Fluorescence and Diffuse Reflectance Spectroscopy and Endoscopy for Tissue Analysis

Vincent Sauvage
Imperial College London
Department of Surgery and Cancer

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Work presented in this thesis is my own work except for where indicated.

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Abstract

Biophotonics techniques are showing great potential for practical tissue diagnosis, capable of localised optical spectroscopy as well as wide field imaging. Many of those are generally based on the same concept: the spectral information they enable to acquire encloses clues on the tissue biochemistry and biostructure and these clues carry diagnostic information. Biophotonics techniques present the added advantage to incorporate easily miniaturisable hardware allowing several modalities to be set up on the same systems and authorizing their use during minimally invasive surgery (MIS) procedures. The work presented in this thesis aims to build on these advantages to design biophotonics instruments for tissue diagnosis. Fluorescence and diffuse reflectance, the two modalities of interest in this work, were implemented in their single point spectroscopic and imaging declinations. Two “platforms”, a spectroscopic probe setup and an optical imaging laparoscope, were built; they included either one of the two aforementioned modalities or the two of them together.

The spectroscopic probe system was assembled to detect lesions in the digestive tract. In its first version, the setup included a dual laser illumination system to carry out an ex vivo fluorescence study of non-alcoholic fatty liver diseases (NAFLD) in the mouse model. Outcomes of the study demonstrated that healthy livers could be distinguished from NAFLD livers with high classification accuracy. Then, the same fluorescence probe inserted in a force adaptive robotic endoscope was applied on a fluorescence phantom and a liver specimen to prove the feasibility of recording spectra at multiple points with controlled scanning pattern and probe/sample pressure (known to affect the spectra shape). This approach proposed therefore a convincing method to perform intraoperative fluorescence measurements. The fluorescence setup was subsequently modified into a combined fluorescence/diffuse reflectance spectroscopic probe and demonstrated as an efficient method to separate normal and diseased tissue samples from the human gastrointestinal tract.

Following the single point spectroscopy work, imaging studies were conducted with a spectrally resolved laparoscope. The system, featuring a CCD/filter wheel unit clipped on a traditional laparoscope was validated on fluorescence phantoms and employed in two experiments.
The first one, building on the spectroscopy study of the gastrointestinal tract, was originally aimed at locating tumour in the oesophagus but a lack of tissue availability prevented us from doing so. The system design and validation on fluorophores phantoms were nevertheless described. In the second one, the underarm of a pig was imaged after injection of a nerve contrast agent in order to test the feasibility of \textit{in vivo} nerve delineation. Fluorescence was detected from the region of interest but no clear contrast between the nerve and the surrounding muscle tissue could be detected. Finally, the fluorescence imaging laparoscope was modified into a hyperspectral reflectance imaging laparoscope to perform tissue vasculature studies. It was first characterized and tested on haemoglobin phantoms with varying concentrations and oxygen saturations and then employed \textit{in vivo} to follow the haemoglobin concentration and oxygen saturation temporal evolutions of a porcine intestine subsequently to the pig’s termination. A decrease in oxygen saturation was observed. The last experiment consisted in monitoring the tissue re-oxygenation of a rabbit uterus transplant on the recipient animal, a successful tissue re-perfusion after the graft was highlighted.
I thank Dr Daniel Elson and Prof. Yang for the trust they put in me. They offered me the opportunity to perform research in medical optics in top notch facilities. A wholeheartedly thank you to my supervisor Daniel whose competence, indefectible optimism and dedication supported my efforts throughout the course of this PhD.

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ABSTRACT ............................................................................................................................... 2
ACKNOWLEDGMENT .............................................................................................................. 4
CONTENTS ............................................................................................................................... 6
LIST OF FIGURES .................................................................................................................... 9
LIST OF TABLES .................................................................................................................... 13
LIST OF ABBREVIATIONS .................................................................................................... 14
1.INTRODUCTION .................................................................................................................. 15
2.LIGHT-TISSUE INTERACTION AND TISSUE SPECTROSCOPY ..................................... 20
  2.1 LIGHT-TISSUE INTERACTIONS ...................................................................................... 20
  2.2 TISSUE FLUORESCENCE SPECTROSCOPY ................................................................. 21
    2.2.1 Fluorescence phenomenon ...................................................................................... 21
    2.2.2 Tissue fluorophore spectra .................................................................................... 23
  2.3 DIFFUSE REFLECTANCE SPECTROSCOPY .................................................................... 25
    2.3.1 Absorption and scattering phenomena ................................................................ 25
    2.3.2 Tissue chromophores ........................................................................................... 27
  2.4 MODELS OF LIGHT TRANSPORT IN TISSUE .............................................................. 31
    2.4.1 Radiative transfer equation ................................................................................... 31
    2.4.2 Analytical models ................................................................................................. 32
    2.4.3 Numerical and experimental models .................................................................... 34
  2.5 IMPLEMENTATION OF FLUORESCENCE AND DIFFUSE REFLECTANCE .................. 36
    2.5.1 Fibre-optic probe based spectroscopy .................................................................. 36
    2.5.2 Importance of the probe geometry ....................................................................... 39
  2.6 FLUORESCENCE AND DIFFUSE SPECTROSCOPY OF DISEASED TISSUE: MAIN APPLICATION TO DATE: DETECTION OF PRECANCEROUS AND CANCEROUS TISSUE ........................................................................................................... 41
    2.6.1 Fluorescence spectroscopy .................................................................................... 41
    2.6.2 Diffuse reflectance spectroscopy .......................................................................... 44
  2.7 SUMMARY .................................................................................................................... 47
  2.8 REFERENCES ................................................................................................................. 48
3.FLUORESCENCE SPECTROSCOPY AS A LIVER DISEASE DIAGNOSTIC TOOL ...... 55
  3.1 DUAL EXCITATION FLUORESCENCE SPECTROSCOPY SYSTEM ................................. 56
  3.2 NON ALCOHOLIC FATTY LIVER DISEASE .................................................................. 58
    3.2.1 Clinical characteristics, prevalence and current diagnostic method limitations .... 58
    3.2.2 Fluorescence spectroscopy to assess liver state: incentive and previous work .. 61
List of figures

Figure 2-1: Different types of light-tissue interactions: specular highlight (reflection), scattering, absorption, fluorescence. ........................................................................................................................................... 21
Figure 2-2: Partial Jablonsky diagram illustrating the electronic and vibrational transitions involved in light absorption and fluorescence emission. Only the two first electronic (ground and first excited) states are represented here. ........................................................................................................... 22
Figure 2-3: Excitation and emission spectra of the main endogenous fluorophores. Reprinted from [7] with permission from John Wiley and Sons................................................................. 24
Figure 2-4: Absorption coefficients of some of the main tissue absorbers between 100 nm and 12000 nm. Reprinted from [24] with permission from American Chemical Society. ................ 28
Figure 2-5: Oxygen dissociation curve, haemoglobin saturation increases with oxygen partial pressure. Reprinted from [29] with permission from BMJ Publishing Group Ltd. .................. 29
Figure 2-6: Molar extinction coefficients of oxyhaemoglobin and deoxyhaemoglobin from different sources. Data compiled from [30, 33, 35]. .................................................................................................. 30
Figure 2-7: Outline of a typical optical spectroscopic system of tissue diagnosis. ......................... 37
Figure 2-9: A common geometry of optical fibre probe. PTD denotes probe-to-target distance and SDS the source-to-detector separation. ....................................................................................... 39
Figure 2-10: (a) Non-normalized and (b) normalized (divided by the area under the curve) fluorescence emission spectra from normal and malignant oesophagus tissues. Reprinted from [73] with permission from John Wiley and Sons. ................................................................. 43
Figure 3-1: Experimental set-up of dual-excitation wavelength fluorescence spectroscopy probe. From [1]. ......................................................................................................................... 57
Figure 3-2: Emission fluorescence spectrum of a FAD solution excited at 375 nm and recorded with a spectrometer (reference) and with the dual-excitation wavelength fluorescence spectroscopy system (measured). ........................................................................ 58
Figure 3-3: Typical H&E stained liver sections at x 400 magnification showing (a) mouse liver fed standard chow, (b) genetically modified mouse liver fed standard chow showing steatosis only, (c) genetically modified mouse liver fed MCD showing steatosis, lobular inflammation and hepatocyte ballooning. From [1] ........................................................................................................ 68
Figure 3-4: Mean malondialdehyde concentration in the livers of the mice ± standard error of the mean (SEM). From [1] ........................................................................................................ 68
Figure 3-5: Mean ALT levels in the plasma of the mice ±SEM. From [1] ........................................ 69
Figure 3-6: Mean spectra at (a) 375 nm and (b) 405 nm excitation and error bars representing standard deviation. From [1] ........................................................................................................ 71
Figure 3-7: Posterior probability of fluorescence spectra from interrogated tissue sites belonging to their correct disease group (as classified by histopathology): control (114 sites), mild steatosis (72 sites), steatohepatitis (114 sites). From [1].............................................................. 72
Figure 3-8: Mean spectra at (a) 375 nm and (b) 405 nm excitation and error bars representing standard deviation.

Figure 3-9: Posterior probability of fluorescence spectra from interrogated tissue sites belonging to their correct disease group (as classified by histopathology): control (252 sites), moderate steatosis (258 sites), severe steatosis (102 sites).

Figure 3-10: (a) Force sensor mechanism including four main elements: a clamp (1) a linear motor which translates backward and forward the probe (2) the force sensor (3) housing (4), moving parts are indicated in red illustration of the robotic endoscope, (b) the probe and the camera are also showed. From [52].

Figure 3-11: (a) Fluorescence spectra acquired at 0 (non-contact), 50, 100 and 150 mN and (b) the corresponding images acquired by the video camera. From [52].

Figure 3-12: Fluorescent spectra acquired by the 6 emission fibres at different points along the probe trajectory traced clock wisely (as indicated by the red arrow) on the phantom consisting of yellow and green fluorescent stripes separated by white paper stripes. Measurement sequences at 3 points along the trajectory are detailed each consisting of a fluorescence spectrum (bottom), an onboard camera image (top-right) and an external camera image (top-left). From [52].

Figure 3-13: Fluorescent spectra acquired by each of the six emission fibres at different points along the probe trajectory on liver and their corresponding images acquired by the onboard camera. From [52].

Figure 4-1: Fibre arrangement at the distal end of the probe consisting of two illumination fibres, one for each modality, and a detection fibre.

Figure 4-2: Fluorescence-diffuse reflectance system featuring an additional white light tungsten-halogen lamp in addition to the two laser diodes of the dual-excitation fluorescence system presented in chapter 3.

Figure 4-3: Standard colour chart containing 24 patches with standardized colours. (X-rite, Inc., USA).

Figure 4-4: Diffuse reflectance spectra recorded from the 24 patches forming the Macbeth colour chart and compared with the mean spectra (error bars represent one standard deviation) measured from the database.

Figure 4-5: Cross-section (top view) of a probe-sample configuration where only the detection and fluorescence illumination probes are in contact with the sample causing an incomplete measurement sequence where the diffuse reflectance spectrum is missing.

Figure 4-6: Mean (a) fluorescence and (b) reflectance spectra of upper GI tissues. Error bars represent standard deviation.

Figure 4-7: Mean (a) fluorescence and (b) reflectance spectra of upper GI tissues and mean fluorescence. Error bars represent standard deviation.

Figure 5-1: Optical imaging as implemented in this thesis: a series of images are acquired at different wavelengths forming a data cube with two spatial dimensions and one spectral
dimension. By recording multiple images, it becomes possible to build the spectrum (fluorescence or diffuse reflectance) at each pixel forming the image. .................................................... 113

Figure 5-2: (a) Front and (b) side views of a typical minimally invasive surgery scene. Small incisions are performed through the abdomen with trocars to enable the passage of laparoscope and surgical apparatus. Adapted from [34]. .................................................... 117

Figure 5-3: Cross-section of a laparoscope. Adapted from [37]. .................................................... 117

Figure 5-4: Schematic of the multispectral laparoscopic imaging system. .................................................... 122

Figure 5-5: Photograph of the laparoscope setup. ............................................................................. 123

Figure 5-6: Spectrum of the metal-halide light source and transmission profiles of the chosen emission and excitation filters. ..................................................................................... 125

Figure 5-7: Location of the solutions of protoporphyrins IX, coumarin 334, FAD, and the control solution (water). ........................................................................................................ 126

Figure 5-8: Raw intensity fluorescence images of the fluorescent phantoms at each excitation/emission filter combination of interest. ..................................................................................... 127

Figure 5-9: Metal-halide source spectrum, fluorescence excitation and emission spectra and transmission profiles of the excitation and emission spectra. .................................................... 129

Figure 5-10: (a) Black and white light image of tubes containing water or the dye solution (indicated by red dots), (b) corresponding fluorescence image. .................................................... 130

Figure 5-11: View of the laparoscope illuminating the brachial plexus at the axilla level with blue light (417/60 nm bandpass excitation filter). .................................................... 131

Figure 5-12: Right axilla looking anteroposteriorly (images acquired with the laparoscope system). (a) White light image featuring a large bifurcating trunk of brachial plexus (green arrow), axillary vein (yellow arrow), medial muscular border of axilla–pectoral/serratus muscle (blue arrow) 5 h after injection and (b) corresponding fluorescence image. .................................................... 132

Figure 5-13: In vivo fluorescence image of an axilla ROI taken with the multispectral laparoscope (left) and ex vivo fluorescence images of the brachial plexus and the axillary vein acquired with the open-field hyperspectral camera (Nuance camera, CRI, Inc., USA) (right).) ............................................................................. 133

Figure 5-14: Temporal evolution of the nerve-muscle contrast (t=0 min corresponds to the time of injection). ........................................................................................................ 133

Figure 5-15: Cross-sectional images of (a) a control rat brachial plexus nerve injected with the formulation agent and (b) the dye-injected pig brachial plexus. Doughnut structures pointed out by red arrows illustrate examples of nerves fibres where contrast agent aggregated. .... 135

Figure 6-1: Anatomy of the lower digestive system showing the colon (also called large intestine or large bowel) and small intestine (also called small bowel). Reproduced from [22]. ............................................................................. 145

Figure 6-2: Hyperspectral laparoscopic system consisting in a CCD camera and a LCTF attached to a regular rigid laparoscope. ..................................................................................... 147

Figure 6-3: Emission spectrum of the Xenon lamp emitting in the visible. Output intensity gradually increases from 390 nm and drops rapidly above 665 nm. ................................. 148
Figure 6-4: 1951 USAF test patterns. This target was used to determine optical system resolution.

Figure 6-5: Example of an intensity profile along one of the 1951 USAF test chart patterns.

Figure 6-6: Sterile sheath used to isolate laparoscope non-sterile camera and video cables from the patient. The red circle indicates the exit window through which the laparoscope is inserted.

Figure 6-7: Evolution of the peak-to-valley ratio with lines spacing of the successive targets.

Figure 6-8: Spectral profiles provided by the LCTF for wavelength set between 420 nm and 660 nm with a 10 nm increment.

Figure 6-9: LCTF bandwidth evolution with wavelength.

Figure 6-10: Linear relationship between measured intensity and (a) exposure time and (b) sample emission.

Figure 6-11: Absorption spectrum between 500 nm and 650 nm (spectral resolution: 10 nm) taken at one pixel within the well containing the 6 g/L solutions of (a) deoxyhaemoglobin and (b) oxyhaemoglobin.

Figure 6-12: (a) Deoxyhaemoglobin and (b) oxyhaemoglobin maps of the well plate containing the haemoglobin phantoms. Dark blue colour corresponds to low concentrations (0 g/L) and dark red colour indicates high concentrations (7 g/L).

Figure 6-13: Comparison of measured haemoglobin concentrations with the real concentrations.

Figure 6-14: Haemoglobin oxygen saturation map. Dark blue colour corresponds to low saturation (0) and dark red colour indicates high saturation (1).

Figure 6-15: (a) Deoxyhaemoglobin and (b) oxyhaemoglobin maps of a region of interest (approximate dimensions: 3.5×5cm) of the porcine colon 6 min after termination. Blue colour indicates low concentrations and red colour indicates high concentrations.

Figure 6-16: Temporal evolution of oxy and deoxyhaemoglobin concentrations and oxygen saturation subsequent to pig’s termination.

Figure 6-17: A schematic of the trinocular laparoscope detailing the specifications of the optics for the two channels for colour stereo vision and the hyperspectral imaging channel. Adapted from [46].

Figure 6-18: (a) RGB image of the uterus in the donor rabbit, (b) corresponding oxygen saturation map, (c) RGB image of the uterus shortly after the transplantation in the recipient rabbit, (d) corresponding oxygen saturation map. The black circle indicates (d) the approximate localisation of the aorta. Colour scale varies between dark blue (0) and dark red (1).
List of tables

Table 2-1: Excitation and emission maxima of some key endogenous fluorophores of interest in this thesis. ............................................................ 23
Table 2-2: Haemoglobin absorption spectra reported in the literature. ........................................ 29
Table 3-1: Methods used to diagnose NAFLD........................................................................ 59
Table 3-2: Details of NAFLD activity scoring. ......................................................................... 66
Table 3-3: Median NAFLD activity score for the mice. From [1].............................................. 68
Table 3-4: Mean malondialdehyde concentration in the livers of the mice ±SEM. From [1]. . 69
Table 3-5: Mean ALT levels in the plasma of the mice ±SEM. From [1]................................. 70
Table 3-6: Confusion matrix presenting the ability of fluorescence spectroscopy to distinguish between control, mild steatosis and steatohepatitis disease groups with the concatenated spectra. From [1] ............................................................. 72
Table 3-7: Confusion matrix presenting the ability of fluorescence spectroscopy to distinguish between control, moderate and severe steatosis disease groups with the concatenated spectra. .................................................................................................................................... 74
Table 4-1: Comparison of the sRGB values of each of the 24 colour patches as provided by the manufacturer and those retrieved from the reflectance spectra. ......................................................... 96
Table 4-2: Number of spectra recorded and patients investigated with each modality and their combination............................................................................................................................. 98
Table 4-3: Distribution of biopsies as classified by histopathology in the upper GI.............. 100
Table 4-4: Distribution of biopsies as classified by histopathology in the lower GI. .............. 100
Table 4-5: Pairs of wavelength chosen after ROC analysis .................................................. 103
Table 4-6: Sensitivity, specificity, positive and negative predictive values obtained after SMLR leave-one-out cross validation with fluorescence, diffuse reflectance and the combination of the two. Modalities providing the best results for each classification are indicated in bold. Grey boxes indicate undefined negative predictive values. ......................................................... 104
Table 5-1: Correspondence between imaging modes and their excitation/emission filter combination................................................................................................................................. 125
Table 7-1: Cost of the four optical systems designed in this thesis........................................ 177
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT</td>
<td>Alanine transaminase</td>
</tr>
<tr>
<td>AOTF</td>
<td>Acousto-optical tuneable filter</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the (ROC) curve</td>
</tr>
<tr>
<td>C334</td>
<td>Coumarin 334</td>
</tr>
<tr>
<td>CCD</td>
<td>Charged-coupled device</td>
</tr>
<tr>
<td>CE</td>
<td>Collection efficiency</td>
</tr>
<tr>
<td>CK-18 fragments</td>
<td>Cytokeratin-18 fragments</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FWHM</td>
<td>Full width at half maximum</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal tract</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HbR</td>
<td>Deoxyhaemoglobin</td>
</tr>
<tr>
<td>HbO₂</td>
<td>Oxyhaemoglobin</td>
</tr>
<tr>
<td>IU</td>
<td>International unit</td>
</tr>
<tr>
<td>LCTF</td>
<td>Liquid crystal tuneable filter</td>
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<tr>
<td>LED</td>
<td>Light-emitting diode</td>
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<tr>
<td>LIFE</td>
<td>Lung imaging fluorescence endoscope</td>
</tr>
<tr>
<td>MCD</td>
<td>Methionine choline deficient</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>MIS</td>
<td>Minimally invasive surgery</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<tr>
<td>NA</td>
<td>Numerical aperture</td>
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<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
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<tr>
<td>NAD(P)H</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
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<td>NAFLD</td>
<td>Non-alcoholic fatty liver disease</td>
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<td>NAS</td>
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<td>Non-alcoholic steatohepatitis</td>
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<td>NOTES</td>
<td>Natural orifice transluminal endoscopic surgery</td>
</tr>
<tr>
<td>NPV</td>
<td>Negative predictive value</td>
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<td>Positron emission tomography</td>
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<td>Protoporphyrins IX</td>
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<td>PPV</td>
<td>Positive predictive value</td>
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<tr>
<td>PTD</td>
<td>Probe-to-target distance</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation</td>
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<tr>
<td>ROC</td>
<td>Research operating characteristic</td>
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<td>ROI</td>
<td>Region of interest</td>
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<tr>
<td>SDS</td>
<td>Source-to-detector separation</td>
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<tr>
<td>SEM</td>
<td>Standard Error of the mean</td>
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<tr>
<td>SMLR</td>
<td>Sparse multinomial logistic regression</td>
</tr>
<tr>
<td>TBA</td>
<td>Thiobarbituric acid</td>
</tr>
<tr>
<td>TBARS</td>
<td>Thiobarbituric acid reactive substances</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organisation</td>
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Chapter 1

Introduction

Lesions of any type are spotted and graded based on their appearance making the surgeon’s eye the main tool to diagnose pathologies. This explains why some of the most important milestones in the history of medicine have been the results of the introduction of instruments extending imaging capabilities by enabling the clinician to visualize regions of the body difficult to access. For instance, flexible endoscopes permitted tumour location in the oesophagus and the colon, and rigid laparoscopes enabled abdominal cavity inspection minimally invasively. Later, medical imaging also had a dramatic impact on disease diagnostics by permitting the non-invasive volumetric visualisation of tissue. X-ray, positron emission tomography (PET), computed tomography (CT) and magnetic resonance imaging (MRI) provide 3-D macroscopic structural views of brain, chest, and bones allowing estimation of the extent of a tumour or diagnosis of heart diseases with very high resolutions.

In spite of the enormous benefits brought by these medical imaging technologies, the most common criticism towards them, besides their cost, infrastructure constraints and inadequacy for infants and old patients, is the absence of functional and molecular contrast. This prevents detection of early stages of diseases characterized by molecular changes which precede and trigger anatomical modifications. Another limitation of the current imaging techniques is the impossibility to use them intraoperatively to provide real time information (with the exception of X-ray fluoroscopy). Consequently, clinicians can only rely on tissue pre-operative images and palpation to spot lesions during an operation. This may either result in tissue regions being wrongly assessed as diseased leading to unnecessary biopsies or missed lesions.
It is proposed in this thesis to enrich the limited spectral information provided by white-light illumination and the eye of the surgeon through the use of biophotonics techniques in order to improve the available diagnosis information. Biophotonics methods consist in studying the interaction between light and tissue that can yield data at microscopic spatial resolution and nanometre spectral resolution, with the added benefit that most biomedical photonics techniques can be extended to an imaging mode. Biophotonics techniques encompass a broad range of modalities and wavelength range (from gamma rays to the infrared). The type of light-tissue interaction (absorption, transmission, diffuse reflection, fluorescence etc.) used may differ, but they share the common goal to supply tissue diagnostic information to the surgeon with a minimum of trauma for the patient and in a minimum time.

Biophotonics techniques are of interest as they have the potential to address the two main shortcomings of current medical imaging techniques. Firstly, some biophotonics modalities such as fluorescence or Raman spectroscopy can probe biochemical alterations presaging diseases. Secondly, they can be used intraoperatively because they can be implemented in a compact way and generate data in real-time, conducive to their use during operations. In fact, as will be demonstrated, photonics systems enable excellent system integration with laparoscopes and endoscopes, which could bring enormous benefits in terms of clinician training length, and cost of installation in hospitals if these technologies are to be brought in operating theatres in the future. Also, biophotonics techniques do not cause trauma to the patients, in contrast to medical imaging, which often uses ionising radiation and biopsy, which is synonym for tissue removal. Finally, another advantage is the possibility to combine several modalities (fluorescence, reflectance, Raman, etc.) in one setup relatively easily giving access to a broad range of functional contrasts.

Several tools stemming from biomedical photonics are now well-established and commonly used in hospitals: endoscopes, pulse-oximeters (which give information on the patient's oxygen saturation); microscopes used to examine histological sections; optical coherence tomography systems provide longitudinal scans of the retina in ophthalmology; colposcopes act as a magnifying binocular to recognize anomalous tissue during cervix cancer screening. Other biophotonics techniques show promising results for diseased tissue detection. Fluorescence and diffuse reflectance spectroscopy and imaging, the modalities of interest throughout this thesis, are among them. Fluorescence spectroscopy and to a lesser extent, diffuse reflectance spectroscopy applications, have been heavily concentrated on cancer diagnosis, which, with ischemic heart disease, is the principal cause of death.
in developed countries. The first major goal of this thesis was to assess the ability of fluorescence spectroscopy to diagnose non-alcoholic fatty liver diseases, a condition associated with diabetes (expected to become the biggest killer together with heart diseases in developing countries over the next decades, according to the World Health Organisation (WHO)). The second objective of this work was to combine fluorescence and diffuse reflectance spectroscopy to attempt to enhance the diagnostic performance provided by the individual modalities. Furthermore, the vast majority of the fluorescence and diffuse reflectance studies to date were performed ex vivo or on external organs, and the question of their translation to theatres for Minimally Invasive Surgery (MIS) has not been addressed despite their appropriateness for these procedures. The third key aim of this work was hence to provide and test solutions to make these technologies amenable to MIS procedures.

The general approach chosen was to apply fluorescence, reflectance spectroscopy and imaging to clinical applications. For each of them, the existing diagnosis methods as well as the fluorescence and reflectance work performed to date (where they existed) were reviewed and their limitations highlighted. Chapter 2 gives a description of light-tissue interaction as well as a review of fluorescence and reflectance spectroscopy studies on diseased tissue diagnosis. The material required for fluorescence/reflectance spectroscopic experiments is also detailed. In the next two chapters, a spectroscopic optical fibre probe was used to detect tissue lesions. The outcomes of a fluorescence spectroscopy study on non-alcoholic fatty liver diseases (NAFLD) diagnosis realized during two mice trials (chapter 3) are presented. Furthermore, a solution for in vivo spectroscopic tissue diagnosis combining the spectroscopic probe with an articulated robotic arm is suggested. A modified version of the spectroscopic probe combining fluorescence/reflectance is used in chapter 4 in a study on precancerous/cancerous lesion detection in human gastrointestinal tract samples.

The transition from spectroscopic point-limited system to imaging systems begins chapter 5. A summary of the different imaging filtering systems alongside a review of endoscopic fluorescence imaging systems is provided. A fluorescence/reflectance laparoscope system of our conception is described and could be used to extend the work presented in the previous chapter to delineate a tumour in a resected human oesophagus. The system design and testing on phantoms is presented as well as the use of the same imaging system in a study which distinguishes itself from the rest of the thesis by using an exogenous dye. The
laparoscopic system was applied to assess the feasibility of in vivo imaging of the brachial plexus nerve of a pig after injection of a contrast agent.

In chapter 6, a hyperspectral laparoscopic system was employed to monitor in vivo tissue blood concentrations and saturation of a porcine colon after termination, as well as the re-perfusion of a uterus transplant on a rabbit recipient. Chapter 7 concludes the manuscript by summarising the thesis contributions and limitations, and by tackling the question of the possible diffusion of fluorescence and reflectance spectroscopy and imaging systems into hospitals.

This thesis was based on results presented in the following publications and conferences (in chronological order):


Chapter 2

Light-tissue interaction and tissue spectroscopy

In this chapter, we describe how light is modified by tissue. The spectral range of interest in this thesis lies in the non-ionizing and richly diagnostic visible domain (400 nm – 700 nm). The light-tissue interaction is a complex phenomenon; a photon incident on a tissue surface can undergo a variety of processes. This chapter outlines some of the most important of them and presents in more detail fluorescence and diffuse reflectance, the two types of light-matter mechanism relevant to this work. The physical principles, biological origins, as well as the existing models which describe them are then presented. The use of fluorescence and diffuse reflectance is then motivated and their implementations, together with their medical applications to date are reviewed.

2.1 Light-tissue interactions

When incident on the tissue, light can either be reflected or refracted. The directly reflected light does not penetrate the tissue and therefore contains no useful information. It is referred to as a specular reflection and does not contain significant diagnostic information, but may result in signal saturation. The amount of refracted and reflected light is governed by the Snell-Descartes law and depends on the refractive index at the air/tissue interface and the angle between the incident light beam and the tissue normal.

Tissue can be seen as a set of scattering and absorption centres [1]. The photons forming the refracted light can be absorbed or scattered by the tissue chromophores and scatterers. The fraction of the photons which emerge from the tissue after multiple scattering events is called diffuse reflectance as the scattering induces the spreading and the loss of directionality of the incident beam. Absorption may result in fluorescence, and in such a case the chromophores are referred to as fluorophores. The fluorescent photons may in turn be absorbed and/or scattered, and only a portion of the fluorescent light escapes the tissue after backscattering. These different scenarios are summarized in Figure 2-1.
Figure 2-1: Different types of light-tissue interactions: specular highlight (reflection), scattering, absorption, fluorescence.

The following paragraphs will detail fluorescence and diffuse reflectance phenomena as they are the two modalities used in this thesis.

2.2 Tissue fluorescence spectroscopy

2.2.1 Fluorescence phenomenon

Fluorescence and absorption are two related phenomena. Fluorescence can be seen as one method by which an illuminated molecule is excited by light before releasing its stored energy. The amount of light (or energy) absorbed by the molecule as well as the quantity of fluorescent light it emits both depend of its molecular structure and the light wavelength, this is described by the molecule absorption spectrum and emission fluorescence spectrum, respectively. Jablonsky diagrams model the electronic energy levels of molecules and provide a convenient way to visualise what may happen once a molecule is excited by light [2].
Figure 2-2 illustrates that a molecule undergoes a transition from its ground state to an excited electronic state after light absorption if the excitation photon energy equals the energy difference between two energy levels. The excited state energy level reached by the electron depends on the wavelength and therefore on the energy of the excitation light. After vibrational relaxation from the initial excited state to the lowest excited state energy level, the molecule may then relax to the ground state by releasing its energy either in the form of light by emitting a photon (fluorescence or phosphorescence) or through a non-radiative relaxation (such as heat) [2]. The wavelength of the emitted photon matches the energy level difference between the excited state and one of the ground state energy levels (Bohr’s frequency condition).

The length of time spent by a molecule in its excited state before returning to its ground state corresponds to its lifetime, whose order of magnitude is one nanosecond for fluorescence and several milliseconds or longer for phosphorescence [3]. Absence of fluorescence may hence be the consequence of a non-radiative relaxation (thermal dissipation for instance) or a slow radiative relaxation (phosphorescence) [4].

Another parameter characterising molecule fluorescence is its quantum yield, that is, the ratio of the number of emitted photons over the number of absorbed photons. Both lifetime and quantum yield can be modified by pH and temperature [2].
One could expect that absorption and fluorescence spectra totally overlap, but the latter is red shifted compared to the absorption spectrum. This phenomenon is known as the Stokes’s shift and comes from the partial loss of energy by intermolecular collisions (also called vibrational relaxations) preceding the fluorescence emission [5].

In summary, the fluorescence spectrum of a given fluorophore is a function of its intrinsic parameters: extinction (or absorption) coefficient at the excitation wavelength and quantum yield at the emission wavelength, which gives each fluorophore a unique spectral signature but also depends on the fluorophore concentration and the intensity of the excitation light [5, 6].

2.2.2 Tissue fluorophore spectra

The fluorescent signal recorded from tissue comes from endogenous fluorophores and the detection of this signal in medical diagnostic instruments has the advantage of not relying on exogenous fluorophores whose toxicity, dose optimization, injection timing need to be considered [4].

The fluorescence spectra of the main endogenous fluorophores responsible for native fluorescence are summarised in Figure 2-3. Their excitation maxima stretch out from 250 nm to 450 nm while their emission maxima lie between 280 nm and 700 nm [7] (Table 2-1).

They can broadly be classified as follows: connective tissue proteins: collagen and elastin; enzymes such as reduced nicotinamide adenine dinucleotide (NADH) and flavins (such as flavin adenine dinucleotide (FAD) involved in the cellular energy metabolism; aromatic amino-acids (tryptophan, tyrosine, phenylalanine); lipopigments that include products resulting from lipid peroxidation (e.g. lipofuscin); and different sorts of porphyrins and vitamins [8].

Table 2-1: Excitation and emission maxima of some key endogenous fluorophores of interest in this thesis.

<table>
<thead>
<tr>
<th>Endogenous fluorophore</th>
<th>Excitation maxima</th>
<th>Emission maxima</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen</td>
<td>325</td>
<td>400</td>
</tr>
<tr>
<td>NAD(P)H</td>
<td>340</td>
<td>450</td>
</tr>
<tr>
<td>FAD</td>
<td>450</td>
<td>515</td>
</tr>
<tr>
<td>Porphyrins</td>
<td>400-450</td>
<td>630, 690</td>
</tr>
</tbody>
</table>
Figure 2-3: Excitation and emission spectra of the main endogenous fluorophores. Reprinted from [7] with permission from John Wiley and Sons.

The excitation spectrum (Figure 2-3-a) is the fluorescence intensity recorded at a fixed emission wavelength for different excitation wavelengths and similarly the emission spectrum (the spectrum of interest in this report, Figure 2-3-b) plots the fluorescence intensities at a fixed excitation wavelength for different emission wavelengths.

The fluorescent signal generated from a tissue is complex to model as it varies with numerous factors such as:

- the nature and the concentration of the fluorophores as well as their depth distribution. Fluorophores are unevenly spread with depth [4, 7]. For instance, the liver’s extracellular matrix is rich in collagen [9] and lies on top of hepatocytes, where NADH, FAD, vitamin A are present [10]. Another example is the layered architecture of the epithelial tissue of the intestinal wall where NADH, FAD and tryptophan are the main fluorophores of the mucosa (first layer [11]) and collagen is the primary fluorophore of the underlying stroma [12].
- the concentration of the scatterers and non-fluorescent chromophores (mainly haemoglobin in the visible (see section 2.3.2.1) surrounding the fluorophores, which can significantly distort the fluorescence with a wavelength-dependent magnitude. Scattering and absorption also tend to decrease with increasing wavelength in the visible [8].
- the excitation light wavelength, which determines which fluorophores will be excited depending on their excitation spectrum, as well as the depth and therefore the fluorophores reached by the light. The fluorescence escaping the tissue reflects therefore the contribution of the fluorophores integrated over excitation light penetration depth.
• photobleaching, which is another important factor affecting the fluorescence signal. It may occur when the excited fluorophores react with surrounding molecules instead of going back to the ground state. This reaction leads to the chemical modification of the fluorophores and may cause, in turn, a permanent fluorescence loss [13].

2.3 Diffuse reflectance spectroscopy

2.3.1 Absorption and scattering phenomena

The diffuse reflectance is a measure of the portion of light backscattered after undergoing a series of absorption and scattering events and hence is a function of both the absorption and scattering coefficients.

Absorption corresponds to the energy transfer of a photon to a molecule which arises when Bohr’s frequency condition is met. The portion of absorbed light depends hence on the concentration of the tissue absorbers (see section 2.3.2) fulfilling the Bohr’s criteria in the visible. Tissue absorption is quantified by the absorption coefficient $\mu_a$, which represents the amount of absorption per unit pathlength [3] and is proportional to the product of the wavelength-dependent absorber extinction coefficient $\varepsilon$ and concentration $c$ according to:

$$\mu_a = \ln(10) \cdot \varepsilon \cdot c$$

Equation 2-1

Measured absorption coefficients range from $0.12|_{\lambda=633\text{nm}} \text{cm}^{-1}$ (in the chicken muscle) to $85|_{\lambda=630\text{nm}} \text{cm}^{-1}$ (in the gallstones) in the visible [14]. The Beer-Lambert law enables the estimation of the portion of the incident light $I_0$ absorbed in a scattering-free medium after having travelled a certain distance $x$:

$$I(x) = I_0 \exp\left(-\frac{\mu_a \cdot x}{\ln(10)}\right)$$

Equation 2-2

When considering several absorbers and using Equation 2-1, Equation 2-2 can be rewritten as follows:

$$I(x) = I_0 \exp\left[-(\varepsilon_1 \cdot c_1 + \varepsilon_2 \cdot c_2 + \ldots + \varepsilon_n \cdot c_n) \cdot x\right]$$

Equation 2-3

The Beer-Lambert was originally developed for transmission through an absorbing-only medium with a known width so that the distance $x$ travelled by the photons is known; whilst this work is concerned with the light diffusely reflected from tissue after
absorption, but also scattering events. However, the Beer-Lambert law remains useable in a scattering media where the pathlength is a priori unknown and wavelength-dependent (since the scattering affects the length travelled by photons and varies with wavelength), provided some adaptations are made.

Biological scattering originates from microscopic variations in refractive index stemming from complex tissue organisation [15]. Collagen fibres [14], the main component of connective tissue, cell nuclei [16], lipid membranes [17], and mitochondria [18] have been identified as contributors to such scattering events. Scattering in the body is mostly elastic, that is without variation in photon energy. It depends on the scatterer concentration and light wavelength, but also on the scatterer refractive index and on the scatterer size/wavelength ratio, which determines the scattering regime. Rayleigh scattering originates from particles much smaller than the wavelength of the visible light, i.e. cell membranes or micelles [3], it is isotropic and its magnitude depends on the wavelength as $\lambda^{-4}$ [19]. Tissue constituents with sizes similar to the visible wavelengths, i.e. cell nuclei and mitochondria as well as collagen fibres [3], scatter light with weaker wavelength dependence ($\lambda^{x}$|0.4≤x≤0.5) and primarily in a forward direction. This regime is referred to as Mie scattering [19]. The equivalent of the absorption coefficient for scattering is the scattering coefficient $\mu_s$ and is defined as the quantity of scattering in a medium per unit pathlength [3]. Measured scattering coefficients span the range of 4.1|$\lambda=633\text{nm}$ cm$^{-1}$ in chicken lungs to 1410|$\lambda=685\text{nm}$ cm$^{-1}$ in whole blood [14]. In other words, light is more diffusive in highly vascularised tissue than in lung tissue.

However, the scattering coefficient is not sufficient to fully describe the scattering phenomenon. The scattering anisotropy factor $g$ is used to take into account the anisotropy of the scattering, and the optical parameter usually chosen to fully describe scattering is the reduced scattering coefficient $\mu_s'$ defined as: $\mu_s' = (1-g)\mu_s$. Values of $g$ close to 1 correspond to forward scattering, a value of 0 to isotropic scattering, and equal to -1 to backward scattering. The values of $g$ in tissue are within the interval [0.74|$\lambda=476\text{nm}$, 0.995|$\lambda=665\text{nm}$], which indicates a predominantly forward scattering, and light emitted from tissue has been therefore multiply scattered [8, 14].

Both the absorption and scattering coefficients affect the depth of penetration of the light $d$. A good approximation of $d$ is given by: $d = \frac{1}{\mu_{s'} + \mu_a}$ [15]. This expression enables, for instance, the estimation of the depth reached in the liver, one of the organs of interest of this work, at the excitation wavelengths of the two lasers used here: 375 nm and 405 nm (see chapter 3). The depth of light penetration at 26
these two wavelengths calculated using the absorption and reduced scattering coefficients measured in a rat liver [20] is 35 µm and 40 µm at 375 nm and 405 nm excitations, respectively.

2.3.2 Tissue chromophores

The tissue absorbers, also called chromophores, and their contribution to the diffuse reflectance is known in more detail than the properties of the scatterers. Chromophores are functional groups of molecules which absorb light, some of which reemit light following absorption, which are the fluorophores mentioned earlier. Chromophores’ light absorption depends on both their concentration and their extinction coefficient (“absorbing power”). For instance, water represents up to 75% of the body weight [21], but its extinction coefficient is very low in the visible making its absorption insignificant in this region of the electromagnetic spectrum. In contrast, protoporphyrin IX extinction coefficients are high in this spectral region, but its concentration is very low also leading to insignificant absorption in the visible [22]. The extinction coefficient changes with wavelength in a characteristic way so that each chromophore has a distinct spectral signature, in a similar way to fluorophores which have distinctive emission fluorescence spectra.

The three main absorbers found in the body are haemoglobin, water and melanin [3, 23]. Proteins and DNA absorb in the ultraviolet (UV), but not in the visible [6]. Melanin is only present in the skin, eyes and the hair, and the water absorption coefficient is negligible compared to haemoglobin in the visible range as illustrated in Figure 2-4.
Haemoglobin: the main absorber in the visible range

Haemoglobin is therefore the main absorbing compound in the body for the visible range. It is present in the body in two main forms: deoxyhaemoglobin and oxyhaemoglobin. Its normal level in blood vessels is 150 g/L [25], which can drop to 120 g/L for anaemic patients [26]. Given its importance in this work, the haemoglobin characteristics are presented in more detail than the other chromophores. Haemoglobin is a protein present in the cytoplasm of red blood cells. Those transport the oxygen from the lungs to the tissue, which is possible thanks to haemoglobin since it has the ability to bind with oxygen [27]. Haemoglobin comprises of four amino acid chains, each containing a heme group which hosts an atom of ferrous iron that connects with a molecule of oxygen [28]. One haemoglobin molecule can therefore host four oxygen atoms to become oxyhaemoglobin. Once it releases its oxygen molecules, it becomes deoxyhaemoglobin. During this process, the colour of haemoglobin changes from bright red, while fully saturated with oxygen to deep purple for deoxyhaemoglobin [27].

The main factor governing oxygen binding to haemoglobin is the partial pressure of oxygen in the blood. The haemoglobin saturation, defined as the ratio of the oxyhaemoglobin concentration over the total haemoglobin concentration raises with oxygen partial pressure with a sigmoid relationship (Figure 2-5) and is normally around 97% in arteries. This behaviour is explained by the preferential oxygen
binding with haemoglobin molecules that are already occupied by at least one oxygen molecule. Temperature and pH are among the other factors influencing the oxygen binding [27].

Figure 2-5: Oxygen dissociation curve, haemoglobin saturation increases with oxygen partial pressure. Reprinted from [29] with permission from BMJ Publishing Group Ltd.

Haemoglobin, a protein, absorbs in the visible which can seem contradictory with Figure 2-4 which shows protein weak absorption in the visible. The absorption actually originates from the heme group of the haemoglobin, which is a variety of porphyrin. Haemoglobin absorption is a well-documented subject, and several groups have measured extinction (or absorption) coefficients of oxyhaemoglobin and deoxyhaemoglobin for various spectral ranges and with different resolutions (Table 2-2) [30-35].

Table 2-2: Haemoglobin absorption spectra reported in the literature.

<table>
<thead>
<tr>
<th>Source</th>
<th>Wavelength range (nm)</th>
<th>Resolution (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prahl</td>
<td>250 - 1000</td>
<td>2</td>
</tr>
<tr>
<td>Zijlstra</td>
<td>450 - 1000</td>
<td>10</td>
</tr>
<tr>
<td>Van Assendelft</td>
<td>350 - 700</td>
<td>2</td>
</tr>
<tr>
<td>Takatani</td>
<td>450 - 995</td>
<td>5</td>
</tr>
<tr>
<td>Wray</td>
<td>infrared</td>
<td>unknown</td>
</tr>
<tr>
<td>Schmitt</td>
<td>630 - 950</td>
<td>10</td>
</tr>
</tbody>
</table>

There are discrepancies in the absorption values between these different sources mainly due to the instrumentation used to record spectra. These extinction coefficients are often used as basis spectra in fitting procedures of diffuse reflectance.
spectra recorded from tissue to extract the concentrations of oxyhaemoglobin and deoxyhaemoglobin, allowing diagnostically useful parameters like tissue oxygen saturation to be obtained. The utilization of different basis extinction coefficient can sensibly impact the retrieved oxygen saturation [36], and therefore a careful choice of the tabulated data is required if absolute values of oxy and deoxyhaemoglobin concentrations are desired. We have chosen to use Prahl’s data (see chapter 6) as it offers the highest resolution in the $[450 – 700]$ nm interval, which is the range of interest in this work. Figure 2-6 illustrates the molar extinction spectra from Prahl as well as the ones from Schmitt, and Takatani to illustrate the deviations between them.

Figure 2-6: Molar extinction coefficients of oxyhaemoglobin and deoxyhaemoglobin from different sources. Data compiled from [30, 33, 35].

Regardless of the source, the same general shape and characteristic spectral features are observed in the 450-700 nm range: the oxyhaemoglobin spectrum peaks at 542 nm and 577 nm, whilst the deoxyhaemoglobin spectrum reaches a single maximum at 555 nm. The spectra intersect five times over the considered spectral region. These crossing points are referred to as isosbestic points, where the total haemoglobin absorption remains constant whatever its composition is. All the optical techniques used to measure tissue oxygenation exploit the marked spectral differences between oxyhaemoglobin and deoxyhaemoglobin.
2.3.2.1 Other chromophores

All the chromophores mentioned in the rest of this paragraph have absorption negligible compared to haemoglobin due to their low concentration and are mentioned for completeness. Methemoglobin, sulfhemoglobin, and carboxyhemoglobin are all haemoglobin derivates absorbing in the visible, but present in the body in low concentration. Myoglobin has a similar absorption spectrum and molecular structure than haemoglobin and provides oxygen to muscles [37]. Lipids, mainly known as scatterers, are also absorbers [38]. Cytochromes, (including cytochromes aa3, b and c) from the hemoprotein family (like haemoglobin) are involved in the electron transfer chain and are found in mitochondria [39]. Bilirubin, a by-product of haemoglobin breakdown localizing in the spleen and liver [40] and β-caroten, a carotenoid present in the skin and the aorta [41] also absorb in the visible.

2.4 Models of light transport in tissue

Spectra recorded from tissue are used to separate normal sites from pathologic sites. There are two main approaches to process them: either the spectral features are directly used to separate normal from diseased sites, or a light-tissue interaction model is used to extract tissue optical properties, which are then used as diagnostic variables. While the former approach was used in this work, the latter approach is described in this section as it permits to further understand the transport of light in tissue.

2.4.1 Radiative transfer equation

Modelling of the light-tissue interaction aims to determine the absorption and scattering coefficients characterizing a tissue which can then be used as a disease marker.

In the classical theory, light is modelled as an electromagnetic wave and its propagation in a medium is analytically described by Maxwell’s equations. However, the highly heterogeneous structure of tissue, and therefore the important spatial variations in its optical properties, makes the application of the classical theory impossible. The radiation transport theory, which ignores fluorescence as well as electromagnetic wave phenomena such as interference or polarization, may be used instead [17]. The radiation transport theory states that in the steady–state regime, the divergence of a non-collimated beam of radiation with intensity \( I \), propagating from a
given position with a given direction in tissue (considered as a homogeneous continuum with randomly distributed scattering and absorbing centres) is decreased by the portion of the beam either absorbed or deviated from its original trajectory by scattering, and is increased by radiation scattered alongside its direction and by neighbouring external light sources.

This model uses the radiative transfer equation, which takes the following form in the steady–state regime when tissue is considered as a homogeneous continuum with randomly distributed scattering and absorbing centres:

\[ \hat{s} \cdot \nabla I(\vec{r}, \hat{s}) = - (\mu_a + \mu_s) I(\vec{r}, \hat{s}) + \mu_s \int_{4\pi} p(\hat{s}, \hat{s}') I(\vec{r}, \hat{s}') d\omega' + \varepsilon(\vec{r}, \hat{s}) \]

Equation 2-4

The interpretation of Equation 2-4 is straightforward and provides a good summary of the events undergone by a packet of photons of energy \( I \) called the specific intensity or radiation (power per unit area, per unit angle more exactly) at a position \( \vec{r} \) and propagating in the direction \( \hat{s} \) in a tissue characterized by an absorption coefficient \( \mu_a \) and a scattering coefficient \( \mu_s \). The first term right hand side represents the drop (hence the minus sign) in the light energy packet caused by absorption and scattering. The energy of this packet is increased by all the photons of initial direction \( \hat{s}' \) scattered along \( \hat{s} \) (\( p(\hat{s}, \hat{s}') \) being the scattering phase function, i.e. the probability that a photon of direction \( \hat{s}' \) will be scattered in the \( \hat{s} \) direction) and by any external source \( \varepsilon(\vec{r}, \hat{s}) \) present in this region [17].

2.4.2 Analytical models

In spite of its apparent simplicity, the solution of the radiation transport is difficult and limits its use. However, it has been analytically solved in the limiting cases of predominant scattering or predominant absorption and isotropic scattering. As stated earlier, scattering is dominant over absorption in most tissues (and hence the absorption coefficient may be set to zero), and the scattering dominant hypothesis, commonly called the diffusion approximation, is therefore the model of interest here. The transport equation (Equation 2-4) simplified with the diffusion approximation is called the diffusion equation.

A more accurate approximation of the transport equation is the \( P_N \) approximation. It consists in expressing the specific intensity and the source distribution in a series of spherical harmonics, and the phase function as well as the scattering angle in a series of Legendre polynomials [42]. The diffusion
approximation is equivalent to the $P_1$ approximation; they both assume that the specific intensity, the source distribution, and the phase function are isotropic. Apart from the $P_1$ approximation, the most common implementation of the $P_N$ approximation is the $P_3$ approximation, which truncates the spherical harmonic series at the fourth order (numeration starting at 0), which can then be simplified in Legendre polynomials [43, 44]. This higher order approximation permits a more exact account of the anisotropy effect near sources and boundaries [44]. The $P_3$ approximation has been shown to be accurate for highly absorbing media and short source-detector separation [44, 45]. The diffusion equation and the transport equation in the $P_3$ approximation may be solved to give an analytical expression of the diffuse reflectance in the improbable assumption that the tissue is an infinite homogeneous medium, but its resolution becomes complicated in the common hypothesis of mono or multi-layered tissue with one or several boundary index mismatches and a fortiori when source-detector geometry is taken into account.

Methods, categorized into the analytical, empirical and numerical ones, have consequently been developed to solve practical situations. The analytical methods are based either on the diffusion or the $P_3$ approximation. The Kubelka-Munk model, equivalent to a one dimension diffusion model, represents one of the earliest attempts to give an analytical expression of the diffuse reflectance [46]. It expresses in one dimension the reflectance and transmittance of a diffuse irradiance within an isotropically scattering medium as a function of the scattering and absorption coefficients and ignoring the reflections at the boundaries. This enables their calculation directly from the measured transmission and reflectance. The numerous hypotheses (matched boundaries, isotropic scattering, and diffuse irradiance) limit nonetheless the applicability of the Kubelka-Munk model [14, 47].

The exponential model is a simple way to calculate the total diffuse reflectance from a semi-infinite homogeneous medium whose essential assumption is that incident and backscattered light are diminished following $e^{-(\mu_a + \mu_s')z}$, where $\mu_a$, $\mu_s'$ and $z$ are the absorption, reduced scattering coefficient and depth of penetration respectively. This is equivalent to neglecting the integral term in Equation 2-4. This method can be generalized to the multi-layered tissue with index mismatches to give an expression of the diffuse reflectance in function of the scattering and absorption coefficients of the different layers [15].

Photon migration [48] and path integral models [49, 50] both propose a probabilistic approach to the analytical estimation of the diffuse reflectance from a semi-infinite continuum using the notion of photon weight. All the analytical models
described so far permit the calculation of the total diffuse reflectance from a semi-infinite medium, yet this quantity is not of interest since, during real experiments, diffuse reflectance is collected with finite size measurement devices at a given location and angle.

Zonios’ model fulfils this requirement as it provides an angularly and spatially resolved expression of the diffuse reflectance from a semi-infinite medium in the diffusion approximation. This makes its use very suitable for modelling the diffuse reflectance measured with an optical fibre probe (probably the most common measurement tool to collect light from tissue) which has finite diameter and numerical aperture [51]. Besides the spatial variables, the diffuse reflectance expression is a function of the absorption and scattering coefficients as well as a constant which is determined after calibration against phantoms with known optical properties. The absorption coefficient is assumed to be only dependent on haemoglobin and the scattering coefficient is compelled to decrease with increasing wavelengths. Then, a non-linear optimisation routine is iteratively used to minimize the dissimilarity between modelled and measured diffuse reflectance spectra to retrieve the absorption and scattering coefficients.

A similar approach referred to as the hybrid $P_3$ approximation is also used to provide a spatially resolved expression of the diffuse reflectance from a semi-infinite medium [45, 52]. It is one type of implementation of the $P_3$ approximation presented in [44] and is called hybrid because it combines elements from both the diffusion and the $P_3$ approximation. Like in the Zonios’ model (based on the diffusion approximation), it uses the Green’s function of the diffuse equation for an isotropic steady-state source, but substitutes the absorption coefficient by a constant denoted $\nu'$ stemming from the P3 approximation and which depends on the absorption and reduced scattering coefficients. Like the Zonios’ model, this approach is applicable to complex optical fibre probe geometries and presents the additional advantage not to require a calibration on phantoms.

2.4.3 Numerical and experimental models

Monte Carlo simulations are a versatile numerical method to simulate the light propagation process. This approach provides the most accurate approximation to the transport equation, while enabling the implementation of complex source-detector geometry, multi-layered tissue with refractive index mismatch boundaries [53], finite size incident radiance [54] and can even account for reemitted fluorescent photons [55-59]. In Monte Carlo simulations, light is viewed as a packet of photons travelling
in tissue which can undergo absorption, scattering within a layer and transmission or reflection at layer interfaces. The inputs of the model are the absorption and scattering coefficients, the refractive indices, the anisotropy factor, the number of photons forming the packet and the geometry of the problem. The photon packet is given an initial weight of 1, which is decreased after each absorption event. The step size of the photon packet between two light-tissue interactions and its deflection angle after a scattering event are both random variables sampled from their probability density functions, which is a function of the absorption and scattering coefficients for the step size and a function of the anisotropy factor for the deflection angle. When the photon group is transmitted through an interface into the air, the up-to-date photon weight is counted as a diffuse reflectance. The photon packet is terminated when the weight goes below a set threshold.

Because the model output of interest is not the diffuse reflectance, but the absorption and scattering coefficients, inverse Monte Carlo methods have been developed to extract these coefficients. They consist of simulating a diffuse reflectance spectrum with an initial guess on the aforementioned coefficients and then comparing it with the measured spectrum. The sum of the squared errors between simulated and acquired spectra is calculated and reduced iteratively with updated absorption and scattering coefficients using an optimization algorithm. The retrieved absorption and scattering coefficients minimise the error [59]. Depending on the geometry complexity and the number of launched photons, the Monte Carlo can be time consuming.

Another means to model the diffuse reflectance is to use empirical methods such as artificial neural networks. After being trained on phantoms, the algorithm is used to extract absorption and scattering coefficients from tissue. The accuracy of this approach depends on the phantoms used during the training [60].

In summary, all the aforementioned methods enable the retrieval of the absorption and scattering coefficients with different degrees of accuracy depending on the hypotheses they are based on. Monte Carlo simulations provide the most accurate solutions as no assumption regarding the tissue optical behaviour is made. It also enables implementation of complex source detector geometry and boundary conditions (multiple layer tissue with index mismatches). Nonetheless, it furnishes numerical solutions only and can be time consuming while real time diagnostic information is sometimes required. It should, however, be mentioned that techniques exist to reduce this processing time [61]. Analytical models have the advantage of speed and help to understand the physics of the light-tissue interaction. Yet, almost all of them provide inaccurate solutions for high absorption tissue since they are
based on the diffusion approximation; this makes them useable in brain or dense connective tissue for instance, which are highly scattering or in low absorption tissue, but they are not valid in highly vascularised tissue such as muscles.

2.5 Implementation of fluorescence and diffuse reflectance

Fluorescence and diffuse reflectance spectroscopy can be employed in a contact and a non-contact way. The two methods each have their advantage; the contact approach allows the probing of internal organs difficult to access like colon, oesophagus, while the non-contact approach enables the acquisition of spatial information by scanning the excitation light beam (see section 2.6.2). Furthermore, fluorescence spectroscopy is implemented either in steady-state mode, which looks at wavelength-resolved intensity or time-resolved mode which examines wavelength resolved-fluorescence lifetime. The latter requires expensive and bulky ultra-short pulsed lasers and time-gated detection. In this thesis, fluorescence and diffuse reflectance has been used in contact and steady state mode.

2.5.1 Fibre-optic probe based spectroscopy

In addition to the optical fibre probe, fibre-optic probe based spectroscopy setups include: an excitation light source, coupling optics to adapt the light source f-number to the numerical aperture (NA) of the probe excitation fibre for optimal irradiance, a second set of coupling optics to match the NA of the probe detection fibres to the disperser f-number for maximum coupling of the collected light from the sample to the disperser, a disperser to separate this light intensity according to its wavelength, a detector to record the dispersed light (Figure 2-7). The rest of this section highlights the different elements required to perform this in clinical settings. The shape of fluorescence and diffuse spectra is known to be modified by changes in tissue biochemistry and biostructure, respectively, which are triggered by disease progression. The two modalities give hence complete information on the tissue condition and their combination is hoped to improve the diagnostic accuracy.
Light sources

Light sources are chosen according to the following characteristics: coherence, bandwidth, spectral range of interest, power, and portability. Lasers are highly temporally and spatially coherent light sources allowing a very narrow spectral output and a high coupling efficiency into optical fibres. They are the typical light sources used for fluorescence spectroscopy. Among the lasers emitting in the UV/visible are the frequency-doubled Nd:YAG emitting at 532 nm; dye lasers emitting between 400 and 800 nm, the argon-ion laser emitting at 488 nm and 514.5 nm, the Ti:sapphire laser tuneable between 690 nm and 1000 nm, and the nitrogen laser (337 nm emission) [19]. Laser diodes (emitting between 350 nm and 1900 nm) are more and more employed as they represent an advantageous substitute to traditional lasers in terms of portability, energy consumption, and usability. Another possible type of light source for fluorescence is a broadband light source normally used for diffuse reflectance spectroscopy in combination with a bandpass filter or a monochromator. This approach has the advantage of wavelength tunability when the broadband light source is employed with a filter wheel.

Optical fibre probe

Fibre probes may be classified as: mono-fibre probes or bifurcated probes. The first type contains a unique fibre which is used for illumination and detection, whereas
bifurcated probes have one or several excitation fibres and one or several detection fibres. They are named “bifurcated” because they have two arms: one excitation arm linked to the light source and one detection arm connected with the disperser/detector system. Mono-fibre probes are the easiest to build and permit the smallest diameter as well as a high light collection efficiency compared to bifurcated probes. However, they require additional optics such as an additional beamsplitter to separate excitation and emission light. More importantly, the mono-fibre approach is restricted because of the large amount of specular light collected, the internal reflection at each internal surface, and the induction of autofluorescence by the generation of parasite autofluorescence by the optical fibre excited by the illumination light [65].

The optical fibres used are multimode for a higher light gathering efficiency and have diameters in the order of hundreds of microns and a numerical aperture typically around 0.22. In order to limit parasitic autofluorescence from the fibre itself, high-grade silica is preferred to silica as the material for the fibre core as silica generates fluorescence when excited in the UV (which is the case in this work see chapter 3) [66]. Also, flection of the fibres and defects mean that the light may exit the core and reach the jacket. Consequently, polyimide is chosen instead of nylon because nylon fluoresces under UV light [65].

The materials constituting the probe are chosen not only to minimize autofluorescence, but also to allow light transmission in the UV/visible. More importantly, the probe is the only part of the fluorescence spectroscopy setup in contact with tissue, and its material must hence be biocompatible. The two probes used in this thesis meet this criterion (see chapters 3 and 4). All the materials used for optical fibre probe packaging are required to meet the standards of the American norms, the USP (U.S. Pharmacopoeia), the European norm ISO 10993 and the Japanese norms, MHLW (Ministry of Health, Labour and Welfare). For instance, substances such as stainless steel 302, polymers, polyimide used for optical fibre housing and glue from the manufacturer Epotek utilized to seal the probe are all biocompatible [65].

**Disperser/detector**

Monochromators and spectrographs are the two main types of light disperser. The former separates light intensity at individual wavelength one at a time, while the latter provides the complete spectrum forthwith, making its acquisition time for full spectrum shorter than the monochromator. Important parameters to consider when
choosing a disperser are: the spectral range and resolution (which depends on the slit width), the transmission efficiency, and the numerical aperture.

The choice of the detector depends on the number of wavelengths and the number of pixels being acquired. Photomultiplier tube or avalanche photodiode used together with one or several bandpass filters or monochromators are utilized for single pixel and single or few wavelengths acquisition. A spectrograph combined with a linear photodiode array enables the instantaneous acquisition of a reflectance or diffuse reflectance spectrum, whilst a spectrograph/ coupled-charged device (CCD) permits the recording of a spectrum for several pixels. Intensified CCDs are sometimes used for fluorescence spectroscopy as fluorescent light levels are low.

2.5.2 Importance of the probe geometry

Probe geometry, and in particular the probe-to-target distance (PTD), source-to-detector separation (SDS), fibre numerical aperture (NA) and diameter, has a dramatic impact on the origin of the excited and collected light (Figure 2-8). As mentioned in 2.4.3, inverse Monte Carlo simulations can quantify tissue optical properties; forward Monte Carlo simulations are also very useful to define the extent of excited and probed tissue volumes, as well as assessing the influence of probe geometry on them.

![Figure 2-8: A common geometry of optical fibre probe. PTD denotes probe-to-target distance and SDS the source-to-detector separation.](image)

In [67], the effect of NA, fibre diameter, SDS and PTD on the emitted fluorescence light at 630 nm from tissue-like optical phantoms after 400 nm excitation was studied. The probe consisted of one excitation fibre surrounded by a ring of detections fibres. Increasing the NA was found to increase collection efficiency (CE) as well as inducing a higher sensitivity to superficial layers, because many of the photons propagating at higher angles were likely emitted directly below the
illumination fibre. The main effect of decreasing the fibre diameter was to increase the spatial selectivity as the diversity of pathlength travelled by the photons is diminished. Raising the SDS led to an increase in the mean path length and the probing depth together with a decrease in the detected fluorescence and the spatial selectivity. Finally, the CE evolution with the PTD was non-monotonic because of the competing effect of illumination and detection area overlap and decay of light collection with distance. In [12], the CE and the probing depth of fluorescent light emitted at 520 nm from a two layer phantom (mimicking epithelial/stromal tissue) after 460 nm excitation were compared between a single fibre probe and a multiple fibre probe. The CE was higher with the single fibre probe but the probing depth was more important with the multiple fibre probe. Also, the CE increased with NA for both probes.

Effects of the illumination and CE have also been assessed for diffuse reflectance. The influence of PTD on the collection efficiency at 337 nm was evaluated for three probe geometries: one single fibre probe (probe 1) and two multiple fibre probes: the first one consisted of one central illumination fibre surrounded by a ring of 18 collection fibres (probe 2), the second one was made up of a 24 fibre central illumination area surrounded by the same number of collection fibres (probe 3) [68]. The evolution of the CE of probes 2 and 3 with PTD was found to be non-monotonic for the same reasons as mentioned above, whereas it decreased monotonically with increasing PTD for probe 1 as illumination and collection areas overlap at all PTD for single fibre probes. Also increased PTD generated increased sensitivity to superficial layers for probe 3 and the average CE was higher with probe 1 than with the two other probes.

The diffuse reflectance CE of a single fibre probe and a bifurcated probe consisting of one excitation surrounded by a ring of collection fibres was computed from a semi-infinite medium with varying optical properties [69]. The main conclusion of the study was that the CE was proportional to \( \sin^2(\text{NA}) \) in the case of highly scattering samples for both the single optical fibre and multiple fibre probe. The CE was highly dependent on the sample absorption coefficient only in the latter case because the mean pathlength of collected photons is longer for multiple fibre probe, (the photons travelling from the source fibre to the detector fibre) giving the absorption a larger distance to wield its effect.

As highlighted above, the probe geometry determines the extent and location of the probing volume as well as the CE. The diameter and NA of the fibres incorporated in the two probes employed in this work were optimized to maximize CE, while ensuring a total probe diameter inferior to 2 mm (for endoscope working
channel compatibility). In both probes, the SDS was minimized to raise the CE. A consequence of this, as mentioned above, is an increased sensitivity to superficial layers, which suits the diagnosis of the pathologies of interest in this thesis as they affect the superficial tissue layers.

2.6 Fluorescence and diffuse spectroscopy of diseased tissue: Main application to date: detection of precancerous and cancerous tissue

2.6.1 Fluorescence spectroscopy

Fluorescence spectroscopy consists in recording and studying the wavelength distribution of a sample’s fluorescence. In a clinical context, the value of fluorescence spectroscopy to make a distinction between normal and diseased tissue through the modifications of spectral features with disease progression has been recognized since Policard in 1924 [70]. He noticed that tumours exposed to UV-visible light emitted red fluorescence relatively higher than normal issue following spectra normalization that was attributed to porphyrins, an observation later confirmed by Gougerot in 1939, who studied skin epithelioma (cancer of the skin superficial layers) [71]. More recently, Alfano provided further evidence of the diagnostic power of fluorescence spectroscopy to discriminate cancerous rat and mouse kidney and prostate from healthy tissue [72]. An argon laser emitting at 488 nm was focused on tissue and the fluorescent light was dispersed by a spectrometer and recorded between 500 nm and 750 nm with a photomultiplier tube. Normal and cancerous spectra peaked in the 520-530 nm range due to flavin fluorescence but cancerous spectra all presented a secondary peak occurring in the 590 nm – 600 nm range originating from porphyrins.

Vo-Dinh proposed fluorescence spectroscopy as a tool to diagnose oesophagus cancer in vivo in 1995 [73]. Two hundred measurements from 48 patients were acquired with an optical fibre inserted in the working channel of an endoscope. The probe included seven illumination fibres linked to a pulsed nitrogen-pumped dye laser tuned at 410 nm, as well as twelve detection fibres picking up tissue fluorescence, which was then dispersed by a polychromator and recorded with a photomultiplier tube. Spectra were displayed between 430 nm and 720 nm. Fluorescence in the 520 nm - 540 nm band and 590 nm – 650 nm band were attributed to flavins and porphyrins respectively. Overall fluorescence intensity (non-normalized) from normal oesophageal tissue was found to be considerably higher
compared to the cancerous ones (see Figure 2-9-a). Spectra were then divided by their area under the curve and subtracted from a “baseline” spectrum determined as the average of a selection of 15 normalized spectra from normal tissue sites (see Figure 2-9-b). This procedure enabled additional spectral differences between normal and cancerous samples to be highlighted such as a relatively higher fluorescence of normal samples compared to cancerous samples in the 450 nm – 550 nm domain. This trend was reversed from 550 nm, and between 640 nm and 670 nm, cancerous fluorescence intensity was markedly relatively superior to normal fluorescence intensity. These relative intensity discrepancies peaked at 480 nm and 670 nm. Intensities at these two wavelengths were therefore chosen as markers to separate normal sites from cancerous ones. Using a linear boundary decision, all the sites except two tumour samples were correctly classified with the intensities at 480 nm and all the sites were correctly classified with intensities at 670 nm making fluorescence spectroscopy a promising approach to diagnose oesophageal cancer that could be used in combination with white light endoscopy to improve its sensitivity and specificity. In the chapter 4 of this thesis, a spectroscopic study of gastrointestinal tissue including oesophageal samples was performed. A larger variety of pathologies was examined than in the Vo-Dinh’s experiment and diffuse reflectance was used together with fluorescence to attempt to enhance the diagnostic power that would be obtained with either modality alone.

In 1996, Ramanujam employed fluorescence spectroscopy to detect early stage cervical cancer in vivo [74]. A seven optical fibre probe consisting of three illumination fibres connected to a nitrogen pumped dye laser provided excitation light at 337 nm, 380 nm and 460 nm. Four detection fibres were used to collect the fluorescence light between 400 nm and 650 nm from 381 cervical sites in 95 patients. A multivariate statistical algorithm used on 161 excitation - emission wavelength pairs allowed discrimination of precancerous lesions form normal sites with a sensitivity of 82% and specificity of 68%. Also, high-grade precancerous lesions were distinguished from non-high grade ones with a specificity and sensitivity of 79% and 78% respectively making fluorescence spectroscopy a promising cervical cancer screening tool.
Figure 2-9: (a) Non-normalized and (b) normalized (divided by the area under the curve) fluorescence emission spectra from normal and malignant oesophagus tissues. Reprinted from [73] with permission from John Wiley and Sons.

The aforementioned examples are just a few of a multitude of fluorescence spectroscopy experiments conducted to detect cancerous tissue on a large diversity of living systems including cell cultures, animal models and human organs. Among the latter are colon, cervix, bronchus, bladder, brain, oesophagus, oral cavity, larynx, skin, bile duct, breast and stomach [4, 7].

It is remarkable that some of the spectral differences between normal and malignant tissue appear consistently across these studies in spite of the differences of biochemistry and biostructure between these organs and despite the variety in the implementation of the fluorescence spectroscopy (excitation wavelength, probe geometry, data analysis, see section 2.5). A drop in the overall fluorescence intensity is the most characteristic dissimilarity between healthy and cancerous tissue [75-85].

Another spectral marker of cancer is a red-shifted spectrum [70, 76, 77, 80, 86-97]. This observation has been turned to good account to build a fluorescence imager (LIFE, Xillix Tech. Corp. (acquired by QImaging Corp.), Canada) which localised tumour in lungs and bronchus based on the red band/green band fluorescence intensity [85, 98, 99]. The fluorophores responsible for the spectral discrepancies between normal and cancerous tissues are collagen, elastin as well as NADH, and flavins which are both involved in the electron transport chain, indicating
disorder in the cell metabolism of cancerous tissue. Also involved are porphyrins, heavily present in the blood (heme), whose relative larger emission in the red together with the overall drop of fluorescence in cancer sites attributed to haemoglobin absorption indicates increased vasculature in tumour [73, 79, 81, 96, 97].

Among other applications of fluorescence spectroscopy are the detection of atherosclerotic plaques (marked by increased presence of collagen) [100], as well as caries enamel characterization [101].

2.6.2 Diffuse reflectance spectroscopy

Diffuse reflectance spectroscopy can be seen as a colour analysis of the excitation light diffusely backscattered from a sample at a higher spectral resolution than the eye, providing richer and more detailed information on the tissue state. The colour of this light depends on the absorption (chiefly from haemoglobin in the visible) and scattering of the sample, which are both altered during disease progression. Consequently, diffuse reflectance spectroscopy can probe the biostructure of the medium under study and provides diagnostic information.

In 1980, Ikeda used a two fibre probe to illuminate and collect the diffuse reflectance from rat brains [102]. He showed that the reflectance intensity varied with brain structures, white matter having a significantly higher reflectance than grey matter. Similarly to fluorescence spectroscopy, diffuse reflectance spectroscopy has been extensively used to identify tumours. In 1991, Marchesini recorded the diffuse reflectance between 400 and 800 nm from different skin lesions and demonstrated statistically significant differences between pigmented nevi and malignant melanomas [103]. Mourant collected diffuse reflectance spectra between 250 nm and 800 nm with a system essentially consisting in a pulsed xenon arc lamp from healthy and dysplastic bladder sites from ten patients [104]. Using the slope of the diffuse reflectance spectra between 330 nm and 370 nm (negative for spectra from cancerous tissue, positive otherwise) as a criterion to separate malignant sites from the non-malignant ones, a sensitivity of 100% and a specificity of 97% were reached. A similar system has been employed by the same group in the gastrointestinal tract and showed promising results as for its capacity to separate normal, inflamed and dysplastic (precancerous modification of the cell development) mucosa [105].

Zonios performed diffuse reflectance spectroscopy during colonoscopy on 13 patients with suspected adenomatous polyps (benign tumourous tissue) with an optical fibre through the working channel of an endoscope [51]. An analytical model
(described in section 2.4.2) was employed to extract four parameters from the spectra recorded between 350 nm and 700 nm: haemoglobin concentration and oxygen saturation, effective scatterer size and density. The knowledge of these parameters allowed not only discrimination of normal from cancerous tissue, but it also enabled an insight into the biological modifications entailed by the development of cancer. Adenomatous polyps exhibited higher haemoglobin concentration, but similar oxygen saturation than normal colons. The effective scatterer density was found to be lower in adenomatous polyps and their effective size more important.

Using the hybrid P3 approximation, Wang also observed an increased haemoglobin concentration in colon polyps and malignant colon tumours compared to normal adjacent tissue, yet he also found a statistically significant lower oxygenation for diseased sites [45]. A similar conclusion to Zonios’ for the increased scatterer size was made while studying adenocarcinoma (cancerous epithelium tissue) in the pancreas [106]. Diffuse reflectance spectra indeed revealed an increased size of the cancerous cell nuclei. Fluorescence spectra were also acquired and showed an increased collagen content in cancerous tissue.

Diffuse reflectance spectroscopy has also been applied in breast cancer tissue diagnosis [107]. Seventy-two spectra (13 cancer sites and 59 normal sites) were collected from 24 patients with an optical fibre probe and recorded with a spectrometer. Sensitivity and specificity of 69% and 85%, respectively, were obtained using artificial neural networks. Reflectance spectroscopy was also utilized to recognize benign neoplasms (tissue characterized by an uncontrolled of production of cells) and cancer in ovary. An optical fibre with three different SDS (1.1 mm, 2.1 mm, and 3 mm) was used to illuminate and collect reflected light, which was dispersed by an imaging spectrograph and recorded with a CCD camera [108]. As mentioned in section 2.5.2, increasing SDS means increasing probing depth, consequently the light collected at higher SDS originated from deeper tissue layers. The study conducted at 64 sites from 16 patients yielded an average specificity and sensitivity of 86±6% and 79±5% to separate normal ovary from benign neoplasms and cancers with a linear boundary decision and spectral features statistically found to maximize differences between disease groups. The same type of probe with four distinct SDS has been used to record spectra from 324 sites in 161 patients to assess diffuse reflectance spectroscopy performance for cervical precancer diagnosis [109]. A principal component analysis was performed to retain the main spectral features among the spectra collected at the four SDS and feature selection and classification were achieved by using Mahalanobis distance with cross-
validation. By doing so, normal epithelial sites were discriminated from high-grade intraepithelial lesions with a sensitivity of 72% and a specificity of 81%. More recently, reflectance spectroscopy has been employed for in vivo paediatric brain tumour detection. Statistical analysis led to the choice of intensities between 600 and 800 nm as optimal spectra parameters to distinguish normal tissue from tumours [110].

Myocardial infarction is another condition studied with diffuse reflectance spectroscopy in the rat model [111]. During the four weeks following the infarct, diffuse reflectance spectra together with fluorescence spectra were collected from normal sites, areas surrounding the infarct and infarcted sites with a system consisting of a tungsten halogen source for reflectance and a nitrogen laser for fluorescence linked to a three optical fibre probe. Reflectance and fluorescence light were dispersed and recorded with a spectrometer. Statistically significant spectral profile differences were found between 500 nm and 600 nm and intensity alteration at 480 nm attributed to changes in local haemodynamic was noticed. In particular, deoxygenation arose in the infarcted area and surrounding region one week post-infarction due to blood flow reduction. Partial reoxygenation of infarcted and surrounding area occurred two weeks post-infarction as shown by the transition to a single valley profile characteristic of deoxyhaemoglobin to a double valley profile characteristic of oxyhaemoglobin.

Figure 2-12 deleted

Point-limited spectroscopy studies were only considered so far. These methods, like the traditional biopsy approach, suffer from spatial under sampling for large tissues and do not allow assessing the extent of tumours. To overcome this limitation, some groups proposed scanning the illumination light beam on the tissue surface with a 2D mirror scanner and record the diffuse reflectance spectrum at each position. By extracting haemoglobin concentration, oxygenation, and scattering coefficient with one of the methods mentioned in 2.4, it is therefore possible to build a pseudo-colour map where the contrast arises from the difference of values of these parameters. A resected colon tissue containing a normal area and a cancerous area below was imaged in this approach [112]. Both reflectance and fluorescence spectra were collected in this study, the reduced scattering coefficient at 700 nm and haemoglobin concentration were retrieved from the reflectance spectra, and the ratio of collagen concentration over the NADH concentration (the two major contributors of colon fluorescence) was deduced from fluorescence spectra. Assuming a multivariate Gaussian prior for these three parameters and using Bayes’ rule, the posterior probabilities of belonging to the cancer class were calculated for each of the
pixel of the image: pixels with posterior probability superior to 0.5 were classified as
cancer and normal otherwise, hence generating the probability map, which enables
delineation of tumour margins. Scattering property changes of murine tumours in the
prostate and the pancreas have been imaged in the same way [113].

2.7 Summary

This chapter highlighted how to measure and extract the diagnostic information
contained in fluorescence and diffuse reflectance light. Typically, a fibre optic probe
is employed to deliver excitation light to, and gather remitted light from a tissue site of
interest. Secondly, a biopsy from the same site is taken in a second time. Then,
fluorescence and diffuse reflectance spectra are sorted in disease groups according
to the diagnostic obtained with the histopathological inspection (the gold-standard
method) of the biopsies. Spectral differences between the groups detected
qualitatively, statistically or via a model to extract the tissue absorption and scattering
coefficients are exploited to discriminate the normal sites from the diseased sites with
a binary or probability based algorithms. As will become apparent in the next chapter,
our approach has been to choose a statistical algorithm to select the best
discriminating spectral features followed by a probabilistic classification scheme.

This chapter focussed on single point spectroscopic methods, of interest in
chapter 3 and chapter 4, a review of the spectral imaging techniques, employed in
chapter 5 and chapter 6, is provided at the beginning of chapter 5.
2.8 References


Chapter 3

Fluorescence spectroscopy as a liver disease diagnostic tool

A novel biophotonic approach to assess liver tissue state is presented in this chapter including building of the optical system and testing it on liver tissue. For the first time, a multi-fluorescence spectroscopy system has been used to diagnose non-alcoholic fatty liver diseases (NAFLD) in mouse model. Non-alcoholic fatty liver diseases (NAFLD), already with high prevalence, are set to increase in the coming years stressing the need of a fast, robust method to diagnose it. In this chapter, after briefly exposing their causes, the current methods of diagnosis of NAFLD are introduced and their limitations highlighted. The results of two experiments, performed in collaboration with Hoa Nguyen, M.Sc. Student and with our clinical collaborators Dr. Adam Levene, Dr. Quentin Anstee, and Pr. Robert Goldin (St Mary’s hospital, Imperial College Healthcare NHS trust) are presented. The first one, looking at separating normal, steatotic and steatohepatitic livers, and the second one, differentiating normal, mild steatotic and severe steatotic tissue were conducted in the mouse model.

The vast majority of studies looking at using spectroscopic probes for tissue diagnosis are performed ex vivo or in vivo, but on external organs easy to access like breasts or the skin. Intraoperative measurements on internal organs like liver raise some challenges among which is the accurate positioning of the probe on a target site and the control of the pressure applied to tissue, which alters the optical properties, and hence the spectra of the probed tissue, which can potentially lead to error in spectroscopic based diagnostic. The contribution of an articulated robotic endoscope, designed by Dr. David Noonan, Dr. Jianzhong Shang, and Chris Payne (Mechatronic group, Hamlyn Centre, Imperial College London) including a force sensor and used in combination with the spectroscopic probe was demonstrated ex vivo on a phantom and liver tissue. This allowed probe pressure control and monitoring, as well as probe scanning.
3.1 Dual excitation fluorescence spectroscopy system

Figure 3-1 presents the optical system used this chapter. It essentially consisted in two laser diodes emitting at 375 nm (model NDU1113E, Nichia Corp., Japan) and 405 nm (model GH04P21A2GE, Sharp Corp., Japan), a custom-built optical fibre probe (Leoni Fiber Optics, Inc., (previously Romack, Inc.), USA), and a CCD (Retiga EXI, QImaging, Canada)/spectrograph (model V10, Specim Ltd, Finland) unit. The probe included one excitation fibre, conveying the laser light to the sample, and surrounded by six hexagonally packed fluorescence collection fibres. The diameters and numerical apertures of the fibres were 200 µm and 0.22, respectively, and they were selected to have low autofluorescence and attenuation in the near UV spectral region. The probe distal tip was covered by a 1.5 mm thick glass spacer allowing excitation and emission beams to overlap and the fibres and window were housed in a stainless steel tube with an external diameter of 2 mm, allowing its insertion into endoscope working channels, needles or small orifices. The proximal end was divided into two arms, the first one contained the excitation fibre and was coupled to the laser optics; the second arm included the six emission fibres arranged in a linear array. The 375 nm beam was reflected by the dichroic mirror (model FF409MDI02, Semrock, Inc.; USA) and was aligned and coupled into the excitation fibre with two steering mirrors. The light from the 405 nm laser was transmitted by the dichroic mirror and coupled to the excitation fibre. It should noted that the angle of incidence of the two laser beams on the dichroic was not 45° as it is normally the case because its cut-off wavelength was 409 nm and was therefore not included between 375 nm and 405 nm (a dichroic mirror with adequate cut-off wavelength was not found at the time of the experiment). The dichroic mirror was orientated to enable the reflection/emission pattern described above. Two beam shutters (one for each laser diode) enabled the selection of the excitation wavelength delivered to the sample. The optical power at the distal end of the probe was 3.5 mW for both lasers. The fluorescence emission from the six fibres was focussed with a lens doublet onto the 80 µm wide input slit of an imaging spectrograph. A 430 nm long-pass filter (model 3RD430LP, Omega Optical, Inc., USA) was inserted between the fibres and the slit to stop the light of the laser diodes reflected by the sample. The light dispersed by the prism-grating-prism element of the spectrograph was then acquired with a sensitive cooled CCD camera. All the optics was assembled together using a cage system and a breadboard, so that the system was robust and portable. A LabVIEW
program controlled the exposure time (typically 100 ms) and beam shutters, and enabled acquisition of fluorescent signals from the laser diodes sequentially with an adjustable number of measurements. Full details of the system can be found in [1].

![Experimental set-up of dual-excitation wavelength fluorescence spectroscopy probe. From [1].](image)

**Fluorescence spectroscopy**

The six normally identical emission spectra recorded for each excitation wavelength were processed from the CCD images and the intensities at different spatial locations on the image were converted into spectra using calibration data obtained with a number of specific laser wavelengths. The spectrum of a tungsten-halogen calibrated light source (Ocean Optics, Inc., USA) was also recorded at the start of the experiment to take into account the system spectral response and the ratio of this spectrum over the real spectrum was used to correct the raw spectra. Spectra were normalized by the area under the curve and truncated between 450 and 750 nm.

Fluorescence spectra at 375 nm excitation from a 3 mM solution of FAD have been acquired with a spectrometer (Ocean Optics, Inc., USA) (the “reference” spectrum) and with our CCD/spectrograph system (the “measured” spectrum) to test the calibration procedure (Figure 3-2). The spectral accuracy defined as the ratio of the area in common under the two spectra over the area under the reference spectrum was found to be equal to 93%. The non-perfect overlap between the two spectra was likely to arise from differences of specifications between the gratings...
detection elements of the spectrometer and the spectrograph/CCD systems, such as the grating’s optical resolution and the detector’s spectral sensitivity.

Figure 3-2: Emission fluorescence spectrum of a FAD solution excited at 375 nm and recorded with a spectrometer (reference) and with the dual-excitation wavelength fluorescence spectroscopy system (measured).

3.2 Non alcoholic fatty liver disease

3.2.1 Clinical characteristics, prevalence and current diagnostic method limitations

NAFLD comprise a large array of progressive liver diseases arising without major alcohol consumption, ranging from steatosis (fatty liver), through steatohepatitis (fat and inflammation) to liver fibrosis, and eventually cirrhosis [2]. NAFLD are characterized by an accumulation of triglycerides (fat) in the liver and are linked to obesity and insulin resistance/diabetes. While steatosis has usually been considered as a benign pathology [2], its evolution into more serious progressive fibrosis and cirrhosis is problematic. NAFLD, already the third most important cause for liver transplantation, are set to become the first indication in coming decades [3]. The global prevalence of NAFLD is estimated to be between 10 and 39% [4] reaching 57% to 74% among the obese population, and 50% among diabetic patients [5]. The general prevalence is predicted to rise with the global growth of obesity and diabetes (obese adult population rise from 400 million in 2005 to 700 million by 2013 [6], and diabetic population augmentation from 285 million in 2010 to 438 million in 2030 [7]). Meanwhile, the waiting list for liver transplantation stretches out, and 354 patients were registered for a liver transplant in the UK in January 2011 [8].
The combined effects of the organ donors shortage and the important prevalence of NAFLD, led the increasingly frequent use of steatotic 'marginal' grafts, i.e. grafts more prone to primary non-function, in liver transplantation [9]. However, severe steatosis and steatohepatitis are associated with increased risk of primary graft non-function [10, 11]. Consequently, to aid effective organ selection, a fast and reliable method of donor organs evaluation for degree of steatosis and the presence of significant oxidative stress is needed as these are the signs of grafts at greater risk of non-function following transplantation.

Table 3-1: Methods used to diagnose NAFLD

<table>
<thead>
<tr>
<th>Method</th>
<th>Marker</th>
<th>Advantages</th>
<th>Drawbacks</th>
<th>Use in clinical settings</th>
<th>Diagnostic length (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Histopathology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Microscopic visualisation of</td>
<td>Reliable (gold standard)</td>
<td>Subjective</td>
<td>Yes</td>
<td>2 (after biopsy)</td>
</tr>
<tr>
<td></td>
<td>tissue section</td>
<td></td>
<td>Time consuming</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AL/ALT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Biochemical hepatitis</td>
<td>Ease of blood sample obtention</td>
<td>Insensitive</td>
<td>Yes but not as specific marker for NAFLD</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>AST</td>
<td>Blood sample obtention</td>
<td>Insensitive</td>
<td>Yes but not as specific marker for NAFLD</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>CK 18 fragments</td>
<td>Blood sample obtention</td>
<td>Not yet at diagnostic level</td>
<td>Research only</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>TBARS</td>
<td>Specific marker of oxidative stress in the tissue</td>
<td>Insensitive, Non-specific, Time consuming, Laborious</td>
<td>No</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Ultrasound imaging</td>
<td>Fat content</td>
<td>High sensitivity &amp; specificity if fat content &gt; 33%</td>
<td>Yes</td>
<td>3 (approx.)</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>Fat content</td>
<td>High sensitivity &amp; specificity if fat content &gt; 33%</td>
<td>No differentiation steatosis/NASH</td>
<td>Occasionnally 3 (approx.)</td>
</tr>
<tr>
<td></td>
<td>MRI</td>
<td>Fat content</td>
<td>High sensitivity &amp; specificity when fat content over 33%</td>
<td>No differentiation steatosis/NASH</td>
<td>Yes 3 (approx.)</td>
</tr>
</tbody>
</table>
Table 3-1 details the existing tests to diagnose NAFLD or non-alcoholic steatohepatitis (NASH). Information available to the practicing clinician when faced with a patient who may have NAFLD includes the medical history and physical examination, routine laboratory investigations, and typically, an ultrasound. Since most patients with NAFLD are asymptomatic and the symptoms, when they occur, are nonspecific (e.g. fatigue, right upper quadrant discomfort, weight loss), the examination of symptoms is an unreliable way of estimating the severity of NAFLD [12]. Biopsy, collected percutaneously or by transjugular catheterization [13], is used as a definitive diagnosis, but is time-consuming and not well suited to peri/intraoperative assessment of fatty liver [13].

Blood biochemistry is another NAFLD diagnostic approach. In practice, the clinical diagnosis of NAFLD is based on the identification of raised alanine transaminase (ALT) and/or aspartate aminotransferase (AST) levels in the blood, indicating liver damage (mostly hepatitis), in patients with features of the metabolic syndrome (e.g. obesity, diabetes) with supporting evidence of fatty liver on ultrasound imaging [3]. Yet, increased ALT and AST levels alone only indicate liver damage without informing on the disease causing it, also normal ALT and AST levels have been found in patients suffering from NAFLD [14] making these tests non-specific and insensitive. Other blood tests include cytokeratin-18 (CK 18) fragment levels and thiobarbituric acid reactive substance (TBARS) assay. CK-18 fragment levels are indicators of cell apoptosis shown to correlate with NALFD presence [15]. TBARS assay is an accepted research method to measure lipid peroxidation as a substitute measure for free radical damage ('tissue oxidative stress') within the liver, a key driver of disease pathogenesis, inflammation and progression in NASH [3]. None of the latter two are used in standard practice.

Several imaging techniques have been tested for NAFLD diagnosis. Ultrasound probes steatosis with a weak sensitivity (67%) and specificity (77%) [16], and becomes more reliable when the severity of the steatosis raises [17]. Computed tomography (CT) imaging enables sensitivity and specificity for steatosis diagnosis of 100% and 82-93% respectively [18]. Magnetic resonance imaging (MRI) allows the most precise triglycerides quantification and hence has the best sensitivity (100%) and specificity (92%) for steatosis diagnosis [10]. Using these imaging techniques, steatosis can be detected optimally only if the liver fat content is more than 33% [19].

Nevertheless, the imaging approach is limited to steatosis grading because all the imaging methods just mentioned only measure the fat content, and can not differentiate steatosis from steatohepatitis or cirrhosis, which are characterized by tissue inflammation and fibrosis, rather than by triglyceride quantity. In fact, in some
livers where steatohepatitis and cirrhosis are well established, fat content may diminish, leading to an underestimation of the pathology gravity [20].

Finally, the length of all the aforementioned diagnostic methods is one day minimum. Therefore, a fast and minimally invasive method able to differentiate normal tissue from steatosis and from steatohepatitis would be desirable.

3.2.2 Fluorescence spectroscopy to assess liver state: incentive and previous work

As mentioned in the last chapter, fluorescence spectroscopy has been widely used for cancer diagnosis in different organs. It could also have potential applications in liver tissue classification. In ex vivo setting, it could be a substitute or a complement to histopathology and would also allow for non-destructive NAFLD diagnosis in liver transplant grafts, which are more prone to primary non-function. In vivo, it could replace biopsy in biopsy intolerant patients. Fluorescence spectroscopic probes could, in particular, be an advantageous alternative to needle biopsy during transjugular liver biopsy. This type of procedure, which consists in the introduction of a catheter in the right hepatic vein after puncturing of the right internal jugular vein and needle biopsy of the liver through the catheter [13], is performed when percutaneous liver biopsy is contraindicated (massive obesity or, coagulopathy for instance) [21]. Samples collected with transjugular liver biopsy are undersized and fragmented, which can prevent diagnosis [13, 21]. The inner diameter of the catheters (in the mm range [22]) would permit the introduction of a fluorescence spectroscopic probe, although effects of the blood absorption on the liver fluorescence properties would remain to be assessed in this particular approach. Fluorescence spectroscopy could also be employed as an adjunct in patients already undergoing an operation, such as bariatric surgery or to evaluate the donor organ at harvest.

Liver autofluorescence has previously been studied by Croce et al [23-27] who demonstrated that NAD(P)H, vitamin A, flavins, porphyrins were major contributors to liver autofluorescence when excited at 366 nm. The main differentiation markers recognized to differentiate healthy, steatotic and fibrotic livers were NAD(P)H, vitamin A and collagen. Importantly, a parallel increase of lipids and vitamin A was demonstrated. Other fluorophores including flavins, riboflavin, arachinoid acid, lipopigments, and porphyrins, all present in liver [28-31] and emitting in the visible range [32], are also expected to contribute to the liver fluorescence. Croce's studies were crucial to comprehend liver fluorescence and to assert
fluorescence spectroscopy as a possible NAFLD detection method. However, the instrumentation used in this work forbids its use intraoperatively as it requires an histological preparation of the excised liver tissues. Sankarankutty et al. proposed fluorescence as a tool to evaluate the viability of liver grafts [33]. The evolution of the fluorescence signal with time has been shown to follow the time course of mitochondrial respiratory capacity, one of the usual indicators used to judge liver viability. De Oliveira et al. demonstrated that the ratio of the maximum fluorescence signal emitted by liver after 532 nm excitation over the amplitude of the backscattered light at this wavelength was linearly correlated with the hepatic fat content with a correction factor of 0.983 [34]. While the most severe steatosis cases were clearly distinguished from the normal ones through this ratio, intermediate study groups could not be differentiated from one another. This study demonstrated the potential of fluorescence spectroscopy for NAFLD analysis, but only exploited a very limited spectral range to separate disease groups as only two wavelengths were used. Furthermore, none of the studies to date quantified the fluorescence diagnosis performance.

Consequently, the building of a fluorescence spectroscopy probe system providing excitation at a wavelength that will allow autofluorescence from vitamin A, collagen, NADH and porphyrins, together with a classification algorithm utilising the whole spectrum appears to be a favourable candidate to swiftly and non-invasively diagnose steatosis and steatohepatitis.

3.3 Data classification with Sparse Multinomial Learning algorithm

For each of the measurements acquired in the NAFLD experiments (see sections 3.4 and 3.5), the spectra from the two excitation wavelengths were concatenated prior to processing rather than treated separately as it was initially assumed that combining the spectra at the two excitation wavelengths might potentially improve the classification. Then, a sparse multinomial logistic regression (SMLR) was applied to classify the concatenated spectra. SMLR has previously been successfully demonstrated to distinguish autofluorescence and diffuse reflectance spectra from healthy, marginal, and tumourous brain tissue [35, 36]. Most of classification algorithms are binary, but more than two disease categories were considered in our study. The SMLR, which enables multi-class separation, was therefore chosen.

SMLR belongs to the family of statistical pattern recognition algorithms; a pattern corresponds here to the set of fluorescence intensities (spectral features)
building up a fluorescence spectrum. Statistical pattern recognition usually involves, firstly, a dimensionality reduction step to select the features or combinations of features which optimize the separation between patterns belonging to the different classes. Secondly, the patterns are classified after decision boundaries separating the selected features have been established. Decision boundaries are defined by assigning probability mass functions to features of a set of training patterns conditioned on the pattern class and by choosing a decision rule; this corresponds to the training of the classifier [37].

In the context of our work, SMLR as previously developed by Krishnapuram et al. [38], was used to separate the 106*2 fluorescence intensities (106 intensities at each excitation wavelength) at the 106*2 wavelengths sampled between 450 nm and 750 nm comprising each concatenated spectrum, into three disease classes (control, moderate steatosis and steatohepatitis in the first experiment, and control, mild steatosis and severe steatosis in the second experiment) with an open source software package [39].

The probability mass function \( P(C_i|s,w_i) \) (Equation 3-1) that a spectrum made up of 212 spectral features belongs to a class \( C_i \) represented by its weight vector \( w_i \) is assumed to follow the multinomial logistic regression model, where the regression coefficients are the numbers of the classifier weight vector \( w_i \) of the corresponding class \( C_i [38]: \)

\[
P(C_i|s,w_i) = \frac{\exp[w_i \cdot s]}{\sum_{i=1}^{3} \exp[w_i \cdot x]} \quad \text{Equation 3-1}
\]

The spectrum is assumed to belong to the class whose weight vector maximises this probability. The estimation of the weight matrix (212 lines, three columns) for the three different classes (in other words the result of the classifier learning process), is calculated using Bayes' decision rule (Equation 3-2) stating that the spectrum \( s \) belongs to class \( C_i \) if:

\[
P(C_i|s,w_i) > P(C_j|s,w_j) \quad \text{for all } j \neq i \quad \text{Equation 3-2}
\]

Equation 3-2 can be rewritten in terms of maximum likelihood decision rule which maximizes the log-likelihood function (Equation 3-3) in order to estimate the weight vectors, from the \( n \) training spectra:

\[
l(w) = \sum_{j=1}^{n} \log(P(y_j|s_j),w) = \sum_{j=1}^{n} \left\{ \sum_{i=1}^{3} y_j^i w_i s_j - \log(\sum_{i=1}^{3} \exp(w_i s_j)) \right\} \quad \text{Equation 3-3}
\]
The sparsity of the classifier is achieved by introducing a prior on the weights. This approach differs from the traditional maximum likelihood estimation (Equation 3-3) in that it is not only the log-likelihood function which is maximised to estimate the weight vectors but the sum of the log-likelihood function and the logarithm of a prior on the weights distribution. A Laplacian prior is chosen because the logarithm of this distribution is proportional to the $l_1$-norm of the weights. Consequently, this maximisation problem is similar to an $l_1$-penalised regression, which presents the major advantage to promote sparse coefficient vectors, i.e. vectors with coefficients either large or null. The details of the maximisation process are beyond the scope of this work, but can be found in [38]. At the end of the optimization step, the estimates of $w$ are known and the spectra posterior probabilities of class membership can therefore be calculated. A spectrum belongs to the class whose weight vector maximizes the estimated posterior probability as stated in Equation 3-2.

The benefits of sparse estimates are numerous: as many coefficients are set to zero, their variance is low, which produce a stable model with high generalisation ability, that is the performance in classifying new spectra [37], inherent feature selection [40], and handling of large dataset as the weights of the spectral features inconsequential for the classification are set to zero [37]. Finally, as the probability of spectrum class membership is known, SMLR presents the advantage to identify “boundary cases” i.e. spectra close to a decision boundary for which the diagnostic need to be more finely-shaded than the spectra with a high-probability of class membership.

### 3.4 Distinguishing healthy liver from steatotic and steatohepatitic livers in the mouse model—Experiment 1

In this experiment, the fluorescence spectroscopy probe described in Figure 3-1 was used to excite different subspecies of the endogenous fluorophores present, and the concatenated emission spectra at the two excitation wavelengths could then be used to distinguish different tissue states. The SMLR algorithm enabled differences
between normal, steatotic and steatohepatitic livers to be detected in the mouse model.

3.4.1 Study design

Research was approved by the local ethics committee and performed in accordance with the UK Animal (Scientific Procedures) Act 1986. Mice were housed under standard conditions and fed with a commercial chow (#801722, SDS, UK) and free access to water. The study was performed to examine the autofluorescence spectroscopy of liver tissue from mice with different NAFLD severities and used mouse models with steatosis and steatohepatitis induced by a combination of genetic and dietary modulation [41]. A ‘control group’ of 8-week old male non-obese mice were fed with a control diet (Charles River, Belgium) and compared to genetically modified mice fed either with a nutritionally replete control diet (Research Diets, USA - ‘mild steatosis group’) or with a choline deficient diet (Research Diets, USA - ‘steatohepatitis group’). There were initially five animals per group but two of the mice from the mild steatosis group died prematurely during the experiment and were excluded from the study.

At the end of the study, animals were culled by exsanguination under general anaesthesia and liver tissue was rapidly harvested. The liver was bisected and half was immediately snap-frozen in liquid nitrogen for subsequent biochemical analysis. Blood was also taken for biochemical analysis. Without any treatment or staining, liver tissues were submitted to measurements immediately after culling to minimize air-exposure effects, cleaned of blood to minimize haemoglobin absorption and brought from the operating table to the optical bench. Emission spectra were recorded and the livers were then fixed in formalin solution for further histopathological study.

Fluorescence data were recorded at between three and five different locations on each liver. At each location, six measurements (one measurement consisted of two spectra – one for 375 nm and one for 405 nm excitation) were recorded from the six emission fibres in the probe, probing an overlapping but different tissue volume. This generated a total of 114 (19 investigated locations × 6 fibres) measurements (also referred to as sites in the rest of this chapter) for the normal group, 72 (12 investigated locations) measurements from the steatotic group, and 114 (19 investigated locations) measurements for the steatohepatitic group. The SMLR classification was completed with a K-fold cross validation rather than with a
leave-one-out cross validation because the six spectra from one acquisition cannot be regarded as being entirely independent. In this approach, the sample pool of 300 spectra was divided in 50 subsets (K=50), each including the six spectra from the same acquisition so that during the classifier learning groups of six were held out at a time.

3.4.2 Measurement protocol and data processing

The liver tissue preparation and analysis described in this paragraph was performed by Dr. Adam Levene, Dr. Quentin Anstee, and Pr. Robert Goldin.

**Histopathology**

Formalin fixed liver tissue was treated into paraffin wax and sections were stained with Haematoxylin and Eosin (H&E). H&E stained sections were examined using the NASH Clinical Research Network histological classification system [42]. The NAFLD activity scoring system (NAS), detailed in Table 3-2, includes the steatosis score (between 0 and 3), which refers to the amount of surface area with steatotic hepatocytes as evaluated on low to medium microscope power, the lobular inflammation score (from 0 to 3), which evaluates the number of inflammatory foci per x200 magnification field, and the hepatocyte ballooning score (0-2), which grades the number hepatocytes with a balloon appearing before their death. The NAS is hence ranging from 0 to 8.

*Table 3-2: Details of NAFLD activity scoring.*

<table>
<thead>
<tr>
<th>NAFLD activity scoring</th>
<th>Steatosis (% of hepatocytes involved)</th>
<th>Lobular inflammation (foci per×200 field)</th>
<th>Hepatocyte ballooning (number of balloon shape hepatocytes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>≤5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>5-33</td>
<td>2</td>
<td>few</td>
</tr>
<tr>
<td>2</td>
<td>33-66</td>
<td>2-4</td>
<td>many</td>
</tr>
<tr>
<td>3</td>
<td>&gt;66</td>
<td>≥4</td>
<td></td>
</tr>
</tbody>
</table>

**TBARS Assay and ALT Biochemistry**

The methionine choline deficient (MCD) mouse model of NASH is characterized by elevated reactive oxygen species production such as malondialdehyde (MDA). A TBARS assay was carried out in order to quantify this. MDA reacts with thiobarbituric acid (TBA) as a thiobarbituric acid reactive substance (TBARS) to produce a red colored complex which has peak absorbance at 532 nm [43]. The amount of TBARS
was expressed as µmol MDA equivalent formed per 100mg cell protein measured. For a quantitative determination of TBARS, MDA standard solutions were employed. About 100 mg of frozen liver was placed into a ceramic beads tube. 300 µl of radioimmunoprecipitation (RIPA) buffer was subsequently added and the tubes positioned into a Precellys rotor at 6500 rpm for one cycle of 50 seconds to homogenise the samples. Then, the supernatant was put on ice and used in the assay. 100 µl of the lysate was put into a 1.5 ml microcentrifuge tube and 200 µl of ice cold, 10% trichloracetic acid was added. The sample was incubated for 15 min on ice followed by centrifugation at 3000 g for 10 minutes. Standards were prepared with distilled water and 500 µM MDA to give known MDA concentrations from 0 µM to 50 µM. 300 µl of the samples and standards were then mixed with 300 µl of 0.67% TBA and incubated at 95 °C for 10 minutes. The samples were then allowed to cool. Using a 96 well plate, 150 µl of each standard and sample was placed into a well, performed in duplicate. Spectrophotometry at 532 nm was measured using a plate reader. For each sample, the MDA concentration in µM per 100 mg of liver was calculated from the absorbance values and standard curve.

ALT levels were also quantified. The blood took when the mice were culled was placed in a plasma collection tube which when centrifuged at 3000 rpm for 10 minutes separated the plasma from the erythrocytes. The supernatant plasma was separated from the blood and stored at -80°C until analysed in an automated biochemical analyser to provide ALT measurements in IU/L.

3.4.3 Results

Histopathology
Marked differences were visible on the H&E stained sections of the three groups. The control group (non-obese mice fed chow) had normal liver histology. The mild steatosis group (genetically modified mice fed chow) displayed features of isolated steatosis without lobular inflammation and steatohepatitis group (genetically modified mice fed a MCD diet) had NASH with marked steatosis, moderate lobular inflammation and numerous ballooned cells (see Table 3-3 and Figure 3-3).
Table 3-3: Median NAFLD activity score for the mice. From [1]

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Steatosis</th>
<th>Lobular inflammation</th>
<th>Hepatocyte ballooning</th>
<th>NAFLD score</th>
<th>activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mild steatosis</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Steatohepatitis</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>7</td>
<td>7</td>
</tr>
</tbody>
</table>

**TBARS Assay**

There were notably raised MDA concentrations in the livers of the steatohepatitis group compared to the two other study groups, as expected - p=0.028 with the control group and p=0.011 with the mild steatosis group, see Figure 3-4.

Figure 3-4: Mean malondialdehyde concentration in the livers of the mice ± standard error of the mean (SEM). From [1]
The mean MDA concentrations (µM per 100mg of liver) were 0.26±0.02 for the control group, 0.28±0.02 for the mild steatosis group, and 1.79±0.46 for the steatohepatitis group (Table 3-4). These results verified that there was increased oxidative stress within the livers of the steatohepatitis group. No major difference was found between the steatosis group and the control group (Figure 3-6).

Table 3-4: Mean malondialdehyde concentration in the livers of the mice ±SEM. From [1].

<table>
<thead>
<tr>
<th>Pilot Study Group</th>
<th>Malondialdehyde Concentration (µM per 100mg of liver) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.26±0.02</td>
</tr>
<tr>
<td>Mild steatosis</td>
<td>0.28±0.02</td>
</tr>
<tr>
<td>Steatohepatitis</td>
<td>1.79±0.46</td>
</tr>
</tbody>
</table>

ALT Biochemistry

There was significantly increased ALT levels in the plasma of steatohepatitis group compared to the two other study groups, as expected (p=<0.001 for the mild steatosis group and control group – see Figure 3-5). The mean ALT levels in the plasma (IU/L) were 26.40±4.07 for the control group, 91.77±4.78 for the mild steatosis group and 307.34±33.38 for steatohepatitis group (Table 3-5).

Figure 3-5: Mean ALT levels in the plasma of the mice ±SEM. From [1]

These observations proved that there was biochemical hepatitis within the liver for the steatohepatitis group which is in keeping with the lobular inflammation and ballooning identified on H&E stain. No important difference was detected between
the mild steatosis group and the control group although there did appear to be a trend towards increased ALT in the steatotic mice.

Table 3-5: Mean ALT levels in the plasma of the mice ±SEM. From [1]

<table>
<thead>
<tr>
<th>Pilot Study Group</th>
<th>ALT biochemistry (IU/L) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>26.40±4.07</td>
</tr>
<tr>
<td>Mild steatosis</td>
<td>91.77±4.78</td>
</tr>
<tr>
<td>Steatohepatitis</td>
<td>307.34±33.38</td>
</tr>
</tbody>
</table>

Fluorescence spectroscopy results

Figure 3-6 presents the mean spectra at 375 nm and 405 nm excitation and error bars (one data standard deviation). At 375 nm excitation, liver autofluorescence peaked at around 475 nm which was attributed to the combined fluorescence of NAD(P)H (emission at around 475 nm) and vitamin A (emission at around 490 nm) [26]. Mild steatotic tissue exhibited higher 475 nm intensity than healthy tissue, with intensity raising together with the steatosis severity in these two groups. The steatohepatitis group showed weaker peak fluorescence than healthy and steatotic tissue. The relative increase in intensity at 475 nm for 375 nm excitation from the normal (steatosis score 0) to the mild steatosis group (steatosis score 2, see Figure 3-6-a) is consistent with an augmentation in vitamin A content associated with the augmentation of lipids [26]. The concentration of NAD(P)H, also emitting in this spectral domain (emission peak at 475 nm), was found to soar with steatosis [44]. Based on these considerations, the steatohepatitis group with a steatosis score of 3, indicating a higher fat content than the two other groups, may hence have been believed to have a greater intensity at 475 nm. Nonetheless, it actually had an inferior intensity (Figure 3-6-a). A possible explanation for this could lie in the biochemical and biostuctural changes provoked by fibrosis in this steatohepatitis model. First, collagen, which is induced by fibrosis, is a well known scatterer and fluorophore and will hence change the fluorescence and scattering properties of the liver tissue. Second, the vitamin A contribution to the fluorescence spectrum of fibrotic liver was found to be half that of normal liver at 366 nm excitation [39]. Consequently, the existence of fibrotic tissue in the steatohepatitic livers is expected to lead to the peak intensity drop in this group. Moreover, the evolution of NAD(P)H concentration with fibrosing steatohepatitis is not known.

The 405 nm excitation spectra revealed a main peak intensity at 500-510 nm and secondary peak at 610 nm attributed to porphyrins, synthesised in liver, whose
excitation spectrum peaks close to 405 nm and emission spectrum peaks at 610 nm [33]. Spectra at both excitation wavelengths included a weak peak at 575 nm, which could come from lipofuscin, a lipopigment present in the liver, whose concentration increases with age [31] and exhibiting a maximum of fluorescence emission near 575 nm [32]. At 405 nm, spectral differences between the three groups appeared visually less noticeable than at 375 nm excitation, although the difference was still statistically significant, as the SMLR analysis will show. Vitamin A and NAD(P)H are poorly excited at 405 nm, their contributions to the fluorescence spectra will appear slightly less important than at 375 nm excitation. The spectral shape also results from the contributions of the other fluorophores present in the liver including flavins [28], which have an emission band between 500 nm and 600 nm [32], and arachidonic acid, a free fatty acid metabolized by the liver [29] that fluoresces between 400 and 550 nm [23].

Figure 3-6: Mean spectra at (a) 375 nm and (b) 405 nm excitation and error bars representing standard deviation. From [1]

The results of the SMLR classification performed with a K-fold cross validation are presented in the confusion matrices in Table 3M6 for the concatenated dataset. The overall classification accuracy obtained reached 95%. This table can be reinterpreted in terms of diagnostically more meaningful parameters by considering the sensitivity, specificity, positive and negative predictive values (PPV, NPV) of one group against the two others. The normal tissue (control group) could be differentiated from the mild steatotic and steatohepatitic tissue with 96% sensitivity, 97% specificity, 95% PPV and 97% NPV. The steatotic tissue was discriminated from the normal and steatohepatitic tissue with 92% sensitivity, 96% specificity, 88% PPV, and 97% NPV. Steatohepatitic tissue was separated from the two other groups with 97% sensitivity, 100% specificity, 100% PPV and 98% NPV.
Table 3-6: Confusion matrix presenting the ability of fluorescence spectroscopy to distinguish between control, mild steatosis and steatohepatitis disease groups with the concatenated spectra. From [1]

<table>
<thead>
<tr>
<th>Disease group</th>
<th>Control</th>
<th>Mild steatosis</th>
<th>Steatohepatitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>114</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mild steatosis</td>
<td>0</td>
<td>72</td>
<td>0</td>
</tr>
<tr>
<td>Steatohepatitis</td>
<td>0</td>
<td>1</td>
<td>113</td>
</tr>
</tbody>
</table>

The posterior probabilities calculated by SMLR of fluorescence spectra belonging to the correct disease group as classified by histopathology are plotted in Figure 3-7. Fifteen sites were spectrally misclassified with a probability below 50%, with the remainder classified with an average probability of 98.9% indicating a strong confidence in the classification of the tissues.

![Figure 3-7: Posterior probability of fluorescence spectra from interrogated tissue sites belonging to their correct disease group (as classified by histopathology): control (114 sites), mild steatosis (72 sites), steatohepatitis (114 sites). From [1]](image)

For single excitation, the classification accuracy of the first experiment was 80% and 95% for 375 nm and 405 nm excitation, respectively. The classification with the combined spectra at the two excitation wavelengths reached 95%, and thus did not enhance the classification achieved with the single 405 laser diode.

In conclusion, this proof-of-concept experiment demonstrated the value of fluorescence as a tool for NAFLD diagnosis. However, the mouse model used here was pretty extreme with clearly distinct differences between disease groups which
might not reflect a real case diagnostic scenario. A second study which included disease groups with more subtle differences was hence conducted to evaluate the diagnostic power of fluorescence spectroscopy in more ambiguous conditions.

3.5 Grading steatosis-Experiment 2

A second experiment was conducted in the mouse model to assess the fluorescence spectroscopy capacity to differentiate steatosis of different severity.

3.5.1 Study design

The protocol included harvesting the mouse livers, recording fluorescence spectra and processing tissue for further histopathology, and was similar to the one indicated in section 3.4.1. No blood tests were performed in this experiment. A total of 25 mice were examined within three groups as follows: Twelve mice in the control group, nine mice in the moderate steatosis group, and four mice in the severe steatosis group. The total numbers of measurements (one measurement consisted of two spectra – one for 375 nm and one for 405 nm excitation) acquired from each of these groups were respectively 252 (42 investigated locations × 6 fibres), 258 (43 investigated locations) and 102 (17 investigated locations), and they were classified using SMLR.

3.5.2 Results

The histopathology scores attributed to the control, moderate steatosis, and severe steatosis after microscopic evaluation of the aspect of tissue sections were respectively, 0, 4, 5-6. Fibrosis was detected in none of the mice. The spectra at 375 nm excitation and 405 nm excitation are presented Figure 3-8. They both peak at the same wavelength as the first experiment, that is 475 nm and 500-510 nm respectively. Like in the first experiment, a secondary peak present at 610 nm attributed to porphyrin was observed in the 405 excitation spectrum, yet the secondary peak at 575 nm present in both spectra of the first experiment and attributed to lipofuscin was absent in both spectra of the second experiment, signifying a difference in lipofuscin content between the mice of the first and second experiment.

As expected, the peak intensity at 475 nm in the mean 375 nm excitation spectra rose with the disease severity. This evolution was attributed to an increase in vitamin A concentration. Spectral differences between the mean 405 nm excitation
spectra of the different disease groups were again visually less obvious, as in the first experiment, yet statistically very significant, as shown in the following paragraphs.

In this experiment, fibrosis was detected in none of the mice, collagen optical properties were hence less likely to impact the shape of the fluorescence spectra as in the first study. The increase of peak intensity observed at 475 nm in the 375 nm excitation spectra is hence consistent with the augmentation of the vitamin A concomitant with the development of the steatosis.

Figure 3-8: Mean spectra at (a) 375 nm and (b) 405 nm excitation and error bars representing standard deviation.

A classification accuracy of 91% was obtained after SMLR K-fold classification. Table 3-7 shows the confusion matrix obtained following classification with SMLR. The control group was discriminated from the moderate and severe steatotic groups with 92% sensitivity, 93% specificity, 94% PPV and, 90% NPV. The moderate steatotic tissue was separated from the normal and severe steatotic tissue with 97% sensitivity, 95% specificity, 93% PPV, 95% NPV. Finally, the severe steatotic tissue was differentiated from the two other ones with 98% sensitivity, 82% specificity, 97% PPV, and 90% NPV.

Table 3-7: Confusion matrix presenting the ability of fluorescence spectroscopy to distinguish between control, moderate and severe steatosis disease groups with the concatenated spectra.

<table>
<thead>
<tr>
<th>Disease group</th>
<th>Fluorescence spectroscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Control</td>
<td>232</td>
</tr>
<tr>
<td>Histopathology</td>
<td>Moderate steatosis</td>
</tr>
<tr>
<td></td>
<td>Severe steatosis</td>
</tr>
</tbody>
</table>
Figure 3M19 presents the posterior probabilities obtained for each site and for each disease group. A high number of measurements were classified with probability close or equal to 100% as indicated by the numerous points clustered close to the value 1 on the graph. The total averaged probability calculated from all the points in was found to be equal to 92% indicating a strong confidence in the classification similarly to the first experiment.

Figure 3-9: Posterior probability of fluorescence spectra from interrogated tissue sites belonging to their correct disease group (as classified by histopathology): control (252 sites), moderate steatosis (258 sites), severe steatosis (102 sites).

As in the first experiment, the classification accuracy obtained in the second experiment with the combination of the lasers did not exceed the classification accuracy obtained with 405 nm excitation alone (91 %), this suggests that the optical system could be simplified by keeping only the 405 nm laser diode, although the combination of the 375 nm and 405 nm might improve the classification performance of other liver diseases. The slightly lower classification accuracy obtained in the second experiment is probably due to smaller differences in the severity of the pathologies between the disease groups than in the first experiment as attested by the histopathological score gaps (0, 2, 7 in the first experiment against 0, 4, 5-6 in the second experiment).

3.5.3 Summary

In conclusion, an excellent discrimination between the disease groups in both studies was obtained with fluorescence spectroscopy which was not feasible with ALT and
TBARS in the first experiment. The added advantage of fluorescence spectroscopy over these blood tests and the gold standard method of histopathology is its speed, as it is nearly real-time. In addition, one of the strengths of the SMLR algorithm lies in its capacity to provide the posterior probability of a sample to belong to a pathology group. According to the probability value of the sample, the clinician could decide to pursue or not further sample analysis. This would contribute to the diminution of the diagnosis procedure duration by exempting samples with low probability of being diseased from biopsy and histopathological examinations, if fluorescence spectroscopy is performed intraoperatively (for in vivo liver diagnosis or graft selection), or from histological examinations only if the technique is performed on freshly excised liver explants or biopsy material.

3.6 Towards in vivo diagnostic of liver diseases with the joint use of the multi-excitation fluorescence spectroscopy probe and a robotic endoscope

The study presents results from the work of Dr. David Noonan, Dr. Jianzhong Shang, and Chris Payne who designed and built the force adaptive robotic endoscope. Our contribution was to incorporate the fluorescence probe in this instrument as the endoscopic robot has the potential to greatly improve the intraoperative use of fluorescence spectroscopy.

3.6.1 Motivation of endoscopic liver disease diagnosis and importance of the control of the pressure applied by the probe on tissue

Transcutaneous and transjugular biopsies, sometimes guided by radiological modalities, are the preferred approaches of gastroenterologists to collect tissue from liver. There are, however, indications for endoscopic liver surgery including staging of cancer [13] and cirrhosis detection [45]. In spite of its lesser use, laparoscopic visual inspection of the liver followed by a forceps biopsy is generally considered as the best liver diagnosis method [45, 46] because it enables:

- inspection of 85% of the liver surface area,
- retrieval of larger size biopsies and therefore increased accuracy of the diagnosis (in a study regrouping 200 patients, 25 patients were diagnosed liver malignancies with laparoscopic biopsy, 8 of these were missed by ultrasound guided transcutaneous biopsy [47])
• monitoring for bleeding
• inspection of liver's neighbouring organs [45].

It is also worth noting that technique stemming from natural orifice transluminal endoscopic surgery (NOTES) (less traumatic than the traditional transabdominal laparoscopic approach) still at the research stage, enabling sampling of liver biopsies via a transgastric approach of the liver [48].

A spectroscopic probe could be beneficial to endoscopic visual inspection of the liver to assess liver tissue state to diminish the number of unnecessary biopsies and help identify suspicious tissue areas, which will require a biopsy for histological confirmation. However, the in vivo use of spectroscopic probe has two main challenges, the accurate targeting of the site of interest and the control of the probe pressure applied to the tissue. The evaluation of the distance separating the probe from the tissue surface can be difficult through an endoscope and can result in an excessive force applied by the probe on the tissue. However, it has been demonstrated that rise of the probe contact pressure induced alterations in the spectra and in turn the diagnostic information extracted from them [49-51]. This is especially true for soft tissue with a low young modulus like liver, whose fluorescence and diffuse reflectance properties were shown to be affected by pressures above 25.8 mN/mm² [49]. It was also reported that a pressure superior to 9 mN/mm² could impact the diagnosis of skin cancer with fluorescence and diffuse reflectance spectroscopy [50]. These spectral modifications have been explained by the creation of a local ischaemia at the tip of the probe, which affects the blood volume and oxygenation (and therefore the diffuse reflectance spectrum), as well as the tissue metabolism (and hence the fluorescence spectrum as the NAD(P)H and FAD are fluorophores involved in the electron chain transport) [49, 50].

3.6.2 Enabling in vivo control of probe pressure applied on tissue and probe guidance

To the best of our knowledge, there is no in vivo study where the probe pressure applied on tissue during the acquisition of spectra was monitored. A system consisting of a spectroscopic probe used in combination with a robotic endoscope (Figure 3-10) including a force sensor could permit such a procedure. Additionally to the force sensing capability, the articulated endoscope allowed for the probe accurate positioning and scanning over the tissue surface so that spectra could be continuously acquired over large areas. It was also possible to relocate the site
where each spectrum was acquired thanks to the evaluation of the position of the distal tip.

The endoscopic robot consisted of three serially attached sections linked to each other with two joints and connected to a 50 cm rigid aluminium tube with a third joint enabling to reach five degrees of freedom, each of them actuated by an embedded rotatory micromotor (Namiki Precision Jewel Ltd, Japan). In practice, the proximal and the medial joints were used to position the probe in a region of interest (ROI), while the distal joint was then employed to scan the probe in the surface of the chosen ROI. The position of the distal joint was assessed by counting the pulses emitting by the micromotor control electronics after each of its rotation. The endoscope contained three channels, one for motors driving electronics, one for the probe and one for a CCD video camera (Medigus Ltd, Israel) (Figure 3-10-b). The fluorescence spectra were acquired at 405 nm excitation with the system described in Figure 3-1.

The fluorescence probe was fastened in a force sensor (ATI Industrial Automation, USA) measuring the load along the probe main axis. A linear actuator was utilized to move the probe forward towards the tissue surface. The joint driving motor controls, the linear actuator motor control, the CCD video camera and the fluorescence spectra acquisition were synchronised so that it was possible to identify the sites where fluorescence spectra and images have been recorded during the scanning of the probe.
Three experiments were conducted \textit{ex vivo}. In a first experiment, the system was used to acquire 405 nm excitation fluorescence spectra at different pressures from a liver and, in a second experiment, fluorescence spectra were continuously acquired while the robotic endoscope was scanning the probe above a phantom made up of alternative yellow and green fluorescent stripes. In the third experiment, the probe was scanned over a liver, with a constant pressure and spectra were recorded. Spectra were acquired with an exposure time of 1 ms when acquiring signal from the fluorescent dye phantom, and of 130 ms when recording spectra from liver tissue.

\subsection*{3.6.3 Results and discussion}

In the first experiment, looking at the influence of the pressure on the acquired spectrum, the probe was advanced towards the liver until a distance of approximately 1 mm separated the tip of the probe from the liver, a spectrum and a picture from the CCD video camera were then acquired (referred to as "non-contact"), the probe was then moved forward further and its pressure on the tissue was increased by increment of 10 mN from 0 mN to 150 mN. Figure 3-11 shows the fluorescence spectra for the non-contact case and for the pressures 50 mN, 100 mN, and 150 mN, together with the simultaneously acquired CCD video camera images, which enabled to note the gradual tissue deformation with increasing pressure. In addition to the autofluorescence signal which reached a maximum at 510 nm, additional peaks originating from the ambient light back reflected from tissue and particularly visible at 490 nm, 540 nm, 595 nm and 670 nm could be observed. Their intensity decreased with the pressure and almost disappeared at 150 mN. Another observation was that the peak intensity slightly increased with pressure from $7.10^{-3}$ a.u. at 50 mN to $7.9.10^{-3}$ a.u. at 150 mN.

In a previous study \cite{49} (see section 3.6.1), it was found that pressures per area unit above 25.8 mN/mm$^2$ could affect liver spectral characteristics \textit{in vivo}; this corresponds to a pressure of 81 mN in this work given that the diameter of our probe was 2 mm, yet the parasite ambient light peaks were still present at 100 mN. This suggests that during the spectra acquisition, endoscopic light source should be switched off, which would deprive the surgeon of visual feedback. Alternatively, a background spectrum acquired with the laser light turned off could be acquired and then subtracted from the fluorescence spectrum. Of note, the shape of the fluorescence spectra barely changed (apart from the magnitude of the ambient light peaks) from 50 mN to 150 mN. The \textit{ex vivo} nature of this study makes this
observation difficult to interpret as the main modifications of fluorescence spectral features with pressure arise from alteration of blood volume and oxygenation as well as metabolism change which can only occur in *in vivo* conditions.

![Fluorescence spectra and images](image)

**Figure 3-11:** (a) Fluorescence spectra acquired at 0 (non-contact), 50, 100 and 150 mN and (b) the corresponding images acquired by the video camera. From [52].

In the second experiment, a phantom with spatially varying fluorescence properties consisting of 1.5 mm large green and yellow dye stripes separated by 1.5 mm gap was created and fluorescent spectra were acquired while the probe was scanned with the robotic endoscope. In Figure 3-12, three sets of measurements consisting in the spectra from each of the six emission fibres, an image from the onboard CCD video (top-right) camera, and an image recorded with a second external camera (top left) were acquired at different points of the trajectory described clock wisely by the probe above the phantom. The first point in the trajectory was situated in a green dye band, whose spectrum had a main peak at 505 nm and a secondary peak at 640 nm.
The second point lied in a “white band” namely paper, which peaked at 460 nm. The last point showed had the particularity to be positioned at the boundary of a paper band and yellow dye band: fibre 1 and fibre 2 were in the paper stripe, fibre 4 and fibre 6 were lying exactly at the frontier between the two bands as their fluorescence spectrum included two peaks, one at 460 nm corresponding to the paper fluorescence, and one at 510 nm corresponding to the yellow dye fluorescence. Finally, fibre 3 and fibre 6 were situated in the yellow stripe. Consequently, because the spectrum acquisition and the robot driving motor were synchronised, it was possible to locate the boundary between two tissue regions of different fluorescence properties. This could be exploited in the future to locate the transition between normal and diseased tissues whose fluorescence properties differ with healthy tissue.

Of note, the peaks caused by the ambient light back-reflection were not visible in this experiment as opposed to the previous one because the set exposure time was 1 ms, that is ten times smaller than the previous time experiment which is too short for the ambient light parasite signal to be recorded.
Figure 3-12: Fluorescent spectra acquired by the 6 emission fibres at different points along the probe trajectory traced clock wise (as indicated by the red arrow) on the phantom consisting of yellow and green fluorescent stripes separated by white paper stripes. Measurement sequences at 3 points along the trajectory are detailed each consisting of a fluorescence spectrum (bottom), an onboard camera image (top-right) and an external camera image (top-left). From [52].

The third experiment was similar to the previous experiment, except that it was performed on a liver and no secondary exterior camera was used. The probe was put in contact with the liver tissue with a 40 mN pressure and scanned along the trajectory presented in Figure 3-13 where the spectra and corresponding images are shown at five points of the probe displacement. The maximum intensity of the fluorescence peak remained stable at $8.10^3\pm1.5.10^4$ a.u., indicating a steady probe pressure along the trajectory. The intensity of the ambient light peaks varied between the points, it might originated from local differences in the tissue surface topology or
stiffness, which caused the probe to bury more or less, impacting therefore its isolation from the ambient light.

Figure 3-13: Fluorescent spectra acquired by each of the six emission fibres at different points along the probe trajectory on liver and their corresponding images acquired by the onboard camera. From [52].
3.7 Conclusion

In summary, we have built a compact dual excitation spectroscopy system using laser diodes and a fibre probe that can be used minimally invasively with endoscopes. Fluorescence spectra at two excitation wavelengths were recorded from healthy, steatotic and steatohepatitic mice and analysed with SMLR. Classification accuracies above 90% in the two experiments with strong confidence (mean posterior probabilities above 90% in both studies) in the classification were obtained, which supports the use of a spectroscopic probe as a tool to quickly assess liver tissue graft ex vivo or in vivo at harvest. Another possible promising application could be to use the spectroscopic probe instead of needle biopsies during transjugular procedures.

In a second time, the same spectroscopic probe was used together with a force sensitive articulated robotic endoscope. This system offers the possibility to circumvent obstacles to reach a region of interest and to perform on it pressure controlled series of spectral acquisitions, opening the door to in vivo wide area covering spectroscopy. Importantly, it was possible to reconstruct the probe trajectory and relocate to a site where a spectrum of interest was previously acquired. A significant application of this system would be cancer tumour delineation.
3.8 References


Chapter 4

Combined fluorescence and reflectance for GI tract tissue analysis

Fluorescence and diffuse reflectance light contain complementary information. The former informs about the fluorophores' distribution, while the latter provides biostructural information such as the scatterers size and density, as well as chromophore concentration. Our major aim here was to evaluate and compare the diagnostic ability for usual pathologies (inflammation, dysplasia, Barrett’s oesophagus, cancer) of upper (oesophagus and oesophagus/stomach junction) and lower (colon and rectum) of the two modalities and assess if their combination enabled enhancement of the spectroscopic diagnostic accuracy. Furthermore, results from this study enabled the selection of adequate filters used in a spectrally resolved laparoscope designed for oesophagus tumour imaging. The move towards imaging (see chapter 5) subsequent to the single point approach followed in this chapter was prompted by the need to assess the spatial extent of tumours. As opposed to NALFD, uniformly spread over the liver, tumours are localized. The knowledge of their boundary is necessary to ensure a complete removal.

A combined fluorescence-diffuse reflectance spectroscopy system is presented. Measurements have been first performed on a phantom with known spectral characteristics to test the diffuse reflectance channel of the system. Fluorescence and diffuse reflectance spectra have then been recorded from upper and lower GI biopsies with various conditions. The performance of both modalities in separating normal biopsies from the diseased ones have been evaluated using an algorithm computing the pair of wavelengths whose ratio maximizes the discrimination followed by a cross-validation performed with SMLR. The wavelength pair selected to discriminate oesophageal normal tissue from the cancer tissue enabled to select bandpass filters used during oesophageal tumour imaging presented (see chapter 5).
4.1 Diagnosis of diseased tissue in the upper and lower GI

Upper and lower GI diseases including pathologies such as inflammation, Barrett’s oesophagus (a precursor pathology of oesophageal adenocarcinoma), dysplasia and cancers are a significant cause of morbidity and mortality. For instance, 7,966 people were diagnosed with oesophageal cancer in 2007 in the UK, making it the ninth most common cause of cancer, and just under 40,000 people were diagnosed with colorectal cancer in 2008 in the UK (third most common cause of cancer. The five years survival rates were 8% and 50% respectively [1, 2].

Improved survival depends on early detection and monitoring of these lesions, which can subsequently be managed at an early stage with surgery, chemo/radiotherapy or even novel new endoscopic therapies [3]. The current gold standard for detecting disease and cancers of the upper and lower GI system is visualization via conventional white light endoscopy, selection of biopsy sites by the endoscopist, followed by histological examination of those biopsies. However, this approach presents several limitations already mentioned in chapter 2, which are: difficulty to visualize precancerous lesions both in the upper GI [4] and the lower GI [3]; subjectivity, potentially leading to sampling error; time taken to process the result, which delays the diagnosis [5].

CT scan of the abdomen and pelvis is another lower GI cancer diagnosis technique, used mainly in elderly patients, as it is non-invasive. Problems arise when occasional spasms of the right side of the abdomen produce an abnormal image, which mimics the appearance of cancer [6]. Faecal occult blood testing is used to screen for colorectal cancer as it is safe and inexpensive. However the sensitivity of the test is low as not all cancers bleed, and therefore the test is negative in 50% of cancers [7].

Fluorescence and diffuse reflectance spectroscopy appear to be an ideal complement to endoscopy since fluorescence serves as an indicator of tissue biochemistry and diffuse reflectance provides morphologic information on tissue biostructure. Both offer the possibility to be performed with an optical fibre small enough to be inserted through the endoscope working channel. The fluorescence and diffuse reflectance spectroscopy in combination with endoscopy could help the surgeon to quickly determine the state of a tissue site without the need to remove it and to perform histopathology.
4.2 Fluorescence-diffuse reflectance setup

4.2.1 System description, data acquisition, and processing

The fluorescence-diffuse reflectance spectroscopy system is mainly an extension of the dual-fluorescence spectroscopy system presented in chapter 3, where a tungsten-halogen lamp (model HL2000HP232R, Ocean Optics, Inc., USA) has been added in addition to the two laser diodes and acts as the diffuse reflectance light source. Only the elements differing from the dual-fluorescence system are described here.

The probe (Leoni Fiber Optics, Inc., previously Romack, Inc., USA) has been modified and consists in two excitation fibres, one for delivering the fluorescence light to the sample, and the other for white light conveyance and one detection fibre for both fluorescence and diffuse reflectance light collection (Figure 4-1). The diameter and NA of the three fibres were 400 µm and 0.22 respectively and the total diameter of the probe distal tip was 2 mm. Unlike the previous probe, this one did not contain a glass window (whose function was to enable excitation and emission beams overlap and hence maximising the amount of collected light), because white light from the tungsten-halogen lamp would be reflected at the glass surface and hence introduce parasitic light in the emission fibre.

The proximal end was divided into three arms, each containing one fibre and each terminated with a SMA connector; the first one was connected to the laser system, the second one to the tungsten-halogen source and the last one to the spectrograph/CCD unit (Figure 4-2). The measured optical powers at the tip of the
A LabVIEW program enabled control of the camera settings and the sequential acquisition of fluorescence and reflectance spectra.

The fluorescence spectra were processed as described in section 3.4.2 and the spectra measured under white light illumination were divided by the spectrum of a spectralon reflectance standard (Labsphere, Inc., USA) to produce the diffuse reflectance spectra.

4.2.2 System testing on a colour chart

Spectral accuracy, understood here as the similarity between measured and real spectra, has been evaluated for the diffuse reflectance channel (the fluorescence channel has been already assessed in chapter 3) to validate the data calibration and processing procedures to produce the diffuse reflectance spectra.

To do so, a Macbeth chart (X-rite, Inc., USA) was chosen as phantom. It is a colour chart used to calibrate digital cameras. It comprises of 24 patches of different
standardized colours (Figure 4-3) numbered between 1 and 24 from left to right and from top to bottom. In order to test the diffuse reflectance spectral accuracy, the spectrum of each colour patch was measured and compared with the average spectrum from a database including twenty spectra recorded by different users (and referred to as mean database spectra in the rest of this paragraph) [8].

Figure 4-3: Standard colour chart containing 24 patches with standardized colours. (X-rite, Inc., USA)

The measured spectra and the mean database spectra are shown in Figure 4-4. The overlap was excellent for the grey patches (last row), demonstrating the system high spectral accuracy. Minor divergences, mostly in the 700 nm - 750 nm spectral domain, appeared in the patches 5-8-10-12-17. Interestingly, the variability of the data base spectra in this domain was higher than in the rest of the spectrum as shown by the larger errors bars. It is important to remember that the mean database spectra are by no mean reference spectra and therefore the dissimilarities between them and our measured spectra can originate from inaccuracies in our measurement protocol and setup, as much as in theirs.

Instead of using the similarity function (see chapter 3) to quantify the system spectral accuracy, the sRGB values (sRGB being one of the several RGB colour spaces) of each colour patch have been computed from the recorded spectra and compared with the sRGB values provided by the manufacturer. This has been done in two steps: the spectral data were first transformed in the CIE (Commission Internationale de l’Eclairage) 1931 XYZ colour space using the three relations of the Equation 4-1 which, for discrete spectral data acquired by reflection, are written as follows [9]:

...
\[ X = \frac{1}{N} \sum_i \bar{x}_i S_i I_i \]
\[ Y = \frac{1}{N} \sum_i \bar{y}_i S_i I_i \]
\[ Z = \frac{1}{N} \sum_i \bar{z}_i S_i I_i \]
\[ \text{with } N = \sum_i \bar{y}_i I_i \]

Equation 4-1

Where \( \bar{x}, \bar{y}, \bar{z} \) are the CIE 1930 colour matching functions, \( S \) the spectrum measured from the chart, \( I \) the spectrum of a reference illuminant (chosen to be D65 because, subsequently, the XYZ values were converted in the sRGB colour space whose reference illuminant is D65 that is a black body heated at 6500 K). This conversion is done using Equation 4-2 [10]:

\[
\begin{bmatrix}
R \\
G \\
B
\end{bmatrix} = \begin{bmatrix}
3.2404542 & -1.5371385 & -0.4985314 \\
-0.9692660 & 1.8760108 & 0.0415560 \\
0.0556434 & -0.2040259 & 1.0572252
\end{bmatrix} \begin{bmatrix}
X \\
Y \\
Z
\end{bmatrix}
\]

Equation 4-2

At the conclusion of the XYZ to sRGB conversion process, negative results and results above 255 were set to 0 and 255 respectively. It is commonly the case that the R value of the patch 18 produces negative values (found to be equal to -40 here) and this was set to zero and ignored in the error calculation.

Table 4-1 summarises the sRGB values round to the nearest integer obtained from the reflectance spectra, together with the manufacturer’s ones. The percent errors (ratio of the absolute difference between the manufacturer’s data and the measured ones multiplied by 100) have been calculated for each of the sRGB values and averaged to produce the averaged error for every colour patch. The mean error ranged from 0.3 % (patch 20) to 27.8 % (patch 13). This was caused by inexactnesses of the spectral measurements but also by the conversion process from XYZ to sRGB values, which introduced additional errors due to the approximation of the conversion matrix constants [11].

The total mean error was found to be equal to 5.6% when averaged over the 24 colour patches indicating a good accuracy in the reproduction of the reflectance spectra of the colour patches by the reflectance channel of the spectroscopic setup.
Figure 4-4: Diffuse reflectance spectra recorded from the 24 patches forming the Macbeth colour chart and compared with the mean spectra (error bars represent one standard deviation) measured from the database.
Table 4-1: Comparison of the sRGB values of each of the 24 colour patches as provided by the manufacturer and those retrieved from the reflectance spectra.

<table>
<thead>
<tr>
<th>Patch #</th>
<th>sRGB</th>
<th>Error (%)</th>
<th>Patch #</th>
<th>sRGB</th>
<th>Error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R</td>
<td>G</td>
<td>B</td>
<td></td>
<td>R</td>
</tr>
<tr>
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<td>82</td>
<td>68</td>
<td>3.4</td>
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<tr>
<td></td>
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<td>73</td>
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</tr>
<tr>
<td>2</td>
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<td>150</td>
<td>130</td>
<td>27.8</td>
</tr>
<tr>
<td></td>
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<td>147</td>
<td>131</td>
<td>27.8</td>
</tr>
<tr>
<td>3</td>
<td>Manufacturer</td>
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<td>122</td>
<td>157</td>
<td>27.8</td>
</tr>
<tr>
<td></td>
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<td>123</td>
<td>152</td>
<td>27.8</td>
</tr>
<tr>
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<td>108</td>
<td>67</td>
<td>27.8</td>
</tr>
<tr>
<td></td>
<td>Measured</td>
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<td>105</td>
<td>68</td>
<td>27.8</td>
</tr>
<tr>
<td>5</td>
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<td>128</td>
<td>177</td>
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<tr>
<td></td>
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<td>129</td>
<td>172</td>
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</tr>
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<td>189</td>
<td>170</td>
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</tr>
<tr>
<td></td>
<td>Measured</td>
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<td>189</td>
<td>169</td>
<td>27.8</td>
</tr>
<tr>
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<tr>
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<td>123</td>
<td>60</td>
<td>27.8</td>
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<tr>
<td>8</td>
<td>Manufacturer</td>
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<td>91</td>
<td>166</td>
<td>27.8</td>
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<tr>
<td></td>
<td>Measured</td>
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<td>89</td>
<td>157</td>
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<tr>
<td>9</td>
<td>Manufacturer</td>
<td>193</td>
<td>90</td>
<td>99</td>
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<td></td>
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<td>90</td>
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<tr>
<td></td>
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<td>163</td>
<td>46</td>
<td>27.8</td>
</tr>
<tr>
<td></td>
<td>Measured</td>
<td>231</td>
<td>162</td>
<td>63</td>
<td>27.8</td>
</tr>
</tbody>
</table>
4.3 GI diseased tissue diagnosis with fluorescence and reflectance spectroscopy

The general goal of this study was to evaluate the ability of fluorescence, diffuse reflectance spectroscopy, as well as the combination of the two to discriminate different types of tissue in the upper and lower GI. This has been done by using the setup described in Figure 4M2 to record spectra of biopsies resected from oesophagus and oesophagus/stomach junction (Upper GI) and colon and rectum (lower GI). This study was conducted in collaboration with Mr. Shalil Solanki, Mr. Hussain Selmi, Iakovos Amygdalos, and Pr. George Hanna, who provided tissue samples and realised spectroscopy measurements.

4.3.1 Study design and measurements protocol

The Royal Free Hospital and Medical School Research Ethics Committee granted ethical approval for this study. Written informed consent was obtained from patients undergoing an upper GI and a lower GI endoscopy at St Mary’s Hospital, Paddington. Biopsies were only taken at the discretion of the endoscopist, and no additional biopsies were taken for the pure purpose of research in accordance to the consent form.

Biopsies were placed in normal saline, in order to maintain hydration. They were all measured when fresh with the fluorescence/diffuse reflectance setup prior to being fixed in formal saline and stained using standard histological procedures. During the measurements, the probe was placed in gentle contact with the tissue. Fluorescence and reflectance spectra were sequentially acquired with exposure times varying between 100 ms and 300 ms for fluorescence and 10 and 50 ms for reflectance. Since the 405 nm laser diode broke down at the start of this study, only fluorescence at 375 nm excitation was acquired. Consequently, it has not been possible to check whether, unlike the NAFLD studies (chapter 3), the combination of two excitations wavelengths would allow the enhancement of the classification performance.

Spectra were recorded between 450 nm and 750 nm with a spectral resolution of 2.9 nm leading to a total of 103 spectral features for each spectrum.

A total of 71 spectra were recorded from 19 patients in the upper GI and 66 spectra from 15 patients in the lower GI. In some pathology groups, the number of spectra and patients differed between fluorescence and reflectance which was
caused by the shape and dimensions of the samples. As illustrated Figure 4-5, GI tract biopsies have the form of a thin filament so that in some instances the biopsy was only cover by the detection fibre and one of the illumination fibre (fluorescence or reflectance) leading to an incomplete dataset consisting of only one spectrum of two

![Cross-section (top view) of a probe-sample configuration where only the detection and fluorescence illumination probes are in contact with the sample causing an incomplete measurement sequence where the diffuse reflectance spectrum is missing.](image)

Nevertheless, one of the aims of this work was to assess whether the combination of the two modalities could increase the spectroscopic diagnostic power which meant that only the biopsies for which both fluorescence and reflectance spectra had recorded were considered for this part of the study. Table 4-2 shows the breakdown of spectra and patients by modalities in the upper and lower GI. Classification accuracy could then be obtained with fluorescence only, reflectance only and the combination of both.

<table>
<thead>
<tr>
<th></th>
<th>Fluorescence</th>
<th>Reflectance</th>
<th>Combined</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>spectra #</strong></td>
<td><strong>patients #</strong></td>
<td><strong>spectra #</strong></td>
<td><strong>patients #</strong></td>
</tr>
<tr>
<td>Upper GI</td>
<td>35</td>
<td>17</td>
<td>36</td>
</tr>
<tr>
<td>Lower GI</td>
<td>42</td>
<td>15</td>
<td>31</td>
</tr>
</tbody>
</table>

Table 4-2: Number of spectra recorded and patients investigated with each modality and their combination.
4.3.2 Data classification

The spectra were processed in two steps, the first one involving a receiving operating characteristic (ROC) analysis for feature selection, and a second one using the SMLR algorithm to perform the classification of the spectra into normal or diseased classes. ROC analysis plots the sensitivity versus 1-specificity obtained with a binary classification scheme using a numerical variable to separate the two classes with different thresholds of separation. The area under the ROC (AUC) is a measure of the performance of the classification algorithm in discriminating two classes. An AUC of 0.5 indicates a non-existent discrimination and an AUC of 1 signifies a perfect discrimination [12]. Spectra from the two classes to be distinguished were analyzed with an algorithm developed in our group by Tobias Wood. It enabled the selection of the pair of wavelengths whose ratio maximized the AUC. Secondly, a SMLR classification was performed on the intensity ratios (or pair of ratios when evaluating the combination of the two modalities) obtained following the ROC analysis using a leave-one-out cross validation. This type of cross validation is equivalent to a K-fold cross validation (see section 3.4.1) where K would be equal to 1. This means that the intensity ratios of the site to be classified was excluded from the training set during the classifier learning, which was then used to classify the excluded sample. This process, repeated on all the sites, allowed assessment of the classification accuracy in an optimal way since all the samples of our restricted dataset were used for the classifier training and in an unbiased manner since test and training sets were separated.

This procedure has been applied for computing the sensitivity, specificity, positive and negative predictive values obtained with fluorescence only, reflectance only and the combination of the two modalities for distinguishing the disease groups in the upper and lower GI.

4.3.3 Results and discussion

Following histopathological procedures, upper GI biopsies were diagnosed with conditions including inflammation, Barrett’s oesophagus and cancer in the upper GI (Table 4-3). Pathologies such as inflammation and dysplasia were detected in the lower GI (Table 4-4).
Table 4-3: Distribution of biopsies as classified by histopathology in the upper GI.

<table>
<thead>
<tr>
<th>Upper GI</th>
<th>Normal</th>
<th>Inflammation</th>
<th>Barrets</th>
<th>Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>spectra #</td>
<td>patients #</td>
<td>spectra #</td>
<td>patients #</td>
</tr>
<tr>
<td>Fluorescence</td>
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<td>7</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>Reflectance</td>
<td>12</td>
<td>8</td>
<td>13</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 4-4: Distribution of biopsies as classified by histopathology in the lower GI.

<table>
<thead>
<tr>
<th>Lower GI</th>
<th>Normal</th>
<th>Inflammation</th>
<th>Dysplasia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>spectra #</td>
<td>patients #</td>
<td>spectra #</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>24</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td>Reflectance</td>
<td>19</td>
<td>7</td>
<td>8</td>
</tr>
</tbody>
</table>

Figure 4-6-a shows the mean normalized fluorescence and reflectance spectra collected from the upper GI biopsies for healthy tissue (blue line), inflamed tissue (red line), Barrett’s oesophagus (green line) and cancer tissue (black dotted line) alongside the standard error displayed every 10 nm. The fluorescence spectra were found to peak in the band 450 nm – 465 nm. For comparison, tissue sites in the oesophagus have been found to peak at 415 nm and 470 nm when excited at 337 nm and 397 nm respectively [13], and around 500 nm when excited at 410 nm [14]. The endogenous fluorophores flavins and porphyrins both absorbing at 375 nm and emitting in the 520-540 nm range and between 590 nm and 650 nm, respectively, are among the fluorophores likely to contribute to the oesophagus fluorescence. The cancerous spectra were most obviously different from the others. Its normalized fluorescence intensity is relatively lower than the other groups in the blue-green region and higher in the red. Also, its total non-normalized integrated intensity was significantly lower than the others, 5.1 times smaller than the normal one, for instance. These two observations have been commonly made (see section 2.1.6) when comparing tumours to healthy tissue in a variety of organs, in particular in the oesophagus [14], and have been linked to differences in the distribution and oxidation states of the coenzymes NADH and FAD between normal and cancerous tissue [15, 16].

Reflectance spectra exhibited characteristic dips at 540 nm and 570 nm caused by haemoglobin absorption (Figure 4-6-b). Differing haemoglobin absorption between tissue types were difficult to interpret due to the ex vivo nature of this study. The normal tissue mean spectrum had a lower intensity in the 450 nm – 575 nm band than in the diseased group spectra which is in accordance with what has been
observed in a previous study looking at dysplasia and cancer detection in the oesophagus with diffuse reflectance spectroscopy [17].

Figure 4-6: Mean (a) fluorescence and (b) reflectance spectra of upper GI tissues. Error bars represent standard deviation.

Lower GI fluorescence spectra are shown Figure 4-7-a (healthy group in blue, inflammation group in red, and dysplasia group in green), they reached a maximum at around 470 nm. Fluorescence spectra obtained following a study aimed at separating healthy tissue from hyperplastic and cancerous tissue with 370 nm excitation were previously found to climax at 465 nm [18].

NAD(P)H, whose maximum intensity is at 475 nm, has been shown to contribute to colonic fluorescence [19]. Contrary to the upper GI reflectance spectra, normal reflectance spectra from the lower GI had higher intensities than the spectra of diseased groups from 450 nm to 600 nm. The normal and dysplasia spectra intersected at 601 nm. A similar observation has been noted in a preceding analysis where the mean diffuse reflectance intensities of colonic dysplastic polyps was found to be smaller than the normal tissue intensities until 605 nm where the two spectra crossed [20].

Figure 4-7: Mean (a) fluorescence and (b) reflectance spectra of upper GI tissues and mean fluorescence. Error bars represent standard deviation.
The classification procedure was performed to distinguish (1) the healthy group from each of the diseased group in the upper GI (Inflammation, Barrett’s oesophagus, cancer); (2) the cancer group from all the other groups (normal, Barrett’s oesophagus, inflammation) in the upper GI; (3) the healthy group from each of the diseased group in the lower GI (inflammation and dysplasia); and (4) the dysplasia group from all the other groups (normal and inflammation).

Table 4-5 summarises the wavelengths pair obtained after ROC analysis, whose intensity ratio maximised the area under the ROC curve. When analysing these results, it is important to remember that this feature selection technique was based on ratios of intensities that measures proportional changes between spectra of two different classes. Therefore, even though there were wavelengths at which two spectra appeared visually well separated, there might be some other spectral region where this difference was proportionally higher. This explains the selection of the wavelength pairs (662 nm, 697 nm) and (659 nm, 697 nm) as optimal wavelengths to discriminate normal sites from inflamed tissue and Barrett’s oesophagus, respectively, instead of wavelengths in the 450 nm - 500 nm region where the spectra overlap seemed less important.

The classification accuracy averaged over all the classifications in the upper and lower GI and over the two modalities was found to be equal to 81%. It is interesting to note that using the full spectra instead of the intensity ratios at the selected wavelengths yielded a classification accuracy of 82%. Consequently, the use of the 88 spectral features constituents of each spectrum did not significantly improve the classification. In fact, when the training sample set is small compared to the number of classification variables, as is the case here (24 training samples at the most -see Table 4-4- against 88 spectral features for single modality spectra and 176 features for the combined spectra), added features do not necessarily improve and can even diminish classification performance. This is referred as the “curse of dimensionality” [21].

In the rest of the chapter, the diagnosis performance was evaluated with the intensity ratios only (and not with the full spectrum).
Table 4-5: Pairs of wavelength chosen after ROC analysis.

<table>
<thead>
<tr>
<th></th>
<th>Fluorescence</th>
<th>Reflectance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal vs Inflammation</td>
<td>662, 697</td>
<td>497, 613</td>
</tr>
<tr>
<td>Normal vs Barrets</td>
<td>659, 697</td>
<td>451, 531</td>
</tr>
<tr>
<td>Normal vs Cancer</td>
<td>491, 662</td>
<td>605, 676</td>
</tr>
<tr>
<td>Cancer versus Normal, Barret's, Inflammation</td>
<td>491, 546</td>
<td>448, 514</td>
</tr>
<tr>
<td>Normal vs Inflammation</td>
<td>526, 572</td>
<td>526, 563</td>
</tr>
<tr>
<td>Normal vs Dysplasia</td>
<td>592, 630</td>
<td>549, 580</td>
</tr>
<tr>
<td>Dysplasia versus Normal, Inflammation</td>
<td>581, 627</td>
<td>613, 647</td>
</tr>
</tbody>
</table>

The performance of fluorescence, diffuse reflectance and the combination of the two in separating pathologies are presented in Table 4-6. This shows the sensitivity, specificity, PPV and NPV obtained following classification of the selected intensity ratios. The best outcomes obtained for each classification are highlighted in bold.

Following classification of normal tissue versus cancer tissue and classification of cancer sites versus normal, Barrett’s oesophagus and inflamed sites with diffuse reflectance data, all the normal sites were correctly classified, yet all the cancer sites were misclassified, which led to a sensitivity equal to 0 and NPV equal to zero divided by zero, hence the grey box in the NPV column.
Table 4-6: Sensitivity, specificity, positive and negative predictive values obtained after SMLR leave-one-out cross validation with fluorescence, diffuse reflectance and the combination of the two. Modalities providing the best results for each classification are indicated in bold. Grey boxes indicate undefined negative predictive values.

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
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<td><strong>Upper GI</strong></td>
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<tr>
<td>Normal versus Inflammation</td>
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<td></td>
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<tr>
<td>Fluorescence</td>
<td>75</td>
<td>60</td>
<td>60</td>
<td>75</td>
</tr>
<tr>
<td>Reflectance</td>
<td>50</td>
<td>80</td>
<td>67</td>
<td>50</td>
</tr>
<tr>
<td>Combined</td>
<td><strong>75</strong></td>
<td><strong>70</strong></td>
<td><strong>67</strong></td>
<td><strong>78</strong></td>
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<tr>
<td>Normal versus Barret's</td>
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<td></td>
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<tr>
<td>Fluorescence</td>
<td>75</td>
<td>88</td>
<td>86</td>
<td>78</td>
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<tr>
<td>Reflectance</td>
<td>88</td>
<td>88</td>
<td>88</td>
<td>88</td>
</tr>
<tr>
<td>Combined</td>
<td><strong>75</strong></td>
<td>100</td>
<td>100</td>
<td><strong>80</strong></td>
</tr>
<tr>
<td>Normal versus Cancer</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>Fluorescence</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Reflectance</td>
<td>100</td>
<td>0</td>
<td>73</td>
<td><strong>67</strong></td>
</tr>
<tr>
<td>Combined</td>
<td><strong>100</strong></td>
<td>100</td>
<td>100</td>
<td>100</td>
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<tr>
<td>Cancer versus Normal, Barret's, Inflammation</td>
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<td></td>
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<tr>
<td>Fluorescence</td>
<td>100</td>
<td>67</td>
<td>96</td>
<td>100</td>
</tr>
<tr>
<td>Reflectance</td>
<td>100</td>
<td>0</td>
<td>90</td>
<td><strong>67</strong></td>
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<tr>
<td>Combined</td>
<td>96</td>
<td>67</td>
<td>96</td>
<td>67</td>
</tr>
<tr>
<td><strong>Lower GI</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Normal versus Inflammation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluorescence</td>
<td>94</td>
<td>71</td>
<td>89</td>
<td>83</td>
</tr>
<tr>
<td>Reflectance</td>
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<td>57</td>
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<td>80</td>
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<tr>
<td>Combined</td>
<td>89</td>
<td>71</td>
<td>89</td>
<td>71</td>
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<tr>
<td>Normal versus Dysplasia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluorescence</td>
<td>94</td>
<td>100</td>
<td>100</td>
<td>80</td>
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<tr>
<td>Reflectance</td>
<td>94</td>
<td>50</td>
<td>89</td>
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<tr>
<td>Combined</td>
<td>94</td>
<td>100</td>
<td>100</td>
<td>80</td>
</tr>
<tr>
<td>Dysplasia versus Normal, Inflammation</td>
<td></td>
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<tr>
<td>Fluorescence</td>
<td>100</td>
<td>75</td>
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<tr>
<td>Reflectance</td>
<td>96</td>
<td>34</td>
<td>89</td>
<td>50</td>
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<tr>
<td>Combined</td>
<td><strong>100</strong></td>
<td>75</td>
<td>96</td>
<td><strong>100</strong></td>
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</tbody>
</table>

Fluorescence spectroscopy outperformed diffuse reflectance spectroscopy in five out of seven classifications. The two cases where diffuse reflectance performed equally or better than fluorescence were, respectively, to distinguish normal from inflamed sites and normal oesophageal tissue from Barrett’s oesophagus tissue sites. Fluorescence has the advantage of the biochemical specificity over diffuse reflectance, because it probes the tissue fluorophore distribution, yet a more likely explanation of the fluorescence superior performance lies probably in the ex vivo nature of this work, which implies a disruption in the blood flow in the investigated tissue sites. This impacts the haemoglobin oxygenation and concentration, both of which contribute to the diffuse reflectance spectrum shape as mentioned earlier. Yet tissue oxygenation and vascularisation have been shown to be important disease markers especially for tumours, which might help to explain the impossibility to recognize cancerous sites in the upper GI with diffuse reflectance data [22]. Overall,
the highest classification accuracy using fluorescence data was obtained when separating normal upper GI sites from cancerous ones (with sensitivity, specificity, PPV, NPV of 100%). The lowest accuracy was when discriminating normal upper GI sites from inflamed ones (sensitivity, specificity, PPV, NPV of 75%, 60%, 60%, 75% respectively). This is expected since cancer entails the most dramatic changes in the tissue biochemistry; however the results of this classification in particular need to be confirmed on a larger patient population as the spectra of cancerous sites have been recorded from only three sites of a single patient.

The perfect classification accuracy obtained for differentiating upper GI cancerous sites from normal sites slightly decreased when comparing cancerous sites from normal, Barrett’s oesophagus and inflamed sites (sensitivity, specificity, PPV, NPV of 100%, 67%, 96%, 100% respectively). It was noted in another study that inflamed upper GI sites were responsible for a decrease of the NPV when separating high grade dysplasia from low-grade and non-dysplastic tissue with fluorescence [23].

In the lower GI, fluorescence was found to be better at discriminating normal sites from dysplastic ones than separating normal sites from inflamed ones, while diffuse reflectance provided similar performances in the two classifications. The classification accuracy obtained for differentiating lower GI dysplastic sites from normal sites with fluorescence (sensitivity, specificity, PPV, NPV of 94%, 100%, 100%, 80% respectively) dropped only marginally when discriminating dysplastic sites from normal and inflamed sites (sensitivity, specificity, PPV, NPV of 100%, 75%, 96%, 100% respectively).

Direct comparisons between studies are always difficult because of the differences in the sample pool size, the instrumentation, and the data processing. However, it is interesting to situate these results in relation with previous work. An in vivo study involving 16 patients, fluorescence spectra from oesophagus tissue excited at 337 nm enabled discrimination of normal and low grade dysplasia from high grade dysplasia tissue with sensitivity and specificity of 100% and 98% respectively, both of which decreased at 79% and 88% when separating low and high grade dysplasia from normal tissue [13]. Another in vivo fluorescence spectroscopy analysis looking at 24 patients at 330 nm excitation reached a sensitivity of 86% and a specificity of 95% to differentiate normal Barrett’s oesophagus sites from neoplastic ones [24]. Sensitivity and specificity of 92% and 60% respectively were obtained with diffuse reflectance to distinguish cancer and high-grade dysplasia from normal tissue and low-grade dysplasia in an ex vivo study with 81 patients presenting Barrett’s oesophagus [17].
Fluorescence spectra recorded at 370 nm excitation from twenty patients in vivo in the colon permitted differentiation of adenoma (benign tumour) from normal and hyperplastic sites with sensitivity, specificity and PPV of 100%, 97% and 94% respectively [18]. Another in vivo fluorescence spectroscopy analysis performed with spectra at 337 nm excitation from 86 normal colonic sites, 35 hyperplastic polyps, 49 adenomatous polyps, and 7 adenocarcinomas led to sensitivity, specificity, PPV and NPV of 80%, 92%, 82%, and 91% respectively to distinguish neoplastic sites from non-neoplastic sites [25].

Finally, the combination of fluorescence and diffuse reflectance showed better performance than the two methods taken separately in two classifications: normal versus inflammation and normal versus Barrett’s oesophagus both in the upper GI, indicating that the combined optical modality approach is required when it comes to accurately discriminate inflamed or Barrett’s oesophagus tissue from normal ones. The combined modalities performed as well as fluorescence alone in all the other classifications except the normal versus inflammation classification in the lower GI. Even if the combination of fluorescence and diffuse reflectance spectroscopy seems natural, the diagnostic improvement brought by this method led to mixed results in previous studies and seems to be organ and pathology dependent. In a study looking at classifying invasive ductal carcinoma, fibroadenoma and normal tissue in the breast, the overall individual classification accuracies of fluorescence and diffuse reflectance were improved from 71% and 72% to 84% with the combined use of fluorescence and diffuse reflectance [26]. In another breast study, the combined modalities approach did not performed better than fluorescence alone at classifying malignant and non-malignant tissue [27]. In a work classifying normal, inflamed and cancerous pancreas tissue, combined fluorescence and diffuse reflectance only slightly improved the classification performance [28].

4.4 Conclusion

In this chapter, the development of a combined fluorescence and diffuse reflectance has been presented. Diffuse reflectance spectra were acquired with a standard colour chart, and the sRGB values retrieved were compared with the manufacturers’ values. The total percentage error between the data sets was found to be equal to 5.6%.

This system was then used to acquire fluorescence and diffuse reflectance spectra from upper and lower GI tissue with various conditions. A two step algorithm has been developed to select spectral features that discriminate between pathology
groups and their diagnostic power has been assessed by cross-validation using the same SMLR classifier that was used in chapter 3 to categorize liver tissue. This approach yielded an overall 81% classification accuracy. Also, the combined use of fluorescence and diffuse reflectance enabled discrimination of normal upper GI tissue from inflamed tissue and the differentiation of normal tissue from Barrett’s oesophagus sites superior to the discrimination provided by these modalities taken separately showing the value of the combined modality approach. This study has been conducted on biopsies from a very limited number of patients. Increasing the patient pool is required to confirm the diagnostic power of fluorescence and diffuse reflectance spectroscopy. However this work provides solid reasons to believe that fluorescence and diffuse reflectance spectroscopy could be a valuable tool to assist clinicians in upper and lower disease detection.

This study has been conducted ex vivo on biopsies, but one of the advantages of biophotonic techniques is that they can probe tissue in vivo by introducing the spectroscopic probe into the working channel of endoscopes. Some challenges will have to be overcome to ensure they can be used in a clinical theatre in combination with endoscopy as pointed out in [29]. For example, the re-localisation of a suspicious site highlighted by spectroscopy for a confirmatory biopsy can turn out to be difficult because of the small field of view of endoscopes and the movement in the GI caused by the heartbeat. Also, it takes time to switch between optical probe and the biopsy forceps, most of the endoscopes having only one working channel. In addition, oesophagus and colon are narrow, which means probes can approach the surface of the mucosa with a limited angle. To solve this problem, it has been proposed to use a “side-view” probe where optical fibres were cut at 45˚ to illuminate and collect light at 90˚ [22].

Finally, the spectral feature selection performed in this study has enabled the choice of suitable emission filters that will be used in chapter 5 in combination with a laparoscope to translate the spectroscopic point limited measurements into imaging for tumour extent assessment.
4.5 References


Chapter 5

Fluorescence and Diffuse Reflectance Laparoscopic Imaging for Oesophagus Tumour Resection Guidance and Nerve Localisation

So far in this work, tissues have been probed with spectroscopic systems furnishing rich spectral information at one point only. As it will be detailed in this chapter, the knowledge of the extent of a lesion is, in several situations, a crucial piece of information to assess its cause and severity and to cure it. Another medical application prompting the need of spatial information is the localisation of some type of tissue that are difficult to spot during surgery, but essential to situate not to damage them. An evolution from probe-based spectroscopy to optical imaging which combines spectral and spatial information is consequently required. The optical components enabling this transition are presented. Their performance in terms of spectral and spatial resolution, acquisition time, optical transmission, and compactness are compared in order to select the dispersive system adequate to our applications. Then, a brief description of minimally invasive surgery (MIS) procedures is given. Finally, our system, a novel multispectral fluorescence/reflectance imaging laparoscope incorporating a filter wheel and a cooled CCD camera is described. Two experiments were conducted with it:

- the optimal wavelengths to differentiate normal and cancerous oesophageal tissue determined in chapter 4 are put into good use to select the appropriate filters to delineate tumourous tissue in the oesophagus
- the multispectral laparoscope is employed in combination with an exogenous fluorophore to image in vivo nerves during a porcine trial.
5.1 Motivation and challenges associated with the transition from point spectroscopy to spectral imaging

Point spectroscopy, often called “optical biopsy” in the context of medical diagnosis, is performed with an optical fibre probe (see chapter 3 and 4) and provides a high-resolution spectrum from the tissue surface, which is then analyzed to rapidly and non-traumatically detect spectral changes inherent to the progression from a normal tissue to a diseased tissue. However, like the traditional biopsy, the point-limited nature of optical biopsy means that it is difficult to use it to assess the extent of the lesion. Yet, the knowledge of the spatial distribution of a lesion is capital information to cure it. For instance, the exact localisation and extent estimation of an ischemic area (insufficient blood supply see chapter 6) in the colon helps to understand which artery is involved. The cure of the Hirschprung’s disease, a hereditary lack of ganglion cells affecting the lower colon leading to severe constipation, consists in identifying the boundary between the aganglionic and normal portion of the colon to section the diseased section of the colon and then stitch the remaining normal part to the rectum. An excessive or incomplete colon resection leads to chronic diarrhoea and constipation, respectively, which highlights the importance of precisely identify in the diseased area [1]. In brain cancer treatment, the amount of tumour removed during a craniotomy correlates with the patient’s survival rate and quality of life [2]. Likewise, the surgical stage of breast cancer treatment requires that no tumourous cells remain at a specific distance of the tumour border, which necessitates accurate location of the tumour margin. In general, the stake of cancer cure is to remove as much tumour as possible without altering the organ function.

While techniques including ultrasound, CT and MRI are useful to localise a tumour, they can not be used to precisely delineate tumour margins because of their poor spatial resolution [3, 4]. Spectral imaging consists in producing a 3D dataset made up of the spectrally resolved images, in other words, it is a spatial extension of the point spectroscopy. Its principle is to utilise the optical contrast between diseased and normal tissue to visualize lesions that would be otherwise invisible to the naked eye. The contrast comes from the difference in intensity or/and line shape of the fluorescent and diffuse reflected light. Apart form the biomedical applications; spectral imaging has been extensively used in remote sensing to classify urban area types (water zone, parking lots, trees, asphalt) [5] or to assess forest health [6]. Spectral imaging is referred to as multispectral imaging where more than one
spectral band is involved and to hyperspectral imaging where spectral bands can be arbitrarily tuned. From the stack of recorded images (an image per each wavelength), it is possible to reconstruct the reflectance or fluorescence spectrum at each pixel, depending on the imaging modality used. Therefore optical imaging combines spectral and spatial information, whereas probe based spectroscopy yields a spectrum at a single point only. Tissue parameters can be extracted from this spectrum making possible the construction of pseudo-colour maps where pixel intensities equal the value of the retrieved tissue parameter.

A variety of strategies has been employed to perform optical imaging. They are described in the following paragraphs.

**Spatial scanning**
The spatial scanning method consists in recording the complete spectrum of a portion of the ROI at a time. The entire ROI is spectrally imaged by moving either the imaging setup or the sample to be imaged. One of the first implementation has been realized with a spectrograph/CCD system with which a region of interest is spectrally imaged line by line by moving either the imaging setup or the sample to be imaged. A line from one of the images of the resulting spatial/spectral cube corresponds hence to the spectrum of a pixel belonging to the imaged line. Such a device was used for tongue area segmentation, an important step of computer aided tongue diagnosis [7]. A hyperspectral cube was recorded between 400 nm and 1000 nm by translating the spectrograph slit, and the cube was then analysed with a support machine vector algorithm. The quality of the tongue separation form neighbouring tissue (lips, face, teeth), was improved compared to current techniques. A similar instrument was employed to identify defects in apples. In this work, it was the fruit to be analysed which was translated instead of the slit to generate the hyperspectral cube [8]. The hyperspectral cube was acquired in the infrared between 954 nm and 1350 nm. The examination of the data cube with a partial least squares algorithm enabled identification of bitter pit lesions on the apple surface. In another work, an hyperspectral setup featuring a spectrograph was used to detect tumours on poultry skins with a classification accuracy of 90.6% [9].

Systems using a two dimensional scanning mirror have been used to obtain fluorescence and diffuse reflectance multispectral images in the colon [10] and the brain [11]. As opposed to the other optical imaging techniques based on multipixel illumination and detection, “flying-spot” scanning systems illuminate only one pixel at a time and the light emitted from it is subsequently collected. Spectral images are generated by the scanning the light beam in the x and y dimension thanks to the 2D
scanning mirror of the full spectral information collected at each point. An advantage of flying-spot instruments is a lower price than multipixel imaging devices, which require expensive high-power light sources needed to illuminate large area with sufficient power density per area unit as well as costly CCD cameras to detect low-level signal. They nevertheless suffer from a slow acquisition speed compared to multipixel systems [11] and are not suited for MIS procedures.

**Spectral scanning**

In this approach, images are acquired one wavelength at a time over the entire ROI. The ROI is imaged at several wavelengths through a filtering element transmitting at selectable wavelength (Figure 5-1). This is the method of choice of this work presented in this chapter and in chapter 6. An alternative to the spatial scanning is to perform a spectral scanning to generate the spatial/spectral cube. An early implementation of this approach was done with motorized filter wheel.

![Figure 5-1: Optical imaging as implemented in this thesis: a series of images are acquired at different wavelengths forming a data cube with two spatial dimensions and one spectral dimension. By recording multiple images, it becomes possible to build the spectrum (fluorescence or diffuse reflectance) at each pixel forming the image.](image)

Skin burn injuries were studied with a setup including four filters (in the blue, green, red and infrared) [12]. Blood volume and oxygenation of the wounds were extracted form the multispectral images with the Kubelka-Munk model and then used to predict
the probability of wound curing. Because of the limited number of filters that a filter wheel can host, detailed spectral information of region of interest can not be obtained if acquisition time is a consideration. The availability of solid state filters, namely the liquid crystal tuneable filters (LCTF) and the acousto-optic tuneable filters (AOTF), considerably increased the number of accessible wavelengths. Furthermore, the wavelength tuning is faster with solid state filters (even though recently released filter wheel models have a switching time of 50 ms i.e. close to LCTF switching time), achieved without moving parts, hence allowing spectral switching without noise and vibration, and eliminating in this way potential mechanical problem. LCTF presents several advantages over the AOTF: it exhibits a higher rejection for out-of-band transmission and it can be constructed with a larger aperture [13]. On the other hand, AOTF has an adjustable bandwidths whereas LCTF bandwidth is fixed (typically 10 nm according to our measurements), and a faster switching time (of the order of the microseconds [14]) compared to that of LCTF (measured typically as of 20 ms) although it should be noted that it is the CCD exposure time which limits the acquisition speed especially when fluorescence signals are recorded. AOTF systems were often used in spectral fluorescence and reflectance animal studies [14-17]. LCTF devices were shown to have promising potential to localise tumours in the breast [3] and the brain [4], to probe the pH of resected tumours [18] and were regularly used in tissue oxygenation monitoring (detailed in chapter 6).

**Interferometry**

Other groups used a Sagnac interferometer to perform hyperspectral imaging [1]. The light beam coming from the sample and entering the Sagnac interferometer was separated into two beams which followed an identical path but in an opposite direction. Subsequent to the introduction of an optical path difference between the two beams, an interferogram of each pixel of the sample image is acquired with a detector [19]. The interferometer is then rotated to select another set of optical frequencies. Once a number of interferograms sufficient to cover the desired spectral range was obtained, a Fast Fourier Transform was applied to transform each pixel interferogram into spectra. Interferometers have a higher optical throughput than spectral scanning systems because they collect light at all the wavelengths simultaneously whereas spectral scanning systems capture only photon from the set spectral window [19]. Nevertheless, a study demonstrated the LCTF system superiority over interferometers in terms of signal-to-noise ratio [20]. Moreover, interferometers, like spatial scanning systems, do not offer the possibility to choose only a subset of pre-selected meaningful wavelengths as swiftly as spectral scanning
systems. Such a feature allows for faster acquisition and lesser size of stored data. Interferometric imagers were mostly used to carry out multispectral chromosome karyotyping [21].

**Snapshot hyperspectral system**

Some newly developed hyperspectral imaging instruments permit the simultaneous capture of full spatial and spectral information. They require large array CCD sensors and generally include sophisticated optics such as diffractive elements [22] or a mirror facet/prism module [23]. The use of the two aforementioned optical elements with a CCD sensor produces a raw image where spatial and spectral information are multiplexed and require unmixing algorithms to reconstruct the data cube. A simpler device was reported, containing a lens plate and a filter block hosting eighteen lenses and filters respectively disposed in front of the CCD camera to record at once eighteen images of the same scene at eighteen different wavelengths [24]. The major advantage of snapshot cameras is their speed of acquisition and elimination of motion artefacts caused by the sample movement during the acquisition. However, this approach imposes a choice between spectral and spatial resolution because of the limited size of the CCD chip.

In summary, the choice of a multispectral imaging system depends on the requirements imposed by the application: the spectral resolution, spatial resolution, speed of acquisition, compactness, optical throughput. For instance, multispectral fluorescence imaging necessitates high throughput systems such as flying-spot devices, filter wheel systems or interferometers because the amplitude of the fluorescence signal is low. Snapshot systems are indicated for experiments with a fast moving scene, thanks to their short acquisition time, but as a technique trading-off spatial resolution versus resolution, may not be appropriate for tumour delineation which requires high spatial resolution.

In this work, fluorescence and reflectance multispectral imaging were conducted with a laparoscope including a camera and the filter component proximally attached. This imposes stringent requirements on the weight and the size of the filter element so that the laparoscope can be comfortably manipulated by the surgeon. Bulky interferometers, snapshot cameras, AOTF were therefore inadequate for this application.

Spatial scanning systems are especially suited for applications where spectral information is only sought for regions of interest within the field of view of the camera. In this situation, the acquisition can be speeded-up by only acquiring spectra from these specific locations. Instead, the primary aim of our work was to identify diseased
regions with some prior knowledge of their spectral features rather than prior knowledge of their positions. The spectral scanning instruments were hence more adapted since they allow imaging the complete FOV at some selected wavelengths. In consequence, filter wheels and LCTF (see chapter 6) were chosen for their wavelengths selection flexibility and their compactness. As described earlier, LCTFs have several advantages over filter wheels, but their low optical transmission prevents them from being used for low level fluorescence imaging, especially when employed in combination with low transmission optics such as the laparoscope.

Of note, some groups, mostly in tissue oxygenation studies, performed multispectral reflectance imaging by filtering the excitation light, that is before reflection from the object to be imaged instead of filtering it at the emission stage (after reflection) [25-27]. This approach, called narrow-band imaging, sometimes enhances image contrast during navigation. For instance, spectral regions where haemoglobin is important compared to others tissue constituents (in the green) can be selected to visualize blood vessels.

5.2 Minimally invasive surgery and laparoscope

Minimally invasive surgery (MIS) is a type of surgery performed in the abdominal cavity through millimetre incisions [28] (Figure 5-2). MIS represents a considerable improvement over open surgery, which consists in executing large incisions to gain direct access to the abdomen. MIS produces reduced pain [29], scars [30], recovery time [31], and infections [32]. A typical MIS procedure involves CO\textsubscript{2} insufflation of the abdominal cavity to produce a working space, introduction of a minimum of two trocars (tool made up of a canula and a sharp triangular end to pierce the abdomen): one for the introduction of the laparoscope and the other(s) for the introduction of surgical instruments, the resection or visualisation of diseased tissue, and finally the removal of trocars [33].
Figure 5-2: (a) Front and (b) side views of a typical minimally invasive surgery scene. Small incisions are performed through the abdomen with trocars to enable the passage of laparoscope and surgical apparatus. Adapted from [34].

The foremost instrument which made the development of MIS possible is the laparoscope. Laparoscopes are long (between 10 cm and 1 m) and narrow (between 0.1 and 2 cm [35]) optical relays allowing clinicians to distantly visualise internal organs. They are made up of a central optical system consisting of an objective, a rod lens relay system, and an eyepiece (or an objective/CCD camera) surrounded by peripheral illumination fibre bundle (Figure 5-3) [36]. Laparoscopes are used in combination with powerful mercury, halogen or xenon light sources connected to the laparoscope light port and a colour CCD capturing the image of the abdomen cavity generally visualized on a monitor [35].

Figure 5-3: Cross-section of a laparoscope. Adapted from [37].
When screening long, narrow, hollow organs such as the colon, rigid laparoscopes are unsuitable and endoscopes are employed instead. The relay system is not anymore rod lenses, but a bundle of thousands of optical fibres of micrometric diameter [35]. Procedures involving flexible endoscopes are called natural orifices transluminal endoscopic surgery (NOTES) as endoscopes in the body introduced through natural orifices.

5.3 Existing multispectral fluorescence endoscopic systems

Apart from [14, 16], all the optical systems used in the applications described in section 5.1 were free space and could not be used in vivo during NOTES or MIS operations. Several multispectral (flexible) endoscopic systems were designed for NOTES operations and were tested mostly for lung, bronchus, and colon tumour localisations. In [38], a conventional bronchoscope was modified to add a fluorescence imaging functionality in addition to the white light imaging modality. Its principle relies on the differences of green and red fluorescence intensities between normal and dysplastic tissue which we briefly recall: the most salient discrepancy is the significantly higher green band fluorescence intensity of normal tissue compared to that of cancerous tissue. Another characteristic feature is the similar or slightly superior relative red fluorescence level of cancerous tissue compared to that of normal tissue (for more details see chapter 2, section 2.1.6). A xenon lamp provided the white light illumination and a helium–cadmium laser (emitting at 442 nm) acted as the fluorescence light source. An optical fibre was connected to the latter and was fed in one of the biopsy channel to convey the laser light at the endoscope proximal end. The fluorescence images were recorded with two intensified CCD cameras while the white light image obtained with a colour CCD camera. This system collects green (500-580 nm), red (≥630 nm) fluorescence images and a colour image. For contrast enhancement, the green fluorescence image which contains most of the healthy/diseased tissue contrast was overlaid on the red fluorescence image, which was used as a (supposed) constant background. A singular capability of this system is the real-time display of the ratio of the red fluorescence image over the green fluorescence image for objective and quantitative colour display in order to minimize false-negative and positive rates. The study presented in [39] exemplifies the importance of this red/green ratio feature. A site in the human tracheobronchial tree was declared cancerous following subjective visualisation of the overlaid green and
red fluorescence images, whilst an offline image processing consisting in dividing the red and green fluorescence image revealed that it was in fact a false positive. This was later confirmed by a biopsy justifying the importance of the live quantitative red/green ratio image display feature. Besides the diagnostic value provided by the ratio of red and green images, it allows reduction of the effects of spatially non-uniform illumination [40], as well as the effects of spatially varying collection efficiency of emitted fluorescence introduced by the illumination angle and distance or uneven surface, for instance [41]. This work served as a base for the development of a commercial system, the Lung Imaging Fluorescence Endoscope (LIFE, Xillix Tech. Corp. (acquired by QImaging Corp.), Canada).

Several others commercial systems from Pentax (Japan), Karl Storz GmbH (Germany) and Richard Wolf GmbH (Germany) were developed based on concepts close to the LIFE device [42]. The Pentax SAFE-1000 endoscope light source is a filtered Xenon lamp allowing white light illumination (no filter) and fluorescence excitation in the blue (bandpass filter with a transmission between 420-480 nm) [43]. The fluorescence is detected in only one spectral band in the green (490-590 nm), which is much larger in normal tissue than in cancerous tissue. The fluorescence mono-channel detection permits the reduction of the system price (one intensified CCD instead of two) and complexity, but its efficiency is doubtful as no ratio is calculated and therefore the geometric effects of illumination are not compensated. Indeed, in the hypothetical situation where a dysplastic tissue is closer to the illumination than adjacent normal tissue and detection channel than a normal site, the fluorescence excitation and collection efficiency of the dysplastic site will be superior to the ones of the normal tissue. This can lead to a green fluorescence recorded from the tumourous site as high as or even larger than that of the normal tissue in spite of the greater intrinsic green fluorescence of the latter potentially leading to a raise of false-negative rates. Conversely, normal tissue distant from illumination and detection channels or overshadowed could be counted as false-positive as it is the case in [44], where an endoscopic fluorescence system similar to the Pentax endoscope was used to demarcate colonic adenomatous polyps. In this study, normal sites overshadowed by mucosa folds were counted as dysplastic sites.

The Storz system takes advantage of a three chip CCD endoscope camera (one chip for each of the R, G, B channels) to perform colour imaging (using the R,G,B channels of the CCD camera), as well as autofluorescence imaging by using the red (600 nm - 800 nm) and green (470 nm – 600 nm) channels [45]. The light source used in the Storz device is a xenon lamp filtered between (380 nm - 460 nm) for fluorescence imaging. As opposed to the Xillix endoscope, the red/green
fluorescence ratio is not directly available in the Storz apparatus. Instead, the dysplastic/normal sites contrast comes from the green fluorescence signal drop observed in dysplastic tissue, which will appear darker than surrounding normal tissue. A particularity of the Storz instrument is that in fluorescence mode, the emission filters leaked 1% of the blue excitation light in order to facilitate navigation, allowing the surgical instruments (such as the biopsy forceps) to be localised in the field of view in fluorescence mode, and improving the contrast. Indeed, the back-reflected blue light excitation is supposed to enhance the healthy/diseased contrast by providing a constant background unaffected by the tissue optical properties. The use of a single three-CCD chips camera means that the Storz system is very compact and can be directly clipped to the ocular of an endoscope; however this CCD camera is not intensified which means that longer integration times are required to detect the fluorescence with an acceptable signal-to-noise ratio.

It was mentioned earlier that the background images of the Xillix and Storz apparatus were the red fluorescence and the backscattered blue light excitation images, respectively. They were assumed to be “constant”, i.e. independent of optical properties change. This is of course inaccurate because even though the differences in red fluorescence between normal tissue and cancerous lesions are less important than in the green range, they still exist. Moreover, should the Xillix system be used in the colon, presence of faecal matter rich in porphyrins, a fluorophore emitting in the red, would create artefacts [46]. Similarly, back-reflected blue light background images are not independent of optical changes, since haemoglobin absorbs highly in the blue spectral range, and the presence of highly vascularised sites could disrupt the signal [44].

These limitations were addressed in a modified DAFE (Diagnostic Fluorescence Endoscopy) system (Richard Wolf GmbH, Germany), which operates in the same way as the Storz apparatus, except that a portion of red light, weakly absorbed in the red, is leaked by the excitation and emission filters [39]. The contrast between healthy and neoplastic tissue in the bronchus using the backreflected red light as background image instead of the red fluorescence image was improved by a factor of 2.7. It is interesting to note that, with the exception of the Storz system, all aforementioned fluorescence imaging units clipped to the eyepiece of the flexible endoscopes, are cumbersome since they include more than one camera and could cause difficulty when used on rigid laparoscopes, which require a compact and light camera unit to be easily manipulated and not to cause excessive strain on the trocars. The system we developed was compact enough to be clipped on a rigid
laparoscope and operated during a surgical procedure. It also combined multispectral fluorescence and reflectance imaging.

Clinical trials performed with Xillix, Pentax, and the Storz systems showed a dramatic improvement of the sensitivity for the detection of cancerous lesions in the bronchus when conventional white light endoscopy and autofluorescence were used in parallel. However, this came with a lower or limited specificity caused by a high false positive rate [44, 47] attributed to non-cancerous yet abnormal sites such as inflamed areas whose fluorescence properties differ from normal sites leading to their misclassification [48]. Cancer detection in the oesophagus is complicated by the abnormal fluorescence of Barrett’s oesophagus sites, which often precedes the apparition of oesophageal adenocarcinoma and is responsible for an elevated number of false positives [48]. In a study of fifty patients suffering from Barrett’s oesophagus, a PPV for neoplasia detection as low as 28% was obtained in the autofluorescence mode with the Xillix instrument [49].

None of these three commercial systems were spectrally optimized, i.e. preceded by a spectroscopic study consisting in recording spectra from sites of the organs and pathologies of interest in order to select the spectral bands that enable the optimal detection of lesions. Such an optimization was performed in the DAFE instrument employed for cancerous lesions in the bronchus [39] as well as on a modified version of the Xillix used for dysplastic lesions detection in the GI [41]. In the study involving the DAFE system and performed on fifteen patients, sensitivity obtained with fluorescence imaging was twice the sensitivity reached with white light imaging. Furthermore, a PPV of 100% was reached with fluorescence and white light modes combined, denoting an absence of false positives. This pilot study indicates therefore, that a spectral optimization improves the diagnostic performances of fluorescence endoscope.

Another strategy to lower the number of false positives limiting the diagnostic performance of autofluorescence imaging consists in, like in spectroscopy, combining several modalities. For instance, narrow-band imaging was used in combination with autofluorescence imaging in a study involving twenty patients with Barrett’s oesophagus and suspected high-grade neoplasia. In the fluorescence imaging mode, all lesions were detected and the false positive rate was found to be 40%, the latter being reduced to 10% when fluorescence imaging was used together with narrow-band imaging [50]. The combination of modalities seems to improve the diagnostic accuracy of neoplastic lesions, since this approach decreased the number of false positives obtained with autofluorescence alone.
5.4 Multispectral fluorescence/ diffuse reflectance laparoscopic imaging system

The schematic of the multispectral imaging laparoscope, used in the two studies presented in this chapter (oesophagus cancer imaging and nerve delineation), is presented in Figure 5-4. It comprises a 200 W metal-halide lamp unit with a built-in six position filter wheel (Prior Scientific, Inc., USA). The 5 mm light guide, originally designed to be coupled with microscopes, is connected to the light port of a laparoscope (0° viewing angle, 10 mm diameter, Olympus Ltd, Japan) via a custom made connector. A second filter wheel (Thorlabs, Inc., USA) containing the emission filters is placed between the laparoscope and a CCD camera (RetigaExi, QImaging, Inc., Canada). In white-light mode, both filter wheels were set on “no filters” positions, and in fluorescence mode, filter wheel slots with the appropriate combination of excitation/emission filters were selected. The number and specifications of the filters used in the two studies are detailed in sections 5.5.1 and 5.6.2.

![Figure 5-4: Schematic of the multispectral laparoscopic imaging system.](image)

During experiments, the laparoscope was used in combination with an articulated arm (Figure 5-5) to minimize the camera movement during the acquisition. The image formed after the laparoscope eyepiece was focussed onto the chip of the camera with a 30 mm focal length objective lens. A LabVIEW program enabled control of the excitation-emission filters combination, the camera settings and the image acquisition.
5.5 Design of a dual-modality laparoscope for oesophagus cancer detection

The initial goal of the work presented in this section was to image ex vivo an oesophagus including tumours, but it has not been possible to do so due to the unavailability of a suitable specimen during the six months that this study lasted. It was possible to get hold of two specimens yet the first one presented a tumour deeply buried and hence inaccessible to fluorescent light. The tumour present on the second specimen was completely resected by the clinician straight after the oesophagectomy for histopathological examination. However, we believed that the design of the system described below was relevant to the thesis.

Spectrally optimized instruments and systems incorporating several modalities were presented in section 5.3. They were shown to enhance the diagnostic performance of autofluorescence endoscopes, which itself improve the
diagnosis power of traditional white-light endoscopy. None of these systems were both spectrally optimized and multi-modalities. The laparoscope presented in this thesis is the first, to our knowledge, to include these two features as it was spectrally optimized thanks to the study presented in chapter 4, and it incorporated fluorescence and diffuse reflectance modalities. It is hoped that the spectral optimization alongside the dual modality capability will further enhance cancerous tissue detection in the oesophagus. Of note, rigid laparoscopic multispectral systems similar to ours exist, but they can only perform either fluorescence imaging [51] or reflectance imaging [52].

5.5.1 Selection of filters

All the filters used in this experiment were acquired from Semrock, Inc., USA. The central wavelengths of the excitation filters were chosen based on the emitting wavelengths of the laser diodes used in the spectroscopy study (375 nm and 405 nm), as well as on the manufacturer catalogue availability. The central wavelengths of the emission filters were chosen according to the spectral analysis conducted in chapter 4 and on the manufacturer catalogue availability. The choice of the bandwidth was the result of a compromise between spectral selectivity, sufficient signal level, and manufacturer catalogue availability. The instrument was designed to host two fluorescence imaging channels, one at 375 nm excitation and the other one at 405 nm, and a diffuse reflectance channel in addition to the white light imaging. For 375 nm excitation generation, a bandpass filter centred at 379 nm with a 34 nm bandwidth (379/34) and encompassing the light source peak at around 370 nm, was chosen (Figure 5-6). For 405 nm excitation, a 417/60 nm bandpass filter was selected, the spectral range covered by this filter included two illumination peaks centred at 400 nm and 435 nm. The optical power densities obtained at the tip of the laparoscope with these filters were measured at 0.25 mW/cm$^2$ and 4 mW/cm$^2$ at 375 nm and 405 nm excitation, respectively, and at 35 mW/cm$^2$ in white light mode (no excitation filter).
Following spectral analysis, the pair of wavelengths whose intensity ratio optimized the separation between cancer tissue from the normal tissue were found to be (491 nm, 662 nm) for 375 nm excitation fluorescence and (605 nm, 676 nm) for diffuse reflectance (see Table 4M5). In chapter 4, no spectral optimization was performed for 405 nm excitation fluorescence yet the pair (480 nm, 660 nm) was found to be optimal to separate normal sites from cancerous sites in the oesophagus in another study [53]. In consequence, the following emission filters (central wavelength (nm)/ bandwidth (nm)) were chosen for the two fluorescence channels: 483/32, and 655/40. For diffuse reflectance imaging, the selected filters were 605/15 (the same one as for fluorescence imaging) and 655/40. Table 5-1 summarizes the excitation and emission filters employed in each imaging mode.

Table 5-1: Correspondence between imaging modes and their excitation/emission filter combination.

<table>
<thead>
<tr>
<th></th>
<th>White-light fluorescence</th>
<th>375 nm fluorescence</th>
<th>405 nm fluorescence</th>
<th>Diffuse reflectance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Excitation filter</strong></td>
<td>None</td>
<td>379/34</td>
<td>417/60</td>
<td>None</td>
</tr>
<tr>
<td><strong>Emission filter</strong></td>
<td>None</td>
<td>483/32; 655/40</td>
<td>483/32; 655/40</td>
<td>605/15; 655/40</td>
</tr>
</tbody>
</table>
5.5.2 Fluorescent phantom imaging

The low magnitude of autofluorescence signal, coupled with the low optical throughput of laparoscopes is the main difficulty to overcome when performing fluorescence laparoscopic imaging. The system validation detailed in this section, was purely qualitative, and simply aimed at verifying the system sensitivity to fluorescence signal by recording images from a fluorescent phantom. As a result, only the fluorescence images are shown and not the diffuse reflectance images. Solutions of 2 µM of protoporphyrins IX (PpIX) and FAD, two fluorophores involved in carcinogenesis [54], coumarin 334 (C334), a fluorophore emitting in the visible, and a control sample of water were prepared. The solutions were transferred to a 100 µL multi-well plate as illustrated in Figure 5-7.

![Figure 5-7: Location of the solutions of protoporphyrins IX, coumarin 334, FAD, and the control solution (water).](image)

Figure 5-8 presents the pair of fluorescence images obtained form the phantom at 375 nm and 405 nm excitation with respective exposure times of 1000 ms and 300 ms. For both excitations, C334 fluorescence signal appeared important with the 483/32 bandpass filter and low with the 655/40 bandpass filter, which was expected as its fluorescence spectrum peaks in the region of 490 nm [55]. Conversely, fluorescence levels from PpIX were visually low with the 483/32 filter and important with the 655/40 filter as fluorescence intensity of PpIX remains low until 600 nm and peaks at 635 nm [56]. FAD fluorescence seemed low or moderate on the four images, as expected since FAD fluorescence maximum is in the region of 550 nm.
(see Figure 2-3). Finally, no fluorescence was detected from the control well filled with water.

The phantom study validated therefore the capacity of the laparoscopic system to excite and capture fluorescence.

<table>
<thead>
<tr>
<th>Excitation</th>
<th>Emission</th>
<th>375 excitation</th>
<th>405 excitation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(379/34 filter)</td>
<td>(417/60 filter)</td>
</tr>
<tr>
<td>483/32</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>655/40</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 5-8: Raw intensity fluorescence images of the fluorescent phantoms at each excitation/emission filter combination of interest.

Last two paragraphs moved to conclusion

5.6 In vivo brachial plexus nerve identification

5.6.1 Nerve visualization relevance and previous work

Unintended nerve damage or transection can occur during surgery leading to chronic pain, atrophy, paralysis, and dysfunction, yet thin and buried nerves in muscles are difficult to visualize. Current methods rely on the surgeon’s experience or electric stimulation (electromyographic monitoring) [57], which is not an imaging technique. Nerves can be labelled with fluorescent dyes injected either directly in the nerve tract or systemically. The former approach presents two drawbacks: it can highlight only one tract and is time consuming as axonal uptake and transport is very slow (a complete nerve labelling can take days) [58].
It is proposed in this work to use fluorescence imaging intraoperatively in combination with a fluorescent dye intravenously injectable. While this thesis aimed to capitalize on differences of tissue autofluorescence distribution as exogenous fluorophores present drawbacks such as toxicity, time for regulatory approval for animal and human use, and length to reach optimal contrast, the lack of fluorescent contrast between nerves and surrounding tissue prompted the need to resort to dyes. Exogenous fluorophores have nevertheless some advantages: their optical properties are well characterized (even though they might vary depending on the biochemical environment) and they are significantly brighter than endogenous fluorophores which facilitates the direct location of the tissue of interest without complex image post-processing to extract the valuable signal. This work is to my knowledge the first reported attempt of fluorescence nerve imaging during a MIS procedure using a laparoscope. In previously published studies, nerves and surrounding tissue were either imaged ex vivo under a fluorescence microscope [59, 60] or in vivo during open field surgery [61].

The nerve dye used here, named GE3111, belongs to a family of nerve contrast agents under development by a team at General Electric Healthcare Global Research and designed to meet the following characteristics: traverse the blood-nerve frontier, bind specifically to the myelin present in nervous tissue, remain in nerves a few hours, and emit fluorescence in the visible/infrared. The dye optical properties were studied and it was successfully used to image nerves in mice and rats with an open-field multispectral camera by Cristina Tan Hehir and Victoria Cotero from General Electric. The objective of the present experiment was to translate this experiment in vivo using the multispectral laparoscope to image the brachial plexus of a pig.

5.6.2 Dye fluorescence properties and filter selection

The excitation and emission filters were chosen to optimally excite and detect the dye fluorescence (Figure 5-9). A bandpass filter (central wavelength: 405 nm, bandwidth: 60 nm, Semrock, Inc., USA) centred close to the dye maximum fluorescence excitation was chosen which, depending on the solvent it is dissolved in, varies between 410 nm and 425 nm. The resulting violet-blue light was within the 375-445 nm range, and contains a peak at 398 nm and a second peak at 430 nm close to the dye absorption maximum.

The selected emission filter was 550 nm long pass (Thorlabs, Inc., USA). It was picked not to overlap with the excitation filter transmission profile to merely
record fluorescent light (and no backreflected excitation light) and to cut as much tissue autofluorescence as possible while enabling the collection of the fluorescence signal within a reasonable timeframe (exposure time of a few seconds maximum). Ideally, a bandpass filter with a small bandwidth centred at the dye peak emission fluorescence around 610 nm should have been used, but this would have led to long exposure times inappropriate for *in vivo* imaging with a moving scene. In other words, spectral specificity had to be compromised to reach acceptable speed acquisition; this is due to the weakness of fluorescence signal.

The optical power densities obtained at the tip of the laparoscope were measured as 35 mW/cm$^2$ and 4 mW/cm$^2$ in white light and fluorescence mode, respectively.

![Figure 5-9: Metal-halide source spectrum, fluorescence excitation and emission spectra and transmission profiles of the excitation and emission spectra.](image)

### 5.6.3 Phantom imaging

The excitation and emission filters described in Figure 5-9 were inserted in the multispectral imaging laparoscope. The imaging procedure involved the acquisition of the white-light image followed by the acquisition of the fluorescence image with respective exposure times of 40 ms and 1000 ms. A phantom consisting of tubes containing either the fluorescent dye formulation or water was imaged. A total volume of 200 mL of dye solution was formulated at a $1.10^{-2}$ g/L concentration. 50 ml of the dye was used for the phantom.
While water tubes were undistinguishable from dye tubes in the white light image (Figure 5-10-a), the distinction between them became clearly apparent in the fluorescence image (Figure 5-10-b). To quantify the contrast between dye and water tubes, the mean intensity of a 20×20 pixels region was calculated for the six tubes, and the average nerve tube intensity was divided by the average water tube intensity leading to a contrast of 8.5. This preliminary experiment indicated that the system illumination channel delivered a power density/ pixel sufficient to excite the dye fluorescence, and validated the system detection channel ability to detect and record the dye fluorescence signal.

5.6.4 In vivo brachial plexus imaging

In this section, the results of the study involving a pig injected with the nerve fluorescent contrast agent are detailed. The aim was to investigate in vivo the fluorescence laparoscope ability to delineate nerves. It was decided to image nerve tracts in the axilla (armpit) region as it hosted a section of a well-known network of nerve fibres, the brachial plexus, and it was easily accessible without disturbing the workflow of other experiments performed in parallel in the porcine abdomen. The brachial plexus is essential to the movements of the arm and the hand; surrounding tissues include the axillary artery and vein, and pectoral/serratus muscle [62]. The resection of tumours located at the top of lungs, referred to as Pancoast tumours, is an example of a surgical procedure during which there is a danger of damaging the brachial plexus and where the approach described here would be of interest [63].
5.6.4.1 Imaging procedure

At the start of the experiment, the totality of the available dye (30 mg) mixed in a volume of 150 ml, was intravenously injected into the pig which correspond to a 0.43 mg/kg dose the pig’s weight being 70 kg. During the pig trial, the brachial plexus and surrounding area were imaged regularly during 5 hours through a mini-incision carried out in the axilla (Figure 5-11) before (for control). The exposure times were between 40 ms and 60 ms for white light imaging and ranged from 2 s to 3.5 s making the total acquisition inferior to 5 s.

![Figure 5-11: View of the laparoscope illuminating the brachial plexus at the axilla level with blue light (417/60 nm bandpass excitation filter).](image)

The working distance between the distal end of the laparoscope and the tissue was maintained at about 10 cm, and the incision (Figure 5-11) was covered with an opaque sheet to isolate the imaged scene from ambient light room. Following the trial, the imaged nerves and axillary vein were harvested, kept in formalin and sent to the GE team for *ex vivo* imaging with an open-field hyperspectral camera (Nuance camera, CRI, Inc., USA). Cross-sections of them were prepared using a reagent system (Ventana Medical Systems, Inc., USA) to be examined under a fluorescence microscope (Carl Zeiss Meditec, Inc., Germany).

5.6.4.2 Brachial plexus images

Figure 5-12 shows a set of white light and fluorescence images acquired 5 h after the contrast agent injection. It features the brachial plexus, the pectoral/serratus muscle region and the axillary vein. Fluorescence signal was collected from both the brachial
plexus and the pectoral/seratus muscle, whilst the axillary vein did not show significant fluorescence levels (Figure 5-12-b). However, the contrast between the brachial plexus and the surrounding pectoral/serratus muscle did not visually appeared more important in the fluorescence image than in the white light image (Figure 5-12-a). The low level fluorescence distinguishable in the axillary vein came from the few straggly wisps of white tissue overlying the vein (clearly visible on the white light image).

![Figure 5-12: Right axilla looking anteroposteriorly (images acquired with the laparoscope system). (a) White light image featuring a large bifurcating trunk of brachial plexus (green arrow), axillary vein (yellow arrow), medial muscular border of axilla–pectoral/serratus muscle (blue arrow) 5 h after injection and (b) corresponding fluorescence image.](image)

Similar observations were made by the GE research team as for the fluorescence intensity recorded *ex vivo* from the harvested fractions of the brachial plexus and the axillary vein with a hyperspectral camera (Figure 5-13): important fluorescence from the different trunks of the brachial plexus was detected, whereas the axillary vein appeared dark as expected since it is a highly absorbing media (strong concentration of haemoglobin).
Figure 5-13: In vivo fluorescence image of an axilla ROI taken with the multispectral laparoscope (left) and ex vivo fluorescence images of the brachial plexus and the axillary vein acquired with the open-field hyperspectral camera (Nuance camera, CRI, Inc., USA) (right).

In order to quantify the nerve-to-muscle contrast progress with time, the fluorescence images were divided by the reflectance images to correct for non-uniform illumination (see section 5.3) and the temporal evolution of the nerve-to-muscle ratio was calculated from the resulting images by averaging the pixel intensity in a rectangular area of 100 over 200 pixels in both the brachial plexus nerve and pectoral/seratus muscle region.

Figure 5-14: Temporal evolution of the nerve-muscle contrast (t=0 min corresponds to the time of injection).
It is apparent from the graph (Figure 5-14) that no clear trend in the nerve-muscle ratio could be observed. The contrast values oscillate around 1, a maximum of 1.37 was reached 71 min post-injection, and a minimum of 0.74, 2h51min post-injection.

Several assumptions can be formulated to explain the absence of contrast. It is possible that no dye was present in the imaged region of interest (ROI). The examination under microscope of the cross-sectional images of a control brachial plexus from a rat injected with the dye formulation agent (Figure 5-15-a) and the brachial plexus resected from the pig injected with the dye (Figure 5-15-b) allowed eliminating this hypothesis. The comparison of the two images revealed indeed on the pig nerve image, bright doughnut structures (some of which are indicated by the red arrows on Figure 5-15-b) easily visible on the nerve periphery and characteristic of contrast agent bound to the nerve myelin sheath. Such pattern was not observable on the control image whose (weak) fluorescence signal came from autofluorescence from all likelihood.

Another hypothesis is that dye was present in the ROI, and that, the absence of increased fluorescence in the nerve is either due to a. an unspecific binding of the dye (i.e. there was dye in both the brachial plexus and the muscle) along with a similar kinetics profile. The examination of the temporal evolution of the fluorescence intensities in nerves and muscles which did not show any rise in fluorescence (data not shown) permitted to eliminate the hypothesis.

Consequently, the absence of nerve-muscle contrast in the fluorescence image was attributed to a low dye concentration leading to a fluorescence signal too low to be excited and/or recorded by the laparoscopic fluorescence imaging setup (fluorescence imaging through low transmission systems like laparoscopes remains challenging because fluorescence signals are generally weak).
Past studies on nerve fluorescence imaging established that a minimal dose of the contrast agent was required to observe nerve-to-muscle contrast [59, 61], below which, the contrast of temporal evolution does not show any trend. In [61], two different contrast agents, both variants of the GE3111 used here, were injected in a mice and the evolution of the contrast with the dose was studied to determine the optimal dose leading to a maximum of contrast for each of the two dyes. Scaling these two values by the body surface area of a 30 kg pig, the dose needed was found to be equal to 2.3 mg/kg and 1.1mg/kg for the first and second dye respectively. At the time of the experiment, a total of 40 mg of contrast agent was available, 10 mg of which was used for the phantom experiment, leaving 30 mg for the \textit{in vivo} imaging of the 70 kg pig trial resulting on the 0.43 mg/kg mentioned earlier. No preliminary study on the optimal dose of the GE3111 dye for maximum contrast has been performed prior to the trial. It is therefore difficult to definitely determine if the contrast agent dose injected the day of the experiment was sufficient. However, while recognizing that the optimal dose is contrast agent specific as it depends on its myelin bonding ability, its solubility, and its blood nerve penetration [62], it can be noted that the dose we administrated to the pig was more than twice below the lowest dose of the study presented in [61].

Another observation is that, although fluorescence was visible throughout the nerve section (Figure 5-15-b), the fluorescence intensity distribution appeared inhomogeneous with higher fluorescence near the nerve border than in the middle indicating an incomplete dye uptake by the nerve. In previous studies using variants
of the GE3111 dye, fluorescence generated by the contrast agents was detected from nerves [60-62], microscope analysis of the resected tissue sections revealed high level of fluorescence evenly distributed throughout the nerve section and not only in the nerve periphery. Figure 5-15 deleted

The previous observations all tend to confirm that the absence of fluorescence signal from the GE3111 contrast agent was due to a quantity of dye too low to be detected. An alternative to using a higher dose would be to enhance the multispectral laparoscope performances by acting on the system illumination power (to reach a power-per-pixel sufficient to excite the dye fluorescence even at a very low local concentration) alongside the system sensitivity. The former could be achieved by using a higher power light source and higher transmission light delivery channel, yet the power lamp employed in this work is already 200 W and light delivery system consisting in the light guide and the laparoscope illumination channel cannot be dramatically improved. As for the system sensitivity, it could be improved by increasing the system collection efficiency and the detector sensitivity. A higher sensitivity and collection efficiency would enable the use of a bandpass filter centred on the dye peak fluorescence instead of using a long-pass filter. A bandpass filter would have the advantage to isolate the contrast agent fluorescence from the background (i.e. the tissue autofluorescence). Indeed, a large portion of the fluorescence emitting by the contrast agent overlaps with the fluorescence emitted by the tissue fluorophores (see figure 2-3, chapter 2) which means that the weak fluorescence stemming from low dye concentration could be “drowned” in the tissue fluorescence. In our system, the collection efficiency was limited by the laparoscope reduced entrance pupil diameter and therefore could not be significantly boosted. Furthermore, the detector employed here was a cooled CCD camera with a very high sensitivity. In consequence, our system collection efficiency and sensitivity can not be dramatically improved and the only way to detect the contrast agent with the fluorescence imaging endoscope is to use higher contrast agent dose.

5.6.5 Conclusion

The development of a fluorescence imaging system adaptable to any commercial laparoscopes was presented. As a follow-up of the spectroscopic studies presented in chapter 4, a multispectral imaging laparoscope was built and validated on fluorescent phantoms. It should be noted that in future oesophagus studies, the pair of images recorded in each of the fluorescence and diffuse reflectance modes
would be divided against each other to generate the ratio image supposed to maximize the contrast between healthy and cancerous tissue since the spectral optimization was performed on intensity ratios in chapter 4. The image ratio has the added advantage to diminish the influence of geometric effects on the intensity values as mentioned in section 5.3.

This system will have to be tested on a significant number of oesophagus specimens to assess its diagnosis performances for tumour detection. It is hoped that, in the future, its suitability to help the surgeon diagnosis will be demonstrated.

Unusually, in this work, the multispectral laparoscope was used with a dye in an attempt to mark nerves out by fluorescence imaging. This dye is still under development and has only been tested on small animals so far. In consequence, many unknowns remained on its kinetics and dose in big animals. Our results indicated that the injected dose was too low to be captured by our laparoscopic system. However, this experiment demonstrated our system ability to capture the contrast agent fluorescence in a phantom, and nerve autofluorescence \textit{in vivo}, and minimally invasively, making it a promising candidate for nerve imaging during minimally invasive procedures, providing the dose of the dye is sufficient.

In summary, the design of fluorescence/reflectance multispectral laparoscope features a six position illumination filter wheel and a six positions emission filter wheels allowing up to 25 excitation-emission fluorescence imaging combinations in addition to white light imaging. Filters and therefore excitation and emission wavelengths can be modified depending on the organ and conditions investigated making the multispectral imaging laparoscope a versatile platform useable in a vast array of applications. In its full potential, this system could be used prior to sigmoidectomy [65], for instance, to simultaneously delineate the tumour in the colon while preserving the pelvic nerve during the tumour removal.
5.7 References


Chapter 6

Hyperspectral Laparoscope for in vivo Vasculature Imaging

In chapter 5, the low intensity of the fluorescence signal, together with the use of low transmission optical system such as a laparoscope led us to trade spectral resolution against signal-to-noise ratio and use high-transmission and large bandwidth filters in combination with filter wheels to record spectrally resolved signals. This chapter describes a spectrally-resolved laparoscope similar to the system described in chapter 5, except that it was designed to record diffuse reflectance images only. Reflectance level signals are several orders of magnitude higher than fluorescence signals which allows reflectance measurements at a higher spectral resolution. The filtering element of the system presented here was modified in consequence.

As highlighted in section 2.3.2, the shape of the spectrum of the visible light reflected from tissue is largely dependent on the haemoglobin concentration and oxygenation, and scattering. This principle is already turned to good account in hospitals with the use of pulse oximeters. Like spectroscopic probes, pulse oximeters do not give spatial information making them inadequate for the assessment of the spatial extent of the ischemic area. In this chapter, a concise overview of the existing blood imaging systems as well as the current methods of ischaemia diagnosis will reveal that none of them can be used intraoperatively. In an attempt to fulfil this need, we built a hyperspectral laparoscope whose design and performance is then described. Phantoms with varying haemoglobin concentration and oxygenation were imaged with the hyperspectral laparoscope to generate haemoglobin concentration and oxygenation maps. The same system was subsequently used in two animal trials: (1) to monitor in situ the colon oxygenation temporal evolution immediately after pig euthanasia and (2) to observe the tissue re-oxygenation in a recipient rabbit following uterus transplantation.
6.1 Current imaging methods for vasculature monitoring

Ischaemia is characterised by an insufficient blood supply to tissue. Its consequences depend on the type of organ affected but, in all cases, results potentially in severe tissue injury due to hypoxia. Two types of ischemic injuries are distinguished, the first one caused by the initial decrease or absence of blood flow and the second one, subsequent to tissue re-perfusion, leading to more serious damage [1]. It is therefore important not only to detect ischemic tissue but also to monitor its re-perfusion.

The first type of ischaemia studied was intestinal ischaemia whose origins and current diagnostics methods are more varied than ischaemia following transplantation. Intestinal ischaemia can be classified according to its cause. Thrombosis and embolism where one or several clots form, can impair or stop the blood flow in the colon [2, 3]. Ischaemia can also be induced from outside the blood vessels and arises during or as a result of an operation on the colon such as anastomosis [4] where two colon parts are joined together. Low blood pressure which may occur during heart surgery [5] or colon strangulation consecutive to a hernia or a volvulus (twisted intestine) can also cause ischaemia [6]. Ischaemia differs in its length: acute or chronic; its localisation: small or large bowel; and its extent: localised or widespread [7].

The second clinical case of ischaemia examined in this work was consecutive to the retrieval of a uterus from a donor rabbit for transplantation. The critical stage of this operation is the uterus re-perfusion in the recipient rabbit during which ischemic injury can cause parenchyma and microcirculatory function impairment [8]. Ultrasound is sometimes used post-operatively to check the uterus perfusion but no intraoperative imaging method exists to monitor uterus vasculature after the transplantation [3]. In the animal model, a pulse oximeter providing oxygen saturation and blood volume is usually employed but only provides point-limited information.

Early diagnosis leads to a significant reduction in the mortality rate of intestinal ischaemia [10]: a study showed that the mortality rate increased from 54% to 95% when acute mesenteric ischaemia was diagnosed more than 24 hours after the symptoms appeared [11]. The diagnosis of intestinal ischaemia is difficult because there is no single set of symptoms [9] clearly related to it. Angiography is considered to be the reference diagnosis modality [12, 13], while use of MRI [14] and CT [15, 16] is becoming more frequent with increasingly good results. However,
these techniques provide information on anatomic modifications resulting from ischaemia, like bowel wall thickening, or bowel lumen dilatation [15], which are consequences of ischaemia but could have been caused by other pathologies [17]. Moreover, the processing time of these techniques is long, whereas early diagnosis is crucial for patient survival. Finally, this time-consuming process means that the use of angiography and MRI is not recommended for chronic mesenteric ischaemia, because the patient’s colon blood flow may return to normal before the diagnostic images are generated [18]. Ultrasound is also used and has a high specificity (92%-100%) but also a limited sensitivity (70%-89%) [19, 20]. Moreover, ultrasound probes are not straightforward to use, and require extensive training. Finally and most importantly, none of these techniques, apart from ultrasound, can be used during an operation.

Methods stemming from biomedical optics could turn out to be useful thanks to their ability to provide intraoperative diagnostic information directly related to haemoglobin concentration and saturation of oxygen. For instance, diffuse reflectance spectroscopy in the visible range has very recently been successfully used to diagnose chronic mesenteric ischaemia during an endoscopic procedure [17, 21].

Figure 6-1: Anatomy of the lower digestive system showing the colon (also called large intestine or large bowel) and small intestine (also called small bowel). Reproduced from [22].
In this approach, the endoscope reaches the colon via the rectum (Figure 6-1) and the small intestine from the oesophagus. The optical fibre probe, used to detect ischaemia is then inserted in the endoscope biopsy channel. This method presents two drawbacks. Firstly, the sinuous track of the small intestine implies that the endoscope can not be inserted very far away from the small intestine extremities making impossible the diagnosis of ischaemia arising in the central portion of the small intestine. Secondly, the measurements are point limited. Extension of this method to an imaging approach may prove useful to help the surgeon to assess the extent of the ischaemia.

Unlike the aforementioned imaging techniques, multispectral or hyperspectral imaging directly probes functional parameters specific of ischaemia (including blood volume and oxygenation) and whose impairments precede the structural change induced by isch (such as bowel wall thickening). Their value can be extracted from the spectrum recorded at every pixel making possible the construction of pseudo-colour maps where pixel intensities equal to the value of the retrieved tissue parameter. This method was employed to gauge oxygenation and/or perfusion in various organs or tissue type including skin [23-25], brain [26-28], kidney [29], eye fundus [30], heart [31, 32], and tumour (known to be hypervascularised and hypoxic) [33]. None of the photonics equipment used in the aforementioned studies of internal organs (brain, kidney, heart) could be applied minimally invasively; therefore the hyperspectral imaging had to be performed through a large incision. A hyperspectral instrument was also employed to diagnose porcine colon ischaemia from the spectral variations between normal and ischemic tissue, but did not relate these differences with tissue parameters such as haemoglobin concentration [35], which can be disconcerting for a doctor. To the best of our knowledge, diffuse reflectance spectroscopy or hyperspectral imaging techniques have not been used during transplantation operation.

It is proposed in this chapter to use a hyperspectral laparoscope as a vasculature imaging monitoring tool during a pig’s termination procedure and a rabbit uterus transplant operation. In the former experiment, colon haemoglobin concentration and oxygenation evolution were monitored during the pig’s termination (blood vessel clamping simulates more closely an ischaemia than pig’s termination but we lacked ethics approval). In the latter experiment, the goal was to more specifically check tissue re-oxygenation in the recipient postuterus removal ischaemia artery and vein re-anastomosis. Results of these experiments are presented after the hyperspectral laparoscope description and performance characterization.
6.2 Hyperspectral laparoscopic system

6.2.1 Instrumentation

The hyperspectral laparoscope (Figure 6-2) included a LCTF (VariSpec, Cambridge Research & Instrumentation, Inc., USA) that transmitted light with a bandwidth of 15 nm in average with a computer controlled central wavelength between 400-720 nm. The Xenon light source (model 20133020, Storz GmbH, Germany) emitted light between 400 nm and 700 nm (Figure 6-3) which was then transmitted by a fibre bundle light guide and coupled into the 12 mm rigid 30 degree laparoscope (Storz GmbH, Germany). The sample was placed 50 mm from the distal tip of the laparoscope and the reflected light was imaged by the central Hopkins rod lens channel of the laparoscope in the usual way. An additional 50 mm focal length lens was inserted at the proximal end to form an image on a monochrome CCD camera (DCU223M, Thorlabs, Inc., USA). This lens was mounted in a helicoid barrel (1 inch linear travel) to adapt the image plan to the laparoscope-sample distance. The LCTF was placed between the lens and the CCD. The laparoscope was attached to the LCTF via a clip which allowed easy removal of the LCTF/CCD block.

![Figure 6-2: Hyperspectral laparoscopic system consisting in a CCD camera and a LCTF attached to a regular rigid laparoscope.](image)

The hardware was controlled with a LabVIEW program which enabled control of the camera settings, as well as a choice of wavelength range and increment at which images were acquired and saved. A live RGB video was also displayed by combining three images at 450 nm, 550 nm and 650 nm. Furthermore, a MATLAB program enabled generation and display of the spectrum at each pixel of the image by storing intensities for all the images of a stack. Each image being acquired at a
known wavelength, it was therefore possible to draw the spectrum by plotting the pair (wavelength, intensity) for every pixel of the image.

![Emission spectrum of the Xenon lamp emitting in the visible. Output intensity gradually increases from 390 nm and drops rapidly above 665 nm.](image)

Figure 6-3: Emission spectrum of the Xenon lamp emitting in the visible. Output intensity gradually increases from 390 nm and drops rapidly above 665 nm.

The instrument spatial and spectral resolutions were measured. The spatial resolution was quantified by acquiring an image of a paper 1951 USAF test pattern (Edmund Optics, Inc., USA) (Figure 6-4). It consists of sets of horizontal and vertical bars separated by decreasing spacings when going clockwise from the left bottom of the target to its centre.

![1951 USAF test patterns. This target was used to determine optical system resolution.](image)

Figure 6-4: 1951 USAF test patterns. This target was used to determine optical system resolution.

The spatial resolution was assumed to be equal to the smallest spacing for which the peak-to-valley ratio of the intensity line profile drawn across image of the test pattern is equal or superior to $\sqrt{2}$ (Figure 6-5).
The spectral resolution, understood here as the bandwidth of the LCTF transmission spectra, was evaluated every 10 nm between 420 nm and 680 nm by recording the light reflected by the Xenon lamp from a spectralon reflectance standard (Labsphere, Inc., USA) through the hyperspectral laparoscope (minus the camera) with a spectrometer (HR4000, Ocean Optics, Inc., USA). The bandwidths were calculated at the Full Width at Half Maximum (FWHM) of the transmission spectra.

Because the principle of measurement of haemoglobin is based on the intensity of the reflected light, it was crucial to ensure the system response was proportional to the sample emitted light and the CCD exposure time (to compare stack of images acquired with different exposure time). The system linearity with sample emission was assessed by recording the light from the standard reflectance target and by inserting neutral density filters of increasing optical density. The linearity with exposure times was checked by acquiring images at increasing exposure times.

6.2.2 Setup considerations for non-invasive intestinal ischaemia diagnosis with hyperspectral imaging

The key element of hyperspectral imaging is a tuneable filter whose band-pass can be electronically tuned. This generates more finely spaced band-pass wavelengths than filter wheels with a lower filter switching time, and without any moving parts. Hyperspectral imaging is implemented by three main technologies: Fourier interferometry, acousto-optic tuneable filter, and liquid crystal tuneable filter (LCTF). A comparative study has demonstrated that even though the Fourier system provides
the highest optical throughput, the LCTF yields the highest signal-to-noise ratio in both fluorescence and spectral imaging [34] (more details section 5.1).

A LCTF is made up of several stages, each of them consisting of a quartz birefringence retarder and layers of liquid crystal, sandwiched between two polarisers. The principle of the LCTF is to select the desired bandpass by adjusting the retardance of the polarized light. The bandpass wavelength can then be selected by modulating the electric field applied to the liquid crystals, thus changing the retardance and therefore the transmission efficiency through the polarisers. The bandpass transmitted by the LCTF is the one whose wavelengths are in phase with the polarisers at each stage.

The LCTF compact dimensions (7.6×7.6×3.1 cm) make the use of the hyperspectral system practical for the surgeon. Additionally, these dimensions allow the hyperspectral system insertion in a sterile plastic sleeve (normally used to isolate the camera of video laparoscope from the patient) which is required as the CCD/LCTF block is non-sterile (Figure 6-6). The hyperspectral laparoscope is introduced in the sheath by one extremity, only the distal end of the laparoscope (sterilised by autoclave) exits the sleeve by a narrow window.

Figure 6-6: Sterile sheath used to isolate laparoscope non-sterile camera and video cables from the patient. The red circle indicates the exit window through which the laparoscope is inserted.
6.3 System characterisation

The peak-to-valley ratios calculated from the intensity profiles of the USAF targets recorded with the hyperspectral laparoscope are presented in Figure 6-7. The limit of $\sqrt{2}$ was reached for the pattern whose line spacing was equal to 56 µm, considered to be the resolution of our system.

Figure 6-7: Evolution of the peak-to-valley ratio with lines spacing of the successive targets.

Figure 6-8: Spectral profiles provided by the LCTF for wavelength set between 420 nm and 660 nm with a 10 nm increment.

Figure 6-8 illustrates the transmission spectra of the hyperspectral system without the camera recorded by the spectrometer for wavelengths tuned with 10 nm increment between 420 nm and 660 nm. The overall increase in the bandwidths with
growing wavelength is apparent and was quantified by taking the spectra FWHM which ranged from 6.3 nm at 420 nm to 28.1 nm at 650 nm (Figure 6-9).

Figure 6-9: LCTF bandwidth evolution with wavelength.

The graphs (Figure 6-10) demonstrate the linearity of the measured intensities with both exposure time (linear regression coefficient $R^2$: 0.998) and sample emission ($R^2$: 0.9917).

Figure 6-10: Linear relationship between measured intensity and (a) exposure time and (b) sample emission.

As the haemoglobin concentration was computed from the measured reflected light, it was crucial to check if the intensity of the recorded signal was linear with the two main factors that affect it: the CCD exposure time, and the sample-emitted light itself. The graphs (Figure 6-10) ensure this is the case so that measured intensities correlated with the haemoglobin concentration and images acquired at different exposure times could be compared.
6.4 Extraction of the haemoglobin maps from the hyperspectral image stack

Generally, oxy and deoxyhaemoglobin concentrations are inferred from the fitting of the measured diffuse reflectance spectra with more or less complex models using oxy and deoxyhaemoglobin as free parameters. The fitted models are broadly classified in two categories: models of light transport as described in section 2.4 like in [36] where a hybrid-P3 model was employed to recover oxygen saturation, or Beer-Lambert law modified to take into account biological scattering. The choice of model depends on the application or the required level of precision on the retrieved haemoglobin parameters (relative or absolute values).

The main challenge in recovering haemoglobin concentration and saturation is to deconvolute absorption (originating mainly from haemoglobin in the visible) from scattering. The precision of the light transport description depends on the inclusion in the models of the scattering effects. They are well described in the numerical or analytical models mentioned in section 2.4, yet they require an exact knowledge of the source detector-geometry making them useful for studies with optical fibre probes but not easily applicable to imaging systems with complex illumination-detection geometry.

In the majority of the studies looking at tissue perfusion, the preferred description of light-tissue interaction is the modified Beer-Lambert law. It is referred as “modified” because its expression is similar to the conventional Beer-Lambert law which describes the transmittance through a transparent medium as a linear combination of the absorption coefficients of the chromophores present in the medium multiplied by a constant pathlength corresponding to the thickness of this medium. However, unlike the conventional Beer-Lambert law, the modified version describes the attenuation of light back-reflected from a turbid sample after multiple scattering events. The light attenuation is related to the reflected light via Equation 6-1 [24, 31, 32].

\[ A = -\log(R) \]  
**Equation 6-1**

The difficulty is to evaluate the distance travelled in tissue by the photons between their emission and their detection. This distance depends on the source-detector geometry (distance between emission and detection fibres, diameter, and numerical aperture), and the photon's wavelength, since scattering is a wavelength-dependent effect. In consequence, the influence of scattering on reflected light attenuation is
two-fold: it affects the magnitude of the light attenuation $A$ and modifies the photon path length with a wavelength dependence which translates in equation as follows [37-39]:

$$A = \varepsilon \cdot C \cdot D(\lambda) + G(\lambda) \quad \text{Equation 6-2}$$

where $\varepsilon$ is the extinction coefficient, $C$ the concentration of the chromophores. As shown in section 2.3.2, the major absorbing chromophore in the visible is haemoglobin in its oxygenated and deoxygenated forms. As a result, $\varepsilon$ is the sum of the oxyhaemoglobin extinction coefficient $\varepsilon_{\text{HbO2}}$ and the deoxyhaemoglobin extinction coefficient $\varepsilon_{\text{Hb}}$ and $C$ the sum of their concentrations. $D$ is usually referred as the mean pathlength and corresponds to the mean distance travelled by photons. $D$ depends on the absorption coefficient, the scattering coefficient and the illumination-detection distance. In order to more precisely highlight the effects of scattering, $D$ is sometimes presented as the product of $DPF$, called the differential pathlength and $x$, the linear source-detection distance. $DPF$ expresses the increase in the distance travelled by photon in scattering media [37]. The factor $G$ models the modification of light attenuation magnitude caused solely by scattering, $G$ depends therefore on the scattering coefficient $\mu_s$ and the source-detector geometry but is independent of the absorption coefficient $\mu_a$ (the product of the excitation coefficient and the concentration).

Most of the studies looking at tissue vascularisation are based on Equation 6-2. It becomes apparent from it that light attenuation is not a linear function of chromophores concentration anymore in turbid media and that knowledge of $D$ and $G$ is required if absolute chromophores concentrations are to be calculated. The estimation of $D$ is complex, but feasible with Monte Carlo simulations if the baseline absorption and scattering coefficients and the source-detector geometry are known. $G$, however, is very difficult to estimate, especially for imaging where the source-detection geometry is not well defined. As a result, various approximations of Equation 6-2 have been applied from the most elaborate ones taking into account the non-linear effects of scattering on $D$ and $G$ [40], to studies modelling scattering as a constant baseline and a slope variable with wavelength [41].

A less refined estimate of Equation 6-2 is to consider $D$ and $G$ wavelength-independent. The latter approximation is reasonable in the visible range [42, 43], the former is more a rough approximation, however, this model, which reduces the effects of scattering to a constant background, was successfully used in several tissue perfusion studies [32, 33, 45] to provide relative values of oxy and deoxy haemoglobin concentration maps. The two models used to process the images
recorded during the uterus transplantation experiment and the pig trial, and chosen for their simplicity and speed, were based on these two approximations.

6.5 Porcine colon vasculature imaging

6.5.1 Haemoglobin phantoms preparation and imaging procedure

Haemoglobin phantoms were imaged to test the hyperspectral imaging system response to haemoglobin concentration and saturation in oxygen. Two series of phantoms at different concentrations and oxygen saturations were prepared in a well plate (Figure 6-11). In each of them, ferrous stabilized human haemoglobin (H0267, Sigma Aldrich, Co., USA) at 0.5, 1, 3, 6 g/L mixed with a solution of phosphate buffer saline were prepared. One group was oxygenated at atmospheric pressure and sodium dithionite, a reducing agent, was added into the second group at a final concentration of 10 g/L to reduce oxyhaemoglobin in its deoxygenated form. In the rest of this section, the phantoms of the first group are referred to as oxyhaemoglobin samples and the ones of the second group are referred as deoxyhaemoglobin samples.

During the pig operation, the hyperspectral laparoscope was used to follow the temporal evolution of the colon’s oxyhaemoglobin and deoxyhaemoglobin concentrations and oxygen saturations during the pig’s termination. It was introduced in the abdomen by a clinician through a trocar and the images were recorded across the visible range from 450 to 680 nm in 10 nm steps or 24 images. Exposure times ranged from 60 ms to 100 ms and the working distance was about 5 cm. The colon was imaged 1 min before the pig’s termination and during 6 min every 1 min after the termination.

6.5.2 Image processing and haemoglobin model

Throughout the experiment, the movement of the instrument and/or the field of view made image registration necessary. In consequence, images were manually registered subsequently to the experiment by applying translations and rotations in order to ensure that all of the spectral points of a given spectrum were from a unique spatial point.

However, the manual registration of a complete image stack consisting of 24 images (and even more so the registration of images from different image stacks),
turned out to be an inextricable task for several reasons. Firstly, the relatively small FOV of the laparoscope meant that even small movement could cause the loss of the ROI on some of the images. Secondly, setup or tissue motion concomitant to the acquisition caused image blurring which, meant that it was not possible to align images due to the loss of sharp features. Finally, rotation and translation are linear transformation which can not correct for soft-tissue deformation of the colon, hence images on which tissue deformation were too significant, could not be accurately registered.

Nevertheless, one particular ROI was found to be consistently appearing on many images throughout the experiment. Using an approach successfully used in a human skin vasculature study [24] and enabling the imaging of haemoglobin parameters from the absorbance at three wavelengths, images at 530 nm, 560 nm, 620 nm were chosen to be aligned as they shared a common ROI with sharp features.

“Brightfield” images from a standard reflectance target (Labsphere, Inc., USA), which reflects isotropically 99% of the light, were acquired to account for the illumination spectrum, instrument response, and illumination non-uniformity. “Darkfield” images were taken with the illumination turned off to take into account for the system dark offset and the room light. The reflectance signal $R$ was generated at each wavelength for the whole image from the sample backreflected signal $S$, the brightfield, $B$, and darkfield, $D$, signals according to this relationship:

$$R(\lambda) = \frac{S(\lambda) - D(\lambda)}{B(\lambda) - D(\lambda)} \quad \text{Equation 6-3}$$

Assuming $D$ and $G$ constant, Equation 6-2 can be written at the three selected wavelengths as follows:

$$A_{530} = -\log( R_{530} ) = \left( \epsilon_{\text{HbO}_2}^{530} \cdot C_{\text{HbO}_2} + \epsilon_{\text{Hb}}^{530} \cdot C_{\text{HbR}} \right) \cdot D + G$$

$$A_{560} = -\log( R_{560} ) = \left( \epsilon_{\text{HbO}_2}^{560} \cdot C_{\text{HbO}_2} + \epsilon_{\text{Hb}}^{560} \cdot C_{\text{HbR}} \right) \cdot D + G \quad \text{Equation 6-4}$$

$$A_{620} = -\log( R_{620} ) = \left( \epsilon_{\text{HbO}_2}^{620} \cdot C_{\text{HbO}_2} + \epsilon_{\text{Hb}}^{620} \cdot C_{\text{HbR}} \right) \cdot D + G$$

with $C_{\text{HbO}_2}$ and $C_{\text{HbR}}$ being the oxyhaemoglobin and deoxyhaemoglobin concentrations respectively. This linear system can be rewritten in function of $C_{\text{HbO}_2}$ and $C_{\text{HbR}}$ which cancel out the geometry factor $G$: 

156
\[ C_{\text{HbO}_2} = \frac{(\epsilon_{\text{Hb}}^{530} - \epsilon_{\text{Hb}}^{620}) \cdot (A_{530} - A_{560}) - (\epsilon_{\text{Hb}}^{530} - \epsilon_{\text{Hb}}^{560}) \cdot (A_{530} - A_{620})}{D \cdot ((\epsilon_{\text{Hb}}^{530} - \epsilon_{\text{Hb}}^{620}) \cdot (\epsilon_{\text{HbO}_2}^{530} - \epsilon_{\text{HbO}_2}^{560}) - (\epsilon_{\text{Hb}}^{530} - \epsilon_{\text{Hb}}^{560}) \cdot (\epsilon_{\text{HbO}_2}^{530} - \epsilon_{\text{HbO}_2}^{620}))} \]

Equation 6-5

\[ C_{\text{HbR}} = \frac{(\epsilon_{\text{HbO}_2}^{530} - \epsilon_{\text{HbO}_2}^{620}) \cdot (A_{530} - A_{560}) - (\epsilon_{\text{HbO}_2}^{530} - \epsilon_{\text{HbO}_2}^{560}) \cdot (A_{530} - A_{620})}{D \cdot ((\epsilon_{\text{HbO}_2}^{530} - \epsilon_{\text{HbO}_2}^{620}) \cdot (\epsilon_{\text{Hb}}^{530} - \epsilon_{\text{Hb}}^{560}) - (\epsilon_{\text{HbO}_2}^{530} - \epsilon_{\text{HbO}_2}^{560}) \cdot (\epsilon_{\text{Hb}}^{530} - \epsilon_{\text{Hb}}^{620}))} \]

Oxyhaemoglobin and deoxyhaemoglobin maps were computed as well as the haemoglobin oxygen saturation map by applying these two formulae at each pixel. The differential pathlength \( D \) is used as a scaling factor in this report. The system and the model were tested on haemoglobin phantoms.

### 6.5.3 Haemoglobin phantoms experiment

As a validation experiment, images from a well plate containing solutions of haemoglobin at different concentrations and oxygen saturations were acquired. The absorption spectra, with a 10 nm spectral resolution, of the 6 g/L deoxyhaemoglobin and oxyhaemoglobin phantoms are presented in Figure 6-11. It can be noticed that the maximum of the deoxyhaemoglobin spectrum was reached at 560 nm; the oxyhaemoglobin spectrum presented two peaks at 540 nm and 570 nm and a secondary minimum at 560 nm. They exhibited the typical shape of deoxyhaemoglobin and oxyhaemoglobin spectra respectively. The deoxyhaemoglobin spectrum comprised a maximum of intensity at 560 nm and the oxyhaemoglobin spectrum included two maxima at 540 nm and 570 nm and a secondary minimum at 560 nm. These values correlated well with [44] (absorbance spectra of oxy and deoxyhaemoglobin with a 2 nm resolution), which reports a maximum in absorption at 556 nm for deoxyhaemoglobin and maxima at 542 nm and 576 nm and a secondary minimum at 560 nm for oxyhaemoglobin. This demonstrates the capacity of this system to recover sample spectral features.
Figure 6-11: Absorption spectrum between 500 nm and 650 nm (spectral resolution: 10 nm) taken at one pixel within the well containing the 6 g/L solutions of (a) deoxyhaemoglobin and (b) oxyhaemoglobin.

The deoxyhaemoglobin and oxyhaemoglobin maps were produced by processing the images acquired at 530 nm, 560 nm and 620 nm (Figure 6-12). On these maps, the intensity of each pixel is equal to the deoxyhaemoglobin or oxyhaemoglobin concentrations. The differential pathlength was arbitrarily set at 1.68 mm to scale the generated concentrations to the real concentrations.

Figure 6-12: (a) Deoxyhaemoglobin and (b) oxyhaemoglobin maps of the well plate containing the haemoglobin phantoms. Dark blue colour corresponds to low concentrations (0 g/L) and dark red colour indicates high concentrations (7 g/L).

It is noticeable from the deoxyhaemoglobin map that the estimated concentration of deoxyhaemoglobin increased from the left to the right in the top row containing the deoxyhaemoglobin samples following the rise in deoxyhaemoglobin concentrations of the prepared samples. This was also the case for the
oxyhaemoglobin samples (bottom row). The oxyhaemoglobin map shows that the estimated concentration of oxyhaemoglobin increased according to the concentration of oxyhaemoglobin of the prepared solutions as expected. An increase in oxyhaemoglobin concentration of the top row samples, (supposed to be deoxygenated by the sodium dithionite), was also observed, and is discussed later in this section.

Then, in order to assess the ability of the system to retrieve the total haemoglobin concentration, the deoxyhaemoglobin map and the oxyhaemoglobin map were summed to produce a “total haemoglobin map”. The concentrations within a rectangle of 40 pixels over 20 pixels were averaged for each of the four wells of the top row.

![Figure 6-13: Comparison of measured haemoglobin concentrations with the real concentrations.](image)

The graph (Figure 6-13) demonstrated a good correlation between estimated and real haemoglobin concentrations. The retrieved mean ± standard deviation concentrations are 0.62±0.24 g/L, 0.90±0.20 g/L, 3.99±0.23 g/L, and 5.94±0.32 g/L, compared to expected concentrations of 0.5 g/L, 1 g/L, 3 g/L, and 6 g/L respectively. Hence, the concentrations were retrieved with a relative error averaged over the four concentrations of 16.5%.

Finally, the oxygen saturation map (Figure 6-14) was created by dividing the oxyhaemoglobin map by the sum of the deoxyhaemoglobin and oxyhaemoglobin maps.
It can be seen from this image (Figure 6-14) that the oxygen saturation was, as expected, lower in the wells of the top row containing the deoxyhaemoglobin samples than in the wells containing the oxyhaemoglobin solutions (bottom row). The oxygen saturation was averaged for each of the eight wells in the same way as described above. It increased from 7% to 27% for the deoxyhaemoglobin samples and was contained between 62% and 71% for the oxyhaemoglobin samples.

A rise in deoxyhaemoglobin concentration of the oxyhaemoglobin samples was also observed on the deoxyhaemoglobin map (Figure 6-12-a) as well as an augmentation of oxyhaemoglobin concentration of the deoxyhaemoglobin samples on the oxyhaemoglobin map (Figure 6-12-b). These observations have to be correlated with the oxygen saturation map (Figure 6-13). Indeed, the fact that the oxygen saturation remained low and stable for the 0.5 g/L and 1 g/L deoxyhaemoglobin samples (7% and 5% respectively) and then increased to 17% and 26% for the 3 g/L and 6 g/L samples respectively, suggests that the 10 g/L solution of sodium dithionite reduced all the oxyhaemoglobin in the 0.5 g/L and 1 g/L deoxyhaemoglobin samples, but was not in sufficient concentration to reduce the whole oxyhaemoglobin in the 3 g/L and 6 g/L solutions. The oxygen saturation of the oxyhaemoglobin samples were found to be contained between 62 % and 73% (Figure 6-14). This means that they are not fully oxygenated, which explains the presence of deoxyhaemoglobin in the oxyhaemoglobin samples (Figure 6-12-a). There was no dissolved oxygen sensor (a device which probes the partial pressure of dissolved oxygen in solution and converts into haemoglobin oxygen saturation with the Hill equation) at disposal to check the oxygen saturation values.

Thus, the hyperspectral imaging system presented here is capable of reconstructing images where the contrast comes from the difference in haemoglobin concentration. Regions with different haemoglobin concentration and oxygen saturation could be clearly visualised.
6.5.4 In vivo imaging of colon vasculature

Subsequent to validation on phantoms, the hyperspectral laparoscope was moved to the operating theatre and in vivo imaging of a pig’s colon vasculature were acquired according to the procedure detailed section 6.5.1, instantly after euthanasia was induced.

The maps, illustrated in Figure 6-15, provide the relative deoxyhaemoglobin and oxyhaemoglobin values 6 min after the pig’s termination. These maps cover a 550*800 pixels area, which was only a portion of the 1024*768 pixel images recorded. This region of interest was chosen because of its important blood vessel density, whose sharp features were helpful for image alignment. The rest of the imaged surgical scene could not be aligned mainly due to the absence of landmarks. As expected, the deoxyhaemoglobin concentration values were found to be higher than the oxyhaemoglobin as the mean deoxyhaemoglobin concentration was 4.9 times higher than the mean oxyhaemoglobin concentration. Both maps also showed that the total haemoglobin concentration was significantly higher in blood vessels than in the surrounding perfused tissue.

![Figure 6-15: (a) Deoxyhaemoglobin and (b) oxyhaemoglobin maps of a region of interest (approximate dimensions: 3.5×5cm) of the porcine colon 6 min after termination. Blue colour indicates low concentrations and red colour indicates high concentrations.](image)

The temporal evolution of the oxyhaemoglobin and deoxyhaemoglobin concentrations alongside the oxygen saturation after the pig’s termination was monitored. The image registration, already difficult within a stack of images acquired at a given time turned out to be more challenging for several stacks of images each acquired at different times subsequently to the pig's termination. This led to a reduction of the size of the studied region of interest from 550*800 pixels area to a 100*50 area to ensure that the same area could be tracked and aligned for the totality of the images. The pixel intensities within this region were averaged and processed to retrieve the haemoglobin concentrations and saturation.
Figure 6-16: Temporal evolution of oxy and deoxyhaemoglobin concentrations and oxygen saturation subsequent to pig’s termination.

The graph, Figure 6-16, underlines the clear fall in oxyhaemoglobin concentration, together with the oxygen saturation which dropped by 77% and 64%, respectively, between the pig’s termination and 6 min after termination. Deoxyhaemoglobin concentration slowly rose by 17.4%. It is important to note that the values indicated on the y-axis are not absolute but relative values. No explanation was found as for the concomitant slight increase of oxyhaemoglobin and the slight decrease of deoxyhaemoglobin between 5 min and 6 min post-termination.

Hyperspectral studies of organs perfusion have already been performed in the past, but none of them in real surgical conditions. In [32] and [45], the perfusion and oxygenation of a resected and arrested heart and a hand respectively were monitored throughout an induced ischemic event. In [34], the colon ischemic area localization was realised in vivo during open surgery, cumbersome flood illuminators were used in the three studies. This work is hence the first to our knowledge to image haemoglobin concentration and oxygen saturation in real MIS conditions endoscopically. The goal and focus were to assemble a MIS hyperspectral system capable of providing real-time information on the tissue vasculature. The model of haemoglobin parameters extraction we used was simple and did not take into account the distorting non-linear effect of scattering on absorbance values. However, it supplied satisfactory results as demonstrated by the phantom experiments and the pig’s trial. The major difficulty in this work was the compensation of the surgical scene movement. This problem was not addressed in the aforementioned studies as the imaged scenes were steady. For example, an isolated beating heart was
hyperspectrally imaged in [32] and motion artefacts were reduced by synchronising image acquisition with the heart cycle. However, this approach increases the acquisition to 4 min and it only corrects for the movement of the heart, yet motion artefacts can also be caused by the setup and/or the clinician movements.

The manual registration in this work turned out to be a very tedious and time consuming task, several of the images being blurred because of motion during the acquisition, which made the tracking of features difficult. Moreover, the program did not permit accounting for tissue deformation as merely translation and rotation could be applied.

Noting the absence of efficient method for registration of hyperspectral images recorded in vivo from organs in motion, Dr. Neil Clancy (Research associate in the biophotonics) is currently developing a trinocular laparoscope integrating the LCTF/black and white camera block presented in Figure 6-2 for hyperspectral imaging in addition to two other channels with colour cameras for stereo vision. The detail of the system is presented in Figure 6-17. It is based on a three-channel laparoscope (Intuitive Surgical, Inc., USA). Beams coming out from two channels were focussed onto two colours CCD cameras (model uEye 2230-C, IDS Imaging, GmbH, Germany) for colour stereo viewing. Light from the third channel was reflected at 45° by a mirror, filtered at chosen wavelengths by the LCTF and imaged on a monochrome CCD camera (model DCU 223M, Thorlabs, Inc; USA). The light source and light guide were the same models than those described for the hyperspectral laparoscope presented earlier in this chapter. The simultaneous acquisition of the pair of stereo colour images and the spectrally resolved image of the same FOV was controlled with a LabVIEW code incorporating the program detailed in section 6.2.1.
It was possible from this set of three images to automatically register the hyperspectral images using techniques stemming from computer vision and developed for 3D reconstruction from stereo images and motion tracking. The details of the registration procedure are available in [47].

In summary, a hyperspectral laparoscope was built, characterized, validated with haemoglobin phantoms, and used during a MIS procedure during which, in spite of motion artefacts, the decrease in oxyhaemoglobin concentration and oxygen saturation alongside an increase in deoxyhaemoglobin concentration subsequent to pig’s termination was observed.
6.6 Transplanted uterine vasculature follow-up

The data collection for this experiment was performed in collaboration with Dr. Neil Clancy, research associate in the Hamlyn Centre who then processed the results. The rationale for its discussion here is to provide an example of vasculature study where the scene to be imaged remained steady enough during the acquisition of the hyperspectral cube to use the totality of the images constituting it, as opposed to the previous experiment.

6.6.1 Image processing and haemoglobin model

The uterus to be transplanted was imaged before removal from the donor rabbit which underwent a laparotomy and after transplantation on the recipient rabbit. 29 images were recorded between 440 nm and 720 nm in 10 nm step. The exposure time was set at 250 ms and the working distance was 15 cm.

In order to correct for the motion artefacts (considerably less pronounced than in the pig’s study) caused by breathing and tissue deformation, an algorithm detecting and tracking features was applied on the images to align them. Details of this algorithm are beyond the scope of this thesis and can be found in [47]. After registration, the reflectance was converted into absorbance according to Equation 6-1. The absorbances were modelled according to Equation 6-2 where $D$, the mean pathlength, and $G$, the geometry factor, were supposed constant with wavelength.

$D$ was incorporated into the oxy and deoxyhaemoglobin concentrations to form the free parameters of a multivariate fitting procedure applied at each pixel to determine the best set of combinations of haemoglobin and deoxyhaemoglobin concentrations minimizing the differences between the measured absorbance spectra made up of 29 points and the linear combination of pure oxy and deoxyhaemoglobin absorption spectra as measured in [44]. Oxy and deoxyhaemoglobin concentration factors (the product of the concentrations with $D$) retrieved at each pixel were used to generate oxygen saturation maps of the uterus before and after transplantation.

6.6.2 Uterus transplant oxygen saturation imaging

Figure 6-18 presents the colour images (computed from the hyperspectral images with a RGB filter) together with their corresponding saturation maps of the uterus pre
and post transplantation. The oxygen saturation map of the pre-transplant demonstrated a well-oxygenated organ with a mean oxygenation calculated at 60±3 %.

The non-uniformity of the oxygen saturation, with for instance a lower oxygenated area in the centre, originates from the inhomogeneous vein and artery density or a less vascularised region. Oxygen saturation map, processed from a hyperspectral image stack acquired immediately after aorta and inferior vena cava re-anastomosis, demonstrated a successful anastomosis as highlighted by the high oxygen saturation regions close to the region where aorta was re-connected (indicated by the black circle Figure 6-18-d). The mean oxygen saturation was found to be 48±6 %. Values of oxygen saturations lower in post-transplant than in pre-transplant were also measured with pulse oximeters in previous operations performed by our collaborators.

![Figure 6-18: (a) RGB image of the uterus in the donor rabbit, (b) corresponding oxygen saturation map, (c) RGB image of the uterus shortly after the transplantation in the recipient rabbit, (d) corresponding oxygen saturation map. The black circle indicates (d) the approximate localisation of the aorta. Colour scale varies between dark blue (0) and dark red (1).](image)

In summary, hyperspectral imaging could be an efficient tool to temporally and spatially follow tissue reoxygenation subsequent to re-anastomosis. Blood flow is gradually re-established in the uterus vessel network consisting of: aorta, uterine
arterial tree, internal iliac artery, uterine arterial and venous trees, and inferior vena cava.

6.7 Conclusion

The system built in this chapter is in its principle similar to the laparoscopic system described in chapter 5: it records spectrally resolved images. However, the nature of the detected signal is reflectance whose intensity levels are higher than fluorescence which means that standard CCD camera and (low transmission) LCTF could be used instead of cooled CCD camera and filter wheel respectively. This leads to a more compact instrument with a much higher spectral resolution. The system spectral and spatial performance was characterized, its linear response with exposure time and sample emission was verified, and its ability to recover haemoglobin parameters were successfully tested on phantoms. The decrease of oxygen saturation subseuent to the pig’s termination was highlighted in spite of the simplicity of the haemoglobin model used, and important motions artefacts that limited the number of useable images and, as a result, decreased the available spectral information. In the uterus transplantation, the minor movement of the surgical scene could be corrected to permit the registration of the complete stack of images. The oxygen saturation maps of the pre and post-transplant could this time be generated from spectral information over the entire visible range, and allowed visualising a highly close region by the aorta/uterus junction sign of successful re-anastomosis of bloods vessels.

Just like the rest of this work, the goal of the work presented in this chapter was to translate biophotonics techniques into operating theatres, therefore the emphasis was given to system development rather than complex haemoglobin model elaboration. The adaptation of hyperspectral imaging to laparoscopes was a step in this direction, yet motion artefacts correction will need to be addressed if this technology is to be used during MIS procedures. The triple endoscope currently in development could tackle this challenge.
6.8 References


Chapter 7

Conclusion

7.1 Thesis accomplishments and limitations

The subject of this thesis was the use of biophotonics techniques to probe tissue and detect lesions. Its purpose was three-fold: (1) enlarge the spectrum of pathologies diagnosable with biophotonics techniques by demonstrating the suitability of the fluorescence spectroscopy for NAFLD diagnosis, (2) combine two biophotonics modalities, fluorescence and diffuse reflectance to improve diagnostic accuracy (3) propose solutions to move fluorescence/reflectance spectroscopy and imaging techniques from the optical bench to the operating theatre to help the diagnosis of a range of pathologies during MIS procedures.

The first goal was reached by performing a fluorescence spectroscopy study of NAFLD in the mouse model in two trials presented in chapter 3, which established its clinical utility for NAFLD diagnosis. In particular, in the first study, fluorescence spectroscopy performed better than the blood tests samples including ALT tests, a diagnosis method already in use in hospitals. While this study constitutes strong evidence in favour of the use of fluorescence spectroscopy for NAFLD detection, the disease model involved NAFLD mice only where the distinction between disease states was unambiguous. In practice, NAFLD severity may need to be distinguished from other inflammatory conditions and from varying degrees of cirrhosis or fibrosis. A follow-up study on a large number of human liver samples is needed to further ascertain the fluorescence spectroscopy performance for NAFLD diagnosis. The addition of the second laser diode, which in this study did not result in better discrimination, might help to improve the classification accuracy when looking at more ambiguous samples.

The second objective was addressed in the context of a fluorescence/reflectance spectroscopy study of lesions in the digestive tract presented in chapter 4. The comparison of the usual diagnostic performance variables (sensitivity, specificity, PPV and NPV) obtained from single modality spectra with the ones obtained with concatenated fluorescence and reflectance spectra, showed that, in some instances, the combined modality approach led to
better diagnosis results. A common weakness of the spectroscopic studies is their retrospective nature where training sets and samples to classify are mixed up, leading to a biased classification [1]. The K-fold cross-validation, where the classifier was trained on all the spectra, except the ones to be classified, ensured the classification was performed in an unbiased manner like in a prospective study. As stated above, studies performed in chapter 3 and chapter 4 included a small number of samples mainly because of time constraints. More specimens are needed not only to ascertain the clinical viability of fluorescence and reflectance spectroscopy, but also to avoid the curse of dimensionality mentioned in section 4.3.3 which arises when the number of classification features is largely higher than the number of samples, and which can result in a degraded classification.

Furthermore, 375 nm and 405 nm might not be the optimal excitation wavelengths to discriminate the pathologies considered here. Further investigation should be conducted with a wavelength tuneable laser for instance to select the laser diodes emitting at the most favourable wavelengths.

The third and last aim was attained by building optical imaging laparoscopes to allow intraoperative fluorescence/reflectance imaging. With the hardware currently available, it is possible to design light and compact spectrally resolved fluorescence and reflectance imaging systems. Our approach to adapt fluorescence and reflectance imaging to intraoperative diagnosis was simple; it consisted in mounting a camera/filter unit onto a laparoscope to make fluorescence and reflectance imaging a de facto useable MIS procedure. To conduct the two imaging studies presented in chapter 5, a cooled CCD camera and a filter wheel were employed. The first experiment was a combined fluorescence/reflectance study originally aimed at locating oesophageal tumours. However, the limited supply of tissue for these two experiments resulted on the impossibility to image a suitable oesophagus specimen. Emission filters were chosen based on the optimal wavelengths selected following the spectroscopic experiment conducted in chapter 4. The laparoscopic system was successfully tested on fluorescent phantoms including some endogenous fluorophores and samples of the nerve contrast agent dye. We hope to demonstrate the efficiency of our system at demarcating tumour in the oesophagus in the future. Of note, we acknowledge that tumour detection in a hollow organ like the oesophagus is done in practice with a flexible endoscope and not a rigid laparoscope, yet the oesophagus was the only organ from which we could hope to obtain tissue samples (for the spectroscopic study) and specimens (for the imaging study) at the time of the study. However, our approach would be applicable to cancer detection in solid organs present in the abdomen that would normally be observed
with laparoscopes such as liver or pancreas. In addition, the filter/CCD unit would be easily adaptable to flexible endoscopes to investigate oesophagus or any other hollow organs.

In the second study, which was on nerve fluorescence imaging, filters were selected according to the excitation and emission filters of the exogenous nerve contrast agent. The nerve imaging study concluded that the cause of the absence of contrast was likely to originate from an insufficient dose. This dye is one of the last iteration of a family of nerve contrast agents under development in a research team of General Electric Global Research. The optimal dose was found to vary greatly between the successive versions of the dye and no analysis looked specifically at the dose needed to be injected in a pig to obtain contrast at the time of our experiment. Such a study is a requisite for future fluorescence imaging studies. Fluorescence laparoscopy is expected to be of great help to the surgeon in order to assist in avoiding nerve tracts during surgery.

The hyperspectral reflectance laparoscope (chapter 6) was similar in its concept to the previous setup, yet different in its implementation. Reflectance signal levels being significantly higher than the fluorescence ones, the filter wheel could be substituted for a LCTF, a filtering element with an optical throughput lower than traditional filters, but much more versatile, and a traditional CCD camera replaced the cooled CCD camera. The objective of the first experiment we presented was to evaluate the hyperspectral laparoscope as an intraoperative ischaemia diagnosis tool. Tests carried out on haemoglobin phantoms proved that the hyperspectral laparoscope could probe haemoglobin concentrations and oxygen saturation. Results during a porcine trial exemplify the effectiveness of the system at monitoring in vivo the temporal evolution of haemoglobin concentration and oxygen saturation of a region of interest of the animal’s intestine after its termination. The disease model of ischaemia in this study was imperfect for three reasons: the exact time of the pig’s death is unknown, the kinetics and the magnitude of the haemoglobin concentration and oxygen saturation evolutions subsequent to the death are not well-known, a pulse-oximeter could not be used as a control measurement because the pig’s heart was not beating anymore after its death. A more realistic and convenient model, for which we did not have ethics approval at the time of the study, would have been to clamp a blood vessel supplying the intestine such as the mesenteric artery.

One of the major challenges of this experiment and of in vivo study in general, is to account for motion artefacts by registering images. In our study, we managed to correctly align images at only one temporal point to produce maps of oxy-haemoglobin and deoxyhaemoglobin concentrations. Devices such as the trinocular
described in chapter 6 appear to be a more realistic approach for intraoperative imaging of moving scenes. The strength of this system and the associated image registration algorithm lies in the possibility to track features on the bright, sharp colour images to then automatically align the dark (due to low LCTF transmission) and motion blurred (caused by long acquisition times) hyperspectral images. Nevertheless, several obstacles have to be overcome if this technique is to be employed intraoperatively: the size and the weight of the trinocular will need to be reduced or used robotically (in combination with the robotic arm of platforms such as the daVinci Surgical Workstation), the current setup being difficult to manipulate at the moment even with the assistance of an articulated arm; the registration technique will need to be real-time which is not the case presently (images are aligned offline); the acquisition duration will have to be shortened to make the system truly real-time. Finally, the acquisition in \textit{in vivo} condition of a complete image set i.e an hyperspectral image and its corresponding pair of colour images takes in average 1 s (mainly because of the light throughput of the hyperspectral channel) in the current system configuration. The shortening of the acquisition procedure would also attenuate motion artefacts.

Providing that they are reduced in size, snapshot camera systems could also represent an excellent solution for intraoperative imaging with the apparition of sensors of increasing size and resolution owing to the possibility to acquire several spectral images in one shot (see section 5.1 for more details). Another ischaemia study on a rabbit uterus transplant demonstrated the hyperspectral imaging laparoscope observing tissue oxygen saturation. The oxygen saturation map processed from the hyperspectral image stack suggested a successful uterus anastomosis in the recipient rabbit. An improvement of this study and the porcine intestine work would consist in developing a more advanced model of the light reflected from vascularised tissue which would take more precisely into account the effects of scattering.

In spite of these limitations, this work showed that (1) fluorescence spectroscopy is a promising tool for NAFLD diagnosis (2) combining fluorescence and reflectance spectroscopy can, in some instances, increase the performance of pathologies diagnosis in the digestive tract (3) optical imaging laparoscopic systems are useful devices for diagnostic imaging during MIS procedures for non or moderately moving scenes. In the rest of the conclusion, we briefly discuss the clinical role of fluorescence and reflectance modalities and their diffusion in hospitals.

Also presented in this thesis (chapter 3), was the manipulation of the fluorescence spectroscopic probe with a robotic endoscope. Fluorescence and
reflectance probes have already been used in vivo, their small diameter permitting their introduction through the working channel of endoscopes. By doing so, clinicians have a limited control on the probe trajectory as well as on the pressure applied by the probe on the tissue surface. While the latter is easily controllable ex vivo, it is not anymore the case in vivo (with the probe inserted in the working channel) where there is a possibility, in the absence of haptic feedback, that the surgeon pushes too hard on the probe to ensure it contacts the tissue surface. The section 3.6.6 showed how the force adaptive robotic endoscope promises to solve these difficulties by authorizing the scanning of the probe on the tissue surface with controlled trajectory and applied pressure. Force sensitive robotic endoscope hence enhances the practicability of intraoperative measurements with spectroscopic probes. To be fully tested, this set up will need to be assessed in real intraoperative conditions in the future.

7.2 Clinical translation

Large-scale human trials are still needed to formulate a definitive answer to the impact of fluorescence and reflectance spectroscopy and imaging on diagnostic procedures. At a minimum, it seems reasonable to think that, in combination with algorithms such as SMLR, they could efficiently complement biopsies by signalling suspicious tissue regions. With a protocol including fluorescence and/or diffuse reflectance screening performed prior to biopsies, more measurements could be taken non-invasively which would decrease the miss rate as well as the number of unnecessary biopsies. Spectra could be then analyzed with algorithms like SMLR and only sites with posterior probability of belonging to a disease class above a chosen threshold would undergo biopsies and histological analysis. The time taken to perform the additional fluorescence-diffuse reflectance measurements would hence be largely regained by diminishing the number of histological examinations.

Besides their intrinsic diagnostic power, the great strength of fluorescence and diffuse reflectance and biophotonics techniques in general is their adaptability to other healthcare technologies (they could easily be integrated in robotic surgery platform for example) or diagnostic methods. We have already mentioned that biophotonics techniques can be used together with biopsies (as explained in the paragraph above), but they could also complement the established clinical imaging modalities. For instance, a hybrid MRI-diffuse reflectance platform was built for breast cancer detection [2]. Tumour masses were localized with MR images to guide the spectroscopic measurements from which oxyhaemoglobin and haemoglobin
concentrations were retrieved. The impact of such an approach in terms of diagnostic accuracy was not assessed, but the addition of information from each modality is likely to improve it.

When looking at the clinical translation of a new technology, its benefits can not be considered separately from its cost, which includes the cost of equipment, the cost of the medical staff time (training and procedure lengths) and the (potential) savings it permits (savings spawned from an earlier disease detection exempting from further treatment for instance) [3, 4]. While it is premature to answer these latter issues, we foresee that expenditure on hardware and training will remain moderate. Table 7-1 shows the price of the four systems designed during the thesis. They are calculated from the sum of the market price of components they incorporate. The price of the more expensive system, the fluorescence-diffuse reflectance spectroscopy setup does not exceed £25,000 (a price that could certainly be decreased by choosing a lower cost spectrograph/CCD unit which account for 75% of the price) whilst the minimum price of the routinely used imaging modalities (MRI, CT, PET) is estimated at £65,000 [5].

Table 7-1: Cost of the four optical systems designed in this thesis.

<table>
<thead>
<tr>
<th>Setup</th>
<th>Dual-excitation fluorescence spectroscopy system</th>
<th>Fluorescence-diffuse reflectance spectroscopy system</th>
<th>Filter wheel laparoscope</th>
<th>LCTF laparoscope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Price (£)</td>
<td>22,820</td>
<td>23,050</td>
<td>17,700</td>
<td>17,200</td>
</tr>
</tbody>
</table>

The straightforward integration of biophotonics into existing clinical instruments suggests that they may not require modification of present clinical procedures [6], and hence will not necessitate lengthy training. The acquisition of additional measurements will nonetheless lead to longer examination time even if biophotonics techniques are fast. However, as stated above, the time saved by decreasing the number of needless biopsies (which then undergo lengthy histological preparation) largely compensates the time lost at taking these data. A study involving 100 patients and including all these cost considerations found that the addition of fluorescence spectroscopy to the traditionally used colposcopes for cervix precancer diagnosis enabled a reduction in the miss rate and also the cost of diagnosis [4].

In conclusion, we hope this thesis has demonstrated the value of fluorescence and diffuse reflectance modalities for probing tissue states and we believe that these could play an important role in standard clinical procedures for
pathologies screening and diagnosis as a stand alone modality or in combination with others.
7.3 References


