Investigation into dual modality fiber probe imaging system

Manu Vaishakh

2011

Manu, V. (2011). Investigation into dual modality fiber probe imaging system. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/51008>

<https://doi.org/10.32657/10356/51008>

INVESTIGATION INTO DUAL MODALITY FIBER PROBE IMAGING SYSTEM

Manu Vaishakh

SCHOOL OF MECHANICAL AND AEROSPACE ENGINEERING

A thesis submitted to the Nanyang Technological University in partial
fulfillment of the requirements for the degree of Doctor of Philosophy

2011

ACKNOWLEDGEMENT

I am thankful to Associate Professor V.M. Murukeshan for giving me the opportunity to pursue my PhD study in this prestigious university. I am thankful for his continuous guidance, suggestion, support and constant encouragement throughout the course of this research work.

I am immensely grateful to Associate Professor L.K. Seah for his support and allowing me to use the facilities in his lab with full freedom.

I am thankful to my colleagues who have helped me in various occasions. Special thanks to Chua Jeun Keey for waking up early in the morning to pick me up when I first arrived in Singapore and making the transition smooth by helping me with the initial formalities. Special thanks to Rakesh for the light and fun discussions we have on the various issues.

I am thankful to the lab staff of Photonics and microsciences (PAM lab), Robotics Research center (RRC lab) and metrology lab for their help. I would like to express my sincere gratitude to the administrative staff, especially Ms. Yati for processing the administrative documents and formalities. I am thankful to the university for the financial support during my stay in Singapore.

I am grateful to the Directors of International School of Photonics (ISP) and Centre of Excellence in Lasers and Optoelectronic Sciences (CELOS), Cochin University of Science and Technology, India who gave me the opportunity to use their laboratory and other related facilities during my attachment programme. I am thankful to the faculty,

administrative staff and students of ISP for treating me as one of their students and making my stay a wonderful experience. Special thanks to Mathew and Linesh for sharing the instruments and for their help in various matters.

I am grateful to my teachers who have taught me in the school, college and university for sharing their knowledge and helping me to improve myself. I am thankful to the professors I have met on various occasions for their encouraging words and support.

I will always be grateful to my parents for their mentoring. They have inculcated the value and beauty of education in me from my early age. They have always guided me towards the right path and taught me to develop myself as an individual in every aspect.

TABLE OF CONTENTS

ACKNOWLEDGEMENT	I
TABLE OF CONTENTS	III
LIST OF FIGURES	VII
LIST OF TABLES	XII
LIST OF ABBREVIATIONS	XIII
LIST OF SYMBOLS	XV
SUMMARY	XVIII
CHAPTER 1 INTRODUCTION	1
1.1 Motivation	2
1.2 Current methods and limitations	3
1.3 Project objectives	4
1.4 Project scope	5
1.5 Organization of the thesis	7
CHAPTER 2 LITERATURE REVIEW	11
2.1 Conventional techniques employed for biomedical studies	12
2.1.1 Non optical diagnostic methods	12
2.1.2 Minimally invasive optical diagnostic methods	15
2.1.2.1 Probe schemes for minimally invasive optical diagnosis	16

2.2	Fluorescence based techniques for biomedical diagnosis	28
2.2.1	Understanding fluorescence	28
2.2.2	Advantages of using fluorescence techniques	32
2.2.3	Fluorescence spectroscopy and imaging studies	33
2.2.4	Two photon induced imaging	37
2.2.5	Fluorescence lifetime imaging	39
2.3	Multi modality imaging systems	44

CHAPTER 3 DIGITAL MICROMIRROR DEVICE BASED PROBE FOR BIOIMAGING APPLICATIONS 48

3.1	Introduction	49
3.2	System details	49
3.2.1	Digital micromirror device (DMD)	49
3.2.2	Image fiber	52
3.3	Effect of individual fibers in the image	54
3.4	Removal of the fiber honeycomb structure from the image	59
3.4.1	Gaussian filter	59
3.4.2	Filtering the spectral component of the honeycomb structure in the Fourier domain	62
3.5	Selective illumination of the sample using DMD	65

CHAPTER 4 FREQUENCY DOMAIN IMAGING: AN INTEGRATED DUAL MODALITY APPROACH 70

4.1	Introduction	71
4.2	Selective suppression of fluorescence emissions based on lifetime differences	72

4.3	Instrumentation details	80
4.3.1	Laser diode	80
4.3.2	Intensified CCD camera (ICCD)	81
4.3.3	Function generator (FG)	81
4.3.4	Fluorescence lifetime spectrofluorometer	82
4.4	Imaging in the frequency domain: non-probe based approach	82
4.4.1	Results and discussions	84
4.5	Image enhancement by removing the unmodulated term	88
4.6	Optical fiber probe based system	91
4.6.1	Results and discussions	93
CHAPTER 5 INVESTIGATION INTO CONFOCAL TECHNIQUE		98
5.1	Introduction	99
5.2	Optical sectioning in image fiber bundle	102
5.3	Wavelength dependence of confocality	111
CHAPTER 6 CONCLUSION AND FUTURE WORK DIRECTION		119
6.1	Contributions	120
6.2	Future work	122
APPENDICES		124
APPENDIX A	SPECIFICATIONS OF THE ICCD CAMERA	125
APPENDIX B	SPECIFICATIONS OF THE EDC 2000-N CAMERA	126
APPENDIX C	DEPENDENCE OF OPTICAL SECTIONING ON THE	

	PINHOLE SIZE	127
APPENDIX D	NONLINEAR OPTICAL CHARACTERISATION OF	
	MICROSPHERES	131
	AC.1 Introduction	131
	AC.2 Theory	133
	AC.3 Experimental setup	134
	AC.4 Results and discussion	135
APPENDIX E	DETERMINING THE SIZE OF FLUORESCENT DYES	138
APPENDIX F	DETERMINING THE RESOLUTION USING	
	RESOLUTION CHART	140
	LIST OF PUBLICATIONS AND AWARD	144
	LIST OF REFERENCES	146

LIST OF FIGURES

FIGURE NUMBER	DESCRIPTION	PAGE
FIGURE 1.1	Flowchart depicting the approach for the realization of the dual modality probe system	7
FIGURE 2.1	Single fiber probe configuration	17
FIGURE 2.2	Multiple fiber probe configuration	17
FIGURE 2.3	Different schemes for oblique-incidence illumination and detection	19
FIGURE 2.4	Different probe configurations	21
FIGURE 2.5	Image obtained using direct contact probe	21
FIGURE 2.6	Experimental setup for imaging skin corneocytes	22
FIGURE 2.7	Comparison of contact and non-contact imaging	23
FIGURE 2.8	Fiber based confocal system	24
FIGURE 2.9	Hyperspectral confocal imaging	25
FIGURE 2.10	OCT in endoscope configuration	26
FIGURE 2.11	Jablonski diagram representing fluorescence	28
FIGURE 2.12	Excitation and emission spectrum of alexafluor 594	31
FIGURE 2.13	Emission property of quantum dots	36
FIGURE 2.14	Jablonski diagram showing one photon, two photon and three photon excitation	37
FIGURE 2.15	Comparison of one photon and two photon absorption	38

FIGURE 2.16	Principle of TCSPC	41
FIGURE 2.17	Schematic representation of frequency domain setup	42
FIGURE 2.18	Multimodality imaging for colon cancer diagnosis	46
FIGURE 3.1	Structure of DMD	51
FIGURE 3.2	Diffraction pattern generated by the DMD	52
FIGURE 3.3	Image fiber bundle with grin lens	53
FIGURE 3.4	Light propagation in the grin lens	54
FIGURE 3.5	Experimental setup for studying the effect of individual fibers of the image fiber bundle in the image	55
FIGURE 3.6	Image of the sample imaged by image fiber probe at various MO magnifications	56
FIGURE 3.7	Experimental setup for freespace imaging of the sample	57
FIGURE 3.8	Image of the sample at various MO magnifications	58
FIGURE 3.9	Schematic representation of a Gaussian filter	59
FIGURE 3.10	Schematic representation of the action of Gaussian filter	60
FIGURE 3.11	Image of the sample imaged by image fiber probe at various MO magnifications after applying Gaussian filter	61
FIGURE 3.12	Intensity profile of the image acquired by image fiber	61
FIGURE 3.13	Intensity profile of the processed image after Gaussian filtering	62
FIGURE 3.14	Fourier Spectrum of the image given in fig 3.6a	63
FIGURE 3.15	Horizontal pattern of the Fourier Spectrum shown in fig 3.14	63

FIGURE 3.16	Effect of bandreject filter	64
FIGURE 3.17	Schematic of the experimental setup for DMD based image fiber probe	66
FIGURE 3.18	Dual modality DMD based image fiber probe	67
FIGURE 3.19	Removal of the honeycomb structure from the probe image and the corresponding fourier spectra	68
FIGURE 4.1	Schematic representation explaining phase angle ϕ and modulation depth m	74
FIGURE 4.2	Effect of modulation frequency on phase shift ϕ and modulation depth m	75
FIGURE 4.3	Representation of signals based on a) Homodyne b) Heterodyne detection schemes	77
FIGURE 4.4	Experimental setup representing the frequency domain imaging.	83
FIGURE 4.5	Details of the experimental setup in the lab	84
FIGURE 4.6	Fluorescence lifetime spectra of a) Basic yellow in acetone b) Rhodamine 6G in acetone	85
FIGURE 4.7	Images representing lifetime based suppression	86
FIGURE 4.8	Images representing better depth perception achieved by the technique	87
FIGURE 4.9	3D representation of images with better depth perception	88
FIGURE 4.10	Enhancing contrast by removal of unmodulated term	90
FIGURE 4.11	Image fiber based frequency domain imaging and	92

	spectroscopy.	
FIGURE 4.12	Fluorescence lifetime spectra of redwop	94
FIGURE 4.13	Peak emissions from cover glass dual fluorescence sample when scanned through the z-axis with steps of 12.5 microns	95
FIGURE 4.14	Selective suppression based on lifetime differences	96
FIGURE 5.1	Schematic diagram for epi-fluorescence excitation	100
FIGURE 5.2	Schematic diagram of confocal set up showing the introduction of pinhole at the focal plane	101
FIGURE 5.3	Schematic representation of an image fiber based endoscope	103
FIGURE 5.4	Details of the Gaussian beam profile	103
FIGURE 5.5	End face of a portion of an image fiber bundle	107
FIGURE 5.6	Definition of the parameters used in the calculation	108
FIGURE 5.7	Effect of neighboring fibers when $D/R=4$	109
FIGURE 5.8	Effect of neighboring fibers when $D/R=20$	110
FIGURE 5.9	Experimental FWHM for $D/R = 4$	111
FIGURE 5.10	Schematic of chromatic confocal microscope	112
FIGURE 5.11	Wavelength dependent effective pinhole size	113
FIGURE 5.12	Effect of variable confocality. Variation in axial FWHM with wavelength	115
FIGURE 5.13	Effect of same confocality. Variation of axial FWHM with wavelength	117

FIGURE AC.1	Schematic of the experimental setup to study the effect of pinhole size	127
FIGURE AC.2	Axial intensity distribution in the confocal setup	128
FIGURE AC.3	Variation of FWHM with pinhole size	129
FIGURE AD.1	Experimental setup for Z-scan	134
FIGURE AD.2	Z-scan plots	137
FIGURE AE.1	Particle size data using particle analyzer	139
FIGURE AE.2	Image of redwop using optical microscope	139
FIGURE AF.1	USAF resolution chart image using image fiber bundle based system at 10X magnification	140
FIGURE AF.2	USAF resolution chart image using image fiber bundle based system at 20X magnification	141
FIGURE AF.3	USAF resolution chart image using free space configuration at 10X magnification	142
FIGURE AF.4	USAF resolution chart image using free space configuration at 20X magnification	143

LIST OF TABLES

TABLE NUMBER	DESCRIPTION	PAGE NUMBER
Table AE.1	Average particle size of the fluorescent dyes	138

LIST OF ABBREVIATIONS

ABBREVIATION	EXPANSION	PAGE
CCD	Charge coupled device	2
GFP	Green fluorescent protein	3
DMD	Digital micromirror device	5
FWHM	Full width at half maximum	9
NLO	Nonlinear optical	9
CT	Computed tomography	13
MRI	Magnetic resonance imaging	13
PET	Positron emission tomography	14
PTD	Probe to target	20
PCF	Photonic crystal fiber	25
OCT	Optical coherence tomography	25
FFOCM	Full field optical coherence microscopy	26
PCR	Polymerase chain reaction	32
NADH	Nicotinamide adenine dinucleotide	33
TCSPC	Time correlated single photon counting	40
PMT	Photomultiplier tube	43
SLM	Spatial light modulator	49

MO	Microscope objective	54
ICCD	Intensified charge coupled device	78
FG	Function generator	81
HRI	High rate imager	81
NA	Numerical aperture	91
FRET	Fluorescence resonance energy transfer	99
FLIM	Fluorescence lifetime imaging	99
TPLSM	Two photon laser scanning microscope	99
SNR	Signal to noise ratio	113
DPSS	Diode pumped solid state	127
BS	Beamsplitter	127
SA	Saturable absorber	135
RSA	Reverse saturable absorber	135

LIST OF SYMBOLS

SYMBOL	DESCRIPTION	PAGE
ε	Extinction coefficient	31
Φ	Fluorescence quantum efficiency	31
τ	Fluorescence lifetime	40
I_{ss}	Steady state intensity	40
t	Time	40
G	Gaussian function	59
σ	Standard deviation	59
ω	Angular frequency	72
m_{ex}	Modulation depth of excitation beam	72
E	Laser intensity	72
F	Fluorescence intensity	72
m_1	Modulation depth of first fluorophore	73
m_2	Modulation depth of second fluorophore	73
m	Modulation depth of fluorescence beam	73
F_1	Fluorescence intensity of first fluorophore	73
F_2	Fluorescence intensity of second fluorophore	73

ϕ	Phase shift	73
ϕ_1	Phase shift of first fluorophore	73
ϕ_2	Phase shift of second fluorophore	73
τ_1	Fluorescence lifetime of first fluorophore	73
τ_2	Fluorescence lifetime of second fluorophore	73
$\Delta\omega$	Small change in angular frequency	76
m_D	Modulation depth of ICCD	78
ϕ_D	Phase angle of detector	78
$D(t)$	Time varying gain of ICCD	78
D_0	Gain of ICCD without modulation	78
$I(r, \phi_D)$	Time averaged image intensity	78
$I_{01}(r)$	Time averaged intensity of first fluorophore	78
$I_{02}(r)$	Time averaged intensity of second fluorophore	78
U'	Principal plane of objective lens (close to sample)	104
U''	Principal plane of objective lens (close to image fiber)	104
I_0	Peak intensity of Gaussian beam	104
x_0	Radius at which lateral Gaussian intensity is reduced to $\frac{1}{e^2}$ of the peak value	104
Z_x	Rayleigh range	104
n	Refractive index	104

z_0	Distance between U' and in focus plane	104
z_0'	Distance between U'' and image	104
f	Back focal length	104
z'	Displacement of image position from z_0'	105
β	Magnification of sample	105
x'	Radius of image at z'	105
x'_{IF}	Radius of beam at image fiber plane	106
I_{IF}	Intensity passing through image fiber bundle	106
D	Fiber Spacing	108
$2R$	Diameter of the fiber	108
h	Point spread function	112
v	Optical unit	112
u	Optical unit	112
$v_p(\lambda)$	Effective pinhole size	113
α_0	Absorption coefficient	133

SUMMARY

Optical techniques like microscopy and spectroscopy have become an indispensable tool in the clinical environment. Interfacing optical fibers into the interrogation system provides a flexible solution for studying regions inside tubular structures and cavities which are inaccessible by conventional techniques. This has resulted in employing optical fiber probes for studying biochemical changes and for medical diagnostics. They can be used both for imaging as well as for spectroscopic studies. Cancerous changes occurring in tissues result in a difference in morphology or abnormal growths. Sometimes, the morphological changes occur at a later stage and abnormal growths can fall into the false positive category. Apart from the visual changes, cancerous progression can also result in a difference in fluorescence wavelength, fluorescence intensity or fluorescence lifetime. Integrating spectroscopic and imaging modalities into one system can improve the sensitivity and accuracy in diagnosis.

To improve the performance of such systems, investigation into dual modality fiber probes is carried out in this thesis. The spatial sensitivity of the system has been improved by using a digital micro mirror device based probe. Investigation on fluorescence lifetime based probe has also been carried out. This probe can distinguish fluorescence lifetime changes in the order of nanoseconds. Both the probes can work in imaging as well as spectroscopic modality simultaneously. As a result, any suspicious changes detected by one modality can be confirmed with the other. Apart from medical

diagnosis, the probes can also be employed for studying other samples or for studying chemical changes in real time.

The thesis also looks into the confocal technique and its effects. Optical sectioning in an image fiber based confocal probe has been investigated theoretically. The effect of neighboring fibers around an illumination fiber on the confocality achieved has been studied taking into consideration the fiber core size and the spacing between the fibers. Wavelength dependence on confocality has also been studied theoretically. This can help in designing and analyzing the performance of chromatic confocal microscope or hyper spectral / multi spectral confocal microscope. The theoretical analyses developed can help in understanding quantitatively as well as qualitatively such systems.

Nonlinear optical response of fluorescent microspheres has been studied during the research period. Such studies can help in identifying markers for nonlinear microscopy. Suggestions for improving the system are mentioned as future directions at the end of the thesis.

Chapter 1

Introduction

The chapter includes the motivation of undertaking the work reported in this thesis. This chapter also briefly discusses the use of optical fiber probes for in-vivo studies. A short note on the limitations of current techniques for biomedical diagnosis is mentioned followed by the objectives and scope. A flowchart depicting the research road map adopted for achieving the targeted objectives is given. The chapter concludes with the organization of the thesis.

1.1 Motivation

Colon cancer is ranked number one and two among the top ten cancers affecting Singaporean men and women, respectively [1]. If the treatment of colon cancer starts at the early stage when the cancer is localized to the point of origin, the five year survival rate is 90 % [2]. If the cancer has spread to adjacent organs, the survival rate drops to 70 % and it drops further to 12 % if the cancer has spread to distant organs. These facts suggest the importance of detecting such biochemical changes at an early stage. It is reported that conventional techniques for detecting such changes can miss some polyps and cancers [2].

Recently, optical techniques are employed for medical diagnosis. Even then, many cell types which are situated within hollow tissue tracts or deep within solid organs are inaccessible to conventional optical techniques. These can be studied using flexible fiber optic devices that can reach such locations in a minimally invasive manner. Our understanding of biological processes of normal as well as pathological cases would be enhanced by relevant concepts/methodologies that allow cellular imaging under conditions where conventional light microscopy cannot be used. An effective interdisciplinary research involving physics, engineering, biology and medicine will be a strong foundation for studies leading to such technological advances. The technology involves an interface between the sample and the device for analyzing the data like spectrometers or charge coupled devices (CCD).

Next section looks at the current methods and its limitations.

1.2 Current methods and limitations

The current methods for studying biological processes involve either external or internal examination of the sample using different techniques like electron microscopy and electron tomography. Such investigations are carried out if the aim is the understanding of the structure of the cells to molecular detail. Such techniques requires sample fixation which can alter the microstructure of the sample even when preparative methods like shock freezing are used. Moreover, it is not easy to label multiple targets inside the cell. Optical techniques like fluorescence techniques overcome the above problems especially after the discovery of green fluorescence protein (GFP) which enables the tagging of every protein. Over the past two decades, fluorescence based methodologies have been widely used for diagnosing diseases like cancer and for understanding biological process. Techniques that monitor quantitative and qualitative differences in naturally occurring cellular fluorophores have been applied to distinguish malignant tissues from normal tissues. More details of these techniques are discussed in chapter 2.

For diagnosis of diseases like cancer, a small slice of the tissue is taken for biopsy [3-11]. Despite being a definitive test of cancer, the biopsy is an invasive and subjective diagnostic technique that requires both trained professionals and significant waiting time. A significant number of false positives may undergo biopsy or many malignant lesions can be overlooked. In such cases, developing minimally-invasive techniques will be highly desirable. Different types of optical probes are employed as endoscopes in minimally-invasive *in-vivo* diagnosis systems. The probes can be made with single mode fibers, multi-mode fibers [12] or image fiber bundles [13]. These probes can function following different modalities like spectral characterization or imaging. Even though,

different types of endoscopes have been reported employing various techniques, they have their advantages and limitations. For example, electronic video endoscopy captures the image using a micro CCD located inside the probe. Early stages of biochemical changes may not show any morphological differences in the tissues. As a result, such endoscopes may not be able to detect the changes at the early stages. Moreover, almost all the endoscopes work in a single modality. This limits the overall sensitivity of the instrument. The present project aims at techniques which can be employed to increase the effectiveness of endoscope based diagnosis. A detailed description regarding the objectives and scope of the project is given in the next section.

1.3 Project Objectives

Multi-modality optical probes have been reported which can be used for colon cancer detection [13]. These probes collect the integrated signal from the sample which reduces the spatial specificity of locating the region of interest. In such cases, incorporating high spatial selectivity and sensitivity into a multi-modality probe will increase the diagnostic accuracy of the system.

The objective of the thesis is the development of dual modality fiber optic probes based on fluorescence for improving the sensitivity of the diagnostic system. Apart from the experimental investigations, the project also focuses on the theoretical analysis of image fiber based probe systems which can be useful in the design and performance analysis of such systems.

The main objectives of the project can be summarized as:

- 1) Investigation into the research and development for improving the sensitivity of fluorescence based image fiber bundle probes for medical diagnostics application

2) Investigation into the resolution aspects in image fiber bundle based systems.

The scope of the whole project to achieve the desired objectives is described below.

1.4 Project Scope

Biomedical changes can be studied quantitatively making use of different parameters like fluorescence intensity variation, spectral change in the fluorescence emission and variation in the fluorescence lifetime. Incorporating two or more such parameters in the instrument will increase the performance and versatility of the system. This will enable in developing a faster and efficient way of medical diagnosis. The Digital micromirror device (DMD) based probe incorporates both fluorescence imaging and the detection of spectral signature. The ability of the probe to selectively illuminate the area of interest will help in locating and tracking the changes in the sample in an efficient way. The image fiber based probe working in the frequency domain incorporates the fluorescence lifetime based concepts to facilitate fluorescence lifetime based imaging and spectral signature detection. Normal and cancerous tissues have fluorescence emissions which differ in their lifetime in the range of nanoseconds. Hence cancerous changes in the tissue can be detected at an early stage by tracking the variation in the fluorescence emission lifetime. The ability of this probe to distinguish fluorescence lifetimes in the order of nanoseconds makes it an ideal candidate for studying biochemical changes in cells and tissues as soon as they start to develop. The experiments revealed that resolution in image fiber based systems is degraded due to the honeycomb structure formed by the individual fibers. By illuminating each fibers sequentially and removing the signal coming from the neighboring fibers will increase the resolution of the system due to the confocal nature. Therefore, a theoretical analysis is carried out to study the effect of

neighboring fibers around the illumination fiber in an image fiber bundle on the confocality that can be achieved.

The scope of the project can be summarized as:

- 1) Experimental investigation of the DMD based image fiber probe working in fluorescence mode for selectively illuminating an area of the sample and obtaining both the morphological and spectral signature of that area.
- 2) Experimental investigation of the dual modality frequency domain probe to detect the changes in fluorescence lifetimes of the order of nanoseconds.
- 3) Theoretical analysis of wavelength dependent confocality in a chromatic confocal system.
- 4) Theoretical analysis of the effect of neighboring fibers in an image fiber bundle based microscope on the confocality that can be achieved in a probe based system.

A flowchart describing the approach for the realization of the dual modality probe is given in Fig. 1.1. A dual modality fiber probe imaging system is investigated. The probe can be used to study the morphology of the sample as well as the biochemical changes occurring in the sample in the microscopic scale. Improvement in the spatial sensitivity of the probe has been achieved using a DMD based image fiber bundle probe. Biochemical changes occurring in the sample can lead to variation in the fluorescence lifetime in the order of nanoseconds. To detect these variations, a frequency domain probe has been developed. Theoretical analysis of confocal based systems has been carried out to characterize the improvement in resolution that can be achieved. Details of the work are given in chapters 3, 4 and 5.

Fig. 1.1 Flowchart depicting the approach for the realization of the dual modality probe system

The organization of the whole thesis is given below.

1.5 Organization of the thesis

The whole thesis is divided into 6 chapters. Each chapter begins with a short note outlining the main contents of the chapter.

Chapter 1 is an introduction chapter which gives a brief overview of the problem. The motivation for the project, the project objectives and the project scope are discussed here.

A general outlook of the current status and limitations of optical fiber based probes is given emphasizing the need for a multimodality system for biochemical studies.

Literature review of the techniques and concepts related to the project are given in **chapter 2**. The current techniques in biomedical studies are reviewed. Beginning with a brief theory of fluorescence, a detailed literature survey on fluorescence based systems for biomedical studies is given. Different types of optical fiber probes, previously reported in literature, for their use in fluorescence imaging and spectroscopy are reviewed.

In **chapter 3**, DMD based image fiber probe for biomedical studies is introduced. Details of the DMD and grin lens coupled image fiber bundle are given. This is followed by the experimental studies on the effect of individual fibers of the image fiber bundle on the image quality. Gaussian filtering and filtering in the Fourier domain for successful removal of the discontinuities in the image captured by an image fiber is presented. Experimental investigation of the dual modality DMD based probe for selective illumination of the sample is performed and the results are given.

In **chapter 4**, theoretical description of selective suppression of fluorescence emissions based on their lifetimes is presented. The system is based on an intensity modulated laser beam for excitation and homodyne scheme for detection and processing. This chapter also includes the instrumentation details for achieving homodyne detection for fluorescence lifetime based imaging. Experimental details of a non-probe based approach for imaging in the frequency domain is presented. Results showing selective suppression of fluorescence emissions whose lifetimes differ in the range of nanoseconds are given. Improvement in contrast and morphology details of the sample imaged by the

technique is shown. This is followed by a theoretical description and experimental result showing enhancement in the image by removing the unmodulated term. The chapter concludes with the results obtained with the dual modality image fiber probe for detecting biochemical changes.

In **chapter 5**, a theoretical analysis on the optical sectioning performance of an image fiber based confocal microscope is presented. A theoretical analysis showing the wavelength dependence of confocality in a chromatic confocal microscope is also presented in this chapter. The analysis showed that the variation in FWHM with the wavelength is dependent on the pinhole size.

A conclusion for the thesis and future work direction is given in **chapter 6** which describes the contributions made in the subject area during the research work. All the theoretical and experimental works related to the proposed research for fulfilling the objectives are concluded here. Recommendations for modifications in the probe design and incorporation of techniques to improve the efficiency of the probe is given. Also, suggestions are given for applying the proposed systems in a real clinical environment.

Although the main content of the thesis is the investigation of dual modality optical probes, studies have been performed for characterizing the fluorescent markers which are used in microscopy. Evaluation of the nonlinear optical (NLO) efficiency of dye encapsulated microspheres is performed. One of the simplest and easiest ways of evaluating NLO efficiency is by the technique called Z-scan. In the appendix, a brief description of open aperture z-scan is presented. This is followed by the experimental details and the results obtained for dye encapsulated microspheres dispersed in water.

The results show that microspheres exhibit a switching from saturable absorber to reverse saturable absorber with concentration.

Next chapter of the thesis will be dealing with a comprehensive review and analysis of the techniques incorporated in the system design like fluorescence methods for detecting biochemical changes and endoscope configurations.

Chapter 2

Literature review

An overview of the work carried out by previous researchers in the areas related to the subject matter presented in this thesis is given in this chapter. The first section of this chapter describes the conventional techniques employed in diagnostic systems for studying the biochemical changes. The advantages of employing optical techniques are highlighted including the discussion on various endoscopic methods developed for in-vivo diagnostics. The second section describes the fluorescence methods which are useful for detecting the biochemical changes. A short review on multi modality imaging systems is given in section 3.

SECTION – A

2.1 Conventional techniques employed for biomedical studies

Based on the methods adopted, the currently available techniques employed for biomedical studies can be classified into two. The first method consists of minimally invasive optical techniques which employs radiations in the visible and near visible wavelength range for diagnosis. The second method consists of non-optical techniques which uses radioactive materials or wavelengths far away from the visible spectrum or biopsy.

2.1.1 Non-optical diagnostic methods

Biopsy is the standard technique applied in medical practice for the examination of cells or tissues. The procedure involves taking a small sample of tissue from the body (skin [3], liver [4], kidney [5], nerves [6], stomach [7] etc.) and examining it under a microscope or testing with chemicals. This method will help in identifying abnormalities or the extent of the damage on the cells due to a disease. Some of the commonly employed biopsy techniques are scraping cells (removing cells from the surface layer of tissue) [8], punch biopsy [9], needle biopsy (obtaining tissue from the body's organs or from tissue underneath the skin) [10], and excisional biopsy (surgery is used to take a large section of tissue) [11]. When an entire lump is removed from the body, the procedure is called excisional biopsy and when only a sample of tissue is removed with

preservation of the histological architecture of the cells in the tissues, the method is called incisional biopsy.

Computed Tomography (CT): In conventional X-ray image, the structures in different planes may overlap with each other. This makes it difficult for the medical practitioner to study the anatomic structure of interest since there will be a certain degree of blurriness in the image. The basic idea of CT is to create a 2-D or 3-D cross-sectional image reconstruction from X-ray images [14]. A narrow beam of X-ray is passed from the source to the detector and recordings are done by steering the beam around a patient at various angles. This results in “sectioned” images without any blurriness.

Magnetic Resonance Imaging (MRI): MRI is a powerful technique to visualize the internal structure of the body. MRI images give better contrast between the soft tissues of the body than in the case of CT images. This technique employs a powerful magnetic field instead of an ionizing radiation. Human body is composed largely of water molecules. When the patient is exposed to the magnetic field of the MRI scanner, the magnetic moments of the protons in the hydrogen nuclei align in the field direction. Electromagnetic field at radio frequency is then turned on. This results in an alteration in the alignment of the protons. When the electromagnetic field is turned off, the protons return to their original alignment. This change in the alignment creates a signal which is detected by the scanner and used for image formation [15].

Positron Emission Tomography (PET): In this technique a radioactive isotope of short lifetime (tracer) which is chemically incorporated into a biologically active molecule is injected into the bloodstream of the patient [16]. The positrons emitted from the isotope during the decay process annihilates with the electrons present in the body to emit a pair of gamma rays moving in opposite directions. These gamma rays are detected by a scintillator in the scanner and create a burst of light which is detected by photomultiplier tubes. Only the photons that arrive simultaneously which are moving in opposite directions are used for image formation.

Ultrasonography: This is an ultrasound based diagnostic procedure to visualize the subcutaneous body structures like vessels and internal organs. Ultrasonography has been effective in imaging soft tissues. In this technique, the ultrasonic waves produced by a transducer is directed towards the tissue. The sound wave falling on the tissue is partially reflected from the layers between different tissues. This reflection is prominent when there is a density change in the body like blood cells in blood plasma or small structures in organs. The reflected wave reaching the transducer is converted into electric signal which is processed to form the image [17]. Typical diagnostic scanners operate in the frequency range 2-18 MHz. The spatial resolution of the image is proportional to the frequency used and the imaging depth is inversely proportional to the frequency. As a result, frequency has to be chosen depending on the region to be imaged and the resolution needed.

2.1.2 Minimally invasive optical diagnostic methods

As discussed above, different non-optical techniques are employed for biomedical diagnosis and studies. Among them, biopsy is still the standard technique for confirming the abnormalities present. The limitation of biopsy is that it is an invasive technique that involves the removal of a slice of tissue from the body. This results in less chance of detecting the abnormalities at an early stage since the region infected can be overlooked during the examination. All the other techniques give the image of the structures inside the body. In these techniques a large number of false positive results may be obtained since random protrusions in the body can be misunderstood to be an abnormal structure. Moreover, early stages of diseases or chemical changes occurring in the tissues cannot be detected by these methods. Some techniques like CT involve using X-rays can be harmful for the tissues under continuous and repeated exposures. Techniques like PET involve the injection of a radioactive material into the body which may turn out to be harmful in the long run.

To overcome these limitations, it is better to develop minimally invasive diagnostic techniques using harmless electromagnetic radiation. Optical techniques are the ideal candidates in such situations. One of the advantages in this case is that light-matter interaction can result in different phenomena like fluorescence, scattering etc., which can be used as an indicator for biochemical changes. This also enables to incorporate various spectroscopic diagnostic methods.

Endoscopes are promising candidates for minimally invasive diagnostic applications. In simple terms, endoscopes are long and narrow optical systems intended for the observation of channels and cavities. Endoscopes can be classified into two main

categories based on the geometrical flexibility namely, rigid and flexible endoscopes. In rigid endoscopes, the optical signal is transferred by relay rod lenses with spherical surfaces [18]. In flexible endoscopes, optical fibers are employed since they can be directed along complicated and curved paths [19]. Since lens systems are used in rigid endoscopes, they give better image quality compared to flexible endoscopes. The limitation is that they cannot be guided through complicated pathways. The next section describes some probe configurations for minimally invasive diagnosis.

2.1.2.1 Probe schemes for minimally invasive optical diagnosis

There are a variety of reported probe designs for obtaining localized or depth-resolved spectroscopic data. Fluorescence collection and imaging employing single-fiber probe configurations [12] as well as with multiple fiber probes have been reported [12, 13]. Fig. 2.1 shows a single-fiber probe configuration for biomedical applications [12]. In this system the light from the excitation source is coupled to the probe employing a single fiber and the fluorescence signal from the sample is collected by the same fiber and is coupled to the detector. The excitation and the fluorescence light are separated by the dichroic beamsplitter. In the case of same fiber being used for illumination and collection it has been reported that they are sensitive to light scattering from superficial tissue regions [20, 21] at the cost of lower signal-to-noise ratios arising due to autofluorescence generated by impurities in the fiber core and by specular reflection from fiber surfaces.

Fig. 2.1 Single fiber probe configuration [12]

Fig. 2.2 Multiple fiber probe configuration [12]

Fig. 2.2 shows a multiple fiber probe configuration [12]. In this configuration, independent fibers are used as the probe for illuminating the target and as collecting fiber of radiation at the detector side. This minimizes the background signal although at the expense of larger probe size. Angled configurations for illumination and collection fibers that can be used to target specific depth regions are also reported [22, 23]. A group has reported an image fiber based probe for autofluorescence collection from colon tissue layers [24]. Other strategies include variation of the diameters of the illumination and collection fibers and variation of the probe-to-tissue distance [25]. Targeting the epithelial layer, however, requires steep angles that may be impractical for clinical probe designs. Most of the fiber based applications involve three components: (1) transfer of light from the probe to the medium, (2) propagation of light in the medium and (3) collection of the medium reemitted light from the sample by the fiber. Fiber optic probes are characterized by their design configurations (single or bifurcated fiber), the core size, numerical aperture of the illumination (source) and collection fibers (detector) and the source to detector distance. Several studies were carried out on this aspect describing how these factors affect the probe's performance (light collection efficiency and volume sampling).

In most of the common applications, researchers use normal incidence illumination-detection scheme as shown in Fig. 2.3a. This configuration is the easiest to design and offers satisfactory results in most of the cases. The limitation of this technique is that if subsurface regions have to be studied, the signal from the top layers will cause a decrease in the efficiency of the system. To overcome this limitation, researchers have employed oblique-incidence illumination and collection for increasing the depth sensitive

detection. Fig. 2.3 shows some of the different schemes employed in this scheme [26]. The red cone shows the region of overlap of the illumination and collection path. Fig. 2.3 (c) represents the convention of defining θ_i and θ_c , the illumination angle and collection angle, respectively. It is evident from the figures that the region of overlap can be adjusted by varying the geometry of the illumination and detection.

Fig. 2.3 Different schemes for oblique-incidence illumination and detection [26]

The bottom row in the figure shows the configurations that will offer better subsurface detection. A prior investigation [27] of angled fiber optic probe designs for fluorescence spectroscopy showed that the variations in relative sensitivity to epithelial layers obtained by changing the illumination angle and fiber separation distance provided control over relative selectivity that was superior to that obtained using normal-incidence probes. The advantage of this technique is that we can selectively probe the subsurface region of our interests with minimum signal from the other layers. This method is suitable for increasing the sensitivity of the probe for subsurface studies.

In another study [28], the effect of probe to target (PTD) distance on the diffuse reflectance was studied. Studies were done using both common illumination and collection channel using a single fiber and by separate illumination and collection channel using two bifurcated fibers. This study showed that PTD and probe design influence the light collection efficiency and the volume from which light is collected. Fig. 2.4 shows the different configurations that were used for the study. The output of a nitrogen laser was coupled into the illumination channel of the probe and targeted to the sample. The reflected light from the sample was collected through the collection channel. Three different configurations were investigated. The single fiber probe (Probe 1) consists of fibers of 600 microns core diameter. The second probe consists of a central illumination fiber with core diameter of 600 microns that was tapered to a distal core diameter of 1025 microns. This illumination fiber is surrounded by a ring of 18 collection fibers, each of 200 microns core size. The source to detector separation is 0.64 mm. The third probe consists of a central excitation core of 24 closely packed fibers each with 200 microns core size. Equal number of peripherally arranged fibers with core size of 200 microns forms the collection channel with the source to detector separation of 1 mm. For probe 1, maximum light collection is effected when the probe is placed in contact with the sample. As the probe to target distance is increased, the signal gets decayed in a quasi-exponential fashion. For probes 2 and 3, maximum signal is collected when the probe to target distance is of several millimeters. Probe 3 had to be at a larger distance from the target to achieve the maximum signal. The results achieved reflect that the overlap between illumination and collection channels is an important aspect in probe development.

Fig. 2.4 Different probe configurations [28]

(a)

(b)

Fig. 2.5 Image obtained using the direct contact probe a) Histological section of the barrel cortex b) Image acquired using the developed probe at the same depth [29]

A direct contact probe was developed using an image fiber bundle to monitor the neuronal and glial calcium activity *in-vivo* [29]. The system consisted of an image fiber bundle working on a lens-free principle connected to a fluorescence microscope. The system employed a two-dimensional raster scan method and the acquisition rate was 2 frames per second. The temporal resolution could be increased to approximately 1 msec by switching the scan to a line scan mode. It was reported that calcium responses acquired by line scan mode were less pronounced than that obtained using two-dimensional raster scan. This was attributed to the noise in the signal. Fig. 2.5 shows a sample histological section of the barrel cortex and the image acquired using the probe at the same depth [29]. Another study compared direct contact *in-vivo* imaging and non contact imaging of human skin corneocytes by an image fiber bundle [30]. Fig. 2.6 shows the experimental setup and Fig. 2.7 shows the images obtained [30].

Fig. 2.6 Experimental setup for imaging skin corneocytes [30]

The direct contact technique was employed by placing the distal end of the image fiber bundle in the stained (fluorescein) region of the skin and collecting the signal and

capturing it using a CCD. To increase the resolution of the system, gradient index lens was attached to the distal end of the image fiber bundle. The improvement is clearly seen in Fig. 2.7. The only limitation is that the area imaged becomes smaller than the contact mode.

Fig. 2.7 Comparison of contact and non-contact imaging a) Image of skin obtained in contact mode b) Image of skin obtained in non-contact mode [30]

In the above discussions, the target was assumed to be flat. In real time scenarios, the tissue surface will be curved. During such cases, the probe design should be modified to incorporate the tissue geometry. A study was done on fluorescence imaging of colon tissue taking into consideration the curvature of the surface and the collection efficiency of the system [18].

Discussions described above focused on the probe design aspects for specialized applications. These configurations have limitation in achieving high spatially resolved information in a probe based system. Integrating conventional high resolution imaging

techniques into probe based systems is a task which medical researchers demand. The next section describes various methods in which this can be achieved. Confocal microscopy technique is a relatively simple design that gives high spatial and axial resolution. A simple approach to integrate this technique into a probe system is shown in Fig. 2.8 [31]. In this setup, an optical fiber is used instead of a pinhole to achieve confocal imaging.

Fig. 2.8 Fiber based confocal system [31]

The light from the illumination pinhole is collimated by a lens and then focused to the sample with the help of a microscope objective. The fluorescent light originating from the focus is collimated by the objective and is then focused by a lens into the detector fiber which acts as the detection pinhole. The flexibility of the fiber allows in-vivo imaging.

This system can be modified for hyperspectral imaging by employing a tunable source. Fig. 2.9 shows the integration of photonic crystal fiber (PCF) into a confocal system for hyperspectral imaging [32]. In this system, the source is a Ti-Sapphire laser pumped through a PCF to generate supercontinuum. The resultant visible continuum can be selectively filtered with the help of optical filters. This system can be designed for in-vivo imaging by employing a fiber before the microscope objective so that the fiber acts as a collection and emission pinhole. Integrating hyperspectral imaging into conventional systems helps in getting details of the sample at high spectral resolution. This can be useful for early detection of abnormalities, especially those which show variation in the spectral signature.

Fig. 2.9 Hyperspectral confocal imaging [32]

The present trend in bioimaging is to incorporate the conventional imaging techniques into probe based schemes. Optical coherence tomography (OCT) is a technique that has made an impact when dealing with depth resolution. Fig. 2.10 shows

the configuration for incorporating OCT into a probe system for whole-field imaging [33]. In this setup, light from a xenon arc lamp is spectrally modulated in a Michelson interferometer prior to illuminating a conventional full field optical coherence microscopy (FFOCM) interferometer. The path length of the source interferometer is controlled by a piezoelectric transducer. Light from the source interferometer was transmitted to the input of the FFOCM interferometer by a multimode fiber. The interference images acquired are transmitted to the CMOS camera by a coherent multimode fiber optic bundle. This design has the advantage that there are no moving parts in the FFOCM probe.

Fig. 2.10 OCT in endoscope configuration [33]

A review of the conventional methods outlined in the above section for biomedical diagnosis indicates the need for better sensitive techniques which can be employed in a minimally invasive manner. The next section deals with fluorescence based techniques which can be employed to improve the sensitivity of the bioimaging instrumentation.

SECTION – B

2.2 Fluorescence based techniques for biomedical diagnosis

Fluorescence is a phenomenon that is being widely employed in biomedical applications. This section discusses the advantages of employing fluorescence based techniques in biomedical studies.

2.2.1 Understanding fluorescence

Fluorescence is a process that results when certain molecules absorb light and reemit radiation at longer wavelength. Absorption of light causes molecules to get excited to the relevant excited state. The de-excitation of the molecule from the excited level results in the fluorescence emission at wavelength higher than that of the excitation radiation.

Fig. 2.11 Jablonski diagram representing fluorescence [34]

The schematic diagram of the phenomenon is illustrated in Fig. 2.11 using a Jablonski diagram [34]. The horizontal closely spaced lines represent the vibrational levels. S corresponds to singlet state and T corresponds to a triplet state. Fig. 2.11 shows the different processes that can take place after a molecule is taken to an excited state by the absorption of light. The processes can be either radiative or non-radiative. In radiative process, a photon is emitted to bring back the molecule to the ground state. In non-radiative process, the energy is dissipated in the form of heat or chemical reaction. The zig-zag lines represent non-radiative transitions and straight lines represent radiative transitions. Figure shows a molecule in the singlet ground state S_0 which absorbs a photon of energy $h\nu_{ex}$ and is excited to one of the upper vibrational levels of the electronic level S_2 . The non-radiative crossing from S_2 to S_1 is called internal conversion (IC) as the crossing is between two states of same spin multiplicity. This is followed by a vibrational relaxation to the lowest level of S_1 . From here, the molecule can return to the ground state S_0 by emitting a photon. This radiative process between two states of the same spin multiplicity is called fluorescence. The lifetime of this process is in the order of nanoseconds. Another process that can happen is the crossing from S_1 to T_1 . This type of non-radiative crossing between two states of different spin multiplicity is called intersystem crossing (ISC). From here, the molecule can reach the lowest vibrational level of T_1 by relaxation and undergo a radiative process to the ground state S_0 . This process is termed phosphorescence and has a larger lifetime than fluorescence. The emission wavelength λ_{EM} during fluorescence is determined by the difference between the energy levels of the two states and is given as [35]

$$\lambda_{EM} = \frac{hc}{E_{EM}} = \frac{hc}{h(\nu_{ex} - \nu_{em})} \quad (2.1)$$

where h is the plank's constant, c is the speed of light , E_{EM} is the energy difference between the two states and ν_{ex} and ν_{em} are the excitation and emission radiation frequencies.

A fluorescent molecule has two characteristics spectra, namely, excitation spectrum and emission spectrum. Excitation spectrum is a plot of the intensity of fluorescence emission at a given wavelength versus the excitation wavelength which gives the relative probability that a given fluorochrome will be excited to different energy levels so as to get fluorescence emission at a given wavelength. The record of the intensity of emitted fluorescence spectrum by fixing the excitation wavelength is the emission spectrum which provides the relative probability that the emitted photon will have a particular wavelength from a particular excited level. The emission spectrum is in general shifted towards the longer wavelength relative to the excitation spectrum. The wavelength difference between the peaks of the excitation and emission spectra is known as Stoke's shift. The Stoke's shift represents the energy dissipated as heat following the fluorescence emission. Fig. 2.12 shows a typical example of excitation and emission spectrum of dye alexafluor 594 with the Stoke's shift around 27 nm [36].

The intensity of the emitted fluorescence depends on the concentration of the fluorochrome.

Let I_0 be the incident intensity being transmitted through a fluorescing sample of length l and concentration C so that the absorbed intensity by the sample will be

$$\begin{aligned}
 I_a &= I_0(1 - e^{-\alpha Cl}) \\
 &\approx I_0\alpha Cl \propto I_0 Cl
 \end{aligned}
 \tag{2.2}$$

Since the intensity of fluorescence emission is proportional to the absorbed intensity, equation (2.2) suggests that fluorescence intensity will vary linearly with concentration of the fluorescing species at low concentration. The emission intensity varies nonlinearly at high concentrations.

Fig. 2.12 Excitation and emission spectrum of alexafluor 594 [36]

The brightness of the fluorescence light depends mainly on two factors, the extinction coefficient ($\varepsilon = \alpha C$) and the fluorescence quantum efficiency (Φ). Extinction coefficient is the ability of a fluorochrome to absorb light and the efficiency with which the absorbed light is converted into fluorescence light is the quantum efficiency. Environmental factors like temperature and pH affects the quantum efficiency and the excitation-emission spectra.

2.2.2 Advantages of using fluorescence techniques

Fluorescence technique has following advantages as far as imaging and spectroscopic applications are concerned.

- **Sensitivity:** Sensitive detection of biological molecules like DNA and RNA can be carried out using fluorescent probes [37-42]. The use of fluorescence probes will be as sensitive as that can be realized by radioisotopes.
- **Low Hazard:** Compared to radioisotopes, fluorescent probes are less hazardous. For example, lead or acrylic shields are required when dealing with radioisotope techniques while the use of a hand glove is sufficient to provide protection when fluorescent probes are employed. Disposing fluorescent materials is easy due to its low hazardous nature while radioisotopes require special storage techniques.
- **Stability:** Applications which involves DNA and protein sizing, enzyme assays, polymerase chain reaction (PCR) based genetic typing assays requires minimal reagent variability between the assays. In such cases, it is better to use fluorescent labels over radioisotope labels since the shelf life of fluorescent labels are longer.
- **Multicolor detection:** Spectrally resolved detection of multiple targets is possible since fluorescent labels are available with different emission wavelengths. The ability to detect multiple targets in the same sample eliminates sample to sample variation. Availability of multicolor fluorescent labels also finds applications in multispectral and hyperspectral studies.

The next section of this chapter discusses some of the spectroscopic and imaging studies carried out by earlier researchers using fluorescence based diagnosis.

2.2.3 Fluorescence spectroscopy and imaging studies

Laser induced fluorescence is a widely accepted technique employed for biomedical studies. It is used to identify pathological conditions based on the spectral signatures [43-52]. The assumption behind the technique is that the absorption and scattering characteristics of the tissue change during the neoplastic growth. Dysplastic changes like increase in the nuclear size of the epithelial cells, increase in DNA content and hyperchromasia with chromatin clumping result in an increase in scattering [53]. It is reported that as dysplasia is developed, the volume fraction of collagen fibers decreases. This leads to a decrease in light scattering in the stroma beneath the epithelium [54]. It is known that the haemoglobin concentration increases during dysplastic changes which results in an increased optical absorption in stroma. Fluorescence spectroscopy identifies the neoplastic growth by detecting the changes in the concentration of intrinsic or endogenous fluorophores. Some of the endogenous fluorophores that play a role during such carcinogenesis changes are tryptophan and tyrosine (amino acids) [55], collagen and elastin (structural proteins) [56], NADH (nicotinamide adenine dinucleotide) [55] and porphyrins [57]. For example, fluorescence studies have been used extensively as a diagnostic tool in organs like colon, bronchus, lung, cervix, brain, bladder, esophagus and skin [58-60]. Results obtained from these studies indicate that the fluorescent signal from malignant tumors is less intense than that from normal tissue. It is worth pointing out that the specificity and sensitivity in detecting the cancerous changes based on the fluorescence spectral data lies in a wide range. This widely varying range could be attributed to many reasons like the broad nature of fluorescence spectra due to the

complexities in the biological tissues, distortion of fluorescence signal due to the high scattering cross-section, artificial dips and peaks in the spectra due to the absorption of the fluorescent photons (self absorption) and the effect of illumination-collection geometry on the intensity and line shape of the spectra [61].

Absorption and scattering characteristics of tissue components like water, fat, melanin, deoxyhemoglobin and oxyhemoglobin determine the light penetration in tissues. In the UV-visible part of the spectrum, light can penetrate tissues to a depth of around a millimeter. In the NIR region (700-900 nm), light can propagate to several centimeters due to the low absorption by the tissues in this “window” region. For wavelengths above 900 nm, the absorption coefficient of water increases creating an upper limit for the “window”. Relying on the endogenous fluorophores alone limits the spectroscopic techniques due to the weak signal and the subtle changes in the spectral information of normal and diseased tissues. The development of exogenous fluorophores or optical biomarkers has resulted in probing the specific signatures of cancer. Optical biomarkers are ligands that have a high selectivity to attach to pathological cells. Contrast agents are bind to the ligands to exhibit fluorescence emission on excitation with light of appropriate wavelength. A powerful contrast agent that is widely used is fluorescent semiconductor nanocrystals or quantum dots [62]. A more detailed description of quantum dot properties can be found in [63]. Colloidal semiconductor quantum dots are single crystals of few nanometers in diameter whose size and shape can be precisely controlled by the duration, temperature and ligand molecules used in the synthesis. This process yields quantum dots that have composition- and size-dependent absorption and emission. Absorption of a photon with energy above the semiconductor band gap energy

results in the creation of an electron-hole pair. The absorption has an increased probability at higher energies (i.e., shorter wavelengths) and results in a broadband absorption spectrum which is in marked contrast to standard fluorophores. For nanocrystals smaller than the so-called Bohr exciton radius (a few nanometers), energy levels are quantized, with values directly related to the quantum dot size (an effect called quantum confinement). The radiative recombination of an exciton leads to the emission of a photon in a narrow, symmetric energy band thereby differing from the red-tailed emission spectra and short lifetimes of most fluorophores. Fig. 2.13 shows the emission maxima and sizes of quantum dots of different compositions. The curves represent experimental data from the literature [63] showing the dependence of peak emission wavelength on quantum dot diameter. In the data shown the range of emission wavelength is from 400 to 1350 nm, with size varying from 2 to 9.5 nm. All spectra are typically around 30 to 50 nm (full width at half maximum). Fig. 2.13 (b) shows the absorption (upper curves) and emission (lower curves) spectra of four CdSe/ZnS qdot samples. The blue vertical line indicates the 488-nm line of an argon-ion laser, which can be used to efficiently excite all four types of quantum dots simultaneously [64]. This property of quantum dots provides the flexibility to choose the excitation wavelength depending on the depth that is to be probed. Development of new exogenous fluorophores have brought benefits to clinical practices like real time detection, therapy at molecular scale and monitoring the response of the therapy.

Fig. 2.13 Emission property of quantum dots a) Size dependent emission property b) Absorption and emission spectra [64]

As an example, the imaging of the development of protease in the mice brain *in-vivo* is described in [65]. The investigators checked whether their technique could resolve the enzyme activity of the protease cathepsin-B, which is linked to orthopic gliomas invasion. They implanted cathepsin-B in the brain of the mice using cathepsin-B activatable fluorochrome as the molecular beacon. Using 675 nm radiation from a laser diode (light was delivered and collected from the tissues through fiber based system) they were able to resolve and quantify pico to femto mole quantities of fluorochromes which targeted tumor specific enzyme activities located several centimeters below the tissue surface.

2.2.4 Two Photon induced imaging

Nonlinear optics based imaging is one of the breakthroughs in laser based diagnostic studies. One of the nonlinear optical effects usually employed in bio imaging is two-photon absorption which is the process in which two photons are absorbed to excite molecule to a higher excited state corresponding to the energy related to the energy of two photons [34]. Fig. 2.14 shows the Jablonski diagram for 1, 2 and 3 photon excitation [66]. Since two photon process is a third-order nonlinear optical process, it has no symmetry restriction (unlike in the case of second order nonlinear optics phenomena) and can occur in any medium irrespective of symmetric or noncentrosymmetric structure.

Fig.2.14 Jablonski diagram showing one photon (violet), two photon (red) and three photon (maroon) excitation [66]

The beauty of the two-photon approach lies in the quadratic dependence of the fluorescence intensity on the incident intensity. This leads to fluorescence emission, which is always confined to the region of focus. This will lead to the system possessing an inherent optical sectioning property as shown in Fig. 2.15 [67]. In the figure fluorescence is observed throughout the laser path (blue arrow) due to one photon excitation whereas fluorescence is localized to a spot (red arrow) in two photon excitation.

Fig.2.15 Comparison of one photon and two photon absorption. Blue arrow represents one photon absorption and red arrow two photon absorption [67]

Other benefits of the two-photon induced fluorescence over the single-photon case include the use of red or infrared lasers to excite dyes which can be excited using one photon pumping near UV radiation, confinement of photo-bleaching to the focal region (the region of excitation) and the reduced effects of scattering and greater penetration. However, it should be remembered that, compared with single photon excitation, the fluorescence yield of many fluorescent dyes under two photon excitation is relatively low

so that we have to use a sensitive detection scheme to observe two photon absorption. From the practical point of view, the two-photon approach also has very important advantages over the one-photon excitation in terms of image contrast when imaging through scattering media apart from the greater depth of penetration afforded by the longer wavelength excitation.

2.2.5 Fluorescence Lifetime imaging

The information obtained from conventional quantitative fluorescence analysis is limited because the intensity at each point in the image is influenced by many factors like fluorophore concentration and the extent of photobleaching. Moreover, the image contrast can be reduced due to autofluorescence. Hematoporphyrin (HpD) is an effective photosensitizer in photodynamic therapy for treating malignant tumors [68]. Studies using picosecond fluorescence spectroscopy has revealed that most of HpD fluorescence originates from monomers that have no tumor localizing ability. The HpD that exists in cancerous tissues form aggregates that fluoresces weakly. As a result, the increase in intensity is minimal which results in a low contrast fluorescence image from cancer cells compared to normal cells. It has been observed that the fluorescence lifetime of the HpD aggregates in the cancerous cells are 0.1 ns and that for the monomers is 4 ns [69]. To overcome the limitations in diagnostics and treatment due to intensity dependent studies, techniques based on fluorescence lifetime were developed. The lifetime of the dyes is sensitive to the environment surrounding the fluorophore. This means that recording

lifetime based images could reveal more valuable information regarding the fluorescing species.

Fluorescence measurements can be classified into steady state and time resolved. Steady-state measurements are the common type in which the sample is illuminated with a continuous light and the emitted fluorescence light is used for imaging or spectroscopic studies. Since the lifetime of the fluorophores are in the order of nanoseconds, the steady-state is achieved as soon as the light falls on the sample. To achieve time-resolved measurements, sample is illuminated with light pulses whose pulse width is smaller than the decay time of the sample. Detection is achieved by detectors with high speed that has nanosecond resolution. Steady-state intensity can be expressed in terms of the decay time τ by the relation [70]

$$I_{ss} = \int_0^{\infty} I_0 e^{\frac{-t}{\tau}} dt \quad (2.3)$$

where I_0 is an intensity parameter that depends on fluorophore concentration.

Two methods of measuring time resolved fluorescence are popular, time domain and frequency domain measurements. Even though the information obtained from the two techniques of time resolved fluorescence measurements (time domain and frequency domain) are the same, they differ in their instrumentation aspects.

In time domain measurements, a pulsed light excites the sample and time dependent intensity is measured following the excitation pulse. The slope of the plot of $\log(I(t))$ versus t is used to calculate the decay time τ . Time correlated single photon counting (TCSPC) is the popular method that is being used in time domain measurements [71-73]. The setup consists typically of a picosecond or femtosecond laser source and high speed

microchannel plate photomultiplier tubes. Fig. 2.16 [70] represents the principle of TCSPC.

Fig.2.16 Principle of TCSPC [70]

After the sample is excited with a light pulse, a waveform similar to the one shown in the top panel of Fig. 2.16 is observed. The shape of the waveform indicates that many fluorophores are excited and detected. For TCSPC, the setup is adjusted so that the detection rate of photons is about 1 in 100 pulses. A histogram is created that has the difference in time between the excitation pulse and the observed photon as the x-axis and the number of photons detected for a time difference as the y-axis. The shape of the histogram represents the waveform of the decay.

In frequency domain measurements, the sample is excited with an intensity modulated light at a frequency in the order of MHz. This results in an emission that is also intensity modulated at the same frequency [74]. The emission shows a time delay and amplitude change which is determined by the intensity decay law of the sample. The time delay can be measured as a phase shift between the excitation and emission. Most of the designs of frequency domain setups are similar to that shown in Fig. 2.17 [70].

Fig.2.17 Schematic representation of frequency domain setup. *P*, polarizer; *SB*, Soliel-Babinet compensator; *F*, frequency; δF , cross-correlation frequency; *PMT*, photomultiplier tube [70]

The light from a continuous source like a laser or arc lamp is intensity modulated using an electro optic modulator or acousto-optic modulator. Typically, two detectors (PMT) are used and cross-correlation detection method is employed. One PMT is for the sample and the other acts as a reference. The gain of the PMT's is modulated at an offset frequency $F + \delta F$ from the excitation light modulation frequency F . δF is in the range of 10-100 Hz. This results in a low frequency signal at δF that contains the phase and modulation information and can be measured easily. Another advantage of cross correlation detection is that higher harmonics present in the signal are suppressed.

This section outlined the concept of fluorescence imaging. The next section reviews the advantages of using multi modality systems for diagnostic applications.

SECTION – C

2.3 Multi modality imaging systems

An imaging technique may be well suited for a particular application while may not suit for others. For example the confocal imaging technique provides higher resolution compared to conventional optical coherence tomography, but suffers from poor depth penetration. Since no single modality can provide information on all aspects of structure and function, a multimodality platform will enable comprehensive diagnosis by combining the complementary strengths of different imaging techniques.. This section provides a brief review on some of the available multi modality imaging systems .

A multi modality fluorescence tomography system was developed combining fluorescence, diffuse optical and x-ray tomography techniques [75]. The anatomical information obtained from x-ray tomography was used as structural priori while optical background heterogeneity information obtained from diffuse optical tomography was used as functional priori. A 2.4 mm diameter fluorescence inclusion was localised with 92 % accuracy of fluorophore concentration using this system. The advantage of multimodality becomes obvious when the system was used without the x-ray tomography modality. In this setup, the fluorophore concentration was recovered with an error as high as 70 %.

Tumor accumulation in mice was studied using a multimodality nuclear and fluorescence imaging system [76]. The system was based on attaching four biotinylated functionalities to a streptavidin nanoparticle. In another study, the imaging capabilities of surface

enhanced Raman spectroscopy and x-ray computed tomography were combined to an *in-vivo* dual modality imaging system [77]. The tumor was first localized by x-ray computed tomography and then assessed at the molecular level by surface enhanced Raman spectroscopy. Studies have been carried out to monitor intracellular organelle transport in living cells using a dual modality system comprising of coherent anti-Stokes Raman scattering microscopy and two photon fluorescence microscopy [78]. The setup was used to monitor the motion of lipid droplet organelles in mouse adrenal cortical cells.

In another study, coherent anti-Stokes Raman scattering, second-harmonic generation and two-photon excitation fluorescence microscopy were incorporated into the same platform to develop a multimodal nonlinear optical microscope [79]. The developed system enabled label-free 3-D visualization of various significant components of plaques such as extracellular lipid deposits, collagen and elastin. This multi modal microscope allowed the diagnosis of different stages of atherosclerotic lesions as well as determination of their vulnerability to rupture. A similar setup was used to investigate the central nervous system in healthy and diseased states [80]. A multimodal optical workstation has been developed for simultaneous linear and nonlinear microscopy [81]. The system allows both confocal and nonlinear modality to work independently and simultaneously. The efficiency of the system was demonstrated by imaging the head of an adult *C. elegans* worm. A group had developed an integrated dual-modality imaging endospeckle fluorescence system as shown in Fig. 2.18 [18]. The probe could be used for simultaneously identifying the presence of abnormalities as well as diagnosing suspicious cancer growths at the detected abnormality sites. In the speckle modality, the system could detect abnormalities of size greater than or equal to 1mm. Since the illumination

channel of the probe delivers diverging light, the area illuminated is considerably large. This limits the system to obtain high spatially resolved information.

Fig. 2.18 Multimodality imaging for colon cancer diagnosis [18]

An image fiber based system has been developed for combined imaging and chemical sensing [82]. The specificity, sensitivity and spatial and temporal resolution of fluorescence microscopy coupled with fluorescent indicators made the system suitable for performing multiparametric analyses on biological cells. Another system was developed in a similar line to acquire both chemical and visual information using an image fiber [83]. The system was used to successfully study mouse fibroblast cells.

The literature survey reveals many works related to multimodality systems incorporating various techniques. However, multimodality imaging systems incorporating fluorescence life time imaging is least attempted. Hence it was thought worthwhile in undertaking work in this direction details of which are given in chapter 4.

Eventhough much works are reported in the laboratory realizations of confocality systems, sufficient theoretical analyses of fiber based confocal systems incorporating

relevant parameters are not available in literature. Two of the most important parameters which are not included in the studies of such systems are core diameters and spacing between the individual fibres in the image fibre bundle. Realization of this gap in the theoretical studies on image fibre bundle based confocality system has led to take up the work in this direction. Analyses of image fibre based confocality systems incorporating these parameters have been carried out details of which are given in chapter 5.

A review of conventional techniques employed for biomedical diagnostics was covered in section 2.1. The advantages of minimally invasive optical techniques were outlined and different probe schemes developed were discussed. It is clear from the literature review that sensitive detection techniques play an important role in early diagnosis of biochemical changes and improvements can be made to the existing techniques. Section 2.2 discussed the advantages of fluorescence based techniques. Fluorescence lifetime based techniques are found to be good candidates for detecting biochemical changes at an early stage. These techniques can detect the abnormalities even before morphological changes start to occur. Section 2.3 provided a brief review of multi modality imaging systems. It is clear that incorporating more than one modality improves the sensitivity of the system. The following chapters describe the work performed to achieve the objectives mentioned in the introduction chapter.

Chapter 3

Digital Micromirror Device Based Probe For Bio Imaging Applications

This chapter describes a digital micromirror device based image fiber probe for spectroscopic and imaging applications. Effect of individual fiberlets of the image fiber on the image quality is studied. Dual modality diagnostic system for selective excitation of the sample is developed which will be useful in bio imaging applications.

3.1 Introduction

Spectroscopy is an accepted tool for the study of biological systems including pathological conditions of the tissues [43-45]. The knowledge of the morphological details as well as the spectral signature is important in such studies. Devices which incorporate both these modalities with high spatial resolution and sensitivity will enhance the accuracy of the in-vivo probe. In a conventional imaging probe, the signal from the full field of view is acquired [24]. As a result, while performing spectroscopic studies, the signal detected is an integrated one from the whole region. By reducing the area of illumination, we get a higher spatial sensitivity in the spectroscopic signal. This can be useful for identifying the region from where the signal is originating more precisely in spectroscopy based diagnostic methods. In this context, a DMD based image fiber probe is developed for selective excitation of an area in the sample. The system gives both structural (imaging modality) as well as spectral characteristics of the sample. The details of the experimental setup and the results obtained are described in this chapter.

3.2 System details

3.2.1 Digital micromirror device (DMD)

For rapid gathering of information by electronic means, direct interface between the electronic information and data processing system is needed. In the field of optical information processing, a large number of devices capable of converting optical signals to electronic form are available. Such devices called spatial light modulators (SLM) are available in two different classes, namely, electronically and optically addressed SLMs

[84]. In the field of optical information processing, different categories of SLM technologies have been explored like liquid crystal SLM, magneto-optic SLM etc. One type of SLM employs electrostatically induced mechanical deformation to modulate a reflected light wave. Such devices come in two ways like membrane device and cantilever beam device. The membrane device corresponds to deformable mirror devices while cantilever beam device work based on motion of cantilever. Both these devices results in the optical beam getting deflected by the tilted micromirror or tilted pixels. In this way, an intensity modulation can be induced at each pixel. The device can be operated either in an analog mode in which tilt is a continuous function of applied address voltage or in a digital mode in which the device has two stable states depending on the bias voltage applied. A major advantage of this type of SLM technology is that it is silicon based and compatible with CMOS drivers. When the device is working in digital mode, we have the SLM named as digital micromirror device (DMD). In the present work, this type of an array of 1024×768 aluminium micromechanical micromirrors with 13.68 micrometer pitch is employed. Each of these mirrors can be individually deflected at an angle of $\pm 12^\circ$ about a hinged diagonal axis [85]. Deflection polarity of the mirrors is controlled by changing the address voltage of underlying CMOS addressing circuitry and mirror reset signals. Data is clocked in on the leading and falling edge of the internal DMD data clock. The device is mounted using a pressure socket, and is equipped with a mounting surface for optical interfacing. Fig. 3.1a shows the photograph of DMD and its controller. Fig. 3.1b is the close-up view of an array of individual micromirrors in the DMD. Figure 3.1c represents the details of a single such micromirror [85].

(a)

(b)

(c)

Fig. 3.1 Structure of DMD a) DMD and its controller b) array of individual micromirrors in the DMD c) Details of a single micromirror in the DMD [85]

When a light beam falls on the DMD, the grid like structure of the micromirror array results in the generation of a diffraction pattern as shown in Fig. 3.2 which resembles

the diffraction of light by a two dimensional grating.

Fig. 3.2 Diffraction pattern generated by the DMD

3.2.2 Image fiber

The fiber optic technology was initiated in the context of development of flexible endoscope for in-vivo imaging. Interfacing the in-vivo region of interest with detection devices is accomplished by coherent fiber bundle, generally named as image fiber. In an image fiber, tens of thousands of fibers are combined in a bundle with active area of the order of about 1 mm^2 . Each fiber transfers a spot of light from the input end to the output side. As a result, these can be used for delivering as well as collecting light from inaccessible areas like colon. With appropriate selection of optical devices and detection schemes coupled with the image fiber, one can study spectroscopic as well as

morphological details of the target samples. Use of image fiber as a probe in microscopy application generally requires microscope objective for sample imaging. This can be miniaturized by using a grin lens at the distal end of the image fiber. In the present work, a Sumitomo image fiber bundle with 50,000 fiberlets and a Grintech focusing grin lens attached to one of its end is employed [86] (Fig. 3.3).

Fig. 3.3 Image fiber bundle with grin lens

The diameter of individual fibers is 2.7 microns and the distance between the neighboring cores is 4.4 microns. This results in an active area with diameter 0.99 mm. The diameter of the grin lens is 1 mm. The grin lens collects the diverging beam from the image fiber and focuses it to the sample as shown in Fig. 3.4. The signal originating from the sample travels back through the grin lens and gets focused on the image fiber plane.

Fig. 3.4 Light propagation in the grin lens

3.3 Effect of individual fibers in the image

Fig. 3.5 shows the schematic experimental setup for studying the effect of individual fibers of the image fiber in the image formed. Laser light from the fiber pigtailed laser was collimated by a lens and guided through a microscope objective (MO) by a dichroic beam splitter. The proximal end of the image fiber was kept at the focal plane of the microscope objective. This results in a group of fibers being illuminated by the focal spot.

The grin lens at the distal end of the image fiber focuses the light onto the sample being studied. The sample was prepared in a glass slide (redwop fluorescence powder in acetone) and was mounted on a translation stage that could be translated through the focal volume of the grin lens. The reflected light from the sample was blocked by the dichroic beamsplitter and only the fluorescent light was imaged by the CCD. Fig. 3.6 shows the result obtained at various MO magnifications (10X, 20X, 50X).

Fig. 3.5 Experimental setup for studying the effect of individual fibers of the image fiber bundle in the image

The interrogation area of Fig. 3.6a, Fig. 3.6b and Fig 3.6c is 435 micron* 330 micron, 217 micron* 124 micron and 87 micron* 57 micron, respectively. It is clear from the figures that the quality of the image is degraded by the cladding around each core. The discontinuity in the image due to the hexagonal pattern of the fiber is more evident in higher magnifications (20X and 50X). It can be understood from the figures that the cladding also reduces the resolution of the image transmitted. The resolution of the system was determined to be 7.81 micron/line pair using resolution chart (details are given in appendix F).

Fig. 3.6 Image of the sample imaged by image fiber probe at various MO magnifications

a) Magnification 10X b) Magnification 20X c) Magnification 50X

Since the core spacing of the fibers is 4.4 microns, the lateral resolution is limited to 8.8 microns (two times the core spacing). To understand the effect of image fiber on the degradation in the image resolution, another experiment was carried out to image the same sample without the image fiber. Fig. 3.7 shows the schematic of the experimental

setup. The experimental setup in Fig. 3.5 was modified by removing the image fiber and bringing the sample to the focal plane of the microscope objective (MO).

Fig. 3.7 Experimental setup for freespace imaging of the sample

Results obtained at various MO magnifications (10X, 20X, 50X) are given in Fig. 3.8. The interrogation area of Fig. 3.8a, Fig. 3.8b and Fig 3.8c is 435.6 micron* 330 micron, 217.8 micron* 124.3 micron and 87.12 micron* 57.2 micron, respectively. From the figures, we can see the morphology of the sample very clearly. Comparing these images with those captured through the image fiber probe, it can be seen that the resolution gets degraded on employing an image fiber bundle. The lateral resolution of the systems

corresponding to magnification 10X, 20X and 50X is 1.064 microns, 0.7 microns and 0.399 microns respectively and is determined by the Abbe criterion $0.6\lambda/NA$. The resolution of the system was studied using resolution chart and the details are given in appendix F. It is worth pointing out that image fiber probe can be used effectively for imaging applications that do not demand lateral resolution in the submicron range. For example, these probes can be used for tissue imaging whose dimensions are around 10 micron.

Fig. 3.8 Image of the sample at various MO magnifications a) Magnification 10X b) Magnification 20X c) Magnification 50X

3.4 Removal of the fiber honeycomb structure from the image

Imaging with image fiber probes results in honeycomb fiber structure in the image. The structure is more prominent at higher magnification as was shown in the previous section. This section looks into the techniques that can be used to remove the honeycomb structure from an image obtained using an image fiber probe.

3.4.1 Gaussian filter

A Gaussian filter modifies the image by convolving it pixel by pixel with a Gaussian function,

$$G(x, y) = \frac{1}{2\pi\sigma^2} e^{-\left(\frac{x^2+y^2}{2\sigma^2}\right)} \quad (3.1)$$

A typical Gaussian filter function is given in Fig. 3.9.

Fig. 3.9 Schematic representation of a Gaussian filter

As a result, the original pixel values are replaced by a weighted average of the neighboring pixel values. The original pixel receives the maximum weighted value and the neighboring pixels receive weighted values which decrease with distance from the central pixel. The honeycomb structure in the image due to the image fiber bundle is more or less periodic. As a result, such an averaging procedure will remove the periodic structure in the image. In order to remove the hexagonal structure of the fiber bundle and create a continuous image, the images acquired with the probe were convolved with the Gaussian filter using the protocols in ImageJ software [87]. This process is schematically represented in Fig. 3.10. Image acquired with the probe at 10X magnification (Fig. 3.6a) was convolved with the filter to get an image without the hexagonal fiber structures of the individual fibers.

Fig. 3.10 Schematic representation of the action of Gaussian filter (a) Convolution operation on the image captured by image fiber probe b) Image without honeycomb structure

Similar procedure has been performed for the other images in Fig. 3.6. Fig. 3.11 shows the result obtained. Compared to Fig. 3.6, the honeycomb structure of the fibers has been removed from the processed images. The intensity profile along a sample line in Fig. 3.6b

is plotted in Fig. 3.12. Well defined discontinuities due to the cores of the fibers are clearly seen.

Fig. 3.11 Image of the sample imaged by image fiber probe at various MO magnifications after applying Gaussian filter a) Magnification 20X corresponding to Fig.3.6 b) Magnification 50X corresponding to Fig. 3.6c

Fig. 3.12 Intensity profile of the image acquired by image fiber a) Fig. 2.6b b) Intensity profile along a sample line

Fig. 3.13 shows the intensity variation along the same sample line of the processed image after applying Gaussian filtering (Fig. 3.11a). Comparing the intensity profile plot of Fig. 3.13b and Fig. 3.12b, it is evident that the discontinuities due to the individual fibers of the image fiber probe have been removed by applying gaussian filter.

Fig. 3.13 Intensity profile of the processed image after Gaussian filtering a) Fig. 3.11a b) Intensity profile along a sample line

3.4.2 Filtering the spectral component of the honeycomb structure in the Fourier domain

Since the individual fibers are more or less spaced at equal distances in the fiber bundle, they create a periodic pattern in the image transmitted through the probe as seen in Fig. 3.6. As a result, the Fourier transform of the image will clearly show the structure of the fibers. Fig. 3.14 shows the Fourier spectrum corresponding to the image shown in Fig.

3.6a. As seen in the Fourier spectrum, a ring structure is visible at a high frequency region. This corresponds to the spectral information related to the structure of the image fiber.

Fig. 3.14 Fourier Spectrum of the image given in Fig 3.6a

Fig. 3.15 Horizontal pattern of the Fourier Spectrum shown in Fig 3.14

Fig. 3.15 shows the horizontal line section taken along the center of the Fourier spectrum shown in Fig. 3.14. taken along the center. Removing this ring structure using a band reject filter and performing the inverse transform will result in an image without the prominence of the honeycomb structure present in the image fiber.

Fig. 3.16 shows the filtered image and the horizontal cross-section of its Fourier spectrum taken along the center. Comparing Fig. 3.15 and Fig. 3.16b, filtering of the sidelobes which corresponds to the spectral information of the image fiber is evident.

*Fig. 3.16 Effect of bandreject filter a) Image after applying bandreject filter on Fig. 3.6a
b) Horizontal pattern of the Fourier spectrum of the filtered image*

Even though the fine morphological details of the sample which were acquired without the image fiber (Fig. 3.8) could not be resolved in the processed image after performing either Gaussian filtering or filtering the spectral component of the honeycomb structure, the filtered images provide visualization of the sample structure without the fiber honeycomb details.

3.5 Selective illumination of the sample using DMD

As indicated earlier the signal detected following conventional imaging is an integrated one from a region of large area which will not be of much help in situations where spatial sensitivity in the imaging is important. We can record spatially sensitive images by reducing the area of illumination. One way to achieve this is by limiting the number of fibers in the image fiber bundle that are used for the delivery of the excitation light. This can be achieved by placing a DMD in the excitation pathway between the image fiber and the excitation source. By controlling the orientation of the individual mirrors of the DMD to either on or off position, we can illuminate selectively the fibers in the image fiber bundle. An image fiber probe that can provide both the morphological and spectroscopic information from the selected area in the sample can be useful for biomedical applications. Fig. 3.17 shows the schematic of the experimental setup for achieving such a dual modality technique employing DMD based image fiber probe.

The diverging beam from the diode laser ($\lambda = 532 \text{ nm}$, 25mW) was collimated by a collimating lens and guided to the DMD as shown in the figure. The light reflected from the mirrors in the DMD gets focused to the proximal end of the image fiber bundle with the help of a fiber coupling unit. The fiber coupling unit consists of a microscope objective (10X) held at one end and a ferrule for holding the image fiber at the other end. The microscope objective or the image fiber can be translated in 3-axis for alignment. The grin lens attached at the distal end of the fiber bundle focuses the light to the sample. Sample (redwop fluorescence powder in acetone) is prepared in a glass slide and mounted on a translation stage.

Fig. 3.17 Schematic of the experimental setup for DMD based image fiber probe

The reflected light originating from the sample is blocked to a large extent by the dichroic mirror. The fluorescence light transmitted by the dichroic mirror is split into two using a beam splitter. One part is used for imaging and the other for spectral characterization. Results obtained are given in Fig. 3.18 which shows the images and corresponding spectra. In Fig. 3.18 a, around 7500 fibers are illuminated. In Fig. 3.18c, around 475 fibers are illuminated.

Spectral emission at 600nm along with the pump beam at 532 nm can be clearly seen in Fig. 3.18. The set up has an advantage of simultaneous recording of image and corresponding spectrum so as to get more information about the target species with high

spatial sensitivity like the identification of the emitter materials since spectral emission will contain the signature of the emitting species.

Fig. 3.18 Dual modality DMD based image fiber probe a) Image with full field of view b) Spectral signature corresponding to the illuminated area in (a) c) Image with a selective area and d) corresponding spectral emission

For example, when a dye is injected which selectively accumulates on specific groups of tissues, their image as well as spectral characteristics can be simultaneously recorded. Depending on the physical and chemical structure of the tissue materials, there will be spectral modifications of the emission spectra. This will provide some indications on the

pathological conditions of the tissues. Fig. 3.19 shows the images after the honeycomb structures are removed by Gaussian filtering and the corresponding Fourier spectrum.

Fig. 3.19 Removal of the honeycomb structure from the probe image and the corresponding Fourier spectra a) Image obtained from the probe b) Image after Gaussian filtering c) Fourier spectrum of Fig. 3.19a d) Fourier spectrum of Fig. 3.19b.

By comparing the Fourier spectrum of the image before and after Gaussian filtering, it is clear that the honeycomb structure created by the fibers in the image fiber probe has been removed by Gaussian filtering.

A DMD based image fiber bundle probe was developed and tested for biomedical applications. The probe can provide simultaneously both spectral information and image of the sample. By performing selective illumination of the sample, the spatial sensitivity of the probe is found to improve. Techniques for removing the honeycomb structure in the image were also studied. Another method which is popular for detecting biochemical changes is the difference in the fluorescence lifetime. Next chapter focuses on using this technique for improving the sensitivity of the probe.

Chapter 4

Frequency Domain Imaging

An Integrated Dual Modality Approach

This chapter describes an integrated dual modality frequency domain imaging system. The proposed system and methodology are found to improve the depth perception and effective in obtaining selective suppression of fluorescence emissions. Investigations and related experimental analysis for conventional and a probe based system were carried out and presented in this chapter.

4.1 INTRODUCTION

In conventional microscopy techniques, only the amplitude component of the reflected or transmitted wave is measured. However, a great deal of additional information can be obtained by measuring the phase [88, 89]. The development of phase imaging in microscopy began in 1935 when Zernike [90] introduced phase-contrast imaging. In phase-contrast imaging, both the amplitude and phase changes of the sample appear as intensity variations in the image. As mentioned in chapter 2 (literature review), in frequency domain lifetime based imaging, the fluorescence lifetime difference can be studied from the phase shift between the excitation and emission light. This change causes a variation in the intensity of the image. By proper algorithms, we can isolate the intensity image due to the phase part alone. This can be used to study biochemical changes in the sample.

Recent developments in optical fiber technology has made a strong platform as one of the most effective and emerging diagnostic means for noninvasive detection of cancers as well as pre-cancerous lesions that originate in the epithelial lining of organs such as the colon. The progression of such cancerous growth in these tissues produces morphologic and biochemical changes in the epithelium and supporting stroma. This eventually alters the concentration and distribution of endogenous fluorophores such as tryptophan and collagen. Though there are optic configurations reported for detecting endogenous chromophores non-invasively *in vivo* by use of fiber-optic fluorescence and reflectance spectroscopy, they are mainly designed to collect the integrated signals [24]. By combining fluorescence lifetime based imaging and spectroscopy into one system, one can obtain high selectivity and precise measurement capability. In this context, a

frequency domain dual modality image fiber based probe is developed for selective suppression of fluorescence emissions. The system can obtain both spectroscopic signature characterizations of the sample as well as fluorescence lifetime based images. A detailed theoretical analysis of the system followed by the experimental procedure is presented in this chapter.

4.2 SELECTIVE SUPPRESSION OF FLUORESCENCE EMISSIONS BASED ON LIFETIME DIFFERENCES

The response of fluorescent molecules with fluorescence lifetime τ is given by [91]

$$fluorescence(t) = \frac{1}{\tau} \exp\left(-\frac{t}{\tau}\right), \quad t > 0 \quad (4.1)$$

where t is time.

In the present experiment, the sample is illuminated with a sinusoidal intensity modulated laser beam with an angular frequency ω and a modulation depth m_{ex} . The time-dependent laser excitation [92] is given by

$$E(t) = E_0[1 + m_{ex} \sin(\omega t)] \quad (4.2)$$

The fluorescence emission $F(t)$ is calculated by the convolution of equation (4.1) and (4.2)

$$F(t) = E(t) \otimes fluorescence(t) \quad (4.3)$$

This results in a sinusoidally modulated fluorescence that is phase-shifted and demodulated relative to the excitation. Therefore, the fluorescence emission intensity falling on the detector is given by,

$$F(r, t) = F_1(r)[1 + m_1(r)\sin(\omega t - \phi_1(r))] + F_2(r)[1 + m_2(r)\sin(\omega t - \phi_2(r))] \quad (4.4)$$

where $m_1(r)$ is the modulation depth of first fluorophore, $m_2(r)$ is the modulation depth of the second fluorophore, $F(r)$ is the time-averaged spatially dependent fluorescence optical power, $F_1(r)$ is the time-averaged spatially dependent fluorescence optical power of first fluorophore and $F_2(r)$ is the time-averaged spatially dependent fluorescence optical power of the second fluorophore. $F(r)$ depends on various parameters such as laser power, absorption of the sample and fluorescence quantum yield.

The fluorescence modulation depth m is given by

$$m_1 = m_{ex} [1 + (\omega\tau_1)^2]^{-\frac{1}{2}} \quad \text{and} \\ m_2 = m_{ex} [1 + (\omega\tau_2)^2]^{-\frac{1}{2}} \quad (4.5)$$

The phase shift angle ϕ for the fluorophore depends on the decay time τ by the relation

$$\tan(\phi_1) = \omega\tau_1 \quad \text{and} \\ \tan(\phi_2) = \omega\tau_2 \quad (4.6)$$

where ϕ_1 and ϕ_2 represent the phase shift of the first and second fluorophore with decay time τ_1 and τ_2 respectively.

Fig. 4.1 schematically represents the phase angle ϕ and the modulation depth m .

Fig. 4.1 Schematic representation explaining phase angle ϕ and modulation depth m

The modulation frequency ω and the decay time τ determine the phase shift ϕ and the modulation depth m of the fluorescence emission. The fluorescence lifetime of dyes lie in the range of nanoseconds (1-10 ns). Fig. 4.2 represents the effect of modulation frequency for a dye with $\tau = 5\text{ns}$ [70].

Fig. 4.2 Effect of modulation frequency on phase shift ϕ and modulation depth m [70]

It is observed from the figure that as the modulation frequency is increased, the phase shift increases while the modulation depth decreases. When the modulation frequency is 2.5 MHz, the period is 400 ns. Since the decay time is 5 ns, the emission follows the excitation with minimum phase shift with almost complete modulation. As a result, the fluorescence decay cannot be observed. When the modulation frequency is increased to

250 MHz, the period is 4 ns. This results in an averaging of the decay during the full cycle. This results in an increased phase shift and lower modulation depth. Therefore, an intermediate frequency must be chosen to trade-off between the depth of modulation and phase shift.

To carry out lifetime sensitive image detection, the phase information has to be obtained. This can be achieved by detecting the output signal using a light detector of fast response and then calculating the phase information electronically. An alternate and easier solution is to use a gain modulated image intensifier employing either homodyne or heterodyne detection approach in which the high frequency signal is converted to a low frequency signal by demodulation [92, 93]. The photocathode of the image intensifier generates an electron image from the light falling on it. The electrons after being amplified by a microchannel plate get reconverted to an optical image at the phosphor screen which is eventually imaged on to the CCD. The voltage between the photocathode and the microchannel plate surface is varied at the desired frequency. In homodyne detection, the gain modulating signal for the intensifier has the same frequency as the laser modulation frequency ω . In heterodyne detection, the gain modulating signal has a slightly different frequency $\omega \pm \Delta\omega$. Fig. 4.3a and 4.3b schematically represents homodyne and heterodyne detection schemes respectively.

(a)

(b)

Fig. 4.3 Representation of signals based on a) Homodyne b) Heterodyne detection schemes.

In the homodyne detection method employed, an electrical sine wave signal with a frequency equal to the laser modulation frequency is applied to the Intensified CCD (ICCD) resulting in a time varying gain,

$$D(t) = D_0[1 + m_D \sin(\omega t - \phi_D)] \quad (4.7)$$

where D_0 is the gain without applying a modulating signal, m_D is the gain modulation depth due to the modulating signal and ϕ_D is the detector phase. The detector phase angle ϕ_D can be shifted relative to the laser modulation phase angle to get phase-sensitive images. The time averaged phase sensitive image is given by multiplying equation (4.4) with equation (4.7).

$$I(r, \phi_D) = I_{01}(r)[1 + \frac{1}{2} m_D m_1 \cos(\phi_1 - \phi_D)] + I_{02}(r)[1 + \frac{1}{2} m_D m_2 \cos(\phi_2 - \phi_D)] \quad (4.8)$$

The resulting image contains only the low-frequency components of the modulated image because high frequencies are filtered out by the slow response of the photoluminescence surface at the output of the intensifier of the ICCD. $I_{01}(r)$ and $I_{02}(r)$ are the average intensity for the first and second fluorophore, respectively which is proportional to the steady-state fluorescence from the samples. It depends on factors such as photon detection efficiency, intensity of excitation and fluorophore quantum yield of the sample. Now another image is taken by changing the detector phase angle by 180° . This results in an image given by

$$I(r, \phi_D + 180^\circ) = I_{01}(r)[1 - \frac{1}{2}m_D m_1 \cos(\phi_1 - \phi_D)] + I_{02}(r)[1 - \frac{1}{2}m_D m_2 \cos(\phi_2 - \phi_D)] \quad (4.9)$$

Adding Equation (4.8) and Equation (4.9) gives

$$I(r, \phi_D) + I(r, \phi_D + 180^\circ) = 2I_{01}(r) + 2I_{02}(r) \quad (4.10)$$

Dividing Equation (4.10) by 2 gives

$$\frac{I(r, \phi_D) + I(r, \phi_D + 180^\circ)}{2} = I_{01}(r) + I_{02}(r) \quad (4.11)$$

This image is stored as background and background subtraction is turned on. Now ϕ_D is changed. When $\phi_D = \phi_2 - 90^\circ$,

$$I(r, \phi_2 - 90^\circ) = \frac{1}{2}I_{01}(r)m_D m_1 \sin(\phi_1 - \phi_2) \quad (4.12)$$

Compared to an image represented by equation (4.8), in the image represented by equation (4.12), fluorescence with a phase angle ϕ_2 or with a lifetime τ_2 is completely suppressed. It is worth mentioning that without background subtraction, equation (4.12) becomes

$$I(r, \phi_2 - 90^\circ) \text{ Without background subtraction} = I_{01}(r) \left[1 + \frac{1}{2} m_D m_1 \sin(\phi_1 - \phi_2) \right] + I_{02}(r) \quad (4.13)$$

It is seen that this image has a term due to the fluorescence with a lifetime τ_2 . Similarly, when $\phi_D = \phi_1 + 90^\circ$, an image with lifetime corresponding to τ_1 is completely suppressed and is given by

$$I(r, \phi_1 + 90^\circ) = \frac{1}{2} I_{02}(r) m_D m_2 \sin(\phi_2 - \phi_1) \quad (4.14)$$

Equation (4.12) and equation (4.14) shows that the technique can selectively suppress the emissions irrespective of whether the lifetime of one fluorescence decay is shorter or longer than the other.

The following section describes the details of the experiment carried out and the results obtained.

4.3 INSTRUMENTATION DETAILS

4.3.1 Laser diode [94]

To perform frequency domain imaging, it is necessary to have an intensity modulated excitation beam which can be modulated at a frequency that can create an appreciable amount of fluorescence modulation and phase shift. To achieve this, a compact current

modulated diode laser module ($\lambda = 405$ nm, Omicron) was employed which can be modulated up to 100 MHz with the aid of an external trigger.

4.3.2 Intensified CCD camera (ICCD) [95]

Normal stand alone CCD cameras are perfect image sensors (Signal to noise ratio is photon limited). In low-light level applications, the image generated by CCD is degraded by the readout noise. Therefore, to study phenomena which are in the scales of fluorescence decay time, it is better to use ICCD since the image intensifier amplifies the incoming light before the camera degrades the image with readout noise. Moreover, it is not possible to modulate the sensitivity of a CCD pixel at high frequencies (RF). However, it is possible to modulate the sensitivity of an image intensifier at RF in either a top-hat or sine function form. Taking this into account, an ICCD camera (Lavision, Picostar HR) was used for detection which could be gain modulated with an external trigger. A high rate image (HRI) controller is used to operate the image intensifier.

4.3.3 Function Generator (FG) [96]

A signal generator is necessary to produce the modulation signals for the illumination beam and the high rate imager (HRI) of the ICCD. The generator must produce two sinusoidally modulated signals with the same frequency and controllable phase shift between the signals. Since the fluorescence lifetime of dyes lie in nanosecond timescales (1-10 ns), the signal generator should be capable of producing frequency in the MHz range. This was achieved by synchronizing two identical single channel function

generators (Agilent, 33250A). The laser diode was modulated at 20 MHz by a sinusoidal signal from one of the FG. The second FG modulates the HRI of the ICCD at the same frequency to enable homodyne detection.

4.3.4 Fluorescence lifetime spectrofluorometer [97]

The fluorescence lifetime of the samples were obtained using a lifetime spectrofluorometer operating in the time domain (Horiba, Fluorocube-01-NL). The system could detect lifetimes of the order of picoseconds and proved to be a good choice for studying fluorescent dye lifetimes. The emission monochromator could be tuned from 200 nm to 800 nm.

4.4 IMAGING IN THE FREQUENCY DOMAIN: NON-PROBE BASED APPROACH

The schematic of the experiment is shown in Fig. 4.4. The laser was intensity modulated at a frequency of 20 MHz using a sinusoidal signal from a function generator. The diverging beam from the diode laser was collimated and was guided through the microscope objective (MO-L1) (20X/0.4) by a dichroic beam splitter as shown in Fig. 4.4. The sample was prepared in a glass slide and was mounted on a translation stage that could be translated through the focal volume of the objective. In order to establish life time discrimination, the test sample was prepared by mixing basic yellow and Rhodamine 6G in acetone. The mixture was applied as a thin coating on the glass slide. The reflected and fluorescent light from the sample passes back through the objective. The reflected light from the sample is blocked by the dichroic beam splitter. Only the emitted

fluorescence passes through the collection objective (MO-L2) (20X/0.5). A pinhole is placed at the focus of the collection objective to minimize the background signal.

Fig. 4.4 Experimental setup representing the frequency domain imaging. MO: Microscope objective, FG: Function generator, HRI: High rate imager

A Z-scan was performed and the images were captured with the help of an ICCD camera whose modulation and signal operation were controlled by the high rate imager (HRI) of the camera. The ICCD was also modulated at the same frequency as that of the laser through an identical function generator that was synchronized with the first. Function generators and the ICCD camera were controlled by an interfaced computer. Fig. 4.5 shows the setup in the lab.

Fig. 4.5 Details of the experimental setup in the lab

4.4.1 Results and discussion

The procedure described in the theory section was carried out and an image corresponding to equation (4.11) was obtained and was set as background for subsequent subtraction. Now ϕ_D was changed till the condition given in equation (4.12) was satisfied and an image with one fluorophore completely suppressed was obtained. The image of the other fluorophore was obtained by changing ϕ_D till a similar condition for the other fluorophore was reached. The dyes used were basic yellow (519 nm, lifetime of 2.41 ns)

and Rhodamine 6G (571 nm, lifetime of 5.22 ns) in acetone. Fig. 4.6 shows the fluorescence lifetime spectra of basic yellow (Fig. 4.6a) and Rhodamine 6G (Fig. 4.6b) in acetone.

(a)

(b)

Fig. 4.6 Fluorescence lifetime spectra of a) Basic yellow in acetone b) Rhodamine 6G in acetone

Fig. 4.7 shows the fluorescence lifetime based selective suppression of the fluorescent dye.

Fig. 4.7 Images representing lifetime based suppression a) Normal image b) Basic yellow suppressed c) Rhodamine 6G suppressed

Fig. 4.7a shows the image obtained by normal detection. Fig. 4.7b and 4.7c shows the image corresponding to equation (4.12) and equation (4.14) respectively. The figure shows that selective suppression of either basic yellow emission or Rhodamine 6G emission is possible. An experiment was also carried out with one fluorophore alone to demonstrate the increased depth perception achieved by this method. Fig. 4.8 shows the improved depth perception achieved by the said technique using basic yellow fluorophore in acetone (519 nm emission wavelength, lifetime of 2.41 ns). Fig. 4.8a corresponds to the normal image. Fig. 4.8b is the image with only the modulated term. In Fig. 4.8b, the features became clearer. The contrast of the image improved and the depth perception increased.

Fig. 4.8 Images representing better depth perception achieved by the technique

a) Normal image b) Image after processing

Fig. 4.9 shows the 3D representation of the images corresponding to Fig. 4.8. The features that are hidden in the normal image are visible in the processed image.

Fig. 4.9 3D representation of images with better depth perception a) normal image (Fig. 4.8a) b) processed image (Fig. 4.8b)

4.5 IMAGE ENHANCEMENT BY REMOVING THE UNMODULATED TERM

Rewriting equation (4.8) for a single fluorophore,

$$I(r, \phi_D) = I_{01}(r) \left[1 + \frac{1}{2} m_D m_1 \cos(\phi_1 - \phi_D) \right]$$

$$\begin{aligned}
 &= I_{01}(r) + \frac{1}{2} I_{01}(r) m_D m_1 \cos(\phi_1) \cos(\phi_D) + \frac{1}{2} I_{01}(r) m_D m_1 \sin(\phi_1) \sin(\phi_D) \\
 &= I_{01}(r) + I_{11}(r) \cos(\phi_D) + I_{21}(r) \sin(\phi_D)
 \end{aligned} \tag{4.15}$$

We can see that the signal consists of an unmodulated term $I_{01}(r)$ along with the modulated term $I_{11}(r) \cos(\phi_D) + I_{21}(r) \sin(\phi_D)$. The unmodulated term affects the quality of the image since it generates a background. Removing this background term will result in an image of better contrast. To achieve this, two more images are acquired with a phase setting of $\phi_D + 120^\circ$ and $\phi_D + 240^\circ$. Consider $\phi_D = 0$, we get

$$\begin{aligned}
 I1 &= I(r, 0) = I_{01}(r) + I_{11}(r) \\
 I2 &= I(r, 120) = I_{01}(r) - \frac{1}{2} I_{11}(r) + \frac{\sqrt{3}}{2} I_{21}(r) \\
 I3 &= I(r, 240) = I_{01}(r) - \frac{1}{2} I_{11}(r) - \frac{\sqrt{3}}{2} I_{21}(r)
 \end{aligned} \tag{4.16}$$

It can be seen that

$$\begin{aligned}
 (I1 - I2)^2 &= \frac{9}{4} I_{11}^2 + \frac{3}{4} I_{21}^2 - \frac{3\sqrt{3}}{2} I_{11} I_{21} \\
 (I1 - I3)^2 &= \frac{9}{4} I_{11}^2 + \frac{3}{4} I_{21}^2 + \frac{3\sqrt{3}}{2} I_{11} I_{21}
 \end{aligned}$$

$$(I_2 - I_3)^2 = 3I_{21}^2 \quad (4.17)$$

Therefore,

$$\begin{aligned} \frac{\sqrt{2}}{3} \left[(I_1 - I_2)^2 + (I_1 - I_3)^2 + (I_2 - I_3)^2 \right]^{\frac{1}{2}} &= (I_{11}^2 + I_{21}^2)^{\frac{1}{2}} \\ &= \frac{1}{2} I_{01}(r) m_D m_1 \end{aligned} \quad (4.18)$$

results in an image with intensity information of only the modulated term. To find out the quality of the image without the unmodulated term, the steps described above were carried out with the configured system. Experimental setup was similar to Fig. 4.4 without employing the pinhole. The sample used was basic yellow in acetone. Fig. 4.10 shows the result obtained.

Fig. 4.10 Enhancing contrast by removal of unmodulated term a) Image with intensity of modulated and unmodulated terms b) Image with intensity of only the modulated term

Fig. 4.10a shows the image with both unmodulated and modulated intensity information (equation (4.15)). It is clear from the image that there is an appreciable amount of background signal due to the unmodulated term which reduces the contrast of the image. Fig. 4.10b shows the image corresponding to equation (4.18). In the image with intensity information of only the modulated term, the background haze has reduced and the contrast has improved.

4.6 OPTICAL FIBER PROBE BASED SYSTEM

The bulk optics based configuration described above is not suitable for *in-vivo* imaging. This methodology can be incorporated into a probe based system and used for in-vivo imaging. In this context, the above design is modified which resulted in an integrated frequency domain imaging and combined spectroscopy probe system. The experimental configuration for image fiber based probe system is shown in Fig. 4.11.

The image fiber used has 30000 fiberlets (fiber pixels) having a numerical aperture (NA) of 0.35 [Fujikura, 0.6 mm core diameter]. The fiber ends were polished and cleaved perpendicular to the optic axis and the fiber is positioned on the central axis of the probe system. A continuous wave (CW) laser (Omicron, $\lambda=405\text{ nm}$, Power = 30 mW) is used as the excitation source and is diverted to the fiber coupling unit using a dichroic beamsplitter.

Fig. 4.11 Image fiber based frequency domain imaging and spectroscopy. MO: Microscope objective, FG: Function generator, HRI: High rate imager

The fluorescence emission collected by the laser beam from the test target is coupled into the image fiber using the custom fiber coupling unit [Newport F-91TS Coupler] employing a translation stage. It is to be mentioned that special custom made ferrule and jig were used for holding the chosen image fiber. The chosen microscope objective (MO) lens is Newport M-20X, 0.4 integrated with the FC coupler at the proximal end of image fiber. The laser emerging from the distal end of the image fiber will be a diverging one and is allowed to pass through the 2-f lens system. The 2-f lens system is configured using two microscope objectives (MO) lenses, (20X/0.4, MO1) and (50X/0.8, MO2) facing

opposite to each other. This distal end face of the image fiber is kept at the focal distance of the first objective lens, MO1. The collimated beam from this MO1 is focused to the target plane by the second objective lens, MO2. This MO unit serves as the collection unit as well. The reflected fluorescence light from the surface plane of the target specimen will be collected and focused at the image fiber plane. The collected fluorescent light as well as the reflected light from the sample will be reaching the dichroic mirror which allows the fluorescence beam to transmit through. A beamsplitter is used to direct a part of the signal to the spectrometer and the other part to the ICCD. The system could simultaneously image and also take the spectral information [Dual-modality]. The integration of spectrometer along with frequency domain imaging gives more information about the sample.

4.6.1 Results and discussions

The sample consisted of basic yellow (519 nm, lifetime 2.41 ns) and redwop (560 nm, lifetime 4.43 ns) powders in ethanol, which is pasted onto a cover glass slide. The fluorescent powders were used as obtained from the suppliers. The powders are not of uniform particle size, but are in the order of tens of microns. It is also possible that the particles aggregate themselves and gives an impression of a larger size. For example, in Fig. 3.6, the size range of the particle (aggregate) redwop is from 15-45 microns. This size range correlates with the size range obtained using an optical microscope (Appendix E). Particle size was also determined using a commercial particle analyzer and the details are given in appendix E. Concentration of the dye was selected appropriately so as to get good fluorescence emission.

Fig. 4.12 Fluorescence lifetime spectra of redwop

Fig. 4.12 shows the fluorescence lifetime spectra of redwop. The sample was moved along z-axis and spectrum was taken at each position. The sample was moved at an interval of 12.5 microns. The experimental data is shown in Fig. 4.13. It is clear from the data that as we do Z-scan, the fluorescence intensity from the two samples change. Initially, emission from basic yellow is prominent and as the sample is moved along the axis, intensity of emission from redwop increases.

4.13 a

4.13 b

4.13 c

4.13 d

4.13 e

4.13 f

Fig. 4.13 Peak emissions from cover glass dual fluorescence sample when scanned through the z-axis with steps of 12.5 microns a) $z = 0$ microns (519 nm is prominent) b) $z = 12.5$ microns (519 nm is prominent, 560 nm is visible) c) $z = 25$ microns (519 nm reducing, 560 nm increasing) d) $z = 37.5$ microns (560 nm is prominent than 519 nm) e) $z = 50$ microns (519 nm disappearing) f) $z = 62.5$ microns (560 nm is prominent)

Fig. 4.14 Selective suppression based on lifetime differences a) Basic yellow suppressed b) Redwop suppressed c) Image with both emissions

Fig. 4.14 shows the selective suppression of the two emissions at one of the planes. Fig. 4.14a and 4.14b show images with one of the emission suppressed. Fig. 4.14c shows the normal image.

The concept of frequency domain imaging for biomedical applications has been analyzed theoretically and experimentally. The effect of removing the unmodulated intensity term by image processing results in an image with better contrast. Experiments revealed that the technique could be used effectively for selective suppression of fluorescence emissions based on the fluorescence lifetime difference. Development of the dual modality probe by integrating spectroscopic modality with lifetime based imaging can find possible applications for detecting biochemical changes. The sensitivity of the probe can be further enhanced by incorporating resolution enhancement techniques like confocal microscopy which will result in optical sectioning and higher axial and lateral resolution. The next chapter describes the studies carried out on confocal technique.

Chapter 5

Investigation into confocal technique

This chapter describes the studies carried out in confocal microscopy. A theoretical analysis on the optical sectioning in an image fiber based endoscope is performed. This chapter also contains the theoretical study on the wavelength dependence of confocality on the pinhole size in a chromatic confocal microscope. Results obtained from the experimental study on the effect of pinhole size on confocality are included in appendix C.

5.1 Introduction

One of the major thrust areas of research and development activities in biophotonics is bioimaging which has applications in the imaging of wide range of specimens from cells to tissue samples and live objects covering broad spectrum of length scales from sub micron viruses to macroscopic sized biological species. Principle of optical bioimaging is based on optical contrast related to difference in light transmission, reflection and fluorescence between the region to be imaged and the background region. The main aim of the investigation is to enhance these contrasts and thereby increasing the signal to noise ratio of images. Out of the various methods, fluorescence microscopic technique is widely employed [98-100]. Different parameters like intensity, life time, etc involved in laser induced fluorescence can be exploited to develop different imaging techniques like FRET [98], FLIM [99], TPLSM [100] etc. Fluorescence emission depends on the excitation wavelength of light and in the case of one photon process, the energy of emitted photons will be smaller than that of the excitation photons. Due to its sensitive concentration dependence and emitter-specificity, fluorescence emission is sensitive to spatial distributions of different emitting species to the limit of very low concentrations. The fluorescence microscope is effective in detecting particular fluorochrome in cells and tissues.

The widely used fluorescence microscope is based on epi-fluorescence excitation schematic diagram of which is given in Fig. 5.1. Fluorescence emission is collected through the same objective lens which is used for the excitation beam making use of a dichroic beam splitter. A serious problem encountered in fluorescence microscope is the out of focus signal which reduces the signal to noise ratio. Photo bleaching of the

imaging object is another problem to be addressed to. These two problems can be eliminated using scanning microscopy. Among various types of scanning microscopy, confocal microscopy is the most popular.

Fig. 5.1 Schematic diagram for epi-fluorescence excitation

In conventional wide field microscope, thick specimens will produce images of poor signal to noise ratio due to the out of focus fluorescence emission. This effect is serious especially under high magnification ($>10\times$). Introduction of a pinhole in the path of the image forming beam (Fig. 5.2) solves this problem. Pinhole used in the microscope will reject out of focus signals thereby increasing the sharpness and resolution of images. In confocal microscopy, a laser beam is focused onto the sample using an objective which will also collect the fluorescence emission from the sample and will be focused onto a pinhole. Pinhole is positioned in a plane such that the focused image converges to it.

Fig. 5.2 Schematic diagram of confocal set up showing the introduction of pinhole at the focal plane.

The pinhole blocks the fluorescence emission from out of focus planes of the specimens. The removal of out of focus signals from the image results into describing confocal image as an “optical section” of the thick object. The thickness of the optical section depends on various factors like excitation wavelength and the size of the pinhole used as the confocal aperture. This chapter focuses on the theoretical and experimental characterization of confocal microscopes.

5.2 Optical sectioning in image fiber bundle

Integrating optical fiber with a microscope helps in imaging areas that are not accessible using conventional light microscopy. One of the earliest works along this line was carried out using a single mode fiber [101]. The advantage was the inherent optical sectioning due to the small size of the fiber core. To get an image, the distal end of the fiber or the sample had to be scanned. This limitation has been overcome by using an image fiber bundle instead of a single mode fiber and placing a pinhole at the detection arm [102]. Endoscopes employing coherent image fiber bundle have become an indispensable tool for biomedical applications due to this reason. Miniaturization of these endoscopes has been achieved using both grin lens and microscope objectives. To improve the optical sectioning capability of the endoscope, a focused laser beam is scanned across the proximal surface of the bundle injecting light sequentially into each fiber. As a result, each fiber acts as a pinhole. Even though the neighboring fibers do not contribute much to the signal from the focal plane, substantial amount of signal from the out of focal planes are coupled into the neighboring fibers. This results in a reduction in optical sectioning and contrast. Fig. 5.3 shows the schematic representation of an image fiber bundle with a miniature microscope objective at the distal end. The details of the circled region are depicted in Fig. 5.4.

Fig. 5.3 Schematic representation of an image fiber based endoscope

Fig. 5.4 Details of the Gaussian beam profile.

In this section, a theoretical investigation is carried out on the effect of neighboring fibers in an endoscope employing an image fiber bundle. The theoretical analysis is developed based on Gaussian optics.

U' is the principal plane of the objective lens close to the sample and U'' is the principal plane of the objective lens close to the image fiber. The TEM_{00} mode output of a laser has a Gaussian spatial profile. The intensity distribution is given by

$$I(x, z) = \frac{I_0}{1 + \frac{z^2}{Z_x^2}} \exp \left[-2 \frac{x^2}{x_0^2} / \left(1 + \frac{z^2}{Z_x^2} \right) \right] \quad (5.1)$$

where I_0 is the peak intensity, x_0 is the radius where the lateral Gaussian intensity is reduced to $\frac{1}{e^2}$ of the peak value and $Z_x = \frac{n\pi x_0^2}{\lambda}$ is the Rayleigh range. If we assume that the image is formed in air, we have

$$\frac{n}{z_0} + \frac{1}{z_0'} = \frac{1}{f} \quad (5.2)$$

where n is the refractive index of the sample, z_0 is the distance between the principal plane U' and the sample in focus, z_0' is the distance between the principal plane U'' and the image and f is the back focal length. From the above equation, we get

$$z_0' = \frac{fz_0}{z_0 - nf} \quad (5.3)$$

If the sample is moved a distance z from z_0 as shown in the Fig. 5.4, equation (5.2) can be rewritten as

$$\frac{n}{z_0 - z} + \frac{1}{z_0' + z'} = \frac{1}{f} \quad (5.4)$$

where z' is the displacement of the image position from z_0' .

At this position the radius of the Gaussian beam on the sample is given by

$$x = x_0 \sqrt{1 + \frac{z^2}{Z_x^2}} \quad (5.5)$$

From equation (5.4), we get

$$z' = \frac{nf^2 z}{(z_0 - nf)(z_0 - z - nf)} \quad (5.6)$$

Magnification of the sample, $\beta = \frac{z_0' + z'}{z_0 - z}$ is given by

$$\beta = \frac{f}{z_0 - z - nf} \quad (5.7)$$

The radius of the image of the illuminating beam at the position given by equation (5.6) is

$$x' = \beta x \quad (5.8)$$

The beam forming the image has at the image fiber plane, a radius of

$$x'_{ip} = \beta x \sqrt{1 + \frac{z'^2}{Z_{x'}^2}} \quad (5.9)$$

The light intensity passing through the image fiber bundle for the defocus distance z is then given by

$$I_{IF}(C) = \int_0^{2\pi} \int_0^C I'(x, z) dx d\phi \quad (5.10)$$

where C is the radius of the area of interest in the image fiber bundle.

The above equation is for the case in which we consider a reflecting sample. If we consider fluorescence, the emission intensity from the sample depends upon the photons absorbed by the sample. If the intensity of the incident light has no temporal dependence, the total number of photons absorbed per unit time is given by [103]

$$N_{abs} = F\sigma \int_V I(r) dr \quad (5.11)$$

where F is the sample fluorophore concentration which is assumed to be constant and σ is the photon absorption cross-section. Assuming no self-quenching and stimulated emission, the number of fluorescence photons detected per unit time is given by [103]

$$F = \frac{1}{n} \phi \eta N_{abs} \quad (5.12)$$

where ϕ is the fluorescence collection efficiency of the imaging system and η is the fluorescence efficiency of the sample. The factor $\frac{1}{n}$ is to reflect the fact that n photons are needed for each excitation event.

Fig. 5.5 shows the microscopic image of a portion of the end face of an image fiber bundle. It is clear from the image that around any individual fiber, there are rings of fibers arranged in a hexagonal pattern.

Fig. 5.5 End face of a portion of an image fiber bundle

The number of fibers inside the fiber bundle follows the relation

$$N = 1 + \sum_{i=0}^m 6i \quad (5.13)$$

where m is the number of rings.

Fig. 5.6 shows the schematic of a section of an image fiber bundle with 1 ring around a central fiber (illumination fiber). From the figure, the intensity passing through the fibers in a ring can be calculated to be

$$I_{IF}^1 = \frac{(\text{number of fibers in ring 1}) \times R^2 \times (I_{IF}(R1) - I_{IF}(R2))}{(R1^2 - R2^2)} \quad (5.14)$$

Fig. 5.6 Definition of the parameters used in the calculation: fiber spacing (D), Diameter of the fiber ($2R$), Distance from the illumination fiber to the outer edge of the first ring ($R1$), Distance from the illumination fiber to the inner edge of the first ring ($R2$).

Therefore, the total signal transmitted from the area up to the first ring is

$$I_{IF}(R1) = I_{IF}(R) + I_{IF}^1 \quad (5.15)$$

This formulism can be extended to any number of rings.

The image fiber parameters used for the analysis has $D/R = 4$ with $D = 8$ microns and $R = 2$ microns. These values resemble with those of commercially available image fiber bundles. It is clear from Fig. 5.7 that the signal coming back through the central fiber (illumination fiber) shows optical sectioning and behaves like a confocal pinhole. As we include the effect of fibers from the neighboring rings, the optical sectioning and the contrast start diminishing. For example, in the case of 4 rings, the signal from the out of focal region is around 60 % of the signal from the focal plane for a defocus distance greater than 2 microns.

Fig. 5.7 Effect of neighboring fibers when $D/R=4$.

To study the effect of spacing between the rings, simulation was repeated for $D/R = 20$ (Fig. 5.8) with the same value of R (2 microns). Increase in the spacing between the rings results in the substantial decrease of the signal through the neighboring fibers. As is clear from the figure, the signal passing through the 4 rings for a defocus distance greater than 2 microns is around 30 % of the signal from the focal plane and reduces still further

following the confocality trend. This will enable illuminating multiple fibers at the same time without losing confocality. Moreover, use of image fiber with multiple fiberlet illumination has better geometrical flexibility in comparison with that of single fiber illumination. Multiple illuminations also will reduce the scanning time considerably since multiple points in the sample will be illuminated instead of single point.

Fig. 5.8 Effect of neighboring fibers when $D/R=20$.

In image fiber bundles, fibers around the illumination fiber will degrade the optical sectioning efficiency. Analysis revealed that this will be prominent when the sample used is thick. By increasing the D/R value of the image fiber bundle, one can substantially reduce the effect of the neighbouring fibers. This enables illumination of multiple points in the sample without losing confocality.

Experiment was performed by replacing the sample and the dichroic mirror shown in Fig. 4.11 with a mirror and a beamsplitter respectively. Data was taken using the spectrometer while moving the mirror along the optical axis. Fig. 5.9 shows the experimental data ($D/R \sim 4$). The FWHM of the system is 78 microns.

Fig. 5.9 Experimental FWHM for $D/R \sim 4$

It is also evident that by increasing D/R , the effect of neighboring fibers around the illuminating fiber to reduce the confocality decreases. As the D/R ratio increases, the FWHM profile will look similar to that shown in [101] and appendix C. However, the image fiber bundles available have D/R similar to the one used to get data in Fig. 5.9.

5.3 Wavelength dependence of Confocality

In chromatic confocal microscopes, different wavelengths are focused at different planes in the sample to do away with the axial scanning as shown in Fig. 5.10. The variation in the spot size within this height interval plays an important role in determining the confocality achieved by the system over a spectral range. Tiziani et.al developed an analysis for finding the range of pinhole sizes over which the variation in axial FWHM is almost constant [104]. This work studies the effect of variable confocality for different wavelengths. Since the size of the pinhole is fixed, the confocality achieved by the pinhole for each of the wavelengths is different. This results in wavelength dependent effective pinhole size. A study is performed on the progression of axial FWHM with wavelength taking into account the wavelength dependent effective pinhole size.

Fig. 5.10 Schematic of chromatic confocal microscope

The detected image intensity of a point object in a confocal system with finite detector sensitivity D , is given as [104]

$$I = |h|^2 (|h|^2 * D) \quad (5.16)$$

where

$$h(u, v) = \int_0^1 P(\rho) \exp\left(\frac{1}{2} i u \rho^2\right) J_0(v \rho) \rho d\rho \quad (5.17)$$

is the point-spread function of the lens in optical coordinates. Optical units v and u is given by

$$v = \frac{2\pi}{\lambda} r \sin \alpha \quad (5.18)$$

$$u = \frac{8\pi}{\lambda} z \sin^2 \frac{\alpha}{2}$$

where $\sin \alpha$ is the numerical aperture. It is clear from equation (5.16) that the size of the detector plays an important role in determining the confocality achieved. In a confocal microscope with monochromatic illumination, the confocal pinhole can be chosen to obtain the required confocality based on the airy disc radius corresponding to

the wavelength used. In a chromatic confocal microscope, a range of wavelengths are used. Therefore, the pinhole will not give the same confocality for different wavelengths since the airy disc radius will vary with wavelengths. When choosing a pinhole for chromatic confocal microscope, it is better to employ a pinhole with radius $v_p(\text{max})$ based on the wavelength that gives the largest airy spot radius among the wavelengths in the spectral range. This ensures that the light loss at the pinhole can be minimized for the entire spectral range to get good signal to noise ratio (SNR). Fig.5.11 shows the effective pinhole size ($v_p(\lambda)$) for the different wavelengths when the size of the pinhole employed is $v_p(\text{max})$.

Fig.5.11 Wavelength dependent effective pinhole size

In this study, the wavelength range was chosen from 350 nm to 750 nm. $v_p(\lambda)$ can be calculated from

$$v_p(\lambda) = \frac{v_p(\text{max}) \cdot \lambda(v_p(\text{max}))}{\lambda} \quad (5.19)$$

where $\lambda(v_p(\text{max}))$ is the wavelength corresponding to the largest airy spot (750 nm in this case). Since the variation in numerical aperture (NA) over the spectral range is small compared to the variation in wavelength, the calculation was performed assuming a constant NA value over the spectral range. In Fig. 5.11, $v_p(\lambda)$ has been normalized with $v_p(\text{max})$. This gives a better representation to the deviation of $v_p(\lambda)$ from $v_p(\text{max})$. We can see from Fig. 5.11 that the effective pinhole size for the smaller wavelengths is larger than the desired size ($v_p(\text{max})$). As a result, the confocality is not a constant over the spectral range. For example, the effective pinhole size for the wavelength 350 nm is around 2.1 times the pinhole size at 750 nm wavelength. This implies that the confocality of the system when imaging the plane corresponding to 350 nm wavelength is worse than the confocality of the plane corresponding to 750 nm wavelength.

To study the effect of the variation in effective pinhole size over the spectral range, the values (non-normalized) for the effective pinhole size obtained from Fig. 5.11 is used in equation (5.16) to find the variation in the axial FWHM with wavelength. The pinhole is considered to be circular ($D = \text{circ}(\frac{v}{v_p})$). Fig. 5.12 shows the variation in axial FWHM for $v_p(\text{max}) = 0.5$ and 2. The FWHM values were normalized with the value at $\lambda(v_p(\text{max}))$ to represent the amount of variation.

(a)

(b)

Fig. 5.12 Effect of variable confocality. Variation in axial FWHM with wavelength for a) $v_p(\max) = 0.5$ b) $v_p(\max) = 2$

From Fig. 5.12, it can be seen that the variation of axial FWHM is 0.53 for $v_p(\text{max})=0.5$ and 0.35 for $v_p(\text{max})=2$ for the chosen wavelength range from 0.35 micrometer to 0.75 micrometer. In Fig. 5.12 a ($v_p(\text{max}) = 0.5$), there is no change of slope. The axial FWHM is almost constant when pinholes of small sizes are used. When the size becomes larger, the axial FWHM starts to vary with size. In the wavelength range used in the present calculation, the pinhole size for the different wavelengths falls in the small - size range resulting into the constancy of the slope. In Fig. 5.12 b ($v_p(\text{max})=2$), there is a change of slope around the wavelength 0.45 microns. This is because from the pinhole size corresponding to this wavelength, axial FWHM starts to vary with pinhole size. To understand the effect of effective pinhole size, calculation was carried out for the variation in axial FWHM with wavelength taking a pinhole size of $v_p(\lambda)=0.5$ and 2 for all wavelengths (physical dimension of the pinhole change with wavelength according to equation (5.19)) and Fig. 5.13 shows the plot. In the case of same confocality for the entire spectral range, the variation is around 0.54 for both $v_p(\lambda)=0.5$ and 2. This is because at these pinhole sizes, the axial FWHM is almost same since both the pinhole sizes fall in the small pinhole size range.

For the smaller pinhole ($v_p(\text{max})=0.5$), the variation in FWHM value due to the variation in effective pinhole size (variable confocality) is almost same as the case of a system having $v_p(\lambda)=0.5$ for all wavelengths (same confocality), whereas for the larger pinhole ($v_p(\text{max})=2$), the variation in FWHM value due to the variable confocality is different from that of a system having same confocality ($v_p(\lambda)=2$) over the entire

spectral range. This shows that the variation in FWHM value due to the variable confocality of the system depends on the size of the pinhole employed.

(a)

(b)

Fig. 5.13 Effect of same confocality. Variation of axial FWHM with wavelength for a)

$v_p(\lambda) = 0.5$ b) $v_p(\lambda) = 2$

In previous chapters, dual modality imaging systems were studied. DMD based system described in chapter 3 has the ability to selectively excite the sample region and obtain the image and spectral signature. The frequency domain system (chapter 4) can be used for distinguishing normal and cancerous region in the specimen based on fluorescence lifetime difference. The resolution of both the systems are limited by diffraction limit. To overcome the diffraction limit and obtain images with higher resolution, confocal technique can be integrated to the system.

A theoretical analysis on image fiber based endoscopes was carried out. The effect of neighboring fibers of the illumination fiber in achieving confocality was studied. Theoretical analysis of wavelength dependence of confocality in chromatic confocal microscope was also studied. It is observed that the variation in FWHM value due to the variable confocality of the system depends on the size of the pinhole employed.

Chapter 6

Conclusion and Future work direction

This chapter describes the conclusions of the work performed in the thesis by pointing out the contributions made during the research period. Recommendations for future work direction are also suggested.

Conventional biopsy techniques can be replaced by optical techniques to minimize the patient discomfort and improve the diagnostic accuracy of the system. Literature survey revealed that incorporating more than one modality to an optical probe can increase the sensitivity of the system in studying biochemical changes. The contributions made during the research period are described in the following section.

6.1 Contributions

The research was focused on the investigation of image fiber based dual modality probes concentrating on the fluorescence methods. Improvement in the selectivity and sensitivity of the system was observed in the DMD based probe scheme. The probe consisted of an image fiber bundle with grin lens incorporated in the distal end. The system was able to examine the sample selectively by turning on/off the individual mirrors in the DMD. This enables the gathering of spectroscopic signal from the sample in a controlled manner. The system also provides information about the morphology of the sample due to its dual modality nature. Information obtained from both the spectral signature and the image of the sample will help in improving the sensitivity in diagnostic applications. It is believed that the percentage of false positive diagnosis can be reduced and real time study of biochemical changes can be performed.

The literature review revealed that some biochemical changes cannot be detected early from the morphology or the spectroscopic signal originating from the tissues. Incorporating techniques in diagnostic systems to increase the chance of early detection of harmful biochemical changes will be useful to reduce the mortality rate of patients by performing early treatment. Taking this into consideration, studies were performed on a

fluorescence lifetime based dual modality probe. The system was able to distinguish between regions whose fluorescence lifetime differed in the range of nanoseconds. This would be helpful in early diagnosis of cancerous changes occurring in tissues. The system also showed an improvement in contrast thereby providing a more detailed morphology of the sample.

Incorporating high resolution techniques into the probes will help in uncovering the finer details of the sample. A theoretical analysis was developed to study the optical sectioning capability of an image fiber based confocal endoscope. The analysis revealed that the spacing between the fibers in the image fiber bundle play a major role in the optical sectioning. The developed theoretical analysis can be used to study the contrast that can be achieved when using image fiber based endoscopes of varying spacing or fiber core size.

Confocal microscope can be modified to reduce the scanning time by using multi wavelength excitation instead of monochromatic illumination. These microscopes (chromatic confocal microscope) do away with the axial scanning since different wavelengths are focused at different planes along the optical axis. Theoretical analysis was performed to study the wavelength dependence of confocality in a chromatic confocal microscope. The study can be used to understand the variation in FWHM of the system for different wavelengths when employing different pinhole sizes.

The contributions can be summarized as follows:

- Prototype dual modality probe to increase the sensitivity and selectivity of the system by incorporating DMD.

- Prototype fluorescence lifetime based dual modality probe for detecting early biochemical changes.
- Theoretical analysis on the optical sectioning performance of image fiber based confocal endoscope taking into consideration the spacing between the fibers and the core size.
- Theoretical analysis on the performance of a chromatic confocal microscope taking into consideration the effect of pinhole size.

The next section gives some suggestions for future work.

6.2 Future work

The future work direction will be to employ the developed probes for studying biochemical changes and for *in-vivo* applications. The concepts and the experimental setup would remain the same for studying biochemical changes. To perform *in-vivo* studies, it is advantageous to automate the motion of the endoscope. MEMS technology can be used to fabricate miniature motors and the necessary mechanical devices for locomotion. It is also recommended to use another channel for passing air and water. Air passage will help in inflating the cavity under inspection (like colon) and the water passage can be used to remove any particles that gets stuck to the lens. This is important especially while performing imaging studies *in-vivo*.

Another aspect will be to increase the resolution and the optical sectioning capability of the system. The work performed in this thesis concentrated on processes involving linear interaction of light with matter. By employing pulsed lasers, it is possible to create nonlinear (intensity dependent processes) interaction of light with matter. Non-linear

processes like two-photon absorption can be used to modify the system to a multiphoton endoscope. The advantage is the inherent optical sectioning capability that can be achieved since fluorescence will be generated only in the focal volume. This also leads to an increase in resolution and contrast. Fluorescent markers like quantum dots and microspheres are employed as markers for studying biological functions. One of the factors to take into consideration when using a marker in a multiphoton experiment is its nonlinear optical efficiency. Future work will concentrate on identifying suitable candidates to be employed as fluorescent markers. Some initial work along this line has been performed during this period and is given in the appendix.

Applying the developed systems in a clinical environment for diagnosis and for real time monitoring of the developments during treatment would be interesting.

APPENDICES

APPENDIX A

SPECIFICATIONS OF THE ICCD CAMERA

Feature	Specification
Active image area	1370 * 1040 pixels
Pixel size	6.45 microns* 6.45 microns
Sensitivity	70 counts/photoelectron @maximum gain
Spatial resolution	8-12 lp/mm
Spectral range	200-800 nm
Lens connector	Nikon F or C mount
Cooling	2 stage peltier with forced air regulated at 12 ⁰ c
Interface	Data transfer (FOL), control output, trigger input

APPENDIX B

SPECIFICATIONS OF THE EDC 2000-N CAMERA

Feature	Specification
Active image area	4.84 mm * 3.67 mm
Pixel size	7.4 microns * 7.4 microns
Lens connector	C mount

APPENDIX C

Dependence of optical sectioning on the pinhole size

To investigate the dependence of pinhole size on the confocality, following experiment was carried out. Fig. AC.1 represents the schematic of the experimental setup.

Fig. AC.1 Schematic of the experimental setup to study the effect of pinhole size

Laser beam of 532nm from a diode pumped solid state (DPSS) laser (Photop Suwtech laser) was collimated and directed to a microscope objective (MO, 50X/0.8, Olympus) with a 50/50 beam splitter (BS). Light beam reflected from the mirror was focused by a lens on to the pinhole. Laser intensity was monitored by a power meter. The mirror was translated along the optic axis at a step size of 0.5 μ m and the intensity of laser beam reaching the power meter was recorded.

a

b

c

d

Fig. AC.2 Axial intensity distribution in the confocal setup with a) pinhole size 20 μm b) 50 μm c) 100 μm and d) in the absence of confocal pinhole. Optical sectioning effect in presence of pinhole is clearly seen.

The procedure was repeated for four configurations (20 μm , 50 μm , 100 μm and no pinhole) and intensity variations recorded are given in Figs. AC.2a-AC.2d.

From figures, FWHM (Full width at half maximum) were calculated corresponding to the three pinhole sizes. It is found that a minimum FWHM value of 1.1 micrometer is obtainable in the present setup when a microscope objective of numerical aperture 0.8, laser of wavelength 532 nm and a pinhole of 20 micron diameter are employed.

Fig. AC.3 Variation of FWHM with pinhole size

Variation of FWHM with confocal pinhole size is given in Fig. AC.3. Within the experimental parameters, FWHM varies linearly with the pinhole size and the resolution of the microscope increases with decreasing pinhole size. However, this should be at the cost of intensity of light falling on the detector. On reducing the pinhole size, the intensity of laser beam has to be increased so that one may have to use laser source of

higher intensity. The optical components have to be chosen with appropriate optical damage threshold so as not to be damaged by optical breakdown.

APPENDIX D

Nonlinear optical characterization of microspheres

AD.1. Introduction

Among various optical phenomena employed in bioimaging, fluorescence based microscopy is the most popular technique due to the sensitivity and resolution enhancement capability of fluorescence based imaging. More over, as noted in previous chapters, fluorescence phenomenon depends on a number of parameters like intensity, lifetime, fluorescence excitation and emission wavelengths which can be used to develop various types of imaging techniques. To utilize fluorescence for biological applications we can use the inherent fluorescence properties of the samples (endogenous fluorescence) or non fluorescent specimens can be labeled with a suitable fluorophore. Such materials are labeled with appropriate exogenous fluorophores. Some fluorophores can stain the sample while others will localize in a particular organelle. In some cases, fluorophores can be chemically conjugated to get targeted onto a specific biological site like cancer cell or an individual organelle in a cell. Such fluorescent probes like texas red, Alexa Fluor dyes, dye coated microspheres having good fluorescence quantum yield can be used for imaging applications. With the availability of different fluorochrome which can be specifically targeted to different parts of the cells, fluorescence microscopy has developed into a major diagnostic tool within a short span of time.

Interaction of light with matter in the low intensity limit is based on the assumption that polarization induced is linear function of the electric field associated with the light.

However, when intensity is increased, one has to consider the induced polarization as a function of the electric field and can be expressed as a polynomial. First order term represents the linear optics while higher order terms give rise to various nonlinear optical phenomena. Optical second harmonic generation, frequency up (down) conversions and optical parametric oscillator belong to second order optical nonlinearity while two photon absorption, four wave mixing, Stimulated Raman Scattering are examples of well known third order nonlinear optical (NLO) phenomena. Phenomena like two photon induced absorption are of much use in bioimaging for enhancement of resolution and target specificity.

For example, when laser beam is focused onto the sample using a Gaussian beam, the focused region will have sufficient intensity to produce two photon induced fluorescence, while the unfocused region will not contribute to the image. Thus two photon induced fluorescence microscopy will enhance the image resolution. The selection of exogenous fluorophores has to be made by considering their fluorescence quantum yield and nonlinear optical efficiency. Thus measurement of nonlinear optical efficiency of fluorophores and dyes employed in bioimaging is important in the context of appropriate selection of probes for imaging applications.

In order to identify suitable materials for NLO based imaging techniques (like two photon induced fluorescence imaging), knowledge of nonlinear optical coefficient will be of much help. One of the simplest but effective tools to study third order optical nonlinearity and the resulting intensity dependent refractive index parameter is the Z scan technique developed by Bahae et.al [105].

AD.2. Theory

Detailed theory of single beam open aperture z-scan technique is available in the literature [105]. The normalized transmission for a Gaussian beam through a nonlinear optical medium with $\alpha = \alpha_0 + \beta I$ is given by

$$T_{norm}(z) = \frac{C}{\sqrt{\Pi} q_0} \int_{-\infty}^{\infty} \ln(1 + q_0 e^{-t^2}) dt \quad (AD.1)$$

where C is a normalization constant and q_0 is given by

$$q_0 = \frac{\beta I_0 (1 - e^{-\alpha_0 l})}{(1 + \frac{z^2}{z_0^2}) \alpha_0} = \frac{\beta I_0 L_{eff}}{(1 + \frac{z^2}{z_0^2})} \quad (AD.2)$$

where I_0 is the maximum incident radiation intensity, l is the path length of the sample and z_0 is the Rayleigh length given by $\frac{\Pi \omega_0^2}{\lambda}$, ω_0 is the beam waist λ is the wavelength of the laser beam, α_0 is the absorption coefficient of the sample in the low intensity limit and $L_{eff} = 1 - e^{-\alpha_0 l}$. For small nonlinear losses, the normalized change in transmitted energy is given by

$$\Delta T(z) \approx -\frac{q_0}{2\sqrt{2}} \quad (AD.3)$$

At z_0 , the value of q_0 is given by $\frac{\beta I_0 L_{eff}}{2}$ so that at the focal point, change in transmitted intensity is given by

$$\Delta T(z_0) = -\frac{\beta I_0 L_{eff}}{4\sqrt{2}} \quad (\text{AD.4})$$

AD.3. Experimental setup

Second harmonic radiation of wave length 532 nm at 7ns pulse width from Q switched Nd-YAG laser (Spectra Physics, LAB-1760) at pulse repetition rate of 10Hz is used as the light source. Dye coated micro spheres suspended in water was the medium. Fig. AD.1 shows a schematic diagram of the experimental set up for single beam open aperture Z- scan measurements.

Fig. AD.1 Experimental setup for Z-scan

A focused Gaussian beam is obtained using a converging lens of 200mm focal length. The sample is moved along the z – axis. The radius of the beam waist ω_0 is calculated to be 35.4 μ m. The Rayleigh length $z_0=\pi\omega_0^2/\lambda$ is estimated to be 7.4mm, which is much greater than the thickness of the sample cuvette(1mm) which is an essential prerequisite for z-scan experiments. The transmitted beam energy, reference beam energy and their ratio are measured simultaneously using an energy ratiometer (Rj7620, Laser Probe Corporation) having two identical pyroelectric detector heads (RjP 735). The effect of the fluctuations of laser power was eliminated by dividing the transmitted power by the power obtained at the reference detector.

AD.4. Results and discussion

Open aperture z-scan measurements were carried out for 1 micron diameter microspheres dispersed in water at various concentrations. Samples of different concentrations were prepared from the stock solution of the microsphere obtained from the suppliers (Invitrogen). Fig. AD.2 shows a couple of z-scan plots at 135 microjoule laser power. Figure show an interesting observation of switching the dip in the plot to a peak at higher concentration. This reveals change in the basic phenomenon causing the optical nonlinearity in the medium. The dip represents decrease in transmission at the focal point corresponding to reverse saturable absorption (RSA) whereas the peak corresponds to increase in transmission related to saturable absorption (SA). The concentration dependence of the switching behaviour can be described using concentration dependent effective length term in equation (AD.5) as shown below.

$$q_0 = \frac{\beta I_0 (1 - e^{-\alpha_0 (\frac{C_t}{C} - 1)l})}{(1 + \frac{z^2}{z_0^2}) \alpha_0} = \frac{\beta I_0 L'}{(1 + \frac{z^2}{z_0^2})} \quad (\text{AD.5})$$

$$\text{where } L' = \frac{(1 - e^{-\alpha_0 (\frac{C_t}{C} - 1)l})}{\alpha_0}$$

We define a threshold concentration (C_t) in equation (AD.5) above which the nonlinear phenomenon is described by saturable absorption and in the case of lower values the reverse saturable absorption is observed. As the concentration is varied from values less than the threshold concentration C_t to values greater than C_t , the sign of q_0 and hence that of ΔT will change from negative to positive values corresponding to lower and higher values of concentration relative to threshold value. The phenomenon described above has potential applications in switching from RSA to SA. For example, the microspheres can be doped in a solid substrate like PMMA rod with concentration gradient so that there will be a gradual increase in concentrations from one end to the other end. When the laser beam is scanned along the polymer rod, there will be enhanced transmission from the region where concentration is above the threshold.

(a)

(b)

Fig. AD.2 Z-scan plots for a) RSA b) SA

APPENDIX E

Determining the size of fluorescent dyes

Particle size of the fluorescent dyes used in the thesis was estimated by using a commercial particle analyzer (nano-ZS, Malvern Instruments). The instrument determines the size by using light scattering technique. The instrument could not determine the size of the large particles, but could determine the size of smaller particles that cannot be resolved using a conventional microscope with nanometer accuracy. The manual specifies that the instrument can measure size ranging from 0.3 nm to 10 microns (sample dependent). Table AE.1 lists the average size of the three dyes: rhodamine 6G, basic yellow and redwop determined from the particle analyzer.

Name of the dye	Average particle size (microns)
Rhodamine 6G	2
Basic Yellow	1.2
Redwop	2.7

Table AE.1 Average particle size of the fluorescent dyes

Figure AE.1 shows the data obtained using the particle analyzer. The image of the sample redwop was captured using an optical microscope (Zeiss, axiotech) (Fig. AE.2). We can see that the particles aggregate themselves and form an almost spherical shape and the diameter is in the range from 20 - 40 microns.

malvern instruments nano-ZS.pdf - Adobe Reader

File Edit View Document Tools Window Help

1 / 5 101% First

Malvern Instruments File name: Size.dts 15 Mar 2011 2:42:21 PM

Record	Type	Sample Name	Measurement Date and Time	T °C	Z-Ave d.nm	PdI	ZP mV	Attenuator
2	Size	1:1	Thursday, January 13, 2011 11:12:35 AM	25.0	870.1	0.071		11
3	Size	rhodamine 1	Tuesday, March 15, 2011 2:16:25 PM	25.0	2022	1.000		10
4	Size	rhodamine 2	Tuesday, March 15, 2011 2:18:29 PM	25.0	1966	1.000		10
5	Size	rhodamine 3	Tuesday, March 15, 2011 2:20:33 PM	25.0	909.1	0.962		10
6	Size	basic yellow 1	Tuesday, March 15, 2011 2:25:38 PM	25.0	1150	0.982		10
7	Size	basic yellow 2	Tuesday, March 15, 2011 2:27:42 PM	25.0	502.7	0.543		10
8	Size	basic yellow 3	Tuesday, March 15, 2011 2:29:46 PM	25.0	477.9	0.511		10
9	Size	redwop 1	Tuesday, March 15, 2011 2:34:45 PM	25.0	2671	0.537		6
10	Size	redwop 2	Tuesday, March 15, 2011 2:37:09 PM	25.0	2527	0.469		6
11	Size	redwop 3	Tuesday, March 15, 2011 2:39:33 PM	25.0	2446	0.441		6

Fig. AE.1 Particle size data using particle analyzer

Fig. AE.2 Image of redwop using optical microscope

APPENDIX F

Determining the resolution using resolution chart

The resolution of the system mentioned in chapter 3 was studied using USAF 1951 resolution chart for 532 nm wavelength. The experiment was conducted for both free space configuration as well as using image fiber bundle based system. Figure AF.1 shows the image of the resolution chart obtained using the system which gives the image shown in Fig. 3.6a (Image fiber bundle based system with 10X microscope objective).

Fig. AF.1 USAF resolution chart image using image fiber bundle based system at 10X magnification

Figure AF.2 shows the image of the resolution chart obtained using the system which gives the image shown in Fig. 3.6b (Image fiber bundle based system with 20X microscope objective).

Fig. AF.2 USAF resolution chart image using image fiber bundle based system at 20X magnification

It is observed from the images (AF.1 and AF.2) that element 1 of group 7 (encircled in red) is just resolved and this corresponds to a resolution of 7.81 micron/line pair.

Figure AF.3 shows the image of the resolution chart obtained using the system which gives the image shown in Fig. 3.8a (Free space configuration with 10X microscope objective).

Fig. AF.3 USAF resolution chart image using free space configuration at 10X magnification

Figure AF.4 shows the image of the resolution chart obtained using the system which gives the image shown in Fig. 3.8b (Free space configuration with 20X microscope objective).

Fig. AF.4 USAF resolution chart image using free space configuration at 20X magnification

It is observed from the images (AF.3 and AF.4) that element 6 of group 7 (encircled in red) is resolved clearly and this corresponds to a resolution of 4.39 micron/line pair.

LIST OF PUBLICATIONS AND AWARD

Journals

1. **M. Vaishakh**, V.M. Murukeshan, and L.K. Seah. (2008). An image fiber based fluorescent probe with associated signal processing scheme for biomedical diagnostics. Laser Physics Letters, **5**, 760-763.
2. **M. Vaishakh**, V. M. Murukeshan, and L.K. Seah. (2008). Quasi-confocal frequency domain imaging for improved depth perception and selective suppression of fluorescent emissions. International Journal of Optomechatronics, **2**, 42-60.

International Conferences

1. **M. Vaishakh**, V.M.Murukeshan, and L.K. Seah. (2008). A homodyne detection based fluorescent microscopy for depth sensitive imaging. Photonics 2008 India: International conference on fiber optics and photonics (biennial conference), 15-17 December 2008 @ IIT Delhi. Cosponsored by IEEE-LEOS, OSA and SPIE (Poster Presentation).
2. **M. Vaishakh**, V.M.Murukeshan, and L.K. Seah. (2008). Improvement in fluorescence confocal microscopy for obtaining better depth perception. IEEE Photonics Global @ Singapore, 8-11 December 2008. (Oral Presentation).
3. Murukeshan V. Matham, and **M. Vaishakh**. (2008). Specialty fiber based fluorescent probe for high resolution multi-dimensional multi-optical modality tissue imaging.

World Molecular Imaging Congress, 10-13 September 2008, Nice, France. (Poster Presentation).

Award

- SPIE student prize for the best paper in the international conference Photonics 2008 held at IIT Delhi, India.

LIST OF REFERENCES

- 1) Singapore Cancer Registry, Interim Report (2004-2008).
- 2) American Cancer Society, Cancer facts and figures 2011.
- 3) R. Gertler, K D Werber. (2009). Management of verrucous carcinoma of the hand: a case report. International journal of dermatology, **48**, 1233-1235.
- 4) N.D. Freedman, J.E. Everhart, K.L. Lindsay, M.G. Ghany, T.M. Curto, M.L. Shiffman, W.M. Lee, A.S. Lok, A.M. Di Bisceglie, H.L. Bonkovsky, J.C. Hoefs, J.L. Dienstag, C. Morishima, C.C. Abnet, R. Sinha, HALT-C Trial group. (2009). Coffee intake is associated with lower rates of liver disease progression in chronic hepatitis C. Hepatology, **50**, 1360-1369.
- 5) S.S. Moon, H.J. Kim, Y.K. Choi, H.A. Seo, J.H. Jeon, J.E. Lee, J.Y. Lee, T.H. Kwon, J.G. Kim, B.W. Kim, I.K. Lee. (2009). Novel mutation of aquaporin-2 gene in a patient with congenital nephrogenic diabetes insipidus. Endocrine journal, **56**, 905-910.
- 6) F.D.A. Gondim, F.P. Thomas, G.R.D Oliveira, L.H.C. Pimentel, B.P.R. Bastos, C.M.D.C. Costa. (2009). On the spectrum of leprosy neuropathies: multifocal inflammatory neuropathy heralding leprosy relapse. Neuromuscular disorders, **19**, 711-713.
- 7) S. Eskaros, V. Ghevariya, M. Krishnaiah, A. Asarian, S. Anand. (2009). Percutaneous endoscopic suturing: an effective treatment for gastrocutaneous fistula. Gastrointestinal endoscopy, **70**, 768-771.
- 8) B.D. Bello, A. Spinillo, P. Alberizzi, S. Cesari, B. Gardella, E.M. Silini. (2009). Validation of the SPF 10 LiPA human papillomavirus typing assay using formalin-

- fixed paraffin-embedded cervical biopsy samples. Journal of clinical microbiology, **47**, 2175-2180.
- 9) N.O.B. Thomsen, E. Englund, S. Tharainsdottir, I. Rosen, L.B. Dahlin. (2009). Intraepidermal nerve fibre density at wrist level in diabetic and non-diabetic patients. Diabetic Medicine, **26**, 1120-1126.
- 10) M. Shimbo, S.Tomioka, M. Sasaki, T. Shima, N. Suzuki, S. Murakami, H. Nakatsu, J. Shimazaki. (2009). PSA doubling time as a predictive factor on repeat biopsy for detection of prostate cancer. Japanese journal of clinical oncology, **39**, 727-731.
- 11) J.L. Caughran, F.A. Vicini, L.L. Kestin, N.S. Dekhne, P.R. Benitez, N.S. Goldstein. (2009). Optimal use of re-excision in patients diagnosed with early-stage breast cancer by excisional biopsy treated with breast-conserving therapy. Annals of surgical oncology, **16**, 3020-3027.
- 12) U. Utzinger, R. Richards-Kortum.(2003). Fiber optic probes for biomedical optical spectroscopy. Journal of Biomedical Optics, **8**, 121-147.
- 13) V.M. Murukeshan, N.U. Sujatha. (2005). Integrated simultaneous dual-modality imaging endospeckle fluoroscope system for early colon cancer diagnosis. Optical Engginering, **44**, 110501.
- 14) C. Lohrmann, N. Ghanem, G. Pache, F. Makowiec, E. Kotter, M. Langer. (2005). CT in acute perforated sigmoid diverticulitis. European journal of radiology, **56**, 78-83.
- 15) R. Kijowski, M. Tuite, M. Sanford. (2004). Magnetic resonance imaging of the elbow. Part 1: Normal anatomy, imaging technique and osseous abnormalities. Skeletal radiology, **33**, 685-697.

- 16) D.E. Cohn, F. Dehdashti, R.K. Gibb, D.G. Mutch, J.S. Rader, B.A. Siegel, T.J. Herzog. (2002)). Prospective evaluation of positron emission tomography for the detection of groin node metastases from vulvar cancer. Gynecologic Oncology, **85**, 179-184.
- 17) T. Kaneko, A. Nakao, S. Inoue, H. Sugimoto, T. Hatsuno, A. Ito, Y. Hirooka, T. Nagasaka, N. Nakashima. (2001). Intraoperative ultrasonography by high-resolution annular array transducer for intraductal papillary mucinous tumors of the pancreas. Surgery, **129**, 55-65.
- 18) T.H. Tomkinson, J.L. Bentley, M.K. Crawford, C.J. Harkrider, D.T. Moore, J.L. Rouke. (1996). Rigid endoscopic relay systems: a comparative study. Applied Optics, **35**, 6674-6683.
- 19) P.H. Tran, D.S. Mukai, M. Brenner, Z. Chen. (2004). In vivo endoscopic optical coherence tomography by use of a rotational microelectromechanical system probe. Optics Letters, **29**, 1236-1238.
- 20) A. Amelink, M. P. L. Bard, S. A. Burgers, H. J. C.M. Sterenborg. (2003). Single scattering spectroscopy for the endoscopic analysis of particle size in superficial layers of turbid media, Applied optics, **42**, 4095-4101.
- 21) B. W. Pogue, G. Burke. (1998). Fiber-optic bundle design for quantitative fluorescence measurement from tissue. Applied optics, **37**, 7429-7436.
- 22) M. C. Skala, G. M. Palmer, C. Zhu, Q. Liu, K. M. Vrotsos, C. L. Marshek-Stone, A. Gendron-Fitzpatrick, N. Ramanujam. (2004). Investigation of fiber-optic probe designs for optical spectroscopic diagnosis of epithelial pre-cancers. Lasers in surgery and medicine, **34**, 25-38.

- 23) L. Nieman, A. Myakov, J. Aaron, K. Sokolov. (2004). Optical sectioning using a fiber probe with an angled illumination-collection geometry: evaluation in engineered tissue phantoms. Applied Optics, **43**, 1308-1319.
- 24) N. Sujatha, V.M. Murukeshan, L.S. Ong, L.K. Seah. (2003). An all fiber optic system modeling for the gastrointestinal endoscopy: design concepts and fluorescent analysis. Optics Communications , **219** , 71-79.
- 25) T. J. Pfefer, K. T. Schomacker, M. N. Ediger, and N. S. Nishioka. (2002). Multiple-fiber probe design for fluorescence spectroscopy in tissue. Applied Optics, **41**, 4712-4721.
- 26) T.J. Pfefer, A. Agrawal and R. A. Drezek. (2005). Oblique-incidence illumination and collection for depth-selective fluorescence spectroscopy. Journal of Biomedical Optics, **10**, 044016.
- 27) C.F.Zhu, Q. Liu, N. Ramanujam. (2003). Effect of fiber optic probe geometry on depth-resolved fluorescence measurements from epithelial tissues. Journal of biomedical optics, **8**, 237-247.
- 28) T. Papaioannou, L. Marcu. (2004). Effects of fiber-optic probe design and probe-to-target distance on diffuse reflectance measurements of turbid media. Applied Optics, **43**, 2846-2860.
- 29) V. Dubaj, A. Mazzolini, A. Wood. (2007). Monitoring of neuronal and glial calcium activity using a novel direct-contact probe. Journal of microscopy, **226**, 83-89.
- 30) T. Dromard, V. Ravaine, S. Ravaine, J.L. Leveque, N. Sojic. (2007). Remote in vivo imaging of human skin corneocytes by means of an optical fiber bundle. Review of scientific instruments, **78**, 053709.

- 31) M. Gu, C.J.R. Sheppard, X. Gan.(1991). Image formation in a fiber-optical confocal scanning microscope. Journal of Optical Society of America A, **8**, 1755-1761.
- 32) G. M. Connell. (2004). Confocal laser scanning fluorescence microscopy with a visible continuum source. Optics Express, **12**, 2844-2850.
- 33) W.Y. Oh. (2006). Spectrally-modulated full-field optical coherence microscopy for ultrahigh-resolution endoscopic imaging. Optics express, **14**, 8675-8684.
- 34) P.N. Prasad. (2003). Introduction to biophotonics, John-Wiley & sons, inc.,New Jersey, ISBN: 0 471 28770 9.
- 35) Tuan Vo-Dinh (2003). Biomedical photonics handbook, CRC Press, ISBN : 0 8493 1116 0.
- 36) “Fluorescence imaging”, <http://www.amershammedia.com/>
- 37) L.M. Smith, J.Z. Saunders, R.J. Kaiser, P. Hughes, C.R. Dodd, C.R. Connell, C. Heiner, S.B.H. Kent, L.E. Hood (1986). Fluorescence detection in automated DNA sequence analysis. Nature, **321**, 674
- 38) J.M. Prober, G.L. Trainor, R.J. Dam, F.G. Hobbs, C.W. Robertson, R.J. Zagursky, A.J. Cocuzza, M.A. Jensen, K. Baumeitser (1987). A system for rapid DNA sequencing with fluorescent chain terminating dideoxynucleotides. Science, **238**, 336.
- 39) W. Ansorge, B. Sproat, J. Stegeman, C. Schwager, M. Zenke (1987). Automated DNA sequencing: ultrasensitive detection of bands during electrophoresis. Nucleic acids research, **15**, 4593.
- 40) M.L. Metzker, J. Lu, R.A. Gibbs (1996). Electrophoretically uniform fluorescent dyes for automated DNA sequencing. Science, **271**, 1420.

- 41) L.G. Lee, C. Connell, S. Woo, R. Cheng, B. Mcardle, C. Fuller, N. Halloran, R. Wilson (1992). DNA sequencing with dye-labeled terminators and T7 DNA polymerase: effects of dyes and dNTPs on incorporation of dye-terminators and probability analysis of termination fragments. Nucleic Acids Research, **20**, 2471.
- 42) B.B. Rosenblum, L.G. Lee, S.L. Spurgeon, S.H. Khan, S.M. Menchen, C.R. Heiner, S.M. Chen (1997). New dye-labeled terminators for improved DNA sequencing patterns. Nucleic acids research, **25**, 4500.
- 43) S.M. Chidananda, K. Satyamoorthy, L. Rai, A.P. Manjunath, V.B. Kartha (2006). Optical diagnosis of cervical cancer by fluorescence spectroscopy technique. International journal of cancer, **119**, 139-145.
- 44) A.M.K. Nilsson, D. Heinrich, J. Olajos, S.A. Engels (1997). Near infrared diffuse reflection and laser-induced fluorescence spectroscopy for myocardial tissue characterization. Spectrochimica Acta Part A, **53**, 1901-1912.
- 45) M.G. Muller, T.A. Valdez, I. Georgakoudi, V. Backman, C. Fuentes, S. Kabani, N. Laver, Z. Wang, C. W. Boone, R. R. Dasari, S.M. Shapshay, M.S. Feld (2003). Spectroscopic detection and evaluation of morphologic and biochemical changes in early human oral carcinoma. Cancer, **97**, 1681-1692.
- 46) G.E. Kochiadakis, S.I. Chrysostomakis, M.D. Kalebubas, G.M. Filippidis, I.G. Zacharakis, T.G. Papazoglou, P.E. Vardas (2001). The role of laser induced fluorescence in myocardial tissue characterization. Chest, **120**, 233-239.
- 47) R.E.N. Shehada, V.Z. Marmarelis, H.N. Mansour, W.S. Grundfest (2000). Laser induced fluorescence attenuation spectroscopy: detection of hypoxia. IEEE transactions on biomedical engineering, **47**, 301-312.

- 48) F. Koenig, F.J. McGovern, H. Enquist, R. Larne, T.F. Deutsch, K.T. Schomacker (1998). Autofluorescence guided biopsy for the early diagnosis of bladder carcinoma. The journal of urology, **159**, 1871-1875.
- 49) E. Koumantakis, W.Q. Liu, A. Makrigiannakis, C. Relakis, E. Unsold, T.G. Papazoglou (1997). Laser-induced fluorescence detection of malignancies in the female genital tract via their natural emission and hypocrellin probing. Journal of photochemistry and photobiology B: Biology, **37**, 96-100.
- 50) S. Mordon, T. Desmettre, J.M. Devoisselle, S. Soulie (1998). Fluorescence measurement of 805 nm laser induced release of 5,6-CF from DSPC liposomes for real-time monitoring of temperature: An in vivo study in rat liver using indocyanine green potentation. Lasers in surgery and medicine, **18**, 265-270.
- 51) E.G. Borisova, T.T. Uzunov, L.A. Avramov (2004). Early differentiation between caries and tooth demineralization using laser induced autofluorescence spectroscopy. Lasers in surgery and medicine, **34**, 249-253.
- 52) M. Panjehpour, C.E. Julius, M.N. Phan, T.V. Dinh, S. Overholt (2002). Laser induced fluorescence spectroscopy for in vivo diagnosis of non-melanoma skin cancers. Lasers in surgery and medicine, **31**, 367-373.
- 53) R. Drezek, M. Guillaud, T. Collier, I. Boiko, A. Malpica, C. MacAulay, M. Follen, R.R. Körtum (2003). Light scattering from cervical cells throughout neoplastic progression: influence of nuclear morphology, DNA content, and chromatin texture. Journal of biomedical optics, **8**, 7-16.
- 54) K.L. Heppner, L.M. Matrisian, R.A. Jensen, W.H. Rodgers (1996). Expression of most matrix metalloproteinase family members in breast cancer represents a tumor

- induced host response. American journal of pathology, **149**, 273-282.
- 55) A. Pradhan, P. Pal, G. Durocher, L. Villeneuve, A. Balassy, F. Babai, L. Gaboury, L. Blanchard (1995). Steady state and time resolved fluorescence properties of metastatic and non-metastatic malignant cells from different species. Journal of photochemistry and photobiology B: Biology, **3**, 101-112.
- 56) T.J. Romer, M. Fitzmaurice, R.M. Cothren, R.R. Kortum, M.V. Sivak Jr, J.R. Kramer (1995). Laser induced fluorescence microscopy of normal colon and dysplasia in colonic adenomas: implications for spectroscopic diagnosis. American journal of gastroenterology, **90**, 81-87.
- 57) F.N. Ghadially, W.J.P. Neish, H.C. Dawkins (1963). Mechanisms involved in the production of red fluorescence of human and experimental tumors. Journal of pathology and bacteriology, **85**, 77-92.
- 58) S.A. Engels, J. Johansson, U. Stenram, K. Svanberg, S. Svanberg (1990). Malignant tumor and atherosclerotic plaque diagnosis using laser induced fluorescence. IEEE journal of quantum electronics, **26**, 2207-2217.
- 59) S.A. Engels, B.C. Wilson (1992). In vivo fluorescence in clinical oncology: fundamentals and practical issues. J. Cell. Pharmacol., **3**, 66-79.
- 60) I.J. Bigio, J.R. Mourant (1996). Ultraviolet and visible spectroscopies for tissue diagnostics: fluorescence spectroscopy and elastic-scattering spectroscopy. Physics in medicine and biology, **42**, 803-814.
- 61) M. Keijzer, R.R. Kortum, S.L. Jacques, M.S. Feld (1989). Fluorescence spectroscopy of turbid media: autofluorescence of the human aorta. Applied Optics, **28**, 4286-4292.

- 62) M.G. Pomper (2001). Molecular imaging: an overview. Academic Radiology, **8**, 1141-1153.
- 63) X. Michalet (2005). Quantum dots for live cells, in vivo imaging and diagnostics. Science, **307**, 538-544.
- 64) D. Gerion, F. Pinaud, S.C. Williams, W.J. Parak, D. Zanchet, S. Weiss, A.P. Alivisatos (2001). Synthesis and properties of biocompatible water-soluble silica-coated CdSe/ZnS semiconductor quantum dots. Journal of physical chemistry B, **105**, 8861-8871.
- 65) V. Ntziachristos, C.H. Tung, C. Bremer, R. Weissleder (2002). Fluorescence mediated tomographic imaging systems. Nature medicine, **8**, 757-760.
- 66) A. Diaspro, P. Bianchini, G. Vicidomini, M. Faretta, P. Ramoino, and C. Usai (2006). Multi-photon excitation microscopy. Biomedical engineering online, **5**:36.
- 67) S. Yazdanfar, T.M. Ragan, P.T.C. So (2003). Emerging technologies and applications in two photon microscopy. Report in global healthcare 2003, 49-52.
- 68) A.E. Profio (1984). Laser excited fluorescence of hematoporphyrin derivative for diagnosis of cancer. IEEE journal of quantum electronics, **20**, 1502-1507.
- 69) M. Yamashita, T. Tomono, S. Kobayashi, K. Torizuka, K. Aizawa, T. Sato (1988). Picosecond fluorescence spectroscopy on incorporation processes of hematoporphyrin derivative into malignant tumor cells in vivo. Photochemistry and photobiology, **47**, 189-192.
- 70) J.R. Lakowicz (2006). Principles of fluorescence spectroscopy, Springer, New york, ISBN : 0 387 31278 1.
- 71) A. Bassi, J. Swartling, C. D'Andrea, A. Pifferi, A. Torricelli, R. Cubeddu (2004).

- Time resolved spectrophotometer for a turbid media based on supercontinuum generation in a photonic crystal fiber. Optics letters, **29**, 2405-2407.
- 72) J. Karolczak, D. Kumar, J. Kubicki, T. Wrozowa, K. Dobek, B. Ciesielska, A. Maciejewski (2001). The measurements of picosecond fluorescence lifetimes with high accuracy and subpicosecond precision. Chemical Physics letters, **344**, 154-164.
- 73) W. Becker, H. Hickl, C. Zander, K.H. Drexhage, M. Sauer, S. Siebert, J. Wolfrum (1999). Time resolved detection and identification of single analyte molecules in microcapillaries by time correlated single photon counting (TCSPC). Review of Scientific Instruments, **70**, 1835-1841.
- 74) J.R. Lakowicz, K.W. Berndt (1991). Lifetime selective fluorescence imaging using an rf phase sensitive camera. Review of scientific instruments, **62**, 1727-1734.
- 75) Y. Lin, W.C. Barber, J.S. Iwanczyk, W. Roeck, O. Nalcioglu, G. Gulsen (2010). Quantitative fluorescence tomography using a combined tri-modality FT/DOT/XCT system. Optics Express, **18**, 7835-7850.
- 76) M. Liang, X. Liu, D. Cheng, G. Liu, S. Dou, Y. Wang, M. Rusckowski, D.J. Hnatowich (2010). Multimodality nuclear and fluorescence tumor imaging in mice using a streptavidin nanoparticle. Bioconjugate chemistry, **21**, 1385-1388.
- 77) M. Xiao, J. Nyagilo, V. Arora, P. Kulkarni, D. Xu, X. Sun, D.P. Dave (2010). Gold nanotags for combined multi-colored Raman spectroscopy and x-ray computed tomography. Nanotechnology, **21**, 035101.
- 78) X. Nan, E.O. Potma, X.S. Xie (2006). Nonperturbative chemical imaging of organelle transport in living cells with coherent anti-Stokes Raman scattering microscopy. Biophysical Journal, **91**, 728-735.

- 79) T.T. Le, I.M. Langohr, M.J. Locker, M. Sturek, J.X. Cheng (2007). Label-free molecular imaging of atherosclerotic lesions using multimodal nonlinear optical microscopy. Journal of Biomedical Optics, **12**, 054007.
- 80) T.B. Huff, Y. Shi, Y. Fu, H. Wang, J.X. Cheng (2008). Multimodal nonlinear optical microscopy and applications to central nervous system imaging. IEEE Journal of selected topics in quantum electronics, **14**, 4-9.
- 81) M. Mathew, S.I.C.O. Santos, D. Zalvidea, P.L. Alvarez (2009). Multimodal optical workstation for simultaneous linear, nonlinear microscopy and nanomanipulation : Upgrading a commercial confocal inverted microscope. Review of Scientific Instruments, **80**, 073701.
- 82) K. A. Hartzell, K.L. Danowski, P. Pantano (2008). Combined imaging and chemical sensing with a disposable microscope objective chemical sensor. Applied spectroscopy reviews, **43**, 1-21.
- 83) K.S. Bronk, K.L. Michael, P. Pantano, D.R. Walt (1995). Combined imaging and chemical sensing using a single optical imaging fiber. Analytical Chemistry, **67**, 2750-2757.
- 84) J.W. Goodman (1996), Introduction to fourier optics, McGraw-Hill Companies, Inc. ISBN: 0 07 024254 2.
- 85) Manual of Texas Instruments DMD Discovery 1100.
- 86) Manual of Grintech, Germany.
- 87) Manual of ImageJ software.
- 88) G.J. Brakenhoff (1979). Imaging modes in confocal scanning light microscopy. Journal of microscopy, **117**, 233-242.

- 89) D.C. Leiner, D.T. Moore (1978). Real time phase microscopy using a phase locked interferometer. Review of scientific instruments, **49**, 1702-1705.
- 90) H.A. Ferwerda (1993). Fritz Zernike- life and achievements. Optical engineering, **32**, 3176-3181.
- 91) E.B.V. Munster, T.W.J. Gadella (2005). Advances in biochemical engineering/biotechnology, Springer-Verlag, Berlin.
- 92) H. Szmazinski, J.R. Lakowicz, M.L. Johnson (1994). Fluorescence lifetime imaging microscopy: homodyne technique using high speed gated image intensifier. Methods in enzymology, **240**, 723-748.
- 93) Z.X. Chao, U.S. Dinish, L.K. Seah, V.M. Murukeshan (2005). Homodyne and heterodyne signal processing assisted phase resolved optical technique for latent fingerprint imaging: a theoretical study. Journal of modern optics, **52**, 119-129.
- 94) Manual of 404 nm laser, Omicron, Germany.
- 95) Manual of LaVision PicoStar HR camera system, Lavision corporation, Germany.
- 96) Manual of Agilent 33250 A, Agilent Technologies, U.S.A.
- 97) Homepage of horiba scientific products.
<http://www.horiba.com/us/en/scientific/products/fluorescence-spectroscopy/>
- 98) R. Koukiekolo, Z. J. Jakubek, J. Cheng, S. M. Sagan, J. P. Pizacki. (2009). Studies of a viral suppressor of RNA silencing p19-CFP fusion protein: A FRET based probe for sensing double-stranded fluorophore tagged small RNAs. Biophysical Chemistry, **143**, 166-169.
- 99) P. J. Tadrous, J. Siegel, P.M.W. French, S. Shousha, E.N. Lalani, G. W. H. Stamp. (2003). Fluorescence lifetime imaging of unstained tissues: early results in human

- breast cancer. Journal of pathology, **199**, 309-317.
- 100) S. Denicke, J.E. Ehlers, R. Niesner, S. Quentmeier, K.H.Gericke.(2007). Steady-state and time-resolved two-photon fluorescence microscopy : a versatile tool for probing cellular environment and function. Physica Scripta, **76**, C115-C121.
- 101) T. Dabbs, M. Glass. (1992). Fiber-optic confocal microscope : FOCON. Applied Optics, **31**, 3030-3035.
- 102) A.F. Gmitro, D. Aziz. (1993). Confocal microscopy through a fiber-optic imaging bundle. Optics Letters, **18**, 565-567
- 103) A. Diaspro. (2002). Confocal and two-photon microscopy. New york: Wiley Liss inc.
- 104) A.K. Ruprecht, T.F. Wiesendanger, and H.J. Tiziani. (2004). Chromatic confocal microscopy with a finite pinhole size. Optics letters, **29**, 2130-2132.
- 105) M.S. Bahae, A.A. Said, T.H. Wei, D.J. Hagan, E.W.V. Stryland. (1990). Sensitive measurement of optical nonlinearities using a single beam. IEEE journal of quantum electronics, **26**, 760-769.