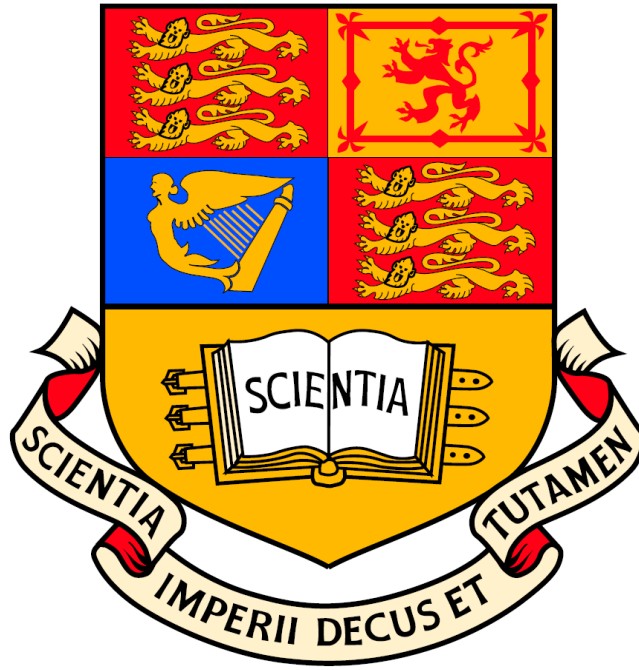




Developing endoscopic instrumentation and techniques for *in vivo* fluorescence lifetime imaging and spectroscopy

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Abstract

Confocal fluorescence endomicroscopes employ fibre optics along with miniaturised scanning and focussing mechanisms to allow microscopic investigation of remote tissue samples with sub-cellular resolution. For this reason they are widely used in biomedical research, both in clinical studies and in small animal imaging experiments. Fluorescence lifetime imaging microscopy (FLIM) has been shown to provide contrast between normal and unhealthy tissue in several diseases including gastrointestinal (GI) cancer. As such, there is significant interest in developing instrumentation that will allow endoscopic confocal FLIM as this would permit the *in vivo* investigation of human GI tissue.

This thesis describes the development and use of several instruments and techniques aimed at clinically viable *in vivo* fluorescence lifetime spectroscopy and confocal endomicroscopy. This research has consisted of two broad branches: the study of the fluorescence signature of healthy and diseased tissue both *ex vivo* and *in vivo*; and the development of a novel method for achieving beam scanning in confocal endomicroscopy.

Firstly the tissue studies are discussed. This begins with the application of a compact steady-state diffuse reflectance/fluorescence spectrometer and a fibre-optic-coupled time-resolved spectrofluorometer to an *in vivo* investigation of the spectral signatures of skin cancer. This study – which involved the interrogation of 27 clinically diagnosed lesions – was carried out in collaboration with researchers at Lund University in Sweden and revealed significant differences between healthy and diseased tissue both in terms of fluorescence lifetime and steady state reflectance and fluorescence spectra.

Further to this study, work is presented charting the development of a clinically viable spectrometer, which measures time-resolved fluorescence spectra with two excitation wavelengths (375 nm and 435 nm) as well as diffuse reflectance spectra. The entire system is contained within a compact trolley (120 x 70 x 55 cm) for easy transportation and safe use in a clinic. It utilises a fibre optic probe to deliver/collect light that can be inserted into the working channel of a medical endoscope meaning that the system can be used to measure diffuse reflectance and time-resolved fluorescence spectra in the GI tract *in vivo*. The development and testing of this system are discussed and data are presented from both *ex vivo* and *in vivo* studies of GI cancer.

The second broad section of this thesis focuses more closely on confocal endomicroscopy. Firstly current methods used in this field are discussed and the sources of several drawbacks are

explained. A novel approach to laser scanning endomicroscopy is then presented, which requires no moving parts and can be implemented without the need for any distal scanners or optics. This technique is similar in concept to the use of adaptive optics to focus through turbid media: it utilises a proximal spatial light modulator to correct for phase variations across a fibre imaging bundle and then to encode for arbitrary wavefronts at the distal end of that fibre bundle. Thus, it is possible to realise both focussing and beam scanning at the output of the fibre bundle with no distal components, permitting extremely compact endoscopic probes to be developed. Proof-of-principle results are presented illustrating the imaging capabilities of this novel system as well as simulations showing the achievable resolution and field of view in several feasible endoscopic configurations.

Overall, this thesis contains work from two quite different projects both aimed at developing novel optical techniques for clinical diagnostic use in endoscopic procedures. The first is aimed at investigating the temporal and spectral properties of the fluorescence and reflectance signatures of cancer, while the goal of the second is to develop improved confocal endomicroscopes.

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Author declaration

All the work presented in this thesis is my own with the following exceptions:

- The development of the spectrometers used in the skin cancer study presented in chapter 4. The diffuse reflectance and steady state fluorescence spectrometer was developed by Mikkel Brydegaard of Lund University in Sweden while the time-resolved instrument was built at Imperial College London by Pieter De Beule.
- Data collection for the skin cancer study discussed in chapter 4 was performed in collaboration with a number of researchers from both Imperial College London and Lund University (Sweden).
- In the investigation of gastro-intestinal diseases presented in chapter 5, collection of data was performed in conjunction with Dr. Sergio Coda and Sister Kim Roche.

To my dad

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List of abbreviations

AFI	Autofluorescence imaging
BCC	Basal cell carcinoma
BS	Beam splitter
CCD	Charge-coupled device
CW	Continuous wave
DA	Discriminant analysis
DAPI	4', 6-diamidino-2-phenylindole
DASPI	2-(p-dimethylaminostyryl)-pyridylmethyl iodide
DCF	Double-clad fibre
DCPCF	Double-clad photonic crystal fibre
ESS	Elastic scattering spectroscopy
FAD/FADH ₂	Oxidised/reduced form of flavin adenine dinucleotide
FFT	Fast Fourier transform
FLC	Ferroelectric liquid crystal
FLIM	Fluorescence lifetime imaging microscopy
FRET	Förster resonance energy transfer
FWHM	Full width at half maximum
GI	Gastro-intestinal
GOI	Gated optical intensifier
GVD	Group velocity dispersion
HDE	High definition endoscopy

HP	Hydroxylysyl pyridinoline
IBD	Inflammatory bowel disease
IR	Infra-red
IRF	Instrument response function
LC	Liquid crystal
LDA	Linear discriminant analysis
LED	Light emitting diode
LP	Lysyl pyridinoline
MCE	Magnifying chromoendoscopy
MPE	Maximum permissible exposure
NA	Numerical aperture
$\text{NAD(P)}^+/\text{NAD(P)H}$	Oxidised/reduced form of nicotinamide adenine dinucleotide (phosphate)
NBI	Narrow band imaging
ND	Neutral density
NMSC	Non-melanoma skin cancer
OCT	Optical coherence tomography
PBS	Polarising beam splitter
PCA	Principal component analysis
PCF	Photonic crystal fibre
PD	Photodiode
PMT	Photomultiplier tube
PpIX	Protoporphyrin IX
PSF	Point spread function

SCC	Squamous cell carcinoma
SHG	Second harmonic generation
SLM	Spatial light modulator
SMF	Single mode fibre
SNR	Signal-to-noise ratio
SPM	Self-phase modulation
SVD	Singular value decomposition
TAC	Time-to-amplitude converter
TCSPC	Time correlated single photon counting
THG	Third harmonic generation
TRANES	Time-resolved area normalised emission spectroscopy
TRES	Time-resolved emission spectroscopy
TRFS	Time-resolved fluorescence spectroscopy
TTL	Transistor-transistor logic
USAF	United States Air Force
UV	Ultra-violet
WLE	White light endoscopy
5-ALA	5-aminolevulinic acid

Chapter 1: Thesis overview

This thesis concerns the development and application of optical instrumentation and methodologies for medical diagnosis. The research presented is separated into two distinct branches, which are both focussed on this central theme and which both utilise fibre-optic technology for *in vivo* applications. The first has involved the development and use of instrumentation – designed for single point measurements of diffuse reflectance and time-resolved autofluorescence spectra of biological tissue – that has been applied to *ex vivo* and *in vivo* clinical studies of skin cancer and gastro-intestinal cancer. The second area of research has involved experiments aimed at developing an ultracompact endomicroscope intended to provide depth-resolved images with sub-cellular resolution. Specifically this has entailed the development of a novel technique (based on adaptive optics) to permit beam focussing and scanning in confocal and multiphoton endomicroscopes. Ultimately this technology is also intended to facilitate *in vivo* studies of autofluorescence for clinical diagnosis. As such, while these branches of research appear quite distinct, both are centred on the same broad theme of developing novel optical instruments for medical applications.

The following two chapters act as an introduction and discuss the main concepts contained within this thesis. **Chapter 2** introduces fluorescence, firstly providing a description of the general phenomenon and then also discussing some relevant properties of fluorescence emission. Finally, the endogenous fluorophores found in biological tissue – which are relevant to the point spectroscopy studies presented later in this thesis – are also described. **Chapter 3** then discusses optical techniques including spectroscopy, microscopy and endoscopy, with an important subset of the latter being endomicroscopy. Spectroscopy and endomicroscopy are the most important techniques within the scope of the work presented in this thesis, while microscopy is discussed mainly to bestow some context upon the reader.

Chapters 4 and 5 then document the development and clinical application of single point fibre-optic probes for diffuse reflectance and time-resolved autofluorescence spectroscopy. **Chapter 4** describes the application of two optical spectrometers to an *in vivo* study of skin cancer, carried out in collaboration with researchers and clinicians from Lund University in Sweden. The first spectrometer provided measurements of diffuse reflectance and steady state fluorescence spectra (with three excitation wavelengths) while the second was a time-resolved fluorescence spectrometer recording fluorescence decay profiles in 16 spectral channels with two excitation wavelengths. Considerable analysis of the data is presented and the results show significant contrast

between healthy and diseased tissue in terms of both their spectral properties and fluorescence lifetime.

Chapter 5 describes the subsequent development of a more compact and clinically practical spectrofluorometer that combined the functionality of the two instruments used in **chapter 4**. The development of this new system is discussed in detail and results are presented from both *ex vivo* and *in vivo* studies investigating the optical signatures of gastro-intestinal disease.

The remainder of the thesis then focuses on the novel endomicroscopy technique mentioned above, presenting a first demonstration and discussing its ongoing and potential future development. **Chapter 6** introduces the concept and presents proof-of-principle results demonstrating focussing, scanning and imaging (with no distal optics) using this technique. The novel approach is also compared to conventional methods and the potential advantages are discussed. **Chapter 7** presents further work on this project involving both numerical simulations of the distal beam profile and experimental progress toward the aim of achieving multiphoton fluorescence endomicroscopy using this methodology. The future direction of this experiment is addressed in both chapters and this involves discussion of the steps required to develop a functioning prototype endomicroscope.

Finally, **chapter 8** summarises the research put forward in this thesis and conclusions are drawn regarding the experimental results presented. Additionally, potential future research – which could be carried out as a consequence of the work presented herein – is suggested and discussed.

Chapter 2: Introduction to fluorescence

This thesis is concerned with the development and use of endoscopic techniques that enable *in vivo* measurements of the optical signatures of human tissue. Most of the results and analysis presented in later chapters focus on fluorescence, although some work is also centred on reflectance spectroscopy. This chapter provides an introduction to fluorescence as this is the key optical signal presented in this thesis. Despite this, much of the discussion of the quantification of fluorescence emission (section 2.2) is also applicable to other optical signatures, including reflectance, and this is indicated where appropriate.

2.1 Fluorescence

Luminescence – which encompasses both fluorescence and phosphorescence – is the emission of light from a substance, corresponding to the decay of an electron from an excited state of an atom or molecule. Fluorescence is specifically the radiative transition between two electronic states of the same spin multiplicity. This typically means that one of two ‘paired’ electrons (having opposite spin) sits in an excited state while the other remains in the ground state. The decay of the excited electron to the ground state is permitted as the spins are balanced (i.e. the total spin, S , is equal to zero). In this example case the spin multiplicity ($M_s = 2S + 1$) is equal to one and both the excited and ground orbitals are singlet states. Fluorescence most often occurs due to transitions like this where an electron decays from the first excited singlet state (S_1) to the ground state (S_0), however, other fluorescence transitions also exist and these include decays from higher excited singlet states (e.g. S_2).

Phosphorescence, on the other hand, involves transitions between states of different spin multiplicity, for example triplet-singlet state transitions. In a triplet state the excited and ground state electrons are unpaired and have the same spin orientation. As such, the decay of the excited electron to the ground state requires a spin reversal. This transition is forbidden and, hence, triplet states are considerably more stable than excited singlet states meaning that phosphorescence lifetimes are significantly longer than fluorescence lifetimes [1-3]. An example Jablonski diagram is presented in figure 2.1, which shows the processes of fluorescence and phosphorescence along with other possible transitions in a typical fluorescent molecule.

For fluorescence to occur, an electron must first be promoted to an excited state. Such excitation is usually caused by the absorption of light and this is indicated in figure 2.1. As shown in the figure, excitation can be caused by the absorption of a single photon of the appropriate energy (i.e. wavelength) or by the simultaneous absorption of several photons. In the figure this is illustrated as the absorption of two photons of identical energy (half the band gap energy) but it could also occur with three or more photons of differing energy. This multiphoton absorption is a non-linear effect as it requires the simultaneous arrival of two or more photons. In the case of 2-photon absorption, the excitation rate is proportional to the square of the intensity of the incident light [1, 3] (unlike single photon absorption in which the excitation rate is simply proportional to the intensity) and this has many implications in microscopy, which are discussed in detail in chapter 3.

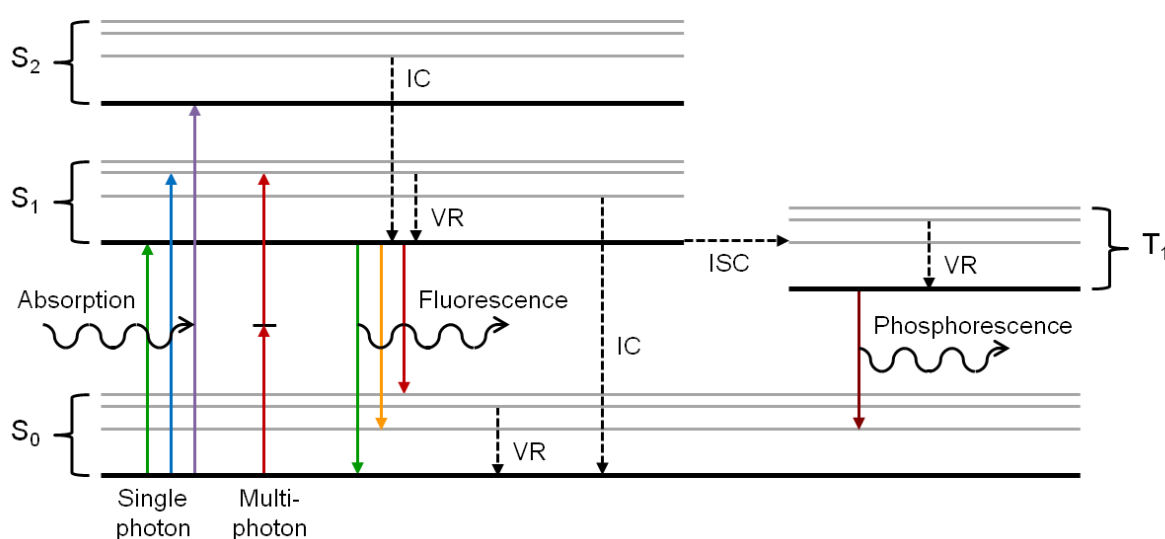


Figure 2.1. Jablonski diagram showing the important energy level transitions (both radiative and non-radiative) between singlet (S_0 , S_1 , S_2) and triplet (T_1) states in a fluorescent molecule. IC – internal conversion; ISC – intersystem crossing; VR – vibrational relaxation.

Within a fluorescent molecule there are also many non-radiative processes, which take place in addition to the radiative decays. After promotion to an excited state, electrons typically relax to the lowest vibrational energy level in that state. This process is known as vibrational relaxation and it occurs over very short time scales meaning that electrons typically relax to the lowest vibrational level within an excited state before decaying further. It is also possible for an electron to decay non-radiatively from one state to another. In the case where the two states have the same spin multiplicity (e.g. S_2 - S_1) this is known as internal conversion. In general, the rate of internal conversion increases as the separation of energy states becomes smaller. As higher excited states tend to be more closely spaced in energy this means that – in the vast majority of fluorescent molecules – an

excited electron will tend to relax to the first excited state before the probability of radiative decay becomes appreciable. Finally, non-radiative transitions can also occur between states of different spin multiplicity, for example from the S_1 state to the first excited triplet state (T_1). Such transitions take place via an inversion of the spin of the excited electron and are referred to as intersystem crossing. This spin inversion results in the unpaired excited and ground state electrons having the same spin orientation and can lead to a subsequent radiative decay via phosphorescence [1-3].

2.2 Quantifying fluorescence

When investigating fluorescence, for example in a disease study or in a medical diagnostic procedure, it is desirable to quantitatively measure some characteristic parameter of the emitted signal. This permits characterisation of the sample under investigation in terms of its fluorescent properties and can provide understanding of its structure and function. Thus, quantification of the fluorescent signal provides opportunities to observe contrast, for example, between healthy and diseased tissue. Other optical signals, such as the reflection or transmission, can also be used to this end and typical optical studies might include measurements of the intensity, wavelength, polarisation and temporal characteristics, all of which are discussed in detail below.

2.2.1 Intensity

The most obvious measurable quantity is the intensity of the emitted fluorescence, which can be assessed by simply directing the light onto a detector such as a photomultiplier tube (PMT), photodiode (PD) or camera. The fluorescence intensity depends upon the excitation power used (i.e. the intensity of the incident light) and upon the absorption cross-section and quantum yield of the specimen. The quantum yield, η , is the fraction of absorbed photons that lead to the emission of a fluorescence photon and is defined as shown in equation (1) where N_a and N_e are the numbers of absorbed and emitted photons respectively.

$$\eta = \frac{N_e}{N_a} \quad (1)$$

The fluorescence intensity is a useful parameter that is simple to measure, therefore, many studies have been carried out involving intensity measurements, for example [4]. Intensity measurements alone, however, are sensitive to variations in excitation power and to heterogeneity in the absorptive and scattering properties of the sample. For this reason, comparing intensity

measurements from several samples is complicated and it is often very difficult to determine the cause of an observed change in the fluorescence intensity.

One approach which circumvents this problem is to measure the quantum yield by comparing the observed fluorescence intensity to that from a known reference standard under matched absorption conditions [2]. This provides a more quantitative readout but it is most often achieved in cuvette measurements and is not readily applicable to investigations of bulk biological tissue. As such, fluorescence experiments are often designed to record other parameters in addition to the intensity, which permit more quantitative analysis.

2.2.2 Wavelength

The wavelength of an emitted photon, λ_{em} , is determined by the energy gap, ΔE , between the two states involved in the electronic transition:

$$\lambda_{em} = \frac{hc}{\Delta E} \quad (2)$$

where h is Planck's constant and c is the speed of light. Clearly, this relationship is true of the wavelength and energy of any photon, emitted or absorbed, therefore, both excitation and emission spectra can infer information about the energy level structure of a sample. For this reason, absorption and emission spectroscopy (which record the transmitted or emitted intensity as a function of wavelength) are both commonly used in optical studies. Typically the emission spectrum will have a similar shape to the absorption spectrum ('Mirror Image' rule) but will be shifted (Stokes' shift) to a longer wavelength (i.e. a lower energy). This similarity occurs because excitation and relaxation pathways are often very similar, however, emission pathways usually have lower energies due to vibrational relaxations preceding and following the fluorescence decay, hence explaining the observed wavelength shift [1]. This is clear in figure 2.1 where each electronic state also has a number of associated vibronic levels, which are more closely spaced in energy.

As stated, the wavelength of absorbed and emitted photons depends on the energy level structure of the sample, which, in turn, can depend on the chemical structure and local environment (e.g. pH) of the fluorescent species in that sample. Thus, it follows that absorption and emission spectra can report on these parameters and, as such, spectroscopy is in widespread use throughout fields ranging from the study of carbon nanotubes (e.g. [5]) to applications in the ecological [6] and biomedical sciences (e.g. [7-8]).

The absolute intensity of fluorescence (or reflectance) spectra can still suffer from fluctuations caused by the factors discussed in section 2.2.1 and this can make it difficult to compare measurements between samples. To avoid such issues, spectrally-resolved ratiometric measurements are often employed, in which a sample is characterised using ratios of the light intensity recorded in two or more discrete spectral bands. Such measurements account for variations in excitation power, detection efficiency, chromophore concentration and other factors that affect the entire spectrum equally and, hence, permit comparison of data from different samples. Examples of the use of spectral ratiometric measurements in medical studies can be found in references [9-11].

2.2.3 Polarisation

Polarisation is another parameter that can be used to quantify an optical signal and has the ability to provide information about both fluorescent and reflected light. Fluorescent molecules absorb light most efficiently when the electric vectors of the photons are aligned parallel to the transition dipole moment of the fluorophore. The fluorescent photons emitted from a fluorophore are then polarised along the axis of its dipole moment. Thus, when illuminating a sample with polarised light, fluorophores of a particular orientation are preferentially excited. Initially, the emission will also be polarised but a depolarisation will occur over a time scale that depends on the rotational mobility of the fluorophore. The degree of polarisation of the emitted light is often referred to as the fluorescence anisotropy [1] and can report on the viscosity of the substance containing the fluorophore and also on changes in its conformation or binding state [12].

Additionally, the polarisation of light can be affected by scattering [13], which has implications for its use in reflectance measurements. As an example, it has been shown that the polarisation can be used to differentiate between light reflected from superficial and deeper layers of tissue. This approach relies on incident polarised light becoming depolarised by multiple scattering events that accumulate if the light penetrates beyond a certain depth in the tissue. Thus, light reflected from superficial layers will retain its incident polarisation while light that penetrates further before detection will become depolarised. As such, this method provides depth discrimination and has been applied to imaging of tissue [14], particularly those involved in skin pathology [15].

2.2.4 Fluorescence lifetime

The fluorescence lifetime, τ , is defined as the average length of time for which an electron remains in its excited state before decaying back to the ground state. This determines the time available for an excited fluorophore to diffuse or to interact with its environment, which, in turn, impacts upon the information available from the emission of that fluorophore. The fluorescence lifetime can also provide useful information regarding the state of a fluorophore and is equal to the reciprocal of the total decay rate as is shown in equation (3),

$$\tau = \frac{1}{k_r + k_{nr}} \quad (3)$$

where k_r is the radiative decay rate and k_{nr} represents the sum of all possible non-radiative decay rates. It is useful to note here that the quantum yield can also be defined in terms of decay rates, as shown in equation (4), where the relationship to the fluorescence lifetime is clear.

$$\eta = \frac{k_r}{k_r + k_{nr}} = k_r \tau \quad (4)$$

The quantum yield returns the fraction of excited electrons that decay via fluorescence. Additionally, a further parameter regarding the decay behaviour of a fluorescent species is the intrinsic (or natural) radiative lifetime, τ_n , which is the fluorescence lifetime in the absence of non-radiative transitions (i.e. when $\eta = 1$) – this is defined in equation (5).

$$\tau_n = \frac{1}{k_r} \quad (5)$$

The effect of a finite fluorescence lifetime manifests itself in the decay of the intensity emitted from a population of fluorophores excited with a short pulse of light. The intensity of light emitted from a population of excited fluorophores with a single radiative relaxation pathway will decay exponentially with time from an initial intensity of I_0 to an intensity of $I(t)$ at time t , as described by equation (6).

$$I(t) = I_0 \exp\left(\frac{-t}{\tau}\right) \quad (6)$$

Moving beyond this simplified case, one can consider the examination of biological tissue, which typically contains a multitude of fluorescent species and each of these may have several radiative decay pathways. Thus, the decay observed is often more complex than the single exponential description put forward in equation (6). A more general approach is, therefore, to use a

multi-exponential model to describe the data with several decay constants, τ_i , each having a pre-exponential amplitude, α_i . These pre-exponential factors represent the fractional contributions of each decay component and are normalised such that their sum is equal to the initial intensity. The intensity profile of a fluorescent sample with n exponential decay components is then given by the following equation [1, 16].

$$I(t) = \sum_{i=1}^n \alpha_i \exp\left(\frac{-t}{\tau_i}\right) \quad (7)$$

Having recorded a fluorescence decay and extracted the decay constants and pre-exponential factors (for example, using non-linear least squares fitting), it is often desirable to parameterise the sample under investigation using a single value. This could be one of the α_i or τ_i values or any combination thereof. Alternatively, a common approach is to calculate the (intensity weighted) mean fluorescence lifetime and this can be achieved according to equation (8).

$$\tau_{mean} = \frac{\sum_{i=1}^n \alpha_i \tau_i^2}{\sum_{i=1}^n \alpha_i \tau_i} \quad (8)$$

An important point regarding the fluorescence lifetime is that it is an inherently ratiometric quantity as it depends on the intensity decay as a function of time and is not dependent on the absolute intensity [1]. As such, measurements of the fluorescence lifetime do not suffer from uncertainties due to variations in fluorophore concentration or excitation power in the same way that intensity measurements do [17-18]. For this reason, research has been directed toward the development of instruments that permit time-resolved fluorescence imaging or spectroscopy, for example [19-23]. Additionally, the fluorescence lifetime has been shown to be sensitive to the local environment of a fluorophore, which means it can report on factors such as pH [24], concentration of ions (e.g. Na^+ [25]), viscosity [26] and temperature [27-28]. Finally, and most importantly from the perspective of this thesis, the fluorescence lifetime has been shown empirically to effectively discriminate between healthy and diseased tissue, for example in various types of cancer [29-37].

2.3 Sources of autofluorescence in biological tissue

As discussed above, fluorescence can be quantified in a variety of ways and this means that it has the potential to provide contrast between disease states in medical research and diagnostics. This contrast can be achieved by introducing exogenous fluorophores to the subject or patient and this

approach is in fairly widespread clinical use – for example see the reviews of fluorescence techniques used in the diagnosis of bladder [38] and gastro-intestinal (GI) cancer [39-41]. An alternative approach is to study fluorescence from endogenous fluorophores, which are intrinsically present in biological tissue. In terms of medical diagnostics this has the advantages of being less invasive – as it does not require the introduction of any foreign substance to the patient – and of reducing the time required for imaging as there is no time delay associated with the uptake of the fluorescent contrast agent. Fluorescence from endogenous substances is commonly referred to as autofluorescence and this is the focus of the studies presented in chapters 4 and 5 of this thesis. The remainder of this chapter introduces and discusses the major fluorophores found in biological tissue.

2.3.1 Pyridine nucleotides and flavins

Pyridine nucleotides, such as nicotinamide adenine dinucleotide, and flavoproteins (for example, flavin adenine dinucleotide) play an important role in cellular metabolism as major electron acceptors or donors. Nicotinamide adenine dinucleotide – an electron acceptor – is fluorescent in its reduced form (NADH) but not in its oxidised form (NAD⁺). Conversely, another major electron acceptor is flavin adenine dinucleotide and this fluoresces when oxidised (FAD) but not when reduced (FADH₂). A further pyridine nucleotide is nicotinamide adenine dinucleotide phosphate (NADPH – reduced; NADP⁺ – oxidised), which is an electron donor that exhibits the same fluorescent behaviour as NADH [1, 42-44].

As the fluorescence intensity (i.e. the quantum yield) of these molecules varies with oxidation state, the fluorescence can be used to infer information regarding their metabolic status. One such approach, which permits mapping of the redox state of tissue, involves measurement of the ratio of the fluorescence intensities of NAD(P)H and FAD [45]. Additionally, the binding state of pyridine nucleotides and flavins affects the fluorescence emission as it alters their conformation and, hence, changes the level of quenching present. This affects both the fluorescence intensity and lifetime of the molecules [1] and, as such, the fluorescence can report on the fractional amounts of free and bound components. As an example, Skala *et al.* used measurements of the fluorescence lifetime to study the behaviour of free and protein-bound NADH in normal and cancerous epithelial tissue in hamsters [46].

NADH and FAD are easily distinguishable spectrally in terms of both their absorption and emission. NADH has an absorption maximum at 340 nm while its emission peaks at around 450 nm. FAD, on the other hand, excites optimally at 450 nm and emits at 515 nm (see table 2.1) [1, 43]. As a

result of this and the other fluorescent properties discussed above, NAD(P)H and FAD are both widely used in studies of endogenous fluorescence from biological tissue.

Table 2.1. Maximum excitation and emission wavelengths of some important endogenous fluorophores found in biological tissue. Data collated from references [1, 42-43, 47].

Fluorophore	Excitation maxima / nm	Emission maxima / nm
NADH	340	450-460
FAD	450	515
Elastin (powder)	350	420
	410	500
	450	520
Collagen		
HP	325	400
LP	325	400
Pentosidine	335	385
Vesperlysine	370	440
Crossline	380	460
Argpyrimidine	320	380
Tryptophan	280	350
Tyrosine	275	300
Phenylalanine	260	280
Porphyrins	400-450	635
		690

2.3.2 Structural proteins

Significant autofluorescence has been observed from the structural proteins elastin and collagen, which are present in many connective tissues. In both cases the fluorescence originates from cross-links between elastin or collagen fibres [42-43]. In elastin, the material responsible for the emission is thought to be a cross-linking tricarboxylic triamino pyridinium derivative [42-43, 48], while collagen contains a number of fluorescent cross-links that are formed via two distinct pathways. The first of these processes is an enzyme-controlled cross-link formation, which results in the production of hydroxylysyl pyridinoline (HP) and lysyl pyridinoline (LP) – two cross-links prevalent in collagen found in load-bearing tissues such as bone and cartilage. In the second mechanism cross-links are

formed via glycation. This is the predominant route for cross-link formation in skin and can result in the production of a multitude of fluorescent cross-links including pentosidine, vesperlysine, crossline and argpyrimidine [42-43, 49-50]. The excitation and emission maxima of elastin and the collagen cross-links mentioned above are shown in table 2.1.

Collagen and elastin are both prevalent in a variety of biological tissue types including bone, cartilage, tendon and skin. Additionally, the abundances of fluorescent cross-links found within them are dependent on both age and metabolism. For these reasons, optical signals from collagen and elastin have been investigated in numerous biological studies and such research has shown, for example, that changes in the distribution of collagen occur in both tumour metastasis [51] and in non-melanoma skin-cancer [52].

2.3.3 Amino acids and porphyrins

Two further sources of endogenous fluorescence in biological tissue are amino acids and porphyrins. The amino acids tryptophan, tyrosine and phenylalanine all contribute to protein fluorescence and tryptophan is usually the dominant source. This is because it has a significantly higher quantum yield than phenylalanine and because energy transfer often occurs between tyrosine and tryptophan. Additionally, tryptophan is the only amino acid that is excited above 295 nm as tyrosine and phenylalanine have peak excitation wavelengths of 275 nm and 260 nm respectively. Emission from tryptophan is highly sensitive to local environment, therefore it is often used to report on protein conformational changes [1, 42-43]. Despite this sensitivity, the short excitation wavelengths required (< 300 nm) mean that *in vivo* studies of amino acid fluorescence are rare due to considerations of the potential for tissue damage caused by ultra-violet (UV) B radiation.

Finally, fluorescence from porphyrins has been shown to be diagnostically useful in tumours. Ghadially was the first researcher to ascribe the observed tumour fluorescence to porphyrins [53] and it was also later shown that the red emission was specifically due to protoporphyrin [54]. It has since been demonstrated that protoporphyrin fluorescence can permit cancer diagnosis and, as such, many clinical procedures are now in place that use pre-cursors to Protoporphyrin IX (PpIX) as contrast agents. An example of such a pre-cursor is 5-aminolevulinic acid (5-ALA) and descriptions of its use in GI endoscopy can be found in references [40-41].

Chapter 3: Spectroscopy, microscopy and endoscopy

As discussed in Chapter 2, the optical signatures of biological tissue can be used to differentiate between disease states. Microscopy and spectroscopy are techniques commonly used to study the reflected light and fluorescence from biological specimens both *in vitro* and *in vivo*. This chapter gives an outline of the basics of both and then also introduces endoscopy – particularly endomicroscopy – which permits the optical investigation of remote tissue samples. Endoscopic techniques are particularly relevant to this thesis as they allow *in vivo* investigation of human tissue, for example in the GI tract.

3.1 Spectroscopy

3.1.1 Reflectance spectroscopy

Reflectance spectroscopy simply involves the study of light reflected from a specimen as a function of wavelength. Reflection of light from biological tissue can take place in either a specular or a diffuse fashion, as indicated in figure 3.1(a). While specular reflections are directly returned from the tissue surface, the diffusely reflected light undergoes several elastic scattering events during propagation through a region of the tissue. The greater the propagation distance is, the higher the likelihood that absorption occurs (also illustrated in figure 3.1(a)). As such, the diffusely reflected light is affected by both the absorptive and scattering properties of the sample.

Diffuse reflectance spectroscopy – also known as elastic scattering spectroscopy (ESS) – aims to study just this diffuse component of the reflected light. In a typical diffuse reflectance spectrometer a white light source is used to illuminate a sample and the reflected light is relayed to a detector via a dispersive optical element (such as a prism or a grating), which acts to separate the light according to its wavelength. Finally, optical fibres are usually used to deliver and collect light and these delivery and collection fibres are separated by a set distance in order to allow the system to preferentially detect diffuse (rather than specular) reflections (as shown in figure 3.1(b)). The resulting data then consists of the diffuse reflectance of the sample as a function of wavelength.

The absorptive and scattering properties of a biological specimen are dependent on a number of factors – such as cell density and nucleus-to-cytoplasm ratio – that can exhibit changes during malignant transformation [55-56]. Additionally, diffuse reflectance spectroscopy is a relatively

simple method that is inexpensive to implement, hence, it has been widely used in clinical studies. Examples of pathologies investigated using diffuse reflectance spectroscopy include cancer of the cervix [57], bladder [9, 58] and skin [59].

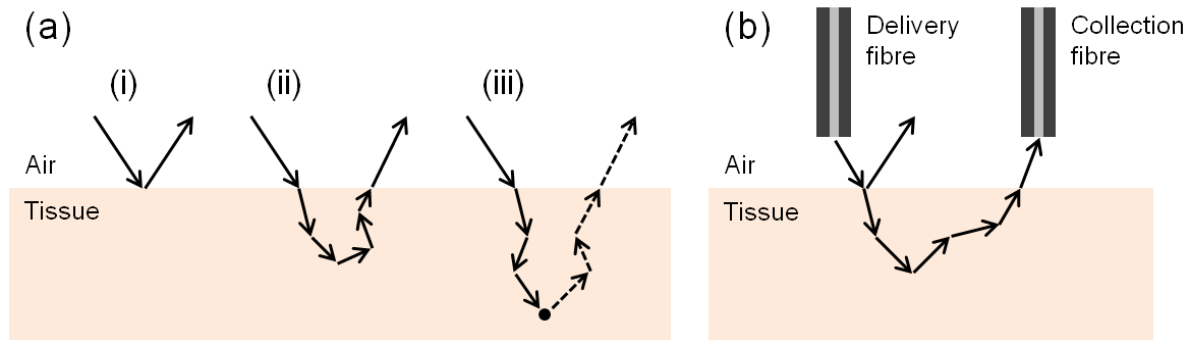


Figure 3.1. (a) Diagram illustrating the interaction of light (signified by the black arrows) with biological tissue showing (i) specular reflection, (ii) elastic scattering as part of diffuse reflection and (iii) elastic scattering followed by absorption, which can lead to fluorescence emission (dashed arrows). (b) shows how the positioning of the optical fibres used for light delivery and collection in a diffuse reflectance spectrometer can be used to reject specular reflections (i.e. direct reflections from the tissue surface) and, hence, allow preferential collection of the diffuse reflectance.

3.1.2 Steady state fluorescence spectroscopy

In steady state fluorescence spectroscopy, a lamp or laser source is used for illumination, an excitation filter is often employed to permit narrowband excitation and an emission filter is used to stop any scattered light reaching the detector. As such, the fluorescence (rather than the reflectance) spectrum is recorded. A schematic diagram of a typical optical configuration used in fluorescence spectroscopy is shown in figure 3.2. In this figure, the sample is excited by a filtered light source and the fluorescence is collimated by a lens then directed through a prism that acts to separate the emission according to its wavelength. Finally, a second lens focuses the spectrum onto a charge-coupled device (CCD) camera for detection. Alternative arrangements involve the use of a diffraction grating instead of a prism to produce the necessary spectral dispersion and the use of single point detectors rather than the wide-area detector shown here. In figure 3.2, the use of a CCD camera permits simultaneous collection of the entire spectrum. If point detection is employed then a scanning mechanism is also required and this usually entails the rotation of the dispersive element or translation of the detector.

As with diffuse reflectance measurements, steady state fluorescence spectroscopy is sensitive to the absorption and scattering coefficients of the sample under investigation. In addition, it also provides information on the fluorophore content, which, as discussed in section 2.3, can

report on the disease state of biological tissue. Thus, fluorescence spectroscopy has been used in a variety of investigations, for example it has been applied to studies of skin malignancies [8, 52, 60-61], lung cancer [10] and breast cancer [62].

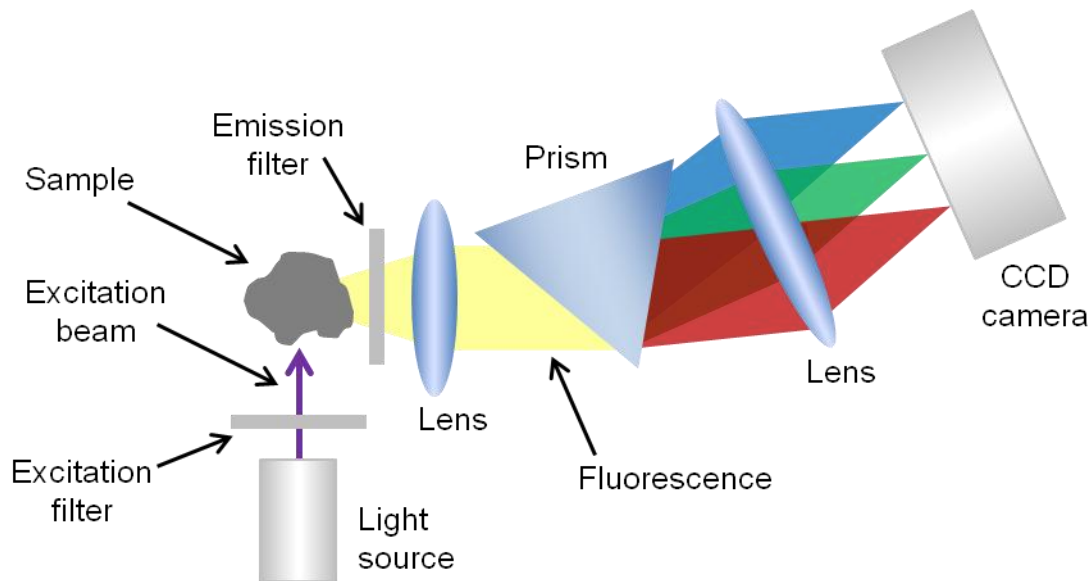


Figure 3.2. Schematic diagram of a steady state fluorescence spectrometer.

3.1.3 Time-resolved fluorescence spectroscopy

Time-resolved fluorescence spectroscopy (TRFS) is similar to the steady state case described above but it typically utilises a pulsed excitation source and a specialised detector (such as a photon counting PMT) in order to permit measurement of the fluorescence decay characteristics as well as the spectrum. The way in which fluorescence decays are recorded is described in detail below (see section 3.2.4), however, a short discussion of TRFS is first presented here.

Some examples of TRFS systems can be found in references [19-21, 63-64] and, in a typical instrument, a dataset consists of fluorescence decays measured in a number of discrete spectral channels. Such systems, therefore, provide both spectral and lifetime-based information. The additional lifetime information provided reveals further opportunities to discriminate healthy from diseased tissue and also yields information that cannot be gleaned from steady state data, even if the spectral resolution is extremely high [65]. For this reason, TRFS has also been applied to a number of tissue and disease studies including investigations of atherosclerotic plaques [66] and of various types of cancer [21, 29-30, 33-35, 67].

While the additional temporal information provides added diagnostic opportunities, it also adds complexity as the data is now three-dimensional (3-D), containing intensity information as a

function of both time and wavelength. For this reason, basic analysis of time-resolved fluorescence spectra can often involve the integration of data across one or more dimensions. While this permits more straightforward analysis, it inherently means that some information is wasted. For this reason, some more detailed analysis techniques have been developed which allow a user to simultaneously assess both the temporal and spectral characteristics of a recorded spectrum. One such technique is Time-resolved emission spectroscopy (TRES) in which the fluorescence spectra are plotted and studied at several discrete time points after excitation. The time-resolved emission spectra are typically normalised to their peak intensity such that it is possible to investigate the variation in the shape of the emission spectrum as a function of time [65, 68].

A further development of TRES is time-resolved area normalised emission spectroscopy (TRANES) [68-70]. In this case the time-resolved emission spectra are normalised such that the area under the curve is the same at all time points after excitation. As before, this permits comparison of the spectral shapes at various time delays and this technique can also be used to prove the existence of exactly two emissive species in a sample [69].

In conclusion, TRFS – and spectroscopy as a whole – are useful techniques, which can provide a wealth of information in biological studies of tissue and disease. Furthermore, the different techniques discussed in this section (i.e. reflectance spectroscopy, fluorescence spectroscopy and TRFS) reveal different properties of a sample and several spectrometers have also been developed that measure multiple optical properties in a single acquisition, for example both the diffuse reflectance and the steady state fluorescence [8, 71-72].

Clearly, while point spectroscopy provides detailed spectral information, it does suffer from an inherent drawback in its inability to record morphological information. To achieve this it is necessary to move to microscopic techniques – which can measure both the spectroscopic and morphological properties of a sample simultaneously – and these are discussed in the following section. It should be noted, however, that while microscopy provides morphological information, it also suffers from an inevitable reduction in the amount of spectroscopic data attained (when compared to point spectroscopy) if a reasonable acquisition time is maintained.

3.2 Microscopy

3.2.1 Wide-field microscopy

The simplest form of microscopy is wide-field microscopy, which involves the illumination of, and collection of light from, an entire field of view simultaneously. A sample is illuminated (typically using a lamp or a laser) and the reflected or emitted light is imaged onto a pixellated detector such as a CCD camera. An example layout of a wide-field microscope used to image fluorescence is shown in figure 3.3.

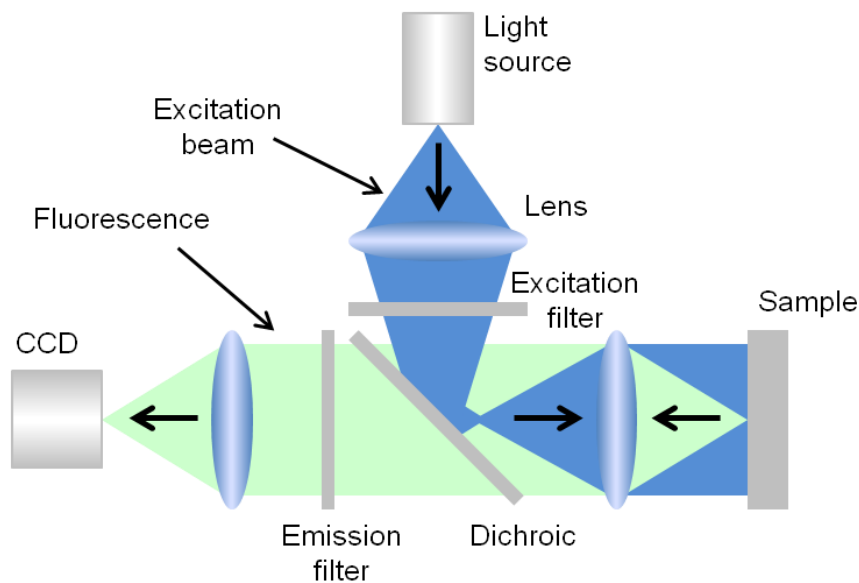


Figure 3.3. Schematic diagram of a wide-field fluorescence microscope.

As is shown in the diagram, the excitation light is directed onto the sample, in this case using a pair of lenses. Fluorescence from all points in the field of view is then imaged onto the CCD using a second lens system. The reflected excitation light is rejected using a dichroic beamsplitter and an emission filter.

The advantages of wide-field microscopes include the fact that they are relatively low in cost and are simple to build and use. Secondly, as light is collected from all points in the field of view simultaneously, image acquisition is fast. The main disadvantage, however, also arises from this method of light collection as it means that out-of-focus light reaches the detector. This acceptance of light from out-of-focus planes leads to a reduction in the contrast of the in-focus image. In some scenarios, it is therefore desirable to only acquire light from the focal plane as this will provide

improvements in both the resolution and the signal-to-background ratio. This is known as optical sectioning and is most commonly achieved using confocal microscopy.

3.2.2 Confocal microscopy

In a confocal microscope a point source – usually a lamp with a pinhole or a laser – is used for illumination. This excitation light is focussed down to a diffraction limited point in the specimen and a confocal pinhole in front of the detector is used to reject out-of-focus light. Typically a point detector, such as a PMT, is used and the detection pinhole only allows light that originated at the focus of the excitation beam to reach this detector. Light from all other points is rejected. This phenomenon is illustrated in figure 3.4 where the out-of-focus light is rejected while light from the focal point is imaged onto the detector. By scanning the focussed excitation spot across the sample in the x- and y-directions, it is possible to build up an image of one plane in the sample – an optical section. A 3-D image stack can then be obtained by also scanning the beam in the z-direction.

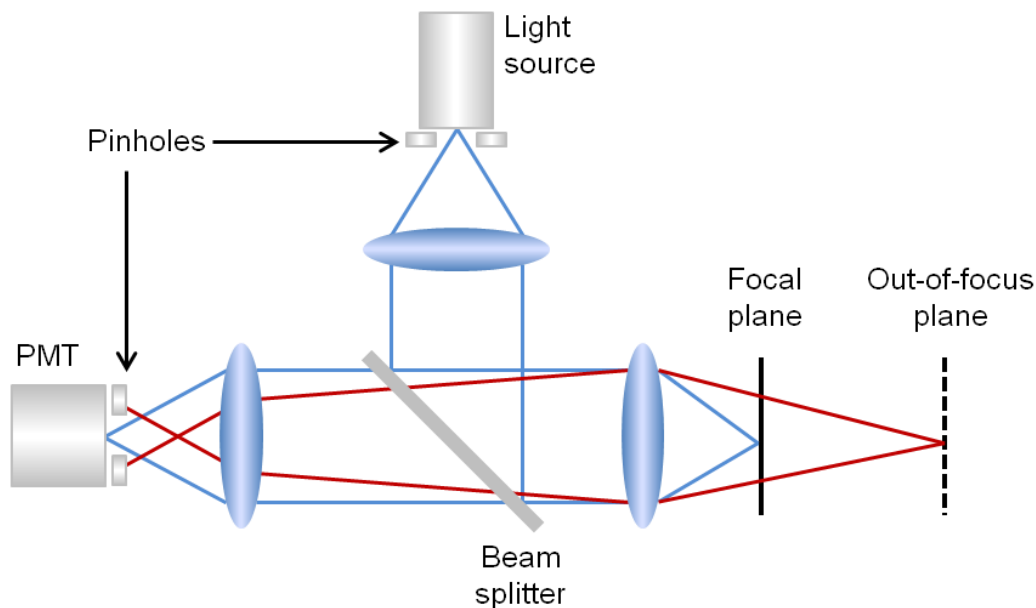


Figure 3.4. Diagram of a confocal microscope demonstrating the rejection of out-of-focus light (red rays) and the collection of light from the focus (blue rays).

The confocal microscope reduces blurring of images due to its ability to reject light from outside the focal plane and, hence, improves on its wide-field counterpart in terms of both resolution and image contrast in the image of the focal plane. It also permits depth resolved imaging and allows for better depth penetration due to the rejection of scattered light [73]. Additionally, the

optical sectioning provided mirrors the physical sectioning that is performed on tissue biopsies in histopathology for medical diagnosis. For these reasons, confocal reflectance microscopy has been used in a multitude of biological studies including, for example, investigations of skin lesions [74-78].

On the other hand, confocal microscopy does have drawbacks, the most important of which is the time needed to scan the excitation source across the sample in order to build up an image. Of course, the scanning speed can be increased through the use of a higher excitation power, however, the maximum excitation power is limited by the need to avoid photobleaching and phototoxicity in the sample. In confocal reflectance microscopy this means that the imaging speed is inherently limited by the speed of the scanning system. Additionally, in fluorescence and fluorescence lifetime imaging modalities, the imaging speed is often reduced further due to low photon count rates. Both of these effects lead to relatively long acquisition times, which can make *in vivo* imaging difficult due to factors such as motion of the sample. While it is not the topic of this thesis, it is interesting to note that there is a degree of interest in addressing this issue of imaging speed using wide-field optically sectioned imaging techniques – i.e. methods that obtain the high resolution and depth penetration of confocal microscopy but with parallel acquisition permitting short collection times comparable to those achievable with wide-field microscopy. Some examples of such approaches include spinning disk microscopy [79] and structured illumination [80].

3.2.3 Multiphoton microscopy

This form of microscopy employs the phenomenon of multiphoton absorption that was discussed in section 2.1. Rather than exciting the sample with short wavelength (e.g. blue or ultra-violet) photons, as is normally the case in confocal and wide-field fluorescence microscopy, a multiphoton microscope uses longer wavelength illumination (infra-red) and fluorescence is excited by the simultaneous absorption of two or more photons. The layout of such a microscope is, therefore, very similar to a confocal microscope (figure 3.4) with the only differences being that an infra-red (IR) pulsed laser is typically used and that the detection pinhole is no longer necessary [81-83].

A laser source providing short pulses (typically less than 10 ps) and high peak power is required because multiphoton absorption is a non-linear effect (proportional to the square of the intensity) that only occurs efficiently in areas of very high intensity. Similarly, it is possible to dispense with the detection pinhole because the non-linear nature of multiphoton absorption means that any fluorescence observed must have originated at the focal volume, as this is the only region at which the intensity is high enough to excite fluorescence. This infers an advantage over

confocal microscopy as there is no need to reject scattered fluorescence, hence, allowing better signal collection efficiencies to be obtained.

The use of IR excitation also permits increased depth penetration due to the reduced scattering and absorption coefficients of biological tissue at these wavelengths (compared to UV and visible wavelengths). Furthermore, photobleaching and phototoxicity outside of the focal plane are reduced because of the decreased absorption in these areas of lower intensity. Of course, multiphoton microscopy also has disadvantages, not least the need for a high peak intensity to generate the multiphoton fluorescence. This means that expensive, ultrafast, pulsed lasers are required as the excitation sources to provide a high peak power but low average power so that damage to the sample is avoided.

The use of longer wavelengths has further advantages, however, including the ability to image signals due to second and third harmonic generation (SHG and THG). These are non-linear processes that occur in non-centrosymmetric media (SHG) or at interfaces between regions of differing refractive index (THG). Both processes involve the instantaneous conversion of two (SHG) or three photons (THG) into one photon of twice or three times the frequency. S- and THG microscopy possess many of the same advantages and disadvantages as multiphoton microscopy as they require a long excitation wavelength (IR) and high intensity in order to occur. Additionally, SHG and THG take place without the absorption of photons, hence, heating and damage of the sample are less likely.

Using a multiphoton microscope it is possible to image both the fluorescence and the harmonic generation signals simultaneously and then separate them according to their spectral or temporal characteristics. This is referred to as non-linear microscopy and has the advantage of providing several datasets in a single acquisition time. Non-linear microscopy (which includes multiphoton microscopy) is a widely used technique and it has been applied to a number of both *ex vivo* and *in vivo* disease studies, for example in brain tumours [36] and in cancer of the skin [84-90], breast [91] and bladder [37].

3.2.4 Fluorescence lifetime imaging and measurement

Fluorescence lifetime imaging microscopy (FLIM) involves the measurement and display of the fluorescence lifetime of a sample at every image pixel within it. While FLIM data is not presented in this thesis, a short explanation of fluorescence lifetime measurement and imaging methodologies is given below. This thesis does cover the development and use of both TRFS (see chapters 4 and 5)

and a novel approach to endomicroscopy (see chapters 6 and 7) and a broader, long term aim of this research will involve combining these two methodologies. As such, it is beneficial for the reader to have an understanding of the concepts behind the techniques used for both fluorescence lifetime measurement and imaging.

In FLIM (and TRFS) the lifetime can be measured using either frequency domain or time domain techniques and the following sections explain the principles behind these two approaches. In imaging modalities, the display is then typically achieved by plotting the fluorescence lifetime on a false colour scale (and, in some cases, weighting each pixel according to its fluorescence intensity).

Frequency domain

The principle of frequency domain fluorescence lifetime measurement is illustrated in figure 3.5. This technique typically employs a continuous wave (CW) excitation source whose intensity is modulated with time at a frequency (ω) that is comparable to the reciprocal of the decay time, τ . As the excitation is modulated, the emission will also vary at the same frequency (as shown in the figure). Due to the finite fluorescence lifetime of the fluorophore, the emission signal will be shifted in time with respect to the excitation signal. This effect manifests itself as a phase shift, ϕ , from which the fluorescence lifetime can be calculated according to equation 9.

$$\tau_{\phi} = \frac{\tan(\phi)}{\omega} \quad (9)$$

A second effect of the finite lifetime of the fluorophore is to decrease the modulation depth of the emitted signal (i.e. the peak-to-peak height as a fraction of the mean) relative to the excitation signal. This demodulation occurs because some of the fluorophores excited during the period of maximum excitation intensity continue to emit through the excitation minimum, hence increasing the fluorescence intensity observed at this point. The same is true of fluorophores excited at the excitation minimum; however, the effect is smaller in this case due to the lower initial fluorescence intensity of fluorophores excited in this period. Therefore, the modulation of the emitted signal is reduced relative to the excitation. The fluorescence lifetime can then also be calculated from measurements of the demodulation factor, m , as shown in equation 10. Note also that m is calculated as shown in figure 3.5.

$$\tau_m = \frac{\sqrt{(m^{-2} - 1)}}{\omega} \quad (10)$$

Clearly it is simple to extend this method to an imaging modality by measuring the demodulation or phase shift at all pixels in a sample and this can provide a relatively low-cost implementation of FLIM [1, 12].

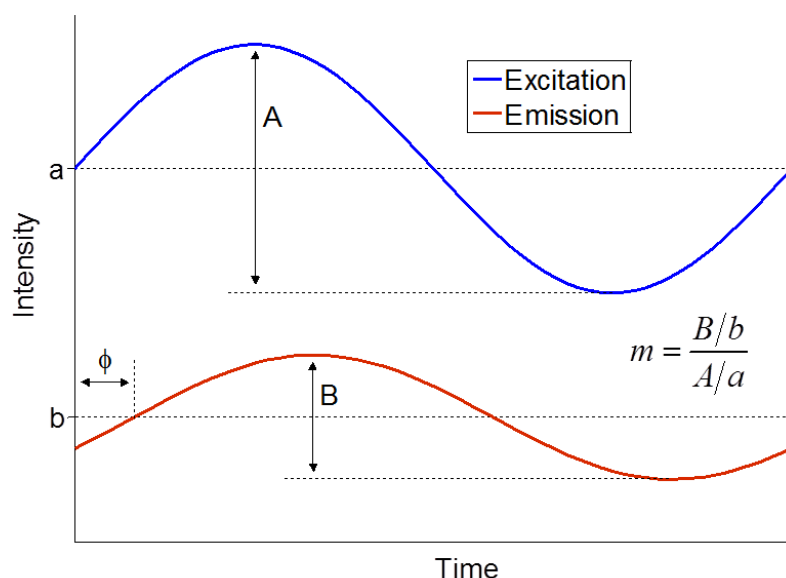


Figure 3.5. Illustration of the principles behind frequency domain fluorescence lifetime measurements showing the phase shift, ϕ , and the definition of the demodulation factor, m .

Time domain

The most common and photon-efficient time domain fluorescence lifetime measurement technique is time correlated single photon counting (TCSPC), which involves recording the arrival times of individual photons with respect to a pulsed excitation source. The detection of a fluorescence photon initiates the charging of a capacitor in a time-to-amplitude converter (TAC). The arrival of the subsequent laser pulse then acts as the stop signal and causes the capacitor to discharge. Using the magnitude of the output voltage from the TAC capacitor it is possible to calculate the time taken for the fluorescence photon to decay. By detecting large numbers of photons in this way it is then possible to build up a histogram of photon arrival times, which represents the decay profile of the sample. The fluorescence lifetime can be extracted from this decay profile in a number of ways, for example by fitting an exponential curve to the decay data (see section 2.2.4) or by implementing phasor analysis [92]. FLIM can then be achieved by scanning the excitation beam across the sample and collecting a decay curve at each pixel. The principles of TCSPC are illustrated in figure 3.6.

Although TCSPC is a very photon-efficient method of measuring lifetimes, it is also quite slow as the rate at which photons can be counted is limited by the dead time of the system's electronics

and, more importantly, by the need to avoid the effect of pulse pile-up (which is explained below). After each laser pulse, the system is limited to detecting the first arriving photon. Thus, if many photons are emitted after each laser pulse then only the early photons are detected and the measured fluorescence lifetime will be biased towards a shorter value. This problem is known as pulse pile-up and to avoid it, excitation intensities need to be low such that, on average, only one fluorescence photon is detected for every 50-100 laser pulses [1].

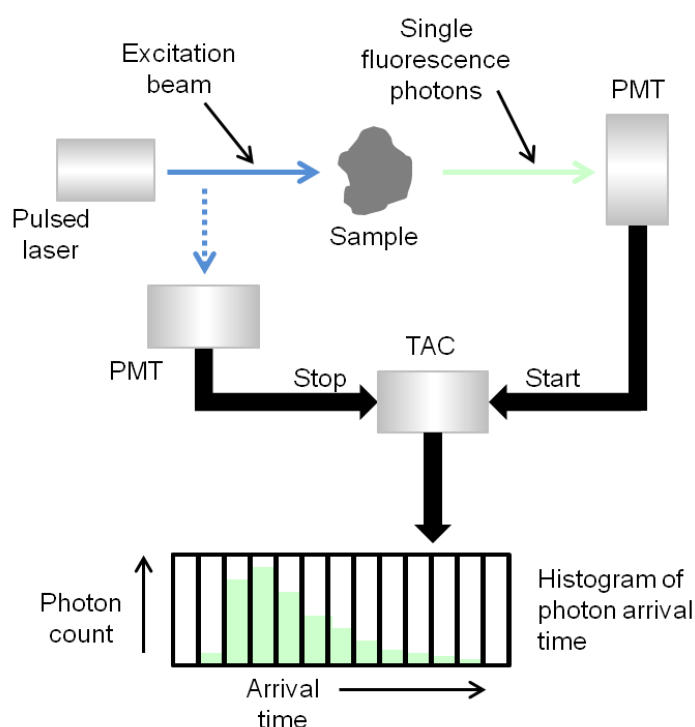


Figure 3.6. Simplified diagram illustrating TCSPC.

Wide-field fluorescence lifetime imaging techniques permit the simultaneous collection of fluorescence decay data from all image pixels and, hence, can considerably reduce acquisition times. Wide-field time-gated FLIM typically employs a gated optical intensifier (GOI) – which can be thought of as a very fast shutter – to take ‘snapshots’ of the decaying fluorescence at distinct time points after the arrival of the excitation pulse. A typical configuration is shown in figure 3.7 where a sample is illuminated in a wide-field geometry by a pulsed excitation source and the fluorescence emission is imaged onto a GOI. At the GOI, the fluorescence photons collide with a photocathode and generate photoelectrons. The photoelectron signal is then amplified in a micro-channel plate and directed onto a phosphor screen where it induces phosphorescence, which is imaged onto a CCD camera. The photocathode voltage can be modulated at high frequencies (up to several GHz) allowing the CCD to image short periods of the fluorescence decay at distinct time points after the

excitation pulse. By varying the delay between the excitation and the ‘opening’ of the GOI it is then possible to collect a sampled fluorescence decay profile at each pixel, from which the fluorescence lifetime can be calculated. Typical gate widths (i.e. shutter speeds) vary from 100-1000 ps while the gate separation is usually on the order of nanoseconds [12, 18, 22].

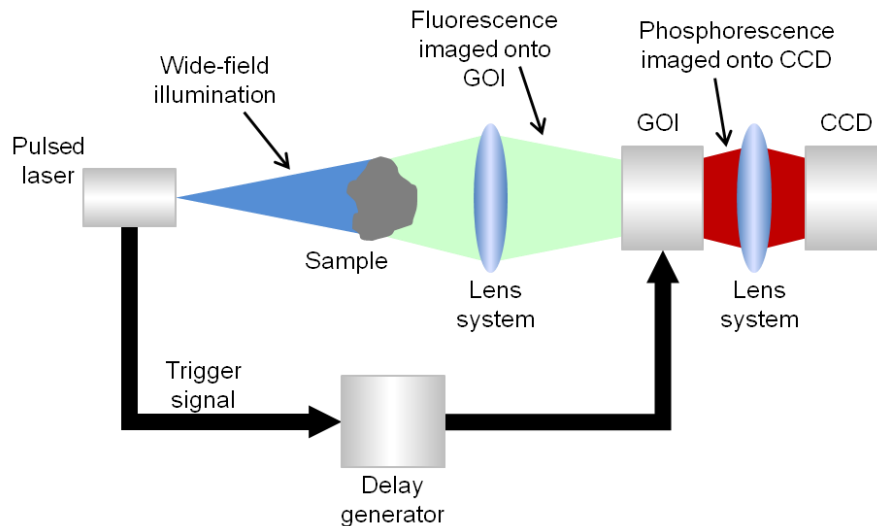


Figure 3.7. Basic layout used in wide-field time-gated FLIM.

Wide-field time-gated FLIM is relatively fast as information from all pixels is obtained in parallel, however, it does have a lower photon efficiency than TCSPC [12]. It also loses the optical sectioning provided by scanning confocal or multiphoton microscopes. As such, there is a degree of interest in the application of wide-field optical sectioning (for example using a Nipkow spinning disk [79]) to FLIM and this has been demonstrated recently, for example see references [93-94].

3.3 Endoscopy

Endoscopy simply implies the imaging of a remote sample and its applications range from medical diagnosis and *in vivo* biological research to the inspection of the interior of heavy machinery used in industry. Common medical endoscopes incorporate wide-field white light illumination (usually provided by a mercury lamp) and can be either flexible (endoscopes) or rigid (arthroscopes). Traditionally, flexible endoscopes consist of an incoherent fibre-optic bundle for illumination and a coherent fibre bundle to relay the image back to a detector or eyepiece. Thus, it is interesting to note that the first clinically useful flexible fibre-optic endoscope used in the lower GI tract was a sigmoidoscope developed by Overholt [95]. In most modern systems, however, miniaturised distal

CCD cameras have replaced the collection bundles as they have significantly higher numbers of pixels and, hence, provide improved resolution [96].

Arthroscopes employ a rigid tube enclosing an array of lenses (usually rod lenses) to relay the image to the eyepiece. The image quality is improved relative to flexible fibre endoscopes as arthroscopes do not suffer from the pixellation introduced by the collection fibre bundle. The obvious drawback, however, is that their use is limited to regions of the body with straight line access and for this reason arthroscopes are most often used in surgical procedures. Arthroscopes are typically 30cm long and less than 1.2cm in diameter. As with flexible endoscopes, the illumination source used is typically a Mercury lamp and this is coupled to the sample via a fibre bundle [96]. Example photographs of flexible and rigid endoscopic systems are displayed in figure 3.8.

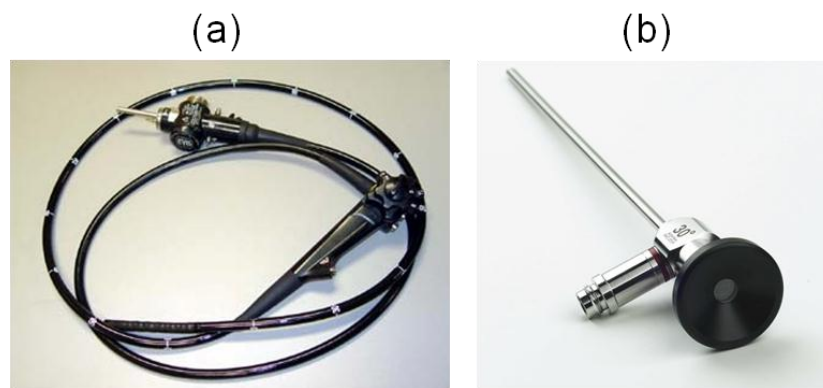


Figure 3.8. Photographs of (a) a flexible endoscope and (b) a rigid arthroscope.

As discussed above, common medical endoscopes permit wide-field, reflected light imaging and, therefore, only provide morphological information and limited (three colour) spectroscopic information. In a typical diagnostic endoscopy, any suspicious regions of tissue need to be biopsied and investigated histologically before a diagnosis can be confirmed. This process is both time-consuming and invasive and can result in the unnecessary excision of benign lesions. While additional endoscopic techniques that provide high resolution or functional information are becoming more widespread – for example, autofluorescence endoscopy [97], narrow band imaging (NBI) [98] and magnification endoscopy [98] – there is still a need for apparatus that allows microscopic, functional analysis of tissue *in vivo* [99]. Such an approach could reduce the regularity with which clinicians have to resort to histopathology and, hence, would reduce the number of unnecessary biopsies. One promising emerging technology is laser scanning endomicroscopy, which

allows microscopic tissue investigation with sub-cellular resolution and permits imaging in either reflectance or fluorescence.

3.3.1 Laser scanning and confocal endomicroscopy

Laser scanning endomicroscopy allows microscopic investigation of a sample by sequentially scanning an illumination spot across it and an image is built up in the same way as in a confocal or multiphoton microscope (see sections 3.2.2 and 3.2.3). Optical fibres are used to deliver and collect light and the resolution achieved is typically on the order of microns meaning that sub-cellular structures can be imaged. Additionally, with appropriate design considerations, endomicroscopy can provide optical sectioning.

At present, there are two distinct methods by which transverse scanning of the focussed illumination spot can be achieved in endomicroscopy: distal and proximal scanning [100]. The former utilises a single mode optical fibre for light delivery and collection, and beam scanning is achieved using a miniaturised distal scanning mechanism. In the case of proximal scanning, the focussed excitation beam is scanned across the input face of a coherent fibre bundle such that light is sequentially coupled into individual fibre cores. Transverse scanning of the output beam is then achieved without the need for mechanical components at the distal tip. These methods are described in more detail below.

Distal scanning

In a distal scanning endomicroscope a single mode fibre (SMF) is used both to deliver the excitation and to collect the emission. After transmission through the fibre the excitation light is focussed to a diffraction limited spot using a system of miniature distal lenses. The reflected or fluorescent light is collected by the same single mode fibre, which also acts as a confocal pinhole as only light from the focus of the illumination source is coupled into the fibre. Hence, optical sectioning is inherently achieved. At the proximal end, the collected light is then directed onto a single channel detector, which records the intensity at each scan position. Figure 3.9 shows a typical configuration of a distal scanning confocal endomicroscope along with examples of distal scanning mechanisms.

As shown in figure 3.9(a), the endomicroscope contains a scanning mechanism at the distal end that allows translation of the focussed beam. This scanning mechanism can consist of a pair of micro-machined scanning mirrors [101] or a scanning head that physically moves the tip of the fibre

[102-104]. Typically the scanning mechanism permits fast 2-dimensional imaging at a set depth in the sample as well as slower adjustment of the focal plane.

Distal scanning endomicroscopes provide high image quality, therefore, they are now in limited clinical use and a commercial system is available (EC3970K, Pentax, Japan). The main drawback of distal scanning, however, is that the minimum size of the endomicroscope is inherently limited by the size of the scanning mechanism. Furthermore, the scanning mechanism is usually the most expensive element of the endomicroscope and, thus, it is desirable to keep this at the proximal end where it is less likely to be damaged. Proximal scanning addresses these issues but, as discussed in the following section, it also brings other disadvantages.

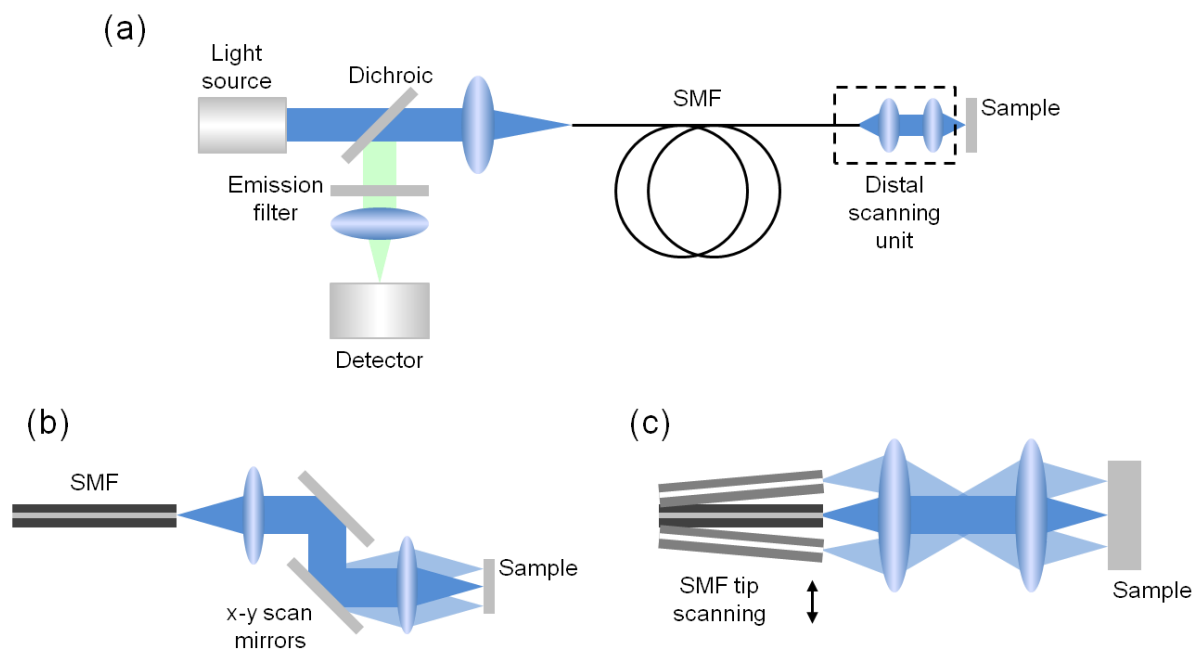


Figure 3.9. (a) Typical setup of a distal scanning confocal endomicroscope. (b) and (c) show example scanning systems incorporating miniaturised scanning mirrors (b) and single mode fibre tip scanning (c).

Proximal scanning

Figure 3.10 shows a proximal scanning endomicroscope (first demonstrated by Gmitro and Aziz [105]), which utilises a coherent fibre bundle – typically containing over 10,000 fibre cores – to deliver light to the sample. As shown in the figure, a proximal scanning endomicroscope consists of a proximal confocal microscope that scans a focussed excitation spot across the input face of the coherent fibre bundle, sequentially coupling light into the individual fibre cores. The distal end of the bundle is directed toward a sample and, as light is coupled into sequential fibre cores, scanning of that sample is accomplished. Light from each scan location returns to the proximal end through the

same fibre core and is imaged onto a single channel detector via a beam splitter. To permit optical sectioning, a pinhole is used to ensure that light collected by adjacent fibres does not reach the detector. In the figure a pair of distal lenses is used to focus the excitation beam. In some cases, however, no distal lenses are used and illumination is achieved by simply placing the probe in contact with the sample. With or without distal lenses, the imaging depth is usually fixed and can normally only be altered by using different endoscopic probes.

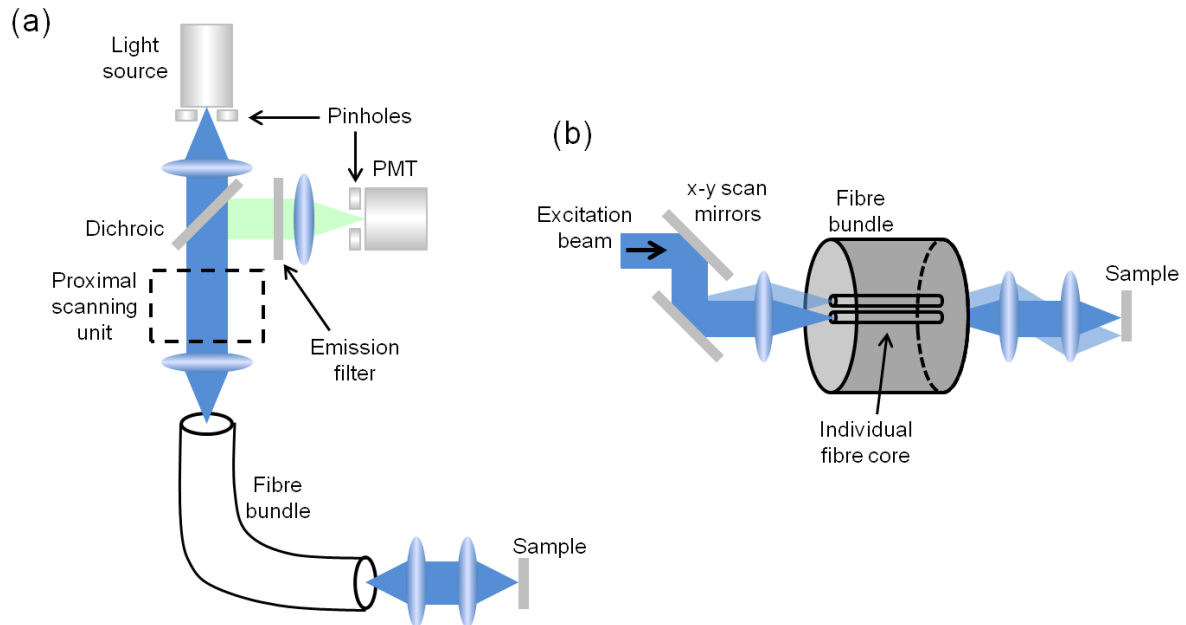


Figure 3.10. (a) Proximal scanning confocal endomicroscope – proximal scanning optics are used to translate a focussed beam across the input face of a fibre bundle, which relays light to the sample. (b) illustrates how scanning of this remote sample is achieved.

As with distal scanning, the use of proximal scanning endomicroscopes is becoming increasingly widespread and they are now commercially available (Cell-viZio™ GI, Mauna Kea Technologies, France). Proximal scanning is in clinical use [106] and has been adapted for FLIM for Förster resonance energy transfer (FRET) experiments [107]. Furthermore, there are also several alternative methods of achieving proximal scanning including slit-scanning [108-109] and the use of a spatial light modulator (SLM) to translate a focus across the input face of a fibre imaging bundle [110].

Clearly, proximal scanning permits the development of smaller and cheaper endomicroscopes as no complex, miniature electronics are required at the distal end. Such systems, however, suffer from reduced resolution due to the limited number of fibre cores (i.e. pixels) and, in

some cases, from undersampling due to the fixed distance between the fibre cores. While both the endomicroscope designs discussed in this section are in regular use, they also both suffer from inherent drawbacks. An ideal endomicroscope should provide the image quality of a distal scanning system while still maintaining the small size permitted by proximal scanning. Chapter 6 of this thesis presents a novel approach to allow beam scanning in endomicroscopy, which aims to address the issues discussed above.

3.3.2 Multiphoton endomicroscopy

Multiphoton endomicroscopy can, of course, also be realised using either of the scanning methods discussed above (section 3.3.1) and the use of multiphoton excitation infers the same advantages as it does in microscopy – for example, increased depth penetration and reduced photobleaching outside of the focal volume (see section 3.2.3). The combination of optical fibres and the short, high energy laser pulses required for multiphoton excitation, however, leads to a significant problem. This problem is temporal broadening of the excitation pulses, which considerably reduces the excitation efficiency.

This temporal pulse dispersion is due to two separate phenomena, the first being group velocity dispersion (GVD). The refractive index of the fibre core varies with wavelength and, therefore, so does the group velocity of the laser pulse propagating through it. In the normal dispersion regime the refractive index decreases with increasing wavelength and so the long wavelength components of the laser pulse will travel faster than the short wavelength components. As the short excitation pulses used in multiphoton microscopy inherently have a large wavelength range and because an optical fibre usually provides a long interaction length (i.e. > 1 m), this effect will cause significant temporal broadening and will also cause the pulse to become chirped (modulated in frequency with time) [13]. GVD can be compensated, however, by ‘pre-chirping’ the incoming laser pulses using a pair of prisms or diffraction gratings [111].

A second contributing effect is self-phase modulation (SPM) – a third order non-linear effect that is more difficult to solve. SPM is caused by an additional phase imparted on an intense optical pulse propagating through a non-linear medium (in this case silicon in a single mode fibre) due to the non-linear refractive index. For ultrashort pulses with high intensity, this self-induced phase is time-dependent and generates an instantaneous frequency shift dependent on the time derivative of the pulse intensity. As such, the leading edge of the pulse experiences a red-shift, the peak is unaffected and the trailing edge is blue-shifted meaning that the resultant pulse is broadened in frequency. As the optical fibre also exhibits GVD, this broadening in frequency leads to further temporal

broadening [111-112]. Although the effects of SPM can be managed to some extent through pre-chirping, to date it has been a limiting factor in multiphoton endomicroscopy [102] (though its effects can be addressed through the use of photonic crystal fibres, as described below).

Despite the problems caused by GVD and SPM, a significant amount of research has still been directed towards the development of multiphoton endomicroscopes. The simplest systems use either single mode fibres [102] or fibre bundles [113-114] but such setups tend to be limited by SPM. An improvement on these designs is to use a double-clad fibre (DCF) to deliver excitation and collect fluorescence [115]. DCFs allow multimode collection of fluorescence through their inner cladding and, therefore, provide better signal collection efficiencies than SMFs. Hence, the same signal levels can be acquired with a lower excitation power and, for this reason, they are less affected by temporal pulse broadening. Ultimately, however, endomicroscopes that utilise DCFs are still limited by SPM. A further development involves the use of photonic crystal fibres (PCFs), in particular double clad photonic crystal fibres (DCPCFs), for light delivery and collection. Light propagating through a PCF typically has a broader mode diameter than in a standard SMF, which means that the peak intensity is lower. In turn, this implies reduced non-linearity and, hence, reduced temporal broadening. Indeed, studies using DCPCFs have first shown that the fluorescence signal is increased relative to that in a SMF for the same excitation power [116] suggesting that pulse broadening is reduced. Further studies have also been carried out using DCPCFs for multiphoton and non-linear endomicroscopy (for example [117]) and it has been shown that DCPCFs do indeed produce lower SPM than DCFs due to a lower level of non-linearity [118].

As discussed, significant developments and improvements have been made in the field of multiphoton endomicroscopy. Nevertheless, to achieve reasonable signal levels, multiphoton endomicroscopes still employ single fibres (usually PCFs) along with distal scanning mechanisms and for this reason their minimum size is inherently limited (see section 3.3.1). As such, further development of multiphoton endomicroscopy – to allow smaller probes and improved excitation efficiencies – is desirable.

3.3.3 Endoscopic fluorescence lifetime imaging and spectroscopy

It was seen briefly in section 2.2.4 and earlier in this chapter that the fluorescence lifetime of intrinsic tissue fluorophores can be used to differentiate between healthy and diseased tissue. This fluorescence lifetime contrast has been observed (both *ex vivo* and *in vivo*) in several diseases including atherosclerotic plaques [18, 66, 119-120] and various types of cancer [29-37, 121]. Due to this observed contrast, significant research has been concentrated on the concept of measuring

fluorescence lifetimes endoscopically, either in imaging or point measurement formats. This can be realised using optical fibres, which permit *in vivo* measurements of fluorescence lifetimes.

Achieving endoscopic FLIM is difficult because it requires the collection of many photons at each image pixel in order to accurately measure the lifetime and, hence, acquisition times can be too long for clinical applications. Nonetheless, several attempts have been made at endoscopic FLIM and the first demonstration utilised a frequency domain method applied to a standard wide-field endoscope, which provided acceptable frame rates for endoscopy (up to 25 Hz) [122-123]. An alternative approach is to employ wide-field time-gated detection – which has also been shown to provide acceptable frame rates (up to 29 Hz) – and this methodology has been used to image *ex vivo* tissue samples [124-125].

Both of the above techniques allow wide-field fluorescence lifetime imaging but, as discussed previously, confocal imaging – which permits optical sectioning – is often desirable, particularly in medical applications. Therefore, FLIM has also been successfully applied to confocal endomicroscopy through the addition of a pulsed excitation source and TCSPC detection [107, 126]. Although these techniques suffer from reduced frame rates due to the sequential acquisition required, they provide high resolution optically sectioned images with functional information (i.e. the fluorescence lifetime).

Some current restraints on the development of endoscopic FLIM techniques are caused by the typical use of UV excitation wavelengths. This permits the excitation of many intrinsic tissue fluorophores but it also generates two significant problems, the first being background fluorescence from optical components. As discussed above, in endomicroscopy, a single fibre is used both to deliver the excitation light and to collect the fluorescence (note that this is true in both distal and proximal scanning – even when using a fibre bundle, for each image pixel a single fibre core delivers the excitation and collects the fluorescence). Normally, the optical fibres used are made from silica doped with germanium (core) and fluorine (cladding) – the dopants provide the refractive index contrast required to allow confinement and guidance of light. Passing UV light through these fibres then excites the germanium centres and generates fluorescence. This background fluorescence can be of comparable (if not significantly higher) intensity to the signal obtained from the tissue. It is also broadband in nature and, therefore, can render the detection of tissue autofluorescence impossible.

Solving this problem requires careful selection of excitation and detection wavelengths (which can have an impact upon the signal and contrast observed) or the use of alternative optical fibres that exhibit significantly reduced background fluorescence. Such fibres are under

development (and include radiation hardened fibre bundles) but are not yet commonly available in commercial endomicroscopes. To illustrate the potential reduction in background, figure 3.11 shows fluorescence images recorded on a confocal microscope of two coherent fibre bundles manufactured by Fujikura. Figure 3.11(a) shows a Fujikura FIGH-30-650S, which has been used for endomicroscopy, including in a commercially available instrument (Cell-viZio™) manufactured by Mauna Kea Technologies [127]. Here the fibre bundle was excited at 488 nm, the detection band was 500-800 nm and significant autofluorescence is observed in the fibre cores. In figure 3.11(b) a Fujikura FIGR-10 (a radiation hardened, low fluorescence fibre bundle with no germanium dopants) has been imaged under identical conditions and a clear decrease is observed in the autofluorescence from the bundle. This implies the potential for suppression of the background fluorescence from the optical fibres, which would permit improved signal detection in fluorescence lifetime endomicroscopy of tissue autofluorescence. It should be noted, however, that the FIGR-10 is not suitable for confocal endomicroscopy because its larger core diameters (approximately 5.5 μm compared to 2 μm in the FIGH-30-650S) do not provide adequate optical sectioning. This is evident in figure 3.11(c), which shows a second image of the FIGR-10 with increased gain and excitation power and with the confocal pinhole open such that the structure of the bundle is visible. All images in figure 3.11 have the same field of view (77.55 x 77.55 μm), hence, the difference in core diameter is clear. Additionally, the FIGR-10 is a larger diameter bundle than the FIGH-30-650S and has a higher minimum bending radius meaning that it is less suitable for endoscopic procedures. Until such fibres are available and are suitable for both confocal detection and application to endoscopy, work towards FLIM endomicroscopy should be aimed at selecting the excitation and emission wavelengths in order to optimise the signal and contrast observed and to circumvent the problem of fibre background fluorescence.

The second problem brought about by the use of UV excitation wavelengths is the potential to cause photodamage to the sample. Maximum exposure limits for optical radiation are well defined for the skin and eyes [128] but not for other tissue types. For this reason, it can be difficult to choose maximum excitation powers for *in vivo* imaging and ethical considerations mean that only very low powers are acceptable for wavelengths below 400 nm. This means it is hard to realise *in vivo* FLIM as the large numbers of photons required lead to long acquisition times that are not suitable for clinical applications. For this reason, many *in vivo* autofluorescence lifetime studies have utilised single point measurements rather than imaging modalities (for example, see references [19-21, 64]). These no longer provide morphological information but do allow determination of the fluorescence decay (and spectral) properties of the tissue under investigation in a reasonable time with reduced excitation power compared to that required for imaging. Thus, such approaches

provide an opportunity for *in vivo* clinical fluorescence lifetime measurements, which are useful in their own right and for determining the optimum excitation and detection wavelengths for imaging technologies. The work presented in chapters 4 and 5 of this thesis centres on single point autofluorescence lifetime measurements in skin and GI cancer.

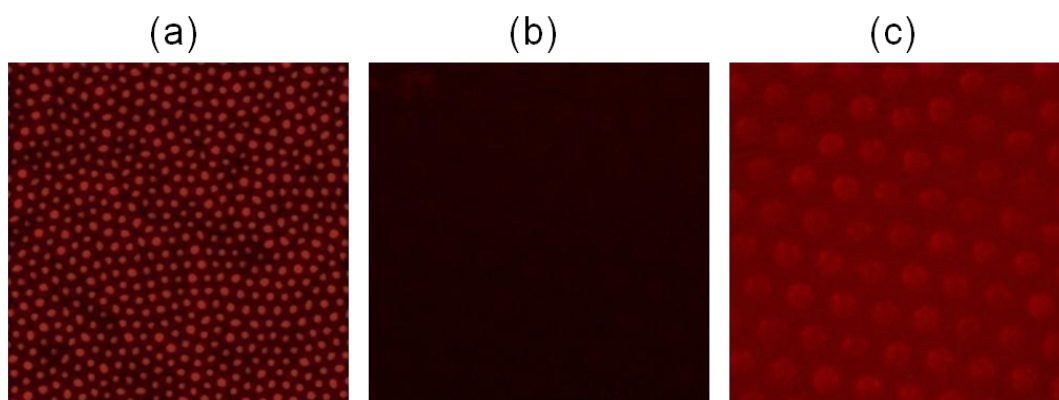


Figure 3.11. Images of the fluorescence intensity from two coherent Fujikura fibre bundles under 488 nm excitation: (a) FIGH-30-650S (pinhole diameter: 85 μm); (b) FIGR-10 imaged under identical conditions as in (a); (c) FIGR-10 with pinhole completely open (600 μm diameter) and with increased detector gain and excitation power such that the structure of the bundle is visible. For all images the emission band was 500-800 nm, the field of view is 77.55 x 77.55 μm and a 20x microscope objective was used.

3.4 Conclusions

This chapter, along with chapter 2, has aimed to provide an introduction to use of spectroscopy and microscopy to study optical signals (i.e. fluorescence and reflectance), particularly for biomedical applications. The remainder of this thesis now utilises some of these techniques in studies aimed at the development of optical instrumentation for medical diagnosis. Chapters 4 and 5 present the use of diffuse reflectance and time-resolved autofluorescence spectroscopy in investigations of the optical signatures of skin and GI cancer. As discussed above, these studies provide information – such as the optimum excitation and emission wavelengths for fluorescence measurements – which can be used to guide the development of future diagnostic equipment.

Chapters 6 and 7 of this thesis are centred on a quite different investigation involving the development of a novel technique to permit beam scanning in endomicroscopy. This novel approach is aimed at improving upon some of the drawbacks of current methodologies used in endomicroscopy (see section 3.3.1). While these experiments are purely optical in nature, their broad aim is still the same as the tissue studies mentioned above: to develop diagnostic instrumentation that utilises measurements of the fluorescence and/or reflectance signatures of

biological tissue. Thus, all of the work presented in the remainder of this thesis builds on the introduction provided by chapters 2 and 3.

Chapter 4: *In vivo* diffuse reflectance and time-resolved autofluorescence spectroscopy of skin cancer

As discussed in section 3.3.3, maximum permissible exposure limits for UV radiation are not well defined for tissues other than the skin and eyes [128]. For this reason, ethical approval for *in vivo* optical measurements using UV illumination is most readily obtained in these external tissues. Such optical measurements have the potential to be clinically useful – for example in the diagnosis of skin cancer – and can also help to provide evidence that optical spectroscopy and imaging could prove useful in the investigation of other internal tissues such as the intestine and oesophagus. When considering the investigation of external tissues such as the skin it does not seem immediately obvious that an endoscopic system would be necessary. None the less, the use of fibre optics for the delivery and collection of light adds substantial flexibility to any imager or spectrometer and, hence, can significantly improve the experience of the patient while also providing access to a wider range of external tissues. This chapter presents results from an *in vivo* spectroscopic study of the diffuse reflectance and time-resolved autofluorescence emission of skin cancer. This study involved the use of two fibre-coupled spectrometers [21, 72] and was carried out in collaboration with researchers at Lund University in Sweden. As mentioned above, this study served to illustrate the clinical applicability of optical spectroscopy for diagnostic purposes and, more importantly, it permitted the investigation of the optical signatures of skin cancer with the overall aim being the assessment of the potential of optical measurements for use in the diagnosis of skin lesions [129].

4.1 Motivation

Non-melanoma skin cancer (NMSC) represents a significant worldwide health problem. The most common forms of NMSC are basal and squamous cell carcinoma (BCC and SCC respectively) and, as a whole, NMSC accounts for 90% of all skin malignancies. Survival prognoses for NMSC are significantly better than those typical for cases of malignant melanoma, however, its incidence is still rising. In the UK and the USA respectively, there are approximately 100,000 and 1,000,000 new cases reported every year [130-131]. Additionally, amongst white populations in Europe, the USA, Canada and Australia the average increase in NMSC has been between 3% and 8% per year since the 1960s [132-133].

Skin cancer is usually diagnosed by a clinician based on a patient's clinical history and on a visual inspection of the lesion. In many cases the diagnosis still remains unclear and a biopsy, followed by histopathological analysis of the excised tissue, is required for confirmation. Taking a biopsy is an invasive procedure that is both painful and scarring, while the histopathological analysis is expensive and time consuming. This method of diagnosis can lead to the unnecessary excision of benign lesions [134] and, additionally, the delineation of NMSCs can be non-trivial meaning that a biopsy usually involves the removal of a significant section of the surrounding healthy tissue to ensure that no cancerous tissue remains. Clearly, a non-invasive method of assessing skin lesions, capable of avoiding unnecessary biopsies, would be desirable.

Optical techniques provide one potential route towards non-invasive diagnostic technologies and a number are being investigated for the detection and delineation of skin cancers. Some examples of optical methods that have been used to study biological tissue, in particular skin, include diffuse reflectance measurements [58-59], polarisation-resolved reflectance spectroscopy and imaging [14-15, 135], confocal reflectance microscopy [76-78], optical coherence tomography (OCT) [136] and Raman spectroscopy [137]. As discussed in chapters 2 and 3, a further opportunity to obtain contrast between healthy and diseased tissue is provided by the endogenous fluorescence of skin. The principal contributors to skin autofluorescence (at the excitation wavelengths used in this study) are NAD(P)H, flavins, keratin, collagen and elastin [42, 138] and, as discussed previously, the spectral and temporal properties of the emission from these fluorophores can report on altered metabolism, protein cross-links and disease state. Accordingly, there is significant interest in the development of instruments that can measure the autofluorescence (and other optical) properties of skin for clinical diagnosis – for example, see references [8, 60-61, 71, 139-141] – and, in particular, several instruments have been presented that are capable of monitoring both the fluorescence lifetime and emission spectrum of a sample [19-21, 64].

There have been several recent examples of the application of such instruments to skin including work on *ex vivo* tissue that showed significant differences between the fluorescence lifetimes of healthy and diseased tissue in NMSC [21, 31]. In addition, Rajaram *et al.* studied non-melanoma skin cancer *in vivo* using a combination of diffuse reflectance and fluorescence spectroscopy [8], while Blackwell *et al.* used an instrument recording fluorescence decays in four spectral channels to compare the fluorescence signature of human skin from diabetic and non-diabetic test subjects *in vivo* [142].

It is important to note here that tissue autofluorescence can be excited using either one- or two-photon absorption (see sections 2.1 and 3.2.3). While two-photon fluorescence microscopy

provides high resolution and permits depth resolved imaging, it is difficult to apply such non-linear excitation to wide-field imaging modalities that can quickly screen fields of view of many centimetres. Fast, wide-field imaging is desirable for clinical applications as it would allow the examination of a large number of patients in a short period of time. Single photon excitation can enable such wide-field imaging and so it is important to determine which combination of spectroscopic parameters – e.g. excitation and detection wavelengths for fluorescence measurements – provide the greatest contrast between normal tissue and (different) disease states. The work presented in this chapter involves the use of two spectrometers, both exploiting single photon excitation for fluorescence measurements, to investigate the optical signatures of skin cancer. The first system is a compact steady state spectrometer, which combines measurements of diffuse reflectance and fluorescence, while the second is a time-resolved fluorescence spectrometer that records fluorescence decays in multiple spectral channels. Both have been applied to an *in vivo* study of skin cancer in 25 patients [129].

4.2 Materials and methods

This study was carried out in collaboration with researchers from the departments of Oncology, Dermatology and Atomic Physics at Lund University, Sweden. The steady state spectrometer was developed at Lund University [72] while the time-resolved instrument was built at Imperial College London [21]. The Imperial system was first transported to Lund (by boat) and then both spectrometers were set up in a room in the dermatology clinic at Lund University Hospital, where all measurements took place. The remainder of this section introduces the two instruments used in the study and also explains the procedures employed in the collection and analysis of data.

4.2.1 Wide-field steady state spectrometer (A)

The first instrument used in this study was a wide-field steady state spectrometer that measured diffuse reflectance and fluorescence with three UV excitation wavelengths. This system (referred to as spectrometer A) comprised a small measurement head, which was coupled to a spectrometer via a 600 μm diameter multimode optical fibre. The spectrometer (USB4000, cylindrical lens and second order rejection filter installed, Ocean Optics, The Netherlands) provided 3 nm resolution and the system as a whole was controlled by a laptop computer running software written in Matlab® (MathWorks™). The spectrometer and measurement head fitted into a small (20 x 15 x 15 cm) carry

case and so the system was compact and easily transportable. A cross-sectional diagram of the measurement head is displayed in figure 4.1(a).

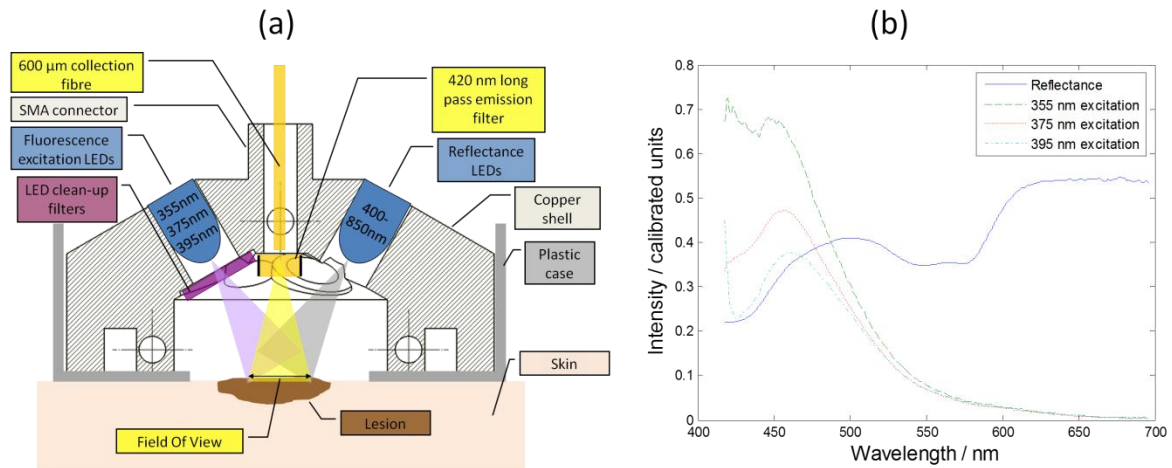


Figure 4.1. (a) Cross-section of the measurement head of the wide-field steady state spectrometer (instrument A) and (b) a healthy tissue dataset from an example patient enrolled in this study. The solid curve in (b) represents the diffuse reflectance spectrum while the dotted curves show the fluorescence spectra with different excitation wavelengths (see key). (a) has been reproduced, with permission, from reference [129].

The instrument incorporated seven CW light emitting diodes (LEDs) as illumination sources (Roithner Lasertechnik, Austria), of which four were used for the reflectance measurements and the remaining three provided excitation for the fluorescence measurements. The reflectance sources consisted of a 430 nm LED, a white light LED (Blue+Ce:YAG conversion, 450-700 nm) and a dual near-IR LED (750 nm and 850 nm) while the fluorescence excitation LEDs had wavelengths of 355 nm, 375 nm and 395 nm. The emission from the UV LEDs was passed through clean up filters (1 mm UG1 or UG11, Schott, Germany) and each source was directed toward the same central measurement site, where the illumination spot was elliptical with major and minor diameters of approximately 8 mm and 6 mm respectively. It should be noted that the fixed size of the illumination spot means that some unwanted signal may be acquired from the surrounding healthy tissue when measuring lesions that are smaller than the illuminated region. In this study, however, the diameter of most lesions was similar to or greater than that of the illumination spot, hence, this did not present a significant problem. Lastly, in order to minimise specular reflections, the system utilised non-contact optics and the illumination was introduced at an angle to the surface normal.

After illumination, the fluorescence or diffuse reflected light passed through a 420 nm long pass emission filter (3 mm GG420) and was collected by the 600 µm diameter optical fibre, which

delivered the light to the spectrometer. The LEDs and filters were housed in a copper shell, and the thermal dependence of the emissive yield was compensated for by measuring the voltage-current characteristics of the LEDs. Dark spectra were recorded by switching off the sources and these were subtracted from the clinical measurements. The reflectance was calibrated with a 50% diffuse grey reference (Oriel Instruments, USA) and the fluorescence intensity counts were corrected by measuring the emission from a calibrated white light source (Oriel Instruments, USA). For an entire measurement, the sample was illuminated by each source sequentially and a single reflectance spectrum over a broad spectral range can be generated by combining the data obtained with each of the four reflectance LEDs. In practice, however, differences in the LED beam profiles led to errors when combining the reflectance spectra, therefore, the data presented in the subsequent sections of this chapter consist solely of the reflectance measured using the white light LED.

In a typical acquisition, the reflectance and fluorescence spectra were recorded at each excitation wavelength in sequence, requiring a total acquisition time of approximately 30 s when averaging over 5 measurements. Thus, a dataset consists of a diffuse white light reflectance spectrum and a fluorescence spectrum for each of the three excitation wavelengths, as shown in the example dataset presented in figure 4.1(b).

This spectrometer was designed and built by Mikkel Brydegaard – a Ph.D. student in the Atomic Physics division of Lund University – and has been previously described in references [72, 129, 143].

4.2.2 Time-resolved fibre-optic probe spectrofluorometer (B)

The time-resolved fluorescence spectrometer (B) was originally designed and built by De Beule *et al.* [21] and, for the purposes of this investigation, it was configured as shown in figure 4.2(a). Excitation was provided by two pulsed laser sources: a frequency tripled ultrafast Yb:glass fibre laser (UVPower355, Fianium Ltd., UK), which provided 10 ps pulses at 355 nm with a repetition rate of 37.1 MHz (referred to as the UV laser); and a diode laser (LDH-P-C-440B, PicoQuant GmbH, Germany), which emitted 50-150 ps pulses at 445 nm with an adjustable repetition rate of up to 40 MHz (referred to as the blue laser). The outputs of the two lasers were first combined using a dichroic beamsplitter and then coupled into the excitation channel of a custom built fibre-optic probe (FiberTech Optica, Canada). This probe was used to both relay excitation light to the sample and to deliver fluorescence to the detection arm of the spectrometer. It comprised seven multimode optical fibres (each with a core diameter of 200 μm and a numerical aperture (NA) of 0.22), of which one was used to deliver excitation and six were used to collect fluorescence. At the distal end of the

probe, the fibres are arranged with the central excitation fibre surrounded by the six collection fibres (inset at bottom left of figure 4.2(a)) in order to provide a reasonable illumination and collection efficiency while maintaining a relatively small probe size.

Spectral bandpass filters were employed in the illumination branch of the system to narrow the emission of the laser diode (Z440/20x, Chroma Technology, USA) and to ensure that none of the fundamental radiation (at 1.06 μm) from the Yb:glass fibre laser was transmitted to the sample (UG1, Schott, Germany). Computer controlled mechanical shutters in the two beam paths were used to switch between the two excitation sources and variable neutral density (ND) filters acted to limit the optical power at the sample to 10 μW of UV light and 50 μW of blue light. Limiting the optical power in this way ensured that no photodamage could be caused as the power levels used were below the maximum permissible exposure (MPE) for skin for exposure times as high as 1000 s.

The output from the distal end of the fibre-optic probe was directed toward the measurement site and a plastic spacer was used to ensure that the distance between the tip of the probe and the sample was fixed (at approximately 1 mm) for all patients. This resulted in an area of approximately 0.2 mm² being illuminated and the fluorescence was collected by the six detection fibres. The output from these collection optical fibres was then imaged onto the input slit of a grating spectrometer (MS125 1/8m, Lot-Oriel, UK), which was attached to a 16 channel multi-anode PMT detector (PML-16-C, Becker-Hickl GmbH, Germany). In the detection branch of the proximal end of the fibre bundle, the six collection fibres are positioned in a line (inset at middle left of figure 4.2(a)) in order to maximise the coupling into the input slit of the spectrograph.

The PMT was linked to a computer equipped with a TCSPC card (SPC-730, Becker-Hickl GmbH, Germany) running custom written LabVIEW (National Instruments, USA) software that recorded fluorescence decay profiles in each of the 16 spectral channels. A motorised filter wheel containing two emission filters (HQ 375 LP and E475 LP v2, Chroma Technology, USA) was also placed in the beam path between the fibre probe output and the spectrograph to ensure that no scattered excitation light reached the detector. The whole system was mounted on a 60 x 60 cm² optical breadboard to facilitate transportation and was fully enclosed for safe use in a clinical setting.

In a typical acquisition data was acquired sequentially, first using UV excitation and then using blue excitation. For both excitation wavelengths the collection time was 5 s and three repeat measurements were made in each case, giving a total acquisition time of approximately 30 s. The raw data obtained with this system thus comprised two sets of 16 spectrally resolved fluorescence decays – one for each excitation wavelength – with three identical measurements performed in all

cases. This is clear in figure 4.2(b) where an example dataset, obtained using 355 nm excitation, is presented.

In order to properly analyse the raw data from this system it was first necessary to determine the wavelength range collected by each PMT channel and to determine their relative sensitivity. By illuminating the distal end of the fibre probe with white light passed through a calibrated monochromator, it was possible to determine the spectral centre and bandwidth of each detection channel. The relative sensitivity of each channel was determined by recording the signal obtained from a calibrated white light source (LS-1-CAL, Ocean Optics, The Netherlands) and this information was then used to correct the intensity measured in each channel [21, 129, 143].

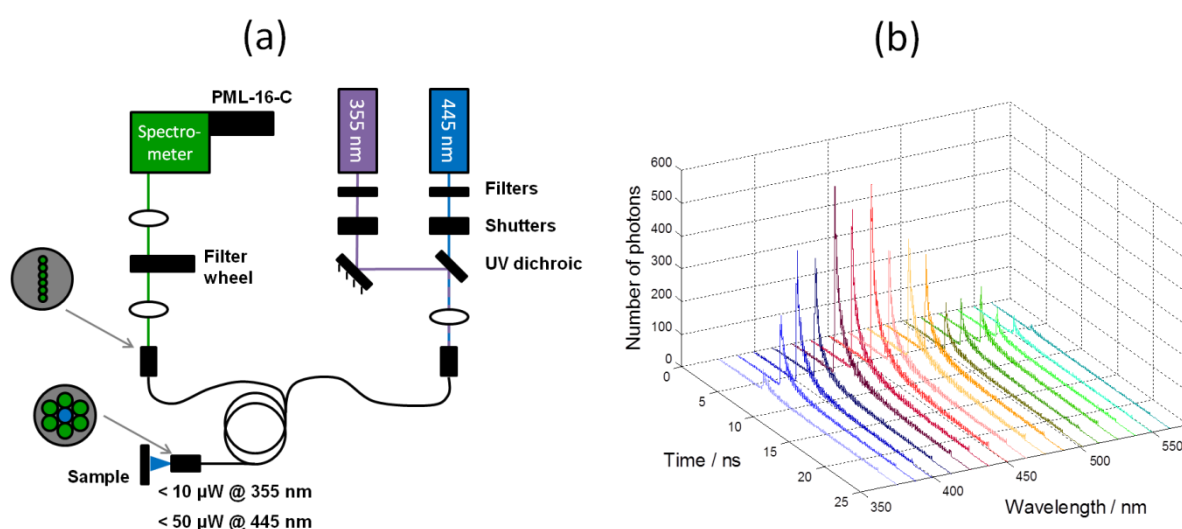


Figure 4.2. (a) Optical layout of the time-resolved fluorescence spectrometer (instrument B) and (b) an example of the raw data acquired with this system. The graph in (b) shows 16 spectrally resolved fluorescence decays obtained from human skin *in vivo* using an excitation wavelength of 355 nm. Similar data is obtained when exciting at a wavelength of 445 nm and, as such, the entire dataset consists of two sets of 16 spectrally resolved fluorescence decays. Diagram in (a) has been reproduced, with permission, from reference [129].

4.2.3 Experimental procedure: *in vivo* clinical measurements of skin cancer

As discussed above, the two instruments described in sections 4.2.1 and 4.2.2 were assembled in the Dermatology clinic of Lund University Hospital. They were then used to investigate the optical signatures of 27 clinically diagnosed skin lesions on a total of 25 patients (all of skin phototypes I-III) prior to surgical excision of the measured region. For each lesion, a measurement of the neighbouring healthy tissue was also made for comparison. Patients were recruited by the clinical

team in Lund and the study was conducted with the approval of the local ethics committee, in accordance with the ethical principles of the declaration of Helsinki.

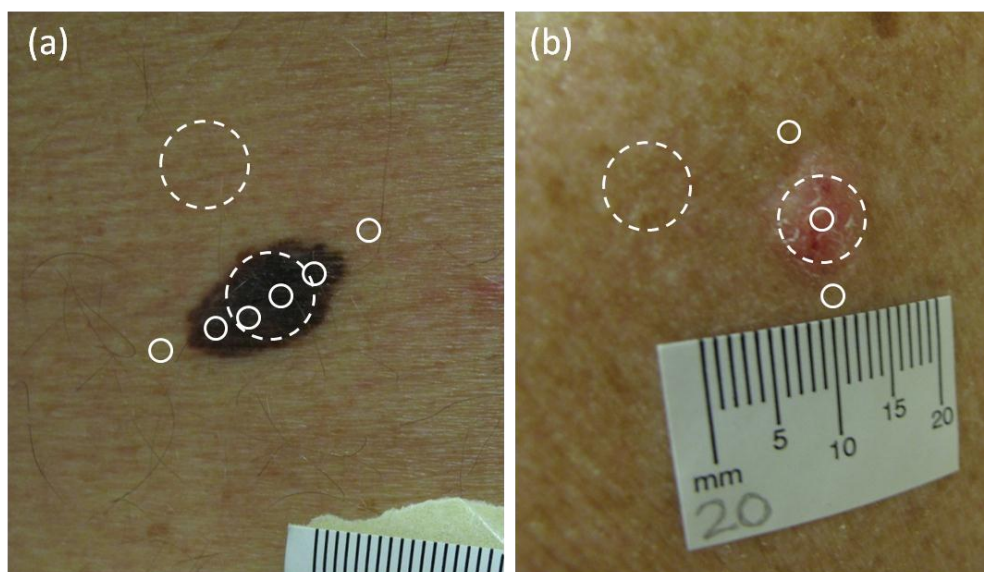


Figure 4.3. Photographs of two example skin lesions indicating the locations of the single-point fluorescence lifetime measurements (solid white circles) and the wide-field steady state measurements (dotted white circles). The images show a malignant melanoma of the back (a) and a Nodular BCC of the left shoulder (b). Figure reproduced, with permission, from reference [129].

With the steady state spectrometer (A), two measurements were made for each lesion: one on the lesion itself and one on the neighbouring healthy skin. Using instrument B, two measurements were performed on the peri-lesional skin surrounding the lesions while between one and four measurements were made on the lesions themselves, depending on their size. Photographs of two example lesions are presented in figure 4.3 where the solid white circles show the locations of the time-resolved fluorescence measurements and the larger dotted white circles indicate the regions at which the steady state spectrometer was applied. After the measurements had been performed, the entire region irradiated with instrument B was excised and sent for histopathology. This ensured that any potentially photodamaged tissue was removed from the patient and also meant that the data recorded could be subsequently correlated with the histological diagnoses.

For identification purposes, each lesion was assigned a unique number and the characteristics of the lesions measured with each spectrometer are summarised in table 4.1. As is clear from the table, not all lesions were measured with instrument A. This was because its collection configuration meant that some lesions were unsuitable for measurement because their highly curved morphology made it difficult to correctly position the spectrometer.

Table 4.1. Summary of the skin lesions studied with each spectrometer, showing the histological diagnoses.

Lesion type (histological diagnosis)	Number of lesions	
	Instrument A	Instrument B
BCC	8	10
SCC	1	3
Benign naevus	2	2
Dysplastic naevus	1	1
Malignant melanoma	3	3
Actinic keratosis	3	3
Other	4	5
Total number of lesions	22	27
Total number of normal skin sites	23	27
Total number of measurements	45	54

4.2.4 Fluorescence decay analysis

As discussed in sections 2.2.4 and 2.3, autofluorescence from biological tissue normally exhibits a complex exponential decay profile due to the multitude of fluorophores present and their different states (e.g. oxidised/reduced and free/protein bound). With instrument B, two sets of 16 spectrally resolved fluorescence decays were collected and it is likely that each decay contains contributions from many different fluorescent species. However, as only a limited number of photons are collected in each spectral channel, a double exponential model was fitted to the fluorescence decay curves. This was performed according to equation (7) with $n = 2$ (see section 2.2.4) and was achieved using in-house software written in Matlab® (MathWorks™). Using the extracted decay parameters it was then possible to calculate the mean fluorescence lifetime in each spectral channel according to equation (8), again with $n = 2$ (section 2.2.4).

In order to accurately fit an exponential model to observed fluorescence decay data it is necessary to account for the instrument response function (IRF), which describes how the instrumentation responds to an input delta pulse. In this experiment, the IRF was determined by measuring a solution of the dye DASPI (2-(p-dimethylaminostyryl)-pyridylmethyl iodide, Radiant Dyes Laser Accessories GmbH, Germany) in water. DASPI is a fluorophore that has a fluorescence lifetime of less than 50 ps, which is significantly shorter than the instrument response time and, therefore, provides a good estimate of the IRF. This measurement did not provide an IRF in all

spectral channels (as the emission spectrum of DASPI does not cover the entire collection range of the spectrograph), so one representative spectral channel was selected and used for fitting all of the spectral channels. This approach is reasonable as the variation in the shape of the IRF between different spectral channels is low [144]. The temporal shift in the IRF across different spectral channels, on the other hand, is significant and this was compensated for by introducing an adjustable temporal offset into the exponential fitting model.

It was observed that the fluorescence lifetime (and the contrast in fluorescence lifetime between healthy and diseased regions of tissue) did not vary significantly with emission wavelength (see section 4.3.3 for details). For this reason, and to summarise the fluorescence decay data from each measurement site, a spectrally averaged mean fluorescence lifetime was calculated. This was achieved by averaging the spectrally resolved mean lifetime values and weighting them according to the fluorescence intensity recorded in each channel. Hence, for each measurement site a single intensity-weighted average fluorescence lifetime was obtained. The difference between these spectrally averaged mean lifetimes was then calculated for the normal and lesional tissue measured on each patient (i.e. $\Delta\tau = \tau_{lesion} - \tau_{normal}$) and this difference was investigated in order to assess its diagnostic potential. The mean emission wavelength was also calculated from the data recorded with instrument B and this was analysed in the same way as the spectrally averaged fluorescence lifetime. As an aside, it is interesting and important to note here that investigating differences in fluorescence lifetime or emission wavelength in this way circumvents potential difficulties introduced by inter-patient variation in the data.

Finally, a statistical analysis was performed on the paired differences in the spectrally averaged fluorescence lifetime and the mean emission wavelength ($\Delta\tau$ and $\Delta\lambda$ respectively). This was achieved using the Wilcoxon signed rank test and it served to determine whether any significant differences existed between the healthy and diseased tissue.

4.2.5 Spectral data analysis: singular value decomposition (SVD) and discriminant analysis (DA)

The spectral data acquired in this investigation consisted of a white light reflectance spectrum and five fluorescence spectra. Instrument A provided the reflectance spectrum and three fluorescence spectra (excited at 355 nm, 375 nm and 395 nm) while instrument B provided a further two fluorescence spectra with excitation wavelengths of 355 nm and 445 nm. The first spectral analysis involved the comparison of the data obtained with 355 nm excitation with both spectrometers and it

was confirmed that the measurements were consistent. Further analysis then focussed mainly on the data recorded using spectrometer A as the spectra provided by this instrument were more finely sampled and covered a wider spectral range than those obtained with system B.

On inspection of the raw data it was clear that there was a large degree of inter-patient variation in the observed spectra. To illustrate this, the mean diffuse reflectance and fluorescence intensity were plotted for each disease type (and for healthy skin) along with the corresponding standard deviations (see section 4.3.1). Despite the observed inter-patient variability, there still appeared to be differences in the spectral signatures of healthy and diseased tissue. In order to quantify these differences and to assess their diagnostic potential, singular value decomposition (SVD) was used to break the acquired data down into a number of base spectral components [145-146]. It was then possible to use the relative contributions of these base components as input parameters for discriminant analysis (DA) [147-149] and this was achieved in one of two ways. Firstly, linear discriminant analysis (LDA) was implemented and, secondly, diagnostic models were generated using multivariate regression. Both methods are explained in detail later in this section.

This approach is similar in concept to the use of SVD or principal component analysis (PCA) for feature selection followed by DA for lesion classification, used in references [150-152]. Additionally, it should also be noted that a number of detailed model-based spectroscopic analysis approaches have been developed, which allow physical optical parameters to be estimated from measured diffuse reflectance and/or fluorescence spectra. Such optical parameters include the reduced scattering and absorption coefficients, total haemoglobin concentration, scatterer size and density, blood oxygen saturation and native fluorophore contributions, and examples of the use of these techniques can be found in references [8, 71, 153-154]. In this study, however, the aim was to establish whether differences exist between different lesion types and not (necessarily) to characterise lesions in terms of real optical properties. For this reason, spectral decomposition was undertaken as it is simpler than modeling spectra from first principles and permits parameterisation of the data (and, hence, the measured regions) without *a priori* knowledge of the constituent chromophores contained within the tissue.

In order to carry out this analysis, the numerical values describing the four spectra from the steady state system (A) were first concatenated (joined together) to produce one 'effective spectrum' for each measurement site. A matrix, M , was then generated that contained the effective spectrum for every measured region, with each row representing a measurement site and each column representing a wavelength band. Software written in Matlab® (MathWorks™) incorporating the inbuilt SVD function could then be used to decompose M into its primary spectral components:

$$M_{n=1\dots N, \lambda} = U_{n=1\dots N, n=1\dots N} \Sigma_{n=1\dots N, n=1\dots N} V_{\lambda, n=1\dots N}^* \quad (11)$$

In equation (11), V^* is the conjugate transpose of V , which is itself a matrix containing a new set of base spectral components (loadings) of the matrix M . U is a matrix describing the relative contribution of each spectral component to the individual measurements (scores), while Σ is a diagonal matrix whose elements represent the overall significance of each base spectrum (the singular values). All three matrices are sorted in order of spectral significance. Thus, by plotting Σ on a log scale it is possible to find the number of components that are significantly contributing to the effective spectra and, hence, decide upon a truncation (tr), which will remove any redundant information (e.g. noise or the same chromophore measured in multiple bands) from the data. The matrix M can then be approximated as shown in equation (12).

$$\begin{aligned} M_{n=1\dots N, \lambda} &\approx \hat{M}_{n=1\dots N, \lambda} \\ &\approx U_{n=1\dots N, s=1\dots tr} \Sigma_{s=1\dots tr, s=1\dots tr} V_{\lambda, s=1\dots tr}^* \end{aligned} \quad (12)$$

The truncated matrix, U , now contains only the scores of the most significant base spectral components for each measurement site. As discussed above, these values could then be used as input parameters for DA. Firstly, LDA was applied to generate a diagnostic model using the data in U from all but one measurement site based on the corresponding histological diagnoses (i.e. BCC = 1, healthy = 0) and this model was then used to diagnose the remaining site. This was achieved with the Matlab® (MathWorks™) software discussed above using the inbuilt LDA function ('classify'). The second approach was similar but, in this case, multivariate linear regression [155-156] was used to generate the diagnostic model according to equation (13), where D_j is a diagnostic parameter for measurement site j and β_i are the regression coefficients for each of the scores in U .

$$D_j = \beta_0 + \sum_{i=1}^{tr} \beta_i U_{j,i} \quad (13)$$

The β_i values are first determined by training the data in U (from all but one site) on their histological diagnoses. In two dimensions this is analogous to fitting a straight line to the data in a plot of diagnosis versus U . For input values of U with unknown diagnosis, the output of equation (13), D_j , is then a continuous variable. Thus, to diagnose a measurement site a threshold was applied to this output parameter in order to produce a discrete value indicating whether the tissue was healthy or diseased. As above, this was accomplished using software written in Matlab.

In order to ascertain the diagnostic potential of the spectral data, this leave-one-out methodology was undertaken for all the measured regions. It should be noted, however, that these

algorithms were only used to differentiate between BCCs and healthy tissue as too few measurements were made of other lesion types to produce reliable prediction algorithms.

This analysis examined the use of a variety of combinations of spectra in the effective spectrum – for example, each individual spectrum alone, all four combined, and all possible combinations of two or three spectra. Additionally, the effect of dividing each fluorescence spectrum by the reflectance prior to their use in the effective spectrum was also investigated. This provides a partial compensation for re-absorption of the fluorescence emission and, hence, goes some way toward estimating the intrinsic fluorescence [157-158]. Again, this was performed using each individual ‘intrinsic’ fluorescence spectrum and using all combinations thereof. Finally, for each effective spectrum, all possible truncation values were tested. Having found the approach with the best diagnostic accuracy (number of correct diagnoses / total number of measurements), the optimum sensitivity and specificity obtained with the spectral data were calculated.

4.3 Results

4.3.1 Steady state spectral analysis

The first analysis applied to the steady state spectra was to compare the data obtained using 355 nm excitation with both spectrometers. Figure 4.4 shows the fluorescence spectra acquired from two patients diagnosed with BCC (lesion numbers 11 and 18 respectively). The two graphs at the top of the figure show the data from the steady state system for healthy (figure 4.4(a)) and lesional (figure 4.4(b)) tissue while the two lower plots show the data from the time-resolved system ((c) – healthy tissue, (d) – lesional tissue). Firstly, these graphs illustrate the improved spectral resolution and detection range of instrument A relative to spectrometer B. More importantly, however, they also demonstrate the qualitative agreement of the spectra recorded with the two different instruments. While the absolute intensities shown are not the same – due to differences in detector sensitivity, collection efficiency and excitation power – the shapes of the curves and the relative intensities are in good agreement across the two instruments. As discussed in section 4.2.5, because of the qualitative agreement of the two systems and the better resolution and detection range of spectrometer A, further analysis of the steady state spectra focussed mainly on the data collected with instrument A.

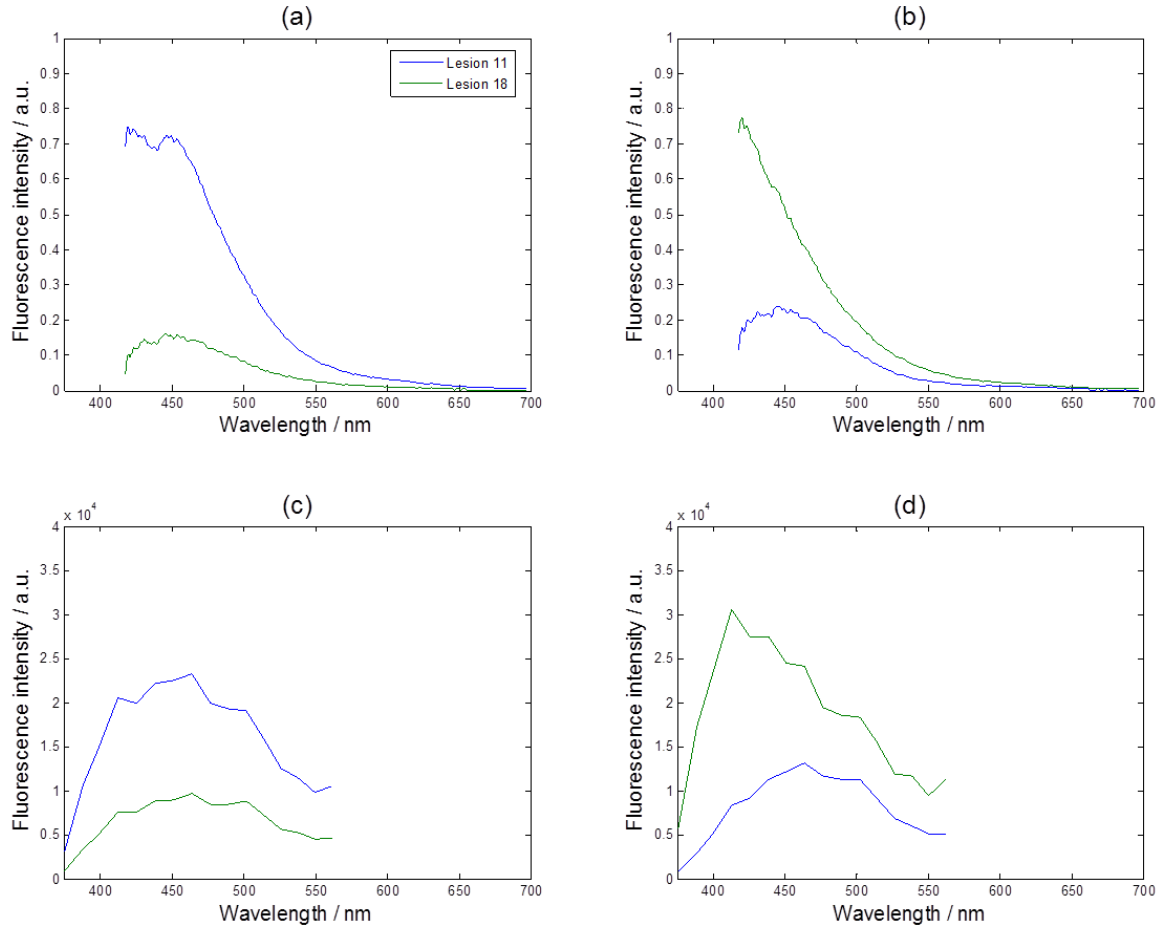


Figure 4.4. Comparison of the steady state spectra acquired with instruments A and B using 355 nm excitation. The upper graphs show the healthy (a) and lesional (b) measurements made with instrument A on two BCCs (lesion numbers 11 and 18). The lower two plots show data acquired from the same regions using the time-resolved spectrometer (instrument B): (c) – healthy tissue; (d) – lesional tissue.

Figure 4.5 presents an example of the spectra recorded with instrument A. This data was acquired from the lesional and adjacent ‘normal’ tissue on a patient subsequently diagnosed with BCC and the graphs show a diffuse reflectance spectrum and a fluorescence spectrum for each of the three excitation wavelengths. The instrument was calibrated as discussed in section 4.2.1, therefore, the spectral intensities can be compared between sites.

It is clear from figure 4.5 (and figure 4.4) that there are spectral differences between BCCs and the adjacent peri-lesional tissue for the examples shown. It should be noted, however, that there was considerable inter-patient variability in the acquired spectra. To illustrate this, figure 4.6 shows the mean diffuse reflectance and fluorescence spectra of healthy and diseased tissue

measured on patients with BCCs (8 lesional and 7 healthy measurements) along with their associated standard deviations (which provide an estimate of the inter-patient variability).

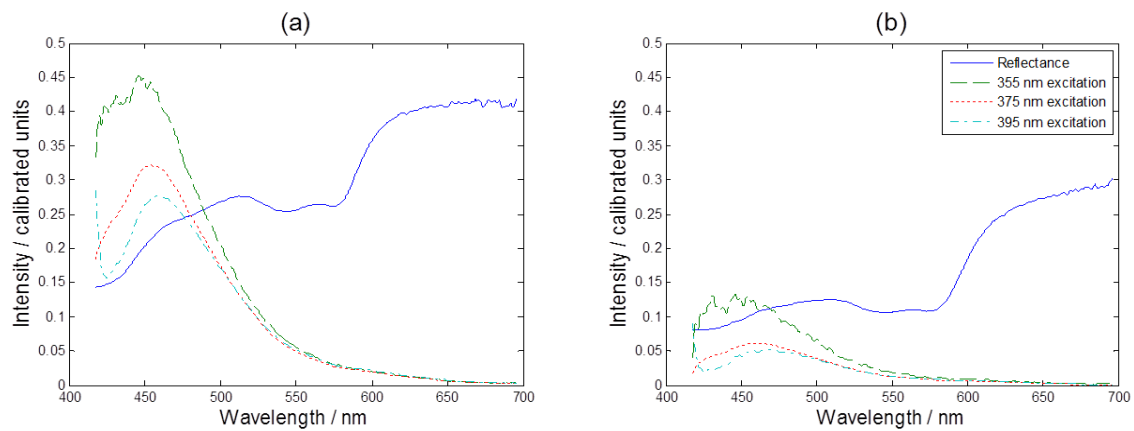


Figure 4.5. Example spectra from normal tissue (a) and lesional tissue with a histological diagnosis of BCC (b) recorded using spectrometer A. Spectral intensities can be compared between sites due to the calibration of the instrument discussed in section 4.2.1. Solid curve – diffuse reflectance spectrum, dashed curves – autofluorescence emission spectra at different excitation wavelengths (see key). Figure adapted, with permission, from references [129, 143].

The plots in figure 4.6 clearly demonstrate that the mean diffuse reflectance and the mean fluorescence spectra are lower for BCCs than they are for the surrounding healthy tissue and this is true for all three fluorescence excitation wavelengths. The same trend was also observed when investigating the spectra from all lesion types together (i.e. when considering all the skin cancer lesions as a single disease type). All of the recorded spectra exhibited a large degree of inter-patient variability, however, and this is also evident in figure 4.6 where the dotted curves indicate a spread of ± 1 standard deviation. Additionally, this variability can be observed in figure 4.4 where the 355 nm excited fluorescence is seen to increase on lesion number 11 relative to the surrounding healthy tissue while the opposite trend occurs for lesion 18. This variation in the measured spectra can be attributed to inter-patient differences in factors such as skin photo-type, level of sun exposure, age and the location of the measurement site on the body [129].

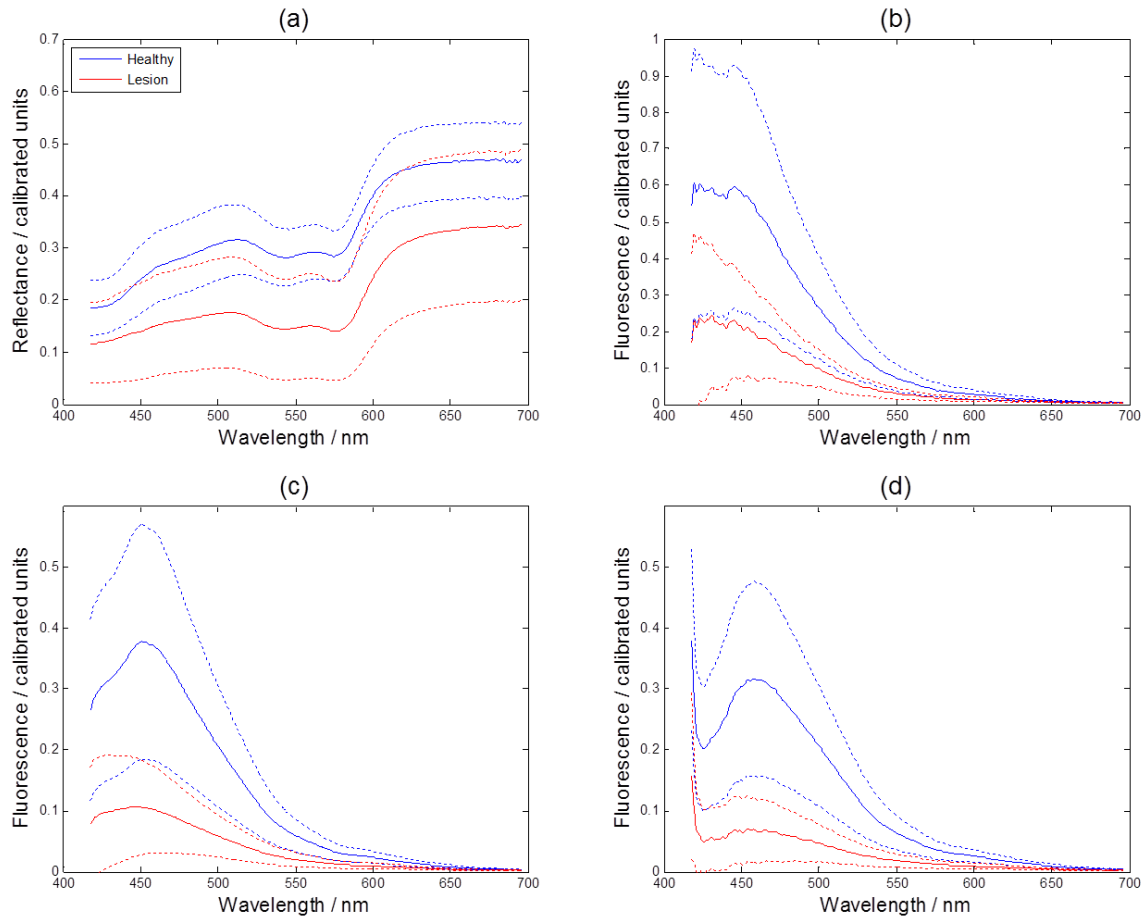


Figure 4.6. Graphs showing the mean diffuse reflectance (a) and the mean fluorescence spectra with excitation wavelengths of 355 nm (b), 375 nm (c) and 395 nm (d) for all BCC measurement sites. Blue curves show data from healthy skin while the red curves represent the lesional measurements. Solid lines show the mean intensity values and the dotted lines give estimated confidence intervals of ± 1 standard deviation. (a) and (c) have been reproduced, with permission, from reference [129].

4.3.2 SVD and DA using steady state spectral data

As discussed in section 4.2.5, SVD was implemented to compress the diffuse reflectance and fluorescence spectra from all 15 BCC measurements made with the steady state spectrometer into a small number of base spectral components (as shown in figure 4.7) in order to permit the subsequent use of DA. It should be noted that various combinations of diffuse reflectance and fluorescence emission spectra were tested in the effective spectrum in this analysis, including ratios of fluorescence to diffuse reflectance, which provide estimates of the intrinsic fluorescence. For BCCs, using both LDA and multivariate regression to generate diagnostic models, the best diagnostic accuracy was obtained when using the ratio of the 375 nm excited fluorescence spectrum to the diffuse reflectance. Hence, this data is displayed in figure 4.7.

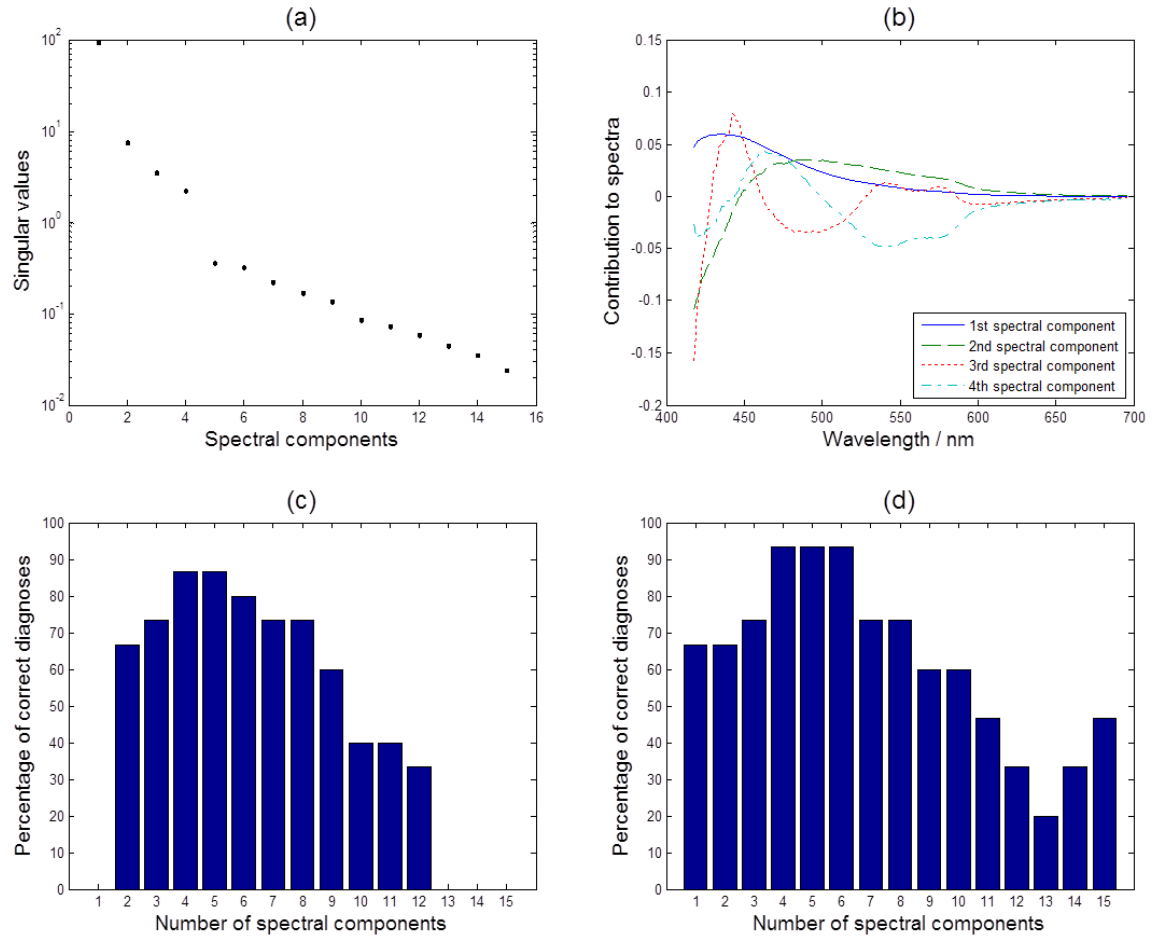


Figure 4.7. Output of SVD and DA – using the ratio of the fluorescence (375 nm excitation) to the reflectance as the effective spectra – for all BCC measurement sites. (a) shows the singular values (i.e. the overall significance) of the individual spectral components generated by the SVD while (b) shows the contributions of the first four spectral components to the fluorescence/reflectance ratio. The bar graphs in (c) and (d) represent the diagnostic accuracy provided by the prediction models as a function of the number of spectral components used (i.e. as a function of the truncation): (c) – LDA; (d) – multivariate regression. (a) and (b) are reproduced, with permission, from reference [129].

The singular values in Σ (figure 4.7(a)) decrease steadily down to the noise floor and, after the 4th spectral component, there is a considerable drop in magnitude indicating that it may be possible to truncate the data here without a significant loss of information. Figure 4.7(b) shows the contributions of the first four spectral components to the fluorescence/reflectance ratio. As is typical in SVD, the first component contains the dominant spectral features and, in this case, has a form similar to a characteristic skin fluorescence spectrum. The remaining components indicate relative changes in either the fluorescence or the reflectance. In particular, the 2nd spectral component implies a redshift of the fluorescence emission (relative to the diffuse reflectance spectrum) while

addition of the 3rd and 4th components will bring about changes in the contributions of the two haemoglobin absorption peaks at wavelengths of 540 nm and 575 nm.

As stated above, figure 4.7(a) shows that truncation of the data after the 4th spectral component is reasonable due to the sudden drop in the magnitude of the singular values. For completeness, however, many different truncation points were tested when implementing the DA in order to find the one with the highest diagnostic accuracy. Thus, the diagnostic accuracy was plotted as a function of the number of spectral components included in the diagnostic model for LDA (figure 4.7(c)) and for multivariate regression followed by appropriate thresholding (d). As is evident in the figure, with both methods the optimum number of spectral components (i.e. the minimum number of components that provides the highest diagnostic accuracy) was indeed found to be four. Using LDA with truncation after the 4th component, the sensitivity, specificity and total diagnostic accuracy were found to be 100%, 71% and 87% respectively, which corresponded to just two false positive diagnoses in a total of 15 measurement sites (8 lesion, 7 normal). Multivariate regression was seen to provide a small improvement in the specificity of diagnosis and, in this case, the sensitivity, specificity and diagnostic accuracy were shown to be 100%, 86% and 93% respectively – this time corresponding to just a single false positive diagnosis.

These results suggest that SVD of steady state fluorescence and reflectance spectra followed by DA has the potential to aid the diagnosis of skin cancer. Future work in this area should now involve the collection of more *in vivo* data – firstly, to confirm the results presented regarding BCCs and, secondly, to permit the investigation of other lesion types, such as SCCs and malignant melanomas. It would then not only be possible to look at the ability of the spectral data to classify a site as ‘healthy’ or ‘unhealthy’ but also its ability to classify a lesion in terms of its type [129]. Additionally, it would also be interesting to investigate the potential of applying spectral decomposition and DA to time-resolved fluorescence spectra. This would add an additional dimension to the diagnostic models described in this section and may provide further improvements in the diagnostic accuracy.

4.3.3 Time-resolved autofluorescence measurements

The first time-resolved fluorescence analysis performed involved investigation of the fluorescence lifetimes recorded in each of the spectral channels. Figure 4.8 shows the mean fluorescence lifetimes measured in each spectral channel for healthy and diseased tissue excited using the UV (a) and blue (b) laser, averaged over all BCC patients. Firstly, it can be observed that the fluorescence lifetime did not vary significantly with emission wavelength over the spectral range containing the

peak fluorescent signal (425-540 nm for UV excitation and 475-550 nm for blue excitation). A second important observation is that the contrast observed in fluorescence lifetime did not vary substantially with emission wavelength. For these reasons, further analysis presented in this section focuses on trends observed in the spectrally integrated, intensity weighted average fluorescence lifetimes for each measurement site (and for both excitation wavelengths). Calculating a spectrally integrated mean fluorescence lifetime in this way increases the accuracy of the lifetime determination by incorporating a larger number of photons and provides a simple, single parameter with which to compare measurement sites.

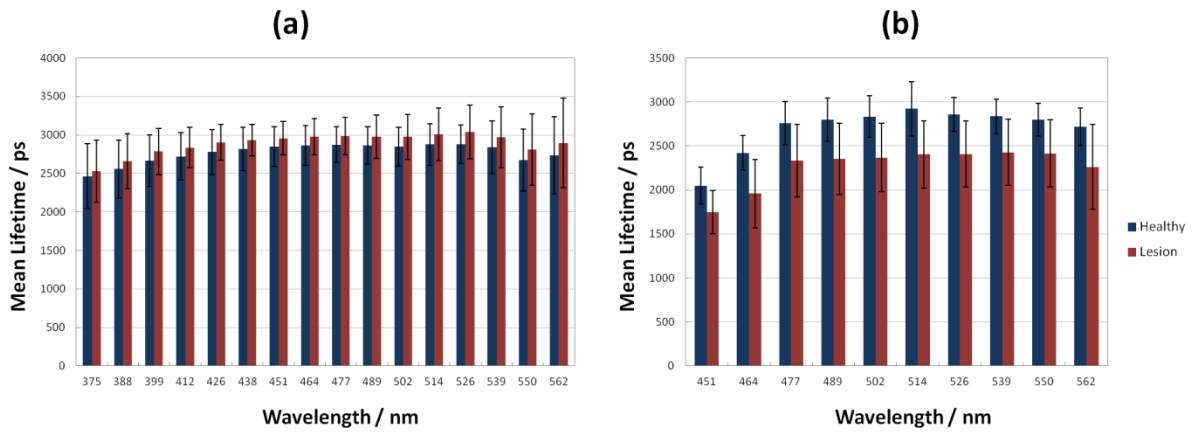


Figure 4.8. Graphs showing the mean fluorescence lifetime in all spectral channels for healthy and lesional tissue (see key on right hand side) for (a) 355 nm and (b) 445 nm excitation. The mean values were calculated over all BCC patients and the error bars represent uncertainties of ± 1 standard deviation.

Using the spectrally integrated mean lifetime values, the intra-patient variability was first quantified by comparing the lifetimes obtained from the two ‘normal’ peri-lesional sites measured on each patient (see section 4.2.3 and, in particular, figure 4.3). The differences in the spectrally averaged mean lifetime (i.e. $\Delta\tau = \tau_{normal1} - \tau_{normal2}$) are shown in figure 4.9. For both 355 nm and 445 nm excitation, the differences are evenly distributed about zero with mean values of -18 ps and 19 ps respectively. Lesion 6 shows the largest difference in mean lifetime between the two normal sites for both excitation wavelengths and this was attributed to the presence of scar tissue in the first peri-lesional measurement site. As an estimate of the error on the fluorescence lifetime, the mean standard deviations of the normal tissue measurements were also calculated and these were found to be 75 ps with 355 nm excitation and 175 ps with 445 nm excitation.

Significant variations in the measured lifetimes were also observed between patients, and this is evident in figure 4.8 where the error bars indicate a considerable spread in fluorescence

lifetime across different patients. This is likely to be due to the factors discussed at the end of section 4.3.1 (e.g. skin photo-type, level of sun exposure, age and body location). However, it is important to note that most of the data presented in this section is concerned with differences in lifetime between the healthy and lesional tissue measured on individuals and this inherently circumvents the issue of inter-patient variability.

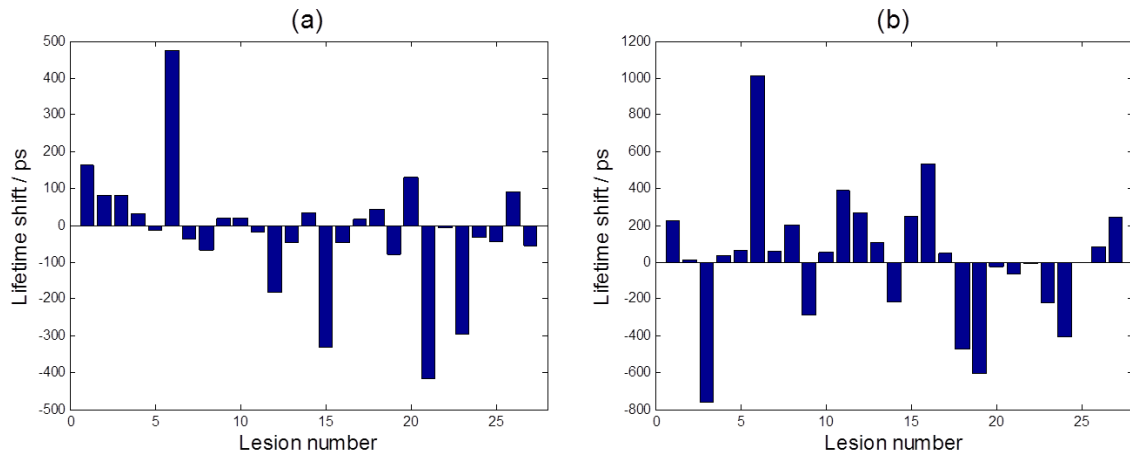


Figure 4.9. Graphs showing the lifetime shifts between the two measurements of the healthy tissue surrounding each lesion (i.e. $\Delta\tau = \tau_{\text{normal } 1} - \tau_{\text{normal } 2}$) for (a) 355 nm and (b) 445 nm excitation. Figure adapted, with permission, from reference [129].

For BCCs, the paired differences in the spectrally integrated mean lifetime between the healthy and diseased regions of tissue measured on each patient were now investigated. The spectrally weighted healthy and lesional lifetimes, averaged across all BCC patients and including all spectral channels, were respectively 2770 ± 250 ps and 2880 ± 409 ps with UV excitation and 3130 ± 413 ps and 2240 ± 480 ps with blue excitation (where the figures report the mean $\pm$ one standard deviation). Additionally, figure 4.10 shows the shift in the spectrally weighted lifetime (i.e. $\Delta\tau = \tau_{\text{lesion}} - \tau_{\text{normal}}$) observed for individual patients.

It is apparent from figure 4.10(a) that there is no clear trend in the lifetime shifts of BCCs observed with UV excitation. For blue (445 nm) excitation (figure 4.10(b)), on the other hand, the fluorescence lifetimes of BCCs are consistently lower than those of the surrounding peri-lesional skin (indicated by negative lifetime shifts for all lesions). The mean lifetime decrease was calculated as 886 ps and the lifetime shifts observed in all but one case (lesion 1) were also seen to exceed the estimate of the mean intra-patient variability reported above (175 ps).

Finally, the mean emission wavelength was calculated for each BCC measurement site and for both excitation wavelengths. Using the time-resolved spectrometer (B), no significant difference was observed in the mean emission wavelength of healthy skin and BCCs for either excitation wavelength (data not shown).

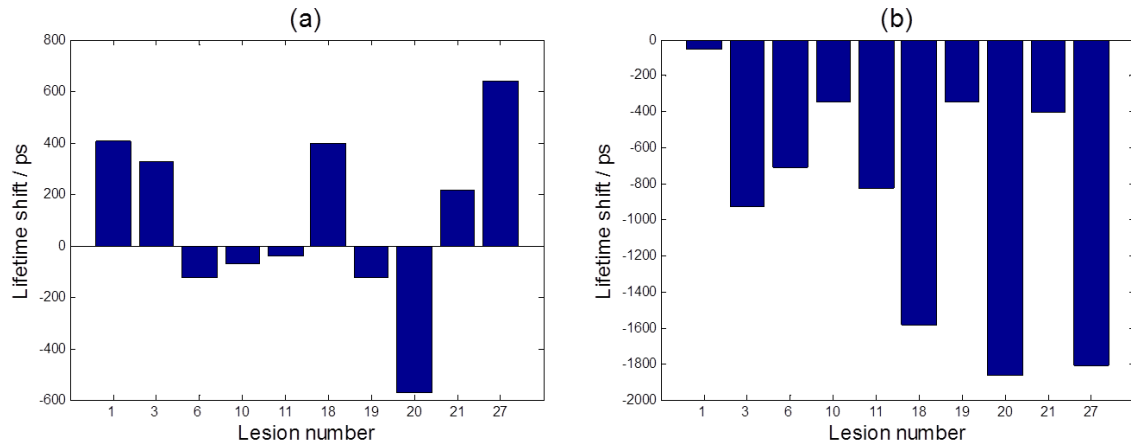


Figure 4.10. Graphs showing the lifetime shifts for all BCC lesions (i.e. $\Delta\tau = \tau_{\text{lesion}} - \tau_{\text{normal}}$) with (a) UV and (b) blue excitation. Figure adapted, with permission, from reference [129].

In general, the trends observed for BCCs were also seen to hold for all other lesion types. Therefore, in order to test for statistical significance, Wilcoxon signed rank tests were performed on the lifetime shifts for each individual lesion type and for all lesion types combined. It should be noted that the null hypothesis in these significance tests was simply that, for each lesion type examined, the lifetime shifts had a mean value of zero. For individual lesion types other than BCCs, there were not enough measurements to reject the null hypothesis and attribute statistical significance to the observed trends. For BCCs alone, however, with 445 nm excitation there was a consistent decrease in fluorescence lifetime (i.e. $\tau_{\text{lesion}} < \tau_{\text{normal}}$) and this was statistically significant with a p-value of 0.002. Furthermore, when investigating all the skin lesions included in this study as an ensemble (i.e. when considering all lesion types together) the same trend was observed, this time with a p-value of 5.6×10^{-6} .

To summarise, when exciting with radiation centred at 445 nm, statistically significant differences have been observed between the fluorescence lifetime of healthy tissue and skin cancer. This is true when considering all of the skin lesions included in this study as an ensemble and when considering only the BCCs. Future work will involve the collection of more data so that it is possible to ascertain firstly whether this is true for more individual lesion types and, secondly, whether the

fluorescence lifetime can be used to differentiate between different lesion types and not just between healthy and unhealthy tissue.

4.4 Discussion and conclusions

This chapter has described the application of two fibre-optic coupled spectrometers to an *in vivo* investigation of the optical signatures of skin cancer. The data from the two systems have been analysed separately in order to evaluate their potential for use in clinical diagnosis. The steady state data obtained with instrument A has shown firstly that the diffuse reflectance and fluorescence intensity excited at 355 nm, 375 nm and 395 nm are all lower in BCC lesions than they are on surrounding healthy tissue (see figure 4.6). This observed difference is in agreement with the work of Rajaram *et al.* [8] who saw decreased diffuse reflectance and autofluorescence (with 337 nm excitation) from BCCs compared to the corresponding normal skin. Similarly Brancalion *et al.* reported reduced autofluorescence intensity in BCCs and SCCs, both *in vivo* (excitation at 350 nm) and in freshly resected frozen samples (360 nm excitation), which they attributed to a (histologically visible) loss of collagen and elastin [52].

In their work, Rajaram *et al.* chose to fit the observed spectra to models and, hence, were able to extract optical parameters of the tissue such as the absorption and scattering coefficients, haemoglobin concentration and the relative contributions of the constituent fluorophores. Using these parameters in a leave-one-out cross validation they were then able to diagnose BCCs with a sensitivity and specificity of 94% and 89% respectively [8]. In the work presented in this chapter, no spectral modeling was performed. Instead, the steady state spectral analysis involved the use of SVD to break down the observed spectra into base spectral components. While this approach does not provide measurements of real optical properties, it does allow each measurement site to be characterised using a small number of parameters – i.e. the relative contributions (scores) from the few predominant spectral components. LDA and multivariate regression were then used – in separate leave-one-out protocols – to build diagnostic models using the scores of the base spectral components as input parameters. Multivariate regression was shown to provide marginally better diagnostic accuracy than LDA and, in this case, the respective sensitivity and specificity obtained were 100% and 86%. This is a similar result to that obtained by Rajaram *et al.* [8], however, it required no *a priori* knowledge of the chromophores contained within the tissue under investigation.

The data acquired with instrument B shows that BCCs exhibit a statistically significant ($p = 0.002$) decrease in their mean fluorescence lifetime relative to neighbouring healthy tissue when excited with 445 nm light (figure 4.10). In addition, it was observed that the fluorescence lifetime (and the contrast in fluorescence lifetime between healthy and diseased regions) did not vary substantially with emission wavelength (figure 4.8). These results are in accordance with previous *ex vivo* measurements made with same system on freshly resected (< 2 hours) NMSC tissue samples [21]. Furthermore, using the blue excitation source, the absolute values observed *in vivo* are in reasonably good agreement with those seen *ex vivo* for both BCCs and normal skin [21]. Finally, in all the BCC lesions investigated, with excitation at 445 nm the fluorescence lifetime was seen to be lower than that of the surrounding healthy tissue and, importantly, in 9 out of 10 cases this decrease in lifetime was greater in magnitude than the mean intra-patient variation in the measured lifetimes. This indicates the future possibility of using time-resolved autofluorescence spectroscopy as a clinical diagnostic tool and suggests a potential sensitivity for BCC diagnosis of 90%.

When envisaging the design of such an optical system for diagnostic purposes, it is sensible to consider moving to an imaging modality as this could potentially be used to rapidly screen multiple lesions in a short period of time. Much work has been done in this field including single-[52] and multi-photon imaging [83-86], fluorescence lifetime imaging [31] and also combinations of several non-linear imaging modalities [87-90]. Clearly imaging holds advantages over point spectroscopy in terms of the spatial resolution it provides. Point spectrometers, such as those described in this chapter, however, represent a significantly lower level of complexity and cost and are able to accurately determine the relevant optical signatures of tissue with relatively short acquisition times. The measured optical signals provide clinically useful information (as shown in this chapter) and may be used to guide the development of future imaging instrumentation.

In conclusion, the two spectrometers described in this study have both been shown to provide useful contrast between skin lesions and the neighbouring unaffected tissue, particularly for BCCs. Future work in this area should now be driven toward the collection of more *in vivo* data. Firstly, this will allow confirmation of the trends reported in this chapter through the investigation of a larger, more significant dataset. It will also permit the detailed study of lesion types other than BCCs meaning that the worth of optical measurements can be assessed over a wider range of skin malignancies. Finally, an increased database of spectroscopic information would also allow the development of more complicated diagnostic models, perhaps including both fluorescence lifetime and spectral information. One possible approach on this front is to perform spectral decomposition on time-resolved fluorescence spectra and then study the time-decaying behaviour of the

predominant spectral components. Parameters describing these temporal decays could then be used in DA to assess their diagnostic capabilities. This would lead to the inclusion of an additional dimension in the diagnostic models described in sections 4.2.5 and 4.3.2, and may provide improvements in the diagnostic accuracy obtained. Once the observed trends have been confirmed to a higher degree of certainty and the best opportunities for contrast between healthy and diseased tissue have been assessed, the development of imaging instrumentation could be accelerated.

Chapter 5: Development of a multidimensional endoscopic spectrometer and its application to *in vivo* and *ex vivo* studies of gastro-intestinal disease

The work presented in chapter 4 showed that measurements of the spectral and temporal properties of light emitted and reflected from tissue can provide useful diagnostic information in non-melanoma skin cancer. In particular, this was seen to be the case in basal cell carcinomas. As discussed previously, this diagnostic potential has been observed in various diseases (for example, see references [8, 29-35, 121]) and it is also interesting to investigate the application of reflectance and time-resolved fluorescence spectroscopy to other tissue types and health disorders. This chapter describes the development of a multidimensional spectrometer designed to combine the key functionality of the two instruments used in chapter 4. This spectrometer records time-resolved fluorescence spectra with two excitation wavelengths as well as the diffuse white light reflectance and it utilises a fibre optic probe to permit *in vivo* use during endoscopy. The system is described in detail below where the development and testing of the instrument are discussed before data are presented from both *ex vivo* and *in vivo* studies of human GI tissue.

5.1 Introduction

5.1.1 Motivation

In section 3.3 it was briefly discussed that typical endoscopic diagnostic procedures simply encompass the investigation of tissue under white light illumination. Using such white light endoscopy (WLE) it is often difficult for a clinician to distinguish between benign and pre-malignant lesions. Additionally, inflammatory disorders, dysplasia (i.e. pre-cancerous growths) and early stage cancers of the GI tract are frequently invisible under inspection with WLE. Thus, the current best clinical practice requires that tissue abnormalities are biopsied and sent for *ex vivo* histopathological analysis to confirm the original endoscopic diagnosis. This inability to confirm a diagnosis at the time of the endoscopic procedure – and the corresponding requirement for histopathology – can lead to the excision of benign lesions and to repeat endoscopies, which would have otherwise been unnecessary [159-161]. Furthermore, the delay in obtaining histological information can place a patient at risk of further disease progression before a planned surgical resection and this can cause significant psychological distress.

Cancers of the GI tract (i.e. the mouth, oesophagus, stomach and intestine) are among the most common in the world, accounting for over 2 million new cases each year. Additionally, survival rates in upper GI cancers are uniformly low – for example, five-year survival rates for oesophageal cancer are usually below 10% – as patients are often only diagnosed once the disease has reached an advanced stage [162-164]. For these reasons, there is significant interest in the development of more sophisticated endoscopic technologies that could improve the sensitivity and specificity of diagnosis. At present there are a number of commercially available optical devices and techniques that can be operated in conjunction with standard WLE. These approaches include high definition endoscopy (HDE) [165-167], narrow band imaging (NBI) [168], autofluorescence imaging (AFI) [97], magnifying chromoendoscopy (MCE) [169] and confocal endomicroscopy [170-171]. Figure 5.1 shows three GI lesions imaged using WLE (a, c, e) along with a second complementary imaging modality (b, d, f). While these techniques are all clinically approved and provide improvements on WLE (as is clear in figure 5.1), they have not yet demonstrated sufficiently specific contrast to allow easy, rapid detection of dysplasia. As an example of the drawbacks of these methods, MCE and NBI only enhance the visualisation of suspicious areas of tissue that must first be detected under normal white light illumination (as the additional time required to inspect the entire bowel with a second imaging modality would be too long for most endoscopic clinics). As such, none of these approaches have yet become widespread in their clinical use and, therefore, the need to develop more sophisticated endoscopic techniques remains. Clearly, the development of a method that could reduce the regularity with which clinicians resort to histopathology – and, hence, cut the number of unnecessary endoscopic biopsies – is desirable.

Several other optical approaches are currently being investigated for their potential to aid diagnosis of GI disease and these include both imaging and point spectroscopy, for example see the reviews presented in references [43-44, 58, 172-173]. In terms of point measurements this has comprised diffuse reflectance and light scattering spectroscopy [174-176], Raman spectroscopy [177] and also a significant amount of research involving steady state autofluorescence spectroscopy. Silveira *et al.* [178] studied fluorescence spectra of benign and malignant *ex vivo* samples of human gastric mucosa with 488 nm excitation. Using the ratio of the intensities recorded at wavelengths of 550 nm and 600 nm they were able to successfully differentiate between healthy tissue and adenocarcinomas (with 100% sensitivity and specificity). Importantly, however, this method was unable to discriminate between carcinomas and areas of inflammation. Kapadia *et al.* [179] investigated fluorescence spectra from adenomatous colonic polyps when excited at 325 nm, again *ex vivo*, and this study utilised multivariate linear regression to classify samples based on fluorescence intensities at six emission wavelengths. This technique permitted discrimination

between normal tissue and adenomatous (i.e. pre-cancerous) polyps but not between normal tissue and hyperplastic (i.e. non-cancerous) polyps. Further *ex vivo* work has been performed by Richards-Kortum *et al.* [180], who measured fluorescence spectra from colonic polyps and normal colon tissue, again demonstrating successful differentiation between normal tissue and regions of adenoma. Additionally, Yang *et al.* [181] used ratios of the fluorescence intensity in several spectral channels to differentiate between cancerous and healthy colon specimens *ex vivo*. Finally, steady state autofluorescence spectroscopy has also been applied to the *in vivo* study of oral dysplasia [182]. In this case, fluorescence spectra were shown to effectively differentiate between healthy and cancerous tissue but, again, classification of benign versus (pre-)malignant regions was not possible.

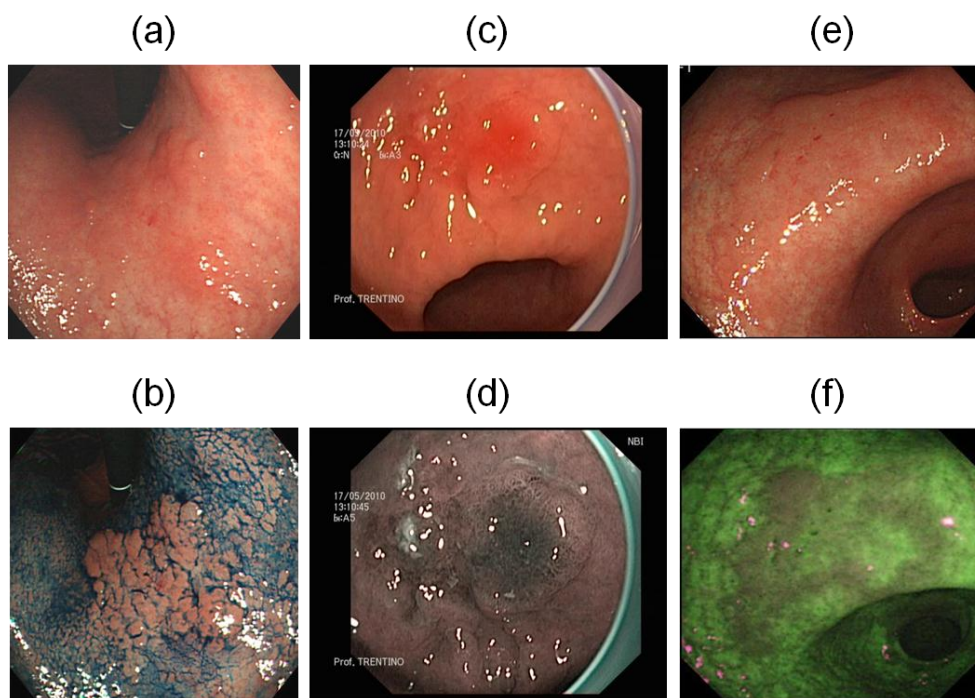


Figure 5.1. Example images from GI endoscopy. (a), (c) and (e) show three regions of tissue imaged using standard WLE. The same three regions of tissue are also shown in (b), (d) and (f) to demonstrate the contrast enhancement provided by chromoendoscopy (b), NBI (d) and AFI (f). Images provided courtesy of Professor Paolo Trentino (Rome, Italy) and Dr. Chizu Yokoi (Tokyo, Japan).

Though not as widely investigated as steady state spectroscopy, time-resolved fluorescence spectroscopy (TRFS) has also been applied to a number of studies of GI tissue and disease. Two studies investigated the temporal and spectral properties of the fluorescence emission in colonic polyps [34] and in Barrett's Oesophagus [35]. Both studies were performed *in vivo* and both found clear differences between healthy and diseased tissue. Furthermore, Chen *et al.* [33] examined the time-resolved autofluorescence emission of human oral mucosa *in vivo* and this investigation

demonstrated successful classification (accuracy $\geq 75\%$) of oral lesions into three categories – normal, hyperplastic (i.e. benign) and dysplastic (i.e. malignant) – using the fluorescence lifetime.

Further to the point spectroscopy work, there have also been a number of applications of fluorescence imaging modalities to the study of GI tissue. Examples of the use of steady state fluorescence imaging include the publications by Chwirot *et al.* [11, 183] and Xiao *et al.* [184]. The latter study utilised confocal microscopy to study freshly resected specimens of gastric cancer and normal stomach tissue. Two excitation wavelengths were used in this study: 488 nm – with a detection band of 505-530 nm (referred to as the green detection band); and 543 nm – with a (red) detection range defined as $\lambda > 580$ nm. The authors showed that the ratio of green to red autofluorescence was significantly lower in cancerous tissue than in healthy tissue [184]. The work by Chwirot, on the other hand, involved fluorescence imaging of gastric [11] and colonic [183] tissue samples in six spectral emission bands using an excitation wavelength of 325 nm. Malignant and pre-malignant colon lesions exhibited reduced fluorescence intensity in all spectral emission bands (compared to neighbouring healthy tissue) [183]. Furthermore, in the investigation of gastric cancer, the difference in the fluorescence intensity measured at 440 nm and 395 nm (normalised to that recorded at 590 nm) was used as a contrast parameter and this was shown to effectively discriminate between healthy and cancerous tissue (with a sensitivity for cancer detection of 96%) [11].

In terms of time-resolved fluorescence imaging, Mizeret *et al.* reported an increased fluorescence lifetime in *ex vivo* human oral carcinoma compared to surrounding uninvolved tissue [123]. This was achieved using a frequency domain technique and the fluorescence lifetime contrast was observed with 417 nm excitation and an emission band ranging from 470 nm to 650 nm. However, this study was mainly concerned with the development of instrumentation and, as such, only a single oral mucosa tissue sample was imaged. A larger scale investigation involving FLIM of *ex vivo* GI cancer (in terms of the number of specimens examined) was reported by McGinty *et al.* in 2010 [32]. This study incorporated wide-field time-gated fluorescence lifetime imaging with 355 nm excitation and demonstrated a statistically significant increase in the fluorescence lifetime of colonic tumours (16 specimens) relative to the surrounding healthy tissue.

All of the autofluorescence studies mentioned above have demonstrated differences between the healthy and (pre-)cancerous tissues investigated. Despite this, further research is still required in order to better determine the spectroscopic parameters that will provide the greatest contrast between healthy and diseased GI tissue. This is most easily accomplished using point spectroscopy – which discards spatial information and, hence, allows the rapid collection of large

numbers of fluorescence photons – and the resulting data could then be used to guide the development of novel autofluorescence based endoscopic devices (for example, endoscopic FLIM instrumentation). Thus, the remainder of this chapter describes the development and use of a multidimensional spectrometer that combines measurements of time-resolved fluorescence spectra and diffuse reflectance spectra. This spectrometer has been designed to be compact, easily transportable and clinically viable and it incorporates a fibre optic probe to permit *in vivo* use during endoscopy. The results presented later in this chapter involve the application of this system to both *in vivo* and *ex vivo* studies of human GI tissue with cancerous and pre-cancerous diseases.

5.1.2 Biology of the human colon

As will be discussed in more detail later, in the tissue studies presented in this chapter the majority of measurements were made on colon tissue. For this reason, a brief description of the human colon is put forward here in order to give the reader a basic understanding of the diseases and regions of the colon discussed later on.

The colon – also known as the large intestine – has a form like that shown in figure 5.2 and can be separated into a number of distinct regions. A human colon begins on a person's right hand side where it is joined by the distal section of the small intestine (ileum). This section of the colon is known as the caecum and the appendix is located at its base. From the caecum, the colon ascends vertically (ascending colon) towards a bend known as the hepatic flexure at which point it extends horizontally across an individual's torso. This length of bowel is deemed the transverse colon and ends at the splenic flexure where the colon continues vertically downward (descending colon) until it reaches the sigmoid colon. Finally, the sigmoid colon becomes the rectum and then the anus, where the colon terminates.

A number of common diseases can develop within the colon and several of these are involved in the study presented later in this chapter. These include inflammatory disorders, cancers and polyps, and the disease type for which the greatest number of measurements was made (both *ex vivo* and *in vivo*) was colonic polyps (see table 5.1 in section 5.3.1). A polyp is simply a region of overgrown tissue found in the GI tract. This overgrowth can be caused by a number of factors including inflammation, hyperplasia (proliferation of otherwise normal cells) or dysplasia (proliferation of pre-cancerous cells) and it is often very difficult to differentiate between these sub-types. Additionally, polyps are often very small (< 0.5 cm in diameter) and so can sometimes be missed altogether during a colonoscopy. As such, investigating the optical properties of polyps is desirable as it may lead to novel diagnostic technologies that can improve the sensitivity of polyp

diagnosis and also provide discrimination between benign and pre-malignant growths. For this reason, and because the most measurements were made on this disease type, the majority of the results presented in section 5.4 regard colonic polyps.

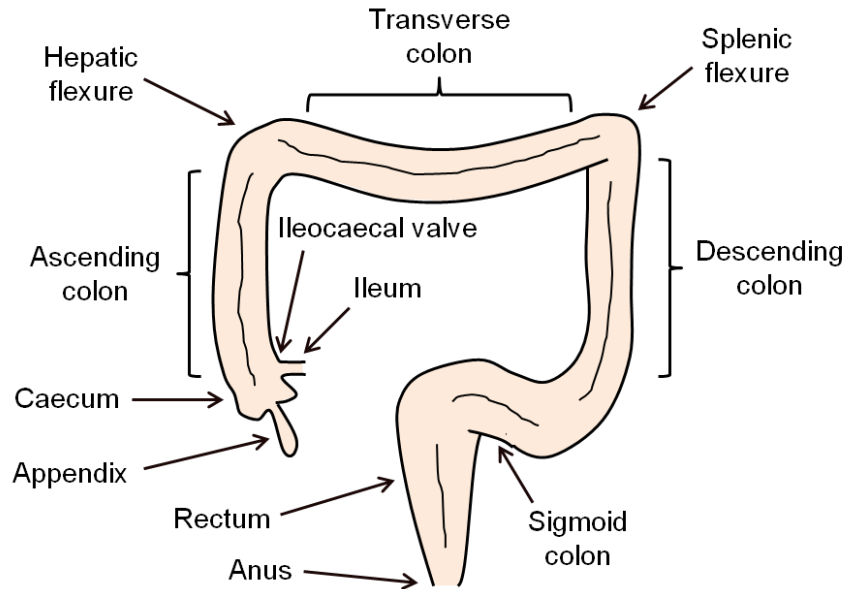


Figure 5.2. Diagram illustrating the anatomy of the human colon.

5.2 Instrument development

This section provides a description of the development of the multidimensional endoscopic spectrometer used in this chapter, including the system itself, the control software and the methods used for data analysis. The individual sub-sections present information regarding the following: 5.2.1 – optical system; 5.2.2 – custom optical fibre probe used for light delivery and collection; 5.2.3 – transportable trolley that houses the system; 5.2.4 – home-written control software; 5.2.5 – detector calibration; 5.2.6 – data analysis.

5.2.1 Optical system

As discussed above, this system was designed to combine functionality from both the spectrometers presented in chapter 4. Thus, it measures a diffuse white light reflectance spectrum and time resolved fluorescence spectra with two excitation wavelengths (375 nm and 435 nm). The optical setup is shown in figure 5.3 where (a) shows a schematic diagram of the system and (b) shows a photograph.

Two pulsed diode lasers are used for fluorescence excitation, both of which emit 50-150 ps pulses and have adjustable repetition rates of up to 40 MHz. The first laser has a central emission wavelength of 375 nm and a maximum average output power of 3 mW (LDH-P-C-375B, PicoQuant GmbH, Germany) while the second emits at 435 nm with a maximum average power of 1 mW (LDH-P-C-440, PicoQuant GmbH, Germany). Hereafter the two excitation sources are commonly referred to as the UV and blue laser respectively. It should be noted that the use of two diode lasers rather than a diode laser and a frequency tripled fibre laser (as used in instrument B in chapter 4) permits significant reduction in the size of the system (compared to the time resolved spectrometer used in chapter 4). Indeed, in this case, all optical components fit onto a 30 x 45 cm² optical breadboard while previously a 60 x 60 cm² breadboard was required (see section 4.2.2).

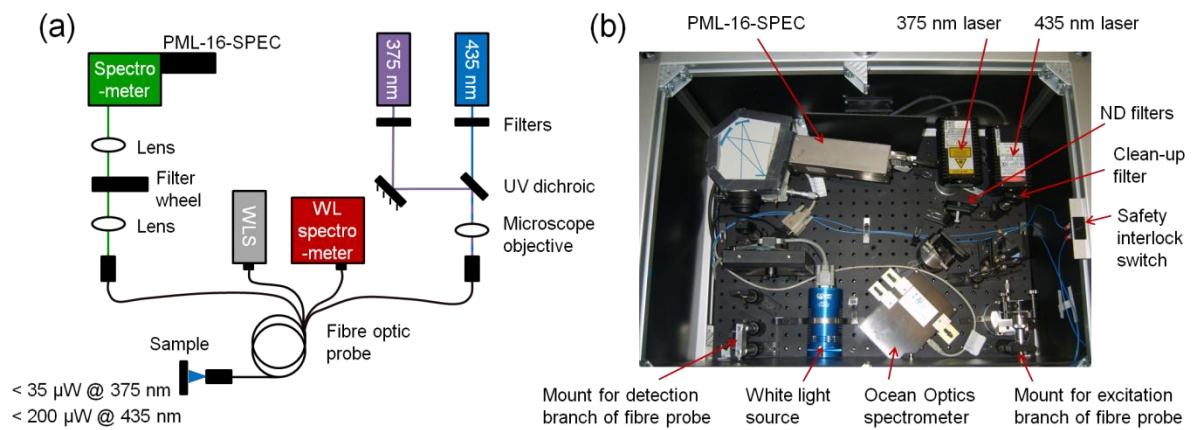


Figure 5.3. Optical setup of the multifunctional spectrometer showing (a) a schematic diagram and (b) an annotated photograph.

As shown in figure 5.3(a) the outputs from the two lasers are first combined using a dichroic beamsplitter (Di01-R405, Semrock Inc., USA) and are then coupled into the fluorescence excitation channel of a custom built fibre optic probe (see section 5.2.2 for details of the fibre probe). Spectral and neutral density (ND) filters are placed in the beam paths of the two lasers to clean up the blue diode emission (Z440/20x, Chroma Technology, USA) and to limit the maximum average optical power that reaches the sample (0.7 ND filter placed in UV beam path) to 35 μ W of UV and 200 μ W of blue light. As in chapter 4, limiting the power in this way minimises the risk of photodamage to the tissue and the power levels quoted are below the MPE for skin (there is no accepted MPE for GI tissue) for exposure times as high as 1000 s.

The distal end of the probe is directed toward a sample and a spacer on the end of the probe ensures that the distance between the output face of the fibres and the sample is fixed at 1.5 mm

(see figure 5.4). As such, the excitation lasers illuminate a fixed area of tissue of approximately 0.9 mm^2 . The fluorescence is collected by 14 optical fibres in the probe and returned to the fluorescence detection arm of the optical system (left hand side of figure 5.3(a)). The output from this arm of the proximal end of the fibre probe is imaged onto the input slit of a grating spectrometer (MS125 1/8m, Lot-Oriel, UK) using a pair of lenses and the spectrometer is attached to a 16 channel multi-anode PMT detector (PML-16-SPEC, Becker-Hickl GmbH, Germany). A motorised filter wheel is placed between the output of the probe and the spectrometer and this contains two emission filters (E400LPv2 and E475LPv2, Chroma Technology, USA) to ensure that no scattered excitation light reaches the 16 channel PMT detector.

As an addition to the fluorescence measurements, this system also records a diffuse white light reflectance spectrum. A white light source (HL-2000-FHSA, Ocean Optics, The Netherlands) is attached to a third arm of the proximal end of the probe (via an SMA connection) and the output from the lamp is coupled into a single optical fibre in the probe. This light is directed toward a sample at the distal end of the probe, as for the fluorescence measurements. The reflected light is then collected by a second optical fibre and delivered to a spectrometer (HR2000CG-UV-NIR, Ocean Optics, The Netherlands) via the fourth and final arm of the proximal end of the probe. It should also be noted that, at the common distal end, the collection and illumination fibres for the reflectance measurements are separated in order to minimise the collection of specular reflections.

The whole system is controlled by software written in-house using LabVIEW 7.1 (National Instruments, USA) and this software is described in detail in section 5.2.4. In short, the software controls both lasers, the lamp and the detectors and a typical acquisition involves the sequential illumination of the sample with each of the three light sources. First the lasers excite the sample and time-resolved fluorescence spectra are recorded at both excitation wavelengths. Acquisition times for the fluorescence measurements are usually 5 s and two or three repeat measurements are made with each laser. After the fluorescence measurements, the white light source illuminates the sample and the Ocean Optics spectrometer is used to record a diffuse white light reflectance spectrum with a typical collection time of 2 s. Hence, in total, an acquisition lasts between 20 and 35 seconds.

5.2.2 Detailed description of custom optical fibre probe

As stated above, a custom built fibre optic probe (FiberTech Optica, Canada) comprising 19 multimode optical fibres (all of 0.22 NA and 200 μm diameter) is used for the delivery and collection of light. This custom fibre bundle has a single distal branch (common to all 19 optical fibres) while the proximal end is split into four individual branches. In the fluorescence excitation branch of the

proximal end of the probe (far right of figure 5.3(a)), an FC connector is used to mount a short (2 cm) length of 600 μm diameter multimode optical fibre. The two laser excitation sources are coupled into this section of multimode fibre, which is then fixed in place in contact with three closely packed 200 μm diameter fibres. The light is transmitted to the three 200 μm fibres and these relay the light to the distal end of the probe. Figure 5.4(a) illustrates the arrangement of the fibres in the excitation branch of the proximal end of the bundle, where both end-on and cross sectional views of the probe are presented.

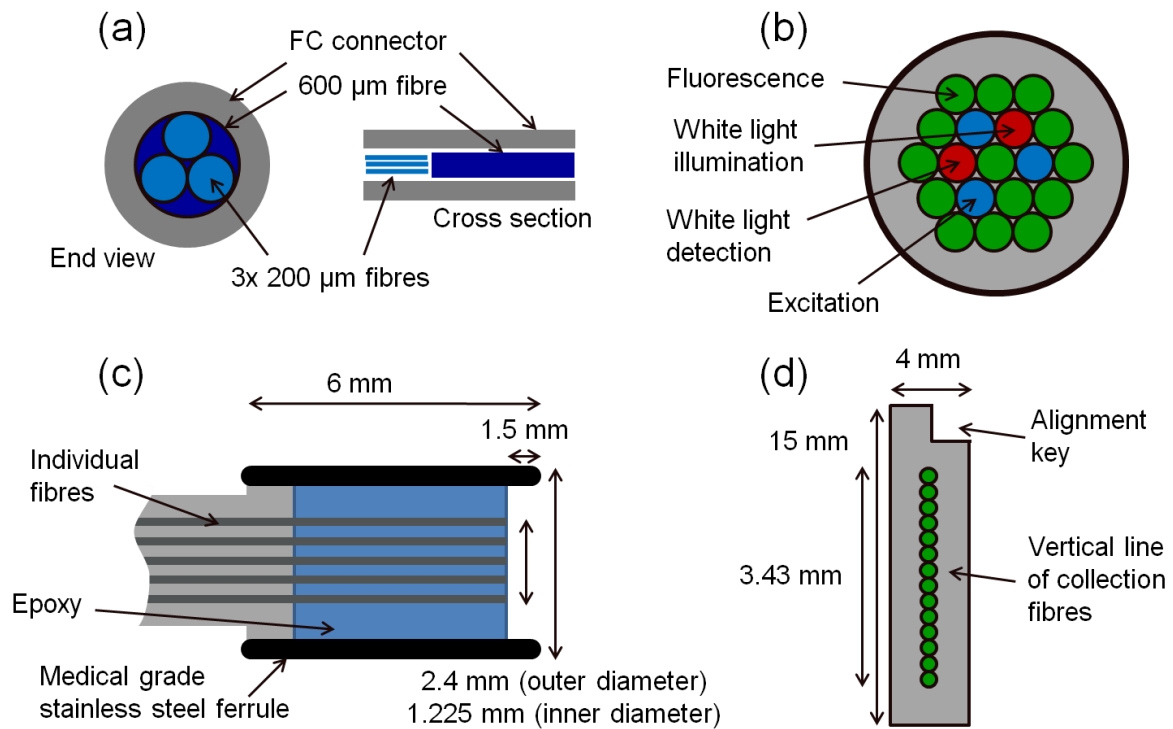


Figure 5.4. Diagrams illustrating the arrangement of fibres at various locations in the custom optical fibre bundle: (a) shows the excitation branch of the proximal end; (b) and (c) respectively show end and cross-sectional views of the common distal end; and (d) shows the fluorescence detection branch of the proximal end.

At the distal end of the probe, the 19 optical fibres are configured as shown in figure 5.4(b) where the excitation fibres are coloured blue, the fluorescence collection fibres are green and the white light illumination and detection fibres are shown in red. The individual fibres are hexagonally packed and secured using epoxy inside a medical grade stainless steel ferrule, which is 6 mm long. The ferrule protrudes 1.5 mm beyond the distal face of the fibre bundle and acts as a spacer to ensure that the sample under investigation is always held at a set distance from the output of the fibre probe. This is illustrated in figure 5.4(c), which shows a cross section of the distal end of the bundle.

The 14 fluorescence collection fibres deliver light to the fluorescence detection branch of the proximal end of the probe (far left of figure 5.3(a)). In the output of this branch, the fibres are arranged in a vertical line to optimise the transmission of the light through the input slit of the grating spectrometer. A custom designed brass connector is used to mount the fibres at this point and this is shown in figure 5.4(d). This connector permits the linear arrangement of fibres discussed above and also has a 2 x 2 mm alignment key cut into it so that the position of the fibres remains constant after the regular removal and replacement of the probe, which is required for sterilisation.

Finally there are two further 200 μm fibres within the probe. These are coloured red in figure 5.4(b) and are used to deliver and collect light for the measurement of the diffuse white light reflectance spectrum. Each fibre has its own branch in the proximal end of the probe, one of which connects to the white light source and the other delivers light to the Ocean Optics spectrometer. In both cases SMA connectors are used. At the distal end of the probe the centres of the two fibres are separated by approximately 350 μm to minimise the collection of specular reflections, hence ensuring that a diffuse white light reflectance spectrum is recorded.

The distal section of the probe has an outer diameter of 2.4 mm, which means it can be inserted into the working channel of most common medical endoscopes. No metallic components are contained within the jacket of the fibre bundle and the fibre assembly as a whole is electrically insulating to ensure that there is no risk of electric shock to the patient. The assembly is also water-tight and resistant to cleaning materials in order to allow sterilisation of the probe prior to use. As such, the probe is suitable for *in vivo* clinical use and permits the application of the multifunctional spectrometer during endoscopic procedures.

5.2.3 Transportable housing

All optical components in the system are mounted on a 30 x 45 cm² breadboard. This, and all the other constituents of the instrument, are housed inside a compact, wheeled trolley to allow easy transportation to (and within) a clinic. The optical breadboard is situated on the top shelf and below this the trolley also contains a driver for the filter wheel (Lambda 10-3, Sutter Instrument Company, USA), a power supply and control unit for the lasers (PDL 828 Sepia II, PicoQuant GmbH, Germany), a USB data acquisition box (USB-DAQ, NI USB-6008, National Instruments, USA), a desktop computer and an isolating medical transformer (REO-MED 600, REO Inductive Components, UK).

The laser control unit connects to the computer via a USB cable and allows control of the repetition rate, output power and on/off state of the lasers through software. It also has a logic

gating input for each laser that permits fast (ms timescale) switching of the on/off state of the lasers using transistor-transistor logic (TTL) voltage signals. This is achieved using the National Instruments USB-DAQ device. The DAQ is also connected to the computer via USB and is programmed to allow a user to send 5 V ('on') or 0 V ('off') signals to each of the lasers and to the white light source individually.

The computer contains a TCSPC card (SPC-730, Becker-Hickl GmbH, Germany), which is used to control the 16 channel PMT detector and permits the collection of the spectrally-resolved fluorescence decays. The filter wheel is also linked to the computer (using an RS-232 cable) and, thus, the entire system can be controlled through software (see section 5.2.4).

The trolley is fully enclosed using a stainless steel lid and front, back and side panels made from a black, optically absorbent, rigid foam PVC sheet material (Foamalux®). It also has a hinged panel (stainless steel) at the front of the optical setup to allow easy connection and containment of the proximal ends of the optical fibre probe. This front panel includes a small opening near the centre where the proximal fibres emerge and the fibre probe splitter (the section of the probe in which the individual optical fibres are routed to the appropriate proximal branches) is clamped in place just below this opening. Having the system enclosed in this way means that the optical system is safe for use in a clinic and that the electrical components cannot be accessed. It also permits easy cleaning of the system in the case of any spillage of clinical liquids. The computer also incorporates a medical grade 'wipe-clean' keyboard and touchpad, again to allow easy cleaning of all external components.

The panels on the side of the trolley have ventilation holes drilled in to them and two fans are installed on the back. The fans run at all times that the system is turned on and create a constant air flow through the instrument. This air flow acts to cool the interior of the trolley and ensures that no problems arise due to overheating.

With the optical setup fully enclosed, the only point at which laser radiation is emitted from the system is at the distal end of the fibre probe. As discussed in section 5.2.1, ND filters in the beam paths of the lasers limit the maximum optical power at the distal end of the probe to 35 μW of UV and 200 μW of blue light. Two interlock switches are also located on the trolley to ensure that the lasers cannot be operated with the optical setup exposed. Thus, the system as a whole has been classified as a Class 1 laser product (by Aurora Radiation Protection Services, UK) and, hence, no protective eyewear is required while the optical setup is enclosed.

All electrical elements contained within the trolley are connected to the isolating medical transformer. Hence, the only electrical outlet from the system is the connection of the medical transformer to the mains electricity. This isolating transformer provides an ungrounded ‘floating’ power supply for the system, which improves the reliability of the supply voltage, reduces the leakage current and minimises the risk of electrocution. The medical transformer is connected directly to the mains and, hence, provides power for the entire instrument. There is also an earthing point on the transformer and this is connected to mains earth via an earth bonding cable which provides a low resistance route to ground in the case of any short circuit fault condition. Additionally, a low pass 7 A electrical filter (FA7-RS, RS Components Ltd., UK) is located between the mains power and the medical transformer and this is used to protect all the electrical equipment from the unwanted effects of any power surges.

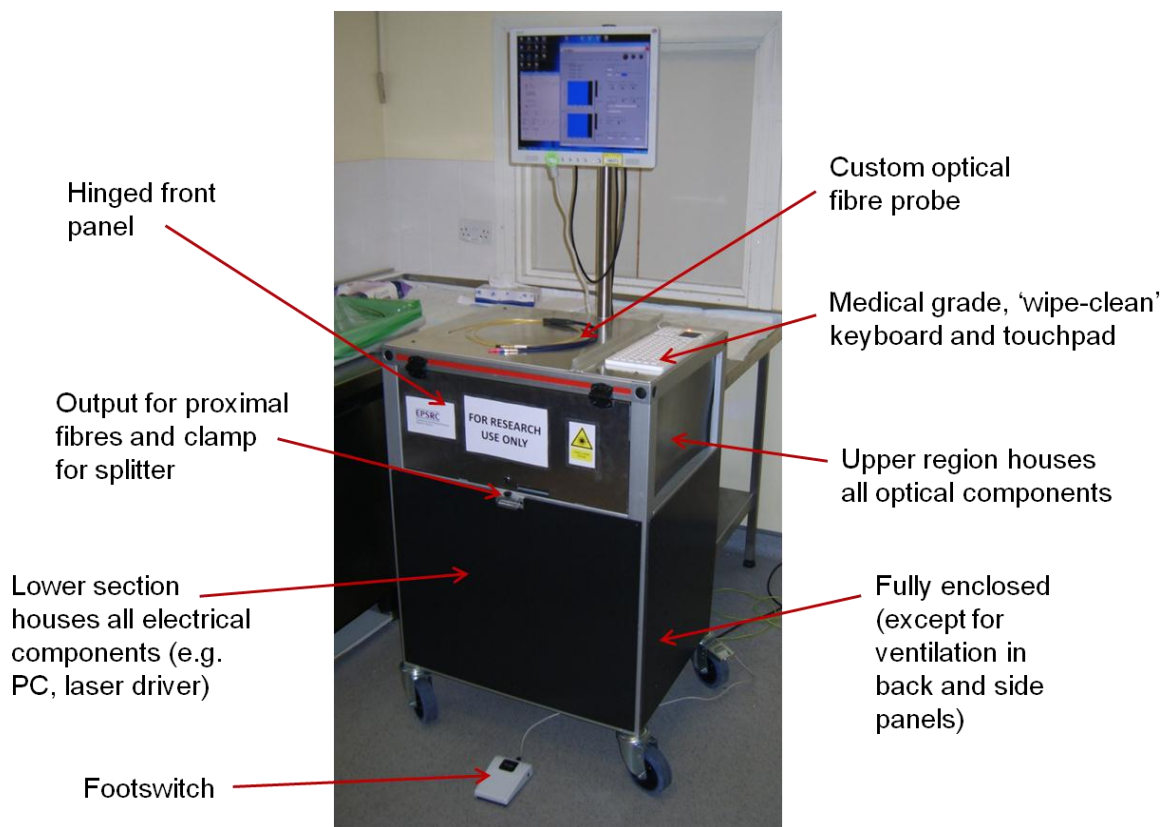


Figure 5.5. Photograph of the transportable trolley that houses the entire spectrometer, showing some important features of the system.

Finally, the computer monitor (also connected to the isolating medical transformer) is mounted on a metal post fixed in place at the back of the trolley. The dimensions of the fully assembled system are as follows: height – 182 cm, width – 70 cm, depth – 55 cm. Thus, the trolley

mounted system is relatively compact and can be easily stored, transported and used within a clinical environment. Figure 5.5 shows a photograph of the trolley indicating some of the important features discussed in this section.

5.2.4 Control software

The spectrometer is controlled solely in software. First, the powers and repetition rates of the lasers are set and fixed using the commercial software provided with the laser control unit. Beyond this, the entire system is controlled by software written in-house using LabVIEW 7.1 (National Instruments, USA). This software comprises a single program that regulates the detectors, light sources (using the USB-DAQ device), filter wheel and all acquisition parameters, and a screenshot of the user interface of the software is shown in figure 5.6.

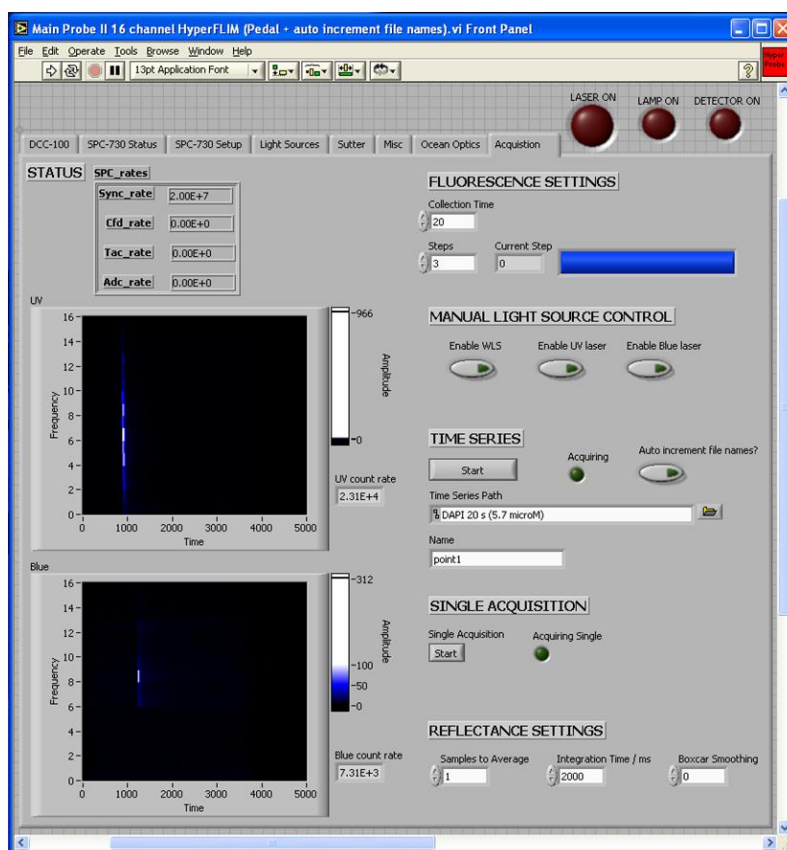


Figure 5.6. Screenshot of the user interface of the LabVIEW software used to control the multifunctional spectrometer.

The software can be used to control each component in the system individually and also allows the user to automatically acquire data in one of three ways: a single fluorescence acquisition (exciting once with both lasers); a single reflectance acquisition; or a time series acquisition, in which

both fluorescence and reflectance data are recorded. In a time series acquisition, time-resolved fluorescence spectra are first recorded using each excitation source in sequence and a chosen number of repeat measurements are made. Secondly, the white light source is used for illumination and a single reflectance spectrum is acquired. In all cases data are recorded as text files showing the fluorescence or reflectance intensity as a function of time and/or wavelength.

Lastly, it should be noted that a USB footswitch is also connected to the computer (indicated in figure 5.5) and this is incorporated into the LabVIEW software. The footswitch is programmed such that depression of the switch creates a new save folder and begins a time series acquisition, saving all data to the newly created folder. Thus, the system can be operated by a single clinician while they carry out a routine endoscopy.

5.2.5 Calibration of 16 channel detector

Before analysing any fluorescence data acquired with this instrument it is necessary to calibrate the 16 channel PMT detector such that the spectral locations and the relative sensitivities of each of the PMT elements are known. This is a similar calibration procedure to that performed on the time-resolved fluorescence spectrometer used in chapter 4 (see section 4.2.2).

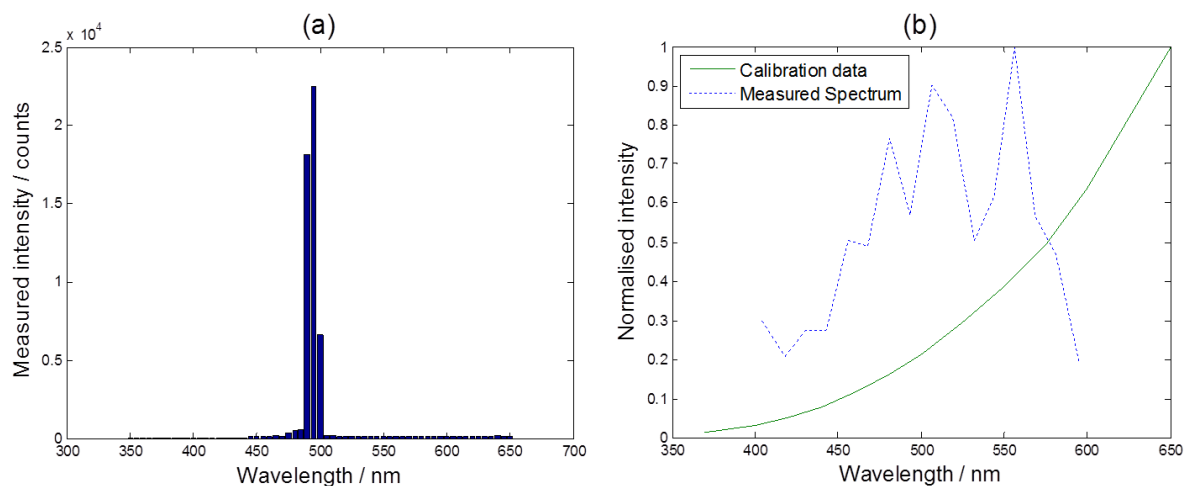


Figure 5.7. (a) Graph showing the intensity measured in the 8th spectral channel as a function of illumination wavelength. From this graph it is possible to calculate the spectral centre of this PMT channel. (b) Spectrum measured from a calibrated white light source (dotted blue curve) along with the calibration spectrum (solid green curve), both normalised to their respective maxima. By comparing the measured and expected values it is possible to calculate correction factors for the intensity recorded in each spectral channel.

First, the spectral centres of each channel were determined by illuminating the distal end of the fibre probe with light of known wavelengths and recording a spectrum with the 16 channel detector in each case. Spectra were recorded at 5 nm intervals over an illumination range of 350-650 nm, with the spectral width of the illumination approximately 2.5 nm at all wavelengths. For each spectral channel, the measured intensity was then plotted as a function of wavelength and, as an example, the intensity observed in the 8th spectral channel at each illumination wavelength is shown in figure 5.7(a). By calculating the intensity weighted mean wavelength for each channel (i.e. by centroiding graphs like the one shown in figure 5.7(a)) it was then possible to determine the spectral location of each PMT element.

Secondly, the relative sensitivities of the spectral channels were accounted for by measuring the spectrum from a calibrated white light source (LS-1-CAL, Ocean Optics, The Netherlands). The measured data was compared to the expected values and correction factors were calculated for the intensities measured in each spectral channel. Figure 5.7(b) shows the measured and expected spectra for the calibrated white light source, both of which have been normalised to their peak intensity.

5.2.6 Analysis of data

Fluorescence decay analysis

In the experiments described later in this chapter, a double exponential model was fitted to the fluorescence decays recorded in each spectral channel. This approach was chosen as the biological tissue under investigation contained a number of fluorescent species. However, only a limited number of photons are collected by each PMT element, hence, fitting to more complex models is difficult.

The fitting was performed in a very similar way to that described in section 4.2.4 but in this case the measurement of the IRF was slightly different. As discussed previously, it is difficult to measure the IRF in all spectral channels using a single specimen (either a scattering sample or a short lifetime fluorophore) because of the wide range of emission wavelengths (approximately 200 nm) and due to the use of two excitation wavelengths. For this reason, reflected light IRFs were measured by placing a diffusely reflecting sample at a short distance (approximately 10 cm) from the distal end of the probe and then adjusting the angle of the grating in the grating spectrometer to direct light onto each PMT element in sequence. Importantly, this provides an IRF in all spectral channels, however, it cannot be performed regularly as the calibration of the spectral locations of

the 16 PMT elements (see section 5.2.5) requires that the grating remains fixed. Thus, this approach relies on the assumption that the IRFs do not vary considerably over time. Additionally, the diffusely reflecting sample was located at a distance from the distal end of the probe and was not in contact with the end of the ferrule as the sample would be in a typical experiment. This introduced a temporal offset between the IRF and the measured data. Further to this, adjusting the position of the grating can also alter the path length of the light in the spectrometer and, hence, change the arrival time of the photons at the detector. Hence, this temporal offset also varies across the 16 spectral channels. Note also that adjusting the angle of the grating can also effect changes in the dispersion, which could alter the IRF. However, this effect is small and, thus, is not considered further.

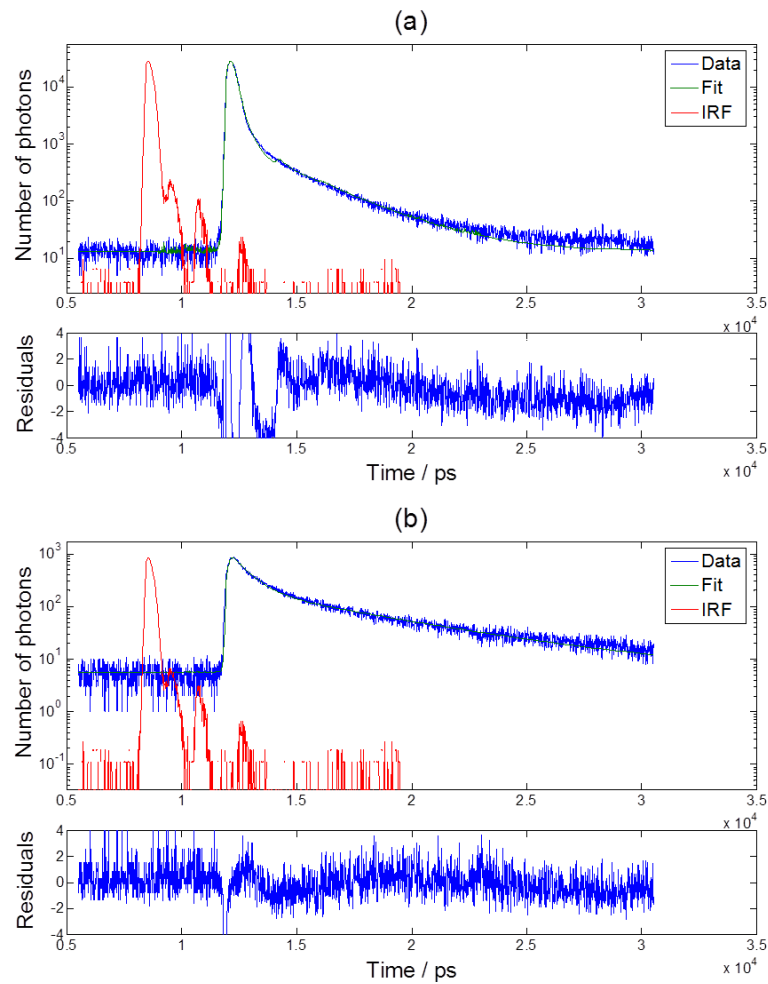


Figure 5.8. Example data and double exponential fits from (a) a solution of DAPI in water and (b) an *ex vivo* specimen of inflamed human colon tissue, excised from the splenic flexure.

To account for the temporal shift, one approach would be to simply include this parameter in the double exponential fitting model. This adds complexity to the fitting process; therefore, instead, the temporal shift was measured using time-resolved fluorescence spectra from a reference fluorophore, which were recorded on every day of measurements. Ideally, this fluorophore would be single exponential in nature and would have a known fluorescence lifetime (to simplify the fitting process). It also needs to be excited by both the UV and blue lasers and to emit across the entire detection range (400-600 nm for UV excitation, 475-600 nm for blue excitation). The decays recorded from this reference sample will have a very good signal-to-noise ratio – one which is considerably better than those typical in the data – and, hence, it is possible to accurately fit the temporal shift using the reference sample. The fitted value in each channel can then be fixed and used in the fitting of the measured data, which has a lower signal-to-noise ratio.

The fluorescent stain DAPI (4', 6-Diamidino-2-phenylindole) was chosen as the reference fluorophore. DAPI is efficiently excited at both 375 nm and 435 nm and emits over the required detection range, however, it exhibits a multi-exponential decay and its lifetime varies significantly with both excitation and emission wavelength. None the less, it is still possible to fit a double exponential decay (with an adjustable temporal offset) to the observed data and this provides a better determination of the temporal shift than would be available from direct fitting of tissue data. Figure 5.8 shows example fitted decays from DAPI (a) and from human GI tissue (b) – both were excited using the UV laser and both decays were recorded in the 7th spectral channel ($\lambda = 481$ nm). Firstly, the temporal offset between the IRF and the data is evident in both cases and it is also clear that the signal from DAPI is considerably higher than the signal from tissue (by more than a factor of 10). The fit to the DAPI decay shows significant structure in the residuals, particularly around the rising edge, however, the time of the rising edge in the fit seems to agree well with the data, suggesting that the temporal offset has been measured correctly. Furthermore, the fit to the tissue data appears good with fairly uniform residuals throughout (note that any deviations from zero in the residuals are probably caused by small contributions from additional exponential components in the decay). This implies that the measurement of the temporal offset and the use of the previously measured reflected light IRFs is a suitable approach that provides a good fit to the measured data.

Analysis of diffuse reflectance spectra

When analysing the diffuse reflectance spectra, one must consider the fact that the data is affected by variations in the optical power transmitted by the probe. Thus, on every day of measurements, the acquired reflectance data are compared to the reflectance obtained from a 99% reflectance

standard (SRS-99-010, Labsphere, USA). Spectra are recorded from the tissue under investigation (R) and from the reflectance standard (R_s) and a dark spectrum is acquired in each case by turning off the white light source (D and D_s respectively). The dark is subtracted from both spectra and then a corrected tissue reflectance spectrum (R_c) is calculated by dividing the tissue data by the reflectance standard spectrum, i.e. $R_c = (R - D)/(R_s - D_s)$. Any differences in integration time between the data from the tissue and the reflectance standard are accounted for by simply multiplying the tissue data by an appropriate scalar (after subtraction of the dark). Example spectra from human skin (measured *in vivo*) and from the 99% reflectance standard are shown in figure 5.9 along with the corresponding R_c curve calculated from these two datasets.

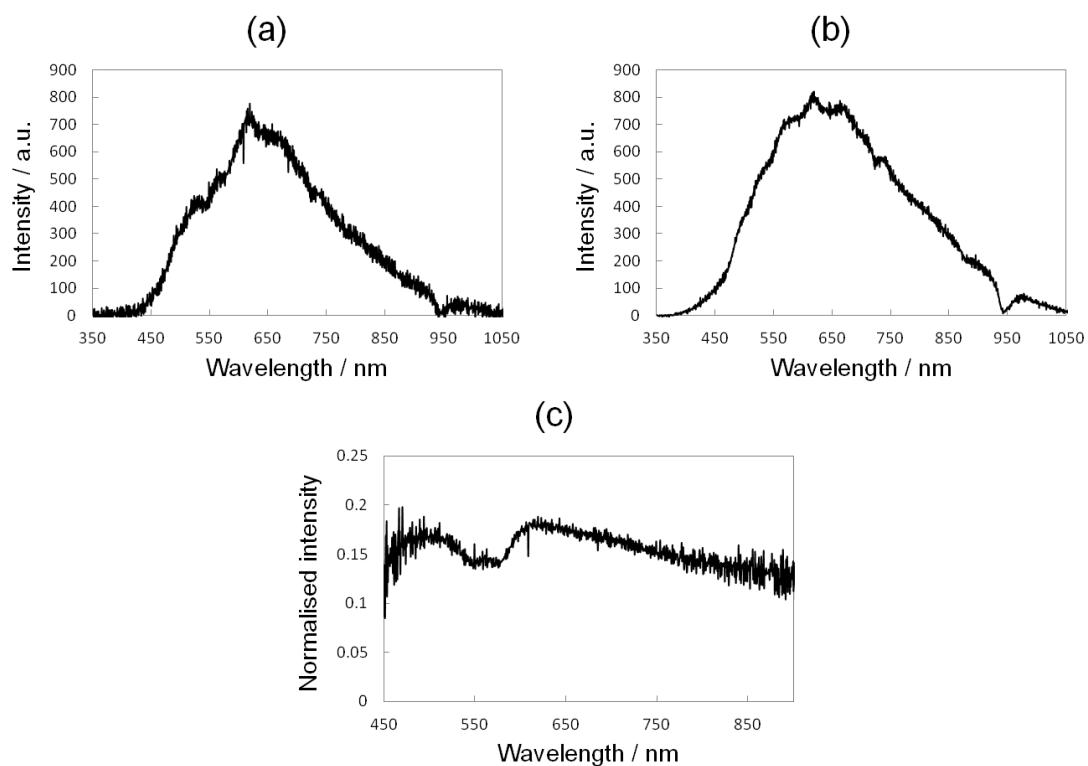


Figure 5.9. Example diffuse reflectance spectra from (a) human skin (recorded *in vivo*) and (b) the 99% reflectance standard. (c) shows the corrected tissue spectrum, calculated by dividing the raw data (a) by the reflectance of the 99% standard (b). In the corrected spectrum, a clear trough is observed around 550 nm, which is indicative of haemoglobin absorption. The data shown in (a) and (b) have both been background subtracted.

5.3 Experimental procedure

5.3.1 *Ex vivo* and *in vivo* measurements of human GI tissue

Once the endoscopic spectrometer was ready to use it was transported by van to the endoscopy unit of Charing Cross Hospital. There it was assembled in a private, lockable room with black-out curtains that was suitable for *ex vivo* tissue measurements and any necessary beam routing. In the endoscopy clinic, the spectrometer was then applied to both *ex vivo* and *in vivo* investigations of the optical signatures of human GI tissue. Patients who participated in the research were recruited by Dr. Sergio Coda and Sister Kim Roche of the clinical team at Charing Cross and the study was carried out with the approval of the local ethics committee (ethics committee REC reference numbers: *ex vivo* - 09/H0706/28; *in vivo* - 11/LO/0728).

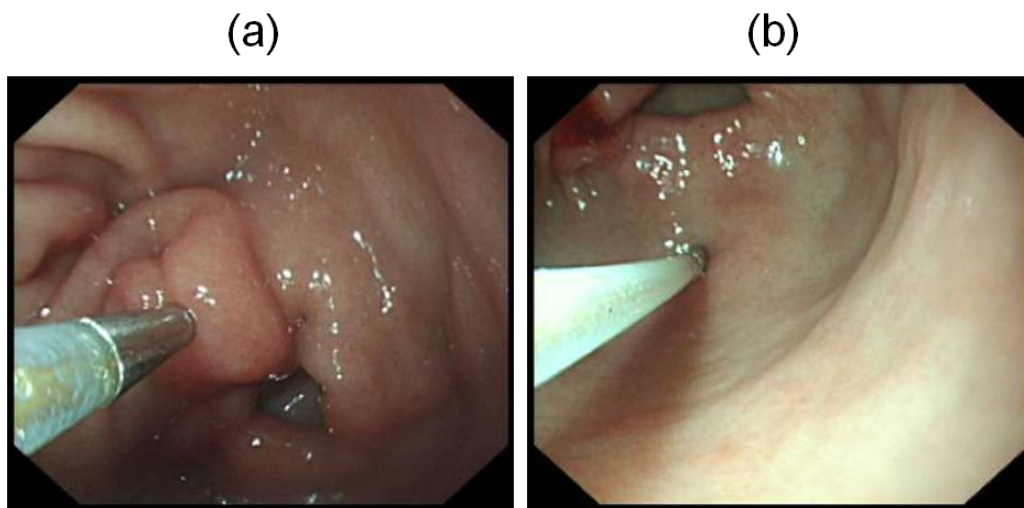


Figure 5.10. Two photographs showing the fibre probe spectrometer being deployed *in vivo* to measure abnormal (a) and normal (b) regions of tissue in the antrum (the distal section of the stomach). Both measurements were made in the same patient.

For the *ex vivo* tissue measurements the spectrometer remained in the private room mentioned above while endoscopic procedures were carried out nearby. During the procedures, endoscopic biopsies were taken of any suspicious regions of tissue and at least one biopsy of healthy tissue was also taken for comparison. After completion of an endoscopic examination, any tissue samples obtained were immediately delivered to the spectroscopy room and measured with the spectrometer. All samples were measured within approximately 15 minutes of excision. Following interrogation with the spectrometer, all tissue samples were sent for histopathology to confirm (or correct) the endoscopic diagnosis.

Table 5.1. Table showing the clinical diagnoses of all the *ex vivo* samples and *in vivo* measurement sites investigated with the endoscopic spectrometer. Note that ‘gastric disease’ incorporates cancer, gastritis and polyps of the stomach.

Tissue type (clinical diagnosis)	Number of <i>ex vivo</i> samples	Number of <i>in vivo</i> measurement sites
Colonic polyps	24	8
Rectal cancer	0	1
Proctitis	0	1
Inflammatory bowel disease (IBD)	6	0
Gastric disease	5	0
Barrett’s Oesophagus	1	2
Oesophageal stricture (reflux)	1	0
Oesophageal nodule	0	1
Oesophagitis	0	1
Normal colon tissue	19	9
Normal stomach tissue	4	0
Normal oesophageal tissue	2	4
Total number of normal tissue measurements	25	13
Total number of diseased tissue measurements	37	14
Total number of measurements	62	27

For *in vivo* measurements the experimental procedure was slightly different. Firstly the spectrometer was moved into the endoscopy suite. Then, during the endoscopy, measurements were made of any suspicious areas of tissue by inserting the optical fibre probe into the working channel of the endoscope and figure 5.10 shows two endoscopic photographs of the probe being deployed *in vivo*. As for the *ex vivo* experiments, at least one measurement was also made of a complementary region of healthy tissue. Lastly, after the measurements were complete, all the irradiated regions of tissue were excised (by endoscopic biopsy) and sent for histopathological analysis. This allows confirmation of the clinical diagnosis and also ensures that any potentially photodamaged tissue is removed from the patient. Note also, however, that the average optical power at the sample was limited to 35 μW of UV and 200 μW of blue light (see section 5.2.1) in

order to minimise the risk of tissue damage – the irradiance at these power levels (for both lasers) is lower than the MPE for skin, even for exposures of up to 1000 s in duration.

The fluorescence acquisition parameters used for the *ex vivo* experiments were as follows: 5 s collection time for each laser with three repeat measurements made in each case. For the *in vivo* measurements, it was desirable to keep the total acquisition time as short as possible, therefore, the fluorescence collection time was also set to 5 s for each laser but only two repeat measurements were performed. The reflectance spectrum was acquired using a 2 s collection time for both *in vivo* and *ex vivo* experiments. Thus, the total acquisition time was approximately 35 s *ex vivo* and 25 s *in vivo*.

After completion of the experiments, each measured region was assigned a unique number for identification purposes. Table 5.1 shows the clinical diagnoses of all the areas of tissue investigated, for both the *ex vivo* and *in vivo* experiments. It should be noted here that the analysis presented in the subsequent sections of this chapter focuses on the clinical (rather than histological) diagnoses. This is because this research is still ongoing and, at the time of writing (April 2012), the histological diagnoses of some measurement sites still remained unknown.

5.3.2 Data analysis

Fluorescence data analysis

As shown in table 5.1, the majority of the data acquired to date has been from *ex vivo* tissue samples and the most common disease type observed has been colonic polyps. For this reason, most of the analysis presented has been performed on the *ex vivo* polyp and normal tissue data, however, some preliminary analysis has also been performed on other tissue types and on the *in vivo* data.

A double exponential model was fitted to the fluorescence decays recorded in each spectral channel and the mean fluorescence lifetime was also calculated (in the same way as described in section 4.2.4). The first step in the analysis of the fluorescence data was then to plot the fluorescence intensity and mean lifetime in each spectral channel, for both excitation wavelengths and for each tissue type. Achieving quantitative comparisons of the fluorescence intensity between samples was difficult because the excitation power incident on the samples varied from day to day. Therefore, the spectra presented in section 5.4 have been normalised to their peak fluorescence intensity, which allows comparison of the shape of the emission profiles but not the absolute intensity. Further analysis then focussed mainly on the fluorescence lifetimes and, in order to simply characterise each measurement site, a spectrally averaged fluorescence lifetime, weighted according

to the intensity recorded in each spectral channel, was calculated (as described in section 4.2.4). Using this parameter, the variation in the fluorescence lifetime was investigated, firstly across different tissue types and, secondly, for measurements made *ex vivo* and *in vivo* on the same tissue type. This was achieved by simply calculating the mean and standard deviation of the spectrally averaged lifetimes over all measurements made of particular tissues.

To investigate whether the fluorescence lifetime held any diagnostic information, the shift in the spectrally averaged lifetime was calculated for each measurement of diseased tissue relative to a corresponding region of healthy tissue measured on (or excised from) the same patient (i.e. $\Delta\tau = \tau_{lesion} - \tau_{normal}$). It should be noted that significant inter-patient variation was observed in the measured fluorescence lifetimes and investigating paired lifetime shifts within individual patients in this way should circumvent any potential problems that this variation could cause. The lifetime shifts were plotted only for the *ex vivo* polyps as this was the only disease type for which a statistically significant number of measurements had been made. Firstly the lifetime shifts were plotted for all the measured polyps and, secondly, the shifts were plotted only for polyps for which a measurement of normal tissue from the same region of colon was available. This acted to ensure that spatial variations in the fluorescence lifetime of normal colon tissue were not adversely affecting the data. Finally, Wilcoxon signed rank tests were performed to test for statistical significance in any trends observed in the paired shifts in the fluorescence lifetime.

Additionally, the mean emission wavelength was also calculated for each measurement. Analysis was then performed on the mean emission wavelength in an identical manner to that described for the spectrally weighted mean lifetime.

Reflectance data analysis

Regarding the reflectance spectra acquired from each sample, the aim was to analyse the data as discussed in section 5.2.6, by comparing the measured spectrum to that observed from a 99% reflectance standard. However, in practice very long acquisition times (> 10 s) were required to obtain appreciable reflectance signal from the GI tissue investigated (both *ex vivo* and *in vivo*). These long acquisition times were not suitable for *in vivo* measurements where each endoscopy must be completed within a short space of time to allow treatment of all patients scheduled for a given day. As such, no analysis of the reflectance data is presented here as the signal levels obtained were too low.

The reason for the lack of signal is simply that the reflectance of GI tissue is much lower than expected and, hence, the sensitivity of the reflectance spectrometer was too low. Thus, to solve this problem, an alternative spectrometer with a wider input slit (USB2000+VIS-NIR-ES, Ocean Optics, The Netherlands) will soon be installed in order to allow more light to reach the detector. Clearly this will provide better signal levels and, hence, further work on this project will involve investigation of the reflectance spectra as well as the fluorescence.

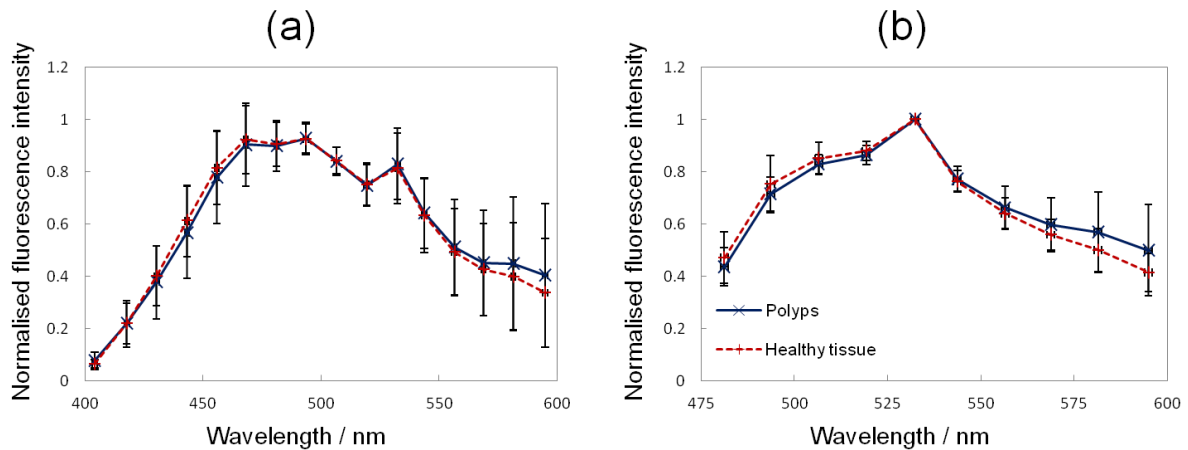


Figure 5.11. Mean normalised fluorescence spectra of colonic polyps and normal colon tissue for (a) UV and (b) blue excitation, observed *ex vivo*. Only measurements where the polyp and the normal tissue were excised from the same region of the colon have been included.

5.4 Results

The first result presented here (figure 5.11) shows the mean fluorescence spectra observed *ex vivo* in normal colon tissue and in colonic polyps, with both excitation wavelengths. As discussed above, variations in the excitation power made it difficult to compare the absolute intensities recorded in different samples, therefore, figure 5.11 shows normalised fluorescence spectra, which allows comparison of the shape of the emission spectra if not the absolute intensity. The values in each spectral channel represent the mean normalised fluorescence intensity averaged over all measurements of colonic polyps and normal colon tissue. It should also be noted that polyps have only been included in the average if a corresponding measurement of normal tissue was made in the same region of colon. This ensures that any differences in the observed spectra are due to changes associated with the development of a polyp and are not simply caused by variations in the tissue structure across different regions of the colon. The error bars on the graphs show ± 1 standard deviation and it is clear that, within these errors, any differences between the fluorescence emission profiles of polyps and normal colon tissue are small. One possible observed difference is that, with

both excitation sources, the normalised fluorescence intensity from polyps is greater than that from normal colon tissue at wavelengths above 550 nm.

Figure 5.12 shows the mean fluorescence lifetimes measured *ex vivo* in each spectral channel for both UV and blue excitation. As for the intensity data, the values represent the mean (± 1 standard deviation) fluorescence lifetime averaged over all measurements of polyps and of normal colon tissue. Again, measurements are only included if the normal tissue biopsy and the polyp biopsy were performed in the same region of colon. With both excitation sources the mean lifetimes of polyps are shorter than those observed in normal tissue. This trend is observed in all spectral channels and is most apparent with blue excitation. With both lasers there is a substantial spread in the lifetimes measured across different patients and this is illustrated by the error bars in figure 5.12, which show ± 1 standard deviation from the mean.

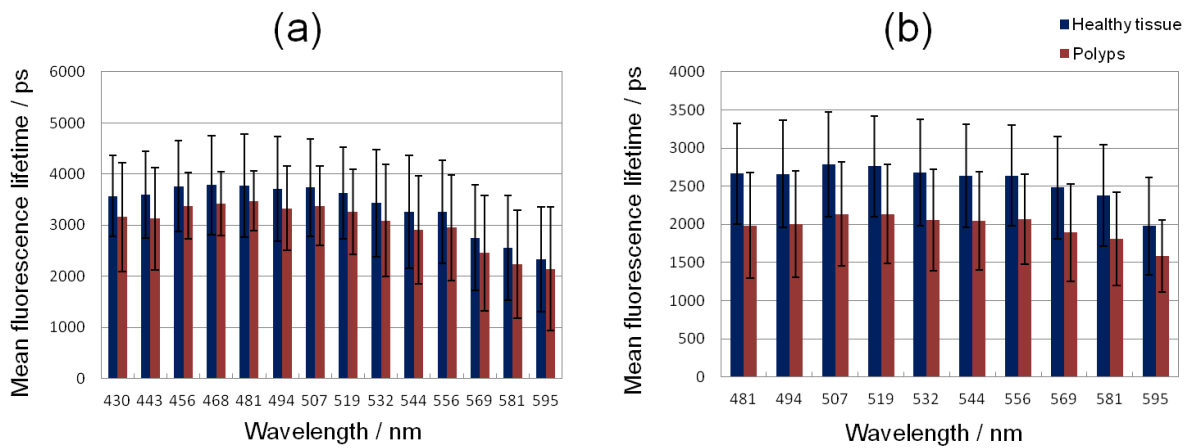


Figure 5.12. Bar graphs showing the spectrally resolved mean fluorescence lifetimes of polyps and normal colon tissue excited using the UV (a) and the blue (b) laser. Mean values were calculated across all polyps for which a normal tissue sample was excised from the same region of colon. The error bars represent ± 1 standard deviation.

As shown in figure 5.12, the contrast in fluorescence lifetime between polyps and healthy colon tissue does not vary significantly across the spectral range investigated. For this reason, and in order to simply characterise each measurement site, a spectrally averaged mean fluorescence lifetime (weighted according to the intensity recorded in each spectral channel) was calculated for each measured region. This provides a single number to simply characterise each measurement site and, hence, also permits straightforward comparison of different tissue types. This parameter was first used to assess the variation in the fluorescence lifetimes of normal tissue from different regions of the colon (as colon was the normal tissue type for which the greatest number of measurements

was made). To this end, figure 5.13 shows the mean spectrally averaged lifetimes (averaged over all samples) for normal tissue excised from different regions of the colon for both excitation sources. The values represent the mean spectrally averaged fluorescence lifetimes from all *ex vivo* measurements of normal colon tissue separated according to the region of the colon from which the tissue was excised, while the error bars show ± 1 standard deviation. Note that the error bars indicate the inter-patient variability in the measured lifetimes, as this variability is considerably larger than the uncertainty in the lifetime determination.

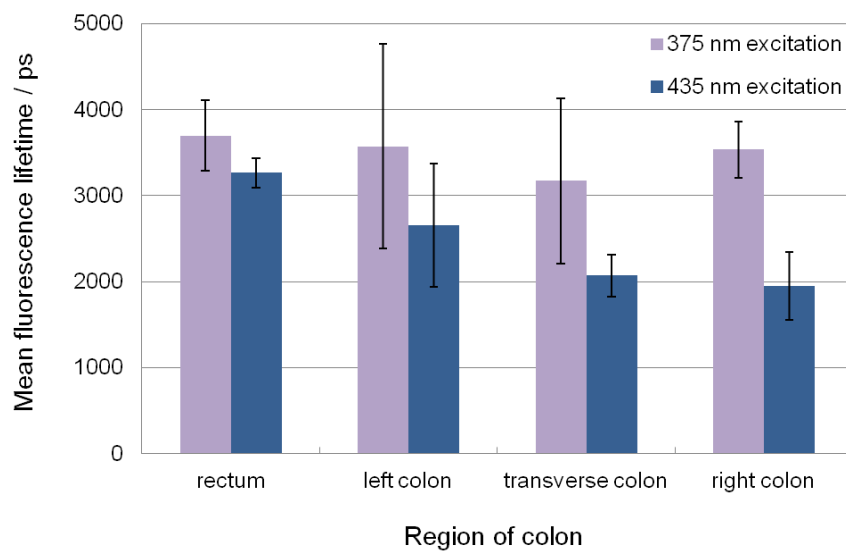


Figure 5.13. Graph showing the mean spectrally averaged fluorescence lifetimes recorded in normal tissue excised from different regions of the colon for both UV and blue excitation. Values represent the mean lifetimes averaged over all *ex vivo* measurements of normal tissue in the appropriate region and the error bars show ± 1 standard deviation. The category entitled ‘left colon’ includes all measurements made in the sigmoid colon, descending colon and splenic flexure while the ‘right colon’ group incorporates measurements from the caecum, ascending colon and hepatic flexure.

It is evident from the figure that the lifetimes measured after excitation with the UV laser do not vary substantially according to the region of the colon. With the blue laser, on the other hand, the different regions of the colon investigated clearly exhibit differences in their fluorescence lifetimes and the spectrally averaged lifetime appears to decrease with increasing distance into the colon (with rectum representing the shortest distance and ‘right colon’ the greatest). This is an interesting result, which suggests that, when comparing healthy and diseased colon tissue, it is important to only study samples from the same area in the colon. This justifies the analysis procedure used in this section where diseased samples have been ignored if the measurement of normal tissue was not made in the same region of the colon. Furthermore, the fact that the lifetime

varies under 435 nm excitation but not with excitation at 375 nm is also intriguing and may be explained by the different penetration depths of the two lasers. The blue light will penetrate further into tissue than the UV radiation, hence, the lifetimes observed with the blue laser are more likely to be sensitive to variations in the thickness of the intestinal mucosa, which is known to vary across different regions of the colon.

In order to further demonstrate how the lifetime varies across different tissue and disease types, table 5.2 shows the mean (and standard deviation) of all the spectrally averaged lifetimes recorded for each tissue type, for both the *ex vivo* and *in vivo* experiments. It is evident from this table that the fluorescence lifetime is affected by a number of factors including tissue type, disease state and excitation wavelength. Indeed, following on from the result presented in figure 5.13, table 5.2 presents further evidence of the variation in fluorescence lifetime across different regions of normal tissue. For example, for UV excitation of all *ex vivo* normal tissue samples it is observed that $\tau_{stomach} < \tau_{colon} < \tau_{oesophagus}$. Additionally, the standard deviations on the quoted mean lifetimes are quite large in a number of cases, indicating that there is also considerable inter-patient variation. Furthermore, while there are some clear differences between measurements made *ex vivo* and *in vivo*, the standard deviations are often large in comparison to these differences and, in some cases, there is even good agreement between the values. For example, for the cases of normal colon tissue and colonic polyps (tissue types where the highest number of measurements have been made) excited at 435 nm, the mean lifetimes measured *ex vivo* and *in vivo* are in relatively good agreement, certainly to within the quoted standard deviations.

A further observation from table 5.2 is that there appear to be differences in fluorescence lifetime between healthy and diseased regions of tissue. This is true, in particular, for colonic polyps measured *ex vivo* (highest number of measurements) using the blue excitation source. For this reason, the shift in the spectrally weighted mean lifetime was investigated for all the measured regions of diseased tissue relative to a corresponding area of healthy tissue measured on (or excised from) the same patient. Figure 5.14 shows the lifetime shifts observed with both excitation sources in all polyps measured *ex vivo*. Firstly, this figure shows the lifetime shifts measured for all polyps and, secondly, it also shows only the shifts observed in polyps where the complementary measurement of healthy tissue was made in the same region of the colon. This ensures that any shift in lifetime is caused by changes associated with the onset of disease and not simply by variations in the tissue structure at different points in the colon (i.e. this acts to reduce the effects of the variation in fluorescence lifetime across different regions of the colon displayed in figure 5.13). It should be noted here that only 18 polyps are shown in figure 5.14 while a total of 24 were included in the

study (see table 5.1). This is because some data was rejected due to very low signal levels caused by poor transmission of excitation light through the fibre bundle, which occurred in a small number of experiments.

Table 5.2. Mean spectrally averaged fluorescence lifetimes observed in all the tissues investigated in this study. Data is presented for both the UV and blue laser and for both *ex vivo* and *in vivo* measurements. Uncertainties denote one standard deviation from the mean. Where no standard deviation is shown, only one measurement was made.

Tissue type	Mean lifetime (<i>ex vivo</i>) / ps		Mean lifetime (<i>in vivo</i>) / ps	
	375 nm excitation	435 nm excitation	375 nm excitation	435 nm excitation
Normal colon (all)	3480 ± 920	2480 ± 650	3070 ± 610	2370 ± 770
Normal colon (polyp patients)	3610 ± 940	2670 ± 680	2950 ± 590	2510 ± 880
Normal colon (IBD patients)	3190 ± 900	2020 ± 230		
Normal stomach	3020 ± 290	2650 ± 920		
Normal oesophagus	4220	2730	3780 ± 670	3820 ± 730
Polyps (all)	3540 ± 1330	2390 ± 870	2700 ± 940	2320 ± 540
Polyps (where normal tissue was also obtained from same region of colon)	3120 ± 1320	2030 ± 640		
IBD	4420 ± 1200	2130 ± 390		
Gastric disease	3190 ± 250	2310 ± 910		
Barrett's Oesophagus	4380	3090	3740 ± 600	3990 ± 390
Rectal cancer			2590	1910
Proctitis			4900	3450

The first observation made from figure 5.14 is that, when using UV excitation, there are no obvious trends in the lifetime shifts between polyps and normal colon tissue. This is true for all polyps (figure 5.14(a)) and for just those where the normal tissue sample was taken from the same region of the colon (figure 5.14(c)). Using 435 nm excitation on the other hand, the lifetime difference results are more promising. When investigating all polyps, it is clear that polyps have a shorter fluorescence lifetime than normal colon tissue and this is true in 14 of the 18 polyps studied. If only polyps with corresponding normal tissue samples from the same region of colon are considered then this trend becomes even more convincing with 13 out of 14 polyps showing a negative lifetime shift (i.e. $\tau_{lesion} < \tau_{normal}$). In this case the magnitude of the mean lifetime shift was calculated as 580 ps and it should be noted that there is a small discrepancy between this value and the difference corresponding to the mean lifetimes quoted in table 5.2 for polyps (2030 ps) and normal colon tissue (2670 ps). This is caused by the occasional use of a single normal sample to calculate the lifetime shifts for numerous polyps within a single patient.

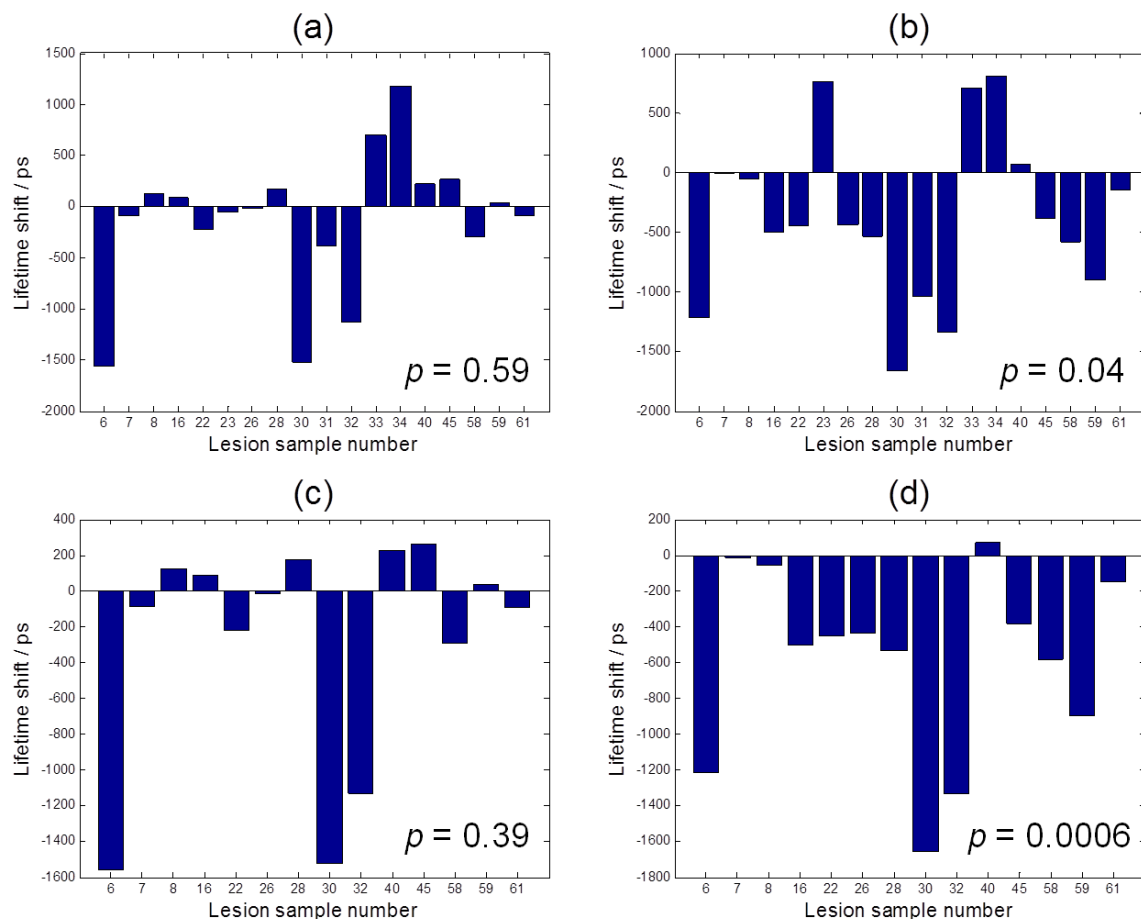


Figure 5.14. Shift in the spectrally averaged mean fluorescence lifetime for UV (a, c) and blue (b, d) excitation of *ex vivo* polyps. (a) and (b) show the lifetime shifts for all polyps while (c) and (d) show the lifetime shifts only for polyps where normal tissue was measured in the same region of colon.

To test for statistical significance in the observed trends in the difference in fluorescence lifetime, Wilcoxon signed rank tests were performed on all the sets of paired difference values (null hypothesis: the mean shift in fluorescence lifetime is equal to zero). The p-values resulting from these tests are shown on the graphs in figure 5.14 and all act to confirm the observations made above. The UV data show no significant trends while the blue data represent a consistent decrease in the fluorescence lifetime of polyps with respect to healthy tissue. As stated, this is most evident when only considering polyps with a normal tissue sample from the same region of colon and in this case the p-value obtained was 6.1×10^{-4} (i.e. $p < 0.001$).

Lastly, the mean emission wavelength was also calculated for every measured region and for each excitation wavelength. The difference in the mean emission wavelength was then calculated for all *ex vivo* polyps (relative to neighbouring healthy tissue) and Wilcoxon signed rank tests were performed to check for statistical significance, as for the spectrally averaged fluorescence lifetime. As was the case for the fluorescence lifetime, no clear trends were observed with UV excitation. With blue excitation, however, when investigating only polyps with a normal tissue sample from the same region of colon, there appeared to be a positive shift in the mean fluorescence emission wavelength (i.e. $\lambda_{lesion} > \lambda_{normal}$). Though this trend was less convincing than that observed in the fluorescence lifetime data – four of the 14 measurements showed the opposite behaviour – it was still seen to be statistically significant to below the 5 % significance level ($p = 0.035$).

Overall, the results obtained thus far show that – when studied *ex vivo* using 435 nm excitation – polyps of the colon exhibit statistically significant changes in their mean fluorescence emission wavelength and, in particular, in their spectrally averaged mean fluorescence lifetime. This is a promising result, which suggests that time-resolved fluorescence spectroscopy (or imaging) may have potential diagnostic applications in GI endoscopy. Further work should now involve the collection of more data to confirm the observations made so far and to allow the investigation of additional disease types. In particular, more *in vivo* data is required to ascertain whether the promising trends seen *ex vivo* still hold before the tissue has been excised.

5.5 Discussion and conclusions

This chapter has described the development of a multidimensional, clinically deployable spectrometer that provides measurements of diffuse reflectance spectra and fluorescence decays in 16 spectral channels. The system utilises two pulsed lasers for excitation of fluorescence with central

emission wavelengths of 375 nm and 435 nm. The entire device is housed within an easily transportable wheeled trolley and has been designed in order to make clinical use both safe and practical. To this end, all components are fully computer controlled by software written in LabVIEW 7.1 (National Instruments, USA) and the system incorporates a USB footswitch to allow use of the instrument by a single clinician during an endoscopic procedure. Furthermore, all optical and electrical components are fully enclosed, the output powers at the distal end of the probe are limited using ND filters and interlock switches are installed on the trolley to ensure that the lasers cannot be operated while the optical setup is exposed.

The spectrometer is now assembled in the endoscopy unit of Charing Cross Hospital in London where it is regularly used to make clinical measurements of human GI tissue, both *ex vivo* and *in vivo*. As discussed in this chapter it is currently being applied to studies of GI disease and, to date, this has involved the investigation of 62 *ex vivo* samples and 27 *in vivo* measurement sites in the oesophagus, stomach and intestine. The majority of the measurements made so far have been of *ex vivo* colonic polyps and the preliminary results presented above indicate differences between polyps and normal colon tissue in terms of both their mean fluorescence emission wavelength and their fluorescence lifetime.

It has been shown that the mean fluorescence emission wavelength from polyps is longer than that observed in normal colon tissue for excitation at 435 nm. This was seen to be true in 10 of the 14 polyps investigated for which a complementary normal tissue biopsy was taken from the same region of the colon. A Wilcoxon signed rank test was performed on this data and it was shown to be statistically significant with a p-value of 0.035 (i.e. $p < 0.05$).

In terms of the fluorescence lifetime data, a negative shift in the lifetime was observed between polyps and healthy tissue (i.e. $\tau_{lesion} < \tau_{normal}$) when excited using the blue laser. In this case, the contrast was more convincing with 13 out of 14 polyps exhibiting a negative lifetime shift. A Wilcoxon signed rank test confirmed this trend and also confirmed the improved significance, this time returning a p-value of 6.1×10^{-4} (i.e. $p < 0.001$).

With UV excitation, no significant trends were observed in the fluorescence lifetime or mean emission wavelength. The observations made with the blue laser, however, are very promising and suggest the potential use of the mean emission wavelength and, particularly, the fluorescence lifetime as clinical diagnostic parameters. In such a scenario, it is logical to consider moving to an imaging modality (rather than point spectroscopic measurements). This would then also provide spatial resolution and morphological information as an addition to the spectroscopic data, and

would be considerably more intuitive for a clinician to use. Future work in this field should eventually be directed towards the development of such imaging instrumentation and this could be guided by the spectroscopic results obtained with instruments like the one described in this chapter. Indeed, work towards realising clinically viable time-resolved endoscopic fluorescence imaging is already underway, for example see reference [107].

More immediate future work is more likely to involve the collection of a larger dataset to allow confirmation of the observations made in the results section of this chapter. While additional *ex vivo* measurements would be useful, it is most important to build a bank of *in vivo* data to permit detailed investigation of the optical signatures of GI tissue before it is removed from a patient. Only at this stage will the true merit of fluorescence lifetime measurement for diagnosis of GI disease be known. On this topic, one promising result discussed in section 5.4 is the observation that the spectrally averaged fluorescence lifetimes measured in polyps and in normal colon tissue do not vary considerably (with 435 nm excitation) depending on whether they are studied before or after excision from the patient. While this result is based on only a small number of *in vivo* measurements, it suggests that the *ex vivo* trends documented here may also be observed *in vivo*.

Chapter 6: A novel endomicroscope design based on adaptive phase compensation in an optical fibre bundle

Up to this point, the work presented in this thesis has been mainly concerned with the spectroscopic study of optical signals in healthy and diseased tissue, with the overall aim being to investigate whether these optical measurements offer any diagnostic capabilities. As mentioned previously, however, when envisaging the development of an instrument for clinical diagnosis, it is logical to consider the use of an imaging (rather than a point measurement) modality because imaging provides morphological information and is more intuitive for a clinician to use. One endoscopic optical imaging methodology that is becoming increasingly widespread is confocal or multiphoton endomicroscopy. This technique can be applied to fluorescence or reflectance measurements and provides high resolution, optically sectioned endoscopic images. It has been used in both medical diagnostic studies and biological investigations involving imaging of small animals (such as rats and mice) and its use is becoming more common. Despite this, current approaches to endomicroscopy still suffer from a number of inherent drawbacks (e.g. size, number of resolution elements, etc. – see sections 3.3.1 and 6.1.1 for details) and, as such, it is important to improve these techniques or to develop new methods in order to solve the current problems. This chapter introduces a novel approach to laser scanning endomicroscopy and presents proof-of-principle results, which demonstrate that this technique permits beam focussing, three-dimensional (3-D) scanning and imaging with no distal optics or moving parts.

6.1 Introduction and background

6.1.1 Current approaches to endomicroscopy and their drawbacks

Section 3.3.1 discussed laser scanning endomicroscopy and presented two distinct approaches that are in common use. The first is based on distal scanning, which incorporates a single mode fibre for delivery and collection of light along with a distal scanning mechanism that can consist of miniaturised scanning mirrors or a scanning head that physically moves the tip of the optical fibre. The second technique is based on proximal scanning, which utilises a coherent optical fibre bundle for beam delivery and, in this case, scanning of the sample is achieved using proximal scanning mirrors that sequentially couple light into the individual fibre cores within the bundle [100].

Laser scanning endomicroscopy is becoming increasingly widespread and commercial systems are now available that incorporate either the distal scanning (EC3970K, Pentax, Japan) or proximal scanning (Cell-viZio™ GI, Mauna Kea Technologies, France) approach. Examples of the use of endomicroscopy include its application to small animal imaging experiments for biomedical studies [185] as well as to clinical studies aimed at diagnosis of disease [104, 170-171]. Furthermore, there is also significant interest in developing confocal endoscopic FLIM techniques for use in Förster Resonance Energy Transfer (FRET) experiments and, therefore, proximal scanning endomicroscopy has recently been adapted to permit such time-resolved fluorescence imaging [107, 126].

Despite the increasing use of endomicroscopy, neither of the current methods is without drawbacks. Distal scanning endomicroscopes require the use of a relatively large distal scanner (typically the outer diameter of the scanning head is greater than 3 mm [186]). This means that the minimum size of the endomicroscope is inherently limited by the scanning mechanism. Additionally, the miniaturised scanner is usually the most expensive part of such an endomicroscope. Hence, having it located at the distal end – which comes into contact with the patient and is regularly sterilised – is not ideal as the risk of damage is increased compared to any components situated at the proximal end. Proximal scanning permits the development of smaller, cheaper endoscopic probes as no mechanical components are required at the distal end, however, the use of an optical fibre bundle brings alternative disadvantages. Firstly, the number of resolution elements in an image obtained with a proximal scanning endomicroscope is restricted by the number of fibre cores in the bundle (typically around 30,000). Furthermore, the fixed separation of the fibre cores means that the sampling rate is also fixed. Thus, if small features are imaged then the endoscopic images acquired can suffer from the effects of undersampling. Finally, both approaches require motorised z-scanning (which is typically slow) and the NA and field of view are usually fixed for a given endoscopic probe.

Clearly, an ideal endomicroscope would provide the imaging quality of a distal scanning system but with the size and cost advantages of a proximal scanning device, whilst also enabling 3-D, optically sectioned imaging. The following section of this chapter now describes the theory behind a novel approach to beam scanning in endomicroscopy. The remainder of the chapter then focuses on the experimental methods used and the results obtained in a proof of concept study based on this new technique.

6.1.2 Proposed novel technique for compact laser scanning endomicroscopy

The novel approach to endomicroscopy discussed in this section is illustrated in figure 6.1. This technique (which could be applied to both confocal and multiphoton endomicroscopes) is similar in its optical configuration to standard proximal scanning endomicroscopes in that it utilises a fibre bundle and has the 'scanning' components at the proximal end (see figure 6.1(a)). In this case, however, rather than scanning the input beam sequentially across each core in the bundle, all cores are illuminated simultaneously using a proximal spatial light modulator (SLM). This SLM firstly pre-compensates for phase aberrations induced by the fibre bundle such that a plane wave (rather than an aberrated wave) can be emitted from the distal end of the bundle. Secondly, the SLM acts to control the phase of the light propagating in each individual fibre core so that it is possible to generate arbitrary wavefronts at the distal end in a manner analogous to phased array radar. Thus, with a single lens at the distal output of the bundle it is possible to create a focussed beam by simply correcting the phase aberrations in each fibre core to produce a plane wave that will be focussed by the distal lens. This focus can then be scanned in the x-y plane by adding tip and tilt to the wavefront using the SLM (as shown in figure 6.1(b)). Furthermore, by adding curvature to the input wavefront (again using the SLM) it is possible to generate a focussing wave without the use of a lens and, hence, distal optics can be dispensed with completely (figure 6.1(c)). Z-scanning can be achieved in both cases by simply adjusting the curvature of the input wavefront. Thus, this approach permits beam focussing and 3-D scanning in endomicroscopy without the need for any mechanical or even optical components at the distal end.

The advantages of this approach over the current methodology are clear. Firstly, the ability to dispense with distal components – both mechanical and optical – means that this technique could permit the development of ultrathin endoscopic probes, where the minimum size of the endomicroscope is only limited by the diameter of the fibre bundle (< 1 mm). As discussed above, this also has advantages in terms of the cost of the endoscopic probes as no expensive scanning mechanism would be required at the distal end. In terms of image quality, the number of resolution elements in a system based on this novel technique would still be ultimately limited by the number of fibre cores within the bundle, however, the problem of undersampling would no longer exist as the location of the focal spot can be arbitrarily controlled and is no longer determined by the positions of the fibre cores within the bundle. Furthermore, as stated above, this approach permits 3-D scanning of the focal spot and will also provide flexibility in terms of both field of view and NA (i.e. spatial resolution). Finally, the use of a SLM also means that there is the potential to apply further phase correction, for example, correction of aberrations induced by the sample as well as the fibre bundle itself, which could allow imaging at increased depths within biological tissue.

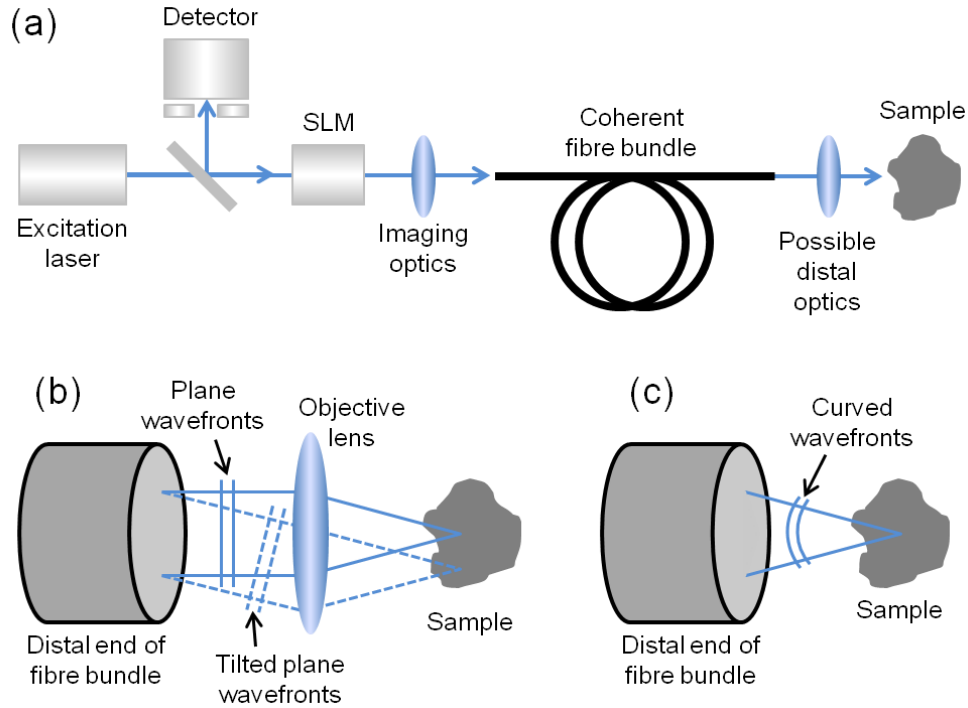


Figure 6.1. Diagrams illustrating the novel concept for beam focussing and scanning in endomicroscopy. (a) shows the concept as a whole (used for confocal reflectance imaging) while (b) and (c) illustrate distal focussing and scanning with (b) and without (c) distal optics.

The pre-correction approach described here is similar in concept to the adaptive optics techniques used to control beam propagation through strongly scattering media that have been extensively studied in the context of atmospheric propagation [187-189]. More recently, such techniques have also been applied to microscopy to allow the focussing of light through turbid media for applications in both imaging and optical micromanipulation [190-194].

The remainder of this chapter first explains the experimental approach used to test this novel endomicroscope design, which utilised a Mach-Zehnder interferometer to measure the phase delay imparted by each fibre core. Afterwards, proof of principle results are presented that demonstrate the capability for beam focussing, 3-D scanning and imaging [195].

6.2 Methodology

6.2.1 Experimental procedure

The aim of the experiments presented in this chapter was to demonstrate the feasibility of the novel endomicroscope design discussed in the preceding section. Thus, the first stage of experimentation

involved measuring the output phase distribution from a coherent optical fibre bundle (FIGH-30-650S, Fujikura Europe Ltd., UK) and then ‘correcting’ the phase using a SLM (SXGA-R2-H1 FLCOS microdisplay, 1280 x 1024 pixels, CRL Opto Ltd., UK). With the phase aberrations corrected, the secondary aim was then to use the SLM to generate a focussed, 3-D scanning beam without the aid of distal optics or mechanics.

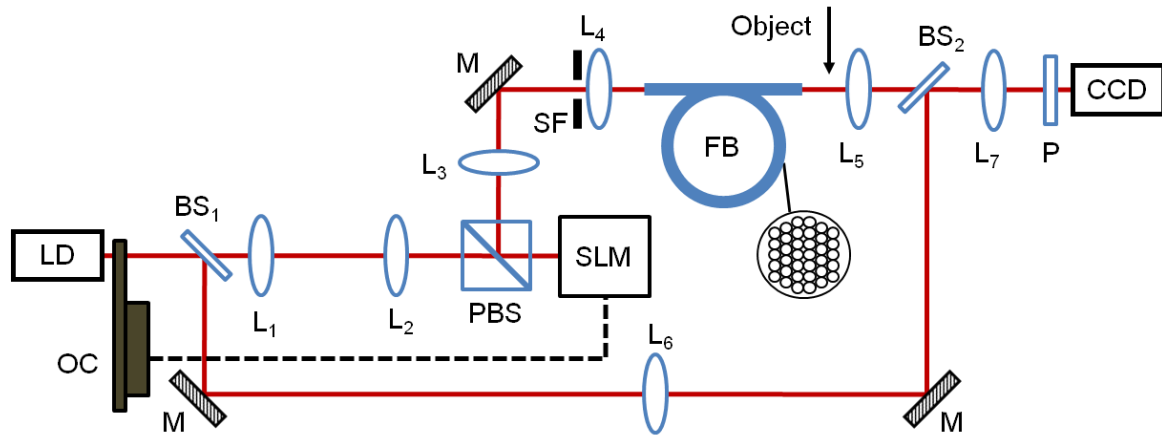


Figure 6.2. Experimental setup used for measuring and correcting phase aberrations in a coherent optical fibre bundle. LD – 840 nm laser diode; OC – optical chopper; BS₁, BS₂ – 50/50 beam splitters; L₁, L₄, L₅ – microscope objectives (10x, 60x and 20x respectively); SF – spatial filter; PBS – polarising beam splitter; SLM – spatial light modulator; M – mirror; FB – fibre bundle; P – polariser; CCD – camera; L₂, L₃, L₆, L₇ – lenses ($f = 100$ mm, 200 mm, 250 mm and 250 mm respectively). Arrow indicates where the object was inserted to record images. Figure adapted, with permission, from reference [195]. © 2012 The Optical Society.

A Mach-Zehnder interferometer setup, analogous to that used by Neil et al. [196], was used to measure the phase distribution at the output of the fibre bundle and the experimental system is shown in figure 6.2. The emission from a single mode diode laser (HL8325G, 840 nm, 40 mW, Hitachi Ltd., Japan) was first collimated and then split into a reference and a sample arm using a 50/50 beamsplitter. The sample beam was expanded and directed onto the SLM, which was programmed to display a binary hologram that coded for the amplitude and phase of the light coupled into each fibre core (see section 6.2.3). The SLM was then imaged onto the input face of the fibre imaging bundle using a 4- f system comprising a lens (L₃, $f = 200$ mm) and a 60x microscope objective (L₄). This 4- f system also contained a spatial filter in the Fourier plane (back aperture of the 60x objective), which acted to reduce the effective NA of the objective to 0.45 and to ensure that only the first order diffracted light was transmitted to the fibre bundle (see section 6.2.3 for details of the generation of wavefronts using the SLM). The distal end of the bundle was then imaged onto a CCD camera (Orca ER, Hamamatsu Photonics, Japan) using a long working distance 20x microscope

objective (L_5) and a tube lens (L_7 , $f = 250$ mm). The sample and reference beams were recombined by a second beamsplitter and two lenses (L_6 and L_7) in the path of the reference beam ensured that it was collimated at the CCD. Thus, the image formed at the CCD was an interference pattern, from which the phase in each fibre core could be calculated (see section 6.2.4 for details). A polariser located in front of the CCD camera acted to remove the effects of polarisation dispersion in the fibre bundle. Finally, because the SLM needed to be operated with a net bias voltage of zero, a synchronised optical chopper was used to ensure that the SLM was only illuminated while receiving a positive drive voltage.

Having measured the phase in each fibre core, it was then possible to program the hologram displayed on the SLM to illuminate the fibre bundle with the appropriate phase distribution to produce a plane-wave output at the distal end. Interferograms were recorded before and after the phase correction was applied to confirm that the phase aberrations had been successfully corrected. With the phase aberrations corrected, the SLM hologram could then be adjusted to add the appropriate curvature, tip and tilt to the output wavefront in order to focus the beam and scan it in the x -, y - and z -directions. To demonstrate this laser scanning capability, the 20x microscope objective (L_5) was defocused to image an object plane a short distance (typically 0.5 mm) from the output face of the fibre bundle and the reference beam was blocked such that only the sample beam reached the CCD. The SLM hologram was then programmed to generate an output wavefront that would focus at this object plane and images of the resulting focal spot were recorded using the CCD. Again, images were recorded with and without a phase correction applied to confirm that the phase correction procedure provided improved focussing. Successive holograms with varied tip and tilt were then generated to exhibit lateral scanning and, similarly, variation of the curvature of the input wavefront was used to achieve axial scanning.

Finally, to investigate the potential for imaging, a United States Air Force (USAF) 1951 resolution test chart was placed in the object plane of the 20x microscope objective. The output beam was then focussed to this plane and laterally scanned (as described above) across the face of the test chart and the transmitted light was recorded at each scan position using the CCD. Firstly, for every scan position all the CCD pixel intensities were summed to produce a single intensity value (i.e. the CCD was operating as a single channel detector), which is equivalent to laser scanning microscopy with no confocal aperture. Secondly, confocal detection was simulated by recording the maximum CCD pixel value for each scanning beam position, rather than binning all of the pixels as before. This acts to improve the contrast of the images recorded by rejecting the background (and consequently eliminating both the photon shot noise and read noise associated with that

background light) and it approximately simulates ideal descanned confocal imaging in transmission with a delta function for the detector.

Images and line profiles illustrating the focussing and scanning capabilities, along with exemplar images of the USAF 1951 resolution test chart, are presented in section 6.3.

6.2.2 Pixel mapping

Before any phase correction can be applied by the SLM, a transformation between the SLM and the CCD frames of reference must be determined – i.e. a mapping scheme is required that calculates where to apply a phase shift on the SLM in order to correct for a delay measured at a particular location on the CCD. This mapping scheme must first include a scaling parameter to account for the magnification of the SLM onto the fibre bundle and the fibre bundle onto the CCD. Secondly it must also account for any rotation imparted by twisting of the fibre bundle and, finally, it should also include a lateral offset parameter. To this end, the locations of the centres of each of the fibre cores at the CCD were first determined using a centroiding algorithm. An affine transformation was then calculated between the CCD and the SLM coordinate frames according to equation (14),

$$\begin{bmatrix} x' \\ y' \end{bmatrix} = \begin{bmatrix} S_x x \cos \theta - S_y y \sin \theta \\ S_y x \sin \theta + S_x y \cos \theta \end{bmatrix} + \begin{bmatrix} t_x \\ t_y \end{bmatrix} \quad (14)$$

where: x' , y' , x and y are the SLM and CCD coordinates respectively; S_x and S_y are scale factors in the x - and y -directions; θ is the angle of rotation; and, lastly, t_x and t_y represent lateral translations in the x - and y -directions. It should be noted that this equation can incorporate a reflection by simply using negative scale factors, however, in the experiments presented here the scale factors were fixed as positive and any observed reflection was accounted for by reflecting the CCD images in software prior to display.

For each set of conjugate x - or y -coordinates (i.e. x' and x , or y' and y), equation (14) has three unknown parameters. Thus, to solve the equation a minimum of three conjugate pairs of CCD and SLM coordinates are required. To find each set of conjugate coordinates, a small hologram was displayed on the SLM that would illuminate just a single fibre core. Using software written in LabVIEW 7.1 (National Instruments, USA) the position of this hologram was then manually adjusted until the transmission through a chosen fibre core was maximised. At this point the central hologram (SLM) coordinates were recorded and the CCD image was centroided to provide the CCD coordinates. This was repeated five times to produce five sets of conjugate coordinate pairs (i.e.

more than three sets, which is the minimum required) and these were then used to calculate the rotation, scale factors and translation parameters as described by equation (14). To illustrate that the pixel mapping scheme worked successfully, figure 6.3 shows two images of the same region of the fibre bundle. In the first the whole area of the bundle is illuminated while in the second an individual fibre – which was not included in the calibration procedure – has been chosen for illumination (highlighted in both images), and it is clear that this fibre alone is transmitting light.

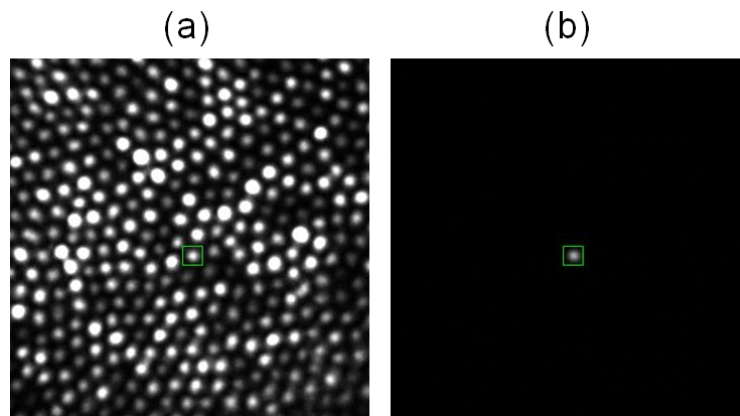


Figure 6.3. Images illustrating successful CCD-SLM pixel mapping. (a) shows an illuminated region of the fibre imaging bundle. (b) shows the same region of the bundle but in this case light has been preferentially coupled into a single fibre core. In both images the single fibre core is indicated by the green square.

6.2.3 Hologram and Wavefront generation

The SLM used in these experiments was a ferroelectric liquid crystal (FLC) device that was arranged to provide binary amplitude modulation. Liquid crystal (LC) molecules typically have different refractive indices along their long and short axes and, as such, they are birefringent and can induce a phase shift between two orthogonally polarised beams of light (see, for example, reference [197] for details of the optics of FLCs). In a FLC SLM designed for binary amplitude modulation, the LC molecules in each SLM pixel have two possible orientations that can be switched by applying a positive ('on' state) or negative ('off' state) electric field of the appropriate magnitude. In the 'off' state, when illuminated with linearly polarised light, the long axes of the molecules in the LC material are aligned parallel to the plane of polarisation of the input beam and, hence, no relative phase shift is induced. In the 'on' state, however, the LC molecules subtend an angle of 45° to the plane of polarisation. The thickness of the birefringent material is then chosen such that, in this orientation, a relative phase shift of π radians is induced between the components of the incident light beam that are parallel and perpendicular to the long axis of the LC molecules. Hence, in the 'on'

state, each SLM pixel acts as a half-wave plate and rotates the plane of polarisation of the input beam by 90° .

In the experiments presented here, the SLM was illuminated through a polarising beam splitter (PBS), as shown in figure 6.2. Thus, the light incident on the SLM is p-polarised. For pixels in the 'on' state, the plane of polarisation is rotated and the light reflected from the SLM is s-polarised. This modulated light will then be reflected by the PBS and directed into the 4- f system that images the SLM onto the input face of the fibre bundle (i.e. this SLM pixel will be bright or 'on'). For 'off' state pixels, on the other hand, the polarisation of the beam remains unchanged and the light is again transmitted by the PBS – thus, after reflection at the PBS this pixel will appear dark ('off'). Hence, it is clear that using this optical arrangement it is possible to achieve binary amplitude modulation and this is illustrated in figure 6.4.

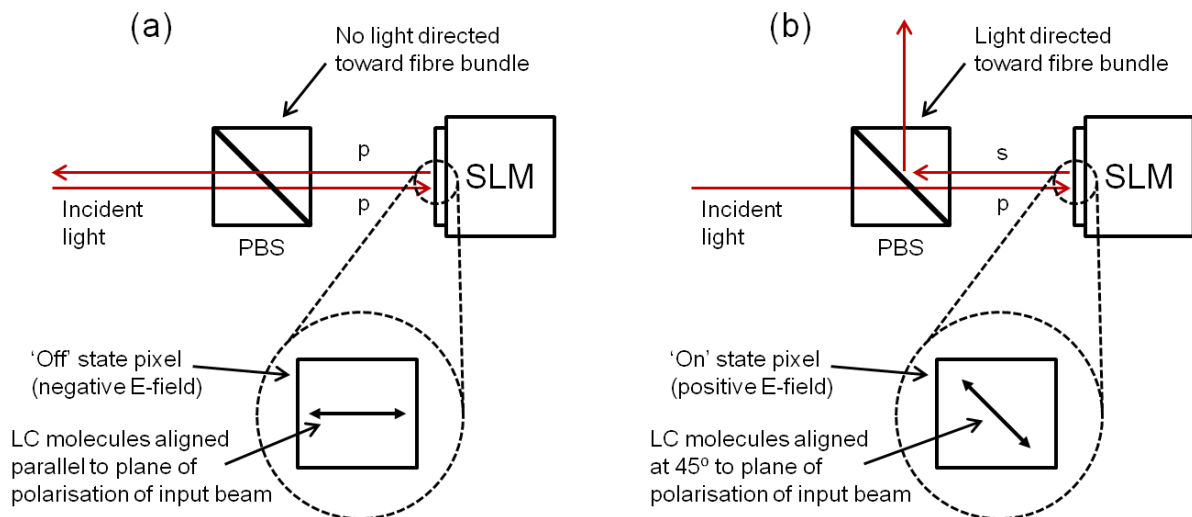


Figure 6.4. Diagrams illustrating how binary amplitude modulation is achieved using a PBS and a FLC SLM. (a) shows incident light reflecting from an 'off' state pixel while (b) shows light impinging on an 'on' state pixel. Inserts in both diagrams show on-axis views (i.e. along the z-direction) of the orientation of the LC molecules in a single pixel.

Of course, the aim here is to use the SLM to correct phase aberrations in a coherent fibre bundle, thus, it is necessary to modulate phase rather than amplitude. The binary amplitude modulated holograms were, therefore, used to control both amplitude and phase according to the method of W.-H. Lee [198-199]. Using this approach the SLM displays binary hologram fringes with a characteristic carrier frequency, which acts to separate the different diffraction orders in the Fourier plane. At specific locations within the hologram, the fringe position then codes for the local phase of the wavefront generated in the 1st diffraction order and the fringe width encodes the amplitude.

Thus, a desired wavefront can be generated by setting hologram pixels to represent ones (i.e. 'on' state) or zeros ('off' state) according to the inequality shown in equation (15) – i.e. a pixel is 'on' if the inequality is satisfied and 'off' otherwise.

$$\cos(2\pi\alpha - \varphi(x, y)) - \cos Q \geq 0 \quad (15)$$

In the above inequality,

$$\alpha = f_x X + f_y Y \quad (16)$$

and

$$Q = \arcsin(A(x, y)). \quad (17)$$

In equation (16) X and Y are matrices representing the coordinates of the pixels in the hologram array in the x - and y -directions respectively. The parameters f_x and f_y are single scalar values that define the desired x - and y -direction carrier frequencies of the hologram. Thus, α determines the angle and carrier frequency of the binary fringes. The variables A and φ are respectively the amplitude and phase of the desired wavefront. Finally, as stated above, the hologram is generated by setting pixel values equal to one if the inequality in equation (15) is satisfied and equal to zero otherwise.

As mentioned previously, this hologram will then code for the desired wavefront in the first (+1) diffraction order. Hence, to produce that wavefront the light reflected from the SLM is directed through a lens (L_3 in figure 6.2) and a diffraction pattern is created in the Fourier plane. A spatial filter located in this Fourier plane is used to block all but the 1st order diffracted light and a second lens (L_4 in figure 6.2) is used to form an image of the SLM on the input face of the fibre bundle. The second lens is positioned so that the 1st diffraction order passes through its centre and this acts to remove the tilt in the wavefront introduced by the hologram carrier frequency. Thus, a desired wavefront can be generated at the input face of the fibre bundle using a simple 4- f system with a spatial filter located in the Fourier plane.

Before the phase measurement and correction is performed, a hologram is designed to generate a wavefront with a Gaussian amplitude and a flat phase profile for each fibre core. The Gaussian amplitude profiles had a full width at half maximum (FWHM) of 8 SLM pixels, which was chosen to match the NA of the individual fibre cores. Both the amplitude and phase are also constant across all illuminated fibres within the bundle. The wavefront generation process is illustrated in figure 6.5 where a typical hologram has been used to numerically simulate the

diffraction pattern observed in the Fourier plane and the complex amplitude profile produced at the input to the fibre bundle. Note that the hologram (figure 6.5(a)) consists of many small circular regions of fringes (approximately 10 x 10 SLM pixels in size) that encode the amplitude and phase in individual fibre cores. Additionally, it should be stated that the carrier frequencies of the hologram shown are 0.15 fringes/pixel in the x -direction (f_x) and 0.13 fringes/pixel in the y -direction (f_y). This was the case for all the holograms used and these frequencies were chosen to provide adequate separation of the +1 and the other diffraction orders in the Fourier plane while also minimising the effects of aliasing.

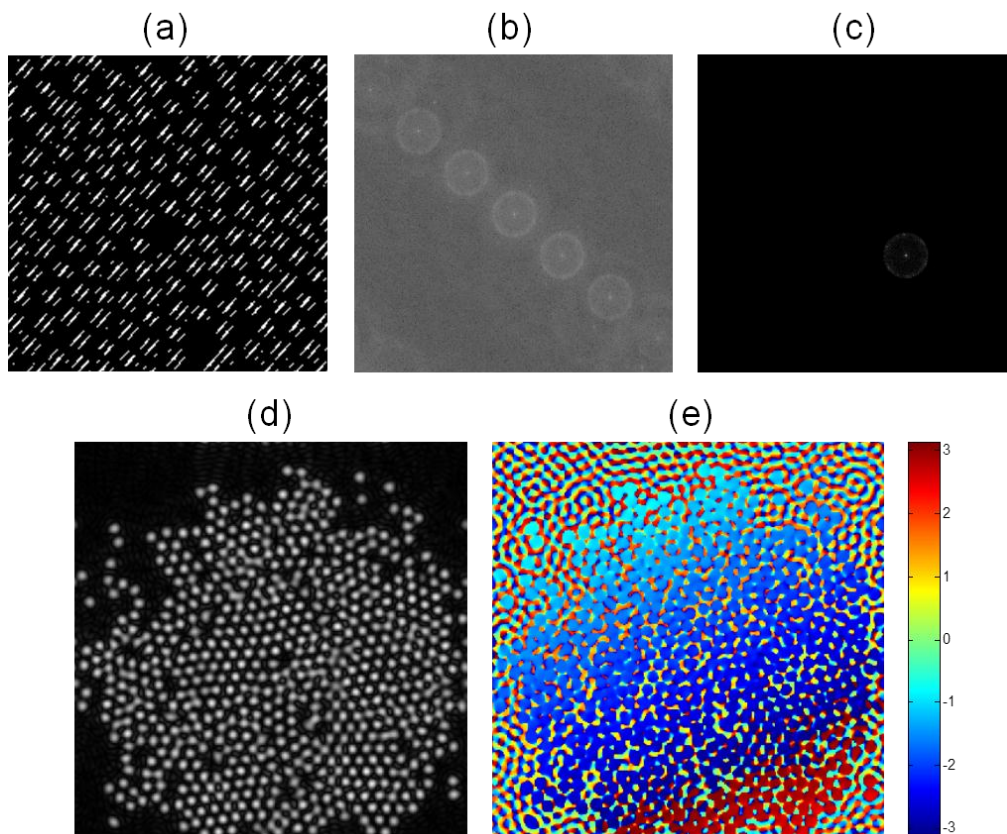


Figure 6.5. Wavefront generation using binary holograms and a 4- f system. (a) shows a section of an example hologram – the small circular regions of bright and dark fringes code for the amplitude and phase in individual fibre cores. Light diffracted by the SLM hologram is passed through a lens to form a diffraction pattern in the Fourier plane. (b) and (c) represent simulated intensity distributions in this Fourier plane before (b) and after (c) the light is passed through a low pass filter centred on the +1 diffraction order. Afterwards, the light is directed through a second lens and an image of the SLM is formed – (d) and (e) respectively show numerically simulated amplitude and phase distributions at this image plane. The tilt (i.e. carrier frequency) of the SLM hologram is removed by positioning the second lens so that the +1 diffraction order passes through its centre. Colour map in (e) represents phase (φ) in the range $-\pi < \varphi < +\pi$.

In this numerical simulation of the wavefront generation process, the intensity distribution in the Fourier plane was first simulated by calculating the square modulus of the 2-D Fourier transform of the hologram. This intensity distribution is shown in figure 6.5(b) where the various diffraction orders are clearly visible. Figure 6.5(c) shows the intensity distribution after the light has been passed through a low pass filter (located in the back aperture of lens L_4 – see figure 6.2). The remaining +1 diffraction order was shifted to the centre of the array shown in (c) in order to simulate the removal of the hologram tilt through the appropriate positioning of the second lens in the 4- f system (L_4). Finally, the inverse Fourier transform was calculated to simulate the complex amplitude profile produced at the input face of the fibre imaging bundle and figure 6.5(d) and (e) respectively show the amplitude and phase of the resulting wavefront. Note that there is a slow ramp in the phase map and this was caused by the fixed pixel size, which meant that, in this numerical simulation, only discrete tilts could be subtracted in the Fourier plane. Ignoring this slow ramp effect (which is only an artefact of the numerical simulation), the complex amplitude profile consists of a flat phase and a Gaussian amplitude profile in each fibre core with the phase and peak amplitude uniform across all fibres.

After calculating the phase (see section 6.2.4), the above approach can be used to generate a hologram that will correct the phase aberrations by simply setting the phase of the desired wavefront (the parameter φ in equation (15)) equal to the complex conjugate of the measured values. This will produce a plane wave at the distal end of the fibre bundle. Focussing and 3-D scanning of the output beam can then be achieved by adding curvature, tip and tilt to the input wavefront. This requires adding an additional phase value to each fibre core such that the overall phase (φ) is given by the sum of the phase applied to correct the aberrations (φ_c) and that applied to permit focussing and scanning (φ_f), i.e.

$$\varphi = \varphi_c + \varphi_f . \quad (18)$$

To generate a wavefront that will produce a focus at a distance, f , from the distal end of the fibre with transverse coordinates (x_c, y_c) , a converging spherical wavefront (of radius f) is required. Such a spherical wavefront can be produced by determining the value of φ_f in each individual fibre core according to equation (19).

$$\varphi_f = \frac{2\pi}{\lambda} \left\{ f + \sqrt{f^2 - (x_{fibre} - x_c)^2 - (y_{fibre} - y_c)^2} \right\} \quad (19)$$

In this equation λ is the wavelength, while x_{fibre} and y_{fibre} are the central coordinates of the fibre core in question. Thus, 3-D scanning of the output beam can be accomplished by simply adjusting the parameters f , x_c and y_c in the calculation of φ_f .

Finally, it should be noted that all the holograms used in these experiments were 512 x 512 pixels in size and these illuminated approximately 500 fibre cores in the bundle. While this represents only a small region of the bundle used (Fujikura FIGH-30-650S has 30,000 fibre cores), it is still large enough to demonstrate phase correction and to produce a focussed, scanning beam at the distal end.

6.2.4 Phase calculation

A Fourier domain method originally described by Takeda *et al.* [200] was used to calculate the phase at the centre of each fibre core and this process is illustrated graphically in figure 6.6. Firstly, a tilt was introduced into the reference beam to provide a small number of interference fringes (2-3) across the image of each fibre core in the interferogram formed at the CCD (see figure 6.6(a)). The fibre bundle was illuminated with a wavefront with a uniform phase distribution and an image of the resulting interference pattern was recorded using the CCD. Software written in LabVIEW 7.1 (National Instruments, USA) was then used to calculate the discrete Fourier transform (which provides an estimate of the continuous Fourier transform) of the interference pattern using the Fast Fourier Transform (FFT) algorithm. The Fourier transform was spatially filtered (in software) to remove all but the +1 diffraction order (figure 6.6(b)) and the filter used had a Gaussian edge to smooth any high frequency noise in the complex amplitude profile. Next, the filtered Fourier transform was shifted so the 1st diffraction order was situated in the centre and this was performed to remove the tilt in the reference beam (figure 6.6(c)). The inverse Fourier transform was then calculated and, from the resulting complex amplitude profile, the phase distribution at the output of the fibre bundle was extracted.

Following this measurement procedure, a phase map was generated that consisted of a flat phase profile across each fibre core with the value equal to that measured at the centre of the core (note that the centres of the fibre cores had previously been located by centroiding during the CCD-SLM pixel mapping process). An example resultant phase map is shown in figure 6.6(d). By applying the complex conjugate (i.e. the negative) of this phase map to the SLM hologram it was then possible to correct the phase distortion measured in each fibre.

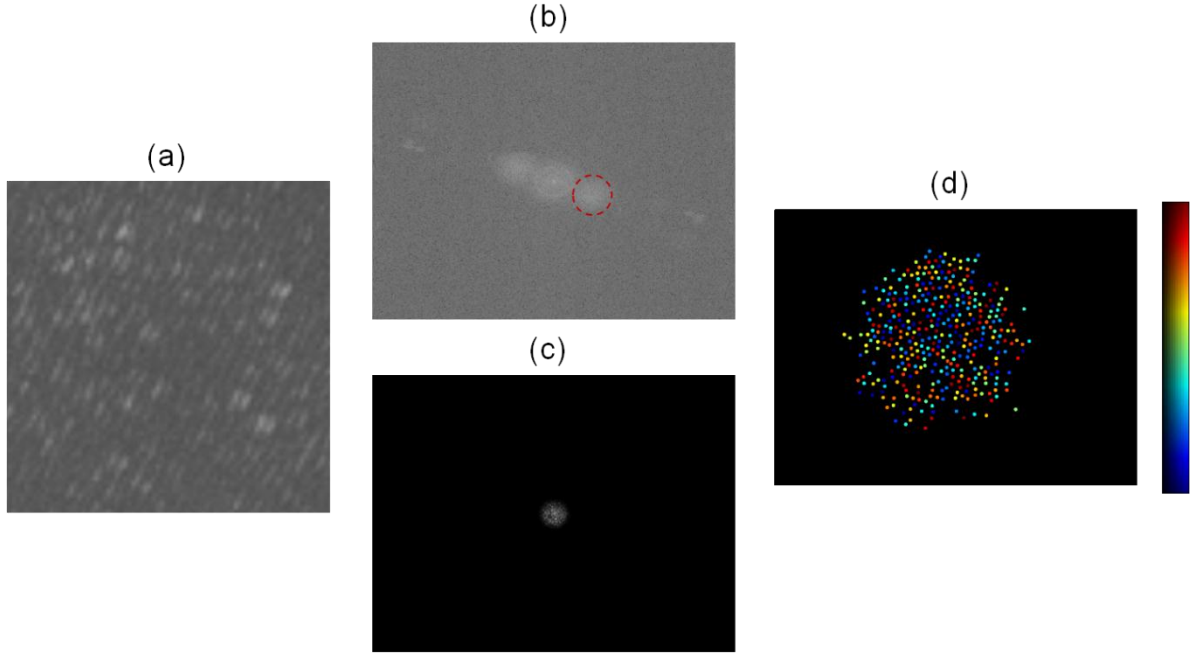


Figure 6.6. Phase measurement procedure. (a) shows a small region of a typical interference pattern where 2-3 interference fringes are observed across the image of each individual fibre core. The phase is obtained by calculating the Fourier transform of the interferogram (b), filtering out all but the 1st diffraction order and shifting this back to the centre of the image (c) and, finally, calculating the inverse Fourier transform. From the resultant complex amplitude profile, the phase at the centre of each fibre core can be extracted. (d) shows an example result of a phase measurement using this procedure. Note that the phase map in (d) has had a binary amplitude mask applied such that the image appears black in all areas in which no fibres are present. The dotted red circle in (b) represents the location of the low pass filter. The colour scale bar in (d) represents phase ranging from $-\pi$ (blue) to $+\pi$ (red).

6.3 Results

6.3.1 Phase correction

To illustrate successful correction of the phase aberrations induced by the fibre bundle, figure 6.7 shows an angular histogram of the phase recorded in each fibre core before and after a phase correction was applied. Figure 6.7(a) shows the phase histogram before correction and it is evident that the phase across the fibre bundle is random with an approximately uniform angular distribution. After correction (figure 6.7(b)) on the other hand, there is a clear bias towards a phase value of approximately 105° , indicating that some correction of the phase aberrations has been achieved. Note that the bias in phase is not necessarily directed towards 0° as the absolute phase may drift between measurement and correction of the phase aberrations due to, for example, variations in the laser output. None the less, as shown in figure 6.7(b), the relative phase across the

fibre bundle is corrected and all fibres have (approximately) the same phase, meaning that a reasonable approximation to a plane wave has been produced at the distal end of the fibre bundle.

One important additional observation from figure 6.7 is that, even after the correction process, not all of the fibre cores have the same phase value – hence the angular spread around the central phase value in figure 6.7(b). This is because, in practice, some fibres exhibit multimode propagation and/or cross-talk, both of which destroy the phase correction using the method described here. When focussing the distal beam, these uncorrected fibres will contribute to a background that will reduce the contrast of the focal spot. Nevertheless, the levels of phase correction shown are still high enough to permit focussing and scanning of the distal beam and results demonstrating this are presented in the following sections.

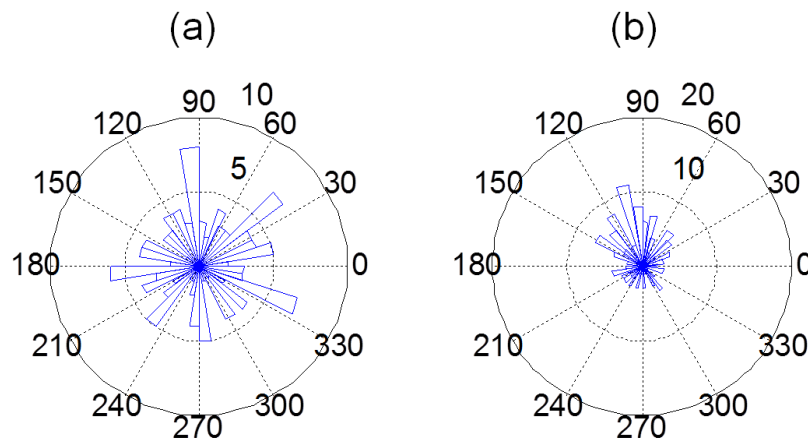


Figure 6.7. Angular histograms of the phase measured in each fibre core before (a) and after (b) correction of the phase aberrations. The radius in the histograms represents the number of fibres with a given phase value while the angle simply represents the phase in degrees. Note that the radial scales are different in the two histograms to allow clear viewing – in (a) the inner (dotted) circle represents 5 fibre cores while in (b) it represents 10 cores.

6.3.2 Beam focussing and scanning

After the phase measurement had been performed, the 20x imaging objective (lens L_5 in figure 6.2) was translated to image a plane 0.43 mm in front of the distal end of the fibre bundle. The appropriate curvature was then added to the input wavefronts – using equation (19) – to produce a focus at this object plane. Figure 6.8 shows intensity distributions at the object plane without (a) and with (b) the SLM applying the necessary phase to compensate for the wavefront aberration imposed by the fibre bundle. In the image shown in figure 6.8(a), the appropriate curvature has been applied to the input wavefront but no phase correction has been attempted (i.e. $\varphi_c = 0$ in all SLM pixels) and it is clear that no focal spot is generated. Instead there is simply a speckle pattern caused by random

interference of the light emitted from each fibre core. This is in agreement with the result of figure 6.7, which suggested that, before correcting the phase aberrations, the phases in individual fibres cores are randomly distributed and uncorrelated with one another. In figure 6.8(b) on the other hand, the phase correction was also applied to the input wavefront and a clear focus is observed in the chosen focal plane. There is still a background speckle pattern produced by interference of the light from fibres that were not accurately corrected (due to multimode propagation and cross-talk as discussed in section 6.3.1), however, the intensity of the focal spot is considerably higher than the background indicating that the phase measurement and correction procedure was successful. Line profiles through the centre of the focal volume are also presented in figure 6.8 and these further demonstrate the phase correction. With the phase correction applied (figure 6.8(d)), the peak intensity of the focus was over 50 times greater than the background and the focussed beam waist had a FWHM of $3.3\ \mu\text{m}$.

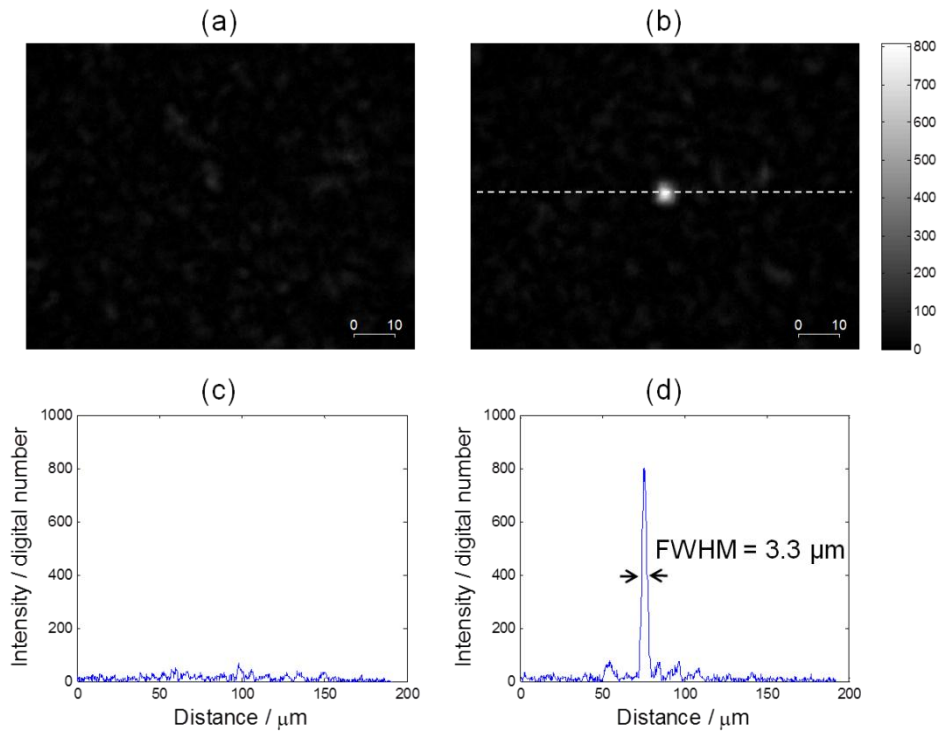


Figure 6.8. Intensity distributions (a, b) and line profiles through focus (c, d) recorded at a focal plane $0.43\ \text{mm}$ in front of the distal tip of the fibre bundle. (a) and (c) were recorded without the phase correction applied while (b) and (d) were recorded with the correction applied. Scale bars are $10\ \mu\text{m}$. The dotted white line in (b) indicates the location of the line profiles. The line profile in (d) has a FWHM of $3.3\ \mu\text{m}$. Figure adapted, with permission, from reference [195]. © 2012 The Optical Society.

Having generated a focus, it was then straightforward to demonstrate 3-D scanning and this was achieved by adjusting the appropriate parameters in the calculation of φ_f accordingly (see equation (19)). Figure 6.9 shows images of the focal plane with the beam focussed to 16 different lateral positions. From the top left to the bottom right image, it is clear that the beam is scanning from the upper left corner to the lower right corner of the field of view. In all images the focal plane was kept constant at 0.43 mm.

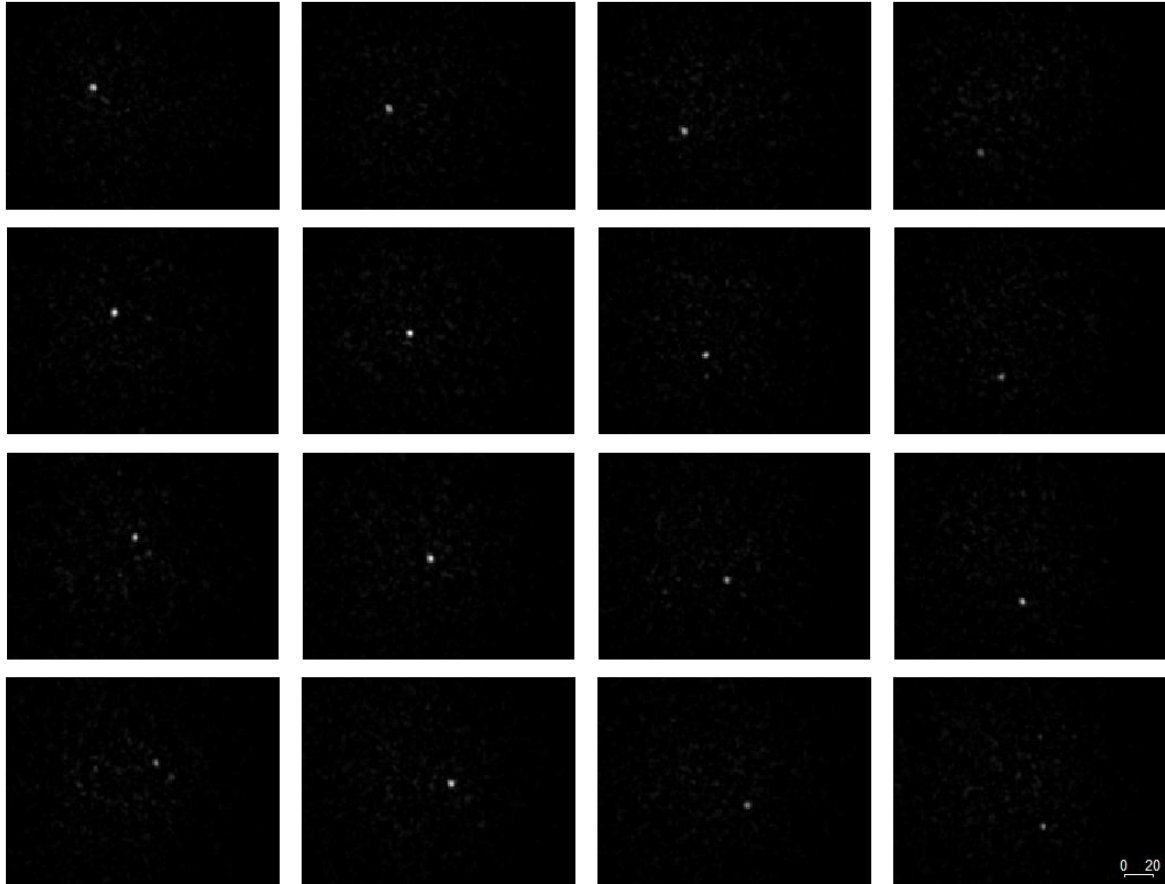


Figure 6.9. Images of a focal plane 0.43 mm in front of the distal tip of the fibre bundle as the beam is focussed to 16 different lateral positions. Moving across and down from the top left image the focal spot is seen to scan from the upper left to the lower right of the field of view. Scale bar shown in bottom right image is representative of all images and is 20 μm in length.

Finally, z-scanning was achieved by varying the curvature of the input wavefronts – i.e. by varying f in equation (19). This was demonstrated by imaging a plane at a fixed distance (0.580 mm) from the distal tip of the fibre bundle while the synthetic focal length was adjusted. Figure 6.10 presents images of this fixed observation plane as the focal length was adjusted to the values indicated in each of the images. It is clear from the figure that a focus is observed when the focal

plane and the observation plane are in agreement (figure 6.10(b)). Conversely, when the focal and observation planes do not correspond, defocus is observed in the focal spot, indicating that axial scanning has been accomplished.

By scanning the focus through a fixed detection plane (i.e. as shown in figure 6.10), it was also possible to investigate the axial extent of the illumination point spread function (PSF). The beam was focussed to 20 axial positions with the focal length ranging from 0.430 mm to 0.715 mm, while the observation was fixed at 0.580 mm from the distal tip of the fibre bundle. An axial line profile through the focus was approximated by plotting the maximum CCD pixel value in the fixed observation plane as the focal length was adjusted. The FWHM of this axial PSF then provided an estimate of the achievable depth resolution (for a true confocal system) and this was found to be approximately 100 μm .

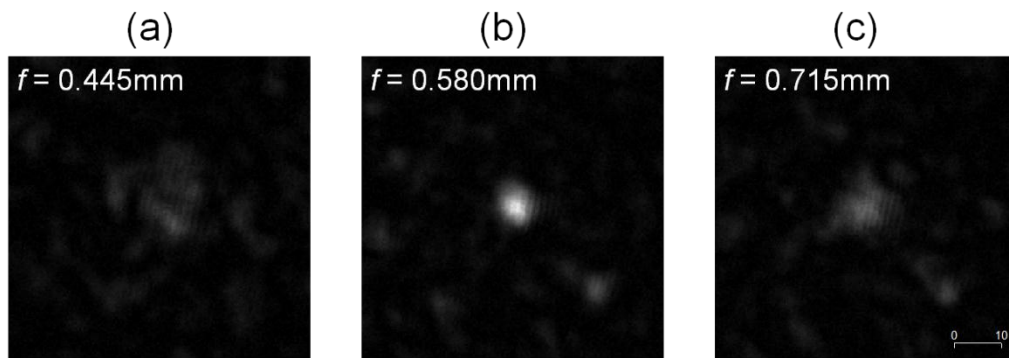


Figure 6.10. Images demonstrating axial scanning of the focus through a fixed observation plane located 0.580 mm from the distal tip of the fibre bundle. The chosen focal length is indicated in each image and the scale bar is 10 μm . Figure adapted, with permission, from reference [195]. © 2012 The Optical Society.

6.3.3 Imaging

To demonstrate the imaging potential, a USAF 1951 resolution test chart was placed in the object plane of the 20x microscope objective. The SLM was programmed to correct the phase aberrations and to scan the focussed beam ($f = 0.43 \text{ mm}$) across the face of the resolution test chart. At each scan position a transmitted light image was recorded using the CCD camera and this series of images was used to generate a scanning microscope image of the test chart in two distinct ways. Firstly, for each scan position all the CCD pixel intensities were averaged to produce a single intensity value, which represented the mean intensity collected by the detector (i.e. the CCD was operating as a large area single channel detector). This is equivalent to laser scanning microscopy with no confocal aperture and the image obtained using this approach is shown in figure 6.11(a). Here it is just

possible to discern group 6, element 3 of the test chart, although the signal-to-noise ratio (SNR) is relatively low. This low SNR is due to the imperfections in the phase correction discussed above, which meant that only 2-3% of the light in the focal plane was contained within the focal spot.

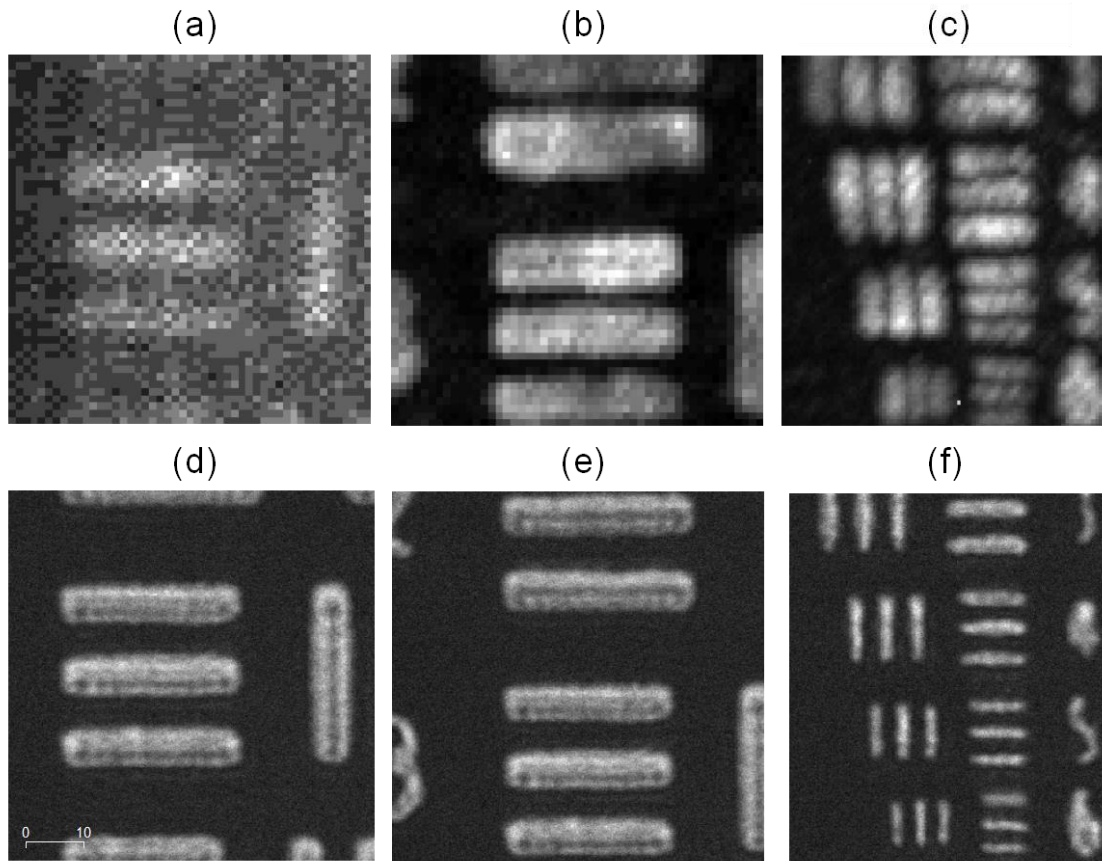


Figure 6.11. (a-c) Scanned transmitted light images of a USAF 1951 resolution test chart showing: (a) group 6, element 3 imaged by recording the mean CCD pixel value; and (b) group 6, elements 2 and 3, and (c) group 7, elements 3–6 imaged by recording the maximum pixel value at each scan position. (d-f) show the same regions of the test chart imaged in (a-c) but with wide-field white light illumination to demonstrate the expected features. Scale bar shown at bottom left of (d) is 10 μm in length. Figure adapted, with permission, from reference [195]. © 2012 The Optical Society.

These scanned transmitted light images can be significantly improved by recording the maximum CCD pixel value for each scanned beam position – rather than binning all the CCD pixels as in the previous approach – and, hence, rejecting all the background light. This approximately simulates ideal descanned confocal imaging in transmission with a delta function for the detector, and it consequently eliminates both the photon shot noise and the readout noise associated with the rejected background. Images obtained with this second approach are shown in figure 6.11(b) and (c) and it is evident that this method significantly improves the contrast. In figure 6.11(b) group 6, element 3 of the test chart is clearly visualised while in (c) the smallest feature on the test chart

(group 7, element 6) has been imaged to demonstrate the lateral resolution achieved. In the latter case, the smallest features are indeed resolved indicating a transverse resolution of less than $4.4\text{ }\mu\text{m}$, which is in agreement with the measured lateral PSF (see section 6.3.2) that had a FWHM of $3.3\text{ }\mu\text{m}$. Finally, it should be noted that the remaining images in figure 6.11 show the same regions of the test chart displayed in (a-c) but with wide-field white light illumination to demonstrate the features that are expected in the equivalent scanned images.

For this proof of principle imaging demonstration, the total acquisition time was approximately 15-20 minutes for a 50×50 pixel image. This long image acquisition time was partly due to the 10 Hz update rate of the SLM holograms (owing to slow computation algorithms) and partly due to the imaging rate of the CCD camera (approximately 14 Hz). One route for further work on this project should now involve improvement of this slow imaging speed. This could be achieved by using a faster single channel detector instead of the CCD and by optimising the computation algorithms to allow more rapid update of the SLM (current hardware is capable of updating at speeds greater than 2 kHz). Additionally, SLM-based phase correction could be combined with rapid proximal scanning of the light diffracted from the SLM (for example using galvanometric mirrors) to generate the tip and tilt in the wavefront that permits lateral scanning. All of these steps would greatly increase the imaging speed and, in the future, could help to develop this technique to a stage at which it becomes clinically applicable.

6.4 Discussion and conclusions

This chapter has presented a proof-of-principle study demonstrating a novel technique to permit beam focussing and scanning in endomicroscopy [195]. This approach utilises a proximal SLM to pre-compensate for phase distortions induced by a coherent fibre bundle. Using the SLM to illuminate the input face of the fibre bundle with the appropriate complex amplitude profile, it is possible to generate arbitrary wavefronts at the distal end in a manner analogous to phased array radar. In the field of endomicroscopy this is useful as it allows the generation of a focussed, scanning beam at the distal end of the fibre bundle without the need for any mechanical or optical components.

The first results presented in this chapter (figure 6.7) illustrated that the phase measurement and correction procedure worked successfully and provided smoothing of the phase profile at the distal end of the fibre bundle. Before correction, the phases of the individual fibre cores appeared randomly distributed while after the application of a correction there was a clear bias in the phase. Despite this bias, the correction was not perfect with some cores still exhibiting

seemingly random phase and being unaffected by the correction procedure. This was attributed to spatial variations in the optical properties of the fibre bundle, which meant that some fibre cores exhibited multimode propagation as well as cross-talk, both of which acted to degrade the phase correction.

Nevertheless, focussing of the distal beam was still achieved without the use of distal optics and the focal spot was seen to be over 50 times brighter than the background speckle pattern (figure 6.8). 3-D scanning of the output beam was also demonstrated and the lateral and axial resolutions achieved in this configuration were estimated as 3.3 μm and 100 μm respectively. Finally, scanned transmitted light images of a USAF 1951 resolution test chart were recorded to demonstrate the potential for imaging. Even though only a small fraction (2-3%) of light was contained within the focal spot, the images still showed contrast between the transmissive and non-transmissive regions of the test chart. These scanned transmitted light images were then significantly improved by simulating confocal detection and rejecting the background light. In this case, very clear contrast was observed and the resolution was seen to be less than 4.4 μm , in agreement with the measured value quoted above.

Overall, the data presented in this chapter has demonstrated focussing and 3-D scanning of a laser beam at the distal end of a coherent fibre bundle without any distal mechanical or optical components. Additionally, it has been shown that the contrast of the focal spot is sufficient for confocal imaging in transmission. The potential advantages of this technique over the current methodology are evident. Firstly, the ability to dispense with all distal components means that this approach may permit the development of ultrathin endoscopic probes, which will also be cheaper than those used in distal scanning endomicroscopes. Furthermore, this technique provides full control of the illumination PSF using a single probe – including the imaging depth, NA, sampling rate and image size – which is not the case in either proximal or distal scanning endomicroscopes. The final advantage is that the SLM provides the opportunity to correct aberrations induced by the sample as well as by the fibre bundle, which could permit endomicroscopic imaging at increased depths within biological tissue.

Further research in this field can now follow a number of routes, with the obvious final aim being to record an image – and to perform the phase correction – at the proximal end of the fibre bundle. Recording an image at the proximal end would involve collecting reflected or fluorescent light back through the fibre bundle and the results presented in this chapter suggest that this will require one of two developments. As shown in figure 6.11, the image contrast obtained in transmission is very low if confocal detection is not incorporated. When imaging in a reflection

geometry (without confocal detection) this will only become worse. Thus, either proximal descanning will be necessary to provide confocal detection or improvements in the level of phase correction will be required to increase the fraction of light directed into the focal volume.

One option to improve the phase correction would be to include a preliminary calibration step in the phase correction procedure. This calibration procedure would involve the initial characterisation of the fibre bundle to generate a metric that is later used to calculate how and where to apply a phase delay to produce a desired phase shift in a specific fibre core. Using this technique the phase measurement process would remain the same, however, the phase correction (for some fibre cores) would then involve the application of phase shifts to several fibres to correct the phase in just one. While this process would still be affected by multimode propagation, it should reduce the effects of cross-talk (provided that cross-talk is not affected by bending of the fibre bundle) and, hence, improve both the level of phase correction obtained and the contrast of the focal spot. An additional advantage of this approach is that, after the initial calibration step, the phase distortions could still be measured and accounted for with the acquisition of just a single interferogram. Indeed, it should be noted that this ability to measure the phase aberrations using a single interferogram provides a key advantage over other methods to image (or focus) through turbid and aberrating media in microscopy [190-194], where at least one interferogram is typically required for every image pixel. Thus, by improving the level of phase correction whilst also retaining the ability to rapidly measure aberrations, it is feasible that the system described in this chapter could ultimately be operated in a closed-loop adaptive optics setup where aberrations caused by the fibre bundle and the sample are repeatedly corrected during real-time endomicroscopic imaging.

Acquiring an image at the proximal end of the bundle could also be achieved by incorporating confocal detection. Clearly, this would not increase the fraction of light contained within the focal spot but it would improve the image contrast provided by rejecting the background light (as demonstrated in figure 6.11). To achieve optically sectioned confocal imaging in reflection would require ‘descanning’ of the collected light using the same SLM and then focussing the corrected wavefronts onto a confocal aperture prior to detection. Confocal fluorescence imaging could be accomplished in the same way but would require an additional proximal SLM operating at the detection wavelength to ‘descan’ the collected fluorescence. It should be briefly noted here that the diffractive efficiency of the SLM used in the experiments presented in this chapter was low (< 10 % of light is contained within the +1 diffraction order). Thus, if one of the confocal detection schemes described above is incorporated (which entail two reflections from the SLM) then an alternative SLM with a higher diffractive efficiency may be required.

In the above confocal detection scenarios, the term ‘descan’ takes a slightly different meaning to its normal use in confocal microscopy – here it entails correcting the phase of the collected wavefronts so that they can be focussed onto a confocal pinhole for detection. Clearly this adds increased complexity to the system when used for fluorescence imaging as a second SLM is required and phase aberrations need to be measured and corrected at two wavelengths; although it may be possible to calculate the phase aberrations at the detection wavelength based on those measured at the excitation wavelength.

A simpler way to achieve optically sectioned fluorescence imaging with this system may be to incorporate multiphoton excitation as the non-linear excitation probability means that fluorescence would only be generated in the focal volume and, hence, descanning of the collected light would not be required. Of course, the use multiphoton excitation also brings additional complications such as the need for high peak illumination powers and the impact of pulse dispersion within the fibre bundle. None the less, work toward achieving multiphoton fluorescence imaging using this system has begun, as discussed in the following chapter.

Finally, a further important aim of future research on this technique is to develop a method to measure the phase distortions at the proximal end of the fibre bundle. Currently, a distal CCD camera is used to measure the phase and this is obviously not feasible in a real endomicroscope. Measuring the phase at the proximal end could be facilitated by the use of a coherent detection scheme, which would be compatible with multiphoton excitation. The pulsed lasers typically used in multiphoton microscopy inherently have very short coherence lengths. Thus, one possible approach to permit proximal phase measurement would be to measure the phase of the light reflected from the distal end of the fibre-optic bundle. This could be achieved using coherence-gated imaging to separate the signal reflected by the distal end of the bundle from the light reflected by the sample (and from other reflections). This approach would be similar in concept to optical coherence tomography (OCT) and would provide a large step toward the development of this system into a clinically useful instrument.

To conclude, this chapter has presented proof-of-concept results for a novel endomicroscope design, including beam focussing, 3-D scanning and imaging without the need for distal components. The following chapter of this thesis discusses further work on this system, involving both numerical simulations of distal beam propagation and work towards achieving multiphoton fluorescence endomicroscopy.

Chapter 7: Towards multiphoton excitation using a SLM-based endomicroscope – simulations of distal beam propagation and experimental progress

In the previous chapter results were presented demonstrating a novel concept to allow beam focussing and scanning in an endomicroscope. This chapter now concentrates on further progress made on this project, which has been centred on two specific tasks. Firstly, a theoretical analysis of the focussing characteristics of the endomicroscope along with the results of numerical simulations of the distal beam profile are presented, both of which act to provide a better understanding of the potential of the novel instrument. Secondly, further experimental work is discussed aimed at allowing phase correction using a mode-locked Ti:Sapphire laser, which could eventually be used to facilitate multiphoton excitation.

7.1 Introduction

This chapter is separated into two distinct sections addressing the numerical simulations and the experimental work mentioned above. The first section begins with a detailed discussion of the focussing and imaging characteristics of the novel endomicroscope in order to impart an understanding of how this system would work in practice. The numerical simulations are then introduced and discussed. In these simulations, beam propagation at the distal end of the fibre bundle was modelled using the theory of the angular spectrum of plane waves [201] with the aim being to study the intensity distributions in (and around) the focal plane under a number of different conditions. This involved investigations of the achievable resolution and the ratio of the focal intensity to the background. As with the theoretical analysis, the numerical simulations acted to provide improved understanding of how this novel instrument would perform as a laser scanning endomicroscope.

The second part of this chapter concentrates on further experimental work. As discussed at the end of chapter 6, an important step in the development of this system is to incorporate optical sectioning in order to provide acceptable image contrast and one way to achieve this is to implement multiphoton excitation. To this end, experiments were carried out initially aimed at replicating the results presented in chapter 6, but using a mode-locked Ti:Sapphire laser. The use of

a pulsed excitation source introduces additional problems caused by the short coherence length of the laser. These problems, which are described later, were addressed and the results presented in section 7.3.4 demonstrate focussing and scanning of the pulsed, low coherence light source at the distal end of an optical fibre bundle, suggesting the potential future use of this technique in multiphoton fluorescence endomicroscopy.

7.2 Simulations of distal beam propagation

7.2.1 Introduction and motivation

As discussed previously, the novel endomicroscope design presented in chapter 6 provides numerous potential advantages over currently available technologies. One possible advantage that was not discussed in detail is the fact that this approach may also relax the constraints placed on the fibre imaging bundle used in the endomicroscope. In typical proximal scanning systems (for example, [106]) the fibre bundle will contain around 30,000 optical fibres with diameters of approximately 2 μm and centre-centre distances of around 3 μm . The large number of fibres and tight inter-core spacing are chosen to allow good imaging resolution and sampling while also minimising cross-talk between fibre cores. In the novel SLM-based approach, the image quality will not depend on the fibre bundle parameters in the same way. Additional factors such as the minimum achievable focal length and the maximum field of view will also be dependent on the fibre bundle. For this reason it is useful to investigate the distal focussing characteristics in the novel endomicroscope in order to better understand the potential of this SLM-based approach.

This section first discusses the way in which focussing is accomplished and also explains the implications this has for the imaging attributes (e.g. field of view, NA, etc.) of any instrument based on this concept. After a description of the focussing characteristics, numerical simulations of the distal beam profile are presented in a number of different scenarios. The simulations were based on propagation of wavefronts using the method of the angular spectrum of plane waves [201]. The theory behind this method is described and results from the simulations are presented. This study involved investigation of the effects of NA, fill factor of the fibre bundle used, the degree of phase correction achieved and, finally, the choice of single- or two-photon excitation.

7.2.2 Discussion of endomicroscope focussing characteristics

In an endomicroscope based on the design presented in chapter 6, focussing and scanning of the illumination beam are achieved by programming the SLM to produce a distal wavefront with the appropriate curvature, tip and tilt. A distal focus is then generated through constructive interference of the light emitted from the individual fibre cores. Thus, in order to generate the optimum focus, it is necessary that light from all fibre cores can overlap in the focal plane and interfere constructively. The minimum distance at which this can occur is determined by the divergence (θ) – i.e. the NA – of the individual fibre cores and the separation of the outermost fibres in the bundle. This is depicted in figure 7.1 where the solid red lines show light emanating from individual fibre cores with a characteristic half-angle, θ . The minimum focal length (f_{min}) is then defined as the distance at which the light from the outermost cores first overlaps. Mathematically this means that

$$f_{min} = \frac{D}{2 \tan \theta} \quad (20)$$

where D is the fibre bundle diameter and, hence, the separation of the outermost cores.

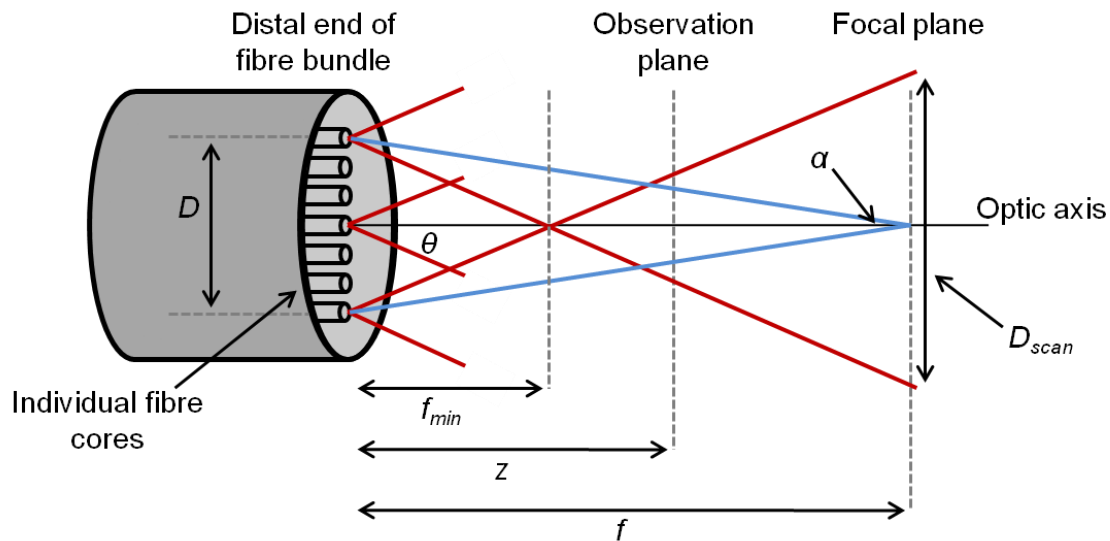


Figure 7.1. Diagram illustrating the achievable focal length, scan range and numerical aperture using an endomicroscope based on the concept proposed in chapter 6. This figure also acts to introduce the coordinate systems used in the numerical simulations of distal beam propagation.

By the same reasoning, for any focal length $f > f_{min}$, there is also a maximum lateral scan range (D_{scan}) that can be achieved without degradation of the focus being observed, as shown in the focal plane in figure 7.1. Furthermore, a minimum focal length also implies a maximum effective NA

(of the illumination beam) and, hence, there is a limit to the resolution that can be achieved using this technique (when no distal lens is used). The effective NA is illustrated in the focal plane in figure 7.1, where the blue lines indicate light coming to a focus with an angle of convergence of α . In this case, the effective NA (NA_{eff}) of the focussing rays is given by the following equation.

$$NA_{eff} = \sin \alpha \approx \frac{D}{2f} \quad (21)$$

It should be noted that outside of the maximum scan range (D_{scan}), a focus can still be generated but the effective NA will be reduced as light from the outermost fibres will no longer reach the focal volume – i.e. the parameter D in equation (21) will be reduced, hence, so will the effective NA.

Of course, $\alpha \leq \theta$ and $f \geq f_{min}$, therefore the maximum illumination NA (NA_{max}) that can be achieved is equal to the NA of an individual fibre core (NA_{core}) and is simply defined as:

$$NA_{max} = NA_{core} = \sin \theta \approx \frac{D}{2f_{min}}. \quad (22)$$

This maximum effective NA can be increased by using a distal lens, however, the simulations presented here focus on the situation where no distal lens is used as this provides a more direct indication of how the fibre bundle utilised impacts upon the image quality obtained.

It is worth stating here that the SLM also places a constraint on the minimum achievable focal length due to the fixed size of its pixels. Thus, this minimum distance was calculated based on the idea of using the SLM to generate a focussing beam by simulating a Fresnel lens. For a 512×512 pixel hologram with a pixel size of $13.62 \mu\text{m}$ (pixel size on the SLM used in chapters 6 and 7) – and after demagnification of the SLM through lenses L_3 and L_4 (see figure 6.2) – the minimum focal length, f_{SLM} , was calculated according to the equation $r_n^2 = n\lambda f_{SLM}$, where r_n is the radius of the n^{th} Fresnel zone. With the n^{th} Fresnel zone having a width of 4 SLM pixels in order to allow a phase shift of 2π radians, the minimum focal length imposed by the SLM was calculated as 0.1 mm. For a region of fibre bundle of the same diameter, the minimum focal length obtained from equation (20) – i.e. that imposed by the fibre bundle – was approximately 0.3 mm, three times greater than that brought about by the SLM. Hence, it is the fibre bundle and not the SLM that constrains the focal length in this system.

A further point to note regarding the generation of a focussed beam is that the light distribution in the focal plane is a diffraction pattern formed from the light distribution at the distal output of the fibre bundle. Thus, the intensity profile in the focal plane may contain higher

diffraction orders. The 0th order diffracted light makes up the desired focal spot and the additional diffraction orders constitute an unwanted background. This focal intensity distribution will have the same structure as the diffraction pattern observed in the far field in the case where the distal output of the fibre bundle has a flat (rather than a curved) phase profile. If the characteristics of the fibre bundle used in the system are altered (e.g. if the core spacing or radius are changed) there will be an impact on the locations and relative intensities of the various diffraction orders. In turn, this will affect the performance of the endomicroscope, for example, in terms of the achievable ratio of the focal intensity to the background.

Finally, it should be noted that the background observed in the focal plane is not just a consequence of the additional diffraction orders and of imperfect correction of phase aberrations. There will also be an inherent error introduced by the fact that this method relies upon the generation of distal wavefronts from a number of discrete actuators (i.e. the individual fibre cores). This inherent discretisation error will also contribute to a background in the focal plane and it is useful to further understand the importance of the background as a whole as well as the limitations it places on this endomicroscope design.

Overall, the characteristics and behaviour of the focus produced in an endomicroscope based on this concept will be different to those observed in conventional systems. For this reason, it is logical to investigate the distal beam profile in more detail. The following sections present an explanation of the theory and methodology used in the numerical simulations of the propagation of distal wavefronts along with the key results from this investigation.

7.2.3 Simulation and propagation of distal wavefronts using the angular spectrum of plane waves

In order to investigate the characteristics of the focus achievable in the novel endomicroscope configuration, wavefronts were numerically simulated at the distal end of a fibre bundle and these were then propagated to chosen distances from the output face of the fibre. This was achieved using software written in-house using Matlab® (MathWorks™). Distal wavefronts were first approximated by generating a square array of hexagonally packed fibre cores each with a Gaussian amplitude profile to imitate the propagation of only the 0th order mode (see figure 7.2(a)). A single phase value was assigned to each core according to the desired phase profile of the bundle and, as discussed in chapter 6, a spherical wavefront was used in order to generate a focussing beam (see figure 7.2(c)). Thus, the phase in individual fibres was defined using the formula:

$$\varphi_{\text{fibre}} = \frac{2\pi}{\lambda} \left\{ f + \sqrt{f^2 - (x_{\text{fibre}} - x_c)^2 - (y_{\text{fibre}} - y_c)^2} \right\}. \quad (23)$$

In this equation: x_{fibre} and y_{fibre} are the centre coordinates of the fibre core; f is the focal length; x_c and y_c are the lateral coordinates of the focus; λ is the wavelength (which was 840 nm in all cases to match the experiments performed in chapter 6); and φ_{fibre} is the phase value in radians. Thus, the simulated wavefronts were designed to mirror the complex amplitude profile applied to the fibre bundle in the experiments presented in chapter 6 (see section 6.2.3).

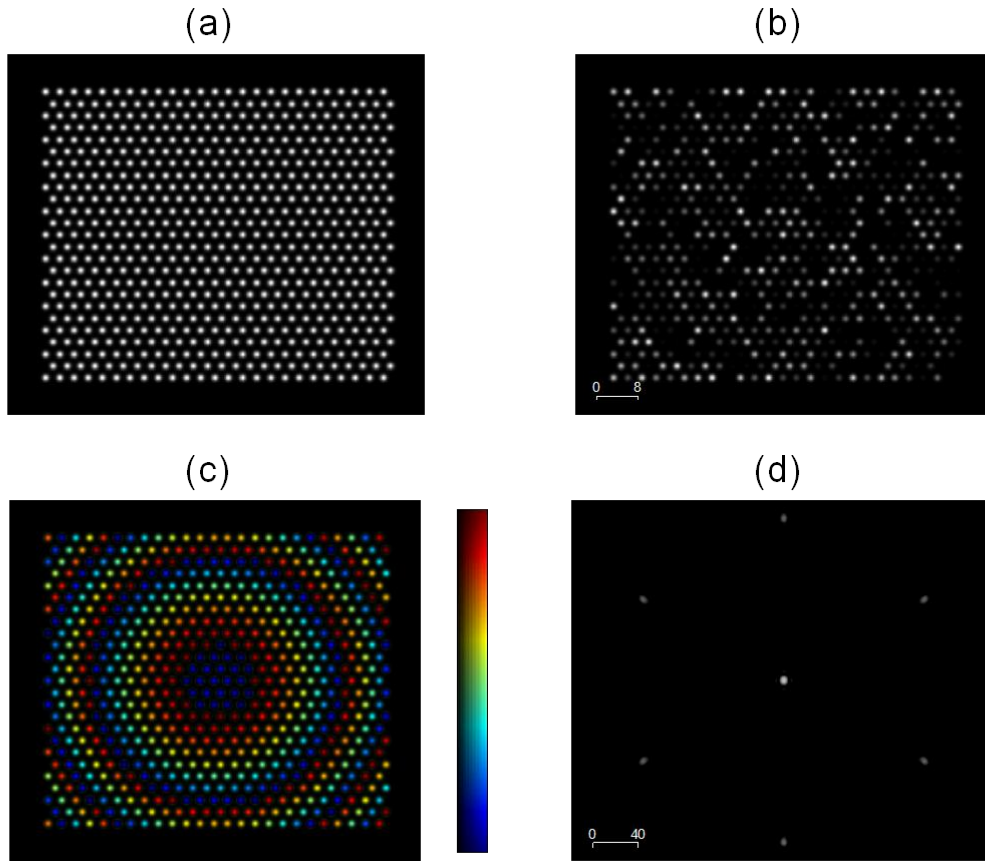


Figure 7.2. Illustration of the numerical simulation of the generation and propagation of distal wavefronts. (a) and (b) show amplitude profiles with uniform and random amplitudes respectively. (c) shows a phase map with no phase errors. (d) shows a simulated intensity distribution in the focal plane arising from a distal amplitude distribution like that shown in (a). Note that the image in (d) is plotted on a log scale so that the higher diffraction orders are also visible. Scale bar in (b) is 8 μm and is representative of images (a-c) while the scale bar in (d) is 40 μm . Colour bar in (c) represents phase in the range $-\pi$ (blue) to $+\pi$ (red).

The wavefronts could be modified in order to allow choice of a variety of parameters including the number of fibres, the diameter and separation of fibre cores and the focal length of the

resulting wavefront. Hence, it was possible to investigate the effects of all of these parameters on the distal beam profile. Furthermore, it was also possible to simulate the effects of variations in the amplitude and phase of the light emitted from each fibre core. In particular, the Matlab® software allowed the generation of wavefronts with either a uniform amplitude profile (i.e. the amplitude is the same in all fibre cores) or a random amplitude profile. In both cases, individual cores were simulated with Gaussian amplitude profiles. The relative amplitudes of the different fibre cores, however, were either uniform (see figure 7.2(a)) or were randomly chosen in the range zero to one (flat distribution), as shown in figure 7.2(b).

The effects of phase variations between cores were also simulated in one of two ways. Firstly, the fraction of cores with the correct phase could be chosen by the user and the phases in uncorrected cores were assigned random values in the range $-\pi \leq \phi_{fibre} < +\pi$ (i.e. rather than the phase being defined by equation (23)). Secondly, it was also possible to choose a spread in the phase, which meant that fibres with the ‘correct’ phase would still have a small error of, for example, $\pm 10\%$ (with a flat distribution). The former simulates the effects of entirely unsuccessful phase correction in certain fibres (caused by, for example, cross-talk or multimode propagation). The latter, on the other hand, models smaller errors in the phase correction caused by factors such as faults in the phase measurement (and in the experiments presented in chapter 6, such an error could have been explained by reduced modulation depth of the interference fringes in certain fibre cores). In practice, for reasonable values (i.e. $\leq \pm 30\%$), the phase spread was found to have little effect on the quality of focus obtained in the simulations. Thus, the results presented later in this chapter regarding the effects of imperfect phase correction were attained using the former method of error simulation. Figure 7.2(a-c) show examples of the simulated amplitude (a, b) and phase profiles (c) used in this investigation.

Having simulated distal wavefronts, the Matlab® software was now also used to propagate these to chosen distances from the output face of the fibre bundle. This was achieved according to the theory of the angular spectrum of plane waves [201]. As shown in figure 7.1, in these propagation simulations the plane at distal end of fibre bundle is defined as $z = 0$, the observation plane is at an arbitrary distance, z , from the fibre output face and the focal plane is at $z = f$. To begin, a wavefront has an amplitude, $t(x, y)$, and a phase profile, $\phi(x, y)$, defined as described above such that the complex amplitude at the distal end of the bundle (i.e. at $z = 0$) is given by the following expression.

$$U(x, y, z = 0) = t(x, y) \exp[i\phi(x, y)] \quad (24)$$

The first step in the propagation of this wavefront involves the calculation of the angular spectrum at $z = 0$, $A(k_x, k_y; z = 0)$. This is achieved by taking the Fourier transform of the input wavefront – i.e. by breaking it down into a series of plane waves propagating at different angles – according to equation (25).

$$A(k_x, k_y; z = 0) = \int \int_{-\infty}^{\infty} U(x, y, z = 0) \exp[-i(k_x x + k_y y)] dx dy \quad (25)$$

In this equation k_x and k_y are the spatial frequencies in the x - and y -directions at each point in Fourier space. To propagate the wavefront by a distance z , the angular spectrum at $z = 0$ is multiplied by a phase factor, $\exp(ik_z z)$, so that the angular spectrum at the point z is then,

$$A(k_x, k_y; z) = A(k_x, k_y; z = 0) \exp(ik_z z), \quad (26)$$

where k_z is related to the x - and y -direction spatial frequencies as follows.

$$k_z = \sqrt{\left(\frac{2\pi}{\lambda}\right)^2 - k_x^2 - k_y^2} \quad (27)$$

The complex amplitude distribution at the observation plane is then found by calculating the inverse Fourier transform of the propagated angular spectrum.

$$U(x, y, z) = \int \int_{-\infty}^{\infty} A(k_x, k_y; z) \exp[i(k_x x + k_y y)] dk_x dk_y \quad (28)$$

Finally, the intensity distribution in the observation plane is simply given by the square modulus of the complex amplitude profile. Intensity distributions in a number of observation planes were simulated for a variety of input wavefronts and these were used to investigate the imaging properties of the novel endomicroscope design. See section 7.2.4 for a description of how the intensity distributions were used to assess the performance of the endomicroscope.

As stated above, the generation and propagation of distal wavefronts were simulated numerically using home-written Matlab® software. This software utilised the equations presented above with all integrals approximated as discrete using the ‘fft2’ 2-D fast Fourier transform (FFT) algorithm of Matlab®.

7.2.4 Assessment of endomicroscope performance

The aim of this study was to characterise the performance of the novel endomicroscope under a variety of different conditions, with those conditions mainly relating to the fibre bundle used in the system. The fibre bundle parameters that were investigated included the radius and spacing of fibre cores, the outer diameter of the bundle and the number of fibre cores it contained. It was possible to simplify most of these parameters into two broad categories: effective numerical aperture (NA_{eff}) and fill factor (F). Equation (21) (see section 7.2.2) demonstrated that the effective NA is dependent only on the diameter of the fibre bundle (i.e. the separation of the outermost fibre cores) and the chosen focal length. Thus, the effects of variations in the effective NA were investigated by independently altering the focal length, f , and the outer diameter of the bundle, D . It should be noted that the latter was simply achieved by varying the number of fibre cores contained within the bundle (while maintaining a constant fibre core radius and inter-core spacing).

Similarly, changing the radius (r) and the spacing (a_s) of fibre cores both affect the fill factor, which is given by

$$F = N \frac{d^2}{D^2} \quad (29)$$

where N is the number of fibres and d is the diameter of individual fibre cores. Assuming that the outer bundle diameter is equal to an integer multiple of the fibre core centre-to-centre distance (i.e. $D = na_s$, where $n = N^{1/2}$ is the number of fibre cores across one dimension of the fibre bundle) then the equation describing the fill factor becomes

$$F = \frac{d^2}{a_s^2} = \frac{4r^2}{a_s^2} . \quad (30)$$

Therefore, as for the NA, the effects of variations in the fill factor were investigated by adjusting both r and a_s individually.

Other than the investigations of the effects of variations in the fibre bundle parameters described above, simulations were also performed to study the consequences of changes in the degree of phase correction achieved as well as the choice of either single- or two-photon excitation. The former served to provide an idea of the robustness of the endomicroscope to errors in the phase correction while the latter elucidated the improvements offered by the use of two-photon excitation.

In all the simulations discussed above the performance of the endomicroscope was assessed by numerically simulating propagated wavefronts at various distances from the output face of the fibre bundle. The planes to which these wavefronts were propagated were centred on the focal plane and, as an example, figure 7.2(d) shows a typical numerically simulated intensity distribution in the focal plane (plotted on a log scale such that the higher diffraction orders are also visible). Using intensity distributions from each of the chosen observation planes it was possible to calculate the peak-to-background ratio in the focal plane as well as the lateral and axial resolution. The peak-to-background ratio was simply found by calculating the ratio of the maximum pixel value in the simulated focal plane to the mean background pixel value. This mean background level was measured by masking out the light in the focal volume (using a circular mask with a diameter of three times the FWHM of the focus) and then calculating the mean of the remaining pixels in the image. The lateral and axial resolutions were estimated by plotting line profiles through the x - y and x - z planes respectively, fitting Gaussian curves to the observed 1-D point spread functions (PSFs) and then extracting the FWHM of the fitted curves. For the axial resolution, the line profile was produced by plotting the intensity value of the pixel at the centre of the focal spot as a function of depth, and it is worth noting that this approximately simulates confocal detection with an infinitely small pinhole. The results presented in the following section use the peak-to-background ratio as well as the lateral and axial resolution to characterise the behaviour of the novel endomicroscope in a number of different configurations.

7.2.5 Results of propagation simulations

The effects of numerical aperture

The first result presented here demonstrates the effects of variations in the effective NA of the system. As discussed above, the NA was varied by independently changing both the synthetic focal length of the distal wavefronts and the outer diameter of the fibre bundle (with the latter simply achieved by altering the number of fibre cores in the bundle). Other than this, all fibre bundle parameters were fixed ($d = 2 \mu\text{m}$, $a_s = 3 \mu\text{m}$) as either the number of fibres was adjusted for a constant focal length or the focal length was varied for a set number of fibres. The focal length ranged from 0.21 mm to 0.63 mm ($N = 625$) while the range of numbers of fibres investigated was 400-2500 (for constant $f = 0.42 \text{ mm}$ and $f = 0.84 \text{ mm}$). The simulated results were plotted as a function of NA and are given in figure 7.3.

Figure 7.3(a) firstly confirms that higher NA infers improved lateral resolution. Secondly it indicates that the dependence of the lateral resolution on the NA is approximately independent of

the way in which the NA variations are produced. Additionally, in all cases the behaviour of the lateral resolution with respect to NA agrees with the theoretical values predicted for a conventional scanning microscope (dotted black line in figure 7.3(a) represents the lateral resolution (Δx) as defined by the equation shown on the graph). As expected, the axial resolution behaves in the same way as the lateral resolution with respect to NA (figure 7.3(b)) and, again, the agreement with the theoretical curve is clear. In this case the dotted black curve (Δz) represents the expected axial resolution for confocal detection with an infinitely small pinhole (for low NA) [202]. Note that this situation was simulated by plotting line profiles through the single pixel at the centre of the focal spot as a function of depth (see section 7.2.4). Finally, it should be stated that the lateral and axial resolution will not follow the theoretical curves indefinitely as the limit to the effective NA of this system (discussed in section 7.2.2) means that the resolution is also constrained. As such, if the simulated curves in figure 7.3(a) and (b) were to be extrapolated, they would reach an asymptote when $NA_{eff} > NA_{max}$ (see equations (21) and (22) in section 7.2.2).

Figure 7.3(c) shows that increasing the NA also leads to an improved peak-to-background ratio and it is clear that, for a given focal length, the greater the size of the bundle (i.e. the greater the NA) the better the signal-to-noise ratio (SNR) will be. That said, this does not mean that increasing the bundle diameter indefinitely will improve the performance of the endomicroscope. In fact there is a clear trade-off here as, for a given focal length, a larger fibre bundle will provide an improved peak-to-background ratio (because light from outer fibres can reach the focus but not necessarily the higher diffraction orders) and resolution (due to the increased NA). It will also improve the light collection efficiency of the system, again, because of the increased NA. However, using fewer fibres will lead to a simpler fibre bundle production process and will also mean that the bundle itself is thinner and more flexible. Additionally, increasing the bundle diameter while maintaining a constant focal length will also reduce the achievable scan range, albeit without degrading the quality of the focus.

As such, figure 7.3 suggests that it should be possible to take one of two routes in the development of the novel endomicroscope. Firstly, as distal mechanics are not required with this approach, it may be feasible to increase the number of fibre cores in the bundle without making the endomicroscope unusually large (in comparison to distal scanning systems). This would mean that the images obtained would have an improved number of resolution elements (defined by the number of fibre cores in the bundle) compared to other fibre bundle based endomicroscopes. Alternatively, figure 7.3 also demonstrates that reducing the number of fibres used – even to below 1000 (i.e. $NA < 0.11$ for $f = 0.42$ mm, $NA < 0.06$ for $f = 0.84$ mm), which is much lower than in current

fibre bundle based endomicroscopes – would not detrimentally affect the resolution or the SNR. This would then permit the production of ultrathin endoscopic probes, albeit it with a reduced number of resolution elements. Lastly, it should be noted that in any endomicroscope configuration that is developed the resolution could be improved simply by including a single distal lens designed to produce a Fourier transform of the output face of the fibre bundle. In this arrangement the NA (and, hence, the resolution) would be determined by the distal lens and not by the fibre bundle.

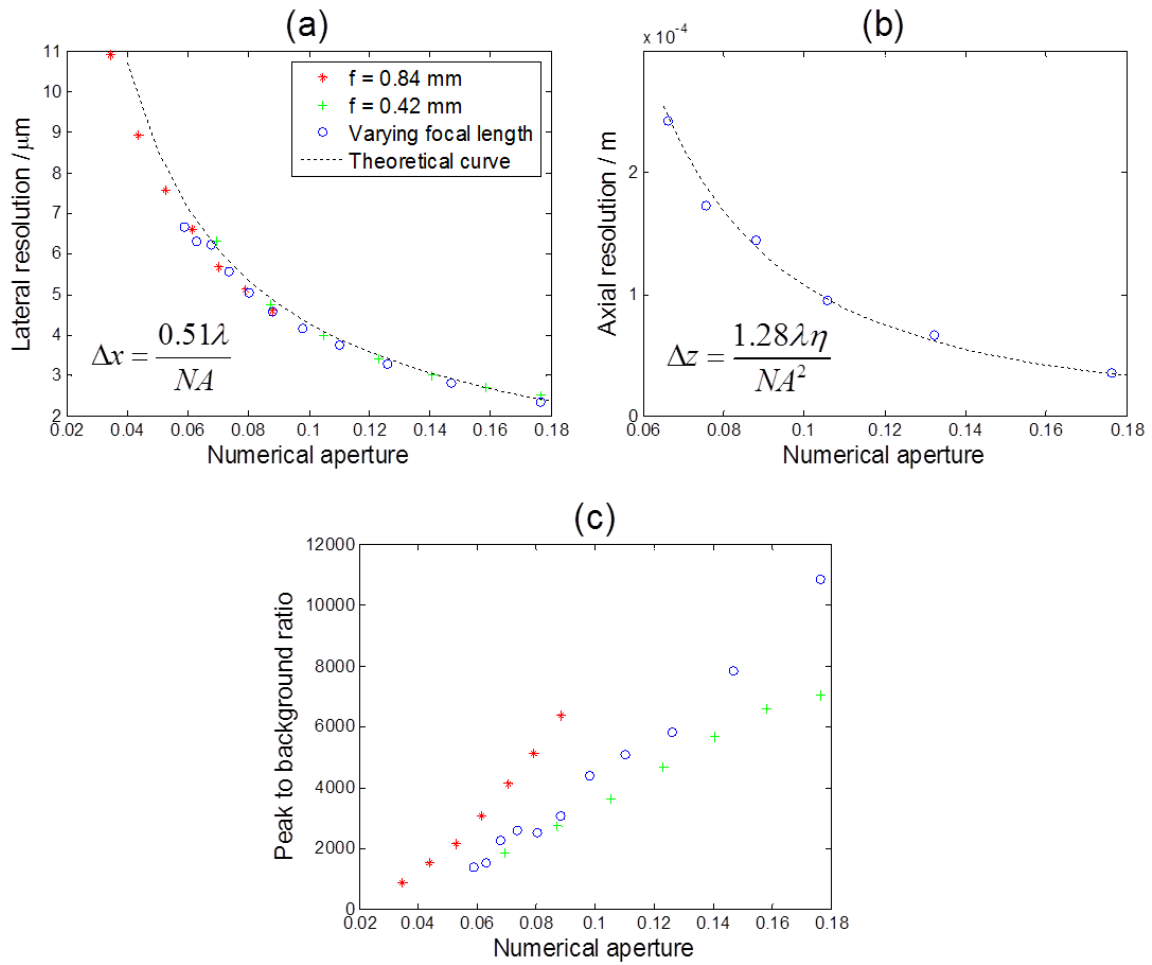


Figure 7.3. Graphs showing the effects of effective NA on (a) lateral resolution, (b) axial resolution and (c) peak-to-background ratio. In (a) and (c) the NA was adjusted by varying both the focal length and the number of fibre cores – see key in (a). In (b) only the focal length was changed. In (a) and (b) the dotted black curves represent the expected behaviour according to the equations shown on each graph – note the good agreement between the simulations and the theory in both cases.

The effects of fill factor

The next stage of the numerical simulations involved the investigation of the effects of fibre bundle fill factor. In this case the fibre core radius and the centre-centre spacing were varied independently.

Firstly wavefronts were generated with $N = 625$ fibre cores, an effective NA of 0.088 and a constant core-core spacing of $7.5\ \mu\text{m}$, and the radii of the fibre cores were varied from $0.5\ \mu\text{m}$ to $3.5\ \mu\text{m}$. The FWHM of the Gaussian beam profile from individual fibres scaled linearly with fibre core size, having been measured in a real fibre bundle (Fujikura FIGH-30-650S) with $r \approx 1\ \mu\text{m}$. Secondly a range of fibre core spacings ($2.5\ \mu\text{m} \leq a_s \leq 6\ \mu\text{m}$) were investigated with a constant core radius of $1\ \mu\text{m}$. Each wavefront was propagated to a distance f , and then the lateral resolution and peak-to-background ratio were calculated as described previously. Note that, in both cases, the focal length was allowed to vary in order to maintain a constant effective NA across all wavefronts. The data from the simulations were plotted as functions of fill factor (F) according to equation (30) and are presented in figure 7.4.

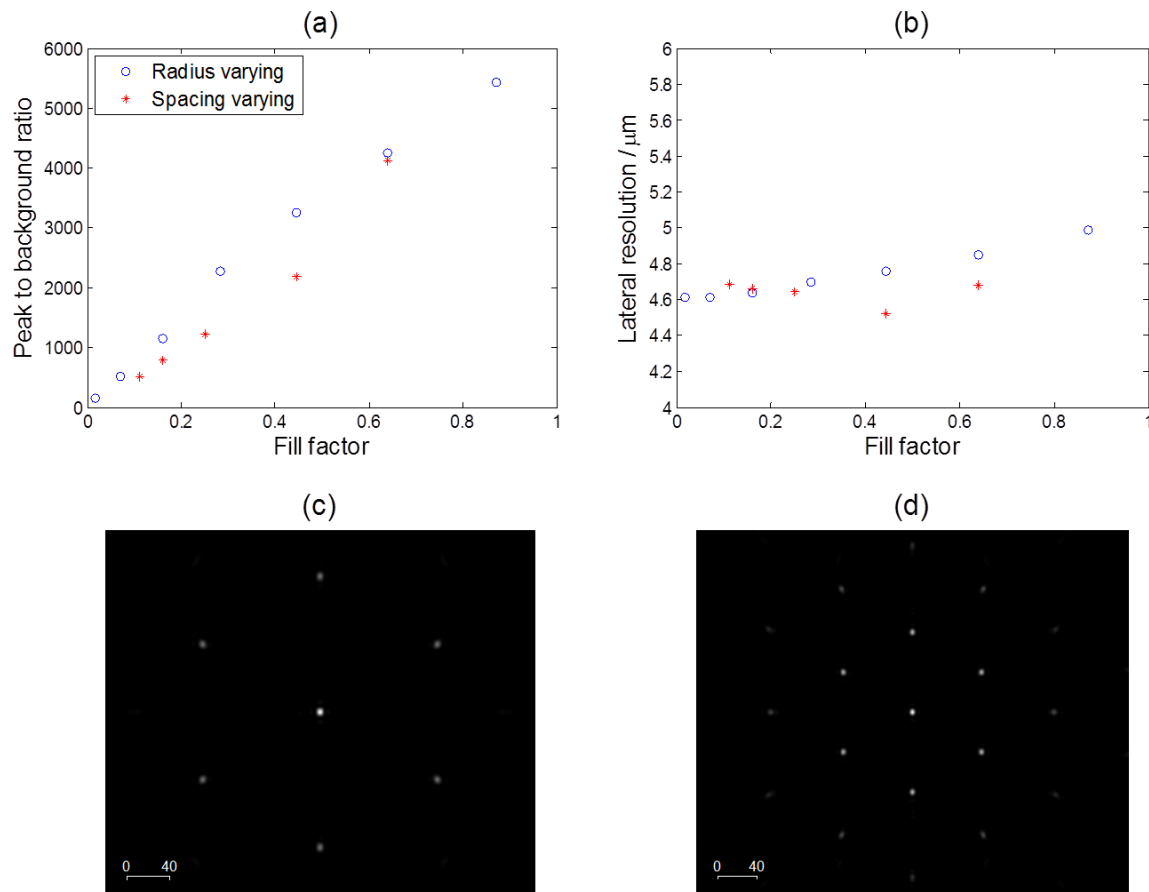


Figure 7.4. Graphs showing the dependence of (a) peak-to-background ratio and (b) lateral resolution on fibre bundle fill factor. The fill factor was varied by changing either the fibre core radius (blue circles) or the core-core spacing (red asterisks), while maintaining a constant NA and total number of fibres. (c) and (d) show example intensity distributions in the focal plane for two simulated bundles with different fibre core centre-centre separations ($4\ \mu\text{m}$ and $6\ \mu\text{m}$ respectively). The fibre core radius was $1\ \mu\text{m}$ and the focal length was $0.5\ \text{mm}$ in both cases. Scale bars in (c) and (d) are $40\ \mu\text{m}$ in length.

It is clear from figure 7.4 that a higher fill factor implies a higher peak-to-background ratio (a) and that fill factor has little effect on the lateral resolution achieved (b). Both of these results seem logical. Firstly, closer packing of fibres will increase the spacing between the 0th and 1st diffraction orders and will also reduce the brightness of the higher orders relative to the 0th order, both of which will act to increase the peak-to-background ratio. This is illustrated in figure 7.4(c) and (d), which show intensity distributions in the focal plane for two simulated bundles with fibre core centre-to-centre separations of 4 μm and 6 μm respectively (core radius is 1 μm for both). Furthermore, closer packing of fibres should have no effect on the resolution as the NA is constant. Despite this, it should be noted that the lateral resolution does appear to worsen slightly as the fibre core radius is increased – blue circles in figure 7.4(b). This is probably due to small variations (i.e. errors) in the NA caused by the use of a square – rather than a circular – array of fibre cores.

Of course, there will be a minimum fill factor in order to ensure sufficient light collection, however, the results presented in figure 7.4 suggest that good resolution and peak-to-background ratio should be obtainable even with fill factors as low as 20% (typical values are 40-50%). This again implies the potential to relax the constraints on the fibre bundle design and it should be noted that reduced fill factors would make it considerably easier to eradicate inter-core cross-talk within the bundle. As discussed in chapter 6, inter-core cross-talk may be one of the limiting factors in the phase correction process, thus, reducing its effects could prove an important step in producing a functioning endomicroscope based on this technique.

Conversely, it should be mentioned here that varying the fill factor also has implications for the field of view obtainable using this endomicroscope design. As discussed earlier in this chapter, the intensity distribution in the focal plane represents a diffraction pattern formed from the light distribution at the distal output of the fibre bundle. Hence, it is clear that increasing the core-to-core spacing will naturally cause the higher diffraction orders to move closer to the central focal spot (for a given focal length) and to increase in relative intensity. This is illustrated in figure 7.4(c) and (d) and explains why larger fill factors produce higher peak-to-background ratios. There will be a stage at which the field of view becomes limited by the separation of the 0th and 1st diffraction orders as the 1st order will also excite significant fluorescence within the field of view and, hence, generate a considerable background. However, only for the largest inter-core distances investigated here ($a_s = 7.5 \mu\text{m}$) did this begin to take effect. As such, this was not seen to be a problem and, in most cases, the field of view would still be limited by the NA of the individual fibres (see section 7.2.2 and figure 7.1) and not by the position of the 1st diffracted order. None the less, in the design of an endomicroscope based on this concept, both effects would still be considered and a final

noteworthy point is that this design could be optimised (i.e. through the appropriate choice of the effective NA of the system, the divergence of the individual fibres and the fibre core spacing and radius) to direct as little light into the higher diffraction orders as possible, while still allowing light from all fibre cores to reach the central focus. Thus, the results presented in this section again suggest that the design of the fibre bundle used in the novel endomicroscope will have a significant impact on its performance.

Imperfect phase correction

As discussed previously, in order to generate a focus it is first necessary to correct for phase aberrations induced by the fibre imaging bundle. In a real endoscopic procedure it is likely that motion of the endoscope will make it difficult to maintain perfect phase correction at all times. Thus, the following results show the effects of varying the level of phase correction achieved. As before, the lateral resolution and peak-to-background ratio have been plotted to characterise the performance of the endomicroscope, this time as functions of the percentage of fibre cores with the correct phase value – i.e. the percentage of cores contributing to the focus rather than contributing to the background speckle pattern. The phases in the ‘uncorrected’ cores were set as random values between $-\pi$ and $+\pi$ (see section 7.2.3) and figure 7.5 shows the results from wavefronts simulated with 2500 cores, a constant NA of 0.088, $r = 1\ \mu\text{m}$ and $a_s = 3\ \mu\text{m}$. The percentage of fibres with the correct phase value varied from 0-100%.

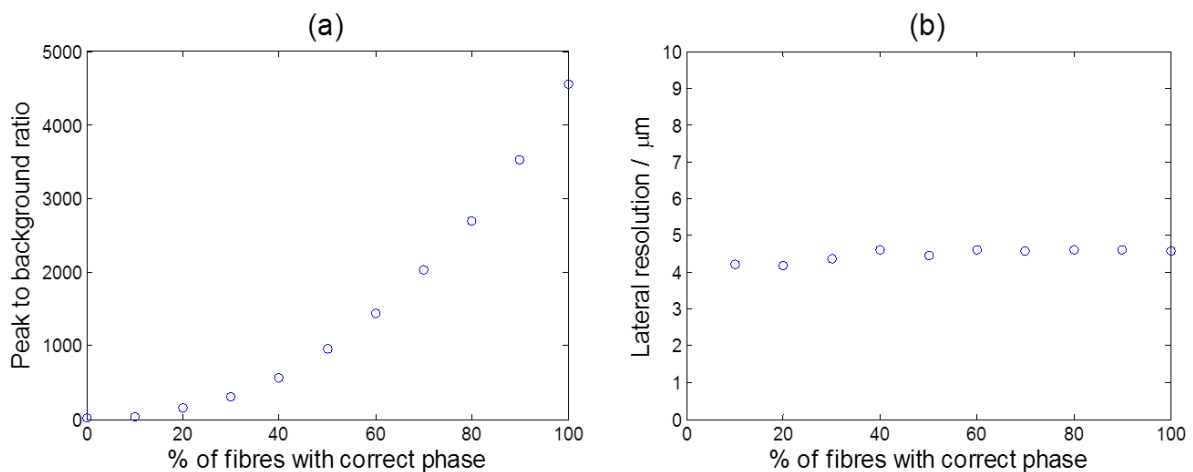


Figure 7.5. Effects of the level of phase correction on (a) the peak-to-background ratio and (b) the lateral resolution.

As expected, the peak-to-background ratio increases with the percentage of cores assigned the correct phase value (figure 7.5(a)) while the lateral resolution is unaffected by the degree of

phase correction (figure 7.5(b)). One feature to note is that there is no point for zero phase correction in figure 7.5(b) as there is no focus in this case and hence, it is not possible to fit a Gaussian to the PSF.

An encouraging result shown in figure 7.5(a) is that peak-to-background ratios of above approximately 100 are observed for degrees of phase correction as low as 20%. This ratio is high enough to facilitate imaging if optical sectioning is incorporated (see results regarding the focal peak-to-background ratio and imaging in sections 6.3.2 and 6.3.3) and suggests that the novel endomicroscope design will be robust to motion artefacts that may act to degrade the level of phase correction achieved in clinical endoscopic procedures.

Single photon vs. two-photon excitation

The final result presented in this section compares the use of one- and two-photon excitation. It was discussed in chapter 6 that multiphoton excitation could be used to provide optical sectioning and, hence, improve the image contrast obtained in the endomicroscope. Thus, figure 7.6 shows a comparison of the simulated lateral PSFs obtained using both one- and two-photon excitation. In this case, the aim was to simulate a fibre bundle like that used in the experiments presented in chapter 6. To this end, a wavefront was simulated consisting of 10,000 fibre cores with radii of $1\text{ }\mu\text{m}$ and centre-centre separations of $3\text{ }\mu\text{m}$. The wavefront had a focal length of 1.7 mm , which corresponded to a NA of 0.088. This wavefront was then propagated to its focal plane and the resulting lateral PSFs are shown in figure 7.6.

The single photon PSF is simply the intensity distribution in the focal plane while the two-photon PSF can be calculated by squaring this intensity distribution. Thus, both PSFs are proportional to the probability of fluorescence excitation and, therefore, the peak-to-background ratio in this case should be thought of as the ratio for the excited fluorescence rather than simply for the intensity of the excitation spot.

Firstly, it is clear from figure 7.6 that two-photon excitation (b, d) provides an improved resolution compared to single-photon excitation (a, c). The measured lateral resolutions are shown in the line profiles in figure 7.6 and, for single photon and two-photon excitation, these were found to be $4.7\text{ }\mu\text{m}$ and $3.5\text{ }\mu\text{m}$ respectively. Furthermore, the background level is suppressed relative to the focus when using two-photon (rather than one-photon) excitation. Again, this is evident in the line profiles in figure 7.6 where there are clear side-lobes in the one-photon PSF but not in the two-photon case. This implies that the SNR will be better in the two-photon case and, in order to test

this, the peak-to-background ratio was calculated for both of the lateral PSFs. These were found to be 2.7×10^4 (single photon) and 2.7×10^6 (two-photon), which suggests that two-photon excitation will provide a significant improvement in image contrast (in this case, by a factor of approximately 100).

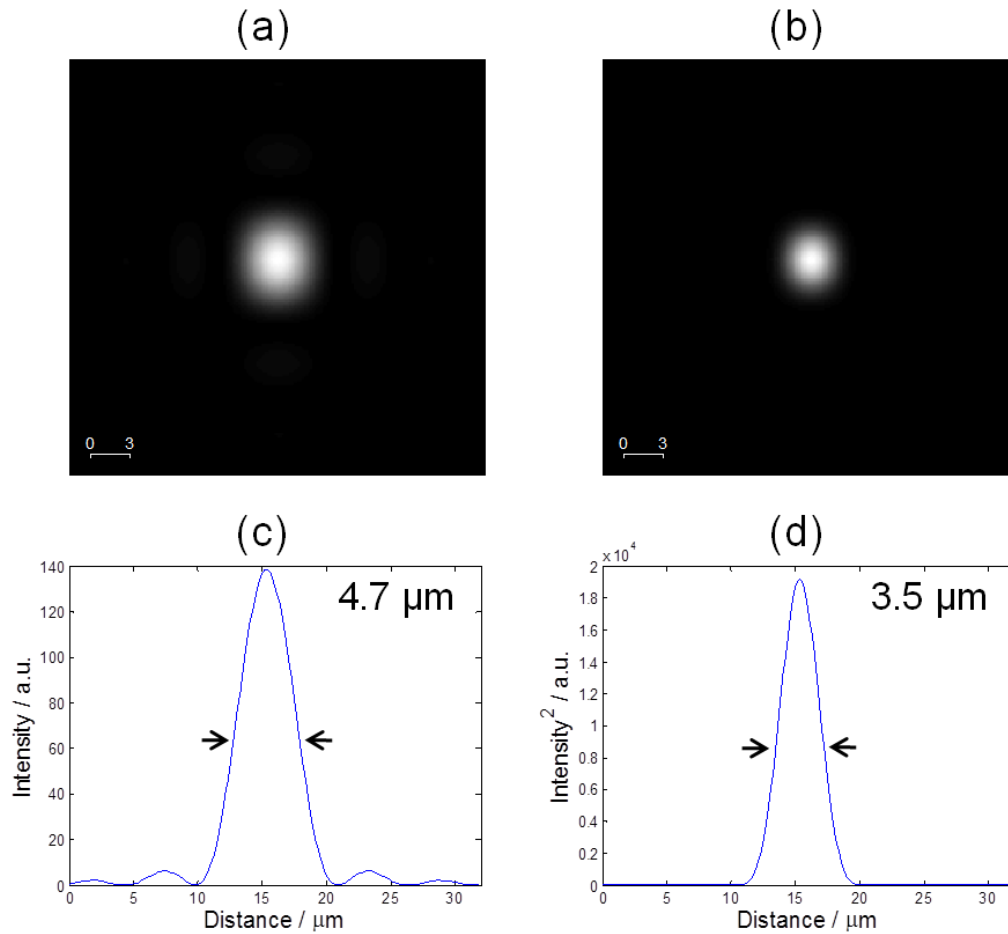


Figure 7.6. Comparison of the numerically simulated PSFs obtained with single photon (a, c) and two-photon (b, d) excitation. (a) and (b) show images of the focal plane while (c) and (d) show horizontal line profiles through the focus. (a) and (c) simply represent the intensity in the focal plane. (b) and (d) on the other hand, represent the square of the intensity. Thus, all the figures provide an approximation of the probability of fluorescence excitation. The arrows and numbers in the two line profiles indicate the FWHM of the two curves, which provide estimates of the lateral resolution. Scale bars in (a) and (b) are 3 μm in length.

7.2.6 Discussion

The results presented in this section (7.2) have firstly demonstrated how the optical focussing characteristics of the novel endomicroscope design depend upon the fibre imaging bundle used in the system. One important result showed that the number of fibres in the bundle could be reduced significantly compared to bundles used in standard proximal scanning endomicroscopes (to as few as

1000 cores) without detrimentally affecting the resolution or peak-to-background ratio achieved. Coupled with the fact that no distal scanning mechanism is required, this suggests that the novel endomicroscope design will permit the development of ultrathin endoscopic probes.

Reducing the number of fibre cores will produce a thinner, more flexible endomicroscope but will also reduce the number of resolution elements (i.e. pixels) in images obtained with such an instrument. An alternative approach is, therefore, to use more fibre cores. In addition to the increased number of resolution elements, this would also provide an improved peak-to-background ratio (see figure 7.3) and, because of the lack of a distal scanner, would not cause an undue increase in the size of the endomicroscope. With both approaches, the resolution and field of view will be dependent on the chosen focal length (for a fixed number of fibre cores) and the resolutions observed in these numerical simulations had typical values of around 5 μm . Using a distal lens would permit significant improvement in the resolution, to the extent that the minimum focal length would be limited by the lens and not by the fibre bundle used in the system.

In terms of the fill factor of the fibre bundle used, the results in figure 7.4 demonstrated that tight packing of fibre cores is no longer necessary, as it is in standard proximal scanning systems. Increased fill factor does provide an increased peak-to-background ratio but acceptable values were observed for fill factors as low as 20% – typical values (e.g. for the fibre bundle used in the experiments presented in chapter 6) are between 40% and 50%. Additionally, the fill factor does not affect the resolution of the system. Thus, this implies the opportunity to relax the design constraints on the imaging bundle. In turn this would lead to a simpler fibre bundle production process and lower fill factors would also mean that inter-core cross-talk (which is a significant hindrance to the phase correction procedure) could be reduced.

Further to the investigation of the fibre bundle, the effects of the degree of phase correction on the focussing characteristics of the endomicroscope were also simulated and studied. This section of the study demonstrated that, with only 20% of fibre cores assigned the correct phase value, it was possible to generate a focus that was acceptable for imaging when incorporating optical sectioning. This indicates the potential of the novel system to operate successfully in a clinical environment (with phase aberrations repeatedly corrected in real time) where the level of phase correction may fluctuate due to motion of the endoscope.

Finally, the effects of two-photon excitation were investigated. As expected two-photon excitation provided improvements in both the peak-to-background ratio and the resolution when compared to single photon excitation. In terms of peak-to-background ratio, two-photon excitation

was seen to improve the SNR by a factor of approximately 100. This suggests that, if implemented into the experimental setup described in chapter 6, two-photon excitation may provide the necessary improvements in image contrast to facilitate proximal endoscopic imaging with light collection as well as delivery performed by the fibre bundle (i.e. imagine the image contrast in figure 6.11(a) increased by a factor of 100). For this reason, the work presented in the remainder of this chapter is focussed on achieving phase correction with a mode-locked Ti:Sapphire laser, which could eventually facilitate multiphoton excitation.

7.3 Towards multiphoton excitation – distal focussing and scanning with femtosecond pulses

7.3.1 Introduction and motivation

It was discussed in chapter 6 that, in order for the novel endomicroscope configuration to become clinically useful, improvements in image contrast are first required. This could be achieved by improving the level of phase correction and, hence, increasing the ratio of the focal intensity to the background. Alternatively, incorporating optical sectioning into the system would also increase the image contrast by rejecting the background light. Again, as discussed in chapter 6, the simplest way to achieve optical sectioning in this system would be to attempt fluorescence imaging with multiphoton excitation (as no descanning is required). Furthermore, the results of the numerical simulations presented in section 7.2 suggest that utilising two-photon excitation would increase the image contrast by a factor of up to 100 compared to single photon excitation. This is a significant improvement in image contrast that may provide the required enhancement to allow collection of an image back through the fibre bundle.

A second reason to attempt multiphoton excitation comes from the fact that the novel endomicroscope may have advantages over current techniques used for multiphoton endomicroscopy. It was seen in section 3.3.2 that multiphoton endomicroscopy has suffered from temporal pulse broadening in the optical fibres used for beam delivery. This pulse broadening is caused by the confinement and propagation of high intensity light through single mode optical fibres. As such, the vast majority of multiphoton endomicroscopes utilise a single PCF for light delivery and collection (as PCFs exhibit less pulse broadening than standard SMFs) with a distal scanner. For this reason, multiphoton endomicroscopy typically suffers from the same drawbacks as distal scanning confocal endomicroscopy. In the novel method described in chapter 6, the excitation is not sequentially coupled into individual cores within a fibre bundle as in the case of a standard

proximal scanning endomicroscope. Instead, all cores in the bundle are illuminated simultaneously and focussing and scanning of the output beam are achieved by independently controlling the phase of the light in each fibre core. This means that, for each scan position, the excitation beam is distributed across the entire fibre bundle and is not coupled into a single fibre core. This distribution of the excitation light implies that the peak power in individual cores will be significantly lower than in the case where all the light is focussed into a single core. In turn this means that the pulse broadening due to non-linearity in the fibre imaging bundle will be dramatically reduced (linearly with number of fibre cores as the spectral broadening due to SPM is linearly dependent on the intensity) and, hence, this novel approach to endomicroscopy may permit efficient multiphoton excitation without the need for a distal scanner.

Both of the points discussed above suggest that it is sensible and interesting to try to achieve multiphoton excitation using the novel endomicroscopy technique. Thus, this section presents experimental work directed toward this end. First the experimental procedure is described (which is very similar to that used in chapter 6) and then results are presented demonstrating focussing and scanning of a pulsed laser at the distal end of a fibre imaging bundle.

7.3.2 Methodology

The experiments discussed in the remainder of this chapter were aimed at replicating the results presented in chapter 6 but with a pulsed, short coherence length laser that could be used to facilitate multiphoton excitation. To this end, the experimental configuration used previously (figure 6.2) was adapted to incorporate a Ti:Sapphire laser (Tsunami Broadband fs, Newport Spectra-Physics Ltd., UK), which provided 100 fs pulses over a tuneable wavelength range of 710-980 nm with an output power of 700 mW. The short coherence length of this laser ($< 100 \mu\text{m}$) meant that it was also necessary to include a SMF in the reference beam of the same length as the fibre bundle to approximately match the dispersion in the two arms. Additionally, a corner cube retro-reflector mounted on a motorised translation stage was also added into the reference beam path to allow adjustment of the reference beam length in order to match the sample and reference paths to within the coherence length of the laser. The amended optical setup is shown in figure 7.7.

It should also be noted that two fibre bundles were used in these experiments. Firstly, the Fujikura bundle (FIGH-30-650S, Fujikura Europe Ltd., UK) used in the experiments in chapter 6 was used and, secondly, a custom made fibre bundle was also investigated. This second bundle was provided by Brian Mangan and Jonathan Knight from the Centre for Photonics and Photonic Materials at the University of Bath and it consisted of 121 fibre cores, each with a diameter of $3 \mu\text{m}$

and with centre-centre separations of $9.5\ \mu\text{m}$. The custom bundle was drawn to provide single mode propagation at $800\ \text{nm}$ and the reasons for its use are described in section 7.3.3.

Using the experimental configuration shown in figure 7.7, experiments were carried out in an identical manner to those presented in chapter 6. Firstly, an interference pattern was recorded from which the phase in each fibre core was calculated. The SLM was used to correct for the phase aberrations and then a second interferogram was recorded. The $20\times$ microscope objective (L_5) was then defocused to image an object plane a set distance (typically $0.4\text{--}1.0\ \text{mm}$) from the distal face of the fibre bundle and the SLM was programmed to produce a focussed scanning beam at this object plane. Finally, the CCD camera was used to record intensity distributions demonstrating focussing and scanning of the output beam without the use of distal optics or mechanics (as in chapter 6) and these results are presented in the following sections.

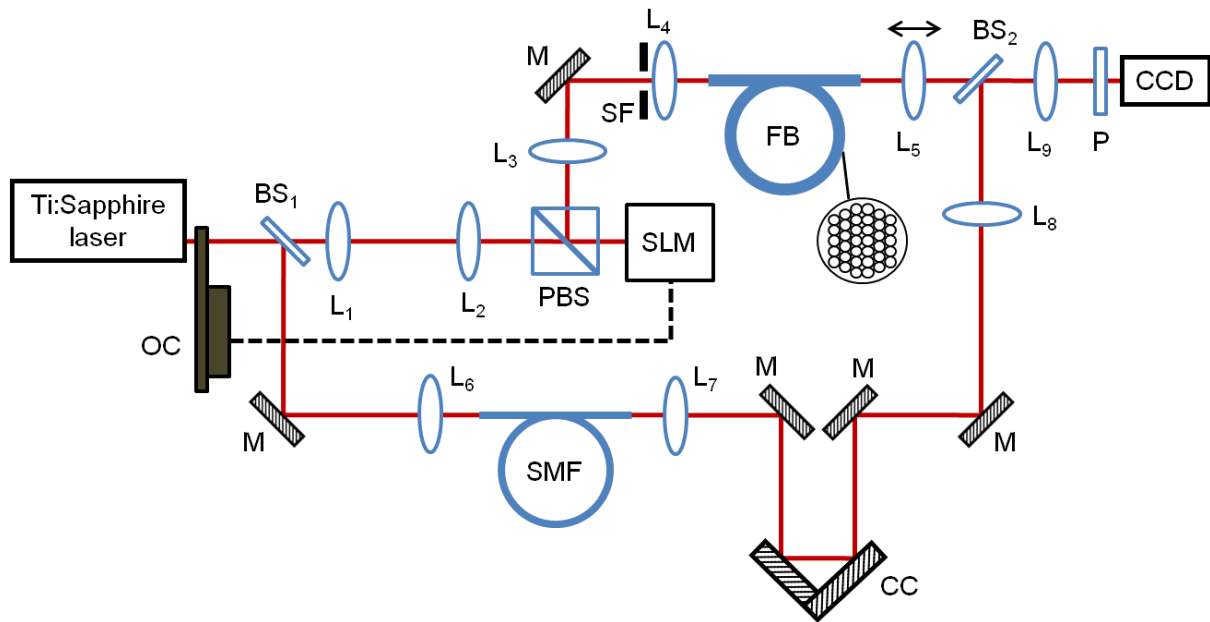


Figure 7.7. Experimental setup used for measuring and correcting phase aberrations in a coherent optical fibre bundle. OC – optical chopper; BS₁, BS₂ – 50/50 beam splitters; L₁, L₄, L₅, L₆, L₇ – microscope objectives (10x, 60x, 20x, 10x and 10x respectively); SF – spatial filter; PBS – polarising beam splitter; SLM – spatial light modulator; M – mirror; FB – fibre bundle; SMF – single mode fibre; CC – corner cube retro-reflector; P – polariser; CCD – camera; L₂, L₃, L₈, L₉ – lenses ($f = 100\ \text{mm}$, $200\ \text{mm}$, $250\ \text{mm}$ and $250\ \text{mm}$ respectively).

7.3.3 Preliminary results

The first stage of the experimentation simply involved investigating the interference fringes obtained using the pulsed, low coherence light source. The wavelength of the laser was tuned to $840\ \text{nm}$ (the wavelength used in the experiments described in chapter 6) and the CCD was used to display a live

image of the distal face of the Fujikura fibre bundle and the reference beam. The reference beam length was adjusted until the path lengths of the two beams were matched and interference fringes became visible. The path length of the reference arm was then altered further in order to maximise the number of fibres exhibiting clear interference. It soon became apparent, however, that for any given reference beam length, only a small number of fibre cores showed interference ($\leq 10\%$).

Clearly, to generate a distal focus using this method, interference fringes are required in all fibre cores (or at least in a large fraction thereof). Thus, the reference beam length as well as the wavelength and spectrum of the laser were all adjusted individually in an attempt to solve this problem and maximise the number of fibre cores exhibiting interference. While continually imaging, the wavelength of the laser was tuned over the range 750-900 nm and the spectrum of the laser was adjusted in an attempt to find a configuration that provided a reduced bandwidth and, hence, an increased coherence length. For all arrangements the result was the same: interference fringes were only observed in a very small number of fibres ($\leq 10\%$). This is demonstrated in figure 7.8(a), which shows an interference pattern recorded at a wavelength of 795 nm. In this image, the green square highlights a fibre core with interference fringes while the red square marks a fibre without fringes and it is evident that very few fibres show clear interference.

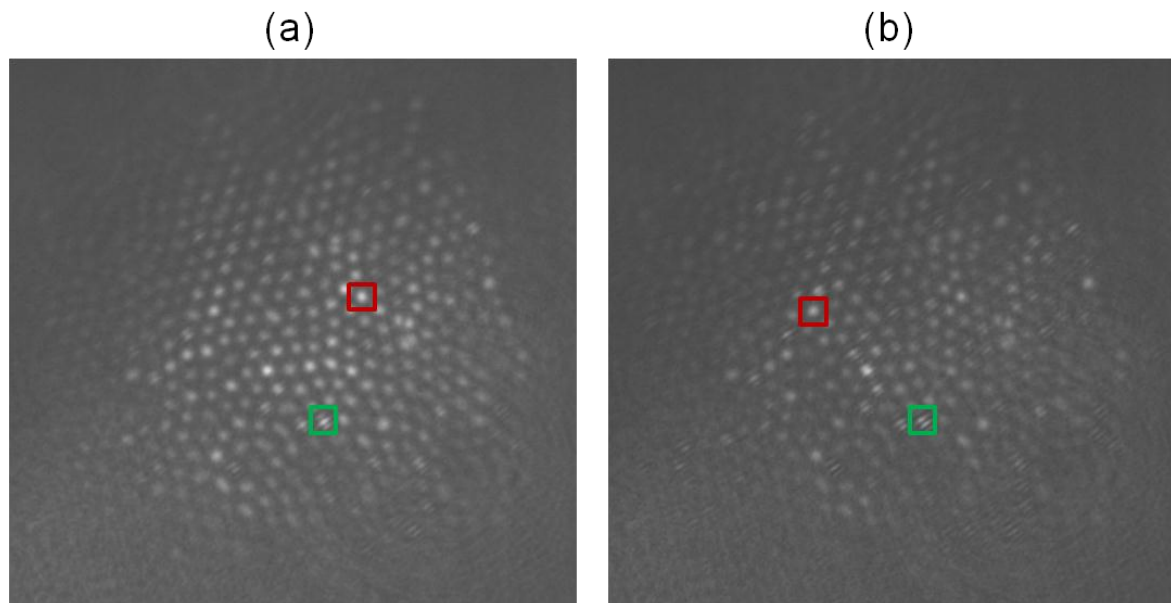


Figure 7.8. (a) an interference pattern observed using the Fujikura bundle at 795 nm. (b) shows the interferogram observed when a 1.5 nm bandpass filter is used to narrow the Ti:Sapphire spectrum. In both cases interference is only seen in a small fraction of the fibre cores for any given reference beam length. The green squares highlight a fibre core in each image showing clear interference fringes while the red squares highlight cores with no fringes.

As a further attempt to improve the interference pattern, a 1.5 nm bandpass filter with a centre wavelength of 795 nm (F1.5-794.7-4-12.5M, CVI Melles Griot, USA) was placed in the Ti:Sapphire beam path (before BS₁ in figure 7.7) in order to narrow the spectrum and increase the coherence length. Figure 7.8(b) shows the interference pattern observed when using this filter and, as before, the green and red squares respectively highlight fibres with and without interference fringes. It is apparent from the two images that using the bandpass filter produces interference fringes in a larger number of fibres. Despite this increase, even with the filter in place the majority of fibres still fail to exhibit interference.

The reason for this lack of interference is in fact the fibre bundle itself. The Fujikura bundle used (like most other modern coherent fibre bundles with over 1000 cores) is designed for imaging and one key design consideration involves minimising artefacts caused by the arrangement of the fibre cores. As such, the fibre cores are packed randomly rather than in a hexagonal fashion and the diameter and separation of fibres also varies from one core to the next. These variations in core diameter mean that the light propagating through each fibre core will experience a slightly different effective refractive index. In turn, this implies that each fibre core has a different optical path length. These variations in path length are evidently significantly greater than the coherence length of the laser (even with the spectrum narrowed using the bandpass filter) and this explains why only a small number of fibres exhibit interference at a given reference beam length.

This problem can be solved in one of two ways. The first option is to use a laser with a longer coherence length. Of course, a longer coherence length implies a longer pulse duration, which may not be suitable for multiphoton excitation. The alternative is to use a different fibre bundle in which the fibre cores are all of the same size and spacing such that the effective refractive index is approximately the same in all cores. As with the Fujikura bundle, the new fibre also needs to provide single mode propagation at 800 nm in order for the phase correction process to work. Many commercial fibre bundles are available for purchase, however, a large fraction of these have the same characteristics as the Fujikura bundle shown in figure 7.8 and, therefore, are not suitable. Others that do have uniform packing and fibre core diameter often have large cores, which are multimode at 800 nm.

For these reasons, a custom made fibre bundle was obtained from the Centre for Photonics and Photonic Materials at the University of Bath. As discussed in section 7.3.2 this bundle contained 121 cores each of 3 μm diameter with centre-centre separations of 9.5 μm . In terms of producing a working prototype endomicroscope, a fibre bundle with thousands (rather than hundreds) of fibres would be desirable, however, this 121-core bundle is sufficient for a proof-of-principle study.

Importantly, the packing and diameter of fibre cores in the bundle are symmetric and, once incorporated into the system, interference fringes were observed in the majority of the fibre cores in the bundle at a single reference beam path length (though some fibres still showed no interference or exhibited fringes with low modulation depth). Thus, all further data presented in this chapter were obtained using this custom fibre bundle and, as an aside, it is also interesting to note that the larger core diameter (compared to the Fujikura bundle used in chapter 6) may also act to reduce temporal pulse broadening (due to the broader mode profile and lower peak intensity), which further assists the development of a multiphoton fluorescence endomicroscope.

7.3.4 Results

Using the custom fibre bundle, the experiments presented in chapter 6 were replicated, as described in the Methodology section above (7.3.2). At first the laser was tuned to 840 nm and the phase was measured and corrected with interferograms recorded before and after the correction process. The 20x microscope objective (L_5) was then defocused to image a plane 0.6 mm from the distal tip of the bundle and the SLM was programmed to generate a focussed beam at this plane. From the interferograms recorded, phase maps were generated showing the phase in each fibre core before and after the aberration correction was applied. Angular histograms of the phase were also plotted before and after correction and both the histograms and the phase maps are shown in figure 7.9.

As shown in figure 7.9, the phases in individual fibre cores are randomly distributed before a correction is applied (a, c). This is demonstrated by the uniform angular distribution in the histogram (c) and the random spread of colours (i.e. phase values) in the phase map (a). After correction, on the other hand, there is a clear bias in the phase distribution with most fibres in the phase map (figure 7.9(b)) appearing blue. Additionally, the histogram after correction is no longer uniform and exhibits a clear peak at approximately 225° (i.e. in agreement with the result from the phase map).

Despite this relatively clear evidence of successful phase correction, it was difficult to discern a focus when studying the images of the focal plane. This suggests that the degree of phase correction achieved was simply not high enough to produce a focus that is clear above the background. This may be due to factors such as inter-core cross-talk and multimode propagation or it may be because the modulation depth of the interference fringes was low in some fibres and this introduced errors in the phase calculation.

To rectify this problem the wavelength of the laser was tuned to 795 nm and this provided two potential advantages. Firstly, and most importantly, at this wavelength it was possible to

incorporate the 1.5 nm bandpass filter discussed in section 7.3.3 into the system. This narrowed the spectrum of the laser, increased its coherence length and, therefore, also increased both the modulation depth of the fringes and the number of cores that would interfere at a given reference beam path length. Furthermore, moving to shorter wavelengths is likely to reduce the inter-core cross-talk (as the fraction of the 0th order mode profile that lies outside of the fibre core increases with wavelength), which acts to degrade the phase correction. Hence, to confirm this effect, the degree of cross-talk was assessed at both 795 nm and 840 nm.

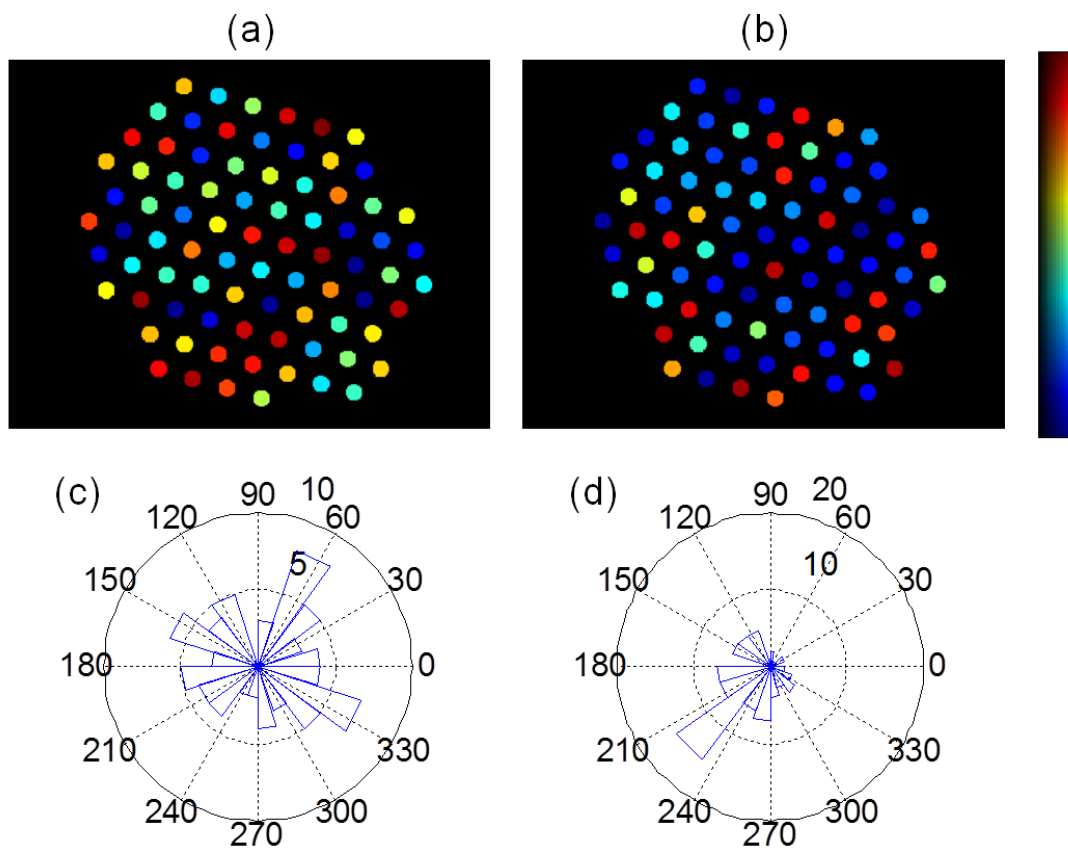


Figure 7.9. Phase maps at the distal tip of the fibre bundle before (a) and after (b) the application of a phase correction along with angular histograms showing the number of fibres with particular phase values (shown in degrees) in each case: (c) histogram before correction, (d) histogram after correction. Colour bar in (b) is representative of phase in the range $-\pi$ (blue) to $+\pi$ (red) for both phase maps. Also note that the radial scales in (c) and (d) are different – the inner ring in (c) represents 5 fibre cores while in (d) it represents 10 cores.

The cross-talk was assessed by illuminating each core in the fibre bundle individually and then imaging the distal output to investigate the number of cores that were transmitting light. For each fibre core, the number of transmitting cores was found as follows. First the transmission of the chosen core was tested by applying an arbitrarily chosen threshold (of 50 digital numbers) to the

image. This was necessary because the polariser in front of the CCD camera completely blocked the light from approximately half of the fibres cores. If the chosen core had an intensity that was higher than the threshold, then the transmitting fibres were counted. At this stage additional fibres were deemed to ‘transmit’ if their intensity was greater than 15% of the peak intensity in the image (i.e. greater than 15% of the intensity transmitted by the main fibre core). If more than one fibre was observed then the chosen core was said to exhibit cross-talk. This analysis was performed for all fibres in the bundle at both 795 nm and 840 nm. Figure 7.10 presents histograms that show the frequency with which one, two or three fibre cores transmit light when only a single fibre is illuminated. Thus, a value of one in the histograms indicates no cross-talk while values of two or three demonstrate the presence of cross-talk. It is clear from the two graphs that the degree of cross-talk is significantly lower at 795 nm than it is at 840 nm and the percentage of cores that exhibited cross-talk was found to be 19.6% at 840 nm (11 out of 56 fibres) and only 6.2% at 795 nm (4 of 65 fibres).

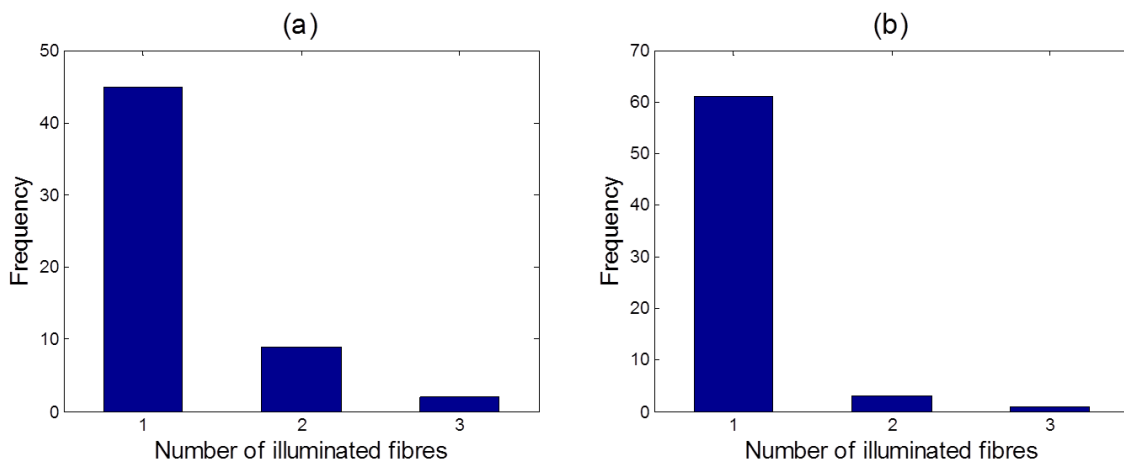


Figure 7.10. Histograms showing the frequency with which one, two or three fibre cores transmit light when only a single fibre is illuminated at wavelengths of 840 nm (a) and 795 nm (b). A value of one indicates no cross-talk while values of two or three demonstrate the presence of cross-talk.

The observed reduction in inter-core cross-talk may act to improve the level of phase correction achieved. For this reason, correction of the phase aberrations and focussing of the distal beam was again attempted, this time at a wavelength of 795 nm (without the use of the 1.5 nm spectral bandpass filter). Figure 7.11 shows phase maps and angular histograms before and after the correction was applied. As at 840 nm, there is clear smoothing of the phase map and an evident bias in the angular histogram after the phase aberrations are corrected. Additionally, in this case a dim focus was discernible after correction and this is illustrated by the two images of the focal plane

($f = 0.49$ mm) shown in the figure. Figure 7.11(f) shows the focal plane imaged with the phase aberrations corrected and the focal spot (indicated by the dotted red circle) is just visible above the background. Furthermore, around the focal spot six additional spots are also just visible above the background and these are the 1st diffraction orders.

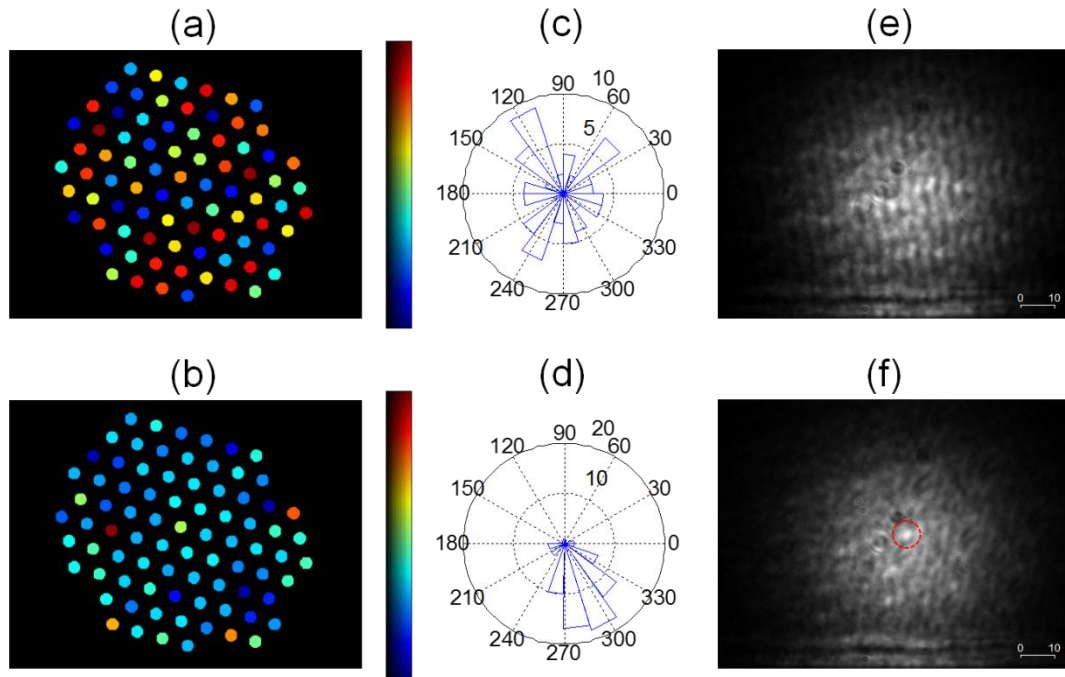


Figure 7.11. Diagrams illustrating improved phase correction at 795 nm (relative to 840 nm). (a) and (b) show the phase in each fibre core before and after correction respectively. Colour bars represent phase in the range $-\pi$ (blue) to $+\pi$ (red). The graphs in (c) and (d) are angular histograms of the phase in individual fibres – (c) before correction, (d) after correction. Finally, (e) and (f) show images of a focal plane 0.49 mm in front of the distal tip of the fibre bundle. Before the phase correction is applied (e) no focus is visible. With the phase aberrations compensated (f), however, the focal spot (0th order) and the 1st diffraction orders are just discernible above the background. The focus is highlighted in (f) by the dotted red circle and the scale bars in (e) and (f) are 10 μm in length.

The fact that the focal spot (and other diffraction orders) are visible indicates that the level of phase correction is slightly better at 795 nm than it was at 840 nm. This may be due to the reduction in cross-talk demonstrated by figure 7.10. Alternatively, the cause could also be small changes in the spectral width of the laser brought about by the adjustment of the prism required to change the wavelength while maintaining the mode-locking. This may have increased the coherence length of the laser, which would have improved the interference fringes and, hence, also reduced the error in the phase measurement. It is not clear which of these is the main cause of the improvement in the phase correction and, in reality, it is likely that both had some effect.

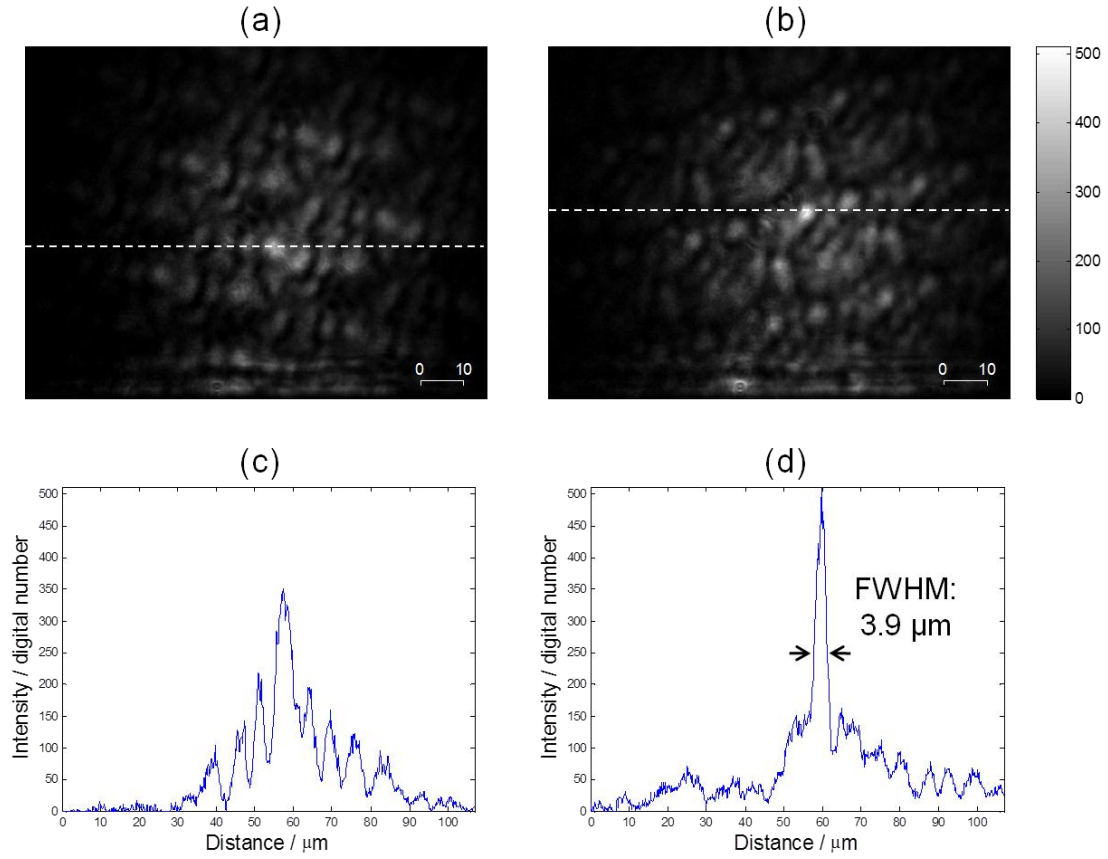


Figure 7.12. Intensity distributions (a, b) and line profiles through focus (c, d) recorded at a focal plane 0.64 mm in front of the distal tip of the custom fibre bundle using the 1.5 nm bandpass filter at 795 nm. (a) and (c) were recorded without the phase correction applied while (b) and (d) were recorded with the correction applied. Scale bars are 10 μm . The dotted white lines in (a) and (b) indicate the location of the line profiles. The line profile in (d) has a FWHM of 3.9 μm and this is also shown on the graph.

One option to further improve the phase correction is to narrow the spectrum of the Ti:Sapphire laser and, in doing so, increase its coherence length. This is most easily achieved using a spectral bandpass filter, therefore, the 1.5 nm bandpass filter discussed in section 7.3.3 was placed in the beam path directly after the output of the Ti:Sapphire. With the filter in place, the experiments described above were repeated in an attempt to generate a focussed beam at the distal end of the bundle. As before, the phase was measured and corrected with interferograms recorded before and after the application of the correction. The SLM was then programmed to focus the beam at a distance of 0.64 mm from the distal tip of the fibre bundle and the 20x microscope objective was defocused accordingly in order to image this plane. Figure 7.12 shows images of the focal plane without (a) and with (b) the phase aberrations corrected along with line profiles through the focus in each case. A dim, blurred focus is visible before the correction is applied (a) and this

implies that the phase aberrations are less predominant in this case than they were in the experiments presented in chapter 6. After the application of the phase correction the focus is significantly improved, appearing both brighter and sharper (see figure 7.12(b) and (d)). Furthermore, its intensity is considerably higher than the background. The line profile with the phase aberrations compensated (d) was found to have a FWHM of $3.9\ \mu\text{m}$ and the peak-to-background ratio was measured as approximately 11. This demonstrates the ability to produce a focussed beam at the output of an optical fibre bundle using a pulsed, short coherence length light source.

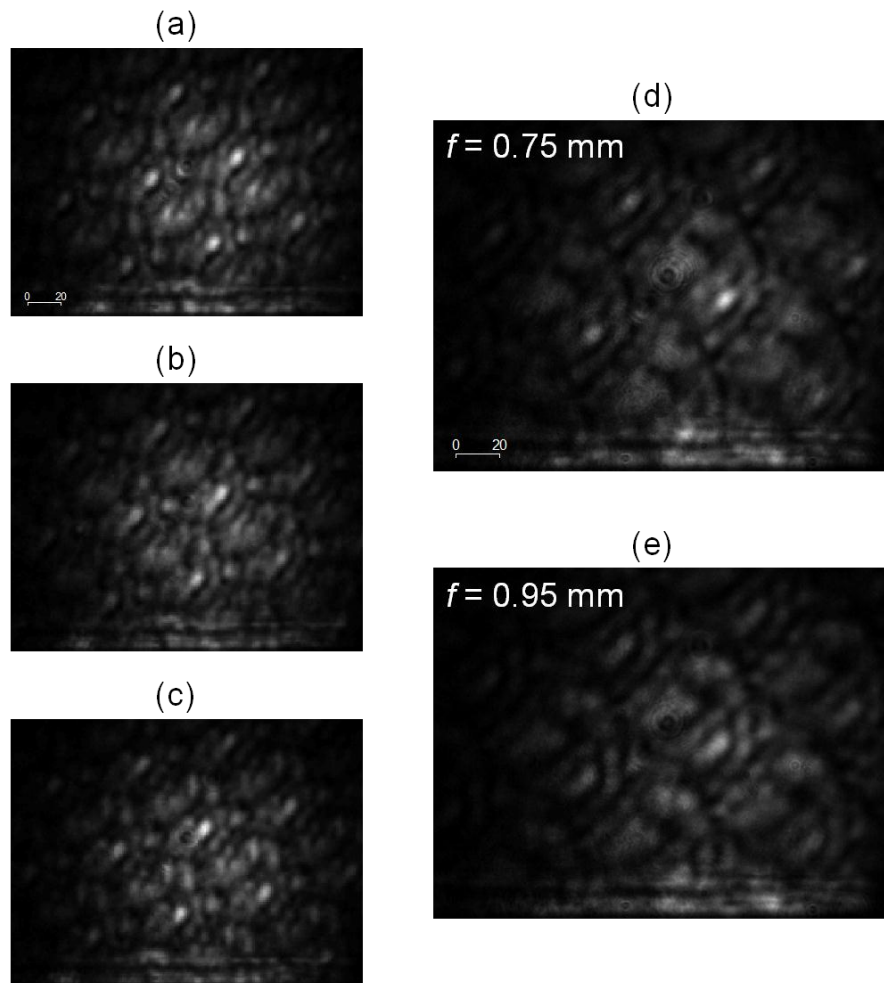


Figure 7.13. Images demonstrating lateral (a-c) and axial (d-e) scanning of the focal spot at the distal end of a fibre bundle. (a-c) show images of a focal plane at a distance of 0.64 mm from the distal tip of the fibre as the beam is scanned from right (a) to left (c). (d) and (e) show images of a fixed observation plane 0.75 mm in front of the output face of the fibre bundle for two different focal lengths (shown at the top left of each image). In (d) the focal plane and the observation plane coincide and a clear focus is observed. In (e) the focal plane is situated beyond the observation plane and defocus is observed in the resultant focal spot indicating that axial scanning has been achieved. Scale bar in (a) is $20\ \mu\text{m}$ and is representative of images (a-c). Scale bar in (d) is also $20\ \mu\text{m}$ and is representative of images (d-e).

As in chapter 6, having demonstrated focussing of the distal beam, it was now straightforward to also demonstrate 3-D scanning of the focus. To this end, after compensating for the phase aberrations, the SLM was programmed to focus and scan the distal beam to a series of lateral and axial positions. First, the beam was again focussed at a distance of 0.64 mm from the output face of the fibre bundle where it was scanned through 16 different lateral positions. Images of three of these focal scan positions are shown in figure 7.13(a-c) where, when investigating the images in alphabetical order, it is evident that the focus is scanning from right to left. The remaining images in figure 7.13 show axial scanning. In both (d) and (e) an object plane is imaged at a distance of 0.75 mm from the distal tip of the fibre. In (d), the focal plane and the object plane coincide, while in (e) the focal length is 0.95 mm meaning that the beam comes to a focus beyond the object plane. When the focal and object planes coincide a clear focus is visible. When they do not coincide, however, the focus is considerably dimmer and significantly blurred (i.e. it is defocused) indicating that axial translation of the focal spot has been achieved.

To summarise, when using the 1.5 nm spectral bandpass filter to increase the coherence length of a Ti:Sapphire laser, it has been possible to generate a focussed scanning beam at the distal output of a fibre bundle containing 121 fibre cores. As before, this was achieved without the use of any optical or mechanical components at the distal end of the fibre bundle. Further work is still needed to improve the peak-to-background ratio and the absolute power (as the spectral bandpass filter considerably reduces the laser power) of the focal spot. None the less, the results presented in this section represent a significant step towards facilitating multiphoton excitation using the novel endomicroscopy technique described in chapters 6 and 7.

7.3.5 Discussion

This section (7.3) has presented work towards the aim of realising multiphoton excitation at the distal end of a fibre bundle using the focussing and scanning methodology discussed throughout chapters 6 and 7. The first step in this study – which has been demonstrated here – is to produce a focussed scanning beam at the distal output of a fibre bundle with a pulsed laser. The laser source used in these experiments was a Ti:Sapphire laser, which provided pulses of approximately 100 fs duration over a spectral range of 710-980 nm. It was first observed that the short coherence length of the pulsed laser presented a significant problem in this investigation in that the effective path length experienced by the light travelling through the individual cores in the fibre bundle varied by more than the coherence length of the light source. This meant that for a particular reference beam

path length, only a small number of fibres would show interference fringes. Hence, it would only be possible to measure and correct the phase in this small fraction of interfering cores.

To solve this problem, an alternative fibre bundle was used. This bundle was a custom made fibre bundle provided by Brian Mangan and Jonathan Knight from the Centre for Photonics and Photonic Materials at the University of Bath. While it contained significantly fewer fibre cores than the previously used Fujikura bundle (121 compared to 30,000), the fibres in the bundle were regularly packed and had uniform diameter and spacing meaning that the variation in effective path length across different cores was likely to be considerably lower.

Using this alternative fibre bundle it was immediately observed that the interference was improved. Furthermore, it was possible to measure and correct the phase aberrations induced by the bundle at both 795 nm and 840 nm (though the contrast of the distal focus generated was low in both cases). It was also observed that – relative to 840 nm – there was an improvement in the phase correction and a reduction in the level of cross-talk when using 795 nm light.

The reduced cross-talk is an interesting result, which implies that the choice of wavelength will be important in the development of the novel endomicroscope. While the level of cross-talk is obviously highly dependent on the fibre bundle used, for a given bundle, it seems that it will be important to carefully select an optimum wavelength. This selection should be aimed at minimising the inter-core cross-talk while maintaining single mode propagation in the majority of fibre cores as these two factors can both significantly degrade the phase correction (see sections 6.3 and 6.4).

Despite demonstrating correction of the phase aberrations at 795 nm, further improvements in the level of phase correction were still required in order to permit the generation of a clear distal focus. To this end, a 1.5 nm spectral bandpass filter was placed in the beam path of the Ti:Sapphire. This acted to reduce the spectral width of the laser emission and, hence, to increase its coherence length. In turn this improved both the modulation depth of the interference fringes and the number of fibre cores exhibiting interference at a given reference beam path length. Using the bandpass filter, focussing and 3-D scanning of the distal beam were demonstrated. As in chapter 6, this was achieved without the use of distal optics or mechanics and it provides a significant step toward accomplishing multiphoton excitation of fluorescence using this novel endomicroscope design.

Of course, the use of the bandpass filter is not ideal and one important problem is that the laser power is significantly reduced by the filter. High powers are required for multiphoton excitation, therefore, any loss of laser power could be problematic. Thus, further work on this

experiment should now be aimed at replicating (or improving) the level of phase correction without the use of the bandpass filter. One option on this front is to narrow the spectrum of the Ti:Sapphire within (rather than outside) the laser cavity – for example, by narrowing the slit used to select the wavelength. This method should permit reduction of the spectral width of the laser to between 2 nm and 3 nm, while still maintaining a pulse duration of less than approximately 300 fs and without sacrificing a large fraction of the total output power. This narrowed spectrum should permit successful correction of the phase aberrations induced by the fibre bundle while the short pulses and high power will still allow multiphoton excitation of fluorescence.

7.4 Discussion and conclusions

This chapter has presented work from two studies, both centred on the novel endomicroscope design introduced in chapter 6. The first study involved numerical simulation of the distal beam profile under a number of conditions (section 7.2) while the second part of the chapter was concerned with experimental work aimed at achieving successful phase correction and distal focussing using a pulsed laser (section 7.3).

The numerical simulations demonstrated the behaviour of the novel endomicroscope – in terms of its resolution and SNR – in a number of configurations. This involved investigation of the effects of the number of fibres in the bundle, the spacing and radius of fibre cores and the chosen focal length. Additionally, the consequences of imperfect phase correction and two-photon excitation (compared to single photon excitation) were also studied. The results are presented and discussed in detail in sections 7.2.5 and 7.2.6. One important result showed that two-photon excitation could potentially increase the peak-to-background ratio by up to a factor of 100. This finding suggests that if two-photon excitation is implemented and the quality of phase correction demonstrated in chapter 6 is maintained, then the focal spot produced should provide adequate contrast to permit imaging in a reflection geometry. In this case, both light delivery and collection would be performed by the fibre bundle and this would signify an important step toward developing a functioning endomicroscope (albeit without descanned detection, which is necessary to provide optical sectioning in single photon or reflectance imaging).

With this result in mind, the remainder of this chapter then went on to present and discuss work aimed at achieving multiphoton excitation. As discussed in section 7.3.5, this work involved the demonstration of focussing and 3-D scanning at the distal end of a fibre bundle using a pulsed excitation source, capable of exciting multiphoton fluorescence. This result provides a further step

toward the development of a functioning multiphoton endomicroscope, however, it was achieved using a spectral bandpass filter to narrow the spectrum – and, hence, increase the coherence length – of the laser used. The use of this bandpass filter is a significant drawback as it limits the power available from the laser. This problem could be addressed by narrowing the laser spectrum intra-cavity rather than using an external filter. An alternative option would be to use an entirely different laser with a narrower spectrum and, therefore, a longer coherence length. Both of these approaches will, of course, suffer from the fact that the pulse duration will increase and this will reduce the efficiency of multiphoton excitation. Ultimately, addressing this issue will involve finding a compromise between the short pulses that permit multiphoton excitation and the long coherence length that will allow improved phase measurement and correction.

The second point that needs to be addressed regarding the focus generated at the distal end of the bundle with the pulsed laser is the peak-to-background ratio (see section 7.3.4). This was measured as approximately 11, which is considerably lower than the values obtained in chapter 6. The reasons for this (and opportunities to improve it) can be understood using the results of the numerical simulations presented earlier in this chapter. Firstly, the short coherence length of the laser is likely to mean that the degree of phase correction obtained in these experiments is lower than that acquired in the investigation presented in chapter 6. As is intuitive and as shown in figure 7.5, the peak-to-background ratio will be lower if the level of phase correction is lower. As discussed in the previous paragraph, careful selection of the excitation source used (and its spectral width) could provide improvements in the level of phase correction and, hence, in the peak-to-background ratio of the focus generated. Additionally, using a fibre bundle with even more uniform fibre core diameters could also improve the level of phase correction achieved. A simpler approach to increasing the peak-to-background ratio, however, is to simply increase the number of fibre cores within the bundle. As shown in figure 7.3, for a constant focal length, increasing the number of fibres in the bundle will also increase the contrast of the focal spot. The custom fibre bundle used in the experiments in this chapter contained only 121 cores while approximately 500 fibres were illuminated in the experiments presented in chapter 6. Thus, there is considerable scope for improvement of the focal contrast simply by increasing the size of the fibre bundle used.

Overall, this chapter has presented further work on the endomicroscope design introduced in chapter 6. This has involved a more detailed discussion of the focussing characteristics, numerical simulations of the distal beam profile and experimental progress toward the control (i.e. focussing and scanning) of a pulsed, short coherence length light source. Immediate future work on this project should now be directed toward achieving multiphoton excitation of fluorescence at the distal

end of the fibre bundle. To do this, improvements are required in both the peak-to-background ratio of the focus and in its absolute power and these can be accomplished as discussed above. More distant future research can then be aimed at the collection of an image at the proximal end of the fibre (i.e. in a reflection geometry) and then, finally, at constructing a phase measurement and correction procedure that can also be implemented at the proximal end of the bundle.

Chapter 8: Conclusions and outlook

The focus of this thesis has been on the development and use of novel optical technologies for clinical endoscopy. This research has followed two separate but related paths, the first of which involved the use of optical spectroscopy (specifically reflectance and time-resolved autofluorescence spectroscopy) in studies of skin and gastro-intestinal (GI) cancer. This section of the research was addressed in chapters 4 and 5 of this thesis. Chapter 4 discussed the application of two optical spectrometers to an *in vivo* study of skin cancer while chapter 5 described the development of a clinically viable trolley-mounted spectrometer that combined the functionality of the two systems used in chapter 4. This trolley-mounted spectrometer was then applied to both *ex vivo* and *in vivo* investigations of GI cancer and the results of these studies were also presented in chapter 5.

The remaining two results chapters of this thesis (6 and 7) then focussed on the second branch of the research. This entailed the development and testing of a novel technique to permit beam focussing and scanning in endomicroscopy, which utilises a spatial light modulator (SLM) and a coherent optical fibre bundle. Chapter 6 first introduced the concept and presented proof-of-principle results demonstrating 3-D scanning of a distal focus without the use of mechanical or even optical components at the distal end. Chapter 7 then discussed further work on this project, including both numerical simulations of the distal beam profile and experimental progress toward the use of this system to achieve multiphoton excitation of fluorescence.

8.1 Investigation of diffuse reflectance and time-resolved autofluorescence emission spectra of skin cancer

Chapter 4 discussed the use of two spectrometers in an *in vivo* clinical study of the optical signatures of skin cancer [129, 143]. The first spectrometer provided measurements of the diffuse reflectance spectrum and steady state fluorescence spectra with three excitation wavelengths (355 nm, 375 nm and 395 nm) [72]. The second system was a time-resolved fluorescence spectrometer, which recorded fluorescence decays in 16 spectral channels with two excitation wavelengths (355 nm and 445 nm) [21]. Both instruments were assembled in the dermatology department of Lund University Hospital where they were used to interrogate 27 clinically diagnosed skin lesions on a total of 25 patients. For each lesion, a complementary measurement of the neighbouring healthy tissue was also made for comparison.

In the analysis presented in chapter 4, the steady state and time-resolved data were assessed separately. For basal cell carcinomas (BCCs) – the disease type for which the greatest number of measurements was made – it was first observed that the mean diffuse reflectance and fluorescence intensity (with all excitation wavelengths) were lower on BCC lesions than they were on the surrounding healthy tissue. This result was in agreement with previously published research [8, 52], however, it should be noted that there was also a considerable degree of inter-patient variation within the data.

In order to analyse the steady state data in a more quantitative fashion, singular value decomposition (SVD) was used to compress the data from all 15 BCC measurement sites into a small number of base spectral components. The relative contributions (scores) of each of the base spectral components were then used as input parameters for discriminant analysis (DA). This involved the use of both linear discriminant analysis (LDA) and multivariate regression to build separate diagnostic models using the spectral data and both approaches were tested using a leave-one-out methodology. Multivariate regression was seen to provide marginally higher diagnostic accuracy than LDA and, in this case, the sensitivity and specificity obtained were 100% and 86% respectively. This corresponded to just a single false positive diagnosis from 15 measurement sites and suggests that SVD of steady state fluorescence and reflectance spectra followed by DA has the potential to aid the diagnosis of BCCs.

Regarding the time-resolved fluorescence data, the most important result showed that – with excitation at 445 nm – BCCs exhibited a statistically significant ($p = 0.002$) decrease in their spectrally averaged mean fluorescence lifetime, relative to the surrounding healthy tissue. Interestingly, this result was in agreement with previous *ex vivo* measurements made with the same system [21]. Furthermore, for all the BCC lesions investigated with the time-resolved fluorescence spectrometer, the fluorescence lifetime was seen to be lower than that of the neighbouring healthy tissue. Importantly, in 9 out of 10 cases this decrease in lifetime was greater in magnitude than the mean intra-patient variation in the measured lifetimes. This indicates the future potential of time-resolved autofluorescence spectroscopy (or imaging) for use in clinical diagnosis and suggests a possible sensitivity for BCC diagnosis of 90%.

Overall, this *in vivo* study of skin cancer revealed differences between healthy and diseased tissue (particularly BCCs) in terms of both spectral profile and fluorescence lifetime. Immediate future work should now be directed toward the collection of more *in vivo* data. Firstly, this will allow confirmation of the trends reported in this thesis to a higher degree of significance. Additionally, it will permit the detailed study of lesion types other than BCCs, which is important as it is clearly

necessary to assess the value of optical measurements over a wider range of skin malignancies. Finally, a larger spectroscopic dataset would also allow the development of more complex diagnostic models – perhaps including the fluorescence lifetime as well as the steady state spectra – and this may provide improvements in the diagnostic accuracy reported above. Once additional data has been collected and the best opportunities for contrast between healthy and diseased tissue have been identified, more distant future work will entail the use of these spectroscopic techniques to produce clinically viable diagnostic equipment. This is likely to involve moving to an imaging modality, as imaging provides additional morphological information and is considerably more intuitive for a clinician to use than point spectroscopy. The results presented in this chapter, along with any further data collected, will be used to guide the development of such imaging systems (in terms of, for example, the excitation and detection wavelengths used) and the aim should be to produce as simple and affordable an instrument as possible, while providing the maximum contrast between healthy and diseased tissue.

8.2 Development of a multidimensional spectrofluorometer and its application to studies of gastro-intestinal disease

Following on from the work presented in chapter 4, the fifth chapter of this thesis first charted the development of a multidimensional spectrometer that combined the functionality of the two systems used in the skin cancer study. This spectrometer provided measurements of diffuse reflectance spectra as well as time-resolved fluorescence spectra (16 spectral detection channels) with two excitation wavelengths (375 nm and 435 nm). In this case, the aim was to produce an instrument that was suitable for regular *in vivo* use in clinical endoscopic procedures. To this end the entire device is housed within an easily transportable, wheeled trolley and has been designed in order to make clinical use both safe and practical.

Having described the design and development of this system, chapter 5 also presented the results obtained to date in both *ex vivo* and *in vivo* studies of human GI disease. These studies are currently ongoing at Charing Cross Hospital and have so far involved the investigation of 62 *ex vivo* samples and 27 *in vivo* measurement sites in the oesophagus, stomach and intestine. The protocol used in these studies was very similar to that described in chapter 4 and involved the comparison of the optical signatures of healthy and diseased regions of tissue measured on (or excised from) the same patient.

First, the variation in the fluorescence lifetime of healthy tissue was assessed and it was observed that the lifetime varied based on the region of the body from which the tissue was excised – even according to different regions within the colon. Thus, in the analysis of the *ex vivo* polyp data (the disease type for which the greatest number of measurements was made), samples were only included if the complementary healthy tissue sample was excised from the same region of the colon. It was then observed that, when excited at 435 nm, polyps exhibited statistically significant changes (relative to the neighbouring healthy tissue) in both their mean fluorescence emission wavelength and their mean fluorescence lifetime. The mean emission wavelength observed in polyps was longer than that recorded in healthy tissue ($p = 0.035$) and this was true in 10 of the 14 polyps investigated. In terms of the fluorescence lifetime data, a negative shift was observed in the spectrally averaged mean lifetime (i.e. $\tau_{lesion} < \tau_{normal}$). In this case, the contrast appeared more convincing – 13 out of 14 polyps showed a negative lifetime shift – and this was confirmed by a Wilcoxon signed rank test, which returned a p-value of 6.1×10^{-4} . These observations are very promising and suggest the potential future use of measurements of the mean emission wavelength and, particularly, the fluorescence lifetime to aid the diagnosis of GI disease.

Future work on this project will be very similar to that discussed in section 8.1. Firstly, a larger dataset is required in order to confirm the trends observed and to permit investigation of further disease types. In this case, a large number of *ex vivo* measurements have been made and it is now most important to increase the number of *in vivo* measurements performed as this will allow detailed study of the optical signatures of GI tissue prior to surgical excision. As before, the spectroscopic data collected can then be used to guide the development of future diagnostic equipment, for example in terms of the optimum excitation and detection wavelengths used. Again, this is likely to involve moving to an imaging modality as imaging systems can provide relative lifetime contrast, are more intuitive to use than point measurement probes and can permit rapid screening of large (i.e. cm^2 rather than mm^2) fields of view.

8.3 A novel technique for laser scanning endomicroscopy based on adaptive phase compensation in an optical fibre bundle

Chapter 6 first described conventional techniques used in endomicroscopy. The concept behind a novel approach to laser scanning endomicroscopy was then introduced and results from a proof-of-principle study based on this system were presented. This novel technique firstly involves the use of a SLM to pre-compensate for phase aberrations induced by an optical fibre bundle and, in this way, it is possible to generate a plane wave at the distal output. The SLM is then also used to further

control the phase of the light propagating through each individual fibre core and this permits synthesis of arbitrary distal wavefronts in a manner analogous to phased array radar. In endomicroscopy this is useful as it allows the generation of a focussed, scanning beam without the need for distal components – either mechanical or optical.

The proof-of-principle study used a Mach-Zehnder interferometer arrangement to measure the phase in each core of an optical fibre bundle. A SLM was used to correct for these phase distortions and then to add the appropriate curvature, tip and tilt to the input wavefront in order to achieve focussing and 3-D scanning at the distal output. Example images of a United States Air Force (USAF) 1951 resolution test chart were also presented to demonstrate the potential for imaging (in a transmission mode). When simulating confocal detection the contrast of the resulting images was high and it was possible to resolve the smallest features on the test chart, indicating a lateral resolution of less than $4.4\ \mu\text{m}$. This was in agreement with the FWHM of the focussed distal beam, which was measured as $3.3\ \mu\text{m}$ [195].

This novel technique has a number of potential advantages over the current methodology. Most obvious is the fact that the ability to produce a focussed, scanning beam at the distal end of an optical fibre bundle – without the use of mechanical or optical distal components – means that the minimum size of the endomicroscope is only limited by the diameter of the fibre bundle used. This represents a significant advantage over distal scanning endomicroscopes, in which the size (and cost) of endoscopic probes are determined by the distal scanning mechanism. Proximal scanning permits the development of smaller and cheaper endomicroscopes, however, it also gives rise to additional drawbacks including a reduced number of resolution elements (due to the limited number of fibre cores) and undersampling (due to the fixed distance between fibre cores). While the novel approach will not improve upon the number of resolution elements, it may solve the problem of undersampling as the focal positions are no longer determined by the locations of the individual fibre cores in the bundle. Furthermore, this novel technique also allows control of the illumination point spread function (PSF) – including choice of parameters such as the numerical aperture (NA), axial position and field of view – which is not the case in conventional systems in which these parameters are usually fixed for a given endoscopic probe. Finally, the use of a SLM also indicates the potential to account for phase aberrations induced by the sample as well as the instrument itself and this may allow endoscopic imaging at increased depths within biological tissue.

Chapter 7 discussed further work carried out on this project including numerical simulations of the distal beam profile and experimental progress toward the use of this technique to permit multiphoton fluorescence imaging. The numerical simulations demonstrated the behaviour of the

novel system under a number of conditions. This involved simulations of how the resolution and peak-to-background ratio of the focus varied according to several fibre bundle parameters (for example, the inter-core spacing, fibre core radius and total number of fibre cores). Additionally, the effects of imperfect phase correction were investigated along with the use of two-photon excitation (compared to single photon excitation). Importantly, it was observed that two-photon excitation will provide an improvement in the peak-to-background ratio (of the excited fluorescence) by up to a factor of 100. For this reason, and because multiphoton excitation provides optical sectioning without descanned detection, the remainder of chapter 7 was focussed on experimental progress toward the aim of achieving multiphoton excitation of fluorescence using this novel endomicroscopy technique.

The methodology used in the second part of chapter 7 was very similar to that presented in chapter 6. The same experimental setup and procedure were used but, in this case, a laser source capable of exciting multiphoton fluorescence was incorporated into the system. The available light source was a pulsed Ti:Sapphire laser that provided 100 fs pulses with a maximum output power of 700 mW. Using this pulsed, low coherence light source, focussing and 3-D scanning at the distal end of a fibre bundle were again demonstrated without the aid of distal mechanics or optics. This was achieved using a spectral bandpass filter to narrow the spectrum (and, hence, increase the coherence length) of the laser but, none the less, it still signifies an important step toward the development of a functioning multiphoton endomicroscope based on the novel scanning concept introduced in chapter 6.

As discussed in chapters 6 and 7, future work on this project will be aimed at developing a functioning prototype endomicroscope. The first stage in this process involves the collection of an image at the proximal end of the fibre bundle (i.e. in a reflection geometry). This requires increased image contrast (compared to that accomplished in chapter 6 without the simulation of confocal detection) and this can be achieved either through improvement of the level of phase correction or through the incorporation of optical sectioning (to reject the background light and, hence, improve the image contrast). The experimental work discussed in chapter 7 was aimed at achieving multiphoton excitation, which would provide optical sectioning (and, thus, improved image contrast) due to the non-linear probability of fluorescence excitation (as a function of excitation intensity). While this experimental work provided a significant step toward the development of a functioning endomicroscope, the use of a spectral bandpass filter represented an important drawback as it limited the available laser power. Thus, the next stage in this experiment will involve attempting to produce a distal focus with high enough contrast and high enough absolute power to permit

multiphoton fluorescence excitation. One option here is to use a laser source with a longer coherence length and this is then likely to involve finding a compromise between the short pulses that permit multiphoton excitation and the long coherence length that allows high fidelity phase measurement.

Once multiphoton fluorescence excitation has been demonstrated, it should be straightforward to collect an image at the proximal end of the bundle (as no descanning is necessary). The final stage in the development of a prototype endomicroscope will then entail developing a phase measurement procedure that can be incorporated at the proximal (rather than the distal) end of the fibre bundle. This will be non-trivial but may be facilitated through coherence-gated detection, which would allow measurement of the phase aberrations through the detection of only the light reflected from the distal face of the fibre bundle. This approach would be similar in concept to optical coherence tomography and, if successful, would provide an important step toward the development of a clinically useful endomicroscope based on this novel technique.

8.4 Final remarks

Overall, this thesis has been concerned with the development and use of optical techniques for clinical diagnostic purposes. On one front this has entailed the application of point reflectance and time-resolved autofluorescence spectroscopy to the *in vivo* study of skin and GI cancer, while the other side of the research has involved the development of a novel method for beam scanning in endomicroscopy. Promising results have been reported on both topics showing significant contrast between healthy and diseased tissue as well as clear progress toward the development of a functioning prototype endomicroscope. Further work in this field has been discussed above and, in both cases, the long term aims will be centred on the development of optical instrumentation for clinical diagnosis. At this stage, the two branches of research discussed in this thesis may begin to merge involving, for example, the use of fluorescence lifetime endomicroscopy in clinical studies. The work presented in this thesis will hopefully lead to improvements in the performance of fluorescence endomicroscopy and has shown clear optical contrast between healthy and diseased tissue in skin and GI cancer. Thus, in the future, the combination of these methodologies may lead to the development of high resolution optical imaging techniques that can provide improvements in the diagnosis of malignancies of the skin, the GI tract and other regions of the human body.

Publications and conference presentations

- **A. J. Thompson**, C. Paterson, M. A. A. Neil, C. Dunsby and P. M. W. French, "Adaptive phase compensation for ultracompact laser scanning endomicroscopy," *Optics Letters*, Vol. 36, No. 9, pages 1707-1709 (2011).
- **A. J. Thompson**, S. Coda, M. Brydegaard Sørensen, G. Kennedy, R. Patalay, U. Waitong-Brämning, P. A. A. De Beule, M. A. A. Neil, S. Andersson-Engels, N. Bendsoe, P. M. W. French, K. Svanberg and C. Dunsby, "In vivo measurements of diffuse reflectance and time-resolved autofluorescence emission spectra of basal cell carcinomas," *Journal of Biophotonics*, Vol. 5, No. 3, pages 240-254 (2012).
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- **A. J. Thompson**, S. Coda, M. Brydegaard Sørensen, G. Kennedy, R. Patalay, U. Waitong-Brämning, P. A. A. De Beule, M. A. A. Neil, S. Andersson-Engels, N. Bendsoe, C. Dunsby, K. Svanberg and P. M. W. French, "Investigation of time-resolved autofluorescence emission spectra and diffuse reflectance of skin cancer *in vivo*," Oral presentation at the Optical Techniques in Clinical Practice meeting, Institute of Physics, London, UK, July 2011.
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- G. T. Kennedy, J. McGinty, **A. J. Thompson**, S. Coda, N. P. Galletly, H. B. Manning, D. S. Elson, M. A. A. Neil, B. Viellerobe, F. Lacombe, M. Brydegaard, N. Bendsoe, A. Thillainayagam, C. Dunsby, K. Svanberg, G. Stamp and P. M. W. French, "Biomedical FLIM: application to tissue autofluorescence and endoscopy," Oral presentation at the Fluorescence Lifetime Imaging Workshop, Imperial College, London, UK, July 2010.

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