levels of engineering tolerability. However, since multiple identical probe systems are not currently available for reproducibility testing, the process of cross system spectral calibration has been explored here. Section 7.5.1 above optimised measures for cross system comparison. In this section, as a theoretical exercise, a sample of ex vivo tissue spectra from 3 patients were acquired using Spectrometer 2.

The Raman probe was used in conjunction with Spectrometer 2 to measure 1 s spectra from 12 homogenous tissue samples for which a consensus histological diagnosis is available. In order to assess the transferability of these data, a cross system calibration was employed and

each spectrum was then projected, as a test dataset, onto the 1 s classification model acquired using Spectrometer 1.

The unprocessed ex vivo tissue spectra recorded by Spectrometer 2 are displayed in Figure 7-13.

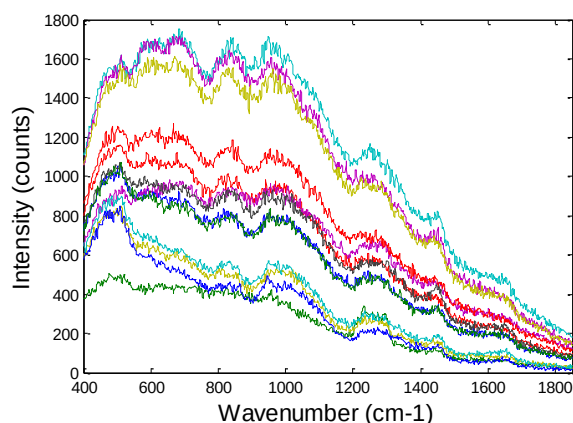


Figure 7-13. Unprocessed 1 s ex vivo tissue spectra measured using the Raman probe in conjunction with Spectrometer 2.

Prior to projection of Spectrometer 2 tissue data onto the model, the pre-defined cross system calibration correction was employed: a background correction was performed (Figure 7-14) followed by normalisation and a linear data stretch.

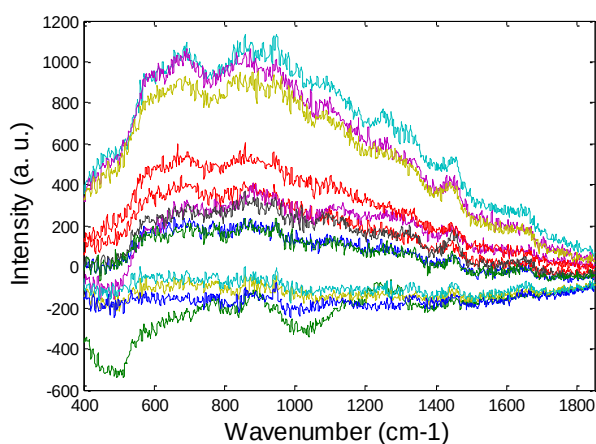


Figure 7-14. One second tissue spectra measured using Spectrometer 2 following air background subtraction.

Following air background subtraction some of the spectra were of very low (or negative) intensity suggesting possible poor contact with the tissue, and/or fluctuations in laser power output at the probe tip between the time of measurement of the air spectrum and the tissue spectra. This finding may also be explained by contamination of the lens leading to reduction in both excitation power and signal collection. All data without significant signal after air background subtraction were excluded from the subsequent proof of principle analysis.

The pre-established correction algorithm was applied to the tissue spectra measured using Spectrometer 2. PCA was then performed on this corrected dataset to generate a set of PC scores which were projected as a test dataset onto the standard 3 group (normal squamous; NDB/LGD; HGD/cancer) classification model generated from data acquired with Spectrometer 1.

The model defined using data acquired with Spectrometer 1 was not able to classify spectra measured using Spectrometer 2, despite the calibration correction. The reasons for this likely reflect the fact that Raman scattering generates only very weak signals. Persisting differences between sets of measurements made on the two different systems, despite the calibration correction, were still much more significant than the subtle biochemical spectral differences between tissue types, causing misclassification. The calibration correction process may also have been hindered by the process of background subtraction which may have introduced significant systematic error.

It must be considered that this was a theoretical exercise since in practice systems would be standardised prior to spectral measurements. However, the ability to correct significant spectral differences even utilising different spectrometers and achieve visually comparable spectra is encouraging. This suggests that minor spectral variations between identically built probes used in conjunction with identically built spectrometers could be overcome using a carefully optimised correction algorithm. When multiple identically built probes are available, further testing will be required in order to evaluate cross-system classification more fully.

7.5 Evaluation of the Potential for Compatibility with Narrow Band Imaging

In a clinical setting Raman measurements must be able to be recorded under the direct vision of the endoscopist. However, the ex vivo spectra displayed in this thesis have all been measured after extrusion of ambient light sources.

Interference of weak Raman signals by ambient light is known to be a significant problem. However, this issue can be relatively easily overcome in the laboratory by switching off lights and shielding windows and computer screens. Similarly, measurements undertaken in published studies of in vivo RS in the upper GI tract have been acquired after switching off the endoscopy light source for up to 10 s at a time.[35; 197; 206]

However, for probe-based RS to be seen as a realistic potential tool for in vivo endoscopic diagnosis, spectra must be able to be accurately acquired in conjunction with real-time white light endoscopy and/or additional wide field imaging techniques. This will enable safe measurements to be recorded from very specific areas of clinical interest.

When working to develop a clinically applicable endoscopic Raman probe it is therefore important to consider and evaluate the effects of endoscopic white light imaging and alternative endoscopic light sources on spectral classification performance.

Modern Olympus endoscopes are capable of both real-time white light (WLE) and narrow band imaging (NBI). NBI illuminates the mucosa of the oesophagus with blue and green light utilizing the principle that longer wavelengths penetrate deeper into tissue than shorter wavelengths. The effect is to enhance the resolution of the mucosal surface potentially helping to differentiate dysplasia and early cancer from benign Barrett's oesophagus.[219]

NBI has been shown to increase the diagnosis of dysplasia in the hands of experienced users.[221; 223] However, considerable inter-user variation and high false positive rates are widely reported.[133; 223]

The great potential of RS is its ability to provide objective tissue classification with high positive predictive values enabling early targeted therapy (see Chapter 5). RS could therefore

ideally complement a wide field imaging technique such as NBI which suffers from subjectivity and poor specificity.[250] A dual modality (RS-NBI) imaging system could potentially limit the high false positive rate associated with NBI and provide objective diagnostic assessment to reduce inter-user variation.

This section aims to assess the potential for endoscopic multi-modality imaging using RS, NBI and WLE.

Ex vivo Raman spectra were measured in the endoscopy department at Gloucestershire Royal Hospital from 14 fresh oesophageal biopsies collected from 3 patients. Spectra measured from one of the biopsies was excluded from the analysis due to excessive fluorescence (see Chapter 5). In this case the excessive fluorescence signal was probably due to considerable contamination of the tissue specimen with blood caused by bleeding at the time of the biopsy.

Tissue spectra were measured using the Raman probe and the custom built portable spectrometer in conjunction with WLE, and NBI, and following exclusion of ambient light (control measurement). Figure 7-15 illustrates air background spectra measured under each of the lighting conditions.

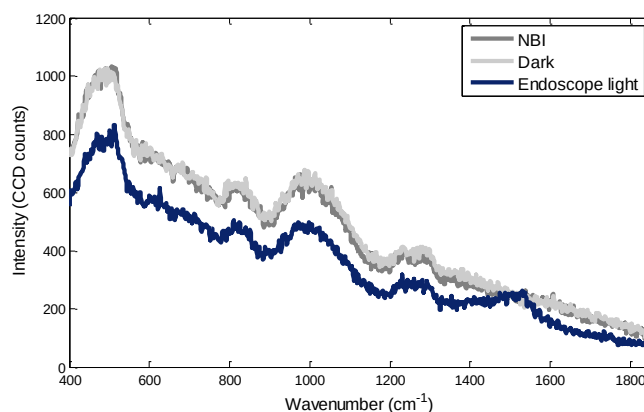


Figure 7-15. Air background spectra measured in conjunction with WLE and NBI, and following exclusion of ambient light (control).

RS measurements in the presence of NBI were visibly consistent with the ‘dark’ control prompting further investigation. A slight difference in background signal was noted in the presence of WLE, most notably in the wavenumber region 1460-1600 cm^{-1} .

Subsequently PTFE and tissue spectra were acquired (Figure 7-16A&B). Visually consistent spectra were demonstrated in the presence of both WLE and NBI, the only prominent area of disparity was again identified in the wavenumber range 1460-1600 cm^{-1} for both PTFE and tissue measurements (Figure 7-17).

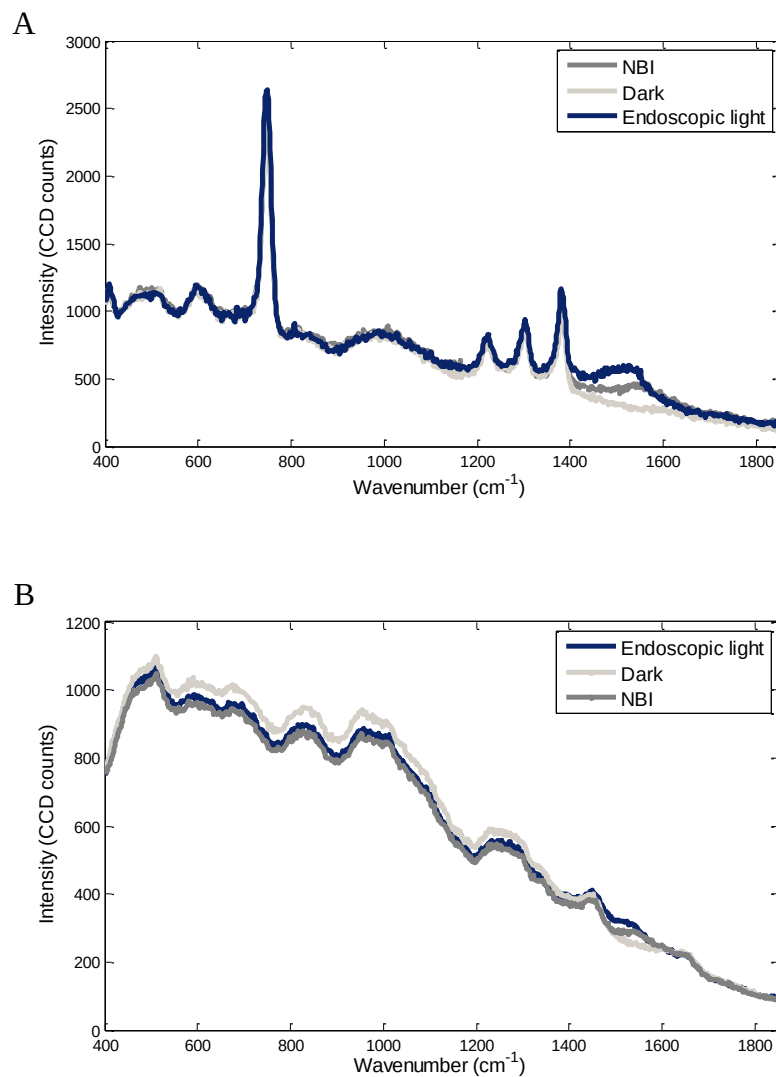


Figure 7-16. Five second PTFE spectra (A); and mean tissue spectra from 13 oesophageal biopsies (B); measured in conjunction with WLE and NBI, and following exclusion of ambient light (control).

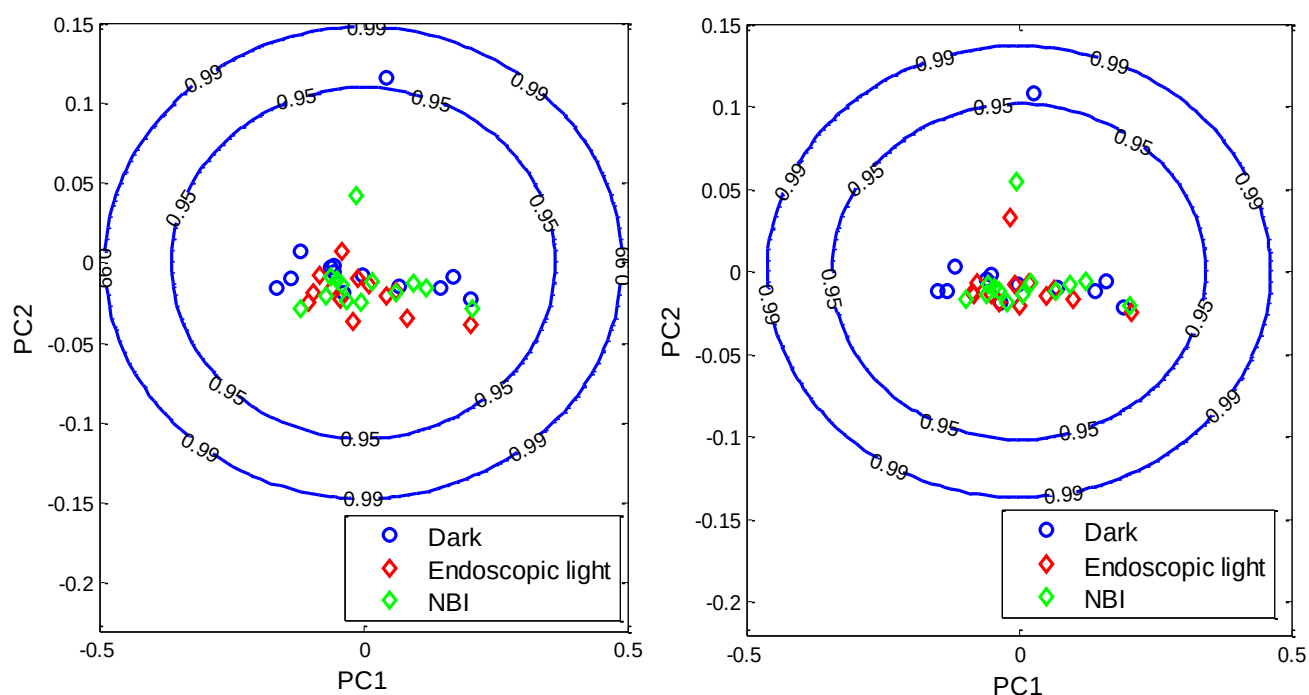


Figure 7-17. Variation in tissue spectra acquired in the presence of endoscopic light and NBI compared to those acquired after exclusion of all ambient light. Confidence intervals for the ‘dark’ control are illustrated. The figure on the right represents a truncated dataset (400:1465); the figure on the left includes the whole spectral range.

7.6 Prediction of Ability to Classify Pathology In Conjunction with NBI Based on Truncated Dataset Analysis

Data presented in Chapter 5 has demonstrated the ability of the Raman probe system to accurately classify normal squamous epithelium, Barrett’s oesophagus, dysplasia and cancer following analysis of 798 ex vivo oesophageal samples from 62 patients. As described previously spectra were acquired in 1 second across the wavenumber range 400-1850 cm^{-1} , and PC-fed LDA algorithms tested using leave one spectrum out cross-validation (LOSOVCV) yielded a sensitivity and specificity of 86% and 88% respectively for the diagnosis of HGD/cancer.

Spectral measurement in conjunction with NBI and WLE has demonstrated spectral disparity in the upper wavenumber range (Figure 7-15 and 7-16). Wavenumbers above 1465 cm^{-1} were

therefore excluded from the 1 s dataset presented in Chapter 5, and multivariate analysis (including LOSOCV) was repeated. Identical classification performance was demonstrated pre and post data truncation (Table 7-3) illustrating the ability of the model to correctly classify spectra across a narrowed spectral range.

		Sensitivity	Specificity
Whole spectral range (400-1850)	NSq	87%	96%
	LGD / Barrett's	72%	91%
	HGD / Adenocarcinoma	86%	88%
Truncated spectral range (400-1465)	NSq	86%	95%
	LGD / Barrett's	73%	91%
	HGD / Adenocarcinoma	86%	86%

Table 7-3. Comparison of diagnostic classification performance when including the full spectral range (400-1850 cm⁻¹); and truncated spectra (400-1465cm⁻¹).

These findings demonstrate that spectra acquired in the presence of both WLE and NBI are free from significant visual interference across the spectral range 400-1465 cm⁻¹. In addition, classification performance across this truncated spectral range has been shown to be feasible and accurate. These data clearly prompt the need for further investigation but raise an exciting possibility for the potential for multimodality imaging using WLE, NBI and RS.

7.7 Is Classification Possible Based on Spectral interference from white light endoscopy or NBI?

PC-fed LDA with LOSOCV was performed to further explore the influence of white light, and narrow band imaging on spectral acquisition. A three group analysis was performed (dark; WLE; NBI) which demonstrated poor classification performance (Table 7-4). The PC loadings are included in Appendix XIV. The obvious visual difference between the three groups of spectra is identified by PC2. Later spectra appear to represent noise, and encouragingly, none display convincing Raman peaks suggesting that both white light and NBI have little impact on the Raman signal.

The classification performance was shown to decrease further, to a point at which it could be stated that no classification was possible, following truncation of the dataset (400:1465) (Table 7-4).

		Sensitivity	Specificity
Whole spectral range (400-1850)	Dark	85%	73%
	Endoscopic light	62%	92%
	NBI	54%	85%
Truncated spectral range (400-1465)	Dark	62%	73%
	Endoscopic light	46%	85%
	NBI	54%	73%

Table 7-4. Classification of spectra acquired in the dark, and in the presence of endoscopic white light and NBI.

7.8 Summary

Prior to a large scale clinical trial of endoscopic Raman spectroscopy convincing data must demonstrate multi-operator, multi-centre and multi-system reliability. The repeatability and transferability of spectral measurements acquired with different Raman systems is essential since clinical application will necessitate the use of pre-defined automated classification algorithms.

Standardisation of spectrometer specifications and probe construction is unlikely to prevent minor spectral variation between different systems. It is therefore necessary to define a clear method for Raman system calibration. This will require careful optimisation of calibration parameters, including clear tolerance levels so that spectral signals measured across all Raman systems can be precisely calibrated.

Although more extensive work is required, the two probe – two operator comparison described here demonstrated encouraging results. In addition, we have demonstrated spectrally consistent data acquired in the presence of NBI and WLE confirming potential for future multi-modality oesophageal imaging.

CHAPTER 8

8 Summative Discussion and Conclusions

The translational research presented in this thesis aims to assess the potential for in vivo endoscopic application of RS in the oesophagus. In light of the data reported in Chapters 5 – 7 it is essential to discuss exactly how oesophageal RS could enhance a clinician's armoury and how this might potentially impact on patient care. The need for further engineering refinements and future clinical testing is also discussed, and scientific and clinical conclusions are drawn.

8.1 *Clinical Perspective*

For years clinicians have relied on the histological appearance of cells in order to confirm the diagnosis of most malignant and inflammatory conditions. Although a trusted technique, histological assessment of tissue is subjective, time consuming and expensive. Furthermore, histological diagnosis necessitates tissue biopsy with subsequent processing and reporting, and therefore does not enable immediate diagnosis and targeted treatment.

In the oesophagus the need for improved diagnosis of early neoplasia is particularly compelling. Oesophageal adenocarcinoma has increased by 500% over the past 40 years while its prognosis has remained relatively constant with current 5 year survival rates of just 8-15%.[28-30]

Early detection of neoplastic disease, ideally at a premalignant stage, is essential to improving cancer outcomes.[28] Successful screening programmes in the colon (adenomatous polyps) and the cervix (cervical intraepithelial neoplasia) have facilitated early intervention and have been shown to prevent malignant progression. However, it is clear that population based, or even targeted endoscopic screening for oesophageal cancer, or its precursor, dysplasia, is inappropriate. Conversely, surveillance of patients with Barrett's

oesophagus is currently recommended due to an increased risk of adenocarcinoma in these patients.[7; 28]

Several recent large studies have demonstrated a lower than previously reported rate of progression from non-dysplastic Barrett's oesophagus to adenocarcinoma.[100; 101; 103; 112] In addition, they have confirmed that only a small percentage (7.3%) of patients diagnosed with adenocarcinoma have a prior diagnosis of Barrett's oesophagus.[101] These important studies have demonstrated that identifying and surveying patients with Barrett's oesophagus is alone insufficient as a strategy to detect and prevent oesophageal adenocarcinoma. In addition, they have fuelled the need for other non-endoscopic screening techniques to identify high risk groups, such as assessment of genome wide association data following blood sampling, or alternatively, molecular or spectral cytology following non-endoscopic oesophageal cell sampling.[145; 146; 252-254]

The true value of surveying all patients with Barrett's oesophagus is highly debatable given this recent evidence, and the results of the Barrett's oesophagus surveillance study (BOSS) are therefore eagerly anticipated. However, it is clear that a cohort of patients with Barrett's oesophagus do have a significant risk of progression to adenocarcinoma and one of the fundamental goals of translational research in Barrett's oesophagus must be to distinguish the small number of patients who will progress to adenocarcinoma from the majority who will not. Currently, there are no validated markers for prognostic evaluation of Barrett's oesophagus that are ready for widespread clinical use other than the presence or absence of dysplasia, particularly HGD, as determined by histopathological assessment. However, other factors such as the length of the Barrett's segment are known to correlate with an increased risk of progression. Until more sophisticated techniques are available, it is possible that future surveillance will be recommended only for those patients with a Barrett's segment >3cm, those with a history of dysplasia, or those with a family history of adenocarcinoma.

It is abundantly evident however that there must be accurate means of assessing and treating those patients who are deemed at higher risk of malignant progression. This treatment must be possible at an early, ideally premalignant, stage.

Surgery is one potential treatment option in patients with early disease. However, in-hospital mortality following oesophagectomy remains around 5% and many older patients, or those

with significant co-morbidities, are not surgical candidates. Endoscopic therapy now offers an alternative approach with lower morbidity and mortality.

Whole segment therapy using 360 RFA is an option for higher risk patients, particularly those with confirmed, macroscopically invisible HGD. RFA is an extremely promising treatment modality particularly for patients with flat lesions although it is not indicated for those with early invasive cancers (T1a or b). In addition, large-scale long-term data (longer than three years) are not yet available.

Where focal HGD and / or T1a lesions are visible endoscopically clinicians can perform single or multiple ER to achieve complete excision followed by whole segment ablation using RFA, to destroy any malignant field change. Although this combined ER and ablation strategy has not been tested in a RCT, it is the policy adopted by the significant majority of experts in the field.[243]

For this treatment strategy to be effective it is crucial that endoscopists are able to target areas of neoplasia for endoscopic resection and that they have a sensitive, and ideally objective, mechanism of defining the margin of the lesion. In addition, to prevent over-treatment, clinicians must be able to confirm a neoplastic lesion with a high degree of specificity. Despite a variety of endoscopic adjuncts, many of which are widely available including NBI and chromoendoscopy, the detection of dysplastic lesions in the oesophagus is often difficult and unreliable precluding successful targeted ER. In a study of 742 ERs from 326 patients with Barrett's neoplasia, an R0 resection was attained in only 26.8% of patients following initial therapy.[8] Subsequent repeated ER achieved R0 resections in 74.5%, but only after several months delay between treatments. The study concluded that if ER is to be used as a viable therapeutic strategy in T1a lesions improved mechanisms of lesion detection and margin definition must be developed.

The difficulty with identifying areas of dysplasia is seen readily in our own practice at Gloucestershire Royal Hospital (GRH). Patients undergoing biennial surveillance endoscopy at District General Hospitals are often referred to GRH for specialist assessment and intervention. These patients typically have had dysplasia or malignancy confirmed following a series of random biopsies. However, on re-endoscopy at GRH it is often difficult to reliably identify and remove all areas of neoplasia.

In this thesis a custom-built Raman probe has been trialed for use as a potential endoscopic tool to improve identification of neoplastic lesions. The Raman probe has demonstrated the ability to discriminate between advanced neoplasia (HGD / adenocarcinoma) and LGD / non-dysplastic Barrett's oesophagus with a sensitivity of 88% and specificity of 82% following ex vivo tissue measurements (Table 8-1). Adenocarcinoma could also be discriminated from normal squamous oesophagus with a sensitivity of 94% and a specificity of 91% demonstrating potential for use in discriminating benign and malignant oesophageal strictures.

These levels of diagnostic accuracy were achieved using just 1 s Raman measurements and performance was increased further still using 5 s measurements. Reduction to 0.1 s acquisition times impaired probe performance significantly, however a degree of classification was still possible prompting optimism that further improvements in probe design and CCD quality could enable near 'real-time' imaging in the future.

		Sensitivity	Specificity
Three group model (Spectra acquired in 1 s)	NSq	87%	96%
	LGD / Barrett's	72%	91%
	HGD / Adenocarcinoma	86%	88%
Two group models (Spectra acquired in 1 s)	NDB / LGD	82%	88%
	HGD / Adenocarcinoma	88%	82%
	IM	91%	80%
	Non-IM CLO (CB/FB)	80%	91%
	NSq	90%	78%
	Buried cancer	78%	90%
	LGD Vs.	100%	100%
	HGD/IMC	100%	100%
	HGD / Adenocarcinoma	82%	80%
	All benign pathologies*	80%	82%
Two group models (Spectra acquired in 5 s)	Adenocarcinoma	94%	91%
	NSq	91%	94%
	NDB / LGD	98%	86%
	HGD / Adenocarcinoma	86%	98%

Table 8-1. The diagnostic performance of the Raman probe is indicated. *Includes all benign pathologies including gastric tissue, fatty or muscular connective tissue, Barrett's oesophagus, and normal squamous tissue.

The probe was shown to be capable of correctly classifying superficial pre-malignant disease in addition to demonstrating the ability to detect invasive cancer beneath a histologically benign squamous or glandular epithelium. Similarly, the potential to distinguish IM, from non-IM CLO was demonstrated (although the numbers of spectra included in this model were small).

These data have confirmed that fibre-optic RS can accurately and objectively classify mucosal oesophageal pathology ex vivo and have clearly demonstrated potential for in vivo diagnosis. Moreover, this ability to classify between pathologies is based on detection of identifiable biochemical differences between normal and benign oesophageal tissue which are supported by the published literature.

The data presented have been rigorously analysed in order to try to derive a true reflection of diagnostic performance and avoid reporting over-optimistic classification. Leave one spectrum out cross validation (LOSOCV) has been employed to ensure that each spectrum in turn is left out from training the model before being projected onto the model in order to validate diagnostic accuracy.

Background subtraction and correction algorithms were not shown to enhance diagnostic classification. This agrees with the conclusions of other published reports and may be a consequence of the introduction of small systematic errors acquired during measurement of the tissue standards.[197; 245]

The ability to record consistent reproducible spectra over 18 months has been demonstrated. In addition, two probes, operated by two independent users, have demonstrated spectral consistency, and mechanisms for calibration of small spectral differences have been explored. Prior to clinical implementation there will also be a need to clearly define levels of engineering tolerance. Engineering tolerance is the permissible limit of variation in a manufactured object that does not compromise its performance. Production of multiple Raman probes and customised spectrometers will inevitably introduce some inherent variation in spectral quality. Tolerance limits must be carefully defined so that measurements falling outside of these limits are rejected as unreliable. The levels of tolerance defined will depend on the levels of performance required.

A clinical trial including large numbers of probes and large numbers of patients, with a full range of pathologies, will enable tolerance levels to be clearly defined. Alternatively, it may be possible to pre-define tolerance levels following mathematical modelling of the existing dataset in combination with additional probe testing once further probes have been constructed.

In this Doctorate, multi-probe analysis of ex vivo tissue was not possible due to a lack of identically built functioning probes. During the course of the work several problems arose with reproducibility of probe construction meaning that probe replicas were unable to measure reliable signal. This was thought to be due to movement of optical components within the probe sleeve and therefore rigid metallic sleeves have been carefully developed in the hope of improving manufacturing reproducibility. Further probe calibration and reproducibility studies will be needed once multiple probes are available to ensure acceptable probe accuracy and to define engineering tolerances.

The ability of RS to establish an objective diagnosis based on precisely measurable biochemical differences between tissue types is a significant advantage of this technology over other competing modalities. Current advanced imaging techniques such as confocal microscopy and narrow band imaging lack this objectivity. Moreover as previously discussed, even histological evaluation may be prone to diagnostic uncertainty - interobserver agreement between pathologists is well recognised to vary widely, particularly for distinguishing HGD from LGD ($k=0.2-0.6$).[85; 188] This is a particularly important clinical distinction as currently patients with HGD are offered therapeutic intervention, whereas those with LGD are managed by 6-monthly endoscopic surveillance.

It is important to note that when only tissue with a consensus histological diagnosis was included as the 'gold standard' the performance of the classification models increased. It must also be noted, that even the consensus of two expert pathologists may not represent a perfect 'gold standard' and therefore the results presented in this thesis must be interpreted in light of this.

Bergman et. al. recently demonstrated that LGD is over-diagnosed by non-specialist pathologists and argued that its true significance might be underestimated by many reported series.[118] In their study, 1198 patients underwent Barrett's surveillance at six non-

specialist hospitals identifying 147 (12.5%) patients with LGD. However, only 8 (0.7%) patients were deemed to have LGD following histological review by two external expert gastrointestinal pathologists. The majority of diagnoses were reclassified as non-dysplastic Barrett's oesophagus. During a mean follow-up period of 51 months, 42% of patients with LGD diagnosed by consensus expert pathologists demonstrated progression to either carcinoma or HGD, and 2.2% regressed to non-dysplastic Barrett's oesophagus.[118] This report demonstrates the importance of accurate classification of dysplasia and supports the need for novel objective diagnostic technology such as Raman spectroscopy. The data presented in this thesis has demonstrated that the custom-built Raman probe could be capable of distinguishing between non-dysplastic disease and LGD; and between LGD and HGD/IMC. This clinically relevant information could prevent inappropriate over- or under-treatment of patients with dysplastic disease. If sufficient diagnostic accuracy can be demonstrated there could also be a case for instigating treatment of LGD, provided the diagnosis was confirmed by a Raman classification model which had been trained by consensus expert GI pathologists.

Similarly, future work could also aim to assess whether susceptible Barrett's phenotypes could be identified using RS. Further advances in diagnostic accuracy may raise the intriguing possibility of using RS to predict disease progression in non-dysplastic Barrett's or LGD since it is possible that biochemical predictors of progression may be detectable before morphological changes manifest.[31]

8.1.1 Potential for Multimodality Imaging

One of the main limitations of fibre-optic RS in its current form reflects the fact that it is a point measurement technique. In order to improve diagnostic yield of early neoplastic lesions within Barrett's segments wide-field imaging techniques are required which enable clinicians to view large areas and detect subtle neoplastic changes. Several large field techniques have been described, some of which are licensed for clinical use, however most are hindered by low levels of sensitivity and specificity.

Recently a promising wide-field technique has been described using visually tagged probe molecules which bind selectively to neoplastic cells.[230; 231][232][2] The report by Bird-

Lieberman from the Fitzgerald group demonstrated that alterations in cell-surface glycans during progression to adenocarcinoma could be identified through changes in their lectin binding properties. Selective binding of a candidate lectin (wheat germ agglutinin) sprayed into a resected oesophagus enabled ex vivo visualization of high-grade dysplastic lesions, which were not detectable by conventional endoscopy.

This technique has considerable potential but is still at the very early stages of clinical development and its true diagnostic accuracy remains to be evaluated. However, wide-field imaging techniques like this one, or others already in clinical use such as NBI, AFI or OCT, could potentially be used in conjunction with RS in the future.[209; 210; 249; 255] [256] Interpretation of these wide-field techniques tends to be subjective and experience governed. However, multimodality tools could enable identification of suspicious areas using the wide-field modality with further detailed and objective assessment of these regions using RS.

The data presented in Chapter 7 demonstrated potential for combining WLE, NBI and RS. NBI filters white light into blue and green wavelengths allowing visualisation of subtle mucosal irregularities and alterations in vascular patterns consistent with dysplasia and intramucosal cancer.[219] NBI can be used by endoscopists at the push of a button and does not require the use of staining or intravenous contrast agents. However, reported rates of diagnostic accuracy vary considerably due to high levels of inter-observer inconsistency.[133; 223]

The data presented here have shown that spectra could be accurately and reproducibly acquired in the presence of NBI. In addition, spectral diagnostic classification was shown to be unchanged when compared to the dark control. Huang et. al. recently described a multimodal imaging system combining endoscopic Raman spectroscopy with AFI, NBI and standard white light endoscopy for use in the oesophagus and stomach.[210] This trimodal wide-field imaging system was used to guide point measurements from suspicious areas which were taken using the Raman system. However, Huang et. al. did not comment as to the added benefit of this *trimodal* imaging system on diagnostic yield.

The concept of combining highly accurate objective diagnostic tools with wide-field imaging techniques is logical and certainly requires further evaluation in vivo. However, when conceiving these multi-modality systems we must remember that they are designed for use as

clinical tools in conscious patients. They must therefore be affordable, user-friendly, and feasible for use during a standard endoscopy session.

8.1.2 Optimising the Classification Model for Purpose

Receiver Operating Characteristic (ROC) curve analysis can allow for alterations in the classification of groups within a model so that sensitivity for detection of neoplasia can be maximised at the expense of specificity or vice versa.

ROC curves plot the true positive rate (sensitivity) against the false positive rate (1-specificity) and therefore each point on an ROC curve represents a sensitivity / specificity pairing at a particular decision threshold. The area under an ROC curve (AUC) is a measure of how well the test can distinguish between the two diagnostic groups (e.g. benign versus neoplastic). AUC values of 0.5 are analogous to a toss of a coin, whereas those of 0.8 are generally considered to be 'acceptable' and those of 0.9 to be 'excellent'. [257]

A test which allows perfect discrimination between two groups has a ROC curve that passes through the upper left corner of the plot (100% sensitivity and 100% specificity). Therefore, the closer the curve is to the upper left corner the greater AUC and the higher the overall accuracy of the test.

ROC curves for a 2 group (NDB/LGD versus HGD/adenocarcinoma) PC-fed LDA classification model validated using LOSOCV are illustrated in Figure **8-1A**. The calculated sensitivity and specificity for this model (as presented in Chapter 5) are 88% and 82% respectively for the detection of advanced neoplasia. The ROC curve for a second classification model (HGD / adenocarcinoma versus all other pathologies) is illustrated in Figure **8-1B**. A sensitivity of 82% and specificity of 80% have previously been reported for discrimination of advanced neoplasia in this model.

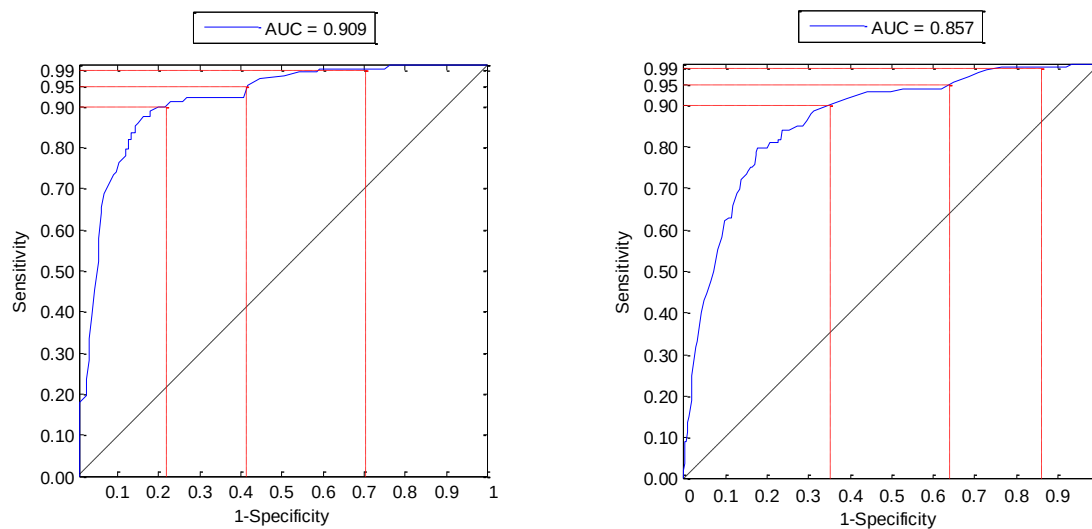


Figure 8-1. ROC curves showing sensitivity / specificity pairings for the discrimination of HGD and adenocarcinoma from (A) LGD and non-dysplastic Barrett's; and from (B) all other pathologies. Note that buried neoplasia has been excluded from these models.

A two group model (all benign pathologies versus LGD/HGD/adenocarcinoma) demonstrates a sensitivity of 84% and specificity of 77% for detecting glandular neoplasia. The specificity has fallen slightly in this model due to difficulties discriminating LGD from non-dysplastic Barrett's. Possible explanations for this have been discussed in Chapter 5. An ROC curve for this model is displayed below.

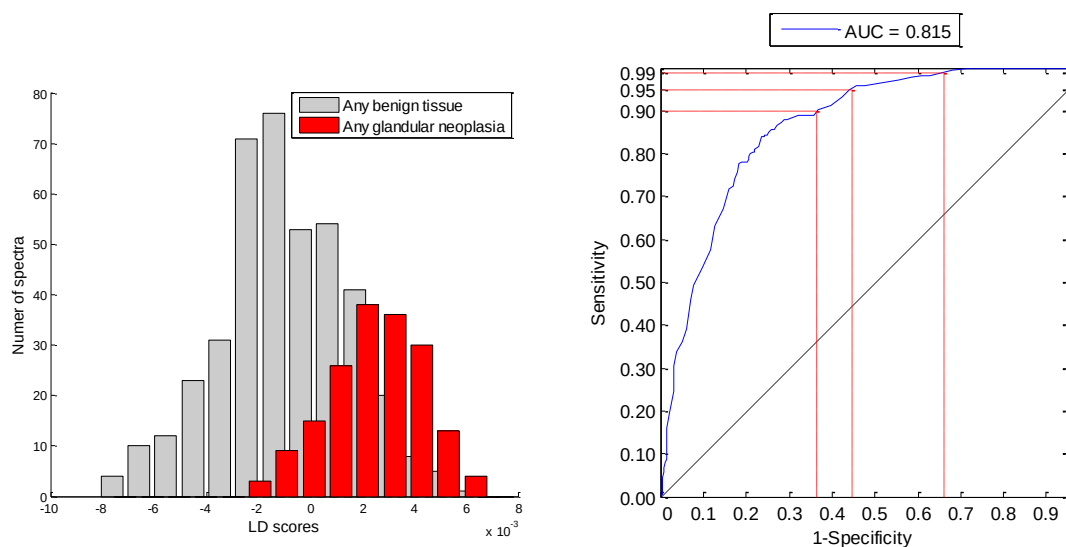


Figure 8-2. (A) An LD scores histogram illustrating probe classification of glandular neoplasia from all other benign tissue. (B) An ROC curve for the same cross validated classification model is also illustrated. Note that glandular neoplasia includes LGD, HGD and adenocarcinoma but excludes disease that was buried beneath a normal epithelium.

If endoscopic RS is intended for use as a surveillance tool ROC curve analysis would need to optimise sensitivity (decreasing false negative results) at the expense of a higher false positive rate. Although prompting increased numbers of tissue biopsies, this modification would serve to reduce the incidence of missed neoplasia.

During endoscopic surveillance there is a clinical need to detect LGD as well as more advanced disease. In this instance, a sensitivity of 95% may be considered acceptable but this would shift the specificity of the instrument to just 56% (Figure 8-2). However, this could potentially enable significantly less biopsies to be taken whilst still ensuring that neoplastic areas are identified. This ability for targeted biopsying could reduce histology processing and reporting costs significantly.

If a Raman probe system were intended for use during endoscopic therapy in patients with known HGD or early cancer the decision line could be shifted to enable a programmed high level of specificity in order to minimise false positive results and therefore reduce the risk of complications caused by resection of non-dysplastic areas. In this setting the probe would need to distinguish HGD and early cancerous lesions (which can be treated endoscopically) from LGD and non-dysplastic Barrett's (which are currently kept under surveillance) (Figure 8-1).

An alternative measure of agreement besides sensitivity / specificity and AUC is the kappa statistic. The kappa statistic takes into account the fact that observers will sometimes agree or disagree by chance. In the case of Barrett's oesophagus, the kappa statistic is often used to report the level of subjectivity between histopathological diagnoses (see Chapters 2 and 3).

The level of agreement between Raman predictions and gold standard (consensus) histopathological diagnosis for the two group model (NDB/LGD and HGD/adenocarcinoma) was $k=0.70$. A kappa score of >0.60 is regarded as a 'substantial agreement', and a score of 0.8 is considered 'almost perfect' agreement.[258] Further reporting of the tissue biopsies by a third independent pathologist could enable comparison of their performance against that of the Raman probe, by comparison to the current gold standard.

8.1.3 Potential Role During Oesophagectomy or Oesophageal Cancer Staging

A Raman probe system could potentially have a role as a surgical adjunct during oesophagectomy. It could enable surgeons to assess resection margins intra-operatively to ensure they are clear of microscopic tumour deposits in order to minimise the risk of locally recurrent disease. Similarly the probe could even act as an adjunct to oesohagogastric cancer staging, through laparoscopic assessment of suspicious peritoneal or lymphatic deposits, or potentially endoscopic assessment of the depth of tumour invasion through the oesophagus. Previously, the ability of RS to stage transitional cell carcinoma of the bladder based on urothelial measurements has been demonstrated[45], and similar potential for tumour depth ‘T-staging’ may be possible in the oesophagus. However modification to the confocal, short focal length design of the probe described in this thesis would be necessary if it were intended for use in T-staging.

8.2 Limitations

This thesis has several methodological limitations which must be highlighted. Several of these have been discussed at relevant stages throughout this thesis but are presented again here for completeness. The limitations relate to the technique of spectral acquisition, the nature and design of the probe, the data pre-processing and analysis, and to assumptions made between the *ex vivo* environment and the real clinical environment at endoscopy.

- Fluorescence – The endogenous fluorophores in blood are well known to induce fluorescence which may mask weak Raman signals. Although water lavage could be performed during endoscopy to remove excess blood, the fluorescence effects may interfere with a clinicians’ ability to take multiple targeted biopsies. This will need further evaluation in vivo. As fluorescence was very rare *ex vivo* (following mucosal lavage) and these spectra were easily identifiable as visual outliers prior to data analysis, the problems caused by fluorescence in this thesis were minimal.
- Probe background – The custom-built Raman probe has a significant background signal due to Raman, fluorescence, and elastic scattering signals being generated within the fibre-optic cables. However, the probe has been shown to be sufficiently sensitive to detect the important spectral signals which enable tissue classification.

Although not all visible above the probe background, PC-fed LDA could extract this data for tissue classification. Background subtraction / correction algorithms were not shown to improve classification performance.

- Exclusion of ambient light – All measurements presented and discussed in Chapters 5 and 6 were acquired following exclusion of all ambient light as visible wavelengths can cause spectral disruption. However, both endoscopic white light and NBI have been shown to be compatible with Raman measurements (see Chapter 7).
- Point measurements – This is a recognised limitation of in vivo Raman spectroscopy which has been discussed previously. It is hoped that further developments may enable real-time imaging which could facilitate assessment of whole Barrett's segments in clinically applicable timescales.
- Pressure effects – Inadequate contact of the probe with the tissue may have affected the spectral signal. However, as explained previously, this was a translational project using a clinically applicable tool which was pressed against the tissue by hand, as would be the case in vivo. No attempt was taken to experimentally regulate probe pressure as this would be impractical in vivo. Initial probe evaluation data demonstrated that adequate probe contact was more significant than minor variation in probe pressure or angulation. This finding is supported by previous studies.[209; 247]
- Small numbers of dysplastic spectra despite spectral analysis of over 700 tissue samples – Many biopsies containing possible dysplastic elements were non-homogenous, or were classified differently by each expert pathologist. These biopsies were therefore excluded from most analyses. Despite relatively small numbers of homogenous dysplastic samples with a consensus diagnosis, diagnostic classification was still possible.
- Neoadjuvant chemotherapy – The majority of patients were treated with chemotherapy prior to surgical resection. This often led to shrinkage and fibrosis of the tumour, which may have affected its spectral signal.
- Previous ablation therapy – Some point biopsies / endoscopic resections were taken from patients who had undergone previous ablation therapy. It is unclear what effect, if any, this may have had on the Raman signal obtained from this tissue. However, in a clinical setting it will be essential that the probe can detect recurrent Barrett's / dysplasia in patients who have undergone previous endoscopic treatment. The ability

to detect buried cancer has been demonstrated. However, the ability of the probe to detect metaplasia beneath neosquamous epithelium was not possible due to lack of tissue samples.

- Ischaemia – The effects of tissue ischemia have not been fully established. This may mean that the results of this thesis cannot be generalised to *in vivo* results with complete accuracy. A large spectral dataset will need to be established following an *in vivo* trial and at that point comparisons with *ex vivo* data can be made.
- Assessment has not specifically evaluated the ability of the probe to identify *macroscopically invisible* areas of HGD / cancer. In order for RS to be a clinically applicable tool, it will need to be shown to improve recognition of the lesions which are undetectable using white light endoscopy and therefore demonstrate the ability to change patient management. This should be considered when designing future *in vivo* clinical trials in order to demonstrate diagnostic benefits.
- All spectra were acquired from a biased population as all patients had a history of Barrett's oesophagus and / or adenocarcinoma. However, these patients represent the target population for this clinical tool.
- It is possible that errors in PCA may have occurred due to systematic error within the dataset, for example systematic drift in the intensity or wavenumber axes. However, careful calibration prior to all sets of measurements was used to obviate these effects. In addition the data presented showed no evidence of systematic drift and green glass correction failed to improve the classification performance suggesting that major systemic error was not a problem.

8.3 Future Work

This research identified several possible avenues for further work. Further optimisation of signal to noise ratio (SNR) could potentially enable diagnostic accuracy to approach 100% and permit real-time imaging. However, the principal aim for the future, which is heavily supported by the data presented here, is translation of the probe into a clinically tested endoscopic tool.

Ideas for alternative research proposals related to oesophageal RS are briefly highlighted below (section 8.3.2).

8.3.1 Translation to an In Vivo Tool

The work presented here demonstrates that the novel fibre-optic probe can accurately classify oesophageal tissue in clinically applicable timescales. In addition, spectra can feasibly be measured under direct vision using WLE and/or NBI. The data also demonstrates that reliable spectra can be acquired using different identically designed probes used by different operators, and that probe performance is reproducible over time.

Previous work, initially by Shim *et. al.* in 2000, demonstrated that in vivo Raman measurements were feasible and safe in the oesophagus, although for a number of reasons diagnostically useful information was not obtained (see Chapter 2).[197] Further subsequent in vivo work by several groups has supported the conclusions that RS is safe and feasible and has demonstrated more encouraging diagnostic data, although detection of early lesions using other probe systems has proved difficult.[35; 206; 209; 212]

The results presented here, and the experience from previous pilot clinical trials, support the need for an in vivo trial using this custom-built Raman probe system. However, prior to commencing such a trial there are several practical considerations which need to be addressed:

- Probe durability – Probes must be sufficiently durable to tolerate multiple passes down an endoscope without it affecting spectral recordings. During the course of this research project a probe was damaged during passage through a scope prompting a change to the design of the external rigid coating of the distal tip of the probe. It has not been possible to undertake a formal probe durability experiment at this stage due to the need to prevent any risk of damage to the current probe. This experiment, which will be quick and straightforward to conduct, can be undertaken after agreement from the probe manufacturers.
- Single or multi-use – It is unclear at this stage whether the probe is intended for single or multi-use. Data presented here has shown potential for multi-use since reliable spectra were recorded over an 18 month period. However, this will require that the probe is packaged in such a way that it can be reliably sterilised between patients and that the probe constituents are adequately protected from this process. Again this

should be relatively straightforward to achieve as with other multi-use endoscopic instruments. However, the decision will need to reflect a number of factors e.g. sales projections, probe build costs and sale price (which are currently confidential).

- Manufacturing – There needs to be an established mechanism for reproducing identically built probes to high standards. During this Doctorate there have been significant issues reproducing multiple identical probes which can acquire reliable spectral signals. This possibly represents the largest current barrier to in vivo use since probes must be straightforward to manufacture and able to record spectra within carefully defined tolerance limits.
- CE marking – this is the mandatory legal conformity mark necessary for products placed on the European market. It confirms that the manufacturer has ensured that the product is not only safe but also functions in a way so as to achieve its intended purpose.
- Commercial support – There needs to be a mechanism for funding a future clinical trial. This will most likely involve a commercial collaboration.

After addressing these factors a carefully planned clinical trial will need to be undertaken. The design of the trial must address the following:

- The aims of the trial must be clearly defined. A small feasibility study will be necessary to demonstrate probe performance in vivo and to build further foundation to support a larger multi-centre trial. Prior to a large, randomised trial, a significant in vivo spectral dataset will be required to generate a robust classification model. Later trials will need to evaluate the potential of RS to alter clinical management - this could be achieved by recording whether mucosal sites detected as abnormal by RS (and subsequently proven to be abnormal histologically) were detectable using standard endoscopic imaging.
- A careful power calculation based on the data presented here, is required to evaluate the numbers of spectra which must be measured from each pathological tissue type in vivo.
- Which patients should be included? Should it be all-comers with Barrett's or should inclusion be limited to those with a history of dysplastic disease. The latter would

slow the rate of data acquisition and also prevent generation of data from patients with non-dysplastic segments, so is probably impracticable.

- A technique for ensuring that probe spectra can be correlated with the current gold standard (consensus expert histology) must be established. Huang et. al. have described the use of an ER cap to ensure that probe and biopsy sites match. However, this requires that all patients are scoped using an ER cap, despite not necessarily needing therapeutic intervention. Alternatively, a double lumen scope could be used so that the probe and the biopsy forceps are passed simultaneously through the scope. This would enable a Raman measurement to be immediately followed by a biopsy at the corresponding site. However, slight adjustment by the endoscopist to allow for the different positions of the instrument channels would be necessary.
- Assessment of cost-benefit in conjunction with a health economist.

8.3.2 Alternative Potential Future Projects Utilising Oesophageal RS

The ability of RS to provide rapid, reproducible, cheap, highly accurate and objective diagnoses of a range of oesophageal pathologies could be utilised in a variety of ways. In addition to this Doctorate investigating the potential for endoscopic diagnosis, other potential uses of oesophageal RS which require further investigation are as follows.

- Correlation of Raman signal with biomarkers of malignant progression. Laboratory, and potentially probe based systems, may have the power to detect subtle differences in non-dysplastic Barrett's or LGD which correlate with an increased likelihood of disease progression. Whilst completing this Doctorate I supervised an MSc student at Cranfield University who began to correlate Raman spectral signals with early neoplastic molecular changes. A summary of this work is included in Appendix XI.
- Assessment of the ability of RS to detect eosinophilic oesophagitis. In this thesis tissue was collected from patients with Barrett's oesophagus and / or oesophageal adenocarcinoma. Further testing could assess patients presenting with dysphagia to assess the ability of the Raman probe to detect spectral changes consistent with a diagnosis of eosinophilic oesophagitis.

- Oesophageal cancer staging using RS. The ability of RS to probe deeper tissues could be utilised to enable an assessment of a tumour's depth of invasion (T-stage). Similarly, endoscopic ultrasound guided Raman probe assessment of para-oesophageal lymph nodes, which lie within 1 cm of the adventitia is potentially feasible. This may be of particular value in the setting of T1sm1 tumours which may or may not be amenable to endoscopic therapy depending on the presence of absence of lymphatic involvement.
- Surveillance for buried metaplasia. Buried metaplasia cannot be detected by other advanced imaging techniques and may be missed by random biopsy. The potential for diagnosis using RS requires further investigation, although the ability to detect cancerous elements beneath histologically normal epithelium is significantly promising.
- Margin assessment during oesophagectomy – whilst this seems a logical step in view of the data presented in this project, this potential will need formal investigation. Particularly, the effects of neoadjuvant chemotherapy on Raman signal may need to be defined.
- Rapid Raman mapping – promising work has reported techniques to aid histological interpretation of biopsies in short, clinically applicable timescales (see Chapter 2). Further on-going work is required to translate this promising technique into the clinical environment.
- Potential for screening – spectral cytopathology (SCP) can accurately assess large quantities of oesophageal cells in short timescales.[252-254; 259; 260] During a British Society of Gastroenterology Travelling Fellowship to Boston, I initiated a collaboration investigating the potential for oesophageal SCP. Early results of a proof of principal study are included in Appendix XII. Further ongoing work is required to determine whether this technology could be combined with the Cytosponge test in order to detect Barrett's, dysplasia or cancer in asymptomatic patients.

8.4 Conclusions

Raman spectroscopy is a highly sophisticated technique with a well established ability to accurately discriminate oesophageal pathology.[35; 43; 49; 191; 206; 209; 212; 237; 261] The potential for endoscopic application of RS has recently been recognised and several

small scale in vivo studies have been undertaken in the oesophagus.[197; 206; 209; 212] However, these have suffered from significant limitations including: a need for long (clinically unacceptable) acquisition times; measurement of spectra only after extrusion of endoscopic light; an inability to detect early superficial pre-malignant disease; or a lack of sufficient diagnostic accuracy.

This translational research thesis has evaluated a novel confocal Raman probe which was built following a multi-unit collaboration. It has been designed to fit through the instrument channel of a standard medical endoscope and has been shown to measure reliable spectra in conjunction with both white light endoscopy and narrow band imaging.

The confocal, short focal length design has facilitated interrogation of the epithelium enabling accurate discrimination between LGD and HGD / IMC. However, in addition the probe has demonstrated the ability to detect invasive cancer beneath histologically benign epithelium.

Endoscopists can usually distinguish normal squamous mucosa from Barrett's oesophagus using standard white light endoscopy. However, identifying and grading dysplastic lesions and early tumours is more troublesome - it is in this setting that endoscopic RS has a potential clinical role. The current clinical division between endoscopic surveillance or treatment occurs between LGD and HGD. That is to say, LGD and non-dysplastic Barrett's is surveyed, whereas HGD typically necessitates early treatment. In this thesis the Raman probe has been shown to distinguish HGD/adenocarcinoma from NDB/LGD and normal squamous oesophagus with a sensitivity of 86% and a specificity of 88%. These diagnostic accuracies were obtained using clinically applicable (1 s) spectral acquisition times. It must also be considered that these figures rely on the assumption that the 'gold standard' - the consensus histological diagnosis of two expert pathologists - is entirely accurate.

There are two main clinical settings in which oesophageal RS could potentially be utilised during endoscopy. These will be addressed in turn:

1. To maximise the detection of dysplasia during endoscopic surveillance of patients with Barrett's oesophagus and minimise the number of biopsies required. The premise for this is that random quadrantic biopsies every 2 cm can miss up to 57% of

dysplasia.[22] Use of endoscopic RS could replace the need for multiple random biopsies by enabling targeted biopsies to be taken from sites with neoplastic spectral signatures.

Using RS to guide biopsies during surveillance could reduce the overall number of biopsies taken, diminish histological processing and reporting costs, minimise the risk of bleeding from biopsy sites, and potentially decrease the incidence of missed neoplasia.

However, the diagnostic accuracies achieved may not be sufficient to support the use of endoscopic RS in Barrett's surveillance at present. Histology is able to identify advanced neoplastic tissue with high levels of accuracy - although histological disagreement between HGD and LGD, and between NDB and LGD is common, it is very rare for histologists to misclassify HGD or adenocarcinoma as non-dysplastic Barrett's. In addition, pathologists often tend to over-call diagnoses to minimise missed pathology, meaning that LGD and 'indefinite for dysplasia' samples are over-diagnosed, but rarely missed. In order for endoscopic RS to be of value to clinicians during Barrett's surveillance it must demonstrate a high sensitivity for detecting all neoplastic disease (including LGD, HGD and cancer). If a sensitivity value of 95% is deemed mandatory ROC curves show that the specificity of the model falls to around 50% (Figure 8-2). This may however, allow for a reduction in the number of biopsies required, so called 'targeted biopsying'.

Perhaps a greater obstacle to the use of endoscopic RS in Barrett's surveillance reflects the fact that it remains a point measurement technique. This will probably limit its use in this setting (as has so far been the case with confocal microscopy). Although probe based RS has the potential to provide rapid, objective diagnosis, its sampling area per measurement (approximately 1 mm²) is less than that of a point biopsy and therefore, as with standard biopsy regimes, 'optical biopsy' could potentially miss neoplastic disease through sampling error. In the future this limitation may be overcome by combination imaging with a wide-field modality such as molecular fluorescence imaging. Alternatively, further technological advances may enable real-time imaging so that future probes can scan across large areas of tissue, although at present this is some way off.

2. In patients with confirmed HGD/IMC following prior endoscopic biopsy who are referred for endoscopic therapy. Targeted endoscopic resection of HGD/IMC enables thorough staging and can completely remove the disease. Where possible, clinicians advise a policy of focal lesion resection followed by whole segment ablation using RFA.[243] However, it is often difficult to identify HGD/IMC lesions prior to treatment. In addition, even where there is a visible mucosal abnormality it may not be possible to truly define the full extent of the lesion.

The advent of ER has enabled complete excision of neoplastic lesions endoscopically. It is essential however, that a surgical philosophy is employed to these therapies. That is to say, clinicians must strive for R0 (complete microscopic) resections. A Raman probe could be used to confirm dysplastic areas prior to targeted ER and enable careful assessment of the margins of the lesion to ensure that no residual disease is left behind following endoscopic therapy. In this setting ROC curve analysis could be employed to maximise sensitivity.

Similarly, during endoscopic therapy a Raman probe could be used to decrease the likelihood of incorrectly excising benign disease by minimising false positive results. In doing so potential complications occurring during unnecessary ER could be avoided.

Currently, endoscopic RS probably has a greater role in facilitating clinicians during endoscopic resection of advanced neoplasia, rather than in surveillance for all-comers with Barrett's oesophagus, due to the limitations discussed above. Patients with known disease who are referred for therapy could be given extra endoscopy time to allow for a thorough Raman probe assessment, aiming to increase the accuracy and oncological quality of the intervention and thus potentially increasing cancer specific survival.

This thesis has demonstrated the exciting potential for objective endoscopic diagnosis of early pre-malignant lesions in the oesophagus using a novel custom built Raman probe. Shorter acquisition times and further improvements in diagnostic performance are expected as the technology continues to move forward. However, even at current levels of diagnostic accuracy, the ability to provide an immediate and objective diagnosis during endoscopy (or

surgery) could have a significant impact on patient care. Further multi-probe testing is required to ensure consistency, optimise system calibration, and define engineering tolerances. However, an in vivo clinical trial is feasible in the near future.

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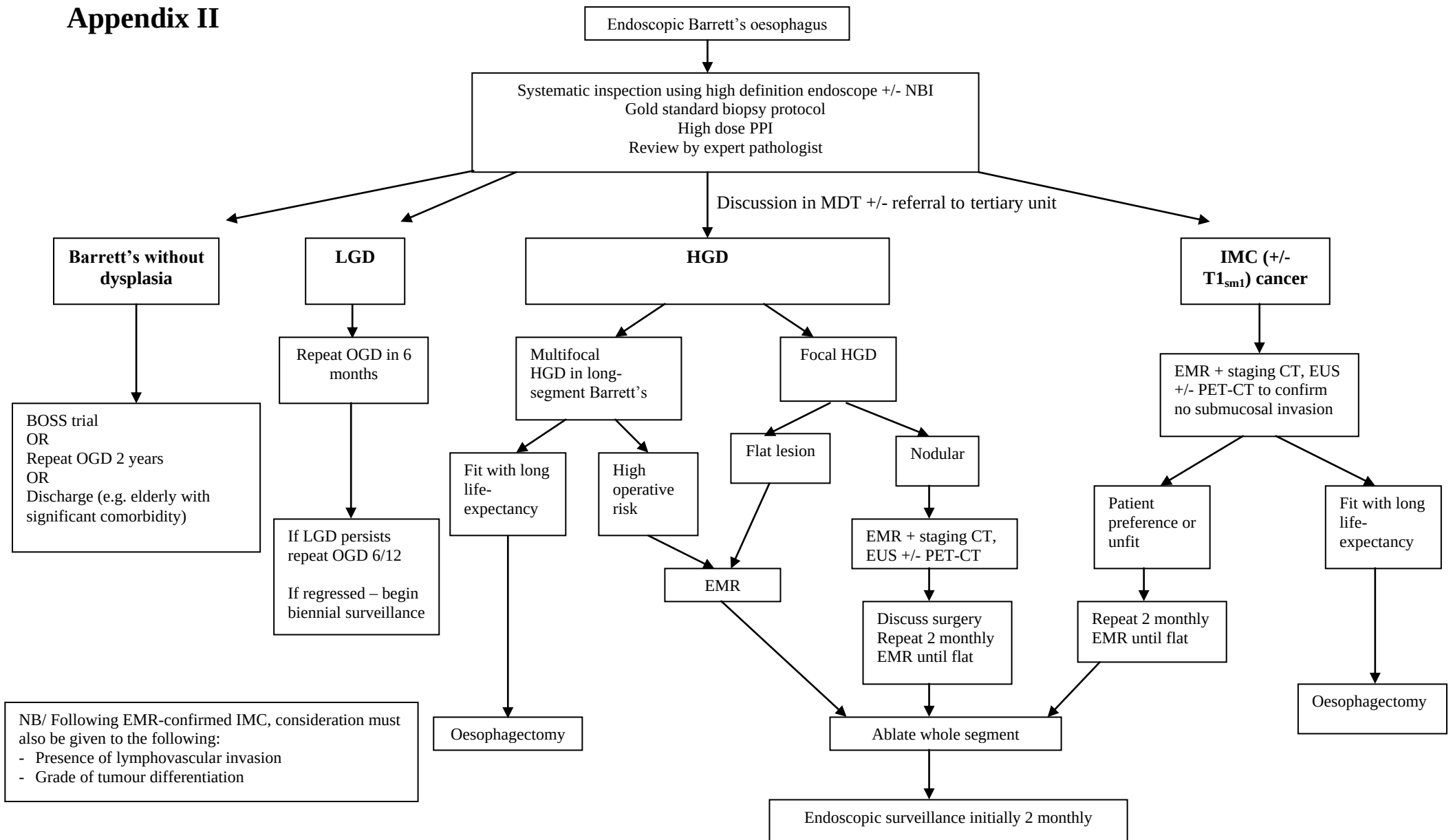
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Appendix I

7th Edition of the AJCC Cancer Staging: Oesophagus and Oesophagogastric Junction[264]

T0	No evidence of primary tumour
Tis	High-grade dysplasia
T1	Tumour invades lamina propria or submucosa
T2	Tumour invades muscularis propria
T3	Tumour invades adventitia
T4a	Resectable cancer invades adjacent structures such as pleura, pericardium, diaphragm
T4b	Unresectable cancer invades adjacent structures such as aorta, vertebral body, trachea
N0	No regional lymph node metastases
N1	1 to 2 positive regional lymph nodes
N2	3 to 6 positive regional lymph nodes
N3	7 positive regional lymph nodes
M0	No distant spread
M1	Distant spread

Appendix II



Appendix III

Raman Peak ID Table

Key:- δ - deformational vibrations ; ν - stretching vibrations; ν_s - symmetric stretching vibrations; ν_{as} - asymmetric stretching vibrations.

	Assignment	Author	Tissue/Substance
207	Calcium carbonate	Wentrup-Byrne 1995	Cholesterol
275	τ CCC	Cassanas 1991	Lactic acid
350	Ribose, cytosine	Hartman 1973	
370	δ CCC	Cassanas 1991	Lactic acid
385	Cytosine	Hartman 1973	
400	Silica	Mahadevan-Jansen 1998a	Fibre probe on cervix
424	δ CCC	Barry 1992	Human stratum corneum
425	δ CCO	Cassanas 1991	Lactic acid
429	Calcium hydroxyapatite	Yu 1996	
430	Cholesterol	Yu 1996	
430	δ (OPO)	Wentrup-Byrne 1997a	Human tooth
435	Ribose	Hartman 1973	
451	O-P-O symmetric bend	Nie 1990	Chicken leg bone
481	Glycogen	Kendall C	Sigma Aldrich G0885
492	vs (SiOSi)	Frank 1994	silicone gel
500	Cytosine, guanine & disulphide	Hartman 1973	
500-550	Disulphide (S-S) stretch	Gnaidecka 1998	Protein
505	ν (S-S)	Fredericks 1995	Bovine serum albumin
507	Disulphide	Schrader 1995	Nails
509	Disulphide (S-S)	Ozaki 1988	
510	Disulphide (C-S-S-C gauche-gauche-gauche)	Gnaidecka 1998	Protein
518	Glucose	Goetz 1995	
525	Disulphide (C-S-S-C gauche-gauche-trans)	Gnaidecka 1998	Protein
526	ν (S-S)	Edwards 1995	Stratum corneum
526	ν (S-S)	Barry 1992	Human stratum corneum
528	ν (S-S)	Yu 1996	Protein
529	Desmosine & isodesmosine (amino acids)	Manoharan 1996	Elastin
530-1	ν (S-S)	Edwards 1995	Callus, psoriatic plaque
533	COC deformation (glycosidic ring)	Maquelin 2000	
533	Glucose	Chaiken 2000	Blood
540	Disulphide (C-S-S-C trans-gauche-trans)	Gnaidecka 1998	Protein
540	ν OCO	Cassanas 1991	Lactic acid
547	Cholesterol	Yu 1996	Protein

553	Glucose	Chaiken 2000	Blood
556	v (S-S)	Manfait 2000	
574	Tryptophan	Siebinga 1992	Eye lens
575	Cytosine, guanine & disulphide	Hartman 1973	
576	Glycogen	Kendall C	Sigma Aldrich G0885
587	Calcium hydroxyapatite	Yu 1996	Protein
591	Apatite	Nie 1990	Human tooth enamel
591	δ (OPO)	Wentrup-Byrne 1997a	Human tooth
600	ρ (CH) wagging	Barry 1992	Human stratum corneum
607	Cholesterol	Yu 1996	Protein
618	Phenylalanine(amino acid)	Frank 1994	Collagen
620	Phenylalanine(amino acid)	Frank 1994	Diseased breast tissue
620	Phenylalanine	Yu 1996	
620-2	v (C-S)	Edwards 1995	Callus, psoriatic plaque
622	Phenylalanine	Puppels 1999	
623	v (C-S)	Barry 1992	Human stratum corneum
623	v (C-S)	Edwards 1995	Stratum corneum
624	Adenine	Mahadevan-Jansen 1996	DNA (Z), RNA(Z)
625	Guanine in the C3' endo/syn form	Mahadevan-Jansen 1996	DNA (Z)
626	Phenylalanine	Mahadevan-Jansen 1995	
626		Mahadevan-Jansen 1998a	Cervix tissue
626	Silica	Mahadevan-Jansen 1998a	Fibre probe on cervix
637	Ribose & disulphide	Hartman 1973	Protein
640	Guanine	Mahadevan-Jansen 1996	RNA(Z)
642	Thymine	Mahadevan-Jansen 1996	DNA (A), RNA (A)
643	v (C-S), amide IV	Edwards 1995	Stratum corneum, Callus, psoriatic plaque
643	Tyrosine	Yu 1996	
644	Tyrosine	Puppels 1999	
644	v (C-S), Amide IV	Barry 1992	Human stratum corneum
650	δ C-COH	Cassanas 1991	Lactic acid
665	Thymine	Mahadevan-Jansen 1996	DNA (B)
668	Guanine in the C3' endo/anti form	Mahadevan-Jansen 1996	DNA (A), RNA (A)
669	v(C-S)	Fredericks 1995	Bovine Serum albumin
669	v(C-S)	Maquelin 2000	
669	Thymine	Puppels 1999	
670	Breathing of aromatic ring of purine bases (guanine & adenine)	Mahadevan-Jansen 1996	DNA
670	Guanine	Mahadevan-Jansen 1996	DNA (C)
670	Guanine	Hartman 1973	

670	Thymine & guanine	Puppels 1991	
681	Guanine breathing ring	Puppels 1991	
682	Guanine in the C2' endo/anti form	Mahadevan-Jansen 1996	DNA (B)
692	Creatine (O-C=O) deformation	Dou 1996	
704	Calcium carbonate	Wentrup-Byrne 1995	Cholesterol
709	Glycogen	Kendall C	Sigma Aldrich G0885
700	Cholesterol	Schrader 1995	Brain
701	Cholesterol	Yu 1996	
710	Adenine	Hartman 1973	
710	vs (Si-C)	Frank 1994	silicone gel
720	Nucleotides	Schrader 1995	Breast cancer in mouse
720	C-N vibration	Mahadevan-Jansen 1996	Phospholipid membrane
722	Adenine	Hartman 1973	
724-7	Adenine	Mahadevan-Jansen 1996	DNA (A), DNA(B), RNA(A)
725	C-S	Siebinga 1992	Eye lens
725	CH2 rocking	Maquelin K	
727	=C-H in plane bend	Frank 1994	Breast tissue, lipid
727	C-C stretch, proline	Frank 1995	Normal breast tissue
727	=C-H in plane bend	Frank 1994b	
728	C-C stretch, proline	Frank 1995	Oleic acid methyl ester
729	Adenine breathing ring	Puppels 1991	
729	Adenine	Mahadevan-Jansen 1996	DNA (Z), RNA(Z)
730		Pilotto 2001	DL-Lactic acid (C3H6O3)
742-748	O-P-O	Mahadevan-Jansen 1996	DNA (Z), RNA(Z)
743	in bovine albumin	Kendall C	Sigma Aldrich A2153
746	$\rho(\text{CH}_2)$	Barry 1992	Human stratum corneum
748	Thymine	Mahadevan-Jansen 1996	DNA (B)
749	Thymine breathing ring	Puppels 1991	
750	δOCO	Cassanas 1991	Lactic acid
750	Cholesterol	Feld 1995	Carotid artery
759	Tryptophan (amino acid)	Yu 1996	
759	Tryptophan (amino acid)	Nie 1990	Human eye lens
760	Tryptophan (amino acid)	Erckens 1997	Rabbit & human cornea & Rabbit aqueous humour
760	Tryptophan & tyrosine	Erckens 1997	Rabbit lens
760	Tryptophan (amino acid)	Hartman 1973	
760	Tryptophan (amino acid)	Ozaki 1988	
760	Tryptophan (amino acid)	Miura 1995	
761	Glycogen	Kendall C	Sigma Aldrich G0885
762	Collagen	Kendall C	Sigma Aldrich C7774
770	Breathing of aromatic	Mahadevan-Jansen 1996	DNA

	ring of pyrimidine bases (cytosine & thymine)		
777	Thymine	Mahadevan-Jansen 1996	DNA (A), RNA (A)
780	Cytosine	Mahadevan-Jansen 1996	DNA (A), RNA (A)
780	Nucleotides	Schrader 1995	Breast cancer in mouse
780	Uracil ring	Maquelin 2000	
782	Cytosine ring breathing	Mahadevan-Jansen 1996	DNA (B)
785	O-P-O	Mahadevan-Jansen 1996	DNA (C)
787	Cytosine & uracil	Hartman 1973	
788	Thymine, cytosine, O-P-O	Puppels 1991	DNA
790	vs O-P-O	Puppels 1999	DNA
790	Acetone (C-C-C) stretch	Dou 1996	
800		Wang 1993	pyruvate
806-813 (809)	vs O-P-O	Mahadevan-Jansen 1996	DNA (A)
811	Nucleotides	Schrader 1995	Breast cancer in mouse
814	vs O-P-O	Manfait 2000	DNA
814	vs O-P-O	Mahadevan-Jansen 1996	RNA (A)
814	C-C stretch, backbone	Frank 1995	Type I collagen (human placenta)
814	C-C stretch, backbone	Frank 1994a	Collagen
814	C-C stretch, backbone in collagen	Kendall C	Sigma Aldrich C7774
815	Phosphate	Hartman 1973	
816	C-C Proline ring	Fendel 1988	Collagen type I, normal skin dermis
817	C-C stretch, backbone	Frank 1995	Infiltrating ductal carcinoma
817	C-C stretch, backbone	Frank 1994a	Diseased breast tissue
818	Haemoglobin	Mahadevan-Jansen 1995	
818		Mahadevan-Jansen 1998a	Cervix tissue
818	Silica	Mahadevan-Jansen 1998a	Fibre probe on cervix
819	Tyrosine	Liu CH 1992a	
820	Structural protein	Manoharan 1998	Breast
825-830	Tyrosine (amino acid)	Mahadevan-Jansen 1996	Histones
825-842	O-P-O	Mahadevan-Jansen 1996	DNA (B)
826	in bovine albumin	Kendall C	Sigma Aldrich A2153
827	δ (CCH) aliphatic	Barry 1992	Human stratum corneum
828	Tyrosine (amino acid)	Kendall C	Sigma Aldrich T8909
830	C-COOH stretch	Wang 1993	Lactate
830	C-COOH stretch	Pilotto 2001	DL-Lactic acid (C ₃ H ₆ O ₃) CH ₃ -CHOH-COOH
830	C-COOH stretch	Wicksted 1995	Lactate
830	Tyrosine (amino acid)	Nie 1990	Human eye lens
830	Tyrosine (amino acid)	Mahadevan-Jansen 1996	

830	Tyrosine (amino acid)	Hartman 1973	
830	Tyrosine (amino acid)	Miura 1995	
830	vC-COOH	Cassanas 1991	Lactic acid
832	Tryptophan & tyrosine	Erckens 1997	Rabbit lens
832	Tyrosine	Puppels 1999	
832	Tyrosine	Ozaki 1988	
833	vas O-P-O	Puppels 1999	DNA
833	Ribose phosphate	Greve 1996	
835	vas O-P-O	Mahadevan-Jansen 1996	DNA (B)
840	Tyrosine (amino acid)	Frank 1994a	
840	Glucose	Chaiken 2000	
840-860	Polysaccharides	Shim 1996	
840-860	Polysaccharides	Gnaidecka 1997a	
840-880	Glucose	Chaiken 2000	Blood
846	Tyrosine (amino acid)	Kendall C	Sigma Aldrich T8909
847	Glucose	Mahadevan-Jansen 1998a	Sigma
849		Frank 1994a	Breast
850	Proline	Liu CH 1992a	
850	Tyrosine (amino acid)	Mahadevan-Jansen 1996	
850	Tyrosine (amino acid)	Miura 1995	
850		Kendall C	Bovine albumin Sigma Aldrich A2153
850	δ (CCH) aromatic	Barry 1992	Human stratum corneum
850-3	δ (CCH) aromatic	Edwards 1995	Stratum corneum, Callus, psoriatic plaque
853	Tyrosine (amino acid)	Nie 1990	Human eye lens
853	Tyrosine (amino acid)	Puppels 1999	DNA
853	Glycogen	Kendall C	Sigma Aldrich G0885
854-6	Tyrosine (amino acid)	Mahadevan-Jansen 1996	Histones
855	Tyrosine	Ozaki 1988	
855	C-C stretch Proline ring	Frank 1994a	Collagen
855	C-C stretch, proline	Frank 1995	Type I collagen (human placenta)
856	Proline	Nie 1990	Chicken leg bone
856		Mizuno 1994	Polysaccharides in glioma grade III (human brain tissue)
856	CC stretch, COC stretch, 1,4 glycosidic link	Maquelin 2000	
856	C-C stretch collagen backbone & Proline ring	Frank 1994a	Diseased breast tissue
856	C-C stretch, proline	Frank 1995	Infiltrating ductal carcinoma (breast)
856	v(CC) skeletal vibrations, keratotic	Carter 1998	Healthy human skin dermis
856	C-C stretch collagen	Lucassen 1998	

	backbone & Proline ring		
856	Tryptophan & tyrosine	Erckens 1997	Rabbit lens
856	C-C stretch Proline ring in collagen	Kendall C	Sigma Aldrich C7774
857	Tyrosine	Yu 1996	
858	C-C stretch in elastin	Kendall C	Sigma Aldrich E1625
859	C-C Proline ring	Fendel 1988	Collagen type I, normal skin dermis
860	Collagen	Redd 1993	Fibrocystic human breast tissue
860	Lactate	Erckens 1997	Human aqueous humour
865	Ribose, Tryptophan (amino acid)	Hartman 1973	Protein
866	Lactic acid	Pilotto 2001	DL-Lactic acid (C ₃ H ₆ O ₃) CH ₃ -CHOH-COOH
868	C-C stretch, hydroxyproline	Frank 1995	Oleic acid methyl ester
870	C-C stretch, hydroxyproline	Frank 1995	Normal breast tissue
870	Proline (amino acid)	Feld 1995	Malignant breast tissue
870		Frank 1994b	Breast
872	C-C stretch of 4-hydroxyproline in collagen	Kendall C	Sigma Aldrich C7774
873	hydroxyproline	Nie 1990	Chicken leg bone
874	C-C stretch, hydroxyproline	Frank 1995	Type I collagen (human placenta)
874	C-C stretch of 4-hydroxyproline	Frank 1994a	Collagen
876	C-C stretch, hydroxyproline	Frank 1995	Infiltrating ductal carcinoma (breast)
876	C-C stretch of 4-hydroxyproline	Frank 1994a	Diseased breast tissue
879	Tryptophan (amino acid)	Nie 1990	Human eye lens
880	Tryptophan (amino acid)	Ozaki 1988	
880	Tryptophan (amino acid)	Miura 1995	
880	Tryptophan (amino acid)	Frank 1994a	
880	Tryptophan (amino acid)	Erckens 1997	Rabbit & human cornea
880	Tryptophan & tyrosine	Erckens 1997	Rabbit lens
880	Tryptophan (amino acid)	Erckens 1997	Rabbit aqueous humour
883	CH ₂ rocking,	Edwards 1995	Stratum corneum,
883	ρ (CH ₂)	Barry 1992	Human stratum corneum
885	Ribose, Tryptophan (amino acid)	Hartman 1973	Protein
890	Structural protein	Manoharan 1998	Breast
890		Frank 1994b	Breast

890-1	CH ₂ rocking,	Edwards 1995	Callus, psoriatic plaque
892	CH ₂ rocking, collagen hydroxyproline	Lucassen 1998	Skin
893		Kendall C	Bovine albumin Sigma Aldrich A2153
895	DNA backbone	Puppels 1999	DNA
896	Proline	Kendall C	Sigma Aldrich T8449
915	Ribose	Hartman 1973	Protein
917	Deoxyribose (CH ₂ deformation)	Mahadevan-Jansen 1996	DNA (B)
920	Glucose	Mahadevan-Jansen 1998a	Sigma
920	C-C stretch in elastin	Kendall C	Sigma Aldrich E1625
920	Lactic acid	Pilotto 2001	DL-Lactic acid (C ₃ H ₆ O ₃)
920	C-C stretch Proline ring	Frank 1994a	Diseased breast tissue, collagen
920	C-C Proline ring	Frank 1995	Type I collagen (human placenta) & infiltrating ductal carcinoma (breast)
920	C-C stretch Proline ring in collagen	Kendall C	Sigma Aldrich C7774
921	C-C Proline ring	Fendel 1988	Collagen type I, normal skin dermis
921	C-C stretch Proline ring	Mahadevan-Jansen 1996	Collagen
925	DNA backbone	Puppels 1999	DNA
928	DNA backbone, α helix	Puppels 1991	Protein
928-940	C-C Proline, valine	Mahadevan-Jansen 1998a	
930	Tryptophan (amino acid)	Hartman 1973	Protein
930	rCH ₃	Cassanas 1991	Lactic acid
931	CH ₃ rocking, collagen proline	Lucassen 1998	Skin
931	ρ (CH ₃), ρ (C-C) α -helix	Barry 1992	Human stratum corneum
932	Skeletal C-C, α helix	Puppels 1999	DNA
932-5	CH ₃ rocking, C-C stretch α helix keratin	Edwards 1995	Stratum corneum, Callus, psoriatic plaque
934	C-C stretch, backbone	Frank 1994a	Collagen
934	C-C Proline ring	Frank 1995	Type I collagen (human placenta)
935-945	(C-C) skeletal vibrations, α helix	Mahadevan-Jansen 1998	α -helix protein secondary structure
935	Collagen	Redd 1993	Fibrocystic human breast tissue
936	C-C stretch backbone, hydroxyproline & collagen	Caspers 1998	Skin
936	C-C stretch, backbone in collagen	Kendall C	Sigma Aldrich C7774
937	Glycogen	Kendall C	Sigma Aldrich G0885
937	C-C stretch, backbone	Frank 1994a	Diseased breast tissue
937	C-C Proline ring	Frank 1995	Infiltrating ductal carcinoma (breast)
937	α -helix	Schrader 1995	nails

938	$\nu(\text{C-C})$ skeletal vibrations, keratotic	Carter 1998	Healthy human skin dermis
938	Peptide backbone stretch	Yu 1996	
938	C-C backbone	Mahadevan-Jansen 1996	Collagen
939	C-C-N stretching	Fredericks 1995	Bovine Serum albumin
940	C-C-N stretching in bovine albumin	Kendall C	Sigma Aldrich A2153
940	$\nu(\text{C-C})$ of proline and valine	Gniakeda 1997	Normal human skin
940	Triple helix vibrations	Fendel 1988	Collagen type I, normal skin dermis
941	C-C stretch	Ozaki 1988	Protein
944	$\nu(\text{C-C})$	Baraga 1992a	Asian skin
950	4-hydroxyproline	Feld 1995	Malignant breast tissue
955	Hydroxyapatite	Manoharan 1992	
956	Carotenoids	Mizuno 1994	Human brain tissue, acoustic neuroma
956	$\rho(\text{CH}_3)$, $\delta(\text{CCH})$ olefinic	Barry 1992	Human stratum corneum
960	Calcification - hydroxyapatite	Mizuno 1994	Human brain tissue, central neurocytoma
960	Calcification - hydroxyapatite	Clarke 1987	Aortic valve leaflets & coronary artery segments
960	P-O symmetric stretch	Nie 1990	Chicken leg bone
960	P-O symmetric stretch	Baraga 1992a	Atherosclerotic plaque calcified salts
960	Hydroxyapatite, P-O symmetric stretch	Yu 1996	Calcified plaque
960	Cholesterol	Keller 1994	
961	Apatite	Nie 1990	Human tooth enamel
961	$\text{Ys}(\text{PO})$ of PO_4^{3-} -in apatite	Wentrup-Byrne 1997a	Human tooth
961	Phosphate groups	Rava 1991	Calcified atherosclerotic plaque
966	Hydroxyapatite	Clarke 1987	Calcified plaque
966	Desmosine & isodesmosine (amino acids)	Manoharan 1996	Elastin
966	Triple helix vibrations	Fendel 1988	Collagen type I, normal skin dermis
968	C-OH	Manfait 2000	
972	$=\text{C-H}$ out of plane deformation	Frank 1994a	Breast tissue
972	C-C Proline ring	Frank 1995	Normal breast tissue, Oleic acid methyl ester
972	$=\text{C-H}$ out of plane deformation	Frank 1994b	Breast
975	Ribose	Hartman 1973	Protein
975	Deoxyribose (CH_2 deformation)	Mahadevan Jansen 1996	DNA (B)
978	Symmetric phosphate ion stretching	Mahadevan Jansen A	Phospholipids, glucose-1-phosphate
978	Symmetric phosphate ion stretching	Mahadevan Jansen A	Human cervix
978	Phosphorylated proteins	Mahadevan Jansen 1995	Human cervical biopsies

	and nucleic acids		
982	CH ₂ rocking	Edwards 1995	Stratum corneum
1000-1100	Glucose	Chaiken 2000	Blood
1000-1150	C-C skeletal vibrations	Mahadevan Jansen 1996	Phospholipid membrane, hydrophobic chains
1000-1250	Phosphate groups	Sajid J 1997	DMPC (dimyristoylphosphatidylcholine), a typical lipid, brain
1000-1200	C-C stretch – lipids	Carter 1998	Healthy human skin
1000	Phenylalanine in bovine Albumin	Kendall C	Sigma Aldrich A2153
1001	Phenylalanine(amino acid)	Kendall C	Sigma Aldrich T8324
1001	Phenylalanine(amino acid)	Feld 1995	Malignant breast tissue
1001		Lieber 2000	Ovary
1001-1004	Phenyl ring breathing mode	Shim 1996	
1002	ν (C-C) aromatic ring	Edwards 1995	Stratum corneum, Callus, psoriatic plaque
1002	(C-C) skeletal vibrations, β sheet	Mahadevan-Jansen 1996	β -sheet protein secondary structure
1002	Phenylalanine(amino acid)	Yu 1996	
1002	Phenylalanine(amino acid)	Frank 1994a	
1002	Phenylalanine(amino acid)	Frank 1994a	Collagen
1002	Hydroxyproline, tyrosine	Frank 1995	Type I collagen (human placenta)
1002	ν (CC) aromatic ring	Barry 1992	Human stratum corneum
1003	ν (CC) aromatic ring stretch of phenylalanine residue in keratin	Carter 1998	Healthy human skin
1003	Phenylalanine(amino acid) breathing mode	Mizuno 1994	Human brain tissue, protein
1003	C-N stretch	Erckens 1997	Urea
1003	C-N stretch of urea	Wicksted 1995	Urea
1003	C-N stretch of urea	Lucassen 1998	
1004	Phenylalanine(amino acid)	Erckens 1997	Rabbit lens, aqueous humour, cornea & human cornea
1004	Aromatic ring (breathing mode)	Gniakeda 1997	Normal human skin
1004	Phenylalanine(amino acid)	Maquelin 2000	
1004	Phenylalanine(amino acid)	Puppels 1999	
1004	Phenylalanine(amino acid)	Puppels 1991	
1004	Phenylalanine(amino acid)	Frank 1994a	Diseased breast tissue

1004	Phenylalanine(amino acid)	Frank 1995	Infiltrating ductal carcinoma (breast)
1004	Phenylalanine(amino acid) + Trp aromatic ring	Fredericks 1995	Bovine Serum albumin
1004	Carotenoid	Redd 1993a	Beta carotene, Human breast carcinoma
1004	8.5 Phenylalanine in collagen	Kendall C	Sigma Aldrich C7774
1004	P-O symmetric stretch	Nie 1990	Chicken leg bone
1005	8.6 Carotenoid	Redd 1993a	Normal human breast tissue
1005	Beta carotene	Frank 1995	Breast
1005	8.7 Carotenoid	Redd 1993b	Human breast tissue Renal cell carcinoma (1003)
1005	Phenylalanine(amino acid)	Hartman 1973	Protein
1006	C-N stretch of urea	Wicksted 1995	Urea, rabbit aqueous humour
1006	Phenylalanine, amino acid	Mahadevan-Jansen 1998	Collagen, histones
1006	Phenylalanine, amino acid	Ozaki 1988	Protein
1006	Carotenoids	Mizuno 1994	Human brain tissue, acoustic neuroma
1008	C-N stretch	Wang 1993	Urea NH ₂ -CO-NH ₂
1008	Carotenoids	Redd 1993b	Carotenoids in normal colon
1010	C-N stretch	Erckens 1997	Human aqueous humour
1013	Urea N-C-N stretch	Dou 1996	
1014	Tryptophan (amino acid)	Mahadevan-Jansen 1998	
1015	Tryptophan (amino acid)	Hartman 1973	Protein
1016	Tryptophan – amino acid	Manoharan 1995	DNA in water
1017	DNA backbone C-O stretch	Puppels 1999	
1030	Collagen	Kendall C	Sigma Aldrich C7774
1031	Proline	Yu 1996	
1031	v(CC) skeletal cis conformation	Barry 1992	Human stratum corneum
1031-32	v(C-C) keratin	Edwards 1995	Stratum corneum, Callus, psoriatic plaque
1032	Phenylalanine(amino acid)	Erckens 1997	Rabbit & human cornea Rabbit lens Rabbit aqueous humour
1032	Phenylalanine(amino acid)	Puppels 1999	
1032	proline	Frank 1995	Type I collagen (human placenta)
1034		Lieber 2000	Ovary

1035	Ribose, Phenylalanine(amino acid)	Hartman 1973	Protein
1035	C-C skeletal cis	Lucassen 1998	
1043	Proline	Frank 1995	Infiltrating ductal carcinoma (breast)
1043	Formalin artefact	Frank 1994a	breast tissue
1046	Lactic acid	Pilotto 2001	DL-Lactic acid (C ₃ H ₆ O ₃) CH ₃ -CHOH-COOH
1048	Glycogen	Kendall C	Sigma Aldrich G0885
1050	vC-CH ₃	Cassanas 1991	Lactic acid
1050	CO stretch, CCC trans skeletal stretch	Carter 1998	Healthy human skin dermis
1057	DNA backbone C-O stretch	Puppels 1999	
1060	δOD	Cassanas 1991	Methyl lactate
1061	Lipid	Caspers 1998	Strateum corneum
1062	ν(C-C) skeletal trans conformation lipid	Edwards 1995	Strateum corneum
1062	ν(CC) skeletal trans conformation	Barry 1992	Human strateum corneum
1064	C-C stretch	Sajid 1997	
1065	C-O stretch & C-O-C sym. stretch - phospholipids	Mizuno 1994	Human brain tissue
1066	C-C stretch	Frank 1994b	Breast
1066	C-C stretch	Frank 1994a	breast tissue
1066	Proline	Frank 1995	Normal breast tissue
1066	C-O, C-O-C stretch	Ozaki 1988	Phospholipids
1066	Lipid	Gnaidecka 1998	
1066		Lieber 2000	Ovary
1067	Proline	Frank 1995	Oleic acid methyl ester
1069	Hydroxyapatite shifted due to environment	Mahadevan Jansen A	Human cervix
1070	Symmetric phosphate ion stretching	Mahadevan Jansen A	Glucose -1-phosphate, collagen
1070	Symmetric phosphate ion stretching	Mahadevan Jansen A	Human cervix
1070	Collagen/elastin	Mahadevan Jansen 1995	
1070		Utzinger 2001	Cervix
1070	Phosphate / carbonate	Baraga 1992a	Atherosclerotic plaque
1070	Phosphate / carbonate	Yu 1996	
1071	apatite	Nie 1990	Human tooth enamel
1071	Yas(PO)	Wentrup-Byrne 1997a	Human tooth
1072	P-O asymmetric stretch	Nie 1990	Chicken leg bone
1074		Manoharan 1994	Adipose tissue
1074	Triglycerides	Baraga 1992a	Adipose tissue from human aorta
1078	C-N stretch	Alfano 1991	Benign breast tissue
1078-1090	C-C, C-O stretch in lipids. C-C, PO ₂ stretch	Shim 1996	

	in nucleic acids		
1079	C-C stretch	Frank 1994a	Breast tissue
1079		Frank 1995	Normal breast tissue
1079	C-C stretch	Frank 1994b	Breast
1080	C-C stretch & PO ₂ -sym. stretch - phospholipids	Mizuno 1994	Human brain tissue
1082	ν (C-C) skeletal random conformation lipid	Edwards 1995	Stratum corneum
1082		Frank 1995	Oleic acid methyl ester
1082	PO ₂ - vibration in phospholipids and nucleic acids	Gniakeda 1997	Normal human skin
1082	8.8 Lipid	Redd 1993a	Normal human breast tissue, Lipid (TPE)
1082	Lipid	Redd 1993	Human breast tissue
1082	Lipid	Lucassen 1998	
1082		Gnaidecka 1998	Protein
1082	ν (CC) skeletal random conformation	Barry 1992	Human stratum corneum
1083	Glycogen	Kendall C	Sigma Aldrich G0885
1084	Lactic acid	Pilotto 2001	DL-Lactic acid (C ₃ H ₆ O ₃) CH ₃ -CHOH-COOH
1085	Calcium carbonate	Wentrup-Byrne 1995	Cholesterol
1085	C-C stretch	Yu 1996	Lipid
1085	Phenylalanine(amino acid)	Hartman 1973	Protein
1085	Phospholipids	Feld 1995	Malignant breast tissue
1086	C-C stretch, PO ₂ stretch	Ozaki 1992	Phospholipids
1087	Symmetric stretching of phosphate groups of the polynucleotide chain	Sajid 1997	DNA
1090	ν CO	Cassanas 1991	Lactic acid
1090	PO-2	Mahadevan-Jansen 1998	DNA (C)
1091	PO-2	Mahadevan-Jansen 1998	DNA (B)
1094	DNA O-P-O	Puppels 1999	
1094	DNA O-P-O	Puppels 1991	
1095	vs of two ionised phosphate oxygens in the diphosphate ester	Mahadevan-Jansen 1998	DNA (B)
1095	PO-2	Mahadevan-Jansen 1998	DNA (Z), RNA(Z)
1095	CC stretch, COC deformation 1-4 glycosidic link	Maquelin 2000	
1099	PO-2	Mahadevan-Jansen 1998	DNA (A), RNA(A)
1100	Formalin	Keller 1994	Formalin
1100	PO ₂ stretch	Hartman 1973	Protein
1100	vs of two ionised phosphate oxygens in the diphosphate ester	Mahadevan-Jansen 1998	DNA (A)

1100	Lipid disorganised	Gnaidecka 1998	
1100-1110	(C-C) skeletal vibrations	Mahadevan-Jansen 1998	Unordered protein secondary structure
1104	C-C stretch lipid	Yu 1996	
1108	Desmosine & isodesmosine (amino acids)	Manoharan 1996	Elastin
1118		Frank 1995	Normal breast tissue
1119		Frank 1995	Oleic acid methyl ester
1119	C-C stretch	Frank 1994a	Breast tissue
1119	C-C stretch	Frank 1994b	Breast
1122	C-C stretch lipid	Yu 1996	
1123	Glycogen	Kendall C	Sigma Aldrich G0885
1123	Glucose	Mahadevan-Jansen 1998a	Sigma
1125		Hartman 1973	
1125		Frank 1995	Infiltrating ductal carcinoma (breast)
1126	C-N stretch	Puppels 1999	Protein
1126	C-C stretch - phospholipids	Mizuno 1994	Human brain tissue
1126	$\nu(\text{CC})$ skeletal trans conformation	Barry 1992	Human stratum corneum
1127	$\nu(\text{C-C})$ skeletal trans conformation lipid & keratin	Edwards 1995	Stratum corneum, Callus, psoriatic plaque
1127	Lipids	Caspers 1998	Stratum corneum
1127		Frank 1995	Type I collagen (human placenta)
1128	C-C stretch	Sajid 1997	
1128	Heme vibrational mode	Shim 2000	
1129	C-C stretch, lipid	Carter 1998	Healthy human skin stratum corneum & epidermal membrane
1130	Trans C-C stretch – phospholipids	Mizuno 1994	Human brain tissue, glioma grade III
1130	Lipid	Carter 1998	Healthy human skin stratum corneum & epidermal membrane
1130	C-C stretch, lipid	Gnaidecka 1998	
1130	C-O stretch glucose	Dou 1996	
1130	Low density lipoproteins	Yu 1996	
1131	Lactic acid	Pilotto 2001	DL-Lactic acid (C ₃ H ₆ O ₃)
1135	νCO , νCH_3	Cassanas 1991	Lactic acid
1150	Carotenoid	Brennan 1997a	Human artery
1150	C=C Carotenoid	Keller 1994	Food
1150	Cholesterol	Feld 1995	Carotid artery
1155	C-C stretch, $\delta(\text{COH})$	Lucassen 1998	
1155	$\nu(\text{CC})$, $\delta(\text{COH})$	Barry 1992	Human stratum corneum
1156	CN stretch	Fredericks 1995	Bovine Serum albumin

1156	Carotenoid	Frank 1994a	
1156	Carotenoid	Redd 1993a	Human breast carcinoma, Beta carotene
1157	8.9 Carotenoid	Redd 1993a	Normal human breast tissue
1157	Carotenoid	Redd 1993b	Human breast tissue Renal cell carcinoma (1155)
1157	Carotenoid	Mizuno 1994	Human brain tissue, acoustic neurinoma, beta carotene
1158	Carotenoid	Feld 1995	Carotid artery
1158	Carotenoid	Redd 1993b	Carotenoids in normal colon
1158	NH2 rocking vibration	Wicksted 1995	Urea
1159	C-N	Siebinga 1992	Protein in eye lens
1160	Carotenoid	Larrson 1974	Blood plasma
1162	Ribose	Hartman 1973	
1166		Frank 1995	Type I collagen (human placenta)
1167		Frank 1995	Infiltrating ductal carcinoma (breast)
1172	v(CC)	Barry 1992	Human stratum corneum
1175	C-O stretching	Mahadevan 1995	Human cervical biopsies
1176	Tyrosine, Phenylalanine	Puppels 1999	Protein
1180		Utzinger 2001	Cervix
1180	Tyrosine	Manoharan 1998	Breast
1180	Tyrosine	Miura 1995	
1180	Cytosine, guanine, adenine	Hartman 1973	
1197	Carotenoid	Frank 1994a	
1200-1700	Collagen	Wentrup-Byrne 1997a	Dentine
1205	Tyrosine + Phenylalanine	Kendall C	Bovine albumin, Sigma Aldrich A2153
1206	4-hydroxyproline (amino acid), tyrosine	Frank 1994a	Diseased breast tissue
1206	4-hydroxyproline (amino acid)	Frank 1994a	Collagen
1206	Hydroxyproline, tyrosine	Frank 1995	Type I collagen (human placenta) & infiltrating ductal carcinoma (breast)
1207	Tyrosine	Nie 1990	Human eye lens
1207	Tyrosine + Phenylalanine (amino acid)	Fredericks 1995	Bovine Serum albumin
1207		Lieber 2000	Ovary
1208	Thymine	Mahadevan-Jansen 1998	DNA (B)
1210	hydroxyproline	Nie 1990	Chicken leg bone
1210		Utzinger 2001	Cervix
1210	Tryptophan, phenylalanine	Hartman 1973	
1211	Tyrosine, Phenylalanine, Thymine	Puppels 1999	Protein
1226-1243	Amide III - v(C-N)	Miura 1995	β pleated sheet protein

			secondary structure in β -Poly-L-alanine
1227	Heme vibrational mode	Shim 2000	
1227-1247	Amide III (C-N stretch, N-H in plane bending) β -sheet	Mahadevan-Jansen 1998	β -sheet protein secondary structure
1230	Uracil in RNA	Yazdi 1999	Cultured breast cells
1230-1240	Amide III β -sheet	Carey 1982	
1230-1245	Amide III - ν (C-N)	Miura 1995	β pleated sheet protein secondary structure in β -Poly-L-glutamate
1234	Antisymmetric stretching of phosphate groups of the polynucleotide chain	Sajid 1997	DNA
1235-1270	Amide III (C-N stretch, N-H in plane bending) unordered	Mahadevan-Jansen 1998	
1237	Amide III	Nie 1990	Human eye lens
1238	Amide III, random coil	Erckens 1997	Rabbit & human cornea
1238	Amide III in elastin	Kendall C	Sigma Aldrich E1625
1238	Cytosine, uracil, Amide III	Hartman 1973	
1239	Thymine	Mahadevan-Jansen 1998	DNA (A), RNA (A)
1240	Amide III - ν (C-N)	Miura 1995	β pleated sheet protein secondary structure in β -Poly-L-lysine
1240	Vibration of pyrimidine bases (cytosine & thymine)	Mahadevan-Jansen 1996	DNA
1240	Amide III ν (C-N)	Alfano 1991	Benign breast tumour
1240	Amide III	Frank 1994a	
1240	Amide III in collagen	Kendall C	Sigma Aldrich C7774
1240	Amide III - ν (C-N)	Liu CH 1992a	Uterus cancer
1240	Amide III	Keller 1994	Caractous lens
1240	Thymine	Puppels 1999	Protein
1240-1260	Amide III unordered	Carey 1982	
1243	Amide III	Keller 1994	Liver - collagen
1243	Amide III	Nie 1990	Chicken leg bone
1243	Amide III	Erckens 1997	Rabbit lens
1243-48	Amide III - ν (C-N)	Miura 1995	irregular protein secondary structure in β -Poly-L-lysine pH4
1244	δ (CH ₂) wagging, ν (CN) amide III disordered	Barry 1992	Human stratum corneum
1245	ν CO, δ OH	Cassanas 1991	Lactic acid
1245	Amide III, random coil	Mizuno 1994	Human brain tissue, glioma grade III
1245	Keratotic	Carter 1998	Healthy human skin dermis
1245		Uttinger 2001	Cervix
1245	Amide III: keratin, disordered collagen	Lucassen 1998	
1245-1305	Amide III (C-N stretch,	Mahadevan-Jansen 1998	Histones

	N-H in plane bending)		
1246	Amide III	Fredericks 1995	Bovine Serum albumin
1246	Amide III ν (C-N)	Mahadevan 1995	Collagen & DNA
1246	Amide III ν (C-N)	Mahadevan 1995	Human cervix precancer
1246	Amide III (C-N stretching vibrations)	Mahadevan 1995	Human cervical biopsies
1246	Amide III (C-N stretching vibrations)	Liu CH 1992a	
1246	Amide III (C-N stretching vibrations)	Gnaidecka 1998	Protein
1247	Amide III	Frank 1995	Type I collagen (human placenta) & infiltrating ductal carcinoma (breast)
1247	Amide III	Frank 1994a	Diseased breast tissue
1247		Keller 1994	Neutrogena sarcoma (brain) - collagen
1247	Amide III	Frank 1994a	Collagen
1247	Amide III - ν (C-N)	Liu CH 1992a	Endometrium cancer
1247	Collagen	Redd 1993	Fibrocystic human breast tissue
1248	Amide III	Manoharan 1998	Breast
1248	PO ₂ - vibration in phospholipids and nucleic acids	Gniakeda 1997	Normal human skin
1248	Amide III (C-N stretch, N-H in plane bending)	Mahadevan-Jansen 1998	Collagen
1249	Amide III - ν (C-N)	Miura 1995	irregular protein secondary structure in β -Poly-L-glutamate pH 11
1250	Cytosine, uracil, Amide III	Hartman 1973	
1250	Amide III	Brennan 1997a	Human artery
1250	Amide III	Ozaki 1992	
1250	Cytosine	Mahadevan-Jansen 1998	DNA (B)
1250	Amide III	Fendel 1988	Collagen type I, normal skin dermis
1252	Amide III	Baraga 1992b	Aorta
1253	Amide III random coil protein	Schrader 1995	Breast cancer in mouse
1254	Adenine	Puppels 1991	Protein
1254	Amide III (C-N stretch, N-H in plane bending)	Mahadevan-Jansen 1998	Elastin
1255	Cytosine	Puppels 1999	Protein
1256	Glycogen	Kendall C	Sigma Aldrich G0885
1258-1304	Amide III (C-N stretch, N-H in plane bending)	Mahadevan-Jansen 1998	α -helix protein secondary structure
1260-1310	Amide III (C-N stretch, N-H in plane bending)	Miura 1995	α -helix protein secondary structure
1259	Amide III	Frank 1994a	Silicone gel
1260	Tyrosine	Miura 1995	Unordered protein secondary structure
1260	Structural protein	Manoharan 1998	Breast
1260	Symmetric CH ₃	Frank 1994a	Silicone gel

	deformation		
1260	Amide III	Nie 1990	Chicken leg bone
1260	Amide III	Feld 1995	Malignant breast tissue
1260-1280	Amide III	Fredericks 1995	Bovine Serum albumin
1261	Lipid	Manoharan 1994	
1262	Amide III - $\nu(\text{C-N})$	Liu CH 1992a	Benign or normal cervix
1262	Amide III - $\nu(\text{C-N})$	Liu CH 1992a	Benign or normal uterus
1262	Amide III - $\nu(\text{C-N})$	Liu CH 1992a	Benign or normal endometrium
1262	Amide III - $\nu(\text{C-N})$	Liu CH 1992a	Benign or normal ovary
1264	Amide III in collagen	Kendall C	Sigma Aldrich C7774
1265-1300	Amide III (C-N stretch, N-H in plane bending)	Carey 1982	α -helix protein secondary structure
1265-1348	Amide III - $\nu(\text{C-N})$	Miura 1995	α -helix protein secondary structure in α -Poly-L-alanine
1265	=C-H in plane deformation	Frank 1994b	Breast
1265	=C-H in plane deformation	Frank 1994a	breast tissue
1265	Amide III	Frank 1995	Normal breast tissue
1267	=C-H in plane deformation	Keller 1994	
1267	Amide III	Frank 1995	Infiltrating ductal carcinoma (breast)
1267	δ (=C-H)	Mahadevan-Jansen 1998	Phospholipid membrane
1267	δ (=C-H)	Keller 1994	Coronary Artery, unsaturated fatty acids
1267	Amide III	Frank 1994a	Lymph node
1267	Amide III	Frank 1994a	Diseased breast tissue
1267	Amide III	Frank 1994a	Collagen
1268	Amide III	Frank 1995	Oleic acid methyl ester
1268	Amide III - $\nu(\text{C-N})$ and $\delta(\text{NH})$ in bovine albumin	Kendall C	Sigma Aldrich A2153
1269	Amide III	Frank 1995	Type I collagen (human placenta)
1269	Amide III α -helix	Mizuno 1994	Human brain tissue
1270		Uttinger 2001	Cervix
1270	Amide III - $\nu(\text{C-N})$	Liu CH 1992a	Endometrium cancer
1271	Amide III - $\nu(\text{C-N})$ and $\delta(\text{NH})$	Gniakeda 1997	Normal human skin
1271	Amide III (C-N stretch, N-H in plane bending)	Mahadevan-Jansen 1998	Collagen
1273	Amide III	Fendel 1988	Collagen type I, normal skin dermis
1274	$\delta(\text{NH})$ wagging, $\nu(\text{CN})$ amide III α -helix	Barry 1992	Human stratum corneum
1280	Amide III	Clarke 1993	Phospholipid membrane
1290	Amide III - $\nu(\text{C-N})$	Miura 1995	α -helix protein secondary structure in α -Poly-L-glutamate

1295	Amide III - $\nu(\text{C-N})$	Miura 1995	α -helix protein secondary structure in α -Poly-L-lysine
1296	$\delta(\text{CH}_2)$	Barry 1992	Human stratum corneum
1296	Keratotic	Carter 1998	Healthy human skin dermis
1296	$\delta(\text{CH}_2)$	Edwards 1995	Stratum corneum
1297	Lipid	Carter 1998	Healthy human skin stratum corneum & epidermal membrane
1300	$\nu(\text{C-C})$ $\nu(\text{C-N})$	Alfano 1991	Benign breast tissue
1300	Amide III	Sajid 1997	Bovine insulin
1300	C-H bending	Liu CH 1992a	Lipid
1300	Phospholipids - CH_2 twist and wagging	Mizuno 1994	Human brain tissue
1300	Lipids – fatty acids	Manoharan 1998	Breast
1300	δCH	Cassanas 1991	Lactic acid
1300	Adenine , cytosine	Hartman 1973	
1302	Lipid	Redd 1993a	Lipid (TPE)
1302	Lipid	Redd 1993b	Human breast tissue
1302	8.10 Lipid	Redd 1993a	Normal human breast tissue
1302	Lipid	Redd 1993	Human breast tissue
1303	CH_2 twisting	Frank 1994b	Breast
1303	Triglycerides	Baraga 1992a	Adipose tissue from human aorta
1303	Adenine	Puppels G	
1303	δCH_2	Mahadevan-Jansen 1998	Phospholipid membrane
1303	CH_2 twisting	Frank 1994a	Normal breast tissue
1303	CH_3CH_2 twisting	Frank 1994a	Diseased breast tissue
1303	CH_3CH_2 twisting	Frank 1995	Normal breast tissue & infiltrating ductal carcinoma
1304	CH_3CH_2 twisting	Frank 1995	Oleic acid methyl ester
1306	Lipid	Redd 1993b	Human colon
1309	CH_2 twisting & wagging in lipids	Gniakeda 1997	Normal human skin
1310-16	$\Delta(\text{CH}_2)$	Edwards 1995	Callus, psoriatic plaque
1316	Histidine	Kendall C	Sigma Aldrich T8776
1316	Guanine	Mahadevan-Jansen 1998	DNA (Z), RNA(Z)
1317	CH_2 twisting	Redd 1993a	Skin
1318	Guanine	Mahadevan-Jansen 1998	DNA (A), RNA (A)
1319	CH_3CH_2 twisting	Frank 1995	Type I collagen (human placenta)
1319	CH_3CH_2 twisting	Frank 1994a	Collagen
1320	Guanine	Hartman 1973	
1321	in bovine albumin	Kendall C	Sigma Aldrich A2153
1322	C-H	Siebinga 1992	
1325	Tryptophan (amino acid) ring vibrations	Liu 1992a	
1325	Nucleic acid ring vibrations	Feld 1995	
1330	Tryptophan (amino acid) $\nu(\text{C-C})$	Liu 1992a	Cervix cancer

1330	Tryptophan (amino acid) v(C-C)	Liu 1992a	Uterus cancer
1330	Tryptophan (amino acid) v(C-C)	Liu 1992a	Ovary cancer
1330	C-H	Mahadevan Jansen 1998a	Nucleic acid bases & DNA, phospholipids
1330	C-H	Mahadevan Jansen 1998a	Human cervix precancer
1330		Utzinger 2001	Cervix
1330	DNA	Yazdi 1999	Cultured breast cells
1330	δ CH	Cassanas 1991	Lactic acid
1333	Glycogen	Kendall C	Sigma Aldrich G0885
1333	Guanine	Mahadevan Jansen 1998	DNA (B)
1334	CH deformation	Maquelin 2000	
1335	Nucleic acids	Manoharan 1995	Colon
1335	Adenine	Mahadevan Jansen 1998	DNA (A), RNA (A)
1337	Nucleic acid, purine bases (adenine, guanine)	Boustany 1999	Colon
1337	Nucleic acid, purine bases (adenine, guanine)	Fodor 1985	
1337	Purine bases (adenine, guanine)	Manoharan 1995	DNA in water
1337	Adenylates	Boustany 1999	Colon mucosa
1338	Tryptophan, amino acid	Mahadevan Jansen 1998	
1339	Adenine	Mahadevan Jansen 1998	DNA (B)
1339-41		Edwards 1995	Stratum corneum, Callus, psoriatic plaque
1340	Tryptophan (amino acid)	Erckens 1997	Rabbit & human cornea
1340	Tryptophan (amino acid)	Erckens 1997	Rabbit aqueous humour
1340	Nucleic acid	Feld 1995	Colon adenocarcinoma
1343	CH ₃ CH ₂ wagging	Frank 1994a	Diseased breast tissue
1343	CH ₃ CH ₂ wagging	Frank 1994a	Collagen
1343	CH ₃ CH ₂ wag	Frank 1995	Type I collagen (human placenta) & infiltrating ductal carcinoma (breast)
1343		Wicksted 1995	Rabbit aqueous humour
1343	Adenine	Hartman 1973	
1350	Glucose	Mahadevan-Jansen 1998a	Sigma
1358		Redd 1993	Normal renal parenchyma & renal cell carcinoma
1359		Redd 1993	Hepatocellular carcinoma
1360		Redd 1993	Normal hepatic parenchyma
1360		Redd 1993a	Myoglobin
1360		Erckens 1997	Human aqueous humour
1361	Tryptophan	Mahadevan-Jansen 1998	
1365	Guanine, tryptophan	Hartman 1973	
1372	Lipid	Redd 1993	Human colon

1375		Redd 1993a	Myoglobin
1376	Thymine, guanine, adenine	Puppels 1991	
1377	Glycogen	Kendall C	Sigma Aldrich G0885
1380	δCH_3 δOH	Cassanas 1991	Lactic acid
1385	$\delta(\text{CH}_3)$ symm	Barry 1992	Human stratum corneum
1400	Uracil, adenine	Hartman 1973	
1400		Utzinger 2001	Cervix
1401	Symmetric CH_3 bending in proteins	Mahadevan 1995	
1410	δOH	Cassanas 1991	Methyl lactate
1412	Antisymmetric CH_3 deformation	Frank 1994a	Silicone gel
1420	δOH , νCO	Cassanas 1991	Lactic acid
1421	$\delta(\text{CH}_3)$	Barry 1992	Human stratum corneum
1421	Guanine, adenine	Puppels 1991	
1438	$\delta(\text{CH}_2)$ scissoring	Barry 1992	Human stratum corneum
1438	CH_2 deformation	Shim 1996	
1439	CH_2 scissoring deformation	Frank 1994a	breast tissue
1439	CH_3 , CH_2 deformation	Frank 1995	Normal breast tissue
1439	CH_2 deformation	McCreery 1995	Human breast biopsy
1439	CH_2 deformation due to lipids and proteins	Mizuno 1994	Human brain tissue
1439	CH_2 scissoring deformation	Frank 1994b	Breast
1440	Lipid	Manohran 1994	
1441	CH_2 bending	Feld 1995	Fibrous atherosclerotic plaque
1441	Lipid	Keller 1994	Liver
1442	CH_3 , CH_2 deformation	Frank 1995	Oleic acid methyl ester
1442	Lipid	Redd 1993b	Human breast tissue Colon tissue
1442	8.11 Lipid	Redd 1993a	Normal human breast tissue
1442	Lipids – fatty acids	Manoharan 1998	Normal breast tissue
1443	elastin	Kendall C	Sigma Aldrich E1625
1444	Lipid	Redd 1993a	Lipid (TPE)
1444	Triglycerides	Baraga 1992a	Adipose tissue from human aorta
1445	$\delta(\text{CH}_2)$ or $\delta(\text{CH}_3)$	Liu CH 1992a	Cervix cancer
1445	$\delta(\text{CH}_2)$ or $\delta(\text{CH}_3)$	Liu CH 1992a	Uterus cancer
1445	$\delta(\text{CH}_2)$ or $\delta(\text{CH}_3)$	Liu CH 1992a	Benign or normal cervix
1445	$\delta(\text{CH}_2)$ or $\delta(\text{CH}_3)$	Liu CH 1992a	Benign or normal endometrium
1445	$\delta(\text{CH}_2)$ or $\delta(\text{CH}_3)$	Liu CH 1992a	Benign or normal ovary
1445	$\delta(\text{CH}_2)$ or $\delta(\text{CH}_3)$	Alfano 1991	Human breast tissue. Benign and Malignant tumours
1445	CH_2 deformation	Frank 1994a	breast tissue

1445	CH2 bending mode	Manoharan 1998	Normal breast tissue
1445	CH3CH2 deformation in collagen	Kendall C	Sigma Aldrich C7774
1447	CH3CH2 bending modes in bovine albumin	Kendall C	Sigma Aldrich A2153
1448	CH2 deformation	Nie 1990	Chicken leg bone
1448	Deoxyribose	Mahadevan-Jansen 1998	DNA (B)
1448-9	Δ (CH2) scissoring	Edwards 1995	Stratum corneum, Callus, psoriatic plaque
1449	CH2 deformation	Frank 1994a	Lymph node
1450	Glucose	Chaiken 2000	Blood
1450	CH bending	Erckens 1997	Rabbit & human cornea
1450	CH bending	Erckens 1997	Rabbit aqueous humour
1449	CH3CH2 bending modes	Fredericks 1995	Bovine Serum albumin
1450	CH3CH2 deformation	Frank 1994a	Diseased breast tissue
1450	CH2 bending	Brennan 1995	Human artery
1450	CH2 bending mode	Manoharan 1998	Diseased breast tissue
1450	δ (CH2) or δ (CH3)	Liu CH 1992a	Endometrium cancer
1450		Liu CH 1992a	Elastin & collagen
1450	CH3, CH2 deformation	Frank 1995	Infiltrating ductal carcinoma
1451	C-H bend	Baraga 1992a	Normal human aorta
1451	CH3, CH2 deformation	Frank 1995	Type I collagen (human placenta)
1451	CH3CH2 deformation	Frank 1994a	Collagen
1451	CH2 bending	Ozaki 1988	
1451-1454	CH2 bending	Mahadevan Jansen 1998a	Histones
1452	Structural protein	Manoharan 1998	Breast
1452	δ (CH2) scissoring in lipids & δ (CH2) δ (CH3) in proteins	Gniakeda 1997	Normal human skin
1452	CH2 bending	Feld 1995	Malignant breast tissue
1452	CH2 in plane bending	Rava 1991	Normal aorta
1453	δ (CH2) or δ (CH3)	Liu CH 1992a	Ovary cancer
1453	δ (CH2) or δ (CH3)	Liu CH 1992a	Benign or normal uterus
1453	C-H bending modes	Baraga 1992b	Aorta
1454	CH3 bending CH2 scissors	Mahadevan Jansen 1998a	Elastin & collagen & phospholipids
1454	CH3 bending CH2 scissors	Mahadevan Jansen 1998a	Human cervix
1454	CH3 asymmetric bending of proteins	Mahadevan 1995	

1454		Utzinger 2001	Cervix
1455	δ CH3	Cassanas 1991	Lactic acid
1455	Glycogen	Kendall C	Sigma Aldrich G0885
1455	CH2 deformation	Frank 1994b	Breast
1456	CH3 bending in elastin	Kendall C	Sigma Aldrich E1625
1456		Lieber 2000	Ovary
1457		Pilotto 2001	DL-Lactic acid (C3H6O3) CH3-CHOH-COOH
1458	Nucleic acid	Feld 1995	Colon adenocarcinoma
1460	Pentose sugar vibration due to δ CH2	Mahadevan-Jansen 1998	DNA
1462	Deoxyribose	Mahadevan-Jansen 1998	DNA (B)
1475	δ CH3	Cassanas 1991	Lactic acid
1480	Vibration of purine bases (adenine & guanine)	Mahadevan-Jansen 1996	DNA
1480	DNA	Yazdi 1999	Cultured breast cells
1485	Nucleic acids	Manoharan 1995	Colon
1485	Purine bases	Manoharan 1995	DNA in water
1485	Nucleic acid, purine bases (adenine, guanine)	Boustany 1999	Colon
1485	Nucleic acid, purine bases (adenine, guanine)	Fodor 1985	
1487	Nucleic acid	Manoharan 1995	Colon
1491	Formalin	Frank 1994a	
1500-1650	Heme	Ozaki 1988	
1509	Phenylalanine	Fredericks 1995	Bovine Serum albumin
1510	Adenine, guanine	Puppels 1991	
1512		Lieber 2000	Ovary
1517	Carotenoid	Frank 1994b	
1520-1670	Tryptophan & tyrosine	Yazdi 1999	Cultured breast cells
1520	C=C Carotenoid	Keller 1994	Food
1520	Carotenoid	Larrson 1974	Blood plasma
1522	Carotenoid	Feld 1995	Carotid artery
1523	Carotenoid	Redd 1993	Human breast tissue Renal cell carcinoma
1523	8.12 Carotenoid	Redd 1993a	Normal human breast tissue
1524	Carotenoid	Mizuno 1994	Human brain tissue, acoustic neuroma
1525	Carotenoid	Redd 1993a	Beta carotene
1525	Carotenoid	Redd 1993a TS	Human breast carcinoma
1525	Carotenoid	Redd 1993a	Human breast carcinoma
1528	Carotenoid	Manoharan 1994	Carotenoids in normal

			colon
1529	Carotenoid	Redd 1993b	Carotenoids in normal colon
1540	Amide II	Sajid 1997	Bovine insulin
1541	Tyrosine	Siebinga 1992	
1547	Tryptophan	Nie 1990	Human eye lens
1550	Tryptophan	Hartman 1973	
1552	$\delta(\text{NH})$, $\nu(\text{CN})$ Amide II	Barry 1992	Human stratum corneum
1553	Tryptophan	Mahadevan-Jansen 1998	
1554		Frank 1994b	Type I collagen (human placenta) & infiltrating ductal carcinoma (breast)
1555	Tryptophan	Manoharan 1995	DNA in water
1556	Tryptophan	Fredericks 1995	Bovine Serum albumin
1570	Vibration of purine bases (adenine & guanine)	Mahadevan-Jansen 1996	DNA
1570	nucleotides	Schrader 1995	Breast cancer in mouse
1575	Guanine, adenine tryptophan	Hartman 1973	
1576	Nucleic acid	Feld 1995	Colon adenocarcinoma
1577	Guanine, adenine	Puppels 1991	
1580	nucleotides	Schrader 1995	Breast cancer in mouse
1580	DNA	Yazdi 1999	Cultured breast cells
1580		Utzinger 2001	Cervix
1585	Nucleic acid, purine bases (adenine, guanine)	Boustany 1999	Colon
1585	Nucleic acid, purine bases (adenine, guanine)	Fodor 1985	
1585	$\nu(\text{C-O})$ olefinic	Edwards 1995	Stratum corneum, Callus, psoriatic plaque
1585	$\nu(\text{C=C})$ olefinic	Barry 1992	Human stratum corneum
1593	Purine bases	Manoharan 1995	DNA in water
1600		Hartman 1973	
1602	Phenylalanine	Siebinga 1992	
1602	Tyrosine	Manoharan 1995	Tyrosine, colon adenocarcinoma
1606	Phenylalanine	Fredericks 1995	Bovine Serum albumin
1607	Phenylalanine in bovine albumin	Kendall C	Sigma Aldrich A2153
1610	Tyrosine	Boustany 1999	Colon mucosa
1610	Tyrosine	Spiro 1987	Protein bands
1612	C=C stretching	Manoharan 1995	Benzene
1616	Tyrosine	Fredericks 1995	Bovine Serum albumin
1617	Tyrosine, phenylalanine	Puppels 1991	
1620	Tryptophan, tyrosine, phenylalanine, uracil	Hartman 1973	

1620	Tryptophan	Boustany 1999	Colon mucosa
1620	Tryptophan	Spiro 1987	Protein bands
1622	Tryptophan	Manoharan 1995	DNA in water
1637	Amide I $\nu(\text{C}=\text{O})$	Frank 1994a	Diseased breast tissue
1637	Amide I $\nu(\text{C}=\text{O})$	Frank 1994a	Collagen
1640	OH bending	Erckens 1997	Water
1642	Amide I $\nu(\text{C}=\text{O})$	Mahadevan-Jansen 1998	Collagen
1648-1661	Amide I $\nu(\text{C}=\text{O})$	Mahadevan-Jansen 1998	Histones
1645	Bending mode		Water
1645	Amide I ($\text{C}=\text{O}$ stretch)	Miura 1995	α -helix protein secondary structure in α -Poly-L-lysine
1645-1660	Amide I ($\text{C}=\text{O}$ stretch)	Carey 1982	α -helix protein secondary structure
1665-1680	Amide I ($\text{C}=\text{O}$ stretch)	Carey 1982	β -sheet protein secondary structure
1660-1670	Amide I ($\text{C}=\text{O}$ stretch)	Carey 1982	Unordered protein secondary structure
1645-1655	Amide I ($\text{C}=\text{O}$ stretch)	Miura 1995	α -helix protein secondary structure
1665-1680	Amide I ($\text{C}=\text{O}$ stretch)	Miura 1995	β -sheet protein secondary structure
1655-1665	Amide I ($\text{C}=\text{O}$ stretch)	Miura 1995	Unordered protein secondary structure
1654-1662	Amide I ($\text{C}=\text{O}$ stretch) α -helix	Mahadevan-Jansen 1998	α -helix protein secondary structure
1665-1680	Amide I ($\text{C}=\text{O}$ stretch)	Mahadevan-Jansen 1998	β -sheet protein secondary structure
1654-1685	Amide I ($\text{C}=\text{O}$ stretch)	Mahadevan-Jansen 1998	Unordered protein secondary structure
1650	Amide I	Baraga 1992a	Normal human aorta
1650	lipid	Boustany 1999	Colon mucosa
1650	Pyrimidine bases (cytidine, thymidine) ?	Manoharan 1995	DNA in water
1650	C=C lipid bands	Boustany 1999	Colon
1650	Amide I $\nu(\text{C}=\text{O})$	Sajid 1997	Bovine insulin
1650		Wang 1993	Ascorbate
1650		Erckens 1997	Rabbit aqueous humour
1650	Amide I ($\text{C}=\text{O}$ stretch) $\nu(\text{C}=\text{O})$	Brennan 1994	Human artery
1650-1666	C=C vibration cis-isomer in fatty acid chain	Mahadevan-Jansen 1998	Phospholipid membrane
1651	Amide I, $\nu(\text{C}=\text{O})$	Alfano 1991	Benign breast tissue and malignant tumours
1651-1664	Amide I, $\nu(\text{C}=\text{O})$	Carter 1998	Healthy human skin
1651	Amide I, $\nu(\text{C}=\text{O})$ in collagen	Kendall C	Sigma Aldrich C7774

1651	Amide I, (C=O stretch)	Liu CH 1992a	Ovarian cancer
1651-4	ν (C-O) Amide I, α helix	Edwards 1995	Stratum corneum, Callus, psoriatic plaque
1652	Lipid	Redd 1993a Zhe Chuan Feng, Kwok to Yue, Gansler TS	Lipid (TPE)
1652	Lipid	Redd 1993	Human breast tissue
1652	Amide I	Miura 1995	α -helix protein secondary structure in α -Poly-L-glutamate
1652	ν (C=O) Amide I α -helix	Barry 1992	Human stratum corneum
1653	Lipid	Redd 1993	Human breast tissue Human colon
1653	8.13 Lipid	Redd 1993a	Normal human breast tissue
1653	Amide I protein	Mizuno 1994	
1653	Amide I (C=O stretch) in bovine albumin	Kendall C	Sigma Aldrich A2153
1654	α -helix	Schrader 1995	nails
1654	C=C stretch Olefinic	McCreery 1994	Breast biopsy tissue
1654	C=C stretch	Frank 1994a	Breast tissue
1654	Amide I	Frank 1995	Normal breast tissue
1654	C=C stretch	Frank 1994b	Breast
1655	C=C stretch of unsaturated fatty acid chains	Baraga 1992b	Aorta
1655	Amide I	Frank 1995	Oleic acid methyl ester
1655	Amide I protein (C=O stretch)	Keller 1994	Coronary Artery
1655	Amide I (C=O stretch)	Frank 1994a	Lymph node
1655	Amide I (C=O stretch)	Liu 1992a	Endometrial cancer
1655	Amide I (C=O stretch)	Miura 1995	α -helix protein secondary structure in α -Poly-L-alanine
1656	Amide I (C=O stretch)	Mahadevan Jansen 1998a	Collagen
1656	Amide I (C=O stretch)	Mahadevan Jansen 1996	Human cervix
1656	Amide I (C=O stretch)	Mahadevan Jansen 1995	
1656		Uttinger 2001	Cervix
1656	Amide I (C=O stretch)	Liu 1992a	Benign or normal endometrium
1656	Amide I (C=O stretch)	Miura 1995	irregular protein secondary structure in β -Poly-L-glutamate pH 11
1657	Amide I (C=O stretch)	Liu 1992a	Cervical cancer
1657	Lipids – fatty acids	Manoharan 1998	Breast
1657	Lipids	Manoharan 1995	Colon
1657	Amide I (C=O stretch)	Feld 1995	Malignant breast tissue
1657	Amide I	Frank 1995	Infiltrating ductal carcinoma (breast)
1657	Amide I (C=O stretch)	Frank 1994a	Diseased breast tissue
1658	Amide I (C=O stretch)	Ozaki 1992	
1658	Amide I	Baraga 1992b	Aorta

1659	Amide I, $\nu(\text{C}=\text{O})$	Alfano 1991	Benign breast tumours
1659	Amide I ($\text{C}=\text{O}$ stretch)	Liu CH 1992a	Benign or normal ovary
1659	Amide I ($\text{C}=\text{O}$ stretch)	Liu CH 1992a	Benign or normal cervix
1659	Amide I ($\text{C}=\text{O}$ stretch)	Liu CH 1992a	Benign or normal uterus
1659	Amide I ($\text{C}=\text{O}$ stretch)	Liu CH 1992a	Uterus cancer
1659	Amide I ($\text{C}=\text{O}$ stretch) α helical	Mizuno 1994	Human brain tissue
1659	Amide I ($\text{C}=\text{O}$ stretch)	Fredericks 1995	Bovine Serum albumin
1659	Amide I ($\text{C}=\text{O}$ stretch)	Greve 1996	
1661	Amide I $\nu(\text{C}=\text{O})$ in elastin	Kendall C	Sigma Aldrich E1625
1661	Amide I $\nu(\text{C}=\text{O})$	Gniakeda 1997	Normal human skin
1662	Nucleic acid	Feld 1995	Colon adenocarcinoma
1662	Triglycerides	Baraga 1992a	Adipose tissue from human aorta
1662	Nucleic acids	Manoharan 1996	Colon
1664	Amide I ($\text{C}=\text{O}$ stretch)	Carter 1998	Healthy human skin
1664		Lieber 2000	Ovary
1665	Amide I ($\text{C}=\text{O}$ stretch)	Miura 1995	irregular protein secondary structure in β -Poly-L-lysine pH 4
1665	Amide I ($\text{C}=\text{O}$ stretch)	Frank 1994a	Collagen
1665	Amide I	Frank 1995	Type I collagen (human placenta)
1665	Amide I ($\text{C}=\text{O}$ stretch)	Keller 1994	Liver - collagen
1665	Carbonyl vibration mode	Feld 1995	Fibrous atherosclerotic plaque
1666	$\text{C}=\text{O}$ stretching modes of pyrimidine bases	Sajid 1997	DNA
1667	Structural protein	Manoharan 1998	Malignant breast
1668	Amide I ($\text{C}=\text{O}$ stretch)	Mahadevan-Jansen 1998	Elastin
1669	Amide I ($\text{C}=\text{O}$ stretch)	Nie 1990	Human eye lens
1669	Protein	Schrader 1995	Breast cancer in mouse
1669	Amide I ($\text{C}=\text{O}$ stretch)	Miura 1995	β pleated sheet protein secondary structure in β -Poly-L-alanine
1669	Amide I ($\text{C}=\text{O}$ stretch)	Rava 1991	Normal aorta
1670-1680	$\text{C}=\text{C}$ vibration trans-isomer in fatty acid chain	Mahadevan-Jansen 1998	Phospholipid membrane
1670	Amide I	Nie 1990	Chicken leg bone
1670	Amide I ($\text{C}=\text{O}$ stretch)	Mahadevan-Jansen 1998	Pure protein - β pleated sheet
1670	Amide I ($\text{C}=\text{O}$ stretch)	Mahadevan-Jansen 1998	Collagen
1670	Amide I ($\text{C}=\text{O}$ stretch)	Frank 1994a	
1670	Amide I ($\text{C}=\text{O}$ stretch)	Erckens 1997	Rabbit lens
1670	Amide I ($\text{C}=\text{O}$ stretch)	Miura 1995	β pleated sheet protein secondary structure in β -Poly-lysine
1672	Amide I ($\text{C}=\text{O}$ stretch)	Miura 1995	β pleated sheet protein secondary structure in β -Poly-L-glutamate
1683	Amide I ($\text{C}=\text{O}$ stretch)	Mahadevan-Jansen 1998	Bound protein - regular uncharacterised secondary structure

1717	C=O stretching modes of purine bases	Sajid 1997	DNA
1725		Wang 1993	Lactate
1725	$\nu\text{C}=\text{O}$	Cassanas 1991	Lactic acid
1726	Lactate	Goetz 1995	
1736	ester stretching mode C=O	Sajid 1997	DMPC (dimyristoylphosphatidylcholine), a typical lipid
1740	$\nu(\text{C}=\text{O})$ -lipid	Keller 1994	Food, coronary artery
1743		Frank 1995	Oleic acid methyl ester, normal human breast tissue
1743	C=O stretch	Frank 1994a	Breast tissue
1743	C=O stretch	Frank 1994b	Breast
1743	$\nu(\text{C}=\text{O})$ lipid	Barry 1992	Human stratum corneum
1747	C=O stretch	Baraga 1992b	Aorta
1750	C=O	Fendel 1988	Normal skin dermis
1752	Triglycerides	Baraga 1992a	Adipose tissue from human aorta
1768	$\nu(\text{COO})$	Barry 1992	Human stratum corneum

Appendix IV

Initial Probe-Spectrometer Evaluation

Experiment 1. Temporal variation in PTFE spectra.

The laser and CCD were switched on at time 0. 5 s PTFE spectra were measured at 3 minute intervals except for the first measurement which was made at 12 minutes (blue spectrum in Figure VI-1).

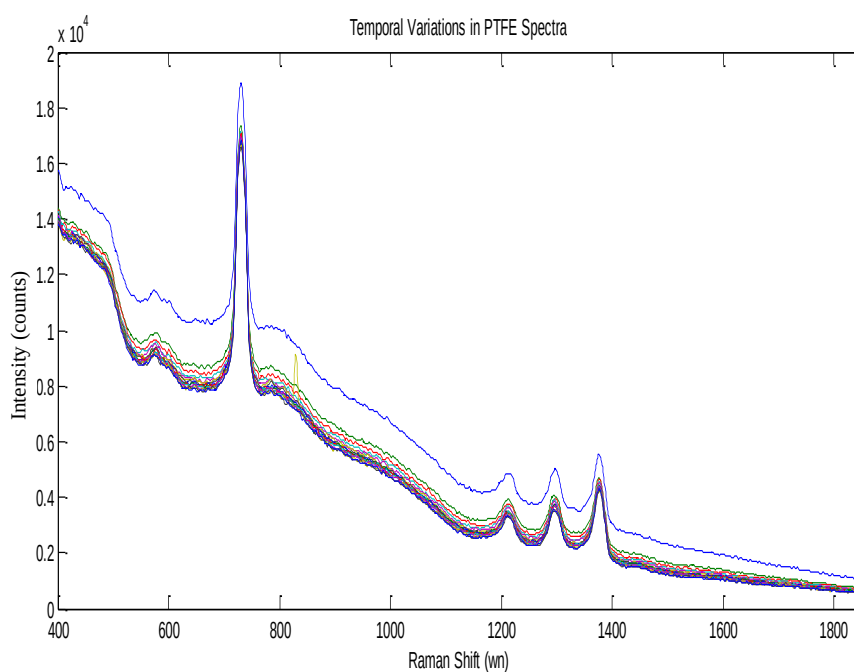


Figure VI-1. Temporal variations in PTFE spectra.

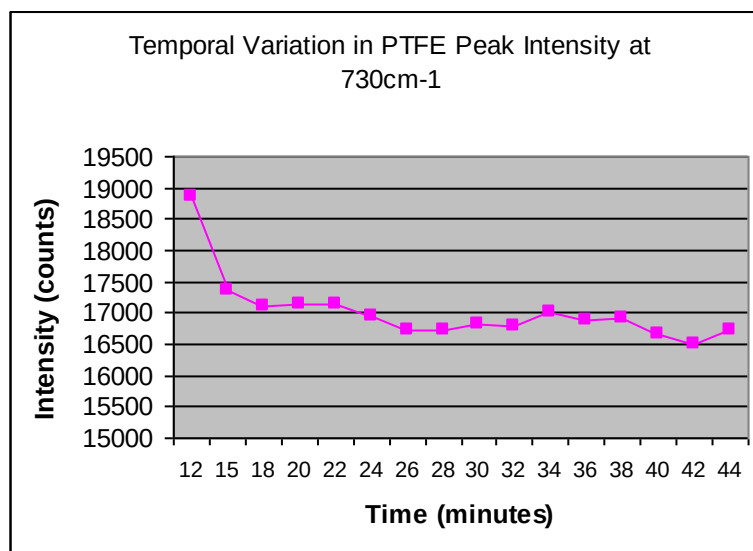


Figure VI-2. Temporal variation in PTFE spectral intensity at 730 cm^{-1} every 3 minutes.

Significant variation occurred over the first 15 minutes. This is due to the laser ‘warming up’ and the CCD stabilising after being turned on. Following this preliminary experiment the system was switched on a minimum of 20 minutes before spectral measurements were taken.

Experiment 2. Switching the laser ‘on’ and ‘off’.

After the laser had ‘warmed up’ the effect of repeatedly switching the laser off for short periods between measurements was evaluated (Figure VI-3). A series of PTFE spectra were measured; the laser was switched off for 2 minutes between each measurement. Consistent spectra were recorded (Figure VI-3).

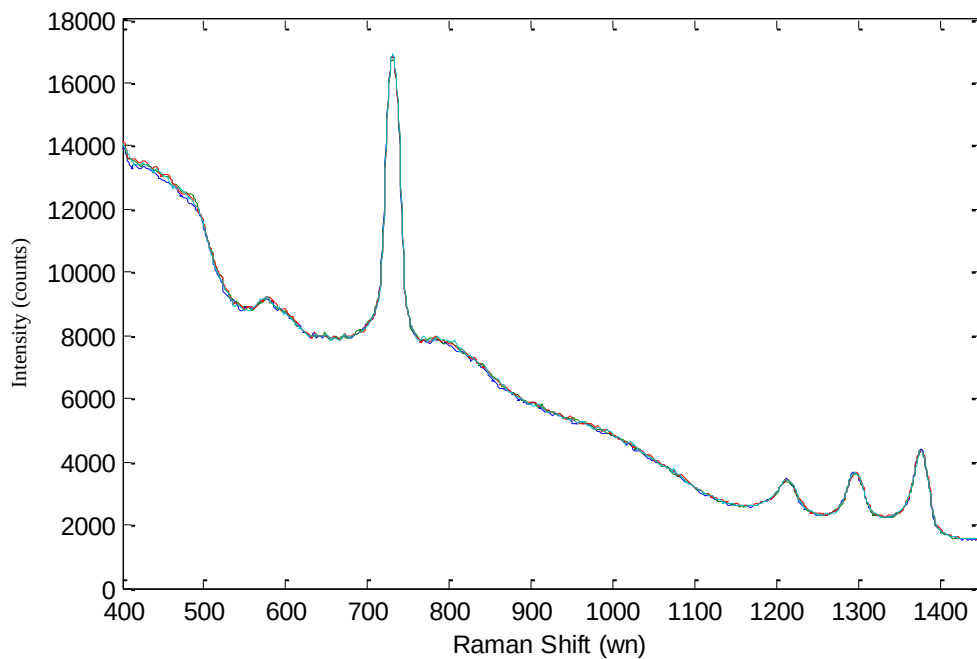


Figure VI-3. PTFE spectra. The laser was switched off for two minutes between each measurement.

Experiment 3. Effect of disconnecting the laser source.

The effect of disconnecting the Raman probe from the laser source between each measurement was evaluated. Figure VI-4 shows that consistent spectra were measured.

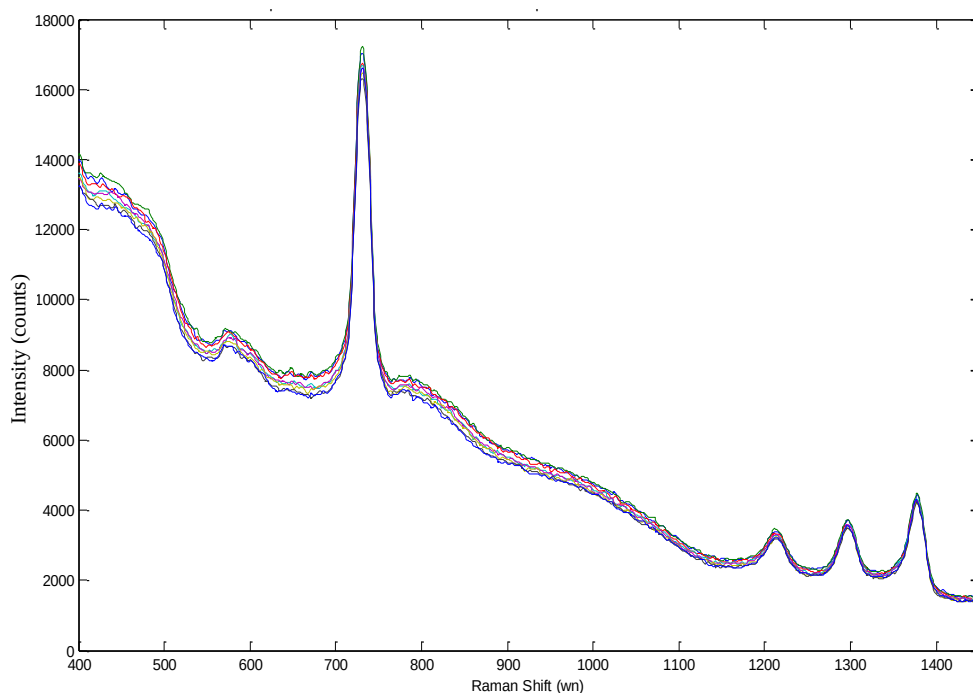


Figure VI-4. PTFE spectra. Between each measurement the probe was disconnected from the laser source.

Experiment 4. Does repositioning of the probe on the PTFE / green glass between readings effect spectral measurement?

Between measurements the probe was removed from contact with the PTFE and green glass standards. Repeat measurements assessed any variation produced by probe contact and probe angle. Figures VI-5 and VI-6 show the effects. Although peak position was consistent, variation in intensity was marked. Adequate contact between the probe and the standards was found to be the significant source of this variation.

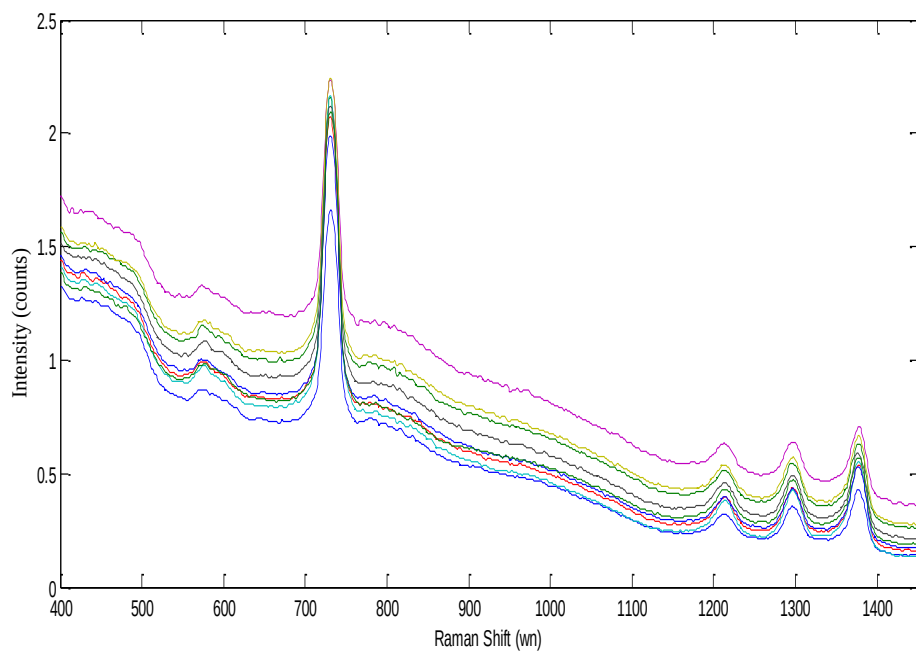


Figure VI-5. PTFE spectra. Between each measurement the probe was moved and then repositioned.

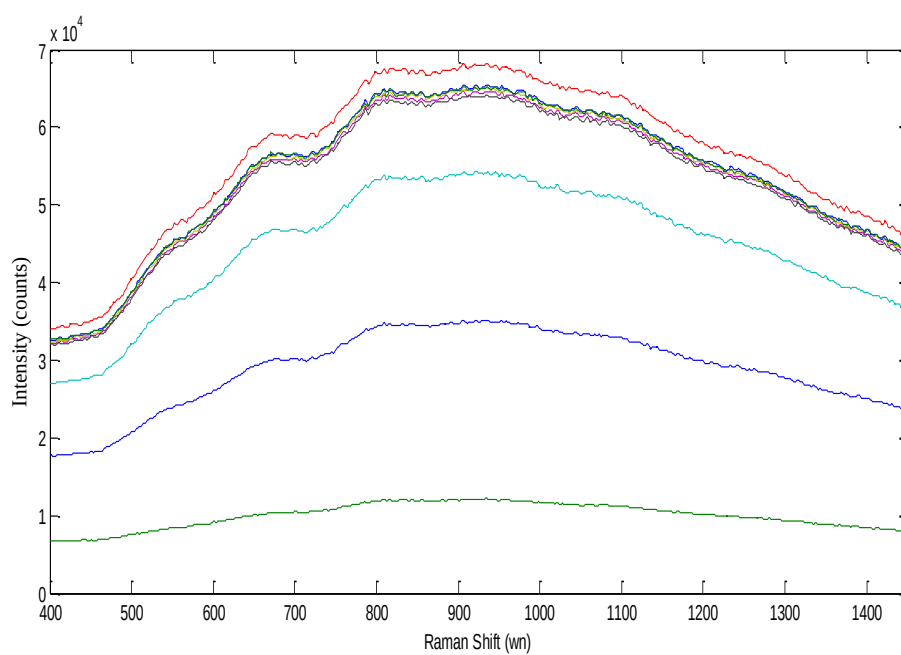


Figure VI-6. Green glass spectra. Between each measurement the probe was moved and then repositioned.

Experiment 5. Can the probe acquire Raman spectra from a tissue phantom?

Prior to taking measurements from human oesophageal tissue, spectra were acquired using the probe-spectrometer system from a porcine tissue phantom. A 830 nm monochromatic laser was used to excite the tissue for 1, 5, and 10 seconds.

Figure VI-7 shows 5 s porcine tissue spectra measured by passage of the Raman probe through a Perspex mapping grid positioned on the surface of the tissue. Note the lipid peaks detected when measuring a lipid-rich portion of porcine tissue (gold spectrum). No evidence of tissue disruption was observed.

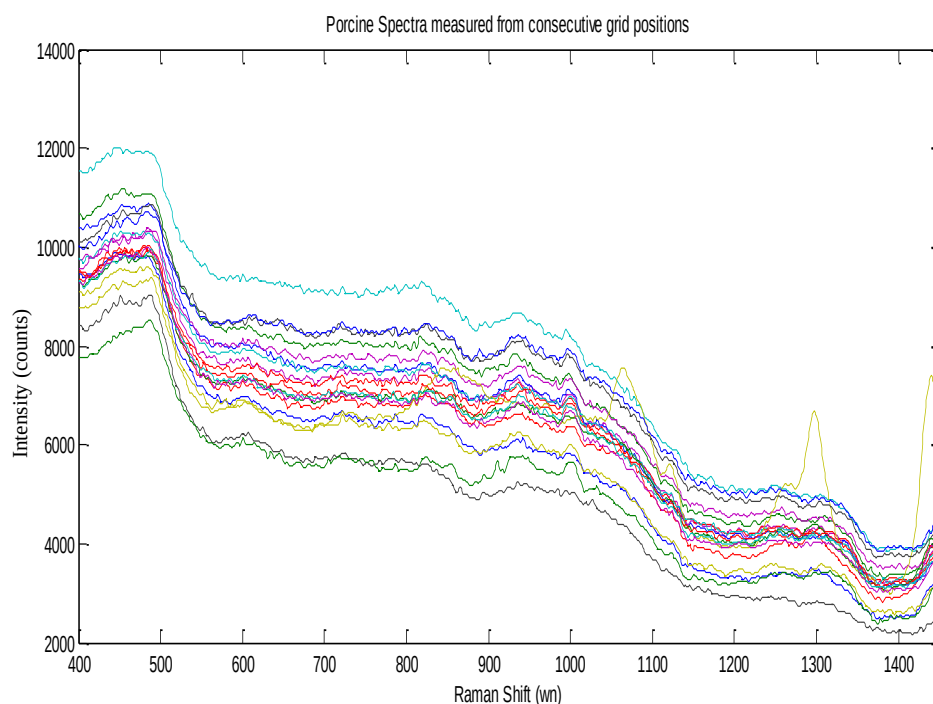


Figure VI-7. Five second spectra measured from adjacent grid positions across a porcine tissue phantom.

For comparison, spectra measured randomly from the porcine phantom (without the use of the grid) are plotted below (Figure VI-8).

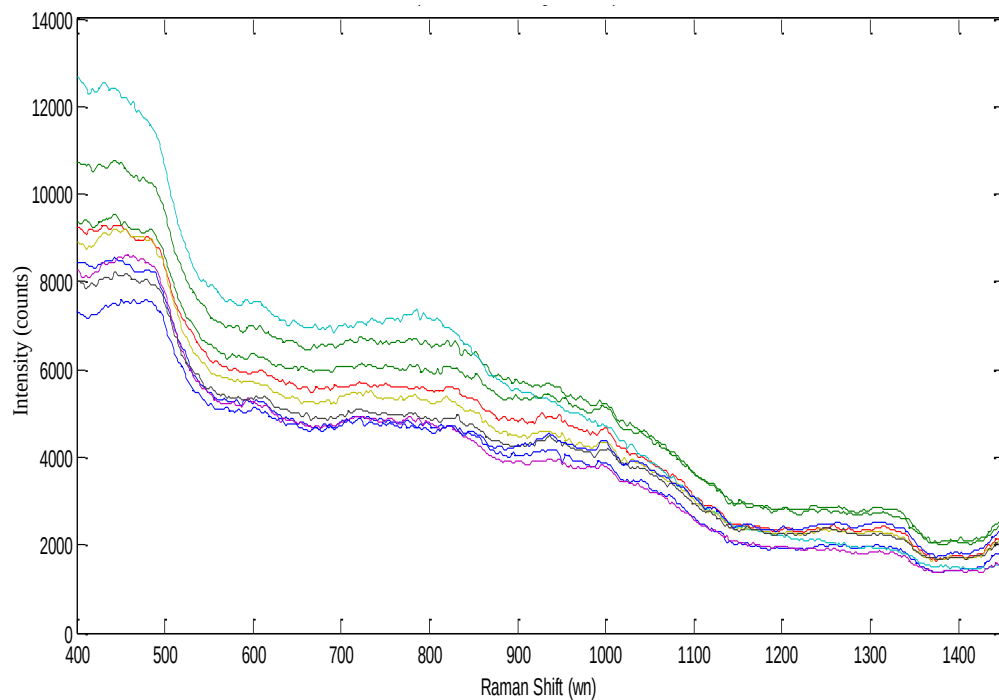


Figure VI-8. Porcine tissue spectra measured at random across the surface of the tissue.

Experiment 6. Effect of saline / water lavage.

Comparison of mean spectra following lavage with saline and spectroscopic grade water. I needed to ensure that removing blood from tissue by washing with water or saline would not cause major spectral disruption. The control was not washed. The spectral differences below may reflect the effects of other variables including tissue contact and probe pressure which were not controlled in this experiment.

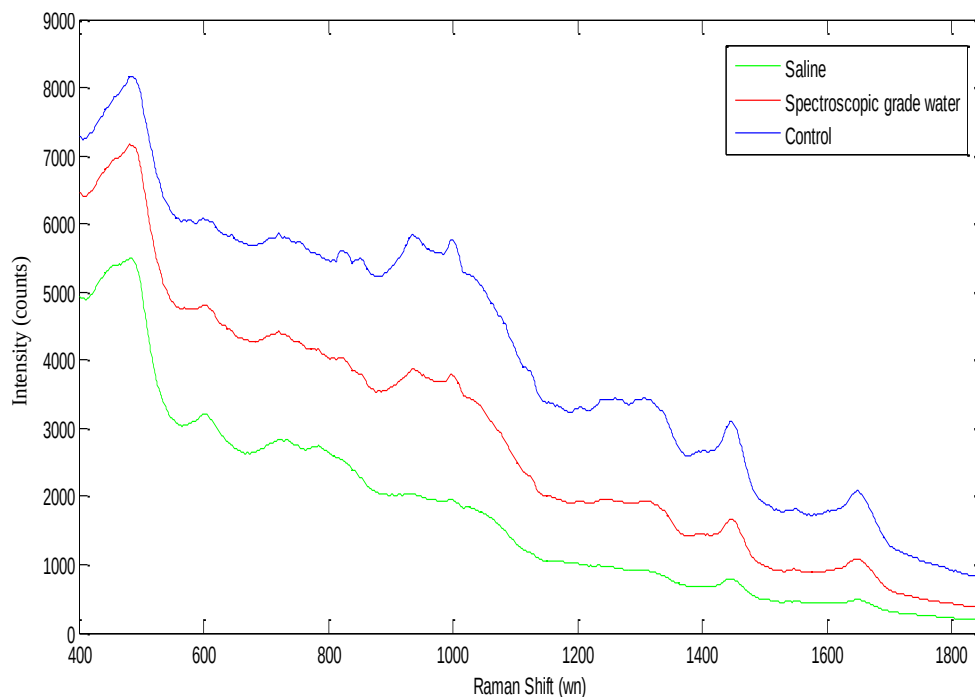


Figure VI-9. Mean 5 s porcine tissue spectra measured before and after lavage with water and lavage with saline.

Experiment 7. Comparison of Current and Original Prototype Probe

The background signal measured using the Raman probe was compared to that of an earlier prototype probe (Figure VI-10). Both probes were used in conjunction with the same laser source and spectrometer. Although the background shapes were similar, the intensity of background signal recorded using the prototype probe was considerably higher than with the current confocal design.

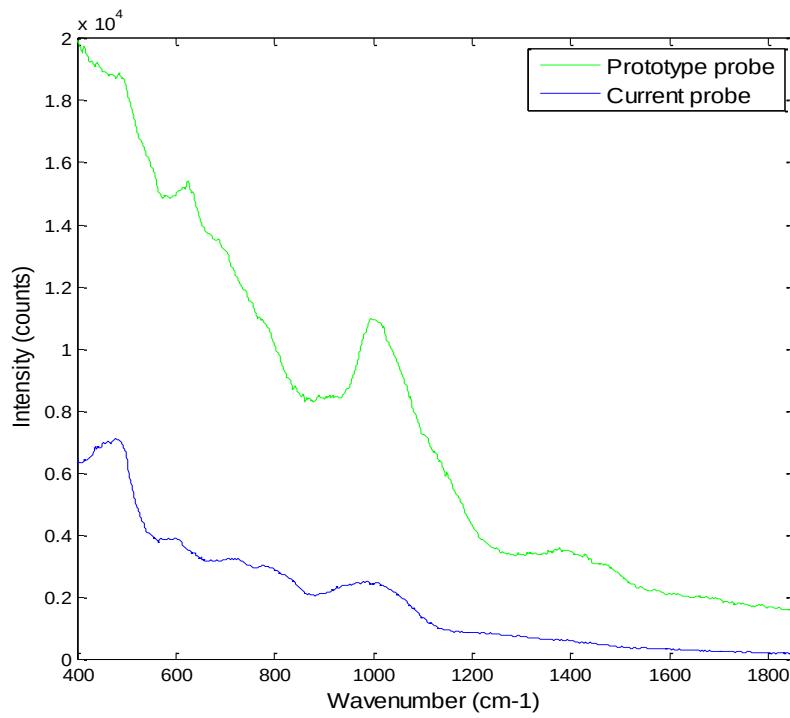


Figure VI-10. Air background spectra acquired in 5 s with the current confocal probe and the earlier prototype probe.

Ex vivo oesophageal tissue spectra were acquired from three patients using the prototype probe. Mean 5 s tissue spectra for 3 pathologies are illustrated in Figure VI-11. Mean 5 s spectra for each pathology acquired using the current probe are also illustrated for comparison. The prototype probe was not used for further tissue measurements as it was not designed for tissue contact (making it less amenable to endoscopic use than the current probe); it did not have a confocal, short focal length design, so could not interrogate oesophageal epithelium without significant signal interference from deeper tissues; and it displayed a significantly higher background signal.

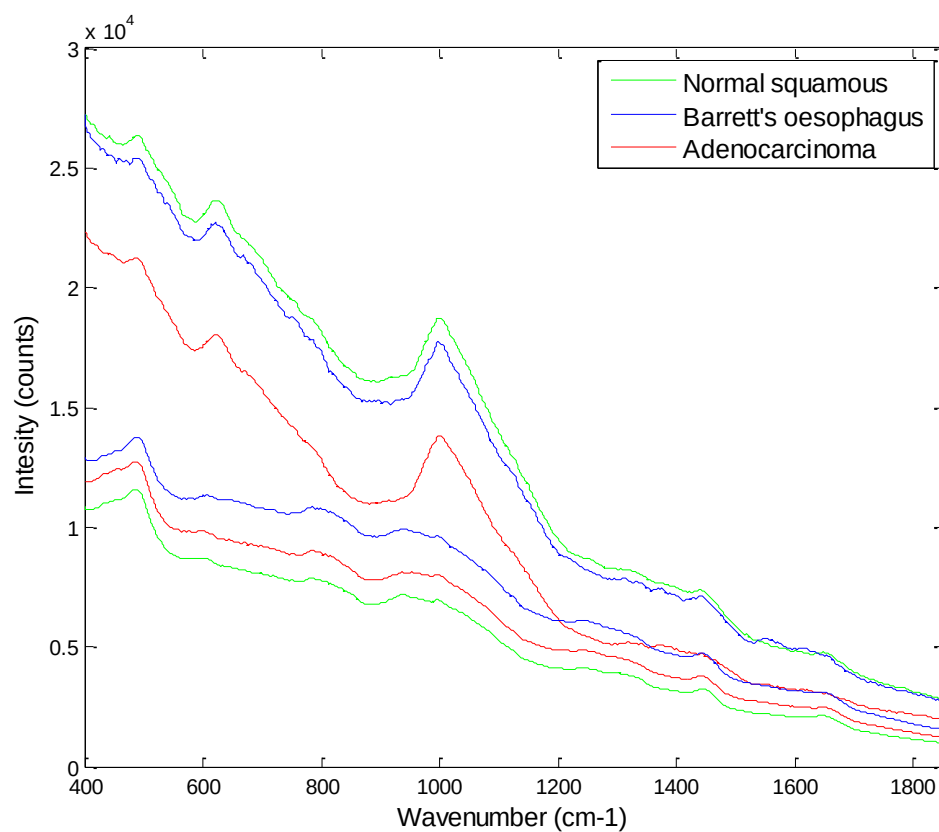


Figure VI-11. Mean tissue spectra acquired in 5 s using the current confocal probe and the earlier prototype probe. The different background signal allows easy visual distinction between tissue measured using each of the two probes.

Appendix V

Temporary Local Rules for the 830nm Laser associated with the Compact Raman Spectroscopy System and Raman Probe

Nature of Hazards to Persons

Innovative Photonic Solutions (IPS) Laser: Class IIIB, >350mW, λ =830nm (Serial # 1002 Model # I0830MM0350MF-EM)

This laser has a shutter which must be closed when the laser is not in use.

Extreme care should be taken when operating these lasers to avoid potentially hazardous exposure to both eyes and skin. Safe use of the lasers depends on strict adherence to the following rules. These Rules apply to each laser equally.

This laser is designed to be used with a fibre optic patchcord attached to the laser output port and should not be operated in an “open beam” configuration for QA measurements. The probes must be kept facing downwards in the jig provided at all times. More than one laser must NOT be operated simultaneously in “open beam” configuration.

These Local Rules have been written such that the laser (and probe system) can only be used in Endoscopy Theatre 3 in Gloucestershire Royal Hospital. The location of the room must be signed off by the LPA prior to use.

Controlled Area Designation and Access

- 1) The room in which the laser is being used is designated a “Controlled Area”, when the laser is switched on. The number of persons in the Controlled Area will be kept to a minimum. The laser will only be used in this area. Approved warning signs will be fitted to the door. The second entrance to the room (single door) will be kept locked at all times. The main door will be manned by an assistant stood outside to prevent anyone from entering the room. Prior to the single designated session of laser use on Friday 21st 1400 – 1800, members of the endoscopy department will be informed of the use of the laser and advised that they will not be able to enter Room 3 whilst the laser is in use. They will be given a written handout to endorse this advice.
- 2) All endoscopy equipment will be moved to the edge of the room and shiny objects will be covered or removed prior to use of the laser.

Laser Protection Supervisor (LPS)

- 3) The Laser Protection Supervisor is Prof. N. Stone. (See (18)). The role of the LPS is to ensure adherence to these Local Rules, that the register of Authorized Users is maintained and liaise with the Laser Protection Advisor (LPA) as required.

Register of Authorised Users

- 4) Authorised users are Dr N. Stone, Dr C.A. Kendall and Dr J. Hutchings and Dr L.M. Almond. They have been trained in the safe operation of the laser and will sign statements

to the effect that they have read and understood these Local Rules. A list of the approved users with the authorisation signature of the Laser Protection Supervisor can be found below.

- 5) It is the responsibility of the Authorized User to be aware of the nature of the hazards involved.
- 6) It is the responsibility of the Authorized User to consider:
 - Location of nearest fire extinguisher – on corridor
 - Control of access to the room – see section 1 above
 - Telephone contact in case of emergencies – extension of room 8228, extension in an emergency 2222

Protective Eyewear

- 7) Protective eyewear, suitable for use with NIR diode lasers (830nm) is provided, in accordance with BS EN 207/208. People in the controlled area must wear protective eyewear.

Methods of Safe Working

- 8) The operator will place a sign on the outside of the laboratory door which states ‘WARNING - LASER ON - DO NOT ENTER WITHOUT SUITABLE EYE PROTECTION’ when the laser is in use.
- 9) The laser output port will not be routinely used in an “open beam” configuration, therefore the beam will be emitted from the probe tip. The probe will be kept facing downwards in the jig to minimize exposure risk.
- 10) The laser should be positioned such that the direct ‘open beam’ is not directed towards the door or windows.
- 11) Do not look directly into the laser or the probe
- 12) The shutter must be closed or the safety cap replaced when the probe is disconnected or reconnected.
- 13) Keep all reflective or shiny components out of the laboratory unless no alternative is available.
- 14) When the lasers are not in use the key will be removed from the laser and stored securely by the nominated key holder.

Custody of the Key

- 15) The Laser Protection Supervisor, Prof. N. Stone, is the nominated key holder. He is responsible for issuing the key to Authorised Operators only (see Point 13).

Adverse Incident Procedure

- 16) If an actual or suspected incident should occur, in which the eyes or skin are irradiated with the laser beam, an examination by an ophthalmologist or dermatologist should be carried out within 24 hours. The Laser Protection Adviser should be contacted as soon as possible.

Laser Protection Advisor

- 17) For further advice or information contact the Laser Protection Adviser Dr Janelle Reyes-Goddard or the Laser Protection Supervisor Professor Nicholas Stone. Biophotonics Research Unit. Gloucestershire Hospitals NHS Foundation Trust.

Signs

- 18) A printed sign shall be fitted to the entrance to the Controlled Area, containing the BS Laser Symbol and the wording, 'Controlled Area, Class 3B Semiconductor Lasers, $\lambda=830\text{ nm}$ '. A second sign will also be fitted stating in larger type: "Please do not enter without knocking".

Appendix VI

Risk Assessment Form

Location:	Ward:/ Department: Biophotonics Research Unit Site: Leadon House: Speciality: Clinical Research	Assessment number:	31
Task:	Raman probe measurements on patient tissue (clinical and research) samples in vitro	Date of Assessment:	8 th July 2011
		Assessor/auditor:	Max Almond

Description of services and situation of the workplace	Which service users does the task affect? Max Almond, Catherine Kendall, Jo Hutchings How often does the above task occur in the workplace? • Daily Weekly Monthly What time of the day does the above task generally occur? Office Hours and Evenings What is the most common type of injury following the above task in the workplace? N/A (No injury has ever occurred). Is there history of staff absence as a result of the above task in your workplace? No	<i>Provide brief description of general working and occurrences of the tasks that may result in harm.</i> Use of laser probe for tissue analysis. Pinning out of ex vivo human tissue (using pins). Fixation of biopsies using formalin. Snap freezing of biopsies using liquid nitrogen. Taking biopsies from tissue specimens using biopsy forceps and scalpel. Handling human tissue.
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Ref no.	What are the Hazards?	Who might be harmed and how?	What are you already doing?	What further action is necessary?	Risk rating			
					G	Y	O	R
	<i>How have the accidents injuries been caused</i>	<i>Review sources of evidence for what has been harmed – e.g. incident accident forms, complaints,</i>	<i>What do you already do to reduce the chance of accidents</i>					
1	Use of laser probe for tissue analysis.	User – damage to eyes / skin.	Eye protection. Limited laser power. Assessment of competency prior to independent use - users are not signed off to use the laser unless assessed by the LPS. Please refer to Biophotonics laser Local Rules.	None.	✓			
2	Pinning out of ex vivo human tissue (using pins).	User – needle-stick injury.	Wear gloves when handling tissue. Refer to needle-stick protocol.	None.	✓			
3	Fixation of biopsies using formalin.	User – contact with eyes / skin.	Wear gloves. Training Well ventilated room	Store large quantities (large 1 L bottles) of formalin in the chemistry lab. Only keep the	✓			

				minimum number of biopsy pots in the Raman lab and use the Chemistry Lab for storage.				
4	Snap freezing of biopsies using liquid nitrogen.	User – contact with body part.	Wear gloves, face protection, long sleeves, apron. Training	None.	✓			
5	Handling of human tissue.	User + others using the lab.	Carefully clean equipment after use. Wear gloves whilst handling tissue. Training Cross reference RA for handling tissue.	None.	✓			
6	Taking biopsies from tissue specimens using biopsy forceps and scalpel.	User.	Wear gloves. Training as a surgeon, training for research	None.	✓			
7	Loss of tissue prior to essential clinical analysis.	Important clinical information may be lost. This could impact on subsequent care of the patient.	Only one patient's tissue is measured at one time. Tissue is taken to the pathology department as soon as Raman measurements have been taken. Ensure that the lab is kept clear from clutter to prevent samples being misplaced.	None.	✓			

8	Laser damage to the tissue sample	A clinical diagnosis may not be possible due to damaged samples. This could impact on subsequent care of the patient.	Measurements are carried out with a constant laser power at the probe tip.	None				
9	Inappropriate handling of patient identifiable information	Patient identifiable information may be accessed by those other than the patient, clinical and research staff involved in the project.	Patient identifiable information is stored in a sample database which is password protected and stored on an office PC. The database is not used on laptops/USB sticks. Spectra are stored with an anonymised patient identifier, Any paperwork (including stickers) with patient identifiable information must be disposed of as confidential waste. Lab books should not contain patient identifiable information. Adherence to the Trust policy on Data protection. Completion of e-learning on Data Protection,	None	✓			

Appendix VII

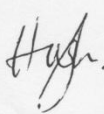
Avon, Gloucestershire and Wiltshire
Health Authority

Gloucestershire LREC
Gloucestershire Royal Hospital
Great Western Road
Gloucester
GL1 3NN
Tel: 01452 395726
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Email: Hazel.Gage@gloucr-tr.swest.nhs.uk

Our Ref: 02_112G(28_4)

28 April 2003

Professor Hugh Barr
Consultant Surgeon
Ward 9
Gloucestershire Royal Hospital
Great Western Road
Gloucester GL1 3NN

Dear Professor Barr 

Study No 02/112G : Investigation of cancerous and pre-cancerous changes in Barrett's oesophagus using techniques to advance the early diagnosis of malignancy

Thank you for your letter dated 11 April 2003 enclosing the amendments for the above study. I have now reviewed the amendments and will be advising the committee that in my view there is now no objection on ethical grounds to the proposed study. Therefore, I am happy to give you approval on the understanding that you will follow the conditions of the approval set out below. The following documents were reviewed by the committee: -

- LREC application form
- Protocol (Version 2, April 2003)
- Consent Form (Version 1, January 2003)
- Patient Information Sheet (Version 1, January 2003)
- Curriculum Vitae

- a) It is the responsibility of the investigator to notify the LREC immediately of any information received by him/her, or of which he/she becomes aware which would cast doubt upon, or alter, any information contained in the original application, a later amendment application or verbal resume submitted to the LREC. The committee should be informed immediately if this information would raise questions about the safety and/or continued conduct of the research.
- b) The need to comply with the Data Protection Act 1998.
- c) You will need to comply with the Research Governance Framework for Health and Social Care (Department of Health 2001). This includes the requirement to

make available records for the monitoring of the study by the research sponsor. Further information regarding this document can be obtained from Gloucestershire Research & Development Support Unit on 01452 395726.

- d) The need to refer proposed amendments to the protocol to the LREC for further review and to obtain LREC approval thereto prior to implementation (except only in cases of emergency where the welfare of the subject is paramount).
- e) The requirement to furnish the LREC with details of the progress of the research project periodically (usually annually) and **failure to do this could result in approval to continue with the study being withdrawn**. Please also inform us of the conclusion and outcome of the research project and inform the LREC should the research be discontinued or any subject withdrawn altogether.
- f) It is the responsibility of the person conducting any Trial to ensure that all professional staff and management of NHS Trusts involved are notified that it is taking place.

Gloucestershire LREC is fully compliant with the International Conference on Harmonisation/ Good Clinical Practice (ICH GCP) Guidelines for the Conduct of Trials Involving the Participation of Human Subjects.

Please indicate your agreement to comply with the requirements outlined in this letter by signing both copies of this letter and returning one to Hazel Gage. Full approval does not commence until the signed copy is returned.

Yours sincerely



Dr M J Richards
Chairman, Gloucestershire LREC

cc Dr Sally Pearson, Director of Clinical Strategy, Glos Hospitals NHS Trust
Catherine Kendall, Cranfield Postgraduate Medical School

I agree to comply with the requirements outlined in this letter.

Signed _____

Date _____

Gloucestershire Research Ethics Committee

C/o North Bristol NHS Trust
Pembroke Room
Beaufort House
Southmead Hospital
Westbury-on-Trym
Bristol
BS10 5NB

Tel: 0117 323 5211
Fax: 0117 323 2832

14 July 2008

02/112G Investigation of cancerous and pre-cancerous changes in Barrett's oesophagus using techniques to advance the early diagnosis of malignancy

Dear Catherine

Thank you for your letter of 4th July outlining the changes to the study which will involve collaboration with Cancer Research UK to develop the molecular analysis of the samples obtained in the study. I can confirm that Standard Operating Procedures, section 5.3 to 5.40 have been consulted. The committee considers that this alteration is a minor amendment. The amendment does not therefore require ethical review by the committee, and may be implemented immediately provided that it does not affect the research governance approval for the research given by the R & D department for the relevant NHS care organisation.

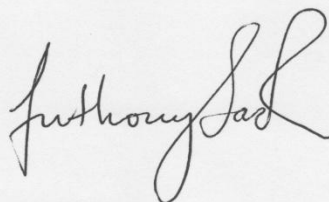
Documents received

Document	Version Number	Date
Covering letter		4 July 2008
Gloucestershire Committee letter		13 May 2004
Letter from Chief Investigator		6 May 2004
Consent Form	3	July 2008
Patient Information Sheet	3	July 2008

Statement of Compliance

The committee is constituted in accordance with the Governance Arrangements for research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK

Yours sincerely



Anthony Sack
Acting Committee Co-ordinator

anthony.sack@nbt.nhs.uk

Appendix VIII

PATIENT INFORMATION SHEET

Version 1 – January 2003

Early Diagnosis of Oesophageal Malignancy

Lead Researchers: Prof H Barr, Dr N Stone, Dr C Kendall

You are invited to take part in a research study being carried out at the Gloucestershire Royal Hospital. Before you decide whether you would like to participate it is important for you to understand why the research is being done, and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Please ask us if there is anything unclear or if you would like further information. Please take time to decide whether or not you wish to take part. Thank you for reading this information.

What is the purpose of the study?

The staff of Gloucestershire Royal Hospital and scientists at Cranfield University are working together to find new ways of providing advanced clinical support for the local community. An area where we feel we can make major advances is in improving our ability to detect oesophageal disease, pre-cancer and cancer.

Why have I been chosen?

We will be collecting samples from many people under surveillance for Barrett's oesophagus. Therefore this request does not mean that your diagnosis is unsure.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to participate you will be given this information sheet to keep and will be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. Your care, treatment or method of assessment will not be altered in any way by your decision to donate or otherwise.

What will happen to me if I take part?

Your doctor has explained to you that he/she will be removing some oesophagus tissue during the course of your care. Specialist staff within the hospital will examine this tissue and your care guided by what they find. If you are willing to allow us, we can use a small number of additional samples to help us develop new diagnostic tests. No additional procedures will be undertaken as a result of your consent. In addition there is no need for any lifestyle restrictions to participate in this study.

What is being tested in this study?

We are developing new techniques using light for the early detection of disease. Using advanced rapid tests we hope to be able to easily detect this disease earlier without the need for long uncomfortable procedures. Before these investigations can be carried out on patients,

it is necessary to assess its suitability with human tissue in the laboratory. The Optical Diagnosis Group based at the Gloucestershire Royal Hospital has over five years experience of working in this field.

What are the possible disadvantages and risks of taking part?

No additional procedures to your routine care will be undertaken as part of this study, therefore there are no associated additional risks.

What are the possible benefits of taking part?

No additional procedures to your routine care will be undertaken as part of this study, therefore there is no immediate clinical benefit to yourself. However, the information collected from this study may help us develop new techniques to improve the treatment of patients with Barrett's oesophagus.

Will my taking part in this study be kept confidential?

All information that is collected about you during the course of the research will be kept strictly confidential. If you consent to taking part in this study you will give permission for Dr N Stone & Dr C Kendall, from the Optical Diagnosis Group Cranfield Postgraduate Medical School to have access to your medical notes, where it is relevant to your taking part in research.

What will happen to the results of the research study?

We hope that the results will be published in scientific and medical journals. You will not be identified in any way in such a publication.

Who is funding and organising the research?

The research is funded by the NHS R&D Executive, Health Technology Development scheme (DoH & DTI) and the EPSRC. It is being carried out by Cranfield Postgraduate Medical School, a link between the Gloucestershire Hospitals NHS Trust and Cranfield University.

Who has reviewed the study?

The Gloucestershire Local Research Ethics Committee has reviewed the study.

Consumers for Ethics in Research (CERES) publish a leaflet entitled 'Medical Research and You'. This leaflet gives more information on medical research. A copy can be obtained from CERES, PO Box 1365, London N16 0BW.

Thank you for your help and co-operation. If you have any questions please contact Professor H. Barr, Gloucestershire Royal Hospital. Tel: 08454 226679 or Dr C. Kendall on 08454 225715

Appendix IX

CONSENT FORM

Early Diagnosis of Oesophageal Malignancy

Lead Researchers: Prof H Barr, Dr N Stone, Dr C Kendall

☐

Please initial each box

1. I confirm that I have read and understand the information sheet, dated for the above study and have had the opportunity to ask questions.

☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. In the event that I wish to withdraw from this study, data collection will cease immediately and I can request that all data collected is confidentially destroyed.

☐

3. I understand that Dr N Stone & Dr C Kendall, from the Optical Diagnosis Group Cranfield Postgraduate Medical School, may access sections of my medical notes, where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.

☐

4. I agree to take part in this study.

☐

5. I understand that you may take additional samples during the course of my care to help you develop new diagnostic tests. These samples may be made available for research after all the procedures required for my clinical management have been completed. I give my consent.

.....

8.13.1.1 Name of Patient

.....

Date

.....

Signature

.....

8.13.1.2 Name of person taking consent **Date**
(if different from researcher)

.....

.....

Signature

.....

8.13.1.3 Name of Researcher

.....

Date

.....

Signature

1 copy for patient, 1 for researcher, 1 for hospital notes

Appendix X

Power Calculation

This calculation aimed to establish the number of samples needed to demonstrate a sensitivity of 86% (attained from the preliminary analysis) for differentiating adenocarcinoma / HGD, from normal squamous, non-dysplastic Barrett's oesophagus and gastric tissue.

$$\begin{aligned} \text{TP} + \text{FN} &= z^2 \times (\text{SN} (1 - \text{SN})) / W^2 \\ \Rightarrow &= 1.96^2 \times (0.86 (1 - 0.86)) / 0.1^2 \\ \Rightarrow &= 185.01 \\ \text{NsN} &= 185.01/P \\ \Rightarrow &= 185.01/0.092 \\ &= 502 \end{aligned}$$

Where:

SN is the sensitivity of the probe for detecting HGD/cancer from my preliminary analysis.

W is the confidence interval (in this case 90%).

P is the proportion of spectral measurements that were from homogenous cancer/HGD.

This calculation determined that approximately 500 samples (NsN) would be required to demonstrate a sensitivity of 86% for cancer/HGD using 90% confidence intervals. This calculation assumes that only 9% of measured spectra will be HGD/cancer (as was the case in the preliminary analysis), and also that only one spectrum will be measured per biopsy. No allowance is made for tissue being excluded due to heterogeneity or diagnostic disagreement between expert pathologists. Also, the number of biopsy samples required to enable classification between grades of dysplasia could not be calculated here.

Given these limitations this power calculation was used only as a guide. The entire spectral dataset presented in this thesis could be used to optimise a more accurate power calculation prior to an in vivo study in the future.

Appendix IX

This appendix summarises the Cranfield MSc Thesis submitted by Ms Marta Losa in August 2011.[259] I co-designed and co-supervised this work and helped with methodology, data collection and interpretation of the results.

Do Raman Spectroscopy Signatures Correlate with Molecular Markers of Progression in Barrett's Oesophagus?

Summary:

Raman spectroscopy is a powerful analytical technique with the ability to distinguish oesophageal pathologies by detecting minute differences in their biochemistry. This technique has the potential to supersede routine histology reporting for establishing pathological diagnosis, however to date the link between the spectral signatures and underlying molecular changes has not been explored.

A thorough literature review was performed to identify a range of biomarkers which are known to correlate with the metaplasia–dysplasia–adenocarcinoma sequence (Table IX-1).

GROUP OF BIOMARKERS	BIOMARKERS
Proliferation markers	PCNA, Ki67, Mcm, Cyclin A
Cell cycle regulatory genes	Cyclin A, E, D1, p16, p53
Telomeres	Leucocyte telomere lengths, hTR and hTERT
Earliest abnormalities	CDX-1 and CDX-2
Growth signals	EGFR, TGF α , TGF β 1, HGF-R, COX-2
Adhesion molecules	E-Cadherin, α -catenin and β -catenin
Inflammation-association markers	COX-2, TNF α , Nf-kB
Vascular endothelial growth factors	VEGF AND VEGFR
Tumour suppressor genes	APC gene, p53, p16, p27

Table IX-1. Biomarkers which are known to correlate with progression from normal squamous oesophagus to adenocarcinoma.[259]

The biomarkers for further study were selected based on the following criteria:

- Detectable at a protein level.
- Detectable using a cheap, relatively straight-forward technique such as immunohistochemistry (IHC).
- Primary antibodies must be commercially available.
- Strong evidence as a biomarker for neoplastic progression.
- Previously well studied and well characterised.

Subsequently five biomarkers (p53, E-cadherin, Cyclin A, CDX-2 And hTERT) were selected for use in the study.

Forty-one biopsies were collected from 17 patients during routine Barrett's surveillance endoscopy. Biopsies were snap frozen in liquid nitrogen and sectioned. Contiguous sections were obtained from each sample (Figure IX-1).

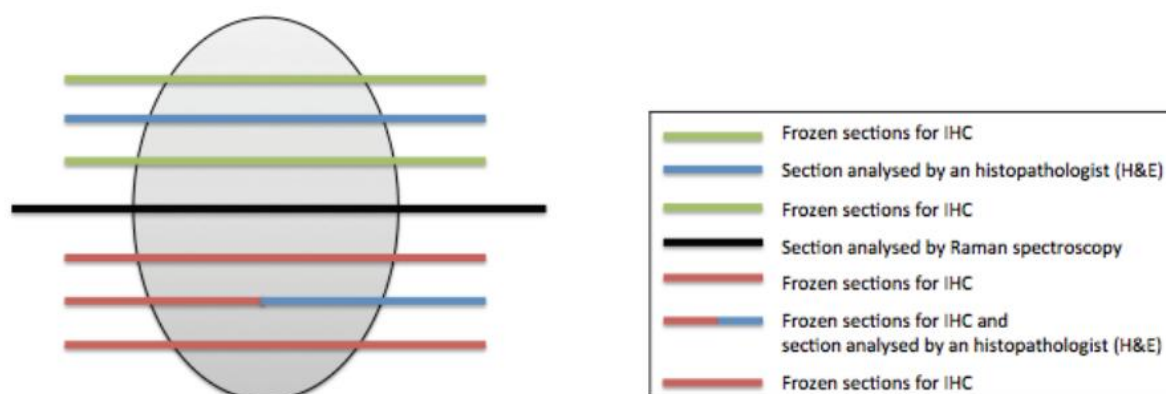


Figure IX-1. Scheme of sections obtained from each tissue sample.[259]

The sections highlighted in green in Figure IX-1 were analysed using p53 and E-cadherin IHC at Gloucestershire Royal Hospital (GRH). IHC analysis of CDX-2, cyclin A and hTERT was performed at Cranfield University (red sections). Standardised IHC protocols were carefully adhered to at both centres. The results of the Cranfield IHC were graded semi-quantitatively as 0, +, ++, or +++ for each of the biomarkers (Figure IX-2).

High quality Raman maps were acquired over 48 hours from two tissue samples. Maps were acquired using a customised Renishaw Raman System 1000 spectrometer coupled to a microscope. An 830 nm monochromatic laser was used for excitation and scattered light was

filtered and then dispersed and measured by a CCD. Figure 3-7 illustrates one of the high quality maps acquired using an x and y step size of 8.4 μm and an acquisition time of 30s per pixel. Mean spectra measured from different areas across the tissue section are illustrated in Figure IX-2.

The two biopsies for which all Raman, histological and IHC analysis was completed both had a histological diagnosis of Barrett's oesophagus. However, a slightly different pattern of molecular markers were identified in each sample:

Sample 1: Cyclin A ++, CDX-2 +, hTERT ++

Sample 2: Cyclin A +++, CDX-2 0, hTERT +++

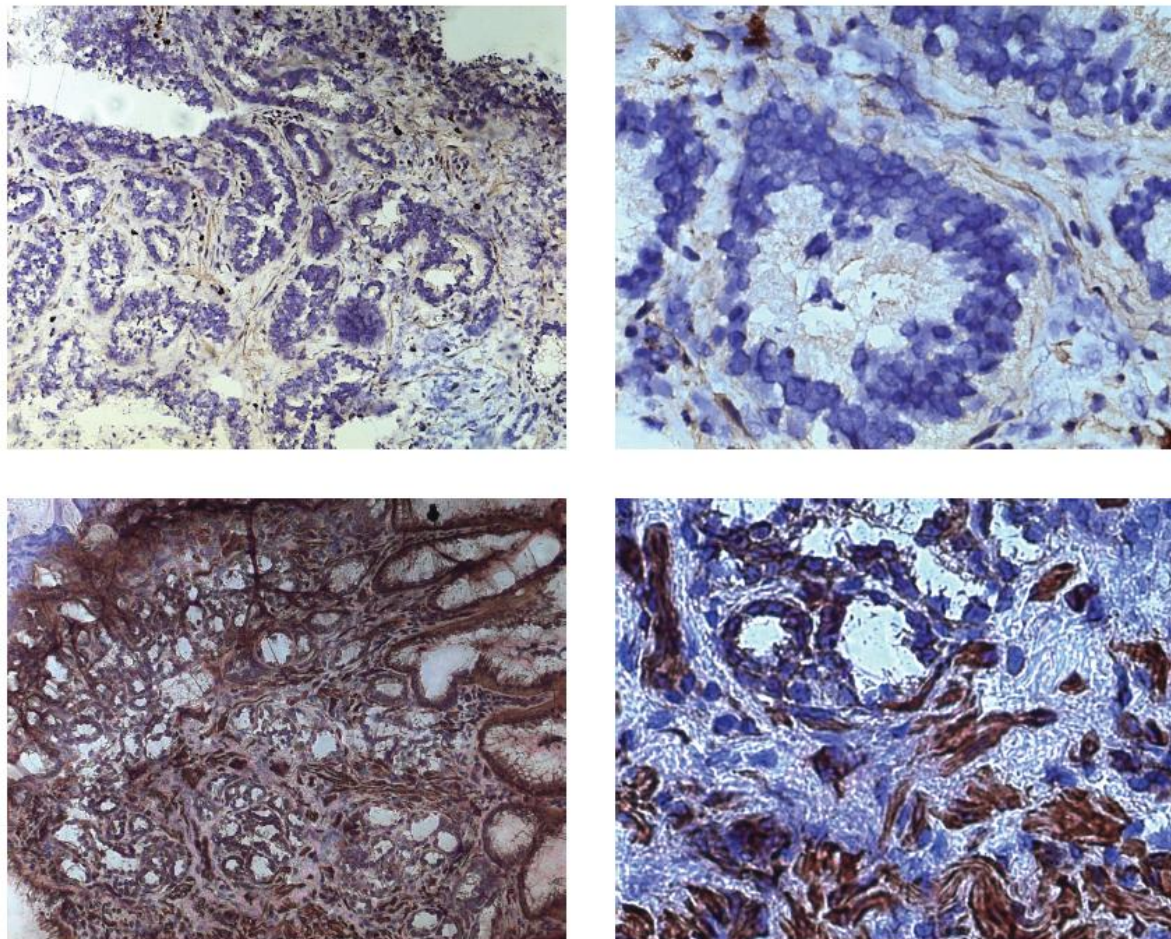


Figure IX-2. Example of positive results for hTERT staining in samples 1 and 2. Sample 1 was graded ++ and sample 2 +++. Photographs on the left were taken at 10x magnification, and those on the right at 40x magnification.[259]

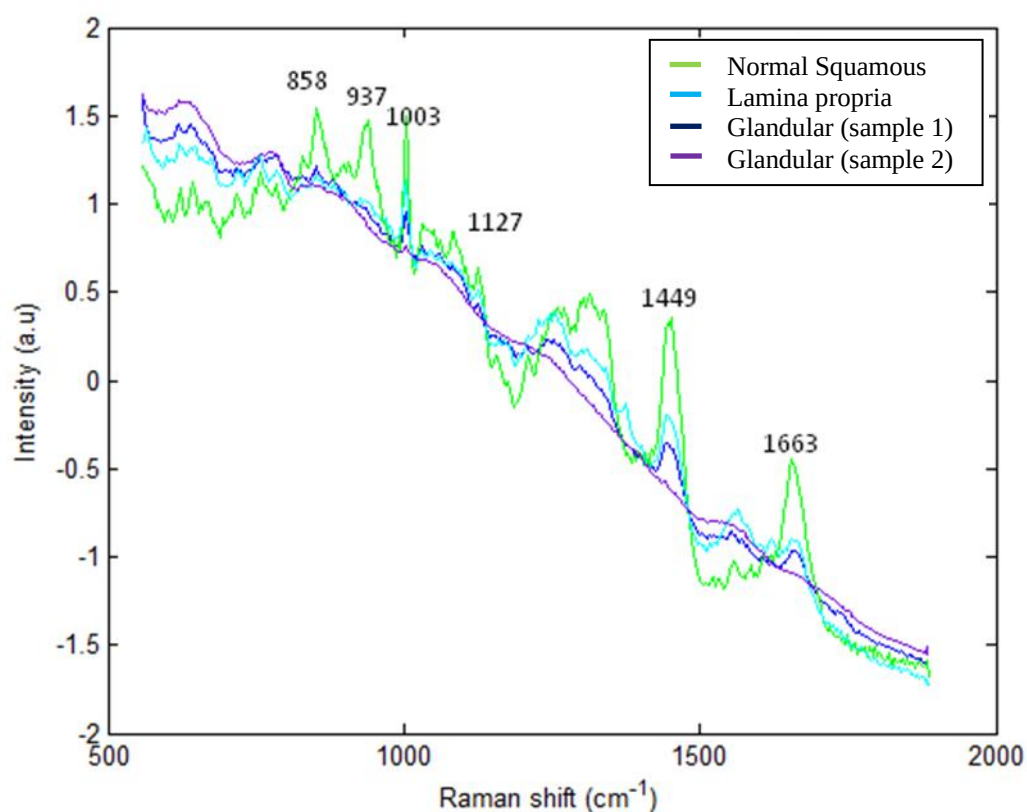


Figure IX-3. Mean Raman spectra acquired from different areas across two mapped tissue sections. Both of these sections had glandular areas – mean spectra for these areas are illustrated individually.[259]

This was a proof of principle study aiming to refine the methodology for identifying Raman signatures which might correlate with molecular markers, prompting further investigation. Within the scope and time-restraints of the study two biopsies were chosen for high quality mapping. IHC for detecting 5 biomarkers, and H&E staining, were performed on sections from the corresponding biopsies as illustrated in figure IX-2. As stated previously, independent, semi-quantitative grading of IHC results was performed for three of the five biomarkers (Cyclin A, CDX-2, and hTERT).

The mean Raman spectra from glandular areas were markedly different from squamous areas, consistent with previously reported studies (Figure IX-3).[34; 38; 43; 49; 245] Slight differences in mean spectra between the glandular areas in each sample were identified, which could potentially reflect biochemical differences corresponding to molecular markers of disease progression. However, in this study, no definite correlation could be identified.

This pilot study had a number of limitations:

- Grading of IHC results was based on staining across the sample as a whole. This could have been improved by defining an area of definite positively or negatively staining glands and proceeding to map that specific area.
- Due to the pilot nature of the study, and short timescales, the numbers of samples with histological, IHC and Raman data available was small.
- Biomarkers were chosen which were known to correlate with disease progression in Barrett's oesophagus. It is possible that several other biomarkers have a more pronounced Raman signal and so may be better 'Raman markers' of progression. To identify these, a larger panel of biomarkers will need to be investigated. It may be that future biomarkers should be chosen based on a known biochemical structure being predicted to generate a significant Raman signal.
- A larger sample size will enable multivariate classification of glandular spectra based on histological and molecular features. In this pilot study no statistical algorithms were developed to explore biomarker-Raman correlations.
- Significant fading of IHC stains was observed in this study, perhaps due to the use of acetone, as opposed to formalin, fixation. Frozen (rather than formalin fixed samples were used here to prevent corruption of Raman signals by unwanted artefacts caused by formalin fixation).

Although no correlations were definitely identified, this pilot study highlighted several potential methodological difficulties which must be addressed prior to further studies. A potential Raman-histology-biomarker classification model has been proposed which will require further study, with much greater sample sizes. Also, alternative techniques for biomarker identification could be employed to enable more specific and quantitative data to be acquired. A much larger group of biomarkers will also need to be evaluated. A PhD position at the Biophotonics Research Unit has been recently advertised to investigate Raman-biomarker correlations further.

Appendix XII

Spectral Cytopathology of Oesophageal Cells

Introduction:

A small proof of principle study assessing the potential for spectral cytopathology (SCP) of oesophageal cells was performed in collaboration with Professor Max Diem and his research group in Northeastern University, Boston. This research followed a BSG travelling fellowship where I visited Professor Diem's team and proposed a role for SCP in the oesophagus.

The ability to reliably and rapidly detect Barrett's oesophagus and / or neoplasia from samples of oesophageal cells could potentially lead to a screening test for these conditions. SCP is a novel technique which uses infrared spectroscopy to probe molecular differences in cell types potentially enabling objective diagnostic discrimination. Several studies have reported the success of SCP in diagnosing neoplasia from other sites including the oral cavity and the cervix but to date SCP of oesophageal cells has not been attempted.[252-254; 260]

Aims:

A proof of principle study to assess the potential for SCP of oesophageal cells to discriminate normal squamous, Barrett's oesophagus and neoplasia.

Ethics:

Ethical approval for this study was provided by the Gloucester Research Ethics Committee.

Methods:

Oesophageal brushings were taken during endoscopy from patients with Barrett's oesophagus with and without neoplasia. Samples were fixed in ThinPrep and transported to Boston. There they were vortexed off the brushes, filtered to remove debris and deposited onto Kevley low-

e slides. Infrared (IR) spectral data maps from a 2 mm x 2 mm spot were acquired at 4 cm⁻¹ resolution and 6.25 µm pixel size with 2 scans per pixel.

Data were processed using PapMap (which excluded spectral data which were not acquired from cells) and PCA.

Results:

Three samples were used for this preliminary analysis in order to ascertain whether SCP was a suitable approach for studying Barrett's Esophagus.

2 samples were taken from an area of normal squamous oesophagus and 1 from an area of Barrett's. 25 individual cells from each of the following samples were used for PCA. Mean absorption spectra for each sample are illustrated (Figure XII-1).

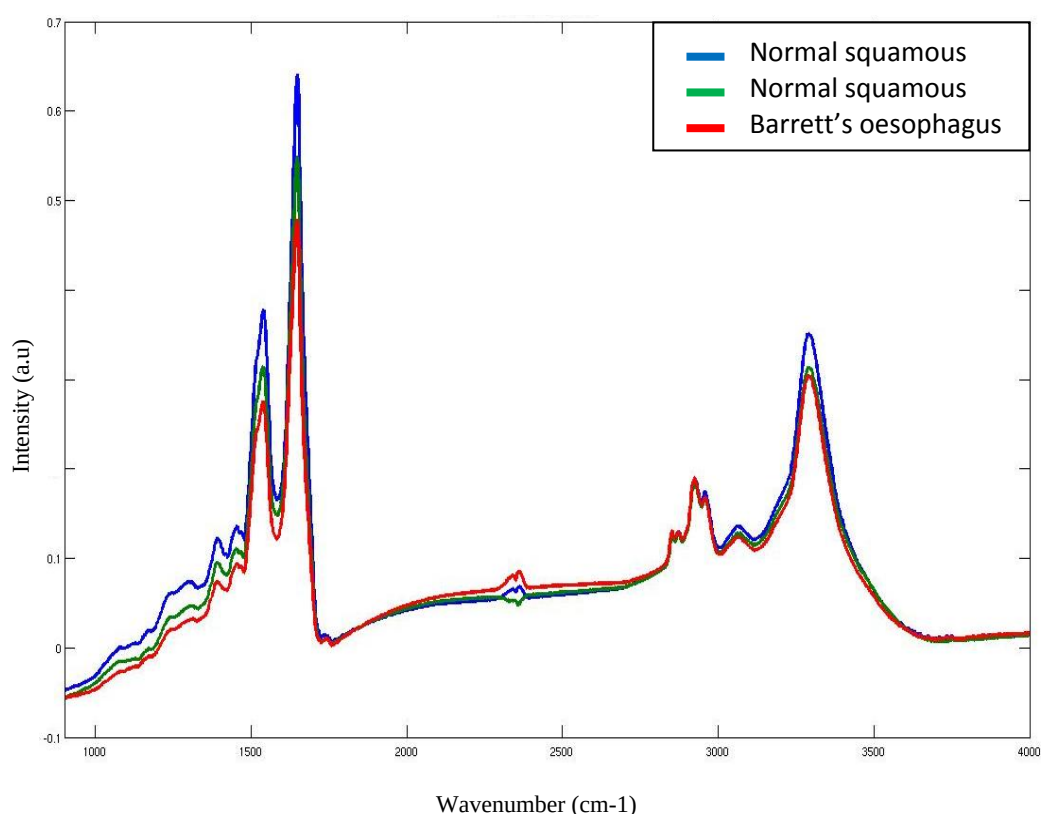


Figure XII-1. Mean absorption spectra for each sample.

PC loadings and PC scores plots are illustrated in Figures XII-2 and XII-3 respectively.

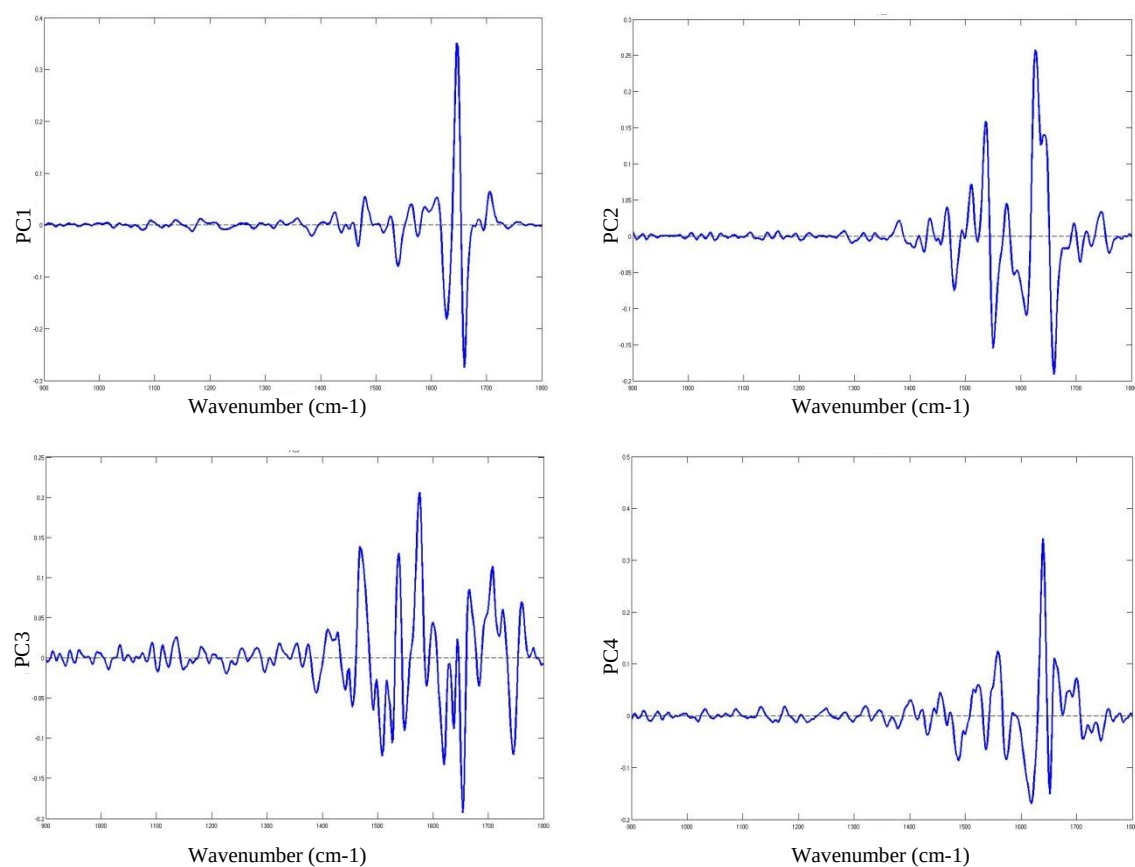


Figure XII-2. illustrates PC loadings 1-4.

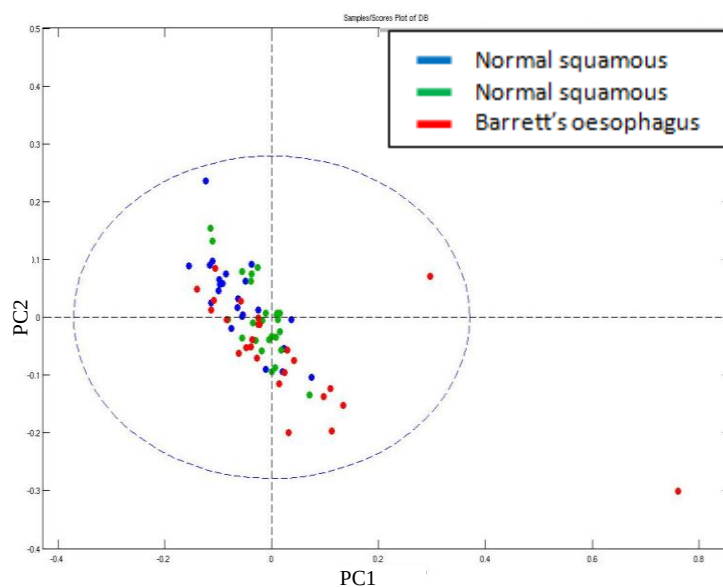


Figure X-3A PC scores plot 1 vs. 2.

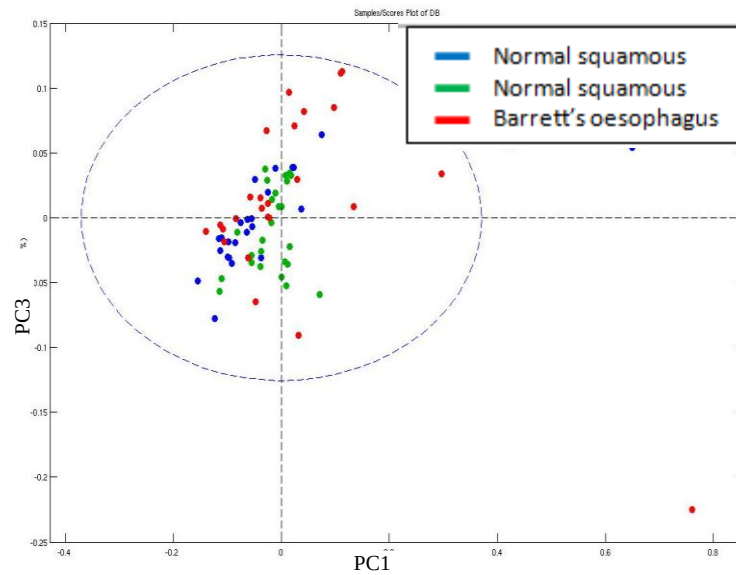


Figure X-3B PC scores plot 1 vs. 3.

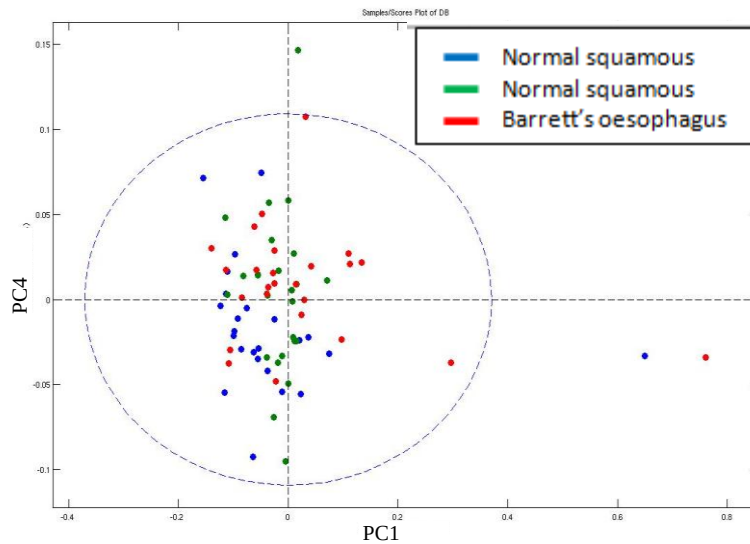


Figure X-3C PC scores plot 1 vs. 4.

Conclusions:

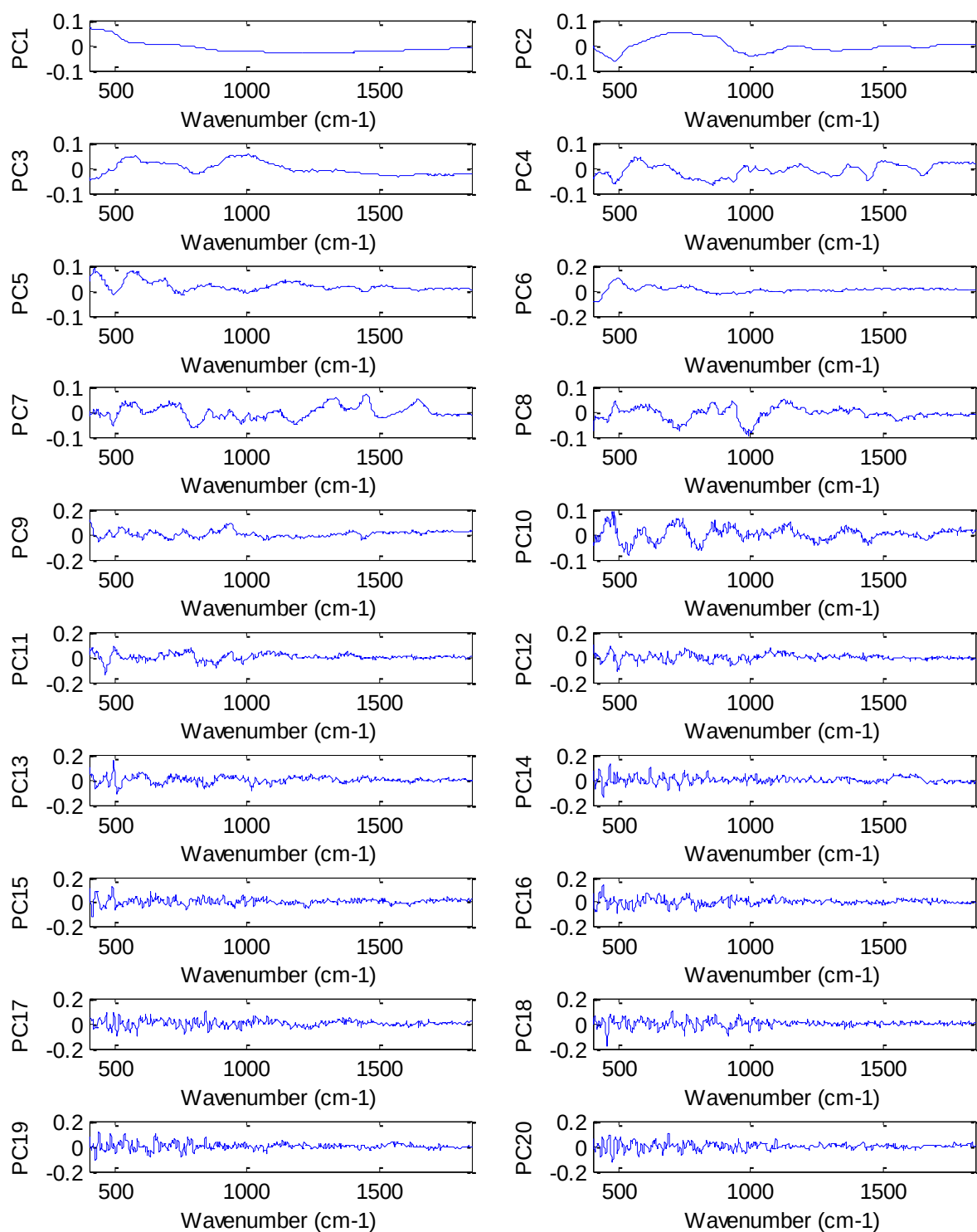
These data show that SCP of oesophageal cells is feasible. Although PCA was not conclusive it is likely that more cells from a larger sample pool may yield a better result. Also, some of the cells from the Barrett's sample were in fact squamous which will have hindered spectral discrimination.

ThinPrep solution did not appear to induce spectral artefacts. However, in future formalin fixation would be preferable. In addition, sample collection must be modified using larger more robust brushes in order to maximize the collection of whole oesophageal cells.

A firm amide I peak was present at 1652 cm^{-1} . More detailed biochemical analysis will be undertaken through further testing.

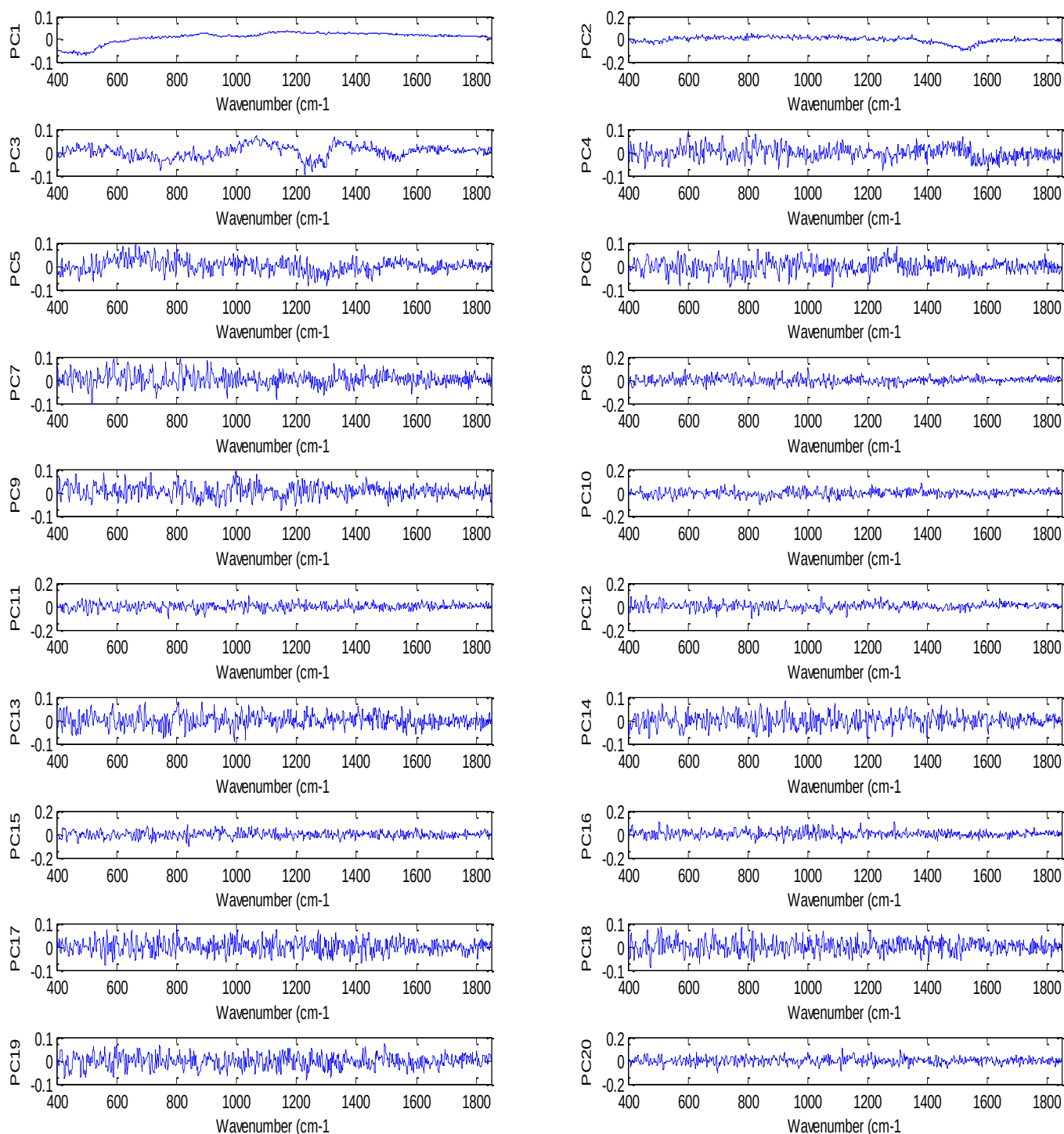
An international collaboration has been initiated between the Biophotonics Research Unit in Gloucester and the Department of Medical Diagnostics in Northeastern University, Boston. There is agreement that there is a clear clinical need for oesophageal SCP and further sample collection is currently in progress.

Appendix XIII



20 PC loadings following PCA on a three group (normal squamous; NDB/LGD; HGD/adenocarcinoma) classification model.

Appendix XIV



PCA was performed on the spectra acquired in the presence of endoscopic white light, NBI and after exclusion of all ambient light. PC loads 1-20 are plotted. No obvious Raman peaks are visible. Most of the PC loads represent spectral noise.

Appendix XV

Grants, prizes, awards, publications and presentations October 2010 – October 2012

Grants

- Royal College of Surgeons of England Research Training Fellowship. October 2011.
- Three Counties Oesophageal Research Training Fellowship October 2010.

Prizes

- First prize for best presentation of original research at the International Laser Europe Conference, London, May 2012.
- Translational Medicine Meeting Oral Presentation Prize. Gloucestershire Royal Hospital. March 2012.
- Winner of the West Midlands Surgical Society prize for best oral presentation. Worcester, November 2011.
- Winner of the best poster prize at the West Midlands Surgical Society. Worcester, November 2011. (2nd author).
- Winner of the Royal Society of Medicine Prize (Surgery Section) for best presentation of laboratory research. London, September 2011.
- Winner of the Twort Prize for best oral presentation at the 5th International Barrett's Symposium, London. April 2011.
- Nominated for The Royal Society of Medicine Trainee of the Year Award 2012. Decision takes place November 2012.

Awards

- The British Society of Gastroenterology John Bennett Travelling Research Fellowship This funded a trip to Harvard University, Boston and the Mayo Clinic, Rochester.

I organised the travelling fellowship myself. It included the following:

- Observation and discussion of complex upper GI endoscopy and Barrett's related research at Massachusetts General Hospital under the supervision of Professor W. Brugge (Director of Endoscopy, Harvard Medical School); and at the Mayo Clinic, Rochester under the supervision of Professor Ken Wang (Professor of Gastroenterology). Clinical cases included diagnostic and therapeutic EUS, ERCP including pancreatic stenting and spyglass, confocal microscopy, chromoendoscopy, EMR, ESD, cryotherapy, RFA and APC ablation, and EUS guided pseudocyst drainage and necrosectomy.
- Presentation and initiation of a collaboration with a group at Northeastern University, Boston, working on oesophageal spectral cytopathology using Raman and infra-red spectroscopy. This included analysis of oesophageal

brushings I had taken from patients in the UK and is part of an on-going collaboration.

- Participation on the biannual Harvard Medical School Advanced Therapeutic Endoscopy course - included performing EUS-guided pseudocyst drainage on porcine stomachs as well as CM, EUS + FNA, 360 RFA and EMR in anaesthetized pigs.

Publications

1. Almond LM, Hutchings, Barr H, Day J, Stone N, Kendall CA. Fibre-optic Raman Spectroscopic Analysis in the Upper Gastrointestinal Tract. *Spectroscopy Europe*, 2011. 23(3): 6-12.
2. Almond LM, Hutchings, Shepherd N, Barr H, Stone N, Kendall CA. Raman spectroscopy: a potential tool for early objective diagnosis of neoplasia in the oesophagus. *Journal of Biophotonics*. 2011. 4(10): 685-95.
3. Almond LM, Bowley DM, Karandikar SS, Roy-Choudhury SH. Role of CT colonography in symptomatic assessment, surveillance and screening. *Int J Colorectal Dis*. 2011 Aug; 26(8):959-66.
4. Almond LM, Bowley DM, Hendrickse C, Karandikar SS, Roy-Choudhury SH. CT Colonography following Colorectal Cancer Resection: a 'one-stop' assessment of metachronous mucosal lesions, local recurrence and distant metastases. *Radiology*, 2011; 260: 302-303.
5. Petal K, Almond LM. Transient Auditory Dysfunction – A Description and Study of Prevalence. Accepted for publication by *ENT Journal*. In Print.
6. Lloyd G, Hutchings J, Almond LM et. al.. Assessing the performance of spectroscopic models for cancer diagnostics using cross-validation and permutation testing. *Proc. SPIE*, 2012. 8219, 821-9.
7. Almond LM, Hutchings J, Kendall C et. al.. Preclinical evaluation of a Raman spectroscopic probe for endoscopic classification of oesophageal pathologies. *Proc. SPIE*, 2012. 8219, 90-7.
8. Almond LM, Barr H. Advanced endoscopic imaging in Barrett's oesophagus. *IJS*. 2012; 10(5):236-41.
9. Barr H, Almond LM. The Future of Upper Gastrointestinal Cancer. *Frontline Gastroenterology*. 2012; 3:i24-i27.
10. Williamson JML, Almond LM et. al.. Current management of Barrett's oesophagus. *Br J Hosp Med (Lond)*. 2012; 73(5):271-7.
11. Green S, Almond LM, Jankowski J et. al.. Surgery versus radical endotherapies for early cancer and high grade dysplasia in Barrett's oesophagus. *Cochrane Syst. Rev*. In Print.
12. Kallaway C, Almond LM et. al.. Advances in the clinical application of Raman Spectroscopy. Submitted to *TCRT*, June 2012. Under review.
13. Torrence AW, Almond LM et. al.. Has The Introduction of Integrated Positron-Emission Tomography and Computed Tomography (PET-CT) Improved The Staging, Survival or Early Recurrence In Oesophageal Cancer? Submitted to *BJS*. 2012. Under review.
14. Almond LM, Dwerryhouse S, Shepherd N, Barr H. Endoscopic Eradication of Early Barrett's Neoplasia. Submitted to *BJS*. 2012. Under review.
15. Almond LM et. al.. Assessment of a custom-built Raman spectroscopic probe for diagnosis of early oesophageal neoplasia. Submitted to *JBO*. 2012. Accepted subject to minor revisions.

Invited Articles

1. Almond LM. Shining Light on the Path to Cancer. The Nursing Times. Invited after winning 1st prize at the International Barrett's Symposium sponsored by Heartburn Cancer Awareness Support. <http://www.nursingtimes.net/nursing-practice/clinical-specialisms/gastroenterology/winning-oesophageal-cancer-presentation-shines-light-on-the-disease/5029108.article>.

Book Chapters

1. Almond LM, Barr H, Jankowski J. Barrett's Oesophagus. Under review by editors Professor Michael Griffin and Professor Simon Raimes. Elsevier Publishing. For inclusion in 5th Edition, A Companion to Specialist Surgical Practice: Oesophagogastric Surgery.
2. Almond LM, Barr H. Endoscopic Detection and Eradication of Dysplastic Barrett's Oesophagus. Accepted for publication by InTech publishing group. In print.
3. Almond LM, Barr H. Prevention of Oesophageal Cancer. Commissioned by Wiley-Blackwell publishing for the GI Oncology Handbook edited by Professor Jankowski and Dr Hawk. In Print.

Published Abstracts:

1. CT colonography after resection for colorectal cancer: safe & feasible. British Journal of Surgery, 2011, 98(S3): 80-217
2. Almond LM, Hutchings J, Kendall C, Stone N, Day J, Shepherd N, Barr H. Towards real-time 'biochemical endoscopy' for diagnosis of early barrett's neoplasia. Gut, 2011;60:A167-A168
3. Almond LM, Torrence A, Wadley M. Pre-operative PET-CT reduces early recurrence of oesophageal carcinoma following oesophagectomy. British Journal of Surgery, 2011, 98(S3): 1-79
4. Almond LM, Barr H et. al.. Prospects for Raman Spectroscopy for Real-time Endoscopic and Intra-operative Diagnosis of Cancer and Pre-cancerous Change.
5. Almond LM, Barr H. Endoscopic Resection offers prolonged survival from High grade Dysplasia and Intramucosal Cancer in Barrett's Oesophagus. British Journal of Surgery, 2011, 98(S3): 1-79
6. Almond LM, Hutchings J, Kendall C, Stone N, Day J, Shepherd N, Barr H. Mapping of the distal oesophagus using fibre-optic Raman Spectroscopy. British Journal of Surgery, 2011, 98(S3): 1-79
7. CT colonography after resection for colorectal cancer: safe & feasible. British Journal of Surgery, 2011, 98(S3): 80-217
8. Almond LM, Hutchings J. Towards Endoscopic Detection of Oesophageal Neoplasia using fibre-optic Raman Spectroscopy. Gut 2012. (In Print).

National / International Oral Presentations

1. Almond LM, Hutchings J, Kendall C, Stone N, Day J, Shepherd N, Barr H. Towards Endoscopic Detection and Targeting of Oesophageal Neoplasia using near-infrared spectroscopy. European Society of Esophagology. 2011
2. Almond LM, Hutchings J, Kendall C, Stone N, Day J, Shepherd N, Barr H. Endoscopic Detection of Oesophageal Dysplasia using Light. 5th International Barrett's Symposium. Winner of Twort prize. 2011
3. Almond LM, Torrence A, Wadley M. Pre-operative PET-CT reduces early recurrence of oesophageal carcinoma following oesophagectomy. Presented at ASGBI International Surgical Congress, '21st Century Surgery', 2011.
4. Almond LM, Barr H. Endoscopic Resection offers prolonged survival from High grade Dysplasia and Intramucosal Cancer in Barrett's Oesophagus. Presented at ASGBI International Surgical Congress, '21st Century Surgery', 2011.
5. Almond LM, Hutchings J, Kendall C, Stone N, Day J, Shepherd N, Barr H. Mapping of the distal oesophagus using fibre-optic Raman Spectroscopy. Presented at ASGBI International Surgical Congress, '21st Century Surgery', 2011.
6. Almond LM, Hutchings J, Kendall C, Stone N, Day J, Shepherd N, Barr H. Towards Endoscopic Detection and Targeting of Early Barrett's Neoplasia using Fibre-Optic Raman Spectroscopy. Royal Society of Medicine, 2011. Winner of 1st prize (surgery section) for best presentation of laboratory research.
7. Almond LM. Potential for Oesophageal Cancer Screening using Spectral Cytopathology? Presented at Northeastern University, Boston, US, 2011.
8. Almond LM. Inaugural John Bennett Travelling Oesophageal Fellowship Award Presentation. DDF (AUGIS / BSG). Liverpool, 2012.

International Poster Presentations

1. Almond LM, Hutchings, Kendall CA, Day J, Shepherd NA, Stone N, Barr H. Towards real-time 'biochemical endoscopy' for diagnosis of early Barrett's neoplasia. BSG, Birmingham, 2011.
2. Almond LM, Hutchings, Kendall CA, Day J, Shepherd NA, Stone N, Barr H. Towards Endoscopic Detection of Oesophageal Neoplasia using fibre-optic Raman Spectroscopy. DDF, Liverpool, 2012.

Courses

1. The 4th *mattu Oesophago-Gastric* Cancer Symposium. Guildford. December 2011.
2. Harvard University Advanced Therapeutic Endoscopy Course. Boston, US. November 2011.
3. Royal College of Surgeons Research Methods Course. London. November 2011.
4. ERCP Workshop. Birmingham, November 2011.
5. Endoscopic Mucosal Resection course. Gloucestershire Royal Hospital. May 2011.
6. Good Clinical Practice course. University of Birmingham. November 2010.
7. Essentials for New Clinical Research Staff. Bath. October 2010.