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Development and characterization of fiber-based systems for biomedical imaging

Andy Yen Hsin Chen

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Physics

The University of Auckland
2011
Abstract

This thesis focuses on the instrumentation aspect of biomedical imaging with the use of optical fiber probes across three different disciplines. This includes the design and development of novel fiber optic based techniques and instruments in fields of optical coherence tomography (OCT), bioremediation and optical mapping of cardiac action potentials.

Although the mechanism behind the imaging techniques of fluorescence and OCT are fundamentally different, the fact that both techniques permit the use of fiber probes gives rise to the possibility of a combined fluorescence-OCT probe. For example, the OCT and fluorescence portions of the integrated system are optically distinct, except for the final optics of the fluorescence-OCT fiber probe. Analog processing electronics for the subsystems can also be distinct, but for the purpose of synchronization and simultaneous data acquisition, both should be controlled by a central computer. Tumlinson and co-workers [140] have already demonstrated the development of such a system for simultaneous optical coherence tomography and laser-induced fluorescence measurement. We begin by demonstrating the feasibility of using the novel source, supercontinuum, in an OCT system. Its capabilities and limitations are also discussed. A prototype all-fiber OCT system was subsequently constructed to meet the design requirements of combined fluorescence-OCT probes.

Current bioremediation methods are hindered by the lack of reliable, non-destructive, and in situ monitoring techniques. We investigate the feasibility of developing a novel spectroscopic technique that can monitor bacterial species in situ. A fluorescence spectroscopy system that meets the design criteria is subsequently built.
Its capabilities and limitations were demonstrated through a series of laboratory controlled experiments which showed promising preliminary results.

Optical mapping of cardiac action potential have proved to be indispensable in the study of arrhythmia. Although optical recordings using optical fibers have already been demonstrated with convincing results, none of which were spectrally resolved. We have constructed a fluorescence setup to make spectroscopic measurement of cardiac action potentials. This has offered more insights into the complex process of spectral modulation which is usually associated with membrane potential and mechanical activity.
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Andy Chen

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Abbreviations

A/D Analog to digital
APD Action potential duration
BDM 2,3-butanedione monoxime
CCD Charge-coupled devices
CFU Colony forming units
CMRR Common mode rejection ratio
CW Continuous-wave
DAQ Data acquisition card
DPSS Diode pumped solid state
ECG Electrocardiogram
eGFP Enhanced green fluorescent protein
eYFP Enhanced yellow fluorescent protein
FWHM Full width at half-maximum
FSD Fourier self-deconvolution
GFP Green fluorescent protein
LB Lysogeny broth
LED Light emitting diode
NA Numerical aperture
NEP Noise equivalent power
OCT Optical coherence tomography
OPD Optical path difference
PAH Polycyclic aromatic hydrocarbons
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>PCF</td>
<td>Photonic crystal fiber</td>
</tr>
<tr>
<td>PMT</td>
<td>Photomultiplier tubes</td>
</tr>
<tr>
<td>PZT</td>
<td>Piezoelectric transducer</td>
</tr>
<tr>
<td>Redox</td>
<td>Oxidation Reduction Potential</td>
</tr>
<tr>
<td>RFP</td>
<td>Red fluorescent protein</td>
</tr>
<tr>
<td>RMS</td>
<td>Root mean square</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron micrograph</td>
</tr>
<tr>
<td>SIF</td>
<td>Step index fiber</td>
</tr>
<tr>
<td>SLD</td>
<td>Superluminescent diode</td>
</tr>
<tr>
<td>SMF</td>
<td>Single mode fiber</td>
</tr>
<tr>
<td>SNR</td>
<td>Signal to noise ratio</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>ZDW</td>
<td>Zero Dispersion Wavelength</td>
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Chapter 1

Introduction

Fiber optic systems have undergone enormous development over the past two decades, particularly with the growth of telecommunication technologies. A major application in the field of medical imaging using such systems has emerged as a result of the new technologies originally developed for telecommunication. These include devices such as couplers, LEDs, solid state lasers that operate over a wide range of frequencies, and fast spectrum analyzers. They are advantageous in that, they make the optical systems more robust and compact by minimizing the use of free-space optics. Optical fibers have also revolutionized medical imaging by providing a means of acquiring information within biological tissues at localized regions. In terms of medical imaging, this thesis was involved in the fields of fluorescence and optical coherence tomography (OCT). In particular, we have focused on the design and development of novel fiber optic based techniques and instrumentation systems.

The use of fluorescence in biological sciences over the past twenty years have seen a tremendous growth, because compared to radioactivity [86] it is a non-invasive way of tracking or analyzing biological molecules. Depending on the technique applied, fluorescence imaging can provide structural or functional measurements at different resolution, ranging from centimeters down to subcellular levels [33]. Moreover, fluorescence offers a number of advantages over competing methods, such as high sensitivity, labeling and detection of multiple fluorochrome, long shelf-life, low haz-
ardous, and relatively low cost due to commercial availability [68]. In particular, we have focused on the utilization of optical fibers in detecting fluorescence as it provides remote sensing of samples at high resolution.

OCT is a non-invasive imaging technique that was first demonstrated in the early 1990s [157, 30]. Although similar to fluorescence, OCT can also provide structural or functional measurements at the cellular level, the principle behind their operation is entirely different. For this reason and depending on the imaging requirements, rather than compete, these two techniques complement each other. One of the main goals of this research was to examine the feasibility of developing a novel portable all-fiber fluorescence-OCT probe which can obtain both the structural (OCT) and functional (fluorescence) information simultaneously. The remainder of this chapter presents a brief overview of the fields related to the research presented in this thesis.

1.1 Fluorescence process

The process of emitted light from a molecule that has absorbed light of a different wavelength is known as fluorescence. While the absorbed radiation usually have higher energy (ie shorter wavelength) than the emitted light, it is possible to have the opposite. In the case of two or even three photon absorption by a single electron, the emitted photon typically carries a higher energy than any of the excitatory photons. As the quantum event of a multi-photon absorption is extremely rare, excitation of the molecule would require a source with high energy flux such as lasers with very short pulses. Since the scope of our thesis is limited to single photon excitation, multi-photon excitation will not be discussed any further.

The process of fluorescence involves three stages: excitation, vibrational relaxation, and emission. These stages are illustrated by the Jablonski energy diagram as shown in Figure 1.1A. When a photon supplied by an external source is absorbed by a fluorophore, its electron jumps from the ground state $S_0$ to an an excited, unstable state $S_1'$. The excited state exists briefly, usually on the order of a few nanoseconds.
1.2. Fluorescence process

During this period, heat is dissipated as the excited electron relax toward the lowest vibrational energy level within the excited state $S_1$. When the electron falls from the excited state to the ground state, light is emitted at a particular wavelength. The energy of the emitted photon is the difference between the energy levels of the two states which also governs the wavelength of the emitted light.

Typical excitation and emission spectra of a fluorochrome is shown in Figure 1.1B. The plot of total emitted fluorescence versus excitation wavelength represents the dependence of wavelength on excitation efficiency. However, it is usually the absorption spectrum that is given as it is much easier to measure and to a first approximation, the absorption spectrum of a fluorophore is identical or very similar to its excitation spectrum. The emission spectra to a certain extent are independent of the excitation wavelength. This is because upon excitation into higher electronic and vibrational levels, any excess energy is quickly dissipated, leaving the fluorophore in the lowest vibrational level of $S_1$ [86]. The electron of the fluorochrome can be in any of several vibrational and rotational energy levels within the ground state, and end up likewise within the excited state. This is reflected by the width of the excitation and emission spectra. For most fluorophores, the emission spectrum is similar to the absorption spectrum as the probability of an electron returning to a particular vibrational energy level in the ground state is similar to the probability of that electron’s position in the ground state before excitation. This concept is known
as the “mirror image rule” [86]. This rule breaks down when excitation is performed far away from the excitation peak, into the emission band.

Although the entire fluorescence process of a single fluorophore can be repeated, it may be irreversibly and photochemically altered in the excited state so that it no longer fluoresces. In time, fluorescence from all the fluorophores manifest in the form of a multi-exponential decay, this phenomenon is known as photobleaching. Although in most cases, the exact physical basis for photobleaching is unclear, it is usually understood as due to the excited state being more chemically reactive than the ground state [136]. Thereby allowing a small fraction of the excited fluorophores to participate in chemical reactions that alter the molecular structure of the fluorochrome. This results in reduced fluorescence. The rate of these reactions depends on the sensitivity of the particular fluorochrome to several factors such as chemical environment, excitation light intensity and exposure time of the excitation beam [68]. For a more in depth description of the photobleaching process, the reader should refer to Goldys [52].

1.2 Principle of fluorescence imaging systems

The design criterion of a general fluorescence imaging system consists of the following key elements: light source and photodetection, excitation and emission filter, light delivery and collection optics. A schematic diagram of such a system is shown in Figure 1.2. An excitation filter is inserted to select the desired excitation wavelength before the specimen is illuminated by the light source via light delivery optics. As the excitation light is absorbed by the fluorophores attached to the sample, they emit light of longer wavelengths. Some of the excitation light and fluorescence is then captured by the light collection optics and directed to the photodetector. An emission filter is inserted in front of the photodetector to ensure no illumination light is leaked into the detection band.

The light source can be divided into two categories: broadband sources such
1.3. Ratiometric dyes

In functional fluorescence imaging, the target physiological parameter in living cells is monitored with fluorescent probes. Any relative change in the fluorescence ex-
citation or emission spectra is a measure of the parameter of interest. Since to a first approximation, the excitation and emission spectra mirrors each other therefore measurement can be performed on either one.

![Figure 1.3: Effects fluorescence excitation/emission spectra variation on target physiological parameter for A: Non-ratiometric, and B: Ratiometric probes. Regardless of the spectral shift, fluorescent excitation/emission remains constant at the isosbestic wavelength.](image)

Strictly speaking, ratiometric probes may exhibit some amplitude modulation and non-ratiometric probes may exhibit some spectral shift. However, for the sake of simplicity, we will categorize fluorescent probes into ratiometric and non-ratiometric. With non-ratiometric probes, the target physiological parameter is measured by the relative change in the fluorescence intensity (see Figure 1.3A). However, it is often difficult to make accurate quantitative measurements using non-ratiometric probes due to various factors such as autofluorescence, intensity noise of excitation light, unequal dye loading and photobleaching. On the other hand, with ratiometric probes, the emission spectra is shifted toward either the shorter or longer wavelengths in accordance with the modulating physiological parameter (see Figure 1.3B). Despite modulation of the spectra, there exists an isosbestic point where the intensity remains constant at a particular wavelength. The windows below and above the isosbestic wavelength is commonly referred to as the short and long wavelength bands, respectively. Since most external factors that can affect fluorescence are of amplitude modulation only, by taking the ratio of the short and long wavelength bands, those common-mode signals are suppressed while the signal associated with the targeted physiological function is amplified. Therefore, as the spectra shifts, the
short and long wavelength bands will see signals that are identical but opposite. The term ratiometric reflects the fact that it is possible to calibrate recordings with such probes and make quantitative measurements.

1.4 Voltage-sensitive dyes

Voltage-sensitive dyes are molecules that can bind to cell membrane with high affinity [120]. Upon excitation and while binding to the membrane of the cells, the dye molecule fluoresce with a spectral shift that is dependent on the transmembrane potential. Electrochromism is responsible for this voltage dependency of the dye. Dye molecules undergo an electronic redistribution upon excitation, and if the direction of the charge shift is parallel to an external field (membrane potential), the energy of the electronic transition is altered resulting in a spectral shift [46]. Herein lies two major advantages of optical recordings, one being the ability to follow any physiological process as the response involves only movement of electrons upon excitation, assuring an almost instantaneous coupling between the external field and electronic states of the chromophore. The other allows the measurement of action potentials in a non-invasive manner, thus minimizing the risk of cellular injury and permits very high spatial resolutions. In particular, we have studied and characterized the commonly used potentiometric dye, di-4-ANEPPS, this will be discussed in detail in Chapter 4.

1.5 Principle of OCT

The most basic form of an OCT system is shown in Figure 1.4. The key components are a Michelson interferometer and a light source with a very broad bandwidth. For Fourier-domain OCT systems, a spectrum analyzer is used as the detector and in
CHAPTER 1. Introduction

Figure 1.4: Schematic of an OCT system based on a Michelson interferometer. The key components are shown in their simplest form. If a spectrum analyzer is used as the detector, z-scanning is not required.

In this case, axial (z-scan) scanning is not required. Here, we will only consider time-domain OCT systems as this is the focus of our thesis.

Light from the source is split into two paths by the beamsplitter, one goes to the reference mirror while the other goes to the sample. A small fraction of light is reflected back from the sample. Through the beamsplitter, it later recombines with the light from the reference mirror with an optical path difference. The combined light incident on the detector will produce a signal such that its magnitude depends on the optical path difference, bandwidth of the source and reflected light from the sample. Optical path difference is achieved by scanning the reference mirror. This produces a 1-D axial reflectivity profile of the sample. 2-D or 3-D image of the sample can be constructed with the inclusion of one or two lateral scans, respectively.

For a much more in-depth discussion on the principle of operation for time-domain OCT systems, please refer to Chapter 2.
1.6 Thesis outline

This thesis focuses on the design and development of novel fiber optic based techniques and instruments in fields of optical coherence tomography (OCT), bioremediation and optical mapping of cardiac action potentials. It contains three distinct experimental chapters that involve the use of optical fibers in the setups for imaging of the specimen. Each of these chapters begins with a general background on the topic relevant to the experiment that is described later, discussion and conclusion will follow.

Chapter 2 demonstrates the feasibility of using the novel source, supercontinuum, in an OCT system. Its capabilities and limitations are also discussed. A prototype all-fiber OCT system was subsequently constructed to meet the design requirements of combined fluorescence-OCT probes.

In chapter 3, we investigate the feasibility of developing a novel spectroscopic technique so that the process of bioremediation can be monitored in situ. A fluorescence spectroscopy system that meets the design criteria was subsequently built. Its capabilities and limitations were demonstrated through a series of experiments which showed promising preliminary results.

In chapter 4, we have constructed a fiber-based fluorescence setup with the inclusion of a spectrometer so that any spectral shifts in the action potential measurements can be resolved. This has allowed for better modeling of motion artifact thus improving post-processing methods that removes this effect. Also, gaining insights into the spectral domain have aided the understanding of mechanisms involved in optical measurements.

Chapter 5 presents a summary of the work done and also provide guidelines for future studies in their respective research areas.
CHAPTER 1. Introduction
Chapter 2

Time-domain optical coherence tomography

2.1 Introduction

Although the technique of interferometry has been well established since the 19th century it was not until the early 1990s that its application in biomedical imaging was first demonstrated [157, 30]. Optical coherence tomography (OCT) is a non-invasive imaging technique based on the theory of low coherence interferometry whereby light reflections within the surface of a sample are resolved to give tomographic visualization. The penetration depth or axial scan into the tissue is dependent on the wavelength of the source but is typically limited to a few millimeters [110]. The axial resolution, typically on the order of 1-10 µm, is determined by the bandwidth of the light source while the transverse resolution is determined by the numerical aperture (NA) of the optics. In terms of penetration depth and resolution OCT fills the niche between ultra-sound and confocal microscopy [1]. OCT first started as a structural or anatomical imaging technique, however development has been rapid and it is now able to obtain functional information for instance blood flow using the Doppler effect [25] or birefringence using the polarization properties of...
light [31]. The ultimate goal of this research was to develop a novel portable all-fiber fluorescence-OCT probe which can obtain both the structural and functional information simultaneously. We have focused on a time domain approach to OCT as it is relatively cheap and simple to implement. Subsequent development of the system into frequency domain is possible with the addition of a spectrometer. The development of such a system is beyond the scope of this thesis hence the initial work done on the OCT part was transferred to another PhD project.

In this chapter, firstly we discuss the fundamental principles of time domain OCT, secondly describe a novel supercontinuum source for OCT that employs a pulsed laser and photonic crystal fiber. This provides a wide-band source resulting in excellent axial resolution [116]. We then detail the layout of our OCT setup and report the sensitivity and axial resolution of this system. Finally, imaging results obtained with a variety of test specimens along with the limitations of the study are presented and discussed. Future prospect includes the construction of an all-fiber OCT system in which the novelty exist in the portability of the design and possible integration with a fluorescence probe.

## 2.2 Principle of time-domain OCT

In order for OCT systems to achieve good axial resolution an incoherent source is indispensable. The coherence of a light source is the degree to which electromagnetic waves maintain a constant phase relationship. Therefore the phase of a broadband light source will lose coherence much faster than the phase of a narrow-band source. This degree of coherence in the spatial domain is called the coherence length.
2.2. Principle of time-domain OCT

2.2.1 The Michelson interferometer

The Michelson interferometer was invented by Albert A. Michelson in 1887 [100]. This optical interferometer was used in the Michelson Morley experiment in 1887, which demonstrated that the speed of light is invariant in different inertial reference frames. Since then it has been widely used. A schematic diagram of a Michelson interferometer is given in Figure 2.1. The light from the source is split into two separate paths by a beam splitter. One goes to a sample arm while the other goes to the reference arm. The two beams recombine after an optical path difference (OPD) is introduced by the scanning reference mirror. Due to this OPD, the two electromagnetic waves experience summation or cancellation at different phase cycles, thus forming an interference pattern. The interferometer in this configuration, where the sample is a mirror, is also referred to as an autocorrelator. It measures the cross-correlation function of an optical source with itself.

In any interferometer, axially moving the reference arm mirror provides a means of scanning through the sample, thereby providing a 1-D axial reflectivity profile of the sample. 2-D imaging is achieved by the addition of transverse scanning, while 3-D imaging requires two orthogonal transverse scanning mechanisms.

![Figure 2.1: The Michelson interferometer.](image)

The power of the light incident on the photodetector $P(z)$ as shown in Figure 2.1
can be written as

\[ P(z) = P_R + P_S + 2\sqrt{P_R P_S} \cos \theta(z) = A + B \cos \theta \]  

(2.1)

where \( P_R \) and \( P_S \) are the power from the reference and sample arm, respectively, and \( \theta \) is the phase difference between the two arms. The term \( A \) is a DC term, and \( B \) is the amplitude of the AC term.

The power difference between the sample and the reference arm defines the fringe visibility \( v \)

\[ v = \frac{2\sqrt{P_R P_S}}{P_R + P_S} = \frac{B}{A} = \frac{P_{\text{max}} - P_{\text{min}}}{P_{\text{max}} + P_{\text{min}}} \]  

(2.2)

where \( P_{\text{max}} \) and \( P_{\text{min}} \) corresponds to the maximum and minimum power detected by the photodetector along the z scan respectively. Note that the fringe visibility \( v \) has a value between zero and one. Zero occurs when no light is reflected back from either the sample or reference arm and one occurs when both arms have an equal amount of power. For a broadband source such as those used in OCT systems the AC signal or fringes start to vanish as the optical path difference between the two arms approaches the coherence length. This is because as the frequency content of the signal increases, the phase relationships of the frequency components become incoherent.

\[ \text{2.2.2 Resolution Limit} \]

The performance of any imaging system is comprised of two important parameters. They are the resolution which determines the amount of details and the signal to noise ratio (SNR) which determines the quality of the data. In OCT systems, the image is made up of multiple axial scans of the sample. Axial resolution is independent of transverse resolution as it depends only on the source whereas the transverse resolution depends on the optics used in the sample arm.
2.2. Principle of time-domain OCT

2.2.2.1 Axial resolution

The axial resolution of an OCT system is governed by the spectral bandwidth of the light source. For a source with a Gaussian spectral profile, the axial resolution $\Delta z$ is given by [14]

$$\Delta z = \frac{2\ln2}{\pi} \frac{\bar{\lambda}^2}{\Delta \lambda}$$  \hspace{1cm} (2.3)

where $\bar{\lambda}$ is the mean wavelength, and $\Delta \lambda$ is the full width at half-maximum (FWHM) of the spectrum. This equation shows that resolution scales with center wavelength. Thus in the visible region, less bandwidth is needed to achieve the same resolution than in the infra-red region.

However, in practice, the axial resolution of an OCT setup is usually determined by verifying the autocorrelation function experimentally. It can be calculated by two simple methods. One is to count the number of fringes within the FWHM of the autocorrelation function. Thus,

$$\Delta z = N \times \frac{\bar{\lambda}}{2}$$  \hspace{1cm} (2.4)

where $N$ is the number of fringes, the constant of $\frac{1}{2}$ is due to the fact that the displacement made by the reference mirror is half of the optical path length traveled.

Another approach is to multiply the velocity $v$ of the reference arm by the time $t$ taken to scan through the autocorrelation FWHM. Thus,

$$\Delta z = v \times t$$  \hspace{1cm} (2.5)

Typically, both methods are used to check for consistency and for validation.
2.2.2.2 Transverse resolution

As in conventional microscopy the transverse resolution in OCT is determined by
the objective lens used to focus the light onto the sample. The Rayleigh’s criterion
for minimum resolvable transverse detail $\Delta x$ is given by

$$\Delta x = 1.22 \frac{\lambda}{NA} = 1.22\lambda \left( \frac{f}{d} \right)$$

(2.6)

where $\lambda$ is the wavelength of the source, and $NA$ is the effective numerical
aperture (NA) of the objective lens, $d$ is the spot size on the objective lens and $f$ is
its focal length.

The depth of the axial scan depends not only on the transmission and scattering
properties of the sample but also to a large degree on the depth of focus. This is
sometimes referred to as the confocal parameter $b$ and is related to the transverse
resolution $\Delta x$ by [14]

$$b = \pi \frac{(\Delta x)^2}{2\lambda}$$

(2.7)

Equation 2.7 shows that higher resolution leads to lower depth of axial scan
hence there is a trade off.

2.2.3 Noise sources

When making any experimental measurement it is necessary to identify the sources
of system noise. Only then can methods be devised to reduce it. In this section we
will discuss the most common sources of noise in OCT systems. The signal to noise
ratio (SNR) is conventionally defined in terms of the electrical power [110] which is
proportional to the photo current squared. The SNR is defined as

$$SNR \equiv \overline{i^2}/\sigma^2$$

(2.8)
where $i$ is the photocurrent averaged over the detector’s response time and $\sigma^2 = \langle i^2 \rangle - \langle i \rangle^2$ is the variance of the photocurrent. The common sources of noise can be individually identified as shot noise, intensity noise and thermal noise of the photo-receiver. These sources of noise are assumed to be independent and we shall outline each of them in turn.

### 2.2.3.1 Shot noise

Shot noise occurs when the detected signal is small enough to give statistical fluctuation due to the stochastic nature of photon arrival time. Assuming a 50/50 splitting ratio between the two arms of the interferometer and that the reflected light from the reference arm overwhelms the sample arm which is usually the case as most samples are weakly reflective, the SNR due to shot noise is given as [130]

$$SNR_{Sh} = \frac{1}{4} \frac{\eta P_0 R_S}{B}$$ (2.9)

where $\eta$ is the quantum efficiency of the detector, $h$ is the photon energy, $P_0$ is the power of the light source, $R_S$ is the reflectivity of the sample and $B$ is the electrical detection bandwidth of the photo-receiver. The optimum value of $B \approx 2 \Delta f = 2 \ln(4)(4/\pi)(v/\Delta z)$, where $\Delta f$ is the FWHM power bandwidth of the signal and $v$ is the axial scan speed [130]. Equation 2.9 shows that in a shot noise limited regime, SNR is independent of the reflectivity of the reference arm. It is proportional to source power and inversely proportional to the electrical bandwidth.

### 2.2.3.2 Intensity noise

In addition to the noise from the source whenever optical fields are mixed together, the detected signal also generates noise [53]. In OCT systems this is commonly referred to as excess intensity noise. The power from the reference $P_R$ and sample
$P_S$ arms in Equation 2.1 can be divided into their respective coherent and incoherent components such that $P_R = P_{Re} + P_{Ri}$ and $P_S = P_{Sc} + P_{Si}$. Mixing these four fields leads to sixteen beat terms made up of four self-beat (source intensity noise) and twelve cross-beat terms, two of which are the OCT signals while the rest arises from unwanted reflections. In general, OCT systems have a strong reference signal and weak residual reflections such that $P_{Re} \gg P_{Ri}, P_S$. Thus, the SNR due to excess intensity noise for a single ended detection can be approximated as [110]

$$SNR_{Ex} = \frac{2R_{Sc}}{R_{Re} \tau_c B (1 + \xi^2)}$$

(2.10)

where $R_{Sc}$ and $R_{Re}$ is the coherent reflectivity of the sample and reference arm respectively, $\xi$ is the degree of polarization (varies between zero and one) and $\tau_c$ is the coherence time of the source. Equation 2.10 shows that increasing the power of the source will not improve $SNR_{Ex}$ as it is not part of the equation. Using unpolarized light increases $SNR_{Ex}$ by a factor of two relative to polarized light. Using a broadband source will increase $SNR_{Ex}$ which is the scenario in any OCT system. Reducing the bandwidth of the receiver will increase $SNR_{Ex}$ as less white noise is contained in the detection band.

Balanced detection schemes can be used in OCT systems to reduce or eliminate common mode intensity noise from the source [2]. In this approach, the OCT signal is split into two paths by a beam splitter. The fringe signal is anti-phase while the intensity noise inherent in the source is still in phase. Subtracting these two signals from each other using two photodiodes doubles the photocurrent of the fringe signal reducing the intensity noise due to the source (self-beat terms) by the common mode rejection ratio (CMRR). However, analogous to the doubling of the fringe signal, any additional noises generated by incoherent beating between fields from sample or reference arm reflections are also doubled. After identifying $P_{Re}$ and $P_{Si}$ as the dominant cross-beat term the SNR after balanced detection is given by [110]
2.2. Principle of time-domain OCT

\[ SNR_{Bal} = \frac{R_{Sc}}{(R_{Sc} + R_{Si}) (1 + P^2 \tau_c R_c B)} \]  \hspace{1cm} (2.11)

where \( R_{Si} \) is the incoherent reflectivity of the sample arm. Comparison of SNR before and after the balanced detection (Equation 2.10 & 2.11) shows that while the \( R_{Rc} \) term in the denominator is reduced by a factor of \( 4CMRR \), a new term \( R_{Si} \) due to the dominance of cross-beat fields is introduced.

2.2.3.3 Thermal noise

Thermal noise or electrical noise is attributed to temperature-dependent random motion of electrons in a conducting medium. It approximates white Gaussian noise and is given by

\[ \sigma^2_{Th} = NEP \times \frac{B}{R_f} \]  \hspace{1cm} (2.12)

where \( NEP \) is the noise equivalent power per Hertz of the receiver which is usually given by the manufacturer and \( R_f \) is the transimpedance gain of the receiver. Assuming a 50/50 split ratio between the reference and sample arm, the associated \( SNR_{Th} \) is given as [110]

\[ SNR_{Th} = \frac{1}{2NEP \times B \rho^2 P_0^2 R_R R_S R_f} \]  \hspace{1cm} (2.13)

where \( e \) is the electronic charge, the term \( \rho \) is the responsivity of the detector and \( R_R \) is the reflectivity of the reference arm. As with intensity noise and shot noise, \( SNR_{Th} \) is also inversely proportional to the electrical bandwidth. \( SNR_{Th} \) can be improved by increasing the source power or using a photo-receiver with less \( NEP \).
2.2.4 Sensitivity

The minimum detectable reflectivity in an OCT system is given by the parameter $S$, also known as the sensitivity. It is commonly expressed in dB and defined as the ratio of the maximum power reflected back from the sample arm to the minimum detectable power equal to the noise of the system [45].

$$S = 10 \log_{10} \left( \frac{P_{\text{max}}}{P_{\text{NEP}}} \right) = 10 \log_{10} \left( \frac{1}{R_{S'}} \right)$$

(2.14)

where $P_{\text{max}}$ is the power reflected from a perfect mirror, $P_{\text{NEP}}$ is the noise equivalent power (NEP) of the system and $R_{S'}$ is the reflectivity of the sample such that SNR is unity. There are many different sources of noise in OCT systems. For a continuous-wave (CW) source under optimal configuration and performance, it is usually shot noise that limits the sensitivity. In a shot noise limited regime, the sensitivity $S$ is given by [45]

$$S = 10 \log_{10} \left( \frac{1}{4 \eta \frac{P_0}{h \nu B}} \right)$$

(2.15)

The sensitivity of OCT systems varies substantially reflecting different design and data acquisition speed requirements. Due mostly to thermal noise, typical sensitivities are in the range of 80-100 dB. However, in the shot noise limited regime sensitivities greater than 110 dB have been reported [36, 45].

2.2.5 Signal demodulation

There are many different methods of demodulating interference fringe signals, each with their own merits and drawbacks. The phase of the fringes contains information on sample scatterer velocity while the envelope is related to the refractive index contrast at different interfaces within the sample. Often when displaying OCT images, only the structural properties are of interest so the extraction of the envelope
is required. Here, we will briefly describe an electronic demodulation method and an analytic Hilbert transform demodulation method, both of which are commonly used.

2.2.5.1 Electronic signal demodulation using logarithmic amplifier

Demodulating fringe signals electronically has the inherent advantage of putting less stress on the computer for signal processing especially if real-time acquisition is required. Detected fringe signals are bandpass filtered at the carrier frequency to remove the DC component as well as any other sources of noise [16]. After bandpass filtration, the fringe signal is then mixed with either a reference signal identical to the carrier frequency with a $\pi$ phase shift (coherent demodulation) or is rectified to exhibit only positive voltages. This is a necessary condition for successful operation of a logarithmic amplifier. Compression of data dynamic range as well as envelope detection is achieved simultaneously through the use of a logarithmic amplifier. After removal of the carrier frequency, a final stage of low-pass filtering is performed before sampling the envelope signal with an analog to digital (A/D) card. The whole electronic demodulation process is illustrated in Figure 2.2. A constraint on this method is that the frequency content of the envelope and the fringes must be sufficiently well separated to enable effective removal of the carrier frequency. This method places a lower bound on the number of fringes within the envelope [110] and hence may not be suitable for ultra-broadband sources.

Figure 2.2: Electronic demodulation process of the OCT signal [110].
2.2.5.2 Analytic signal demodulation using Hilbert transform

Digital signal processing provides a robust means of demodulating OCT signals when analog methods are not suitable. The envelope of a signal $f(t)$ is $|f_a(t)|$, where $f_a(t)$ is defined as the analytic signal of $f(t)$. The analytic signal $f_a(t)$ is defined as twice the inverse Fourier transform of the positive frequency part of the Fourier transform of a real signal $f(t)$.

$$f_a(t) = 2 \int_0^\infty F(v) \exp(j2\pi vt) dv$$  \hspace{1cm} (2.16)

The Hilbert transform of $f(t)$ is defined as the imaginary part of the analytic signal $f_a(t)$ and is denoted by $\hat{f}(t)$. It can be shown that the real signal $f(t)$ is related to its analytic signal $f_a(t)$ by

$$f_a(t) = f(t) + j \hat{f}(t)$$  \hspace{1cm} (2.17)

The envelope of $f(t)$ is $|f_a(t)|$, thus

$$|f_a(t)| = \sqrt{f(t)^2 + \hat{f}(t)^2}$$  \hspace{1cm} (2.18)

This is a simple yet effective method of extracting the envelope of the fringe signals.

2.2.6 Supercontinuum sources

When intense light travels through a nonlinear dispersive medium such as silica, new frequency components can be generated through a cascade of nonlinear processes such as four-wave mixing (FWM) and Raman scattering. The output of such processes exhibits substantial spectral broadening of the pump, which is often known as a supercontinuum. Although supercontinuum can be generated by various
techniques, the largest bandwidths are currently achieved by launching a high peak power, pulsed laser into a photonic crystal fiber (PCF) [91, 154, 114, 116, 59].

2.2.7 Photonic crystal fibers

Photonic crystal fibers (PCFs) are a special class of optical fibers that were developed in the early 1990's [77]. Since then, there has been increasing interest in the optical properties of PCFs and their applications. PCFs can be divided into two categories, namely index-guiding PCFs and photonic bandgap PCFs. The first type guides light by means of total internal reflection as in conventional fibers, while the second type guides light by the photonic bandgap effect. In this thesis we consider only index guiding PCFs. PCFs consist of a silica core surrounded by a periodic arrangement of air holes held in place by fine silica struts running through its longitudinal axis. PCFs can be fabricated from a single material and this allows the design of a wide range of refractive index profiles without introducing problems of material compatibility [78] such as doping. The large contrast in refractive index between core and cladding minimizes leakage of light into the cladding. This means that light can be confined to a much smaller core area in PCFs than in conventional SMFs, resulting in high nonlinearity which is fundamental to the generation of a supercontinuum. Another novel feature of PCFs is that their zero dispersion wavelengths (ZDW) can be shifted away from the natural position of bulk silica at 1272.6 nm due to the large contribution of dispersion from the geometry of the waveguide itself [103, 117]. The capacity to select a PCF that has a ZDW at almost any wavelength makes them very useful indeed in nonlinear optics.
2.3 System configuration

A prototype OCT system was set up during a five week period spent as a PhD intern in the Optical and Biomedical Engineering Laboratory (OBEL), School of Electrical and Electronic Engineering, University of Western Australia. This laboratory has established expertise in the development of novel OCT systems. However, the short period spent in Perth meant that it was necessary to use equipment available in the laboratory since there was insufficient time to source and purchase new instrument.

Design considerations and specifications were as follows. We opted to construct an OCT system based on a bulk Michelson interferometer design working in the time domain. In comparison with frequency domain implementations, this approach is cheap and easy to set up. In order to achieve the best axial resolution, we chose to use a supercontinuum source. Our pulse-synchronized OCT system schematic setup is shown in Figure 2.3.

![Figure 2.3: Pulse-synchronized OCT system schematic diagram.](image)

The supercontinuum light source was generated by a pulsed laser and a PCF. The output light was collimated via an objective lens and a bandstop filter was inserted to block residual pump frequencies. Axial scanning was performed by 1-
D translation of a retroreflector mounted on a piezoelectric transducer stage and driven by a triangular wave from a function generator. 1-D transverse scanning was achieved by mounting specimens on a translation stage, which was driven by a DC high resolution linear actuator. A balanced detection scheme was used to minimize common mode noise. Since the light source is pulsed and pulse-to-pulse interval can vary by up to 50%, it was necessary to synchronize signal acquisition. This was done, by bleeding some of the output of the supercontinuum with a beamsplitter prior to the interferometer, detecting pulses using a fast photodiode and triggering the sampling after an appropriate time delay. The system was controlled with a laboratory PC equipped with a standard A/D interface. The computer (1) triggered the axial scan generator (2) drove the transverse scan motor, and (3) acquired the output of the balanced detector and the position of the axial scanning translation stage. As shown in the diagram, the two beam splitter cubes were slightly tilted as reflections from their facets lower SNR.

2.3.1 Supercontinuum source

Our supercontinuum source (see Figure 2.4) is consisted of a pulsed Yag laser and a piece of PCF around 20m long. The laser emits high peak power (pulses < 1 ns) in the order of 6 kilowatts at the wavelength of 1064 nm. The output from the PCF was collimated with an objective lens (60X NA=0.8, Olympus).

Short pulse widths limit the nonlinear interaction time between the pump pulse and newly generated spectral components as different wavelengths of light propagate at different group velocities due to dispersion of the fiber. This phenomenon is referred to as the walk-off effect. To maximize the efficiency of supercontinuum generation, the laser is injected to the PCF at the ZDW thus reducing the walk-off effect between different frequency components as much as possible [23]. In addition, a half-wave plate is placed in front of the PCF to rotate the polarization of the
laser to align it with either the slow or the fast axis of the birefringent fiber for best coupling efficiency [154, 23].

The spectra obtained from such a setup when the pump is completely depleted is shown in Figure 2.5. The peak wavelength is at 630 nm and the FWHM is 460 nm. The walk-off effect (see Figure 2.6) could be the reason for the sharp cut-off at 460 nm. Moreover, Figure 2.8 shows that at short wavelengths the fiber becomes multimode thus lowering the peak power of the pulse in the fundamental mode. These effects will be explained in detail in the next section.

Figure 2.5: Spectra of the supercontinuum after complete depletion of the pump at 1064 nm.
2.3. System configuration

2.3.1.1 Laser

The laser used in our OCT setup is a diode-pumped solid state Cd doped Yag laser at 1064 nm (NP-10620-x00, Teem Photonics) with output pulses at a repetition rate of 7 kHz and with a peak power 6 kW. The pulse energy is at more than 6 µJ with a pulse width of less than 1 ns. This repetition rate inherently limits the sampling rate of the OCT scan, while the pulse duration limits the length of the PCF that can be used. Figure 2.6 illustrates the walk-off effect between different frequency components of the supercontinuum with respect to the pump. The walk-off between the center pulse at 630 nm and a violet pulse at 460 nm is computed at 70 ps/m. Thus, using a piece of fiber longer than 20 m to increase the bandwidth of the supercontinuum in the short wavelength range is redundant as there are no nonlinear interactions between the center pulse and the newly generated high frequency components since they are more than 1.4 ns apart.

![Figure 2.6: Computed walk-off for our PCF at the pump wavelength of 1064 nm. The core diameter used in this graph is 5 µm with an air-filling fraction of 0.2 in the cladding (computed from Figure 2.7).](image-url)
2.3.1.2 Photonic crystal fiber

The PCF used in this experiment consists of a circular silica strand enclosed by a cladding of symmetrically micro-structured air capillaries supported by silica struts running continuously along its entire length. It can be viewed as a solid central core surrounded by a lattice of circular air holes. This together with its refractive index profile and scanning electron micrograph (SEM) is shown in Figure 2.7.

![Figure 2.7: Schematic illustrations of the (A) cross section, (B) refractive index profile of a photonic crystal fiber, and (C) a SEM image of the cross section of the PCF (SC-5.0-1040) used [117].](image)

The parameters associated with PCFs are the diameter \(d\) of the air-holes and the pitch \(\Lambda\), which is the spacing between the centers of adjacent air-holes. The refractive index of the cladding can be viewed as the effective index \(n_{\text{eff}}\) as a result of the combination of struts and air holes in the cladding [78]. Since the core can be seen as a defect air hole, the pitch \(\Lambda\) is widely used instead of the core radius [79]. In analogy with step-index fibers (SIFs), the number of guided modes in PCFs can also be determined by the \(V\)-parameter [11]. It is a parameter known as the normalized frequency \(V\) which determines the number of confined modes within the core of the fiber at any particular wavelength. For PCFs the \(V\)-parameter is given by [11]

\[
V_{\text{PCF}}(\lambda) = \frac{2\pi\Lambda}{\lambda} \sqrt{n_{\text{core}}^2(\lambda) - n_{\text{eff}}^2(\lambda)}
\]

Where \(n_{\text{core}}^2(\lambda)\) is the core index of the fundamental mode. Similarly, \(n_{\text{eff}}^2(\lambda)\) is
the effective index seen by the fundamental mode at the cladding. The second order modal cut-off normalized frequency $V_{co}$ is at $V = 2.405$ for SIFs [155]. Recently, it has been shown that $V_{co}$ for PCFs is at $\pi$ [105].

The core of our PCF is 4.8 µm in diameter with a zero-dispersion wavelength (ZDW) at 1040 nm, and a cut-off wavelength at 1000 nm (SC-5.0-1040, Crystal Fiber). As mentioned earlier, in PCFs, the dispersion profile can easily be tailored via the geometry of the waveguide. Figure 2.8 shows that a PCF can be endlessly single mode at any given wavelength as it is only dependent on the $d/\Lambda$ ratio [104]. In terms of supercontinuum generation, it is desirable for a PCF to be endlessly single mode as any occurrence of higher order modes lowers the power in the fundamental mode required for nonlinear interactions. However doing so may shift the ZDW away from the pump thus careful design of the fiber dispersion should be taken.

Figure 2.8: Diagram illustrating "phases" with singlemode and multimode operation. The solid line separating the single mode from multimode corresponds to $V_{PCF} = \pi$. According to the SEM image, the $d/\Lambda$ value lies close to 0.49 as shown by the thin dotted line. The phase change between single and multi-mode regime on the graph agrees with the manufacturer’s cut-off wavelength at 1000 nm [104].

### 2.3.1.3 Performance of supercontinuum

Problems were uncovered with the performance of the supercontinuum assembled and steps were taken to address the most pressing of these issues. Incomplete depl-
tion of the pump possibly caused by insufficient pump power have exhibited significant residual pump components in the PCF output (see Figure 2.9). The residual pump frequencies introduce laser line fringes which negates the use of the supercontinuum as a broad bandwidth OCT source. A compromise was made by inserting a band-pass filter (850-1150 nm). This further reduces power, but removes residual pump frequencies. The average power after the band-pass filter was measured as 2 mW. Using the walk-off curve in Figure 2.6, the pulse duration after dispersion broadening was estimated to be 1-2 ns which corresponds to a peak power of around 190 W. A further problem was that the repetition rate of the laser was unstable and varied in the range of 6-9 kHz. This was overcome by detecting pulses and synchronizing acquisition with them (see Sections 2.3.4 & 2.3.5).

![Figure 2.9: Comparison of the supercontinuum with and without the bandpass filter (850-1150 nm) inserted.](image)

### 2.3.2 Reference arm

The reference arm directs light to the reference mirror via a corner cube retroreflector and a microscope objective (UPLSAPO 10X/0.4 inf/0.17/FN26.5, Olympus). Axial scanning (see Figure 2.3) was performed by displacement of the retroreflector, which was mounted on a piezoelectric transducer (PZT) stage (P625.1, Physik
2.3. System configuration

Instrumente). The PZT stage was driven by a triangle wave signal from a function generator (33250A, Agilent Technologies) and operated in closed-loop mode to remove the hysteresis intrinsic to piezo actuators. The axial scan period was around 4 seconds and the PZT driver outputs a position voltage from a capacitor built into the PZT stage. This signal was acquired at the same time as the OCT output.

2.3.3 Sample arm

In the sample arm, light was directed at the sample via a microscope objective matching that in the reference arm (UPLSAPO 10X/0.4 inf/0.17/FN26.5, Olympus). The sample was placed on a mounted stage where 1-D transverse scanning was performed. It was driven by a linear DC motor (M-511.HD, Physik Instrumente).

2.3.4 Detector

A balanced detection scheme was chosen as the intensity noise of the source can be greatly reduced by cancellation of the common mode noise in the two receiving arms. The light exiting both facets of the cube beamsplitter was directed to a free-space large-area silicon photodiode balanced receiver (2307, New Focus). A variable attenuator was inserted into the receiving arm with higher power and set to a position such that the average output of the balanced detector in the absence of OCT fringes was zero. The balanced detector used was the only such device readily available, but was poorly matched to the requirements of a pulsed OCT system. While a large area photodiode is easy to align, its rise time is compromised. For this device, the rise time was 500 ns, while FWHM of the detected pulse was two orders of magnitude shorter than this. This is shown in Figure 2.10 where the response
of the balanced detector to a laser pulse (duration ~1 ns, onset indicated by purple trace) is presented. Detector output (orange trace) slowly rises after a fixed time delay to reach a maximum 520 ns later. Acquisition was synchronized with this delay to maximize signal output (see next section). It is evident that better matching of the rise time of the detector to the pulse duration of the pump would markedly improve detection sensitivity.

There are three gain/bandwidth (BW) settings. The LOW (gain $2 \times 10^3 \text{ V/A}$, BW 1 MHz), the MEDIUM (gain $10^5 \text{ V/A}$, BW 200 kHz) and HIGH (gain $2 \times 10^6 \text{ V/A}$, BW 150 kHz). Depending on the sample, the medium and high gain setting were mainly used. The common mode rejection ratio (CMRR) of the detector was quoted at 40 dB by the manufacturer.

![Figure 2.10: Pulse synchronized detection scheme. Scale: Purple trace: Fast photodiode. Green trace: Pulse generator. Yellow trace: Balanced receiver.](image)

2.3.5 Data acquisition

The system was controlled and data was acquired using a data acquisition card (NI 6115, National Instruments) connected to the PCI bus of a laboratory PC. This data acquisition (DAQ) card has a 12-bit A/D converter with four simultaneously sampling analog inputs (10 MS/s/channel). It also has two 12-bit D/A converters
and eight lines of TTL-compatible digital I/O. Acquisition and control programs were written in the Labview programming language.

Data acquisition was synchronized with pulse timing as shown in Figure 2.10 above. Part of the incoming light was reflected by an initial cube beamsplitter to a fast silicon photodiode (1601, New Focus). The oscillations at the rising edge of the photodiode (purple trace) were probably due to electrical ringing rather than different propagating modes as the walk-off between the modes cannot be this great. The trigger was produced with a digital pulse generator (HP8112A, Agilent Technologies) and delayed by around 520 ns so that the peak of the balanced interferometric signal was sampled by the DAQ card. The position output of the axial scanning PZT stage was acquired at the same time.

2.3.6 Frame rate

In our system, acquisition rate was the limiting factor as sampling was synchronized with the source pulses which had a repetition rate that varied between 6 to 9 kHz. As analog circuit envelope detection is not possible when sampling individual pulses, the full fringe information must be fully sampled. For reasonable extraction of the envelope we must capture say 10 samples per fringe. The maximum axial scan rate \( f_{A\text{-scan}} \) is then given by

\[
f_{A\text{-scan}} = \frac{\bar{\lambda}}{4} \frac{f_{\text{pulse-rep}}}{10 L_{\text{scan}}} \tag{2.20}
\]

The optical path difference is four times the displacement of the PZT. If we assume the mean wavelength \( \bar{\lambda} \) is 700 nm (see Figure 2.5 & 2.9) then for a scanning length \( L_{\text{scan}} \) of 500 \( \mu \)m (in air), with pulse repetition rate \( f_{\text{pulse-rep}} \) at 7 kHz, the axial scan rate is 0.245 Hz. To capture a frame of 512 axial scans this requires 2090 seconds or 35 minutes.
2.4 System performance

2.4.1 Resolution

2.4.1.1 Axial resolution

The axial resolution in air was experimentally verified by measuring the displacement of the FWHM of the coherence function (Equation 2.5). The steps in this process are illustrated in Figure 2.11. The FWHM was estimated as 1.54 µm from the envelope of fringes by performing Hilbert transformation (Equation 2.18). There were 4.5 fringes within the FWHM which corresponds to a center wavelength of 684 nm (Equation 2.4). This agrees with the spectra shown in Figure 2.5 & 2.9. Moreover the side-lobes in Figure 2.11 were minimal, unlike some other supercontinuum sources which exhibit extensive coherence function side lobes that preclude their use in OCT [124]. This demonstrates that the supercontinuum source used in our prototype OCT system is capable of providing very high spatial resolution. Dispersion mismatch between sample and reference arms can blur the coherence function and at the limit this may be a more important constraint on axial resolution than source bandwidth [35]. In addition, dispersion mismatch is more critical in the visible region than the infra-red where the zero dispersion of water is located.

![Figure 2.11: Experimentally measured coherence function of the OCT system. The envelope is calculated by performing the Hilbert Transformation (Equation 2.18).](image)
2.4. System performance

2.4.1.2 Transverse resolution

The transverse resolution is determined by the microscope objectives used in the reference and sample arms\(^1\). If we assume a beam size of 3 mm then the corresponding NA is 0.17 for the given sample objective and therefore the resolution is approximately 5 µm (Equation 2.6). However, we must point out that this is an estimate only, as the transverse resolution was not experimentally measured. Given a 5 µm transverse resolution the depth of field is calculated at 57 µm (Equation 2.7). Again, this was not verified experimentally.

2.4.2 Sensitivity

2.4.2.1 Theoretical limit

The sensitivity of OCT systems depends on the noise sources as described earlier in Section 2.2.3. To find the sensitivity in each noise regime, we set the SNR in Equations 2.9, 2.10, 2.11, and 2.13 to unity and solve for the coherent reflectivity of the sample arm \(R_{Sc}\).

![Figure 2.12: Sensitivity S VS Reference reflectivity \(R_{Re}\) for various noise regimes. Left: \(P_0 = 100\ W\). Right: \(P_0 = 10\ mW\). Dashed line represents the value of the \(R_{Re}\) we were operating at. Parameters: \(R_f = 2\times10^6\ \text{V/A}, \ R_{Si} = -40\ \text{dB}, \ \lambda = 650\ \text{nm}, \ \Delta\lambda = 300\ \text{nm}, \ \text{NEP} = 3.3\ pW/\sqrt{\text{Hz}}, \ B = 700\ \text{Hz}, \ \rho = 0.6\ \text{A/W}, \ CMRR = 40\ \text{dB}, \ P = 1\).](image)

\(^1\)Since Olympus reference tube length = 180 mm & Focal length = Ref tube length / Mag, thus Focal length=18mm.
Figure 2.12 shows sensitivity $S$ in different regimes plotted as a function of the coherent reflectivity of the reference arm $R_{Re}$. The dashed line at -14 dB represents the $R_{Re}$ used in our system. Left and right panels compare the effects when a pump power of 100 W (typical of pulsed OCT systems such as ours) with a pump power of 10 mW (typical of a CW OCT system). All the parameters used are actual values obtained either from estimates, experiments, or quoted by manufacturers. The main difference between the graphs is that the thermal noise of the receiver is the factor that limits sensitivity with a source power of 10 mW. On the other hand, at 100 W, intensity noise is the limiting factor. In both cases, when balanced detection is employed the dominant self-beat terms of the intensity noise were drastically reduced by roughly the CMRR leaving only cross-beat terms. This shows that sensitivity can be greatly improved via the use of balanced detection when intensity noise is the limiting factor. Moreover, pulsed OCT systems exhibits very high sensitivity (greater than 150 dB) in a shot noise or thermal noise regime because the peak power is high, a result of the short pulses.

It should be noted that the actual sensitivity achieved with our prototype OCT system was significantly less than the theoretical limits given here as a result of the slow rise time of our balanced detector. This results in lower detector output relative to the high peak power delivered than would be achieved with a “fast” detector.

However, this does not alter the outcome of this analysis. For a pulsed OCT system where the source is strong, sensitivity is limited by the intensity noise. That is self-beat noise in a single ended detection scheme or cross-beat noise in a balanced detection scheme.

### 2.4.2.2 Measured sensitivity

The sensitivity of our OCT system was measured at different gain settings (Low, Medium, and High) of the balanced detector. As expected, sensitivity was greatest at high gain. We calibrated the system by placing a neutral density filter that
introduced 30 dB of attenuation in front of the sample mirror and then recorded the interferogram. With this as a reference, we removed the filter and attenuated the signal until it was comparable to the noise floor by adjusting the angle of the sample mirror to make it out of focus. Figure 2.13 shows the results of these steps. The sensitivity can be determined by comparing the SNR measured in these two cases using Equation 2.14. On this basis, we calculated a sensitivity of 98 dB with High gain. This is very high compared to most other OCT systems thanks to its high peak power which greatly improves the SNR of shot noise or thermal noise limited regime.

![Figure 2.13: Left: Filtered interference fringes measured on High Gain by placing a 30 dB neutral density filter in front of the sample arm mirror. Right: The mirror was adjusted out of focus to make the filtered signal comparable to noise.](image)

### 2.4.3 Signal demodulation & image reconstruction

Signal demodulation was performed via software (Labview) using the Hilbert transform (Equation 2.18). There was a significant problem with jitter in the axial scan which arises from the irregular arrival times of the individual source pulses. For this reason post-experiment correction of the image was required. This is achieved by replacing the position signal of the axial scan (triangle wave) with a fitted line segment, then re-sampling to find the corresponding interpolated envelope signals.
CHAPTER 2. Time-domain OCT

Figure 2.14 shows an image of 5 layers of 3M Scotch sticky tape with no transverse scanning. The left and the right panels compare the image before and after jitter correction respectively. The five lined up layers of the sticky tape demonstrates that sampling jitter was removed effectively, thus ensuring that any 2-D images taken would be an accurate representation of the sample. Although our jitter correction was performed post-experiment via signal processing, at this low sampling rate it should be possible to correct on the fly.

![Figure 2.14: The sample used was 5 layers of 3M Scotch sticky tape. Left: Image with no transverse scanning showing the effect of jittering due to irregular sampling rate. Right: Jitter corrected image after the re-sampling of the position signal to match the interpolated axial scan.](image)

2.5 Results

2-D images of specimens including slices of onion and a sardine fish eye were acquired. Because of the long acquisition time, these specimens dehydrated during the imaging process, reducing quality.
2.5. Results

2.5.1 Onion cells

Figure 2.15 shows an image of onion cells acquired with our OCT system. While pleomorphic mesenchymal cells in the onion close to the surface were easily identified, image reconstruction of structures deeper than 200 µm into the sample was not possible.

2.5.2 Fish eye

The image of a fish eye was taken with the orientation at the center where the cornea lies. Figure 2.16 compares the image acquired with our OCT system to a conventional system which uses a superluminescent diode (SLD) as the source. The image acquired with our system showed significantly more detailed structures such as the layered features found immediately within the air-sample interface.
CHAPTER 2. Time-domain OCT

Figure 2.16: Image of a sardine fish eye (cornea). Top: Acquired with our supercontinuum OCT system at an axial resolution of 1.54 µm. Bottom: Acquired with a conventional SLD OCT system [76] at an axial resolution of 20 µm.

2.6 Discussion

The purpose of the work described in this chapter was to develop a prototype broadband OCT system using a supercontinuum and to establish key design criteria for the components. Because we were required to use existing equipment to establish the feasibility of our OCT system there are a number of features that could be refined.
2.6. Discussion

2.6.1 Sampling rate

The most critical drawback of our system is the low repetition rate of the laser which leads to a low sampling rate. Ti-Sapphire lasers are the most obvious candidate for a replacement laser. These lasers have very high peak power and also have repetition rates starting from MHz to the low GHz. Most importantly, they are tunable within the infra-red range from 650 to 1100 nm thanks to their large gain bandwidth. This would enable the PCF to be pumped exactly at the ZDW for a wide variety of fibers. Even if one were to operate at a relatively low repetition rate of 7 MHz this would represent a thousand folds increase, in the sampling rate compared to our system. Moreover, sampling jitter would be avoided as these lasers are mode-locked and not Q-switched like our microchip laser thus enabling a very stable pulse repetition rate.

2.6.2 Image quality

In our images of biological structures, we were unable to penetrate more than 200 µm into the sample. This was most likely due to the limited depth of field, estimated at 57 µm. Solutions to this problem include using an objective lens with lower magnification, but this would reduce transverse resolution. An alternative approach would be to move the sample mirror together with the reference mirror during axial scanning such that their optical path difference is always zero. This procedure maximizes the signal reflecting back from the sample at any given depth as it is always exactly at the coherence gate.

2.6.3 Sensitivity

Our current sensitivity limit is probably due to the thermal noise of the balanced receiver. As mentioned earlier, the rise time of our current receiver is 500 ns which means that the advantage of having a high peak power was not fully utilized. A
fast photodiode with a rise time of 100 ps should be sufficient to fully resolve the
detector output, thus changing the sensitivity limiting factor to cross-beat intensity
noise (see Figure 2.12).

2.6.4 Power efficiency

From the schematic diagram of our OCT system (see Figure 2.3), it is clear that
the source power is not efficiently used. A more efficient use of the power could
be obtained by replacing the first beamsplitter with a polarizing beam splitter and
placing a quarter-wave plate after it [93]. This would ensure that no light was
wasted. That is 100% efficiency from the double pass (incident & return light) of
the beam splitter as opposed to 25% efficiency.

2.6.5 Dispersion

Last but not least, dispersion becomes more of a critical factor when the bandwidth
of the source is broad. This is because any dispersion mismatch between the reference
and sample arms leads to a lengthening of the coherence function which is now
much more comparable to the nominal axial resolution. For this reason, dispersion
mismatch lowers axial resolution (ie more fringes within FWHM of the coherence
function) thus degrading the image quality. When dispersion is matched by using
a mirror as the sample (see Figure 2.11) the resolution is equivalent to 4.5 fringes.
When dispersion is not matched as with a tissue-air interface (see Figure 2.17),
the resolution degrades to the equivalence of 7 fringes. Moreover, dispersion mis-
match increases with increasing axial penetration of the sample. There are various
dispersion compensation techniques [137, 83, 150, 152] that can reduce dispersion
mismatch such as insertion of a prism pair, a piece of glass plate, or a grating in
the reference arm. The disadvantage of these techniques is that they are usually
2.7 All-fiber design

difficult to implement into the setup, and may not fully compensate dispersion. Numerical techniques have also been used to compensate for dispersion mismatch without any modification to the existing setup [92, 44, 97]. The disadvantage of numerical techniques is that they typically require intensive computation which may not be suitable for real-time imaging.

Figure 2.17: The coherence function of the OCT system at the air-onion interface. Compared to air-mirror interface Figure 2.11, the extra 2.5 fringes comes from mismatch of the dispersion.

2.7 All-fiber design

All-fiber probes offer a number of advantages in a combined fluorescent OCT system, in that they are robust, compact and minimize the need for alignment. The Mach–Zehnder interferometer configuration as shown in Figure 2.18 is an excellent candidate for such application, because it conserves pump power [119]. We have designed and built an all-fiber OCT system on this basis. A CW SLD with a center wavelength of 840 nm and a bandwidth of 40 nm (IPSDD0803, Inphenix) was used as our source. The first coupler splits the pump into the reference and sample arm. The sample arm has a lensed fiber tip which focuses the light onto the sample. The delay line of the reference arm was comprised of fibers wrapped around a PZT (P-840.60, Physik Instrumente) which stretches the fiber when a voltage is applied to it creating an optical path difference with respect to the sample arm. The polar-
ization controller (FPC560, Thorlabs) in the reference arm was used to match its polarization to the sample arm thus giving maximum fringe signal. Although the second fiber coupler in the sample arm results in a power efficiency of only $25\%$, it can be boosted to $100\%$ if replaced with a circulator. However, because the performance of a circulator is wavelength dependent, this could render it ineffective when an ultra-broadband source such as supercontinuum is used. The mechanical fiber stretcher on either side of the two arm ensures their optical path lengths were easily matched. Light from both arms recombines at the third coupler where interference occurs and the two output ports goes to a balanced receiver (PDB120A, Thorlabs) for data acquisition.

As this design is only a prototype, dispersion compensation methods have not been implemented yet. However, it will be easy to insert some kind of dispersion compensation components in future thanks to the all-fiber design.

Figure 2.18: Schematic diagram of a prototype all-fiber OCT setup.

The OCT part of the thesis was taken over by another PhD student. Since then the setup has evolved with the replacement of the source to another SLD at a center wavelength of 870 nm and a bandwidth of 180 nm (T870, Superlum). Dispersion compensation was achieved by splicing a piece of PCF into one of the mechanical fiber stretchers. The dispersion properties of PCFs and normal single-

\footnote{Assuming no insertion loss}
mode fibers are drastically different thus maintaining a zero optical path difference between the two arms while scanning through different optical path lengths, the zero dispersion length can be found. In practice after the zero path difference is found, dispersion imbalance can be fine tuned to zero by stretching both fiber stretchers simultaneously. Detailed description of the technique can be found at Iyer et al [71].

2.8 Conclusion

We have demonstrated the use of a supercontinuum source in an OCT system to achieve ultra-high resolution. Thanks to the high peak power of the source, sensitivity of 98 dB was achieved. Our short wavelength (visible range) OCT system could complement other OCT systems whose sources are almost exclusively in the infrared. The major drawback of this system lies in the low sampling rate due to the low repetition rate of the laser. This can only be improved by the use of alternative lasers.

This has led to the development of an all-fiber OCT system that used the design requirements of combined fluorescence-OCT probes.
Chapter 3

In Situ spectroscopy techniques for monitoring bioremediation

3.1 Introduction

Soil contamination from pollutants such as Polycyclic Aromatic Hydrocarbons (PAHs) due to oil spills poses a mounting threat to global environments. Increasingly, bioremediation of such pollutants is being explored as a treatment approach and this is also the subject of our research. Bioremediation is the process of introducing micro-organisms to contaminated environments which accelerates natural degradation processes [27]. In order to employ this approach more optimally, it is necessary to develop techniques that will enable bioremediation processes to be better understood and monitored over time. The research described in this chapter investigates the feasibility of using a novel spectroscopy technique to monitor the effectiveness of bioremediation in situ.
3.1.1 Bioremediation

Bioremediation is a restoration process whereby pollutants are biologically degraded by microorganisms to remediate the environment. Although biodegradation of pollutants occurs naturally in a wide range of environments, the process can be accelerated with the aid of bioremediation. Bioremediation was reportedly invented by George M. Robinson during the 1960s. He demonstrated that petroleum contamination was reversed by various mixes of microbes [95]. The conventional approach to restoring polluted environments is to remove contaminated soil but bioremediation can be used in situ in areas that are inaccessible to excavation. Typically this approach is less expensive, site disruption is minimal, waste is eliminated permanently and it has greater public acceptance. However, bioremediation technology is not without its limitations as some chemicals are not amenable to biodegradation, for instance, heavy metals, radionuclides and some chlorinated compounds. In some cases, microbial metabolism of contaminants may produce toxic metabolites. Bioremediation requires extensive scientific background research and preparation as it must be tailored to site-specific conditions. This often means that treatability studies must be carried out on a small scale before the actual clean-up of the sites [12].

3.1.2 Bacterial degradation of Polycyclic Aromatic Hydrocarbons

Polycyclic Aromatic Hydrocarbons (PAHs) are a major component of global anthropogenic soil pollution that increasingly poses a threat to the quality of global landscapes and the sustainability of resources [24, 122]. PAHs are produced in a wide variety of ways, but their major source is fuel combustion and industrial processes [58, 147]. Due to their highly stable, recalcitrant hydrophobic chemical properties they accumulate in soil, compromising the quality of environments and living systems. PAHs pose serious risks to human health as they are carcinogenic,
3.1 Introduction

Some microorganisms have the genetic capability to feed off PAHs as a carbon source, facilitating their degradation into non-toxic end products [109, 94]. This makes PAH contamination an ideal candidate for bioremediation. In order to develop effective systems for bioremediation, it is necessary to characterize the way in which bacterial species interact with PAHs. For the purposes of this research, the catabolism of naphthalene by bacterial species will be investigated. Naphthalene is the most readily degraded of the PAHs and a wide range of bacteria have the genetic capability to produce enzymes which can readily break down this compound [139].

3.1.3 Conventional technique for monitoring bioremediation

The main objectives in any bioremediation technique are to enhance microbial growth, biomass and metabolism for faster biodegradation of the contaminated sites [82]. There are many examples, such as landfills, hazardous wastes, diesel and petroleum spills, where bioremediation has been successfully employed to degrade recalcitrant compounds [3]. To achieve successful bioremediation outcomes, it is necessary to monitor the biological activity and survival of the introduced bacteria as this provides feedback on the effectiveness of the technique. However, acquisition of this information is currently hindered by the lack of reliable in situ monitoring methods [29]. At present bioremediation is most commonly monitored indirectly by measuring the Oxidation Reduction Potential (Redox) in soil and groundwater, together with pH, temperature, oxygen content, etc. Redox potential measurements that indicate depletion of electron acceptors are an indication of active microbial activity. Chemical analysis is required to determine if the levels of contaminants have been reduced to below regulatory limits and it is also necessary to sample adequate points at the contaminated site to obtain a contour of the bioremediation results [95].

Conventional methods do not provide reliable measures of both bacterial growth
and degradation, and they do not readily translate to the laboratory. This limits their use for optimization of bioremediation processes.

3.1.4 Novel spectroscopic technique for detecting biosensors

As mentioned above, current methods for monitoring the survival and activity of microorganisms are limited. Here, we outline a novel in situ method in which the degrader bacterial species is tagged with fluorescent proteins. Excitation with light at an appropriate wavelength will therefore stimulate fluorescent emission with intensity proportional to the concentration of the tagged bacteria. The schematic of a fiber optic system that could be used for this purpose is illustrated in Figure 3.1. Excitation light is launched into a fiber coupler. One of the output arm of the fiber coupler is inserted into the soil or soil sample providing the conduit for delivering excitation light and collecting fluorescence emission. The other output arm can be used to monitor the excitation power. The collected light is then guided to a spectrometer via the fiber coupler. A similar method has been demonstrated with relative success using optical filters and a photomultiplier (PMT) tube instead of a spectroscopic configuration [135].

Since we do not know whether the tagging of bacterial species with fluorescent proteins affects the function of the bacteria, it is important to address this issue in a scientifically rigorous manner. For instance, a study which also used the same bacterial species as us (P. Putida) have shown that there is no significant difference in the parameter of their interest between green fluorescent protein (GFP) and red fluorescent protein (RFP) labeled strains of the same species or between wild-type and labeled strains [49]. Our method will only become viable if we can also show similar results. This is discussed under the future work section of this chapter.
3.1 Introduction

Figure 3.1: Simplified schematic of the fiber probe setup

3.1.5 Selection of fluorescent proteins

Each GFP variants are derived from naturally occurring fluorescent proteins and can be delivered into target organisms using viral or plasmid vectors. They have distinct excitation and emission spectra as shown in Figure 3.2. Although the efficiency of the emission spectra is decreased as the excitation wavelengths moves away from the peak, the spectral characteristics are largely preserved [70]. The left panel of Figure 3.2 also shows that excitation with a 473 nm laser will excite all three fluorophores (eGFP, eYFP and DsRed) while a 532 nm laser is suitable only for eYFP and DsRed.

Figure 3.2: Excitation and emission spectra of BD Living Colors™ Aequorea victoria GFP variants. The vertical blue and green lines on the excitation plot represent 473 and 532 nm laser excitation [10].

Tagging bacterial species with multiple fluorophores can offer further insight into the bioremediation process. For instance, the bacteria can have a constitutive GFP gene to monitor the growth and a RFP gene linked to degradation to monitor the activity. This would enable bacterial population and the effectiveness of the
degradation process to be monitored simultaneously, provided that the spectra can be resolved into their spectral components. Biosensor technology therefore offers a number of advantages for gathering reliable information relating to bioremediation.

One of the most obvious criteria for resolving multiple spectra is that the selected fluorescent proteins must have discernable peak emission maximums. As shown in the right panel of Figure 3.2, \textit{eGfp} (enhanced GFP) and \textit{eYfp} have very closely related emission peaks and characteristics suggesting they are not ideal candidates for use in a mixed fluorescent protein sample. For this reason, \textit{eGFP} and \textit{DsRed} are well suited for taking simultaneous measurement in a sample as their peaks are well separated.

### 3.1.6 Bacterial growth curve

For batch-cultured bacteria, the time-course of population growth is shown in Figure 3.3 Bacterial growth occurs in four distinct stages: lag phase (A), exponential phase (B), stationary phase (C), and death phase (D). In the lag phase, growth is slow at first while the bacteria acclimate to the nutrients in their new habitat. In the exponential phase, their population increases rapidly as a result of metabolic adaptation. In the stationary phase, bacterial population stabilizes as a result of competition for dwindling nutrients and in the death phase, bacteria die out because of the build up of toxic waste and depletion of nutrients.
3.2 Optical setup

A dedicated fluorescence system for monitoring bioremediation was designed and constructed as shown in Figure 3.4. The system was developed with the focus on portability, ease of use, robustness, and minimal maintenance. The entire system was mounted inside a regular aluminum 19” rack style light-proof enclosure with a dimension of 483 mm × 335 mm × 128 mm. Once the system had been aligned during construction, the only adjustment was to manually select the appropriate emission filters to suppress the excitation light. The system was controlled by a computer via two USB connections, one to the spectrometer and the other to the data acquisition card (DAQ). The input/output of the system used standard SMA and FC/PC connections so that it could be readily interfaced with fiber optic components.
Figure 3.4: Schematic of “fluorescence spectroscopy system” for monitoring bioremediation. The major components consist of two diode pumped solid state (DPSS) lasers at 473 and 532 nm, two multimode fiber (MMF) coupler, one spectrometer, one data acquisition card (DAQ) and one photodiode.

Below we describe each component utilized in the setup in detail.

3.2.1 Laser Sources

Diode pumped solid state (DPSS) lasers were used as the excitation source as they present high output power and are cost effective as well as stable. A 473nm blue laser (100 mW, power stability 5%, noise < 3%, CNI Tech.) and a 532nm green laser (100 mW, power stability 1%, noise < 0.5%, CNI Tech.) were built into the system, and these two wavelengths provide adequate excitation for fluorophores within the visible region.

3.2.2 Fiber

Optical fibers were chosen as the means of light delivery within the system as they are highly stable, alignment-free, and compact. The fiber used (CF04406-03, OFS) has an all silica pyrocoat, high OH− and multimode properties. The high OH−
3.2 Optical setup

Concentration results in efficient power transmission in the ultraviolet (UV) through to the visible range. The fiber core, cladding and coating diameters are 200, 220 and 250 μm diameter respectively with a numerical aperture (NA) of 0.22. Attenuation across the visible range is less than 14 dB/km (quoted by the manufacturer), and the fiber length used in the entire setup was less than 10m making loss of power negligible during delivery.

3.2.3 Spectrometer

A scientific-grade 16-bit spectrometer (QE65000, Oceanoptics) with thermal electric cooling (-15 °C) was used for this low-light level application. The detection unit is a 2-dimensional charge-coupled device (CCD) array (sensitivity = 0.065 counts/e⁻), this makes use of the width of the spectrum which is spread over the height of the CCD pixels. The quantum efficiency is greater than 80% over the wavelength range we were interested in. The dark noise of the spectrometer is 3 root mean square (RMS) counts. There is no slit on the spectrometer because the NA of the fiber acts as a slit.

3.2.4 Fiber launcher

An Ozoptics custom made fiber launcher was used to launch the laser beam into the fiber. It consists of an achromatic lens (f = 10mm), an electronically controlled shutter, and a receptacle for FC/APC connection. An Ozoptics custom made fiber patch cord with FC/APC connector was used to connect to the launcher. The ferrule of the FC/APC connector can be adjusted along the optical axis of the fiber making it possible to place the fiber at the focal point. A coupling efficiency of up to 90% can be achieved using this configuration.

3.2.5 DAQ card

A 16 bit DAQ card (USB1608FS, Measurement Computing) was used to syn-
chronize the shutter with the spectrometer’s acquisition time so that the effect of photobleaching can be minimized. It also samples the signal from a photodiode (output voltage = 60 V/µW^{-1}mm^{-2}, 10530DAL, IPL) which monitors the stability of the laser during acquisition to account for any power fluctuations. We achieved this by measuring the average power for each spectrum, then all spectra were multiplied by a factor of \frac{10}{\text{ave. power}} individually so that they all equal to a pump power of exactly 10 mW.

3.2.6 Software

LabVIEW software (National Instruments) was chosen to run the system as it is relatively easy to encode and provides an intuitive interface platform for end users. The developed program controls and synchronizes the DAQ card, triggers the spectrometer, saves the raw data as well as performing real-time and post experimental analysis.

3.2.7 Fiber probe

Any fluorescent signals originating from substances other than the fluorophore of interest is regarded as autofluorescence or background signal. The signal of interest is determined by subtracting the background from the fluorescent measurement made. Background readings were taken by acquiring the spectrum of a blank sample (see Section 3.3.1). The level of autofluorescence ultimately determines the limit of detection as any weak fluorescent sample will suffer from this background offset. During our experiments, sources of autofluorescence mainly come from instrument components such as optical fibers and filters [141]. In addition, the bacteria itself also fluoresce weakly and this was confirmed by measuring the fluorescent signals from the wild-type bacteria. Simple methods of lowering the degree of autofluorescence include the use of excitation sources with less noise or fibers that exhibit lower autofluorescence. A more drastic measure to effectively remove autofluorescence from instrumentation would be to change the design of the probe completely. This
could include the use of different fibers for excitation and collection of fluorescence [115], or a double-cladding optical fiber in which the core and inner cladding can be used as independent channels for excitation and collection [144].

3.2.7.1 Single fiber probe

Single fiber probes are relatively easy to fabricate and work well in opaque environments such as soil sediment. In single fiber mode, excitation is delivered and fluorescence is collected via the same fiber, and the return path of the coupler is connected to the spectrometer. A major drawback of this regime is that a large background signal may cause the spectrometer to saturate as the integration time is increased. This sets an upper limit on the capacity to resolve low level fluorescence with single fiber probes.

3.2.7.2 Dual fiber probe

A dual fiber probe was constructed as shown in Figure 3.5. Two optical fibers were held in place with epoxy and cleaved at the same length. Fluorescence collected by one of these two parallel fibers was delivered directly to the spectrometer. The advantage of this design is that since background is drastically reduced, integration time can be extended. A drawback is that due to the orientation of the collection fiber it is suitable only for taking measurements from liquid media.

The fiber probe end (single or dual) was prepared by carefully removing the outer coating of the fiber end using a flame then flat cleaved. The end was fed into an amber 1.5 mL microcentrifuge probe tube by making a small opening at the top and bottom of the tube. The fiber was held in place by adhesive at the top of the tube. A second amber microcentrifuge sample tube provided a light proof environment surrounding the sample and probe end. Due to the conical shape of the tube, the probe tube maintained a snug fit inside the mouth of the sample tube, leaving enough space for the end of the fiber to be fully introduced inside the sample. To maintain the consistency of background readings between samples, the
probe was always placed at the exact same position. To avoid contamination, the end of the fiber holder tube was always positioned above the sample surface and the fiber tip was rinsed thoroughly in acetone, and distilled water then left to dry between readings.

![Diagram of fluorescence probe](image)

Figure 3.5: Operation of the fluorescence probe [41].

### 3.2.7.3 Fluorescence collection volume

In order to interpret the data collected with our system, information about fiber illumination and fluorescence collection volumes is required. The illumination and fluorescence collection fields for a flat-cleaved multimode fiber are the same as long as the wavelengths of excitation light and fluorescent emission are similar because their optical paths in both directions are matched [133]. Tai and co-workers [132] have shown that not only does the collection volume have a cubic dependence on fiber diameter, but it also varies inversely with fluorophore concentration. From their results, we estimate that for the fiber we used, the collection volume of the single fiber probe in non-scattering fluid would approximate a cylindrical shape with a dimension of 900 μm in length and 200 μm in diameter. In the case of opaque media such as soil, the collection volume is expected to be reduced to a fraction of that in non-scattering fluid as most of the illumination light is immediately absorbed by the surrounding soil when it leaves the fiber.

Assuming integration time and photobleaching is accounted for, the area under
the spectrum is directly proportional to the number of cells detected which is given by the concentration of the cells multiply by the collection volume.

\[ \text{No. cells detected} = \text{Con. cells} \times \text{Collection volume} \] (3.1)

In opaque media such as soil, penetration of excitation light is limited and therefore there is little overlap between illumination and fluorescence collection fields with dual fiber probes. This limits their \textit{in situ} use in soil sediments. On the other hand, single fiber probes are well suited for such purposes as since their illumination and fluorescence collection fields are closely matched.

3.3 Methods and analysis

3.3.1 Sample preparation

Fluorescently labeled bacterial isolates were grown overnight in sterile 50 mL centrifuge tubes with 10 mL of Lysogeny broth (LB, a nutritionally rich medium) while shaking at 200 rpm. \textit{E. coli} were grown at 37°C and \textit{P. putada} at 28°C. The cells were harvested and resuspended, twice in equal volumes of saline. 600 µL of the resuspended cells were transferred to 1.5 mL amber microcentrifuge tubes for fluorescence readings at the predetermined concentration.

Sand microcosms were created by combining 1.6 g of sterile sand (particle sizes 0.0625 to 2 mm) with 400 mL of washed cells resuspended in saline at the desired concentration. Mixing was achieved by inverting the specimen 10 times. The resultant fluid level was below the surface of the sand which therefore has the characteristics of damp soil.

Background elimination was achieved by taking readings of prepared blank samples containing 600 µL of saline (the solution in which the bacteria were resuspended), or in the case of soil trials, 1.6 g of sterile sand with 400 µL of saline was used. Several blanks were prepared and checked for consistency in terms of emission
intensity and normalized spectral characteristics.

## 3.3.2 Data analysis

Custom applications written in LabVIEW software (National Instruments) were incorporated into a flexible user interface for acquisition and analysis of spectroscopic data. The three key analysis functions are outlined below:

1) Spectral comparison: for plotting and comparing raw data as well as normalized spectra from individual samples.

2) Photobleaching comparison: to assess and plot the effect of photobleaching.

3) Spectra deconvolution: to resolve for individual spectral components of a mixed sample. It also provides CFU (colony forming units)/mL estimates based upon fluorescence data and CFU/mL of initial reference samples.

### 3.3.2.1 Photobleaching

Photobleaching is the photochemical destruction of a fluorophore under intense light. This phenomenon manifests itself in the form of a reduction in fluorescence over time and can occur in almost all fluorescent dyes. Fluorescence decays in either a single or a multi-exponential function manner [153, 146, 40, 113]. Multi-exponential functions are typically found for ensemble measurements while single exponential bleaching times are measured on a series of single entrapped molecules. It is important to characterize the photobleaching behavior of each dye because if not accounted for, it may affect the results. Photobleaching characteristics are usually recorded via a series of time resolved signals or spectra in which the sampling intervals are much shorter than the photobleaching rate.

In general, photobleaching results are best fitted with a multi-exponential curve as they are not events from a single entrapped molecule. However, to simplify comparison between different fluorophores, concentrations and/or environments we have used a single exponential curve with a DC component that approximates the steady state reached after prolonged exposure to excitation. The photobleaching
rate is described as

$$I(t) = I_0 e^{-kt} + C$$  \hspace{1cm} (3.2)

where $I(t)$ is the intensity at time $t$, $I_0$ is the amplitude of the decay, $k$ is the emission intensity decay rate constant, and $C$ is the steady state intensity. The normalized version of this equation is

$$I_N(t) = (1 - C)e^{-kt} + C$$  \hspace{1cm} (3.3)

whereas the intensity $I_N(t)$ is normalized with respect to $I(0)$ and is illustrated in Figure 3.6.

The “half life” $T_{1/2}$ is the time at which the signal intensity decays to half of the difference between the initial intensity and steady state intensity. It is given as

$$T_{1/2} = \frac{\ln(2)}{k}$$  \hspace{1cm} (3.4)

The area of the acquired spectrum $S_{TN}(\lambda)$ for an integration time of $T_N$ equals to the area under the curve $I(t)$ from zero to $T_N$. As depicted by Figure 3.6, this is the total accumulated energy over the integration time of $T_N$ and can be expressed by

$$\int S_{TN}(\lambda) \, d\lambda = \int_0^{T_N} I(t) \, dt$$

where $\lambda$ is the wavelength. When comparing raw spectra, their integration times
were normalized to the same value of $T_N$. We calculate their energies as $I_0T_N$ rather than the area under the curve so that the effect of photobleaching can be removed. For this reason, only one spectrum were acquired per sample and only for an illumination time that is much smaller than the $\frac{1}{2}$ life time of the photobleaching curve. However, if it is necessary to illuminate the sample for a prolonged period (ie to obtain as much signal as possible at low concentrations), then the normalized photobleaching curve $I_N(t)$ together with the accumulated energy $S_{TN}(\lambda)$ is used to recover the integrated energy $I_0T_N$.

3.3.2.2 Resolving multiple spectra

Resolving overlapping spectra is an important yet challenging problem in various applications of spectroscopy. This may include absorption and emission curves, nuclear magnetic resonance (NMR), bioluminescence, etc. The problem involves extracting individual spectral components from an experimental curve where the intensity at any given wavelength may be contributed by multiple bands. The common approach is curve-fitting based on algebraic methods. The principle of curve-fitting is to represent peaks by certain analytical functions with some undetermined parameters so that they can be optimized to fit the experimental curve. Nonetheless, an accurate fit is usually based on priori information about the form of individual spectral bands such as their number, positions, shapes and widths, etc [64].

Common algebraic methods may include finding the second derivative of the curve to determine the number and the position of overlapping peaks [55]. This method is less suitable for spectra with multiple broad bands as the derivatives of the peaks are less prominent. Fourier self-deconvolution (FSD), is a linear deconvolution method based on measured data in the time domain [72]. FSD performs better than techniques needing high-order numerical derivatives, but it requires estimates of peak shapes and widths at half-height for proper deconvolution [32]. Although FSD can accommodate spectra with broad bands it is most common only used with multiple Lorentzian functions which may not be suitable for our data. At this point, we only
3.3 Methods and analysis

need to resolve two spectral components, which greatly reduces the problem. We have therefore chosen to use the Alentsev-Fok \cite{56, 47} technique and a simple least squares curve fitting method to resolve our measured spectra. These methods are complimentary and operate best under different conditions.

3.3.2.2.1 Least squares method The measured spectrum is decomposed into the following components

\[ RAW(\lambda) = \alpha GFP_{Ref}(\lambda) + \beta RFP_{Ref}(\lambda) \] (3.5)

where the asterisk * indicates that spectra are normalized, \( RAW \) is the measured spectrum, \( GFP_{Ref}(\lambda) \) and \( RFP_{Ref}(\lambda) \) are the reference spectra of green and red fluorescent spectra, respectively. The coefficients \( \alpha \) and \( \beta \) are the parameters that best fit the measured spectrum in a least squares manner. The least squares solution \( \hat{x} \) is given by

\[ \hat{x} = (A^T A)^{-1} A^T b \] (3.6)

where \( \hat{x} = \begin{bmatrix} \alpha \\ \beta \end{bmatrix} \), \( A = \begin{bmatrix} GFP_{Ref} \\ RFP_{Ref} \end{bmatrix} \) and \( b = RAW \).

The advantages of this method are that it works for any arbitrary shape and only one spectrum is required to resolve for its constituents. The main disadvantage is that it is necessary to specify the individual spectral components fully prior to decomposition, whereas there may be changes in the spectra of these component fluorophores over time.

3.3.2.2.2 Alentsev-Fok Method The Alentsev-Fok method for solving complex spectra is analogous to solving simultaneous equations. Here, since the spectra we wish to resolve are made up of two components, only the case of solving for a pair of simultaneous equations is considered. The description of this method along with an illustration (see Figure 3.7) are given as follow.

Let green fluorescent protein \( GFP(\lambda) \) and red fluorescent protein \( RFP(\lambda) \) be
the individual spectral components that make up the compound spectrum $f_1(\lambda)$ such that

$$f_1(\lambda) = GFP(\lambda) + RFP(\lambda)$$  \hspace{1cm} (3.7)

where $\lambda$ is the wavelength. The individual components can be determined if the following criteria are satisfied.

1. Their spectra are such that there exist two ranges in the wavelength in which one of the amplitude becomes zero, while the other remains nonzero. Or simply

$$GFP(\lambda) \neq 0; \quad RFP(\lambda) = 0 \quad \text{for} \quad \lambda_1 < \lambda < \lambda_2$$

$$GFP(\lambda) = 0 \quad RFP(\lambda) \neq 0 \quad \text{for} \quad \lambda'_1 < x < \lambda'_2$$

2. One can determine a spectrum $f_2(\lambda)$ that consists of the same spectral components $GFP(\lambda) + RFP(\lambda)$ but with different weights.

$$f_2(\lambda) = aGFP(\lambda) + bRFP(\lambda) \quad a/b \neq 1$$  \hspace{1cm} (3.8)

When these two conditions are met, the graph of the ratio $F(\lambda) = f_2(\lambda)/f_1(\lambda)$ should have two horizontal parts whose ordinates are $a$ for $\lambda_1 < \lambda < \lambda_2$ and $b$ for $\lambda'_1 < \lambda < \lambda'_2$ (see Figure 3.7). Once $a$ and $b$ are deduced the individual components of $GFP(\lambda)$ and $RFP(\lambda)$ can be determined by solving the simultaneous equation using Equation 3.7 and 3.8 such that

$$GFP(\lambda) = \frac{bf_1(\lambda) - f_2(\lambda)}{b - a} \quad \text{and} \quad RFP(\lambda) = \frac{af_1(\lambda) - f_2(\lambda)}{a - b}$$  \hspace{1cm} (3.9)
The advantages of this method are that it works for any arbitrary shape and that no information is required prior resolving the spectra. However the drawbacks are that at least two spectra are required and when resolving for more than two components, fulfilling the criteria becomes increasingly difficult. To check for consistency, a comparison of Alentsev-Fok to the least squares method can be made by substituting Equation 3.9 into 3.5. For the ease of comparison, the coefficients of $\alpha$ and $\beta$ obtained with both methods can be compared using only one coefficient which can be $\beta/\alpha$. Theoretically speaking, the values of the coefficient $\beta/\alpha$ with both methods should match.

3.4 Results

Prior to performing full scale bioremediation strategies, it is necessary to establish an effective small scale “model” of the process. This is best done under controlled laboratory conditions where it is easier to monitor and, if possible, quantify biore-
CHAPTER 3. In Situ spectroscopy monitoring techniques of bioremediation

The location, distribution, degradation activity and survival of introduced degrader organisms can potentially be monitored in situ through the creation and use of biosensors [34]. The objective of this research was to test the feasibility of using our novel spectroscopic approach for this purpose and to identify limitations.

Wild-type and genetically modified bacterial species such as *E. coli* and *P. Putida* were used in this investigation. *E. coli* offers nothing for the purposes of bioremediation, but was chosen for preliminary studies because the fluorescent proteins labeled in the plasmid can be made very bright. This enables measurements to be made at high signal to noise ratio (SNR). *P. Putida* is a strong candidate for bioremediation site clean-up and was selected for this reason. However in this bacteria, the fluorescent protein is tagged in the chromosome and fluoresces relatively weakly. We have used these two species because it is highly likely that bacteria with appropriate bioremediation properties which can also be labeled with high yield fluorescent protein will be identified. More details on the motivation for choosing these bacterial species and methods for cultivating them can be found in [41, 62].

When comparing spectra it is important to maintain consistency in the variables used. In all our measurements, the excitation power was kept at 10 mW. If not otherwise specified, the integration time was 100 ms. This enabled photobleaching to be minimized while maintaining adequate SNR.

3.4.1 Spectral characteristics

In this section we characterize the emission spectra of isolates labeled with green and red fluorescent proteins. The effects of 473 and 532 nm excitation are also investigated. These are important first steps toward developing a viable bioremediation monitoring technique.

3.4.1.1 GFP and RFP

Emission spectra for GFP labeled *E. coli* and DsRed labeled *E. coli* are shown
in Figure 3.8. The emission spectrum of E. coli- GFP showed characteristics similar to those demonstrated by other groups when excited at 473 nm [21, 22, 123, 138]. Peak emission occurred at a wavelength of 521 nm with a shoulder around 545 nm. Superimposed on the same figure is the emission spectrum for E. coli-DsRed excited at 532 nm. This has a peak at 586 nm but no shoulder which is also in agreement with previous reports for this fluorescent protein [123, 8].

![Figure 3.8: Normalized emission spectra of E. coli-GFP and E. coli-DsRed, subjected to excitation at wavelengths of 473 and 532 nm respectively.](image)

**3.4.1.2 Wild-type and GFP transformed bacterial species**

When modifying bacterial species genetically with fluorescent proteins, it is important that they can be distinguished from their parental wild-types on the basis of fluorescence emission. In Figure 3.9, emission spectra for GFP-tagged *P. putida 852-137* and the parental wild-type strain *P. putida 852* are presented, together with the subtraction spectrum. These confirm that the spectral response of GFP protein can be separated relatively easily from the autofluorescence of wild-type isolates.
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Figure 3.9: Emission spectra of fluorescently (GFP) labeled *P. putida* 852-137, wild-type *P. putida* 852 and the resultant GFP spectrum following subtraction of the wild-type from *P. putida* 852-137.

Depending on the nature of GFP expression, bacterial species can exhibit very high levels of GFP induced fluorescence. The fluorescence from *E. coli*-GFP in Figure 3.10 is two to three orders of magnitude greater than its wild-type counterpart. This is because the plasmid transfection of *E. coli* with GFP is much more efficient than the chromosomal DNA transfer that applies with *P. putida* 852-137. In Figure 3.11, normalized emission spectra for *E. coli*-GFP and *P. putida* 852-137 isolates are superimposed. There is an up shift of 8-10 nm for the latter across the 500-540 nm wavelength range and this was observed in all such comparisons.

Figure 3.10: Emission spectra of fluorescently labeled *E. coli*-GFP and *E. coli* MG1655 (wild-type).
3.4 Results

Figure 3.11: Normalized emission spectra of E. coli-GFP and P. putida 852-137 after their respective wild-type spectra subtraction.

These results show that GFP transformed bacterial variants can be distinguished from corresponding wild-type bacteria on the basis of their differing emission spectra. However, the emission spectra for different bacterial species containing the same GFP variant are not identical.

3.4.1.3 Soil and solution

To determine if our system could successfully detect fluorescence in soil microcosms, samples of sand and E. coli-GFP were mixed as outlined in Section 3.3.1 and spectroscopic readings were acquired. These data were compared with results for the same suspended cell concentrations in saline solution alone. The emission spectrum of E. coli-GFP was three times less in the soil microcosm than in saline solution (see Figure 3.12, Top). This was expected as soil is opaque. Identical spectral characteristics were observed when the E. coli-GFP emission spectra obtained from soil and saline are normalized and superimposed (see Figure 3.12, Bottom). These measurements demonstrate that it is possible to remove soil background from collected fluorescence thus revealing an unaltered GFP spectrum which can be used to quantify the amount of fluorescent organisms in the soil environment. Further measurements from different types of soils are required.
3.4.1.4 Excitation of \textit{E. coli}-DsRed using 473 and 532nm

Emission spectra of \textit{E. coli}-DsRed at an initial concentration of approximately $1 \times 10^9$ CFU/mL were taken at excitation wavelengths of 473 and 532 nm. Then, to establish the differences in their relative excitation efficiencies and spectral shapes, these two spectra were compared. Figure 3.13 shows that there is a high degree of similarity both in terms of the peak emission and spectral shape between the normalized emission spectra of \textit{E. coli}-DsRed using the two excitation wavelengths. Although there is residual pump "leakage" at 532 nm with the green laser, it does not affect the emission spectrum. The 532 nm laser is closer to the peak excitation of \textit{DsRed} at 556 nm (Figure 3.2) and the emission spectrum was five times greater than at 473 nm.

We found that it was not possible to excite GFP (reported excitation peak at 489 nm [125]) with a 532 nm laser. No signals were detected in \textit{E. coli}-GFP samples and only \textit{DsRed} signals were detected in mixed samples of \textit{E. coli}-GFP and \textit{E. coli-
3.4 Results

*DsRed*. This confirms that the identification of *E. coli-DsRed* within a mixed sample is possible based upon the selective choice of appropriate excitation wavelengths (see Figure 3.2).

Using our system it is possible to excite fluorescent protein variants with multiple lasers at different wavelengths and this may aid in resolving complex spectra through deconvolution. For instance, since *E. coli-GFP* is not excited by 532 nm and the emission spectrum of *E. coli-DsRed* is independent of excitation wavelengths, it is possible to detect only *E. coli-DsRed* in a mixed sample via 532 nm excitation. Using this spectrum, the complex spectrum collected with the 473 nm excitation can easily be resolved.

![Figure 3.13: Emission spectra of *E. coli-DsRed* using 473 and 532 nm excitation. Left: Raw spectra. Right: Normalized spectra.](image)

3.4.2 Photostability

All fluorescent proteins will eventually photobleach upon extended excitation. In addition, there is substantial variation in the rate of photobleaching between different fluorescent proteins, and even between fluorescent proteins with otherwise very similar optical properties [123]. Photostability of fluorophores is an important issue in this study as instability will impact on our ability to make reliable measurements based on fluorescent emission. Data on the photostability of fluorescent protein variants are impossible to compare directly. The photobleaching of these fluorophores is complex, depending on excitation wavelength, intensity and exposure time. To
date no systematic characterization of fluorescent protein photostability has been available in the scientific literature [123]. To provide a practical basis for comparing the photostability of different fluorescent proteins, we have measured photobleaching curves under different conditions for fluorescent proteins listed in Table 3.1 and fitted parameters are given.

In this study, the photostability of fluorescent proteins was measured by characterizing the photobleaching curve of the fluorescent protein of interest. Photobleaching rates were estimated to determine the effect of dilution media had on the fluorescently labeled isolates. The first step in this process was to take a background reading from a sterile blank sample containing the same media used for the fluorescent protein so that it could be subtracted from the emission spectra. During the data acquisition process the sample was subjected to constant excitation at 10 mW. Consecutive spectra were acquired over a period of a few seconds at an integration time of 8 ms to provide for adequate resolution in the time domain. To evaluate the effect of photobleaching, a simple single exponential decay curve was fitted to the data (see Section 3.3.2.1 and [111]) and fitting parameters were compared between different samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution factor</th>
<th>Decay constant ( (k) )</th>
<th>Steady state ( (C) )</th>
<th>Half-life ( (T_{1/2} \text{s}) )</th>
<th>Signal intensity at ( T_{1/2} )</th>
<th>Figure reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{E. coli-GFP in saline}</td>
<td>1</td>
<td>0.21</td>
<td>0.90</td>
<td>3.20</td>
<td>0.95</td>
<td>3.14 A</td>
</tr>
<tr>
<td>\textit{E. coli-GFP in soil}</td>
<td>1</td>
<td>0.37</td>
<td>0.61</td>
<td>1.87</td>
<td>0.81</td>
<td>3.14 C</td>
</tr>
<tr>
<td>\textit{E. coli-DsRed in saline}</td>
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<td>0.77</td>
<td>0.70</td>
<td>0.90</td>
<td>0.85</td>
<td>3.15 A</td>
</tr>
<tr>
<td>\textit{E. coli-DsRed in saline}</td>
<td>1/10</td>
<td>0.61</td>
<td>0.70</td>
<td>1.14</td>
<td>0.85</td>
<td>3.15 C</td>
</tr>
<tr>
<td>\textit{E. coli-DsRed in saline}</td>
<td>1/100</td>
<td>0.72</td>
<td>0.66</td>
<td>0.96</td>
<td>0.83</td>
<td>3.15 E</td>
</tr>
</tbody>
</table>

Table 3.1: Fitted parameters of the photobleaching curve of \textit{E.coli} isolates in different media and concentration.

3.4.2.1 Photobleaching in soil and saline

The photobleaching curves varied substantially between \textit{E. coli-GFP} isolates in soil and in saline (see Table 3.1 and Figure 3.14). At steady state, the fluorescence
emitted by freely suspended cells in saline was 90% of the initial intensity and for cells mixed with soil it was much lower at 61%. Moreover, the half-life extended from 1.87 to 3.2 seconds, respectively. This is expected as in saline the cells are freely suspended, whereas in soil this is not the case. In the former case, cells diffuse through the collection volume of the probe during the acquisition time which produces higher steady state than the cells. In the latter, they are immobilized on sand particles and subjected to the full radiation over the acquisition time. These results indicate that photobleaching of E. coli-GFP isolates is negligible in solution microcosms, but needs to be accounted for in soil, if the integration time is comparable to the half life.

Steady state fluorescence emission for E. coli-DsRed in saline was around 70% of initial intensity (compared with 90% for E. coli-GFP). On this basis, we would expect of E. coli-DsRed in soil to be much greater than 70%. However, this requires further investigation as no measurements were made to confirm this.

Figure 3.14: Photobleaching rate of E. coli-GFP samples in solution and soil using 473 nm excitation. Left: Plots of exponential decays fitted to the acquired spectra in saline and soil. Right: First and last spectra acquired in saline and soil.
3.4.2.2 Photobleaching in different cell concentrations

The light emitted by organisms containing fluorescent proteins can be directly related to their concentration only if photobleaching is negligible or has been accounted for. We have shown that considerable photobleaching of \textit{E. coli-GFP} occurs in soil so it cannot be neglected. In order to estimate cell concentration in an unknown sample, therefore, it is necessary (i) to relate fluorescence emission to known cell concentration in a reference sample, and (ii) to characterize the photobleaching curve. In addition, it is also necessary to demonstrate that photobleaching is not concentration-dependent.

We have measured the photobleaching curves for \textit{E. coli-DsRed} in saline at dilution factors of 1, 10 and 100. These were found to be independent of dilution within detectable limits. Steady state was achieved around 70\% of the initial intensity and the half-life was around 1 second (see Table 3.1). Photobleaching measurements for \textit{E. coli-GFP} in soil have also exhibited similar photobleaching curves at all concentrations although signals were weaker at low concentrations. These results indicate that it should be possible to estimate cell concentration on the basis of fluorescent emission.
3.4 Results

Figure 3.15: Photobleaching rate of *E. coli*-DsRed in solution with 473nm excitation. Plots (A), (C) and (E) are the first and last spectra acquired at neat, 1/10 and 1/100 dilutions respectively. Plots (B), (D) and (F) are exponential decay fitted to the acquired spectra at neat, 1/10 and 1/100 dilutions respectively.

3.4.3 Predictability of cell concentration

The relationship between dilution and emission signal intensity readings was investigated using *P. putida* 86-1, *E. coli*-GFP, and *Salmonella* SM022. These three bacterial species were all tagged with GFP and samples were prepared from the initial concentration to 1/512, with progressive dilution by a factor of two. In order to minimize the effect of photobleaching, integration times were progressively shortened from 100 ms to 8 ms as the bacteria concentration of the samples were increased. The total intensity (area of the spectrum) measured from each sample was normalized with respect to the integration time before it was plotted against the dilution factor. The data plotted in Figure 3.16 demonstrated the expected direct proportional relationship between dilution factor and signal intensity. This suggests that the fluorescent cell concentration of any sample can be derived from a known
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3.4.4

Figure 3.16: Integrated spectral intensity of bacterial species plotted against the dilution factor from initial concentration.

Figure 3.17: Reference spectra of *E. coli*-DsRed and *E. coli*-GFP. The dilution ratio at these concentrations are designated as 1 GFP to 1 DsRed.

In this experiment, we excited mixtures of *E. coli*-GFP and DsRed at 473 nm. The peak intensity for *E. coli*-GFP was around 2.5 times greater than *E. coli*-DsRed for equivalent concentrations. Reference spectra for each were acquired with *E. coli*-GFP diluted by a factor of 2.5 relative to *E. coli- DsRed so that each culture
emitted light of comparable brightness. Figure 3.17 illustrates the emission spectra acquired. The peak intensity of the GFP spectrum was initially around 68% of the DsRed spectrum. Overnight culture samples for mixed sample trial were collected via centrifugation (4000 rpm (2316g), 5 min) and then resuspended in saline. The saline volume for *E. coli*-GFP was 2.5 times greater than for *E. coli*-DsRed for the reasons outlined above. Mixtures were prepared from these stock solutions in the following ratios (GFP:DsRed) 0:10, 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1, and 10:0. Spectra acquired from these mixtures were decomposed into their GFP and DsRed components using both the Alentsev-Fok and Least squares methods (see Section 3.3.2.2). When using these two methods, two assumptions are made. Firstly, when fluorophores are mixed together their respective spectra are not altered by the presence of the other fluorophore. Secondly, the photobleaching rate of each fluorophore is independent of the presence of the other fluorophore. Because fluorescent proteins are produced and sequestered within bacterial cells, the direct interaction of different fluorescent proteins is likely to be limited. If the deconvolved GFP and DsRed ratio are consistent with the dilution ratio, it proves that the method used is effective and validates the assumptions made.

In Figure 3.18, we compare the Alentsev-Fok and Least squares methods by superimposing the deconvolved GFP and DsRed spectral components. These plots show that the resolved GFP and DsRed spectra are very similar regardless of dilution ratio. Figure 3.19 examines the effectiveness of the two methods by plotting the parameter $\beta/\alpha$ (Equation 3.5 & 3.9) against the ratio GFP:DsRed. The actual value of $\beta/\alpha$ were determined independently from reference spectra and dilution ratios. The linear relationship in the log-log plot shows that both spectral deconvolution methods were equally effective across the range of the dilution ratios tested.
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Figure 3.18: A and C: Deconvolved *E. coli*-GFP and *E. coli*-DsRed spectra using the Alentsev-Fok method at dilution ratios of (GFP:DsRed) 3:7 and 7:3 respectively. B and D: Deconvolved *E. coli*-GFP and *E. coli*-DsRed spectra using the Least squares method at dilution ratios of (GFP:DsRed) 3:7 and 7:3 respectively.

Figure 3.19: Log-log plot of the parameter $\beta/\alpha$ value against the dilution ratio of GFP to 1 DsRed. Both the Alentsev-Fok and Least squares method are employed as well as the actual $\beta/\alpha$ predetermined by the reference spectra and dilution ratios.
3.4.5 Probe sensitivity

3.4.5.1 Single VS dual fiber probe

A factor that limits the sensitivity of fiber optic spectroscopy systems is background autofluorescence which prevents the spectrum of the "signal" from being resolved with respect to the "noise". Background can be reduced using a dual fiber probe in which excitation light is delivered to the specimen in one fiber and fluorescent emission is collected with a second adjacent fiber. In this case, autofluorescence generated along the excitation fiber pathway is not returned to the spectrometer. Background spectra recorded with a dual probe in blank saline specimen tubes were reduced 15 fold with respect to those acquired with a single fiber probe (see Figure 3.20).

To our surprise, we were not able initially to demonstrate any significant sensitivity improvement over the single fiber probe with GFP labeled bacteria. To explain this it is necessary to recapitulate the procedures used. Background spectra were acquired from a small set of blank samples and the mean background was then subtracted from test spectra. In Figure 3.21, we plot the difference between two successive background spectra recorded from the blank saline specimens. While the SNR of the resultant curve is much greater for the dual fiber probe than the single fiber probe, the variation in the background is similar in both cases. This is consistent with variation in the background between samples. We demonstrated that the position of the probe in the specimen tube (depth and location with respect to central axis) can change the background spectrum dramatically. We tried to minimize this by always placing the probe at the same position and depth each time a measurement was taken. However, we still experienced significant variation in the background readings between blank samples and attribute this to differing degrees in autofluorescence in the specimen tubes.
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Figure 3.20: Comparison of background spectra in saline between single and dual fiber probe. Single fiber’s signal is near saturation with a prominent spike while the dual fiber probe has much less signal and without a spike.

With the cells suspended in saline and soil, GFP signals were acquired for different culture concentrations using both probes. Concentration series (progressive dilution by a factor of 4 from an initial concentration of $1 \times 10^9$ CFU/mL) were tested to determine the minimum detectable concentration. For saline samples, fluorescent emission from *E. coli*-GFP could be detected down to a dilution of 4096. In soil samples, the GFP spectrum could be detected with a single fiber probe to a dilution of 1024, but no detectable signals could be recorded with the dual fiber probe. Typical low level results are presented in Figure 3.22.

Figure 3.21: Variation in background photon counts between different sample tubes.
3.4 Results

On the basis of Equation 3.1, we estimate that single and dual fiber probes have a detection limit around sixty nine cells within the collection volume (in the order of \(0.03 \text{ mm}^3\)) in saline. We believe this detection limit also holds true for single fiber in soil.

![Emission spectra of E. coli-GFP](image)

**Figure 3.22:** Emission spectra of *E. coli-GFP* at a dilution factor of 4096 and an integration time of 2700ms.

Ayotamuno and co-workers [4] reported degrader bacterial concentrations on the order of \(1 \times 10^5 - 10^6 \text{ CFU/mL}\) during in situ bioremediation of crude oil-polluted agricultural soil at Port Harcourt, Nigeria. Another study conducted by Margesin and Schinner, which assessed the natural attenuation and biostimulation of native degrader organisms in diesel oil-contaminated soil in an alpine glacier skiing area, identified culturable heterotrophic microorganisms on the order of \(1 \times 10^7 \text{ CFU/g (dry weight) of soil}\) [96]. Within this context, the fact that we can detect *E. coli-GFP* at concentrations down to \(2.5 \times 10^5 \text{ CFU/mL}\) in saline and \(1 \times 10^6 \text{ CFU/mL}\) in soil is promising. However, the effectiveness of our fluorescence-based bacterial detection system for actual field work will depend on engineering fluorescent protein-labeled degrader organisms that are as bright or brighter than *E. coli-GFP*.

In summary, with current procedures, the sensitivity of our system is not limited by the SNR of the spectra, but rather by the variation in background fluorescence between samples. Dual fiber probes have the capacity to improve SNR in saline suspensions. The results presented in Figure 3.21 indicate there is far less RMS
noise with the dual fiber probe than the single fiber probe. In this case, sensitivity is limited by the background fluorescence of the specimen tubes used in this study. The dual fiber probes that we have constructed do not work well with soil, because absorption and scattering markedly reduce the depth of excitation and collection volume, minimizing the overlap between them.

3.4.6 In situ monitoring of naphthalene degradation

The feasibility of using our fiber optic spectrometer system to monitor bioremediation was investigated using the promoter linked bioremediating bacteria P. putida pPG7-GFP. This bio-indicator strain was obtained from the Swiss Federal Institute of Technology Laboratory of Soil Science in Lausanne, Switzerland. It has been developed and used successfully as a bio-indicator in naphthalene degradation trials by Tecon et al [134]. Salicylate is one of the main intermediary products of naphthalene breakdown and the promoter gene pPG7-GFP was linked to the salicylate end products. Therefore P. putida pPG7-GFP fluoresces when breaking down naphthalene, but only in the presence of salicylate end-products. Low level constitutive fluorescence is also seen in these bacteria in the absence of biodegradation.

3.4.6.1 Preparation of promoter linked fluorescence trials

The fluorescent response of P. putida pPG7-GFP to naphthalene availability was assessed as follows. The bio-indicator was cultured in different concentrations of naphthalene and in a range of combinations of naphthalene, salicylic acid and glucose as an alternative carbon source. This enabled us to quantify responses of growth and fluorescent gene expression under different conditions. Cultures were prepared in triplicate for each sample type in each of the three days, the study was conducted over a total of nine samples for each culture as follows:

1) Glucose 2%
2) Naphthalene 500mg/L + Glucose 2%
3) Salicylate 500mg/L
3.4 Results

4) Naphthalene 500mg/L + Salicylate 500mg/L
5) Naphthalene 500mg/L
6) Naphthalene 400mg/L
7) Naphthalene 300mg/L
8) Naphthalene 200mg/L
9) Naphthalene 100mg/L
10) Naphthalene 50mg/L
11) Negative control (background, saline)

Fluorescent measurements were made before inoculation to ensure that the additives along does not affect the spectra. To prevent photobleaching due to repeated illumination from affecting our results, samples were used only once. Measurements were made in the three samples taken from each culture every 24 hours.

3.4.6.2 Bacteria growth in different concentrations of naphthalene

Figure 3.23 depicts the emission spectra of *P. putida pPG7-GFP* and *P. putida 852-137-GFP* cultured in naphthalene and broth, respectively. This shows that even for the same type of bacteria, having a different GFP strain attached to it can result in emission spectra that are significantly different.

![Emission spectra of different P. putida GFP strains.](image)

We characterized the relationship between naphthalene concentration and promoter-
linked GFP production by comparing fluorescent emission of cultures grown in different naphthalene concentrations over three days. The results suggest that GFP production was highly correlated to naphthalene availability (see Figure 3.24). Bacterial growth was minimal with the exception of the naphthalene concentration of 500 mg/L. In this case, growth reached the exponential phase after 24 hours and leveled off within 48 hours.

This suggests that bacterial growth was probably in the lag phase for the first 72 hours at naphthalene concentrations of 400 mg/L and under. During this stage, growth is slow as bacteria acclimate to the carbon source in their new habitat. For naphthalene concentrations over 400 mg/L, bacterial growth enters the exponential phase after 24 hours as metabolic rate picks up. For a concentration of 500 mg/L, bacterial growth stagnated within 48 hours suggesting that the source of naphthalene has depleted as the stationary phase was reached.

Figure 3.24: Fluorescence produced by *P. putida pPG7-GFP* under different concentrations of naphthalene over 72 hours.

3.4.6.3 Bacteria growth in different culture media

The effects of different culture media on *P. putida pPG7-GFP* are presented in Figure 3.25. Bacteria cultured in 2% glucose in the absence of naphthalene or
naphthalene breakdown products had the highest levels of GFP production. At 2% glucose concentration, the carbon source available to bacteria is vast and rapid population growth is expected. Under these conditions, constitutive GFP would be expected to become significant. Although constitutive GFP production per cell, is minute in comparison to promoter-linked GFP production, the cumulative signal due to it will be amplified at very high concentrations of bacteria in glucose. In addition, GFP fluorescence from bacteria cultured in media containing naphthalene and/or salicylate increased with respect to background over 72 hours. However, we would have expected GFP production for 2% glucose and naphthalene to have been greater than 2% glucose alone. Likewise, we expected samples containing naphthalene and salicylate to give stronger fluorescence signals than naphthalene alone as salicylate is an enzymatic stimulant of the naphthalene degradation pathway. However, the data in Figure 3.25 do not support this. Within this context, it seems important to correct for the confounding effects of constitutive GFP production and to reduce background when using relatively weakly labeled bio-indicators such as *P. putida* pPG7-GFP. Additional work is required to resolve these issues.

![Figure 3.25: Fluorescence produced by *P. putida* pPG7-GFP under different growth media.](image)
3.5 Discussion

In this chapter we have outlined a novel optical approach that could be used to monitor the degradation activity of micro-organisms via fluorescence induced by promoter-linked genes and have completed preliminary trials to assess the feasibility of this method. In terms of the findings and outputs achieved in this study, a brief summary is outlined below.

We have constructed an all fiber spectrometer system which enables multiple fluorescent signals to be extracted. The feasibility of the system was tested first with *E. Coli*, a non biodegrader organism which can be engineered to express both GFP and DsRed with high efficiency. We demonstrated that it is possible decompose GFP and DsRed spectra faithfully in this organism with a single excitation at 473 nm. We have also demonstrated for *P. putida pPG7-GFP* that both population growth and biodegradation can be monitored. However, two fluorescent proteins would need to be used for this purpose, one indicating population growth and the other biodegradation. This would enable correction to be made for the effects of constitutive fluorescent protein production.

The limiting factors with the approach we have demonstrated are (i) the low efficiency of promoter linked fluorescent protein production with *P. putida* and other potential biodegrader organisms, and (ii) fluorescence background. It seems likely that improved techniques for transfection of biodegrader organisms with fluorescent proteins will be developed. However, independent of development of bioreporter technologies, our results demonstrate that substantial improvements in sensitivity could be made immediately by removing sample-to-sample variation in background that are due to the specimen tube. In addition, SNR could be further improved with the development of a dual fiber probe in which the overlap between excitation and collection volume is optimized. On this basis, we estimate that improvements in SNR on the order of 10-100 folds could be achieved relatively simply.

We argue that the methods developed in this study provide a foundation for the *in situ* monitoring of bioremediation in the laboratory. Through numerous
experimental trials we have identified various drawbacks of the system and our methodology. In the next section, we will discuss each of these in turn and suggest possible improvements that could be made.

3.5 Discussion

3.5.1 Optical setup improvements

The two lasers built into the system can only be switched on or off, they do not support variable output power. The desired output power is currently achieved by the attenuation with a manual shutter built onto the laser head. This is a cumbersome and slow process, recordings were therefore usually made at the output power of 10 mW. This has limited our options in designing experimental protocols. Lasers that support variable output power via an analogue input voltage are better alternatives. Not only can their output power be adjusted to the desired value via the DAQ card, power stabilization can be improved via a feedback loop with the inclusion of photodiode to monitor laser power.

As mentioned earlier in Section 3.2.7 the main difficulty with our current procedure lies in the high level of autofluorescence and/or background variation which restricts the dynamic range and lowers the sensitivity, respectively. Although we have developed a dual fiber probe specifically for the purpose of reducing background, due to its orientation, it is not suitable for use in opaque media such as soil. A simple approach would be to use fibers with lower autofluorescence while a more drastic approach would be to change the design of the probe completely. Possible probe design for use in soil may include a double-cladding optical fiber as it offers independent channels for excitation with the core and fluorescence collection with the inner cladding [144], or have the collection fiber adjacent to the excitation fiber with their optical axes aligned.
3.5.2 Spectral deconvolution

Numerous spectral deconvolution techniques from literature have been considered. Since in this study only two components were needed to be resolved, we have chosen two simple methods. Both the Least squares and Alentsev-Fok methods successfully resolved the complex spectra into their respective components. Although the criteria for each of these methods are different, it is possible to use both techniques simultaneously to check for consistency.

As the techniques for genetic modification of bacterial species develops, it may be possible to introduce more than two fluorophores to a single cell and hence report more information on the bioremediation process. In such a scenario, more complicated spectral resolving techniques may be required.

The Levenberg-Marquardt algorithm provides a numerical solution to the problem of minimizing a function over a space of parameters of the function [90]. It has successfully resolved overlapping peaks in Fourier transform-infrared spectra [50] and thermoluminescence glow curves [20]. One of the main drawbacks is that the software may not provide the resolved spectra in real time as this algorithm uses an iterative procedure to find the best solution which may require intense computing power.

Indirect hard modeling is another curve fitting method in which the algorithms are actually physically motivated [85]. It does not just treat spectral data as vectors and matrices, but also exploits the underlying physical structures of the spectrum. This approach allows the consideration of various nonlinear effects such as peak variations or spectral shifts. Since this technique also uses an iterative procedure, intensive computing power may be required.

3.5.3 Sample preparation

In Section 3.4.5, we have shown that currently it is the variation of background signal between samples that limits our sensitivity. This variation is mainly due
3.5 Discussion

to the uniqueness of each sample tube. In future this may be reduced by taking a background measurement first then using the same tube to record a fluorescent measurement. This is a tedious method of making fluorescent measurements which will only work for solution samples.

Taking background signals from soil samples is especially difficult as their variation is much greater than solution samples. The only way to minimize this variation is to make certain the position of the probe remains intact with respect to its surroundings when making background and fluorescent measurements.

3.5.4 Time series measurement of bacteria growth

Results from these initial trials of bacteria growth under different concentrations of naphthalene provides proof of concept that \textit{in situ} monitoring of bioremediation with a fluorescence system is a viable technique. We have demonstrated that \textit{P. putida pPG7} degrades naphthalene by measuring the fluorescence of the promoter-linked GFP gene. Characterization of the bacterial growth curve can aid in designing an effective bioremediation method by predicting the time required to “clean up” the contaminated site. Future trials should include the use of same samples each time a measurement is taken instead of destroying it to avoid photobleaching; measuring at concentrations higher than 500 mg/mL; sampling of the bacterial growth curve over a longer period but at shorter intervals so that each phase of the growth curve from lag, exponential, stationary, to death can be fully characterized. Simultaneous measurements of naphthalene degradation (for instance, by using gas chromatography to establish remaining concentrations of naphthalene in cultures, and therefore how much has been degraded) alongside GFP production should also be recorded to estimate the amount of GFP produced per naphthalene degradation.
3.5.5 Bacteria growth under different culture media

Experiments were conducted to determine whether bacterial degradation activity could be measured in PAH cultured media via promoter linked fluorescence. Since constitutive fluorescence is always present, we need to compare the amount of fluorescence from culture media with and without the presence of PAH carbon source. Although fluorescence was recorded from PAH cultured media, we were unable to compare it to non-PAH cultured media as the concentration of bacteria in each culture media was not known. For this reason, we could not determine whether promoter-linked fluorescence per cell is actually much greater than its constitutive counterpart. Further trials including measurements of bacteria concentrations are required to establish the relationship between constitutive and promoter-linked fluorescence.

3.5.6 Future work

Work is currently underway on a bench-scale soil column trial in order to investigate and model transport parameters within amended bioremediation microcosms. Figure 3.26 shows that two glass columns were set up in parallel for use in comparative experiments. Various openings are available throughout the entire column so that its pressure can be monitored and fiber probes can be inserted to record fluorescent measurements. The target bioremediating bacteria can be introduced to the microcosms via fluid pumped from the bottom of the column. This approach should allow us to maintain complete saturation and stable pressure within the columns. In this proposed experiment, we hope to demonstrate the use of a novel fiber-optic \textit{in situ} fluorescence monitoring technique as a viable real-time method for monitoring biological activity, dispersion and gene expression of the bioremediating bacteria in sub-surface environments.
3.5 Discussion

Figure 3.26: Soil column setup showing empty column (left) and column packed with silica sand (right). The column has 6 pressure monitoring ports where pressure transducer components are attached, and four sampling ports for fluorescent measurements.

Although the exact spatial resolution of the fiber probe is unknown it can be quantified by measuring the fluorescence collection volume. We currently know factors affecting the resolution include fiber size, concentration of fluorophores [133], and the type of medium it is in. Further experiments are required to determine exactly how each of these factors contribute and affect the resolution. Due to its fine resolution, the fiber optic probe gives a localized measurement only, it is necessary to take various readings at different locations within the contaminated site. The overall effectiveness of the employed bioremediation technique can then be based on the collected statistical data.

To determine whether the amount of fluorescence emitted by the bacteria of interest is above the detection limit, it will be necessary to conduct preliminary trials before an actual in situ bioremediation monitoring experiment takes place.

Because our technique requires an excitation source, background signal or autofluorescence is unavoidable. In weakly fluorescent samples this can lead to ambigu-
ous results as background cannot be effectively removed. In this respect, we have also begun to investigate the possibility of using bioluminescent bacterial species. Since there is no background, the accuracy is only limited to the sensitivity of the detector.

Since fluorescence can be associated with a certain bacterial gene expression, this is a novel real time way to monitor biological activity of a bioremediation system via gene expression at a very fine level rather than by just looking at the “before and after” performance which is the typical approach that does not show exactly what the bacteria is doing, how and why. This new technique monitors the response of bacterial activity in real-time as we make changes to the degradation system by looking at the level of fluorescence in a particular strain. Furthermore, as discussed above in the proposed soil column experiment, it allows the comparison between two bioremediation systems by using the same bacteria simultaneously while comparing their fluorescence to the predicted model behavior.

It is currently unknown whether the genetic transcription of fluorescent protein places increased energy demands on labeled organisms thus affecting their performance and reducing comparability to wild-type strains for bioremediation applications. Future experiments should include the comparison of biodegradation performance of wild-type to fluorescent protein labeled bacteria.

Once this optical bioremediation technique has matured, it should be possible to devise a standardized series of experiments such that just by following protocols, the bacterial species of interest can be completely characterized in terms of its growth curve, emission spectra, brightness, effectiveness in biodegradation, etc.

Further applications in molecular biology and environmental engineering should also be explored such as biosensors for detecting estrogen-like molecules and protein biomarkers [149].
3.5 Discussion

3.5.7 Limitations

Currently, in terms of limitation of the system, we are affected by the reduced collection volume when measurement is conducted in soil. This, coupled with variation in background fluorescence can limit the capacity to detect the presence of protein labeled bacteria. Therefore the key features in further developing this optical biosensing approach are as follows.

1) Increase the fluorescence yield of bacterial species via the establishment of a superior protein labeling method.

2) Increase the collection volume of the probe possibly via a light gathering lens mounted to the fiber tip.

3) Develop a dual fiber probe in which the overlap between excitation and collection volume is considerable.

4) Examine the possibility of using bioluminescent bacterial species as this will eliminate background fluorescence completely.

In terms of limitation of the study, although bacteria can be labeled with fluorophores to report on the activity of interest, the amount of fluorescence to the level of activity may change over time due to mutation or evolution. This may limit our precision if we attempt to quantify the gathered results because the relationship between fluorescence intensity and level of activity is never determined for certain.

At last, fluorescent bacterial species are genetically engineered in a way that their use in actual “field applications” would never be permitted. They are only cultured in confined facilities under controlled conditions. Although they will never be employed in an actual field application they are excellent for investigation into designing model based bioremediation systems and optimization of such models.
3.6 Conclusion

In this chapter, we have outlined a novel optical spectroscopic detection system and techniques that can monitor the degradation activity of micro-organisms via fluorescence expressed by promoter linked genes. Moreover, results gathered from the preliminary trials have established the feasibility of this approach. Following are concluding remarks regarding the objectives and outcome of this research.

The initial goal of our research was to monitor the bacterial activity of a degrader that contains a fluorescent protein gene linked to growth and a different color fluorescent protein gene linked to degradation activity. While novel techniques for genetic transformation suggest that there is a strong potential for the creation of a biosensor organism with both constitutive and activity linked fluorescence, these methods have not yet been perfected in the \textit{P. putida} 852 strain. Although such bacteria were not created, we are still convinced that our fluorescence system is capable of the proposed task through the results of related experiments such as the growth curve of bacteria plotted via the time series measurements and the ability to resolve spectra of mixed fluorescent protein samples.

We have successfully demonstrated that our fluorescence system provide biologists with a technique for monitoring the complex range of bacterial behaviors such as growth and degrader responses. Controlled experiments in batch trials are essential in developing an understanding of how bioremediation processes work. Optimizing bioremediation in batch trials is a fundamental precursor of its progression into successful field applications. The results and findings from in this study provide proof of concept of a novel optical technique to assist the understanding and optimization of bioremediation. This is an important step towards the goal of achieving a complete \textit{in situ} biodegradation monitoring system that could provide information related to activity, viability and other various aspects in defining each bioremediation strategy. In terms of future research, areas required includes the refinement of this technique and pseudo bioremediation trials that mimic actual field applications.
Only then can we develop a comprehensive understanding of this technique that is mature enough to be widely adopted.
CHAPTER 3. In Situ spectroscopy monitoring techniques of bioremediation
Chapter 4

Spectrally resolved measurement of cardiac action potentials

4.1 Introduction

Optical techniques are being used increasingly to study cardiac function of the intact heart at cellular level. For instance, measurement of membrane potential using fast stryl dyes such as di-4-ANEPPS [54] and more recently di-4-ANBDQBS [145, 98], while intracellular release of calcium using a variety of ratiometric and non-ratiometric calcium indicators [73, 88]. Fluorometric systems have employed a variety of light sources such as tungsten halide, UV, lasers and LEDs. Optical filters are used for band separation, while CCD and photodiode arrays have been used for spatially resolved imaging systems. In some cases, dual measurements of membrane potential and intracellular calcium concentration have been demonstrated [26]. For the most part, surface imaging has been employed using these techniques. However, fiber optic probes have been used to record optical potentials and calcium release signals at individual [106] and multiple sites [63, 19, 18, 131] intramurally. Unlike surface measurement systems which image light scattered from a variety of sites within the tissue, fiber optic probes emit and collect light from a limited collection volume. Such fiber optic probes can be incorporated into all-fiber systems employing solid state lasers to excite fluorescence and are easily interfaced with a
fast spectrometer.

Spectroscopic imaging, as described in Chapter 3, provides a flexible way of extracting more than one functional fluorescence signal from specific intramural sites. Fast and relatively inexpensive spectrometers have become available over the past five years. This means that it is possible to acquire sequential spectra at a sampling rate sufficient to resolve the cardiac action potential. In addition, solid state lasers and LEDs now provide a wide range of different excitation wavelengths. The purpose of the work described in this chapter was to demonstrate the utility of spectral imaging with this context and to investigate the spectral characteristics, and responses of di-4-ANEPPS-stained myocardial tissue over a range of excitation wavelengths.

4.1.1 Cardiac action potential

In quiescent cells, charge is separated across the cell membrane, with positive charge on the outside and negative charge on the inside. The membrane potential is the electrical potential difference across the cell membrane. Normally, the resting membrane potential is between -50 and -90 mV with respect to the extracellular potential. In excitable cells (nerve and muscle), short-lived local electrical stimulation can give rise to an action potential in which the cell membrane depolarizes and then repolarizes or returns to its resting state. This is due to changes in voltage and time-dependent transmembrane ion channel conductivity as well as the operation of ion pumps and transporters in the cell membrane [75]. Typically, the action potential will propagate along the membrane of individual cells (in the case of nerves or skeletal muscle) or across multiple electrically coupled cells (cardiac muscle or smooth muscle). In nerves, action potentials transmit frequency encoded information and in muscle they trigger the intracellular release of calcium ions, which causes contraction.
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Figure 4.1: Typical example of a cardiac action potential showing five distinct phases. 0: Rapid depolarization. 1: Early repolarization. 2: Plateau. 3: Repolarization. 4: Resting state.

The different “phases” of an action potential cycle for ventricular myocytes are shown in Figure 4.1. The cardiac action potential is customarily subdivided into five distinct phases: 0: Rapid depolarization, 1: Early repolarization, 2: Plateau, 3: Repolarization, 4: Resting state. The biophysical basis of each is well understood [107].

Figure 4.2: Left: Schematic of the structures of the heart. LA, RA, LV and RV refer to left and right atrium, left and right ventricle respectively. Right: Schematic of action potential waveforms in different regions of the heart. Action potentials are displaced in time to reflect the temporal sequence of propagation [107].

Normal cardiac electrical activation involves the coordinated spread of depolar-
ization of cells throughout the heart (see Figure 4.2). The sino-atrial (SA) node on
the upper surface of the right atrium (RA) is the pacemaker that drives this process.
Cells in this region spontaneously generate repeated action potential which subse-
quently propagates throughout the cardiac chambers where they trigger atrial and
ventricular contraction. The right panel of Figure 4.2 shows the temporal relation-
ship of cardiac action potentials at their respective heart regions. The electrocar-
diogram (ECG) is shown on the bottom right panel reflects the potentials generated
at the body surface by the electrical activity of the heart. The P wave represents
atrial depolarization, the QRS complex represents ventricular depolarization and
the T wave represents ventricular repolarization. Although we can correlate ECG
with the action potential waveform, its spatial resolution is limited when compared
to optical recordings. This is because the surface potential of ECG is due to current
flows over a relatively large volume [61, 67, 126].

4.1.2 Recording cardiac electrical activity with membrane-bound fluorescent dyes

As stated previously, cardiac action potentials can be recorded using fast potential-
sensitive dyes such di-4-ANEPPS and di-8-ANEPPS bound to the cell membrane.
This can be achieved because the spectral characteristics of these dyes are modu-
lated by changes in transmembrane membrane under these circumstances. Typical
absorption and emission spectra of di-8-ANEPPS bound to phospholipid bilayer
membranes (similar to di-4-ANEPPS) are shown in Figure 4.3 [69]. At peak exci-
tation efficiency (around 480 nm), depolarization produces a shift of the excitation
spectrum toward shorter wavelengths [80]. It has also been shown [48] that the
excitation spectrum undergoes a similar "blue" shift with depolarization. The shift
of the emission spectrum (see Figure 4.4) with depolarization demonstrates the ra-
tiometric characteristics of these dyes. That is, there is a wavelength (the isosbestic
4.1 Introduction

Figure 4.3: Absorption and emission spectra of di-8-ANEPPS (similar to di-4-ANEPPS) bound to phospholipid bilayer membranes [69].

point) at which emission is unchanged. Below this wavelength, depolarization increased fluorescent emission, while emission is reduced above it.

Photobleaching and uneven dye distribution are problems associated with most fluorescent imaging techniques. Ratiometric imaging provides an established method for calibrating fluorescent signals and correcting for the effects of photobleaching or motion artifact. All three will be discussed in more detail in the sections that follow.

Figure 4.4: Fluorescence emission spectrum and spectral response of di-4-ANEPPS during an action potential in rabbit heart recorded with a spectrofluorometer. A: Relative intensities at various wavelengths during diastole. B: Fractional change in intensities during action potential phase-zero depolarization [80].
4.1.3 Origins of photobleaching

With most dyes, the intensity of fluorescence emission decreases in a multi-exponential fashion which is dependent on the excitation wavelength and power. This phenomenon is known as photobleaching. In the case of transmembrane voltage-sensitive dyes, progressive internalization of the dye to the inner leaflet of the membrane contributes to a decline in effective concentration [80]. Depending on the objective of the experiment, this phenomenon usually does not pose a major problem as it is a slow time-varying function relative to the fast events of action potentials.

4.1.4 Origins of motion artifact

Motion artifact arises from the movement or deformation of the heart wall during each cardiac cycle and it presents a major problem in the optical mapping of cardiac electrical activity. The contraction and associated heart wall motion affects the fluorescence intensity detected thus introducing artifacts to the recovered action potentials. Although the characteristics of motion artifact are very similar for intramural and surface measurements, their origins are somewhat different. Optical imaging of the surface of the heart is carried out with a stationary camera and the heart moves with respect to this frame of reference when it contracts. Therefore the region of the heart addressed by any pixel can alter throughout the cardiac cycle. A further source of the artifact is the fact that the extent of staining varies regionally across the heart and contraction gives rise to variation in fluorescent intensity across individual pixels that has nothing to do with functional modulation of the emission spectrum. Planar transformation of successive images to preserve the registration of key structural features provides a means of correcting for physical translation of the heart regions [118, 129]. That is, if we have separate images of the heart surface $A$ and $B$ which differ as a result of contraction, it is possible to correct for the effects of heart wall motion using established image registration methods that transform $B$ to best match $A$. Svreck and co-workers [128] have shown that it is possible to
suppress motion artifact substantially using this image registration approach. However, motion artifact cannot be removed completely because 3D heart motion is being projected as a 2D translation. For intramural measurements, the origin of motion artifact is less straightforward as the optical probe moves along with the contracting heart wall [63]. While translation of the myocardium with respect to the optical probe contributes to motion artifact, due to much higher resolution than 2D surface imaging, cell membrane deformation and redistribution of intramural vascular volume associated with contraction may also play a role [133]. Moreover, wavelength dependent processes such as absorption, transmission, and scattering of excitation and fluorescent light by the tissue are also likely to vary throughout the cardiac cycle [15].

In optical recording of cardiac electrical activity, motion artifact is usually suppressed either mechanically or pharmacologically to prevent masking of the signals. For epicardial surface imaging, the heart can be stabilized mechanically by pressing it against the imaging window which minimizes gross movement of the heart during contractions [39, 87]. Such mechanical strategies have the advantage of avoiding any electrophysiological side effects associated with pharmacological suppression of cardiac motion, however they are better suited for short or intermittent rather than continuous recordings. In particular, for the example mentioned above, this procedure can lead to local ischaemia if maintained for long periods (>10s) [74, 120].

For intramural measurements, motion artifact is usually suppressed pharmacologically using mechanical uncoupling agent such as 2,3-butanedione monoxime (BDM), cytochalasin D or blebbistatin as physical stabilization is less effective and more difficult to employ [18, 63, 43, 151]. Although BDM has no significant effects on takeoff potential, action potential amplitude, $dV/dt_{max}$ (only for concentrations below 10 mM/L [9, 28]), and peak plateau amplitude, it can significantly shorten action potential duration (APD) in a dose dependent manner [9]. This is because to various extents, they can affect transmembrane ion channel kinetics. It starts from concentrations as low as 5 mM/L to one that result in an almost complete elimination of
the isometric force. Therefore, methods that enable action potentials to be retrieved reliably in the absence of BDM or even at low concentrations of it are of great interest in the study of electrical repolarization of the heart. The technique of ratio imaging fits this criterion as it suppresses motion artifact substantially via signal processing and for this reason, it is commonly employed.

4.1.5 Ratio imaging

Optical recordings of ratiometric dyes are often used for the correction of unequal dye loading, photobleaching, and motion artifact as the ratio does not depend on the absolute intensity of the signal [7, 15, 18, 63, 80]. Ratiometry exploits the fact that, with dyes such as di-4-ANEPPS, it is possible to acquire simultaneous recordings in which modulation by the "signal" of interest (in this case membrane potential) is opposite in direction, while "noise" due to photo-bleaching or motion artifact is common in both. Under these circumstances, formation of a ratio will amplify the signal and attenuate the noise.

Ratiometry can be performed in two ways, Excitation ratiometry usually involves dual sequential excitations, above and below the isosbestic wavelength in the excitation spectrum, while emitted light is collected in one detection band using a synchronized photodetector. For instance, Bachtel and co-workers [5] measured membrane potentials using di-4-ANEPPS with pulsed LEDs at blue (450+10 nm) and cyan (505+15 nm) with a CCD camera. Lee and co-workers [89] made simultaneous measurements of membrane potential and intracellular free calcium using di-4-ANBDQPO and Fura-2, respectively. They employed LEDs at four different wavelengths for the excitation of the two ratiometric dyes while detection was achieved with a camera synchronized to all LEDs. Emission ratiometry, on the other hand, involves excitation at a single wavelength and detection of dual spectral bands, above and below the isosbestic wavelength in the emission spectrum. Examples of this technique are outlined in a number of references [80, 63, 131]. In these cases, membrane potentials were measured using di-4-ANEPPS with an excitation wavelength
4.1 Introduction

at 488 nm. This is close to the peak of the excitation spectrum and it provides sufficient bandwidth between emission isosbestic point and excitation wavelength to separate short and long wavelength bands easily.

Although ratiometry provides a simple means of minimizing the effects of photobleaching and motion artifact [15, 63, 80], when heart wall motion is substantial, full recovery of voltage-dependent signal is often not possible [63]. Brandes and co-workers [15] have shown that signal components due to motion were not simply proportional in the short and long wavelengths which suggest that motion artifact cannot be completely removed with ratiometry.

Another important aspect of ratio imaging is that if properly calibrated, it can be used to make quantitative measurements [133]. This is because the signal is measured as a function of spectral shift rather than the absolute intensity. The spectral shift is generally measured by comparing the fractional change of power below and above the isosbestic point of the excitation or emission spectra. The fractional change of fluorescence $\Delta F/F$ within the specified spectral window gives the membrane potential which is independent of dye concentration and excitation power. It is given as

$$\Delta F/F = (F_p - F_r)/F_r$$

(4.1)

where $\Delta F/F$ is commonly known as the fractional fluorescence, $F_p$ is the fluorescence signal at the peak of depolarization and $F_r$ is the fluorescence signal at the resting state.

4.1.6 Validation of intramural optical technique

Salama and co-workers pioneered the use of membrane potential-sensitive dyes to map electrical activity in the heart [121]. They compared optical signals with extracellular potential measurements at adjacent sites on the epicardial surface of the
heart and demonstrated a close resemblance between the timing of depolarization and repolarization. Moreover, several studies have demonstrated that the fluorescent signal from voltage-sensitive dyes such as di-4-ANEPPS is not only proportional to changes in the transmembrane potential but is also sufficiently fast to reproduce this fast upstroke of the cardiac action potential during depolarization. Several groups [148, 42] have measured optical upstrokes in single isolated cells that are as fast as a few hundred microseconds, which is comparable with the upstroke velocities recorded with microelectrodes.

Investigations initially carried out by Caldwell and co-workers [19] and then by Tai and colleagues [133] are of greater relevance to this study since validation was performed intramurally with a fiber optic probe. In the first of these studies, optical potentials at sites through the LV wall in a pig heart were compared with adjacent extracellular potential measurements. Although the correspondence between activation times estimated with both techniques was good, greater scattering was observed in the repolarization times recorded optically. It is believed that these differences are due to the spatial resolution of the two techniques. While optical recordings address only a small number of cells, extracellular potential measurements are integrated over a much larger tissue volume [19]. Tai et al [133] reported the rate of rise at the action potential upstroke is consistently twice as great for intracellular action potentials than for optical recordings. Similar results were also observed across a few comparable studies [102, 106, 51]. It is argued that the lengthening of the depolarization time in optical measurements compared with intracellular measurements is due to the weighted average of individual action potentials over a greater number of cells [66, 51].

4.2 Methods

4.2.1 Isolated heart preparation

Rats were anesthetized and their hearts were rapidly excised and immersed in cold (4°C) saline. The aorta was cannulated, and the heart was mounted on a Lai-
4.2 Methods

gendorff system. It was perfused with modified Krebs-Hensleit solution containing (in mM) NaCl 118, KCl 4.75, MgSO4 1.18, KH2PO4 1.18, NaHCO3 24.8, Glucose 10, CaCl2 2.5 in room temperature at a constant pressure ∼80mmHg. The hearts were stabilized for 30 minutes. The coronary circulation was loaded with the voltage sensitive dye, di-4-ANEPPS (Molecular Probes) at ∼100 µM for 5 minutes. When required, mechanical activity was suppressed by adding the electromechanical uncoupler 2,3-butanedione monoxime (2,3 BDM) to the perfusate to achieve concentrations in the range of 0-10 mM.

4.2.2 Optical layout

A schematic diagram of our fiber optic fluorescent setup is shown in Figure 4.5. The delivery of excitation light and collection of fluorescence was achieved using high OH−, multimode, optical fiber (OFS fiber, CF04406-03), which resulted in efficient power transmission in the ultraviolet (UV) through to the visible light range. The fiber core, cladding and coating diameters were 200, 220 and 250 µm diameter respectively with a numerical aperture (NA) of 0.22. As the attenuation across the visible range is less than 14 dB/km and the fiber length used was less than 10 m, the loss of power was negligible. The excitation source used was a 473 or 532 nm diode-pumped solid-state (DPSS) laser (tunable to 200 mW, noise < 3%, Beijing Viasho Technology Co., Ltd.). Light from this laser was injected into a 2x2 multimode fiber coupler (excess loss 0.5 dB, Opneti Communications Co., Ltd) via an injection stage. The shutter, controlled by a DAQ card (USB1608FS, Measurement Computing Co.), was placed in front of the injection stage to synchronize the exposure time of the sample to the spectrometer so that photobleaching could be minimized. Half of the excitation light was guided to the sample via one of the output arms of the coupler while the balance was directed through the other arm to a photodiode for monitoring laser noise. A fraction of the fluorescence from the illuminated sample was collected at the tip of the excitation fiber and returned to a compact fiber-coupled spectrometer (HR2000+, 2048 pixels across 410-850 nm, A/D resolu-
tion 14 bit, up to 1 kHz sampling frequency, 41 photons/count at 600 nm, Ocean Optics, Inc.) via a $2 \times 2$ (50/50) multimode fiber coupler. A colored glass long pass emission filter (FGL495S or FGL550S, Thorlabs) was used to suppress the laser line. The spectrometer was connected to a computer via USB connection, the setup can perform data acquisition and analysis in real-time if necessary. For portability and robustness, the assembly was mounted on a small breadboard which could be enclosed in a 19” light-proof box.

Figure 4.5: Time-resolved fluorescence spectroscopy setup. DPSS is acronym for diode pumped solid state laser.

4.2.3 Data acquisition & signal processing

A graphical interface was written in the LabVIEW™ programming language (National Instruments Corp). Spectra were recorded continuously for six seconds, then saved, processed and displayed before starting the next acquisition cycle. We opted to acquire data in this sequential quasi real-time manner because it enabled us to achieve high burst sampling rates. For each spectrum, the following were determined and presented as a time series (i) short and long wavelength power, estimated by integrating spectral intensity across specified short and long wavelength bands (ii) total power, estimated from the integral of spectral intensity across all wavelengths, and (iii) amplitude corrected short and long wavelength power, constructed by dividing short and long wavelength power by total power. Amplitude correction minimizes the effects of temporal variation in overall spectral intensity due, for instance, to photobleaching and/or laser noise. Finally, frequency analysis was performed on the
total power signal and a running average of the spectrum was also displayed.

4.2.4 Spectral characterization

In conventional emission ratiometry, the functional signal is recovered by taking the ratio of the fluorescence integrated over short and long wavelength windows. Here, we have the entire emission spectrum with sub-nanometer resolution at our disposal and this has enabled us to characterize the emission spectrum during the cardiac action potential more comprehensively. Amplitude modulation and spectral shift can be determined throughout the cardiac cycle and fluorescent signals can be integrated across specified wavelength windows. This provides a powerful means of separating and analyzing spectral changes associated with membrane potential changes, photobleaching and heart wall motion.

Amplitude modulation is, by definition, the proportional scaling of spectral components across all wavelengths. Amplitude modulation was removed by normalizing successive spectra with respect to the integral of the spectrum across all wavelengths. This reduces "common mode" and enabled frequency modulation to be characterized.

4.3 Results

4.3.1 Spectral characteristics of di-4-ANEPPS in LV myocardium with 473 nm excitation

The objective of the work described in this section was to characterize optical "potentials" in the absence of mechanical activity, to establish a baseline data. Wall motion was suppressed by adding 2-3, BDM incrementally to the perfusate until cardiac movement was abolished. Experiments were carried out at using a 473 nm DPSS laser set to 10 mW as the excitation source.
4.3.1.1 Photobleaching

Figure 4.6 shows the effect of prolonged exposure to 473 nm excitation on di-4-ANEPPS-stained LV myocardium. Spectra for the resting state (immediately prior to the onset of the cardiac action potentials see Section 4.3.1.2) acquired at the same site after 0, 5 and 10 seconds of sustained excitation are superimposed. The spectral shape is approximately Gaussian bounded within the range 480 to 780 nm with a peak at around 590 nm. These curves reveal that as the emission spectrum decays, a subtle spectral drift is occurring towards the red with a shift of around 2 nm over a period of 10 seconds. The total power signal over this period is shown in Figure 4.7. Fluorescent emission declines rapidly for the first 100 ms followed by an exponential decay with a half-life estimated at around 25 seconds. The decay indicates that photobleaching is taking place and the fact that there is no evident cyclic variation suggests mechanical activity is minimal. This decay induced by photobleaching typically does not pose a problem as this is a slow time-varying phenomenon compared to the fast events of action potentials. One may choose to remove this effect first by characterizing the decay envelope then subsequently subtracted it from the recovered action potential signal [131].

Figure 4.6: Emission spectra of di-4-ANEPPS at 0, 5 and 10 seconds of exposure time showing the effects of photobleaching. Spectra shown are for resting state with excitation at 473 nm and an integration time of 1 ms. Left: Relative spectra. Right: Normalized spectra.
4.3 Results

Figure 4.7: Effects of sustained excitation at 473 nm on the total power signal (fluorescence emission) for di-4-ANEPPS-stained LV myocardium.

4.3.1.2 $V_m$ modulation

Short and long wavelength power signals are shown in Figure 4.8 (these are integrated across wavelength bands of 500-580 nm and 590-750 nm, respectively, see Figure 4.9). Modulation of the emission spectrum by cardiac electrical activity is evident in the action potential deflections that are evident in both signals. The faster decay in the short wavelength signal is consistent with a time-varying red shift in the emission spectrum due to photobleaching (see Figure 4.6).

Normalized emission spectra for resting and depolarized states are superimposed in Figure 4.9A. This shows that, after the removal of amplitude modulation, a spectral shift of around 1-2 nm occurs between the resting and depolarized state. Also indicated are the approximate wavelength bands used to construct short and long wavelength signals. Figure 4.9B shows the corresponding fractional fluorescence $\Delta F/F$ and difference in fluorescence $\Delta F$. Maximum and minimum $\Delta F$ are at around 560 nm and 620 nm, respectively. The maximal fractional fluorescence appears to lie close to the edge of the spectrum, but SNR in this range is poor. Conversely, noise is least for signals close to the isosbestic point, but $\Delta F/F$ is minimal. A spectroscopic system such as ours provides the freedom to adjust the spectral bands over which signals are integrated, thus allowing SNR to be optimized. Figure 4.9C shows amplitude-corrected short and long wavelength signals. Compared with the substantial decays seen over the same time in the raw short and long wave-
length signals (21% and 15%, respectively, over 5 seconds, see Figure 4.8), removal of amplitude modulation substantially reduces the effects of photobleaching. The corresponding decay in Figure 4.9C is 5% and -4% reflecting a time-varying difference in relative rates of decay in short and long wavelength bands. Photobleaching occurs more rapidly in short than long wavelengths, consistent with previously reported [133, 80] decay characteristics and as indicated in Figure 4.5. It is noteworthy that the decay in the short wavelengths is much faster for the first 100 ms or so.

Careful examination revealed some negative amplitude modulation (0.4%) between the resting state and peak depolarization. This was confirmed by the timing of the small deviation in the total power signal that aligns with the upstroke of action potentials recovered as depicted by Figure 4.10. The bottom panel of the figure shows not only a blue shift in the spectrum, but also a minute amount of negative amplitude modulation during depolarization. This is not evident in the spectra as shown above in Figure 4.9, since they are amplitude corrected.

Figure 4.8: Normalized raw signals of the short and long wavelengths.
Figure 4.9: Comparison of normalized emission spectra at resting state and peak depolarization with excitation at 473 nm. A: Normalized emission spectra for resting state (black trace) and peak depolarization (gray trace). B: Corresponding difference $\Delta F$ (gray trace) and fractional fluorescence $\Delta F/F$ (dotted trace) as a function of wavelength. C: Corresponding amplitude-corrected short (black trace) and long wavelength (gray trace) signals.
4.3.2 Spectral characteristics of di-4-ANEPPS in LV myocardium with 532 nm excitation

In this section, the experimental procedures were identical to those already outlined for 473 nm excitation.
4.3 Results

4.3.2.1 Photobleaching

In Figure 4.11, we present total power as well as short and long wavelength signals during sustained exposure of di-4-ANEPPS stained LV myocardium to 532 nm excitation (their corresponding emission spectra are shown in Figure 4.12). These signals show the effects of electrical activation with regular negative deflections in each of 9% at regular intervals and this is discussed in the next section. However, the decay envelope in all three signals is less than 5% over 5 seconds, demonstrating that photobleaching with 532 nm excitation is much less than with 473 nm excitation. This is consistent with previous reported results [133]. Moreover, it was not possible to distinguish any difference in the extent of decay over 5 seconds in short and long wavelength signals at 532 nm excitation.

Figure 4.11: Integrated signals from a di-4-ANEPPS stained rat heart with excitation at 532 nm. A: Signals integrated from the entire spectrum. Short (B) and long (C) wavelength signals constructed from their respective bands (see Figure 4.12).
4.3.2.2  $V_m$ modulation

As has already been stated, regular negative deflections of 8-12% associated with cardiac electrical activation are evident in both short and long wavelength signals (see Figures 4.11B & C, respectively). This indicates that membrane potential modulation of fluorescence at 532 nm excitation is similar in short and long wavelength bands, although deflections in the long wavelength band were always around 3% greater than in the short wavelength band. Relative emission spectra for resting and peak depolarized states are superimposed in Figure 4.12A. The wavelength windows (540-590 nm and 610-740 nm, respectively) used to construct the short and long wavelength signals are indicated. These results show that it is amplitude modulation rather than frequency shift that plays the dominant role in producing changes in fluorescence emission with membrane depolarization at 532 nm excitation. Figure 4.12B presents the corresponding $\Delta F$ as a function of wavelength and the absolute magnitude of $\Delta F$ is greatest at 615 nm. Normalized emission spectra for resting and depolarized states are shown Figure 4.12C. This suggests that there is also a slight blue shift with membrane depolarization and this is reinforced in Figure 4.12D where the corresponding $\Delta F$ is plotted as a function of wavelength.

The relative effects of amplitude and frequency modulation at 532 nm excitation are demonstrated in Figure 4.13, where amplitude-corrected short and long wavelength signals (see Figures 4.13 A & B, respectively) are compared with the total power signal (see Figure 4.13C) The change in fluorescence $\Delta F$ at peak depolarization for the short and long wavelength bands is around 2% and -1%, respectively, while the corresponding change in the peak power signal is around -9\%.
Figure 4.12: Emission spectra of action potentials upon 532nm excitation. A: Resting state and peak of depolarization. B: $\Delta F$ of A. C: Normalized spectra of A. D: $\Delta F$ of C.
4.3.3 Comparison of fluorescence emission at different excitation wavelengths

In terms of pump power, excitation of di-4-ANEPPS in the blue region [80, 17] provides high fluorescence efficiency as this is where the peak absorption lies. Excitation in the green region offers advantages over the blue region, such as cheaper light sources and less photobleaching [143, 108, 127]. However, the responses of the dye, to different excitation wavelengths have not been studied in detail in the heart as far as we are aware. Here, we compare the spectral responses of di-4-ANEPPS stained LV myocardium to depolarization at excitation wavelengths of 473nm and 532 nm and add comparable results acquired by Tai and coworkers [133] for 488 nm excitation. In all cases, 2,3 BDM was added to the perfusate at concentrations to suppress mechanical activity.

Relative and normalized emission spectra with excitation at 473 nm and 532 nm are superimposed in Figure 4.14. Excitation efficiency at 473 nm was around 4 times
4.3 Results

Figure 4.14: Comparison of relative (Left) and normalized (Right) emission spectra with excitation at 473 and 532 nm from a di-4-ANEPPS stained rat heart in the absence of heart wall motion.

greater than at 532 nm. The normalized spectra show that there is a red shift of the emission spectrum when the excitation wavelength is increased from 473 and 532 nm, with peak emission shifted from around 585 to 600 nm.

We have already analyzed the differing extents of amplitude and frequency modulation with membrane depolarization at excitation wavelengths of 473 nm and 532 nm.

In summary, with 473 nm excitation there was a significant spectral shift with peak depolarization toward shorter wavelengths as well as a small reduction in amplitude (<1%). On the other hand, at 532 nm excitation the spectral shift with peak depolarization was minimal, but the amplitude reduction was substantial (~9%). From Tai's data [133], we estimate a 3% reduction in total fluorescence during peak depolarization with changes of +1.65% and -4.4% for the short and long wavelengths, respectively. This is consistent with the results of previous studies [80, 133] using 488 nm excitation in which the amplitudes of action potential signals were much less pronounced in short than long wavelength windows. These trends are represented in Figure 4.15, where $\Delta F$ associated with a shift in membrane potential from resting state to peak depolarization is plotted as function of emission wavelengths for excitations at 473, 488 and 532 nm. This indicates that there is a progressive shift in emission characteristics with depolarization from spectral shift to amplitude modulation for increasing excitation wavelengths. As a result, although the excitation
efficiency is much less with 532 nm than with 473 nm excitation, the fractional fluorescence with depolarization is markedly greater. On the other hand, the ratiometric characteristics of fluorescent emission with 473 nm and 488 nm excitation can be exploited to amplify the response of the dye to depolarization. However, this requires optimization of the wavelength ranges over which short and long wavelength signals are integrated.

Figure 4.15: Comparison of normalized $\Delta F$ (fitted curves) at the excitation wavelength of 473 nm, 488 nm and 532 nm. Note that the curve for 488 nm was based on data extrapolated from Tai’s results [133].

4.3.4 Optimization of action potential signals

4.3.4.1 Choosing appropriate spectral bands

We have previously shown that when choosing the wavelength bands for short and long wavelength signals at 473 nm excitation, there is trade off between obtaining large fractional fluorescence $\Delta F/F$ and maximizing SNR. In our optical setup, it is possible to adjust the boundaries of short and long wavelength windows to optimize SNR. The upper and lower boundaries of short and long wavelength windows were varied systematically and optimal settings were defined as those which gave rise to the greatest SNR across the wavelength band for a typical action potential.
4.3 Results

Figure 4.16 shows the SNR for an action potential in the short wavelength window is greatest when lower and upper wavelengths are set to 510 nm and 580 nm, respectively. Likewise, SNR in the long wavelength window is optimized for a wavelength band from 590 nm to 700 nm. As no spectral shift is induced at the isosbestic wavelength, it should not be encompassed within either short or long wavelength windows. The analysis above suggests that the isosbestic wavelength lies between 580 nm and 590 nm.

![Figure 4.16: A: SNR of the short wavelength window by fixing the lower boundary while varying the upper boundary. B: SNR of the short wavelength window by fixing the upper boundary while varying the lower boundary. C: SNR of the long wavelength window by fixing the lower boundary while varying the upper boundary. D: SNR of the long wavelength window by fixing the upper boundary while varying the lower boundary.](image)

4.3.4.2 Reduction of noise via low-pass filtering

The sampling rate required for adequate digital representation of any signal is determined by its frequency content. Girouard and co-workers [51] have shown that 99% of the total energy in the action potential recorded with a microelectrode is contained in frequencies below 150 Hz. Therefore it is reasonable to assume that the
action potentials recorded with our optical system will be captured within a bandwidth of 150 Hz. The results in Figure 4.17 are consistent with this. The sampling rate for our system was 1 kHz and this means that SNR could be improved by using low-pass filter to attenuate noise above 150 Hz. However because depolarization occurs within a few milliseconds, filtering too close to 150 Hz may delay during the upstroke of the action potential.

Figure 4.17: Normalized frequency components of action potentials compared to noise floor.

4.3.4.3 Beat averaging

Because action potentials signals have very similar morphology in successive cardiac cycles, it is standard practice to employ beat averaging to improve SNR [133]. Signals can be synchronized across successive cycles by aligning the maximum first derivative \((dF/dt)_{\text{max}}\). Figure 4.18 shows an averaged action potential signal \((n = 6\) and \(\text{SNR} = 40\)). In this case, short and long wavelength signals with optimized wavelength bands were acquired at 473 nm excitation and low-pass filtered with a cutoff frequency of 200 Hz. These were further processed using the subtraction technique outlined by Tai [133]. For both short and long wavelength signals, the effects of decay were corrected and steady state offset was removed before subtracting long from short wavelength signal. The optical potential presented here is very similar to
4.3 Results

Figure 4.18: Recovered action potentials of a rat heart via optimization of spectral bands, low-pass filtered, and six beats averaged. SNR of 40 was achieved.

action potentials that have been directly measured in the rat heart. The rise time of the action potential upstroke is 4 ms and $APD_{50}$ (the time for which the membrane potential is greater than 50% of the maximum deviation from resting levels during activation [133]) is 21 ms.

4.3.5 Motion artifact

Motion artifact arises from the mechanical activity of the heart as discussed in Section 4.1.4. Much of this is manifest in the form of amplitude modulation (see Figure 4.19) although mechanical activity can also give rise to wavelength shifts in the emission spectrum. This is shown in Figure 4.20, where the normalized emission spectrum can also show a red shift in the presence of heart wall motion as oppose to only a blue shift when depolarization occurs.

Ratiometry [15] and subtraction [131] have been used to minimize the effects of contraction where the response of potential sensitive dyes is ratiometric (that is, membrane potential change produces a wavelength shift of the emission spectrum). For both techniques, short and long wavelength signals are formed in which signal components associated with electrical activity are opposite in sign, whereas components associated with motion are assumed to be temporally similar. With ratiometry, the short wavelength signal is divided by the long wavelength signal whereas, with
subtraction, the motion component in short and long wavelength signals is matched and then subtracted. In principle, correction of short or long wavelength signals for the effects of amplitude modulation should be equally effective in removing motion artifact.

Because spectral imaging enables us to characterize the effects of amplitude modulation and wavelength shifts separately, we hypothesize that it may enable more effective analysis of and correction for the effects of motion artifact. This is addressed as follows.
Figure 4.21: Recovery of action potentials in the absence of 2,3 BDM. A: Short and long wavelength signals B: Amplitude-corrected short and long wavelength signals C: Summation of amplitude-corrected short and long wavelength signals.

In Figure 4.21, we demonstrate that removal of amplitude modulation markedly reduces the effect of motion artifact in an experiment where 2,3 BDM was not used. Figure 4.21A shows uncorrected short and long wavelength signals with electrical activity completely masked by motion artifact. However, after removal of amplitude modulation the effects of heart wall motion are markedly reduced in short and long wavelength signals. The underlying features of the cardiac action potential are clear in amplitude-corrected short and long wavelength signals although some residual motion artifact remains, presumably due to contraction-related wavelength shifts. Summation of corrected short and long wavelength signals further reduces the motion artifact although at the cost of reduced amplitude of the electrical signals.

Figure 4.22 presents another case in which electrical signals in short and long wavelength bands are recovered from substantial motion artifact by correcting for amplitude modulation (see Figure 4.22B). In this case, though, the relative ampli-
CHAPTER 4. Spectrally resolved measurement of cardiac action potentials

Figure 4.22: Recovery of action potentials in the absence of 2,3 BDM. A: Short and long wavelength signals B: Amplitude corrected short and long wavelength signals C: Summation of amplitude corrected short and long wavelength signals.

Figures of action potential and residual motion waveforms are comparable in both amplitude-corrected short and long wavelength signals. Summation therefore attenuates action potentials and residual motion artifact equally (see Figure 4.22C).

An alternative approach of removing the residual motion artifact for the data in Figure 4.22 is illustrated in Figure 4.23. Here, because it is straightforward to identify the upstroke and approximate duration of the action potential, estimated motion throughout the cycle can be reconstructed from its characteristics during diastole prior to the upstroke [133]. In other words, the effectiveness of this technique depends largely on the clear separation between the frequency components of action potentials and motion artifacts. Reconstructed motion waveforms in amplitude-corrected short and long wavelength signals are given in Figure 4.23B. Figure 4.23C shows the effect of subtracting amplitude-corrected long from short.
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Figure 4.23: Recovery of action potentials for study in Figure 4.22. A: Amplitude-corrected short and long wavelength signals. B: Residual motion artifact estimated for amplitude-corrected short and long wavelength signals. C: Subtraction of amplitude-corrected short and long wavelength signals after removal of residual motion artifact.

In both cases, (see Figure 4.21 and Figure 4.23), the action potential duration $APD_{50}$ was measured as 40 ms compared to 20 ms (see Figure 4.18) when BDM was used to prevent movement. This was expected as BDM can significantly shorten APD in a dose dependent manner.

In both cases, the action potential duration $APD_{50}$ was measured as 40 ms compared to 20 ms (see Figure 4.18) when BDM was used to prevent movement. This was expected as BDM can significantly shorten APD in a dose dependent manner.

The analysis presented here demonstrates that it is possible to substantially remove the effects of photobleaching and motion artifact using amplitude correction. With conventional dual channel measurements this may be achieved with ratiometry or subtraction. However, with spectral analysis it can be done directly, and short and long wavelength signals are preserved. We have also shown that it is pos-
Figure 4.24: Recovered action potential after the removal of motion artifact in Figure 4.23. Left: Five superimposed action potentials. Right: Action potential beat averaged five times.

possible to characterize residual motion waveforms present after amplitude correction provided that the onset of the action potential can be distinguished. Therefore, the effectiveness of this approach relies on the clear separation between the frequency components of action potential and residual motion artifact. Subtraction of amplitude-corrected long wavelength from short wavelength signals after removal of residual motion artifact means that SNR can be further increased.

4.3.6 Action potential rise time

Action potential rise time is measured as the time taken for the upstroke to change from 10 to 90% of its maximum amplitude. Reported action potential rise times for optical mapping studies are commonly in the range of 5 to 30 ms [19, 63, 84, 6, 133, 38, 88], whereas the corresponding intracellular measurement in normal cells [51] is less than 1 ms. Optical signals are integrated over the fluorescence collection volume and the propagation of electrical activity through this volume is thought to blur the upstroke of action potential recorded in this way [51, 65, 133]. In this context, the effect of light scattering can be lumped together with absorption which reduces fluorescence collection, provided that the dimensions of the volume of interest are much greater than the scattering mean-free-path length [112, 156].

From literatures, there appear to be a direct relationship between reported action potential rise time and collection volume [133]. Caldwell et al [19] and Tai [133] reported rise times in the range of 5-8 ms with a fiber core diameter of 100 μm.
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Figure 4.25: Histogram of action potential rise times for a sample size of 80.

However, we have estimated rise times of $3.7 \pm 1.6$ ms with a fiber core diameter of $200 \, \mu$m. These results are summarized in Figure 4.25, where a histogram of rise times is given for 80 action potentials recorded in the absence of mechanical activity. Even in the presence of full mechanical activity, the rise time for Figures 4.21 and 4.24 was measured as 3.1 ms.

Figure 4.26 shows four unfiltered raw signals of action potentials with rise times less than 1 ms (left panel) and at 3.7 ms (right panel). When calculating the rise time, only raw data were used as filtering and/or averaging inevitably lengthens the rise time. For our data sets, rise times approaching 7 or 8 ms were a result of noise fluctuation and probably should be regarded as outliers. It seems likely that the four cases in which rise times less than 1 ms were also outliers, although the source of this is less clear. A possible explanation for this is that there is occasional jitter in the spectrometer sampling rates. However, this requires further investigation.
4.4 Discussion

In this study, we describe a fast spectrally resolved imaging system that has been successfully used for the intramural functional imaging of the heart. Within this framework, spectroscopic imaging has a number of advantages. These include the capacity (i) to define multiple wavelength windows over which fluorescent emission is integrated and (ii) to decompose amplitude and wavelength modulation of acquired spectra. We have used these features to characterize the fluorescent response of membrane-bound di-4-ANEPPS to membrane potential, contraction and photobleaching at a range of excitation wavelengths. These studies confirm that both the nature of the modulation of fluorescent emission of di-4-ANEPPS by changes in membrane potential and the rate of photobleaching vary markedly with excitation wavelength. We have also demonstrated that it is possible to recover action potentials in the presence of very substantial motion artifact (ie, no mechanical uncoupling agent) using spectral imaging. These issues are now discussed in greater detail.
4.4 Discussion

4.4.1 Comparison of fluorescence emission and photobleaching of di-4-ANEPPS at different excitation wavelengths

Excitation efficiency is around 4-fold greater at an excitation wavelength of 473 nm than at 532 nm and the peak of the emission spectrum moves from around 585 nm to 600 nm (see Figure 4.14). The rate of photo decay was much greater at the shorter wavelength excitation (around 7% in 5 seconds at 473 nm compared with <1% in 5 seconds at 532 nm, at pump power of 10 mW). In the former case, decay rates were marginally greater at shorter than at longer wavelengths resulting in a time-dependent change in the shape of the emission spectrum with sustained excitation.

The decay in fluorescence emission of membrane-bound dyes such as di-4-ANEPPS is thought to be due to light-induced destruction of fluorophores and it seems reasonable to argue that such destruction will be increased with excitation efficiency. The excitation wavelength 473 nm is close to the peak of the excitation spectrum for di-4-ANEPPS and as a result, we have observed much faster decay rate for 473 nm than 532 nm excitation. Other groups [133, 80] have also reported rapid photobleaching with 488 nm compared to 532 nm excitation. Interestingly, we have observed that the decay rate is faster for short wavelengths than the long wavelengths. With 473 nm excitation, we have recorded a decay date difference of 7% over the first five seconds. For those groups [133, 80] using 488 nm excitation, it was 4-5% over ten seconds. Although it is unclear what excitation power they had used, it seems that the difference in decay rates can also be attributed to different excitation wavelengths.

The source of the shift in the emission spectrum with sustained excitation at 473 and 488 nm is unclear and warrants further investigation. In practice, though, photobleaching is not a problem in optical measurement of electrical activation of the heart, since it can be removed in a number of ways. However, sustained photobleaching may lead to unacceptable reductions in signal intensity. In this respect, excitation of di-4-ANEPPS at 532 nm has the advantage that it produces stable
fluorescence output albeit at lower intensity, because excitation efficiency is much less than for 473 nm.

4.4.2 Modulation of di-4-ANEPPS emission with membrane potential change at different excitation wavelengths

We have demonstrated marked differences in the spectral response of di-4-ANEPPS to changes in membrane potential at 473 and 532 nm excitation. Comparable results presented by Tai [133] at 488 nm excitation were consistent with the trend that we have observed. At 473 nm excitation, membrane depolarization of 100 mV produced a blue shift in the emission spectrum, together with minute amplitude modulation (<1%). Tai's data [133] also show a blue shift in the spectrum with depolarization using 488 nm excitation, but it is difficult to estimate the extent of amplitude modulation, since the spectra presented are normalized. With 532 nm excitation, the spectral shift with depolarization was much less evident, but amplitude modulation was substantial (up to 9%).

As mentioned in Chapter 1, the characteristics of excitation and emission spectra for most fluorophores mirror each other. A systematic characterization of voltage sensitivity of di-4-ANEPPS in dissociated Retzius cells (leech neurons) has been presented by Fromherz and Lambacher [48]. While the wavelength resolution of their measurement system was limited, these workers characterized excitation and emission spectra, and their modulation with changes in membrane potential. They reported that spectral changes induced by depolarization can be described by a blue shift of the absorption spectrum, a weaker blue shift of the emission spectrum and a difference in fluorescence yield. Bachtel and colleagues [5] recently presented a phenomenological model based principally on the findings of Fromherz & Lambacher [48], but also on other data from the literature. This was used to design a novel excitation ratiometry method for recording membrane potential. The model developed
4.4 Discussion

by Bachtel et al [5] is illustrated in Figure 4.27. They incorporated the voltage-dependent shifts of excitation and emission spectra for di-4-ANEPPS described by Fromherz & Lambacher [48]. However, they assumed that emission spectra were Gaussean in shape with peak emission wavelengths dependent on excitation wavelength. For excitation at 450 nm, Figure 4.27 indicates that excitation efficiency will be increased by membrane depolarization, thereby producing positive amplitude modulation of the emission spectrum as well as a blue wavelength shift, with depolarization. In contrast, with excitation at around 520 nm, depolarization give rise to negative modulation of the emission spectrum together with a blue wavelength shift. Bachtel et al [5] used this analysis to reconstruct emission responses with depolarization expected for excitation with blue and cyan LEDs (450+10 nm and 405+15 nm, respectively). On this basis, they determined the emission bandwidth over which depolarization would produce positive and negative $\Delta F$ when excited by blue and cyan LEDs, respectively. This was used to specify a single emission filter that would enable ratiometric recovery of action potentials from fluorescence recorded in interleaved video frames and excited by synchronously pulsed blue and cyan LEDs.

The Bachtel model was adapted to fit the changes in emission spectra with depolarization that we observed at different excitation wavelengths and the results of this analysis are presented in Figure 4.28. We estimated a blue shift of the emission spectrum of around 2 nm and amplitude modulation $\Delta F/F < -1\%$ with peak depolarization at 473 nm excitation. With 532 nm excitation, the spectral shift of the emission spectrum was estimated at around 3 nm, but the amplitude modulation $\Delta F/F$ was around -9%. These amplitude modulation results were used to specify values of $\Delta F/F$ for the excitation spectrum as shown in Figure 4.28C. For Figure 4.28A, the excitation curve was estimated using the emission spectrum of 473nm excitation, assuming that the mirror image rule applies (see Section 1.1). The center of the curve was fitted using the experimentally obtained values of $\Delta F/F$ at 473 and 532 nm. On this basis, along with the resting emission spectra for both
Figure 4.27: Model of di-4-ANEPPS voltage sensitivity. A: Excitation spectra for the resting and depolarized state. B: Difference spectrum taken by subtracting the resting excitation spectrum from the depolarized excitation spectrum (top). The spectrum for blue and cyan LEDs (bottom). C: Emission spectra at resting and depolarized state for blue and cyan excitation. D: Difference spectra taken by subtracting the resting emission spectra from their respective shifted and amplitude modulated emission spectra [5].

For 473 and 532 nm excitation, we have predicted the emission spectra for depolarization and corresponding results for $\Delta F$ as a function of wavelength, as shown in Figures 4.28 B and D, respectively. The latter figure presents the difference in fluorescence which results from the combined effects of amplitude and wavelength modulation for excitation at 473 and 532 nm. These results match the experimental data presented in Figure 4.15 and are consistent with the comparable findings presented by Tai [133] for 488 nm excitation.

Full characterization and quantification of the spectral response of di-4-ANEPPS is required to gain a comprehensive understanding of the behavior of the dye. This could be achieved using an excitation source that is tunable across a broad range of wavelengths such as a supercontinuum (see Chapter 2). Recording fluorescence changes during the action potential gives only a qualitative measurement of the membrane potential. For comprehensive characterization, it would be preferable to record excitation and emission spectra at several fixed levels of membrane potential.
4.4 Discussion

Figure 4.28: Voltage sensitivity model of di-4-ANEPPS illustrating the voltage-dependent spectral shift of the excitation and emission spectra. A: Excitation spectra of di-4-ANEPPS for the resting and depolarized state. B: Emission spectra of di-4-ANEPPS for the resting and depolarized state with excitations at 473 and 532 nm. C: Fractional fluorescence $\Delta F/F$ as a function of excitation wavelength. D: Difference in fluorescence $\Delta F$ as a function of emission wavelength.

that can be measured independently. This could be achieved in many ways. For instance it is possible to vary membrane potential in the isolated, Langendorff-perfused, non-beating heart by changing the potassium ($K^+$) concentration of the perfusate. With such a preparation, intracellular (transmembrane) potential can be measured using a micropipette. Alternately, a voltage clamp could be used to control membrane potential in an artificial lipid bi-layer preparation [46]. In both cases it would be necessary to measure the excitation and emission spectra for membrane-bound di-4-ANEPPS at a range of membrane potentials and at a full range of excitation wavelengths.
4.4.3 Optimization of action potential signals

A major advantage of spectroscopic imaging systems is that it is relatively easy to define multiple wavelength bands which fluorescence signals are acquired. With di-4-ANEPPS at 473 nm excitation, this has enabled us to set long and short wavelength bands in which modulation by membrane potential change is maximized.

We have also demonstrated that for di-4-ANEPPS, 532 nm excitation has advantages in situations where it is not necessary to exploit the ratiometric characteristics of the dye. Although excitation efficiency is substantially less at 532 nm than 473 nm, this is offset by the fact that the change in fluorescence with altered membrane potential (amplitude modulation rather than a spectral shift) is much greater. Moreover, the extent of photobleaching is much less at 532 nm than 473 nm.

A 50/50 fiber coupler is used in our imaging system and fluorescent detection efficiency is therefore only 50% of the total collected fluorescence. This could be be improved to 90% if a 90/10 coupler were used, albeit at the expense of using only 10% of the available excitation power. For example, if the optimum excitation power exposed to a particular specimen is 5 mW at the probe end, for a 50/50 coupler, this equates to 10 mW of input power with 2.5 mW of usable excitation power (10 mW × 0.5 × 0.5 = 2.5 mW). However, for a 90/10 coupler, this would reduce 50 mW of input power to 4.5 mW of usable excitation power (50 mW × 0.1 × 0.9 = 4.5 mW).

4.4.4 Motion artifact

It is not possible to record cardiac action potentials with optical techniques unless motion artifact is suppressed. There are several mechanisms which can give rise to motion artifact with optical mapping. In the case of surface mapping, complex 3D movement of the heart with respect to a fixed 2D imaging system may lead to artifact that is particularly apparent when there is variation in background fluorescence intensity due to nonuniform staining. For intramural recording, similar
processes occur as the heart contracts, thereby altering the arrangement of cells within the fluorescence collection volume. The modulation of light due to scattering and absorption are wavelength dependent processes which vary throughout the cardiac cycle. The mechanisms described are merely factors contributing to motion artifact, the detailed mechanisms involved and their relative contributions are by no means clear. We have assumed a simple empirical model of optical cardiac action potential measurements in which the effects of photobleaching, electrical activation, and mechanical activity can be represented as individual components in the form of amplitude or spectral modulation. In previous studies where attempts were made to correct for motion artifact [131, 15], it was assumed that the fractional fluorescence associated with contraction was the same for all wavelengths. That is, the morphology of the motion waveform in short and long wavelength bands was viewed as identical, and thus removable using ratiometry or subtraction. However, these workers pointed out that, in the presence of substantial motion, there were residual motion artifacts that could not be removed in this way. Through observation of fluorescence emission spectra throughout the cardiac cycle, we found mechanical activity can be decomposed into two distinct spectral components. The major component is manifest in the form of amplitude modulation and is identical across all wavelengths. A smaller component, associated with frequency modulation gives rise to motion signals that are similar in shape, but opposite in phase in short and long wavelength bands. Attempts to recover action potentials by ratiometry or subtraction reinforce this residual component, which can mask electrical activity if motion is substantial.

We have proposed two alternative approaches to minimizing artifact that works well with excitation that produces wavelength shift of the emission spectrum rather than amplitude modulation. In both approaches, amplitude correction was used to remove the major motion component from short and long wavelength bands. In the case where the frequency components of action potential and residual motion artifact are sufficiently separated, the motion component could be estimated and hence re-
moved. The extraction of the motion component were performed on the basis that its temporal variation during electrical activation is predicted by the background variation at rest. In the case where $\Delta F/F$ of action potential and residual motion artifact are sufficiently different, summation of the short and long wavelengths by matching the amplitudes of their motion component will result in the removal of motion artifact, albeit at the cost of reduced amplitude of the electrical signals. The two outlined approaches are entirely empirical, in the case where $\Delta F/F$ of motion artifact is similar to electrical activations and that their frequency components overlap substantially, it is not possible to fully recover action potentials.

The approaches outlined above for minimizing motion artifact are less easy to apply when di-4-ANEPPS is excited at 532 nm. At this wavelength, membrane potential change produces substantial amplitude modulation of the emission spectrum, which means that amplitude correction would remove most of the electrical signal as well as the dominant motion waveform. In principle, it should be possible to separate these two components by integrating across a wavelength band in which the modulation due to depolarization sum to zero (for instance from 520-550 nm in Figure 4.15). In practice, the signal to noise ratio across this band is very low. A disadvantage of using 532 nm excitation with di-4-ANEPPS is therefore that the ratiometric character of this dye cannot easily be exploited to reduce motion artifact.

### 4.4.5 Action potential rise time

We have previously stated that optical action potential rise times scale with respect to the effective fluorescence collection volume. The most obvious explanation for this is the spread of electrical activation through the region addressed by the imaging system. To examine this, Tai [133] simulated the temporal responses to step functions of altered fluorescence activity propagating as a plane wave through the collection volumes of different optical fibers at an angle of 90° with respect to the fiber axis. The time course of fluorescence change was estimated for multimode fibers.
with 100 and 200 µm core diameter at velocities ranging from 0.01-10 m/s. Propagation velocities in normal ventricular myocardium range from 0.2-1 m/s [37, 57, 142]. Tai predicted upstroke rise times less than 3 ms for optical fibers with a core diameter of 200 µm, when propagation velocity was greater than 0.2 m/s. We have observed action potential rise times of 3.7±1.6 ms with our spectroscopic imaging system, whereas corresponding intracellular or single cell measurements [42, 148] report time delays of 600 ns to 1 ms. Tai's model [133] suggests that the significant (19-97%) delay in the rise time can be explained by velocity blurring given the range of propagation rates in normal ventricular myocardium. Other possible explanations include (i) discrete delays occurring in a stochastic fashion or (ii) a contribution to fluorescent emission by membrane structures that are passively coupled to myocytes and respond slowly to potential change. Kohl [81] have argued that fibroblasts contribute a large population of cells in the heart that contribute heterogeneous cell coupling. Likewise, Miragoli and co-workers [101] have recently demonstrated that fibroblasts can give rise to slowed impulse propagation in cardiac cell culture at densities less than those observed in the heart in vivo.

It is surprising to us that the action potential upstrokes which we have recorded with a 200 µm multimode optical fiber are faster than those reported by others using similar techniques with 100 µm fibers [19, 63, 133]. This difference is most likely explained by differences in the instrumentation used and the fact that action potentials were averaged over 8-16 cycles in the previous studies cited.

4.5 Conclusion

While optical recording of cardiac electrical activity using the ratiometric dye di-4-ANEPPS is well-established, fast spectral imaging at sub-nanometer resolution has not previously been used for this purpose. We have demonstrated that this approach provides a robust and effective means of recording intramural electrical activity in the heart that can be used with all fiber optical assemblies which do not require dichroic mirrors or specific emission filters. Moreover, the acquisition of
Spectral information provides additional degrees of freedom in the processing and analysis of fluorescent signals. This has opened new insights into various aspects of action potential measurement such as the removal of motion artifact, optimization of wavelength windows and the effect of excitation at different wavelengths. In particular, we have confirmed that there are substantial differences in the fluorescence characteristics of di-4-ANEPPS with membrane depolarization at excitation wavelengths of 473 and 532 nm. There is a ratiometric shift in the emission spectrum in the former case, but a relatively large reduction in the amplitude of the emission spectrum in the latter. We have also shown that when amplitude or frequency components of the spectral shift caused by mechanical activity is dissimilar to that due to altered membrane potential, action potentials may be recovered faithfully from spectral recordings in the absence of any mechanical uncoupling agent. Thus, reliable information about repolarization at the heart wall can be obtained with less or no alteration on the neurohumoral profile of the heart [133].

Future study may include the addition of a calcium indicator to di-4-ANEPPS, so that calcium transients and action potentials can be recorded simultaneously in the intact heart. Although fluorescent dyes are usually used to measure these mechanisms separately, simultaneous measurements in cardiac tissue have been carried out [73, 88]. Johnson and co-workers [73] used a 488nm argon laser line to simultaneously excite both di-4-ANEPPS and Fluo-4 (a non-ratiometric calcium dye), and a 16 element photodiode array was used to record the emission spectra. Because this measurement system had poor wavelength resolution it was not possible to decompose the component spectra reliably and this introduced large errors. Laurita and co-workers [88] used a different calcium dye (ratiometric, Indo-1) and a dual excitation approach in order to minimize spectral overlap. Consequently, sequential bands of fluorescence emission were collected rather than resolving the spectra. Although ratiometric dyes were used, ratiometric imaging was not employed. Our prototype imaging system can accommodate either single or dual excitation with ease as in a spectroscopic configuration, the wavelength bands over which fluores-
cence is integrated can be adjusted in software rather than by replacing a filter. Moreover, detecting fluorescent emission at high resolution allows the optimization of any spectral bands and offers analysis that are not possible for fixed wavelength bands. For instance, with a ratiometric dye it is possible to separately characterize the extent of amplitude and frequency modulation and use this information reduced motion artifact and optimize signal to noise ratio.
Chapter 5

Conclusion

In this thesis, we have explored a range of fiber optic based biological imaging applications. A common theme of this work is that fiber optic systems provide a robust and flexible means of probing both function and structure within relatively small regions and local biological environments. We have designed, developed and tested the feasibility of novel fiber optic based techniques and instrumentation systems in the fields of OCT, bioremediation and optical mapping of cardiac electrical activity. Briefly, the objectives we set out to achieve are (1) to design and develop an OCT system using a supercontinuum source and evaluate its capabilities and restraints, before developing an all-fiber OCT system that may be combined with a fluorescence probe, (2) to design and develop a fiber based fluorescence imaging system for monitoring bioremediation and (3) to construct a fiber based fluorescence setup that can resolve cardiac action potential measurements spectrally. Principle outcomes in each of these areas are summarized below.

(1) We designed and built a prototype, visible range OCT system incorporating a supercontinuum source. We demonstrated that ultra-high axial resolution (\(\sim 1.54 \mu m\)) can be achieved with a supercontinuum source and that this approach complements other OCT systems which use light sources almost exclusively in the infrared. This work led to the development of an all-fiber OCT system.

(2) We have successfully built a fiber optic fluorescence system for the \textit{in situ} monitoring of bioremediation processes. This system allows for detailed visualization of biological processes in real-time, providing valuable insights into the effectiveness of remediation efforts.

(3) A fiber based setup was constructed for the spectral resolution of cardiac action potentials. This setup demonstrates the potential for high-resolution imaging of cardiac electrical activity, enabling detailed analysis of cardiac function.

These outcomes represent significant advancements in the field of fiber optic biomedical imaging, offering new tools for scientists and researchers to explore and understand complex biological systems.
monitoring of bacterial species used in bioremediation. Controlled experiments performed with this system on cultures of fluorescent labeled bacteria (E. coli and P. putida) have provided “proof of concept” that fiber optic spectrometers can provide information about biodegradation activity and bacteria population as well as other functional measures. Continuous in situ recording of such information under laboratory conditions is necessary to optimize biodegradation in general and in specific environmental conditions. The work described here is an important step toward this end.

(3) Finally, we have used a fast fiber optic spectrometer to record cardiac action potentials in the heart wall using the membrane potential sensitive dye, di-4-ANEPPS. The ability to resolve fluorescent signals spectrally enables amplitude modulation and spectral shifts to be decomposed on a sample-to-sample basis across a range of different emission spectra. As a result of this flexibility, it is possible to minimize noise, compensate for photobleaching and substantially remove motion artifact. Moreover, this approach has provided a new insight into fluorescence emission at different excitation wavelengths. Not only does it allow us to observe the interplay between amplitude modulation, spectral shift, wavelength dependency of photobleaching, and excitation wavelength, it can also differentiate the real physiology effect from the effect of all the above on the spectrum.

5.1 Future work

The remainder of this chapter provides a guide for the continuation of the research presented in this thesis. However, the suggested future work here, is by no means limited to the fields we have studied.
5.1 Future work

5.1.1 Development of a fluorescence-OCT probe

A prototype all-fiber OCT system has been designed and constructed. In principle, it should be possible to combine this with an all-fiber fluorescence system so that both structural and functional information can be obtained simultaneously from the same tissue site. The development of an appropriate fiber optic probe is an important step toward this end. In the first instance, a dual fiber probe or double cladding optical fiber seem to be the appropriate approaches.

5.1.2 Design of new fiber probes for in situ bioremediation

A limiting feature of the bioremediation studies is the low fluorescence intensities of bioremediation species. This is exacerbated by the fact that fluorescence illumination and collection volumes in soil are markedly reduced by absorption and scattering. At low signal levels, sensitivity can be increased through the use of dual fiber probes which minimize background autofluorescence. However, collected fluorescence intensity was reduced by the lack of overlap between illumination and collection fields. Detection of weak fluorescent signals such as bioluminescence, having collection optics attached to the end of the fiber probe can significantly improve the amount of fluorescence collected. Or, when detecting bacteria in soil, the use of a dual fiber probe in which the illumination and collection fields overlap would certainly improve sensitivity.

5.1.3 Simultaneous measurement of multiple fluorescence indicators

Provided the correct excitation wavelengths and appropriate selection of emission filters are employed, the spectroscopic fluorescence setup described in this thesis
allows the simultaneous measurement of multiple fluorescence indicators. In the case of bioremediation, this includes the tagging of additional fluorescent proteins to the target bacteria so that multiple information such as population, activity and behavior can be extracted. In the case of cardiac transmembrane measurements, the addition of a calcium dye to di-4-ANEPPS would allow simultaneous recordings of calcium transients and action potentials from the intact heart.

5.1.4 Development of novel functional probes and techniques

Viral delivery of fluorescent proteins can be used to track related viral delivery of other therapeutic agents (adeno-virus and lenti-virus) such as gene therapy. This may include treatments for cancers and degenerative brain diseases. Fiber optic probes can be introduced into and left in organs for a period to monitor the time course of fluorescent protein expression and hence track expression of a particular functional responses targeted by the gene therapy.
Bibliography


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