Investigation into phase-resolved optical technique for latent fingerprint detection and bioimaging

Dinish Unnimadhavakurup Soudamini Amma

2005

Dinish U. S. A. (2005). Investigation into phase-resolved optical technique for latent fingerprint detection and bioimaging. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/5680>

<https://doi.org/10.32657/10356/5680>

Nanyang Technological University

Downloaded on 26 Apr 2023 18:09:57 SGT

Investigation Into Phase-Resolved Optical Technique for Latent Fingerprint Detection and Bioimaging

Dinish Unnimadhavakurup Soudamini Amma

School of Mechanical and Aerospace Engineering

**A Thesis submitted to the Nanyang Technological University
in fulfillment of the requirement for the degree of
Doctor of Philosophy**

2005

ABSTRACT

Fluorescence spectroscopy and imaging has lot of applications in forensic science and biomedical areas. Sensitive detection techniques are needed when dealing with very weak fluorescence signals from the test samples. Moreover, in fluorescence based imaging, separation of fluorescence often raises challenging problems when it is overshadowed by the strong background fluorescence or when the background fluorescence lies in close wavelength range as that of sample fluorescence.

In this context a sensitive Phase-Resolved (PR) imaging technique is proposed to overcome the above-mentioned major limitations. A theoretical formulation of PR imaging was carried out by incorporating the homodyne and heterodyne concept of signal processing. Theoretical analysis shown that separation and imaging of sample fluorescence can be achieved by suppressing the background fluorescence, which are in close wavelength ranges. Also, imaging of sample fluorescence can be achieved even if the fluorescence lifetime is longer or shorter than the lifetime of background fluorescence emission. Sensitivity improvement for the homodyne assisted PR imaging was carried out by incorporating the ‘pi’ shift method along with ‘even-step-phase shift’ method.

An experiment was set up to validate the formulated theory. The imaging of latent fingerprints and specific bio sample such as microlitre volume of DNA in capillary was carried out using the proposed technique. Often, when fingerprints deposited on strongly fluorescing backgrounds, their separation offer a challenging problem to fingerprint experts. Similarly, in the case of fluorescence-based detection of

microlitre DNA samples, presence of strong back-scattered light as well as unwanted background fluorescence from the sample environment limits the detection sensitivity.

Experiments were carried out for imaging the latent fingerprints, which are deposited on strongly fluorescing backgrounds, even when background fluorescence lifetime is longer than that of fingerprint fluorescence. Imaging of two years old fingerprints were also carried out with improved contrast. The advantage of homodyne assisted PR imaging over heterodyne assisted PR imaging was both theoretically and experimentally demonstrated in the context of latent fingerprint imaging. An Image quality evaluation method for the fingerprint image obtained using PR technique was proposed and carried out. It quantitatively proves the better quality of fingerprint image obtained by PR technique.

In order to further demonstrate the high sensitivity and ability of proposed PR technique, experiments were carried out for the imaging of fluorescein chemicals having same emission wavelength but differing in their lifetime values. Suppression of either of the emissions by selective imaging of the other validates the superiority of the proposed technique. Imaging of the sample fluorescence was carried out with sub nanosecond resolution. This imaging principle can find potential applications in fluorescence lifetime imaging along with fluorescence microscopic research in biochemistry, biophysics, and cell physiology.

ABSTRACT

Imaging of DNA samples in micro capillary tubes was also successfully carried out with the help of PR technique. Detection of weak fluorescence from DNA sample with 5-microlitre volume proved the high sensitivity of PR technique when dealing with weak fluorescence emissions. This is significant for cost effective fluorescence detection by minimising the expensive chemicals. Initial experiments were also carried out to show the feasibility of applying PR techniques in detecting DNA fluorescence amplification due to Polymerase Chain Reaction (PCR).

The proposed PR technique can detect weak fluorescence from the sample and also enables to separate sample fluorescence such as fingerprint fluorescence when it is overshadowed by the strong background fluorescence. The ability of PR technique to resolve and separate fluorescence emissions, which are close in wavelength and lifetime value, can be applied for the detection of cell abnormalities such as early detection cancer growth in living cells.

Dedicated to:

My family members, they always inspired me

ACKNOWLEDGEMENTS

In fact, this thesis is not mere results of my few years research work; rather it is the essence of knowledge I gradually acquired during my entire academic career. In this proud moment, it is my great pleasure to say that the basic, but the greatest lessons that I have ever learned was from my parents. Since then, thousands of people, including my teachers, relatives, colleagues, friends and even some strangers have directly or indirectly influenced me in achieving this goal. I thankfully remember each of them in this proud occasion.

First of all, I would like to express my sincere gratitude to my supervisors, Associate Professor Seah Leong Keey and Assistant Professor Murukeshan Vadakke Matham for their wonderful guidance, support and help throughout the course of this research work. Their patience for answering my doubts and giving me invaluable advices was the key to all good results that I managed to achieve. Apart from the research guidance, their constant encouragement was always a source of inspiration for me. Also, the influence of their positive criticism was so strong that I will never forget them.

It is a pleasure to convey my indebtedness and sincere thanks to Associate Professor Ong Lin Seng for his support and encouragement. His valuable suggestions really guided me during the course of my research work. Sincere words of appreciation goes to Dr. Chao ZhiXia, Research Fellow and Mr. Aditya Singh, Project Officer, for their suggestions, help and support while carrying out the experiments.

ACKNOWLEDGEMENTS

Sincere thanks to Mr. Lim Seng Kim, head of Scene of Crime Unit (SCU) at Criminal Investigation Department (CID) of Singapore Police Force, for his help and support by providing the facility to carry out some initial experiments related to the project at SCU. I also express my sincere gratitude to Mr. Stephen Tay and all other staffs at SCU, Singapore for their invaluable help.

I acknowledge gratefully the great help from Associate Professor Gerald Seet, director of RRC lab of MAE school, for providing me the space to carry out my experiments. Many thanks to all the technicians of RRC lab of MAE school, for their help and support. I am so grateful to the helping hands of technicians Mr. Lim Eng Cheng, Mrs. Agnes Tan, Mr. You Kim San and Miss. Toh Yen Mie.

I am grateful to TEC (The Enterprise Challenge) committee for their interest and funding in this project and Nanyang Technological University for its AcRF (Academic Research Fund) support. I also express my sincere gratitude to the Nanyang Technological University for the research scholarship.

I wish to thank all my friends and well wishers at this pleasant moment for their support and encouragement throughout the course of this work. I am grateful to some of the constructive suggestions that I received from my friends, which helped me a lot during my experimental research. They always motivated me to do successful research and at this happy moment I sincerely acknowledge their words of consolation and inspiring suggestions to meet the challenging research life.

ACKNOWLEDGEMENTS

Lastly, but not least, I attribute the success of this research to my family members for their continuous support and inspiration. At this moment, I can happily tell them that “I did it wonderfully well” for their query of “how is your research”.

Above all, I bow my head with respect to the power that controls all of us, without whose blessings and kindness we cannot achieve anything in this world.

<u>TABLE OF CONTENTS</u>	
	Page. No
ABSTRACT	I
ACKNOWLEDGEMENTS	IV
TABLE OF CONTENTS	VII
LIST OF FIGURES	XIII
LIST OF TABLES	XXI
LIST OF SYMBOLS	XXII
1. INTRODUCTION	1
1.1 Background	1
1.2 Existing fingerprint detection techniques	2
1.3 Fluorescence based techniques for bioimaging	4
1.4 Objective	6
1.5 Scope	6
1.6 Organisation of the thesis	7
2. LATENT FINGERPRINTS: AN OVERVIEW	10
2.1 Introduction	10
2.2 Types of fingerprints	10
2.3 Historical background of fingerprinting	11
2.4 Constituents of the fingerprint deposits	13
2.5 Classification of fingerprint patterns	15
2.5.1 Fixed points in fingerprints	15
2.5.2 Different fingerprint patterns	16
2.6 Fundamental principles of fingerprint identification	18

TABLE OF CONTENTS

2.7	Conventional fingerprint detection methods	19
2.7.1	Chemicals used for conventional fingerprint detection	19
2.7.2	Limitations of chemicals based conventional fingerprint detection methods	26
2.8	Summary	27
3.	FLUORESCENCE TECHNIQUES FOR FINGERPRINT DETECTION AND BIOIMAGING	28
3.1	Introduction	28
3.2	Fluorescence phenomena	28
3.2.1	Fluorescence measuring techniques	32
3.2.2	Basics of frequency domain spectroscopy	33
3.3	Fluorescence based fingerprint detection methods	34
3.3.1	Fluorescence examination and optical filtering	35
3.3.2	Fingerprint detection using semiconductor nanocrystals	36
3.3.3	Time-resolved imaging	37
3.3.4	Limitations of fluorescence based fingerprint detection techniques	41
3.4	Frequency domain technique for biochemical and biomedical applications	43
3.5	Fluorescence detection of rapid cycle DNA amplification	46
3.6	Limitations of current phase-resolved technique	49
3.7	Summary	50
4.	THEORETICAL FORMULATION OF PHASE-RESOLVED OPTICAL TECHNIQUE	51
4.1	Introduction	51
4.2	Imaging of fluorescent sample deposited on fluorescing backgrounds	52

TABLE OF CONTENTS

4.2.1	Extraction of sample fluorescence based on homodyne assisted phase-resolved method	55
4.2.1.1	Filtering out DC terms in homodyne signal	57
4.2.1.2	Suppression of background fluorescence	59
4.2.2	Extraction of sample fluorescence based on heterodyne assisted phase-resolved method	60
4.2.2.1	Filtering out DC terms in heterodyne signal	62
4.2.2.2	Suppression of background fluorescence	64
4.2.3	Imaging of fluorescent sample deposited on non-fluorescing backgrounds	65
4.3	Choice of modulation frequency and optimisation of fluorescence emission	68
4.4	Sensitivity improvement for phase-resolved imaging by combining even-step-shift and pi-shift methods	72
4.5	Comparison of homodyne and heterodyne assisted PR imaging techniques	75
4.6	Summary	77
5.	PHASE-RESOLVED OPTICAL IMAGING	
	TECHNIQUE: INSTRUMENTATION DETAILS	78
5.1	Introduction	78
5.2	Phase-Resolved experimental setup	78
5.2.1	Components of the PR optical system	79
5.3	Spectrometers and broadband light source	83
5.3.1	Poly light source	83
5.3.2	Fluorescence spectrometer	83
5.3.3	Fluorescence lifetime analyzer	84
5.4	Experimental procedure	85
5.4.1	Lifetime measurement	85

TABLE OF CONTENTS

5.4.2	Phase-Resolved imaging	87
5.4.2.1	Homodyne assisted phase-resolved imaging	87
5.4.2.2	Heterodyne assisted phase-resolved imaging	89
5.5	Summary	93
6.	PHASE-RESOLVED IMAGING OF LATENT FINGERPRINTS	94
6.1	Introduction	94
6.2	Lifetime characterisation of fingerprints treated with fluorescent powders	94
6.3	Homodyne assisted phase-resolved imaging	101
6.3.1	Imaging of fingerprints deposited on porous surfaces	102
6.3.2	Imaging of fingerprints deposited on non-porous surfaces	110
6.3.3	Imaging of older fingerprints	112
6.3.4	Imaging of fingerprints on difficult surfaces	114
6.4	Fingerprint image quality evaluation	117
6.5	Sensitivity improvement for phase-resolved imaging	119
6.6	Heterodyne assisted phase-resolved imaging	122
6.7	Comparison of homodyne and heterodyne assisted PR imaging techniques	125
6.8	Summary	126
7.	PHASE-RESOLVED IMAGING FOR BIOMEDICAL APPLICATIONS	128
7.1	Introduction	128
7.2	Separation of fluorescence emissions with sub nanosecond resolution	128
7.2.1	Sample preparation	129
7.2.2	Phase-resolved imaging of the sample	129
7.3	Detection of DNA sample fluorescence in micro capillary tube	137

TABLE OF CONTENTS

7.3.1	Sample preparation	137
7.3.2	Phase-resolved imaging of DNA fluorescence	138
7.3.3	Detection of DNA amplification due to PCR	140
7.4	Summary	143
8.	CONCLUSIONS AND RECOMMENDATIONS	144
8.1	Conclusions	144
8.2	Recommendations for future work	147
APPENDIX A. CONVENTIONAL METHODS OF FINGERPRINT		
	DEVELOPMENT	149
A.1	Introduction	149
A.2	Fingerprints on porous surfaces	149
A.3	Fingerprints on nonporous surfaces	151
A.4	Fingerprints on blood contaminated surfaces	154
A.5	Fingerprints on adhesive surfaces	155
A.6	Fluorescence examination and optical filtering	156
A.7	Summary of attachment training at SCU	158
APPENDIX B. TIME-RESOLVED IMAGING OF LATENT FINGERPRINTS		
	WITH NANOSECOND RESOLUTION	159
B.1	Introduction	159
B.2	Samples tested	159
B.3	Experimental set up and procedure	160
B. 4	Results and discussions	162
B. 5	Summary	170

TABLE OF CONTENTS

APPENDIX C. SPECIFICATIONS OF ICCD CAMERA	171
LIST OF PUBLICATIONS	172
REFERENCES	175

LIST OF FIGURES

	Page. No
Figure 2.1 'Delta' points on fingerprints	16
Figure 2.2 Loop fingerprint pattern	16
Figure 2.3 Whorl fingerprint pattern	17
Figure 2.4 Arch fingerprint pattern	18
Figure 3.1 Jablonski diagram for fluorescence	30
Figure 3.2 Phase shifted fluorescence emission due to modulated excitation	33
Figure 3.3 Time-resolved imaging prototype and image intensifier gating scheme	40
Figure 3.4 Time-resolved imaging system for latent fingerprint imaging-Block Diagram	41
Figure 3.5 Schematic diagram showing the principle of time-resolved imaging	41
Figure 4.1 Graphical representation of modulated excitation and homodyne detector modulation	53
Figure 4.2 Graphical representation of modulated fluorescence emission from the sample	55
Figure 4.3 Graphical representation of even step phase shifting to cut off DC term of signal from fluorescence emission	57
Figure 4.4 Graphical representation of fluorescence emission from sample after removing the DC term	59
Figure 4.5 Graphical representation of modulated excitation and heterodyne detector modulation	61
Figure 4.6 Graphical representation of the time-varying fluorescence signal at heterodyne frequency, which includes both DC and AC terms	62
Figure 4.7 Graphical representation of the ICCD camera exposure time function	63

LIST OF FIGURES

Figure 4.8	Graphical representation of phase-resolved fluorescence emission from the sample after removing the DC term	64
Figure 4.9	Graph showing the variation of linear frequency versus $\log(\tau)$ with respect to different m_s	68
Figure 4.10	Theoretical result of optimum modulation frequency at different background lifetimes when $\tau_s = 8 \text{ ns}$	71
Figure 4.11	Theoretical result of optimum modulation frequency at different background lifetimes when $\tau_s = 4 \text{ ns}$	72
Figure 5.1	Schematic diagram of Phase-resolved optical imaging system	79
Figure 5.2	Schematic diagram of the experimental setup used for determination of fluorescence emission wavelengths	84
Figure 5.3	Schematic diagram of fluorescence lifetime measurement system	86
Figure 5.4	Schematic diagram of homodyne assisted PR imaging set up	88
Figure 5.5	Schematic diagram of heterodyne assisted PR imaging set up	91
Figure 6.1	Fluorescence decay plot for Blitz-green treated fingerprint sample	97
Figure 6.2	Fluorescence decay plot for Greenwop treated fingerprint sample	97
Figure 6.3	Fluorescence decay plot for Redwop treated fingerprint sample	97
Figure 6.4	Fluorescence decay plot for Blitz-red treated fingerprint sample	97
Figure 6.5	Fluorescence decay plot for Ultra-yellow treated fingerprint sample	98
Figure 6.6	Fluorescence decay plot for Ultra-orange-yellow treated fingerprint Sample	98
Figure 6.7	Fluorescence decay plot for Ultra-red treated fingerprint sample	98
Figure 6.8	Fluorescence decay plot for Ultra-gold treated fingerprint sample	98
Figure 6.9	Fluorescence decay plot for Ultra-green treated fingerprint sample	99
Figure 6.10	Fluorescence decay plot for Ultra-azure-blue treated fingerprint sample	99
Figure 6.11	Fluorescence decay plot for Ultra-white treated fingerprint sample	99
Figure 6.12	Fluorescence emission spectra of Blitz-green treated fingerprint sample on fluorescing green paper at 476 nm excitation	102
Figure 6.13a	Fingerprint deposited on fluorescing paper substrate:	

LIST OF FIGURES

	Conventional digital image	104
Figure 6.13b	Fingerprint deposited on fluorescing paper substrate: TR image	104
Figure 6. 13c	Fingerprint deposited on fluorescing paper substrate:	
	Homodyne assisted PR image	104
Figure 6.14	Fluorescence emission spectra of greenwop treated fingerprint sample on smooth fluorescing paper at 476 nm excitation	105
Figure 6.15a	Fingerprint deposited on fluorescing paper having emissions at very close lifetime and wavelength values: Conventional digital image	106
Figure 6.15b	Fingerprint deposited on fluorescing paper having emissions at very close lifetime and wavelength values: Homodyne assisted PR image	106
Figure 6.16a	Fingerprint deposited on fluorescing magazine paper: Conventional digital image	107
Figure 6.16b	Fingerprint deposited on fluorescing magazine paper: Homodyne assisted PR image	107
Figure 6.17	Decay plot and lifetimes values of multi fluorescing calendar paper	108
Figure 6.18a	Fingerprint deposited on multi fluorescing calendar paper: Conventional digital image	109
Figure 6.18b	Fingerprint deposited on multi fluorescing calendar paper: Homodyne assisted PR image	109
Figure 6.19	One-week-old Fingerprint deposited on glass substrate: Homodyne assisted PR image	111
Figure 6.20.	Two-week-old Fingerprint deposited on glass substrate: Homodyne assisted PR image	111
Figure 6.21a	One weak old fingerprint deposited on aluminum clamp substrate: Conventional digital image	111
Figure 6.21b	One weak old fingerprint deposited on aluminum clamp substrate: Homodyne assisted PR image	111
Figure 6.22a	Sweat fingerprint deposited on aluminum substrate: Conventional digital image	113

LIST OF FIGURES

Figure 6.22b	Sweat fingerprint deposited on aluminum substrate: Homodyne assisted PR image	113
Figure 6.23a	Oily fingerprint deposited on aluminum substrate: Conventional digital image	113
Figure 6.23b	Oily fingerprint deposited on aluminum substrate: Homodyne assisted PR image	113
Figure 6.24a	Fingerprint deposited on fluorescing fruit juice cover: Conventional digital image	114
Figure 6.24b	Fingerprint deposited on fluorescing fruit juice cover: Homodyne assisted PR image	114
Figure 6.25a	Fingerprint deposited on currency: Conventional digital image	115
Figure 6.25b	Fingerprint deposited on currency: Homodyne assisted PR image	115
Figure 6.26a	Ultra-azure blue treated Fingerprint deposited on transparency sheet: Conventional digital image	116
Figure 6.26b	Ultra-azure blue treated Fingerprint deposited on transparency sheet: Homodyne assisted PR image	116
Figure 6.26c	Blitz-green treated Fingerprint deposited on transparency sheet: Conventional digital image	116
Figure 6.26d	Blitz-green treated Fingerprint deposited on transparency sheet: Homodyne assisted PR image	116
Figure 6.27	Fingerprint image with the representative diagonal sampling line	117
Figure 6.28	Representation of the pixel arrays that undergoing diagonal sampling	117
Figure 6.29a	Fingerprint image of the first sample obtained by normal homodyne assisted PR imaging	121
Figure 6.29b	Fingerprint image of the first sample obtained by pi shift Combined homodyne assisted PR imaging	121
Figure 6.30a	Fingerprint image of the second sample obtained by normal homodyne assisted PR imaging	122
Figure 6.30b	Fingerprint image of the second sample obtained by pi shift combined homodyne assisted PR imaging	122

LIST OF FIGURES

Figure 6.31a	Oily fingerprint deposited on aluminum substrate: Conventional digital image	124
Figure 6.31b	Oily fingerprint deposited on aluminum substrate: Heterodyne assisted PR image	124
Figure 6.32a	Fingerprint deposited on fluorescing paper substrate: Conventional digital image	124
Figure 6.32b	Fingerprint deposited on fluorescing paper substrate: Heterodyne assisted PR image	124
Figure 6.33a	Fingerprint deposited on multi fluorescing calendar paper: Conventional digital image	125
Figure 6.33b	Fingerprint deposited on multi fluorescing calendar paper: Heterodyne assisted PR image	125
Figure 7.1	Fluorescence emission spectra of fluorescein sample at 402 nm excitation	130
Figure 7.2	Fluorescence decay plot of fluorescein sample at 10 ml/lit concentration	131
Figure 7.3	Fluorescence decay plot of fluorescein sample at 40 ml/lit concentration	131
Figure 7.4	Fluorescence decay plot of fluorescein sample at 60 ml/lit concentration	131
Figure 7.5	Fluorescence decay plot of fluorescein sample at 100 ml/lit concentration	131
Figure 7.6a	Homodyne assisted PR image of fluorescein sample: Suppression of emission with lifetime 5.80 ns by selectively imaging 4.49ns emission	133
Figure 7.6b	Homodyne assisted PR image of fluorescein sample: Suppression of emission with lifetime 4.49ns by selectively imaging 5.80 ns emission	133
Figure 7.7a	Homodyne assisted PR image of fluorscein sample: Suppression of emission with lifetime 5.80 ns by selectively imaging 5.39 ns emission	135

LIST OF FIGURES

Figure 7.7b	Homodyne assisted PR image of fluorescein sample: Suppression of emission with lifetime 5.39 ns by selectively imaging 5.80 ns emission	135
Figure 7.8a	Homodyne assisted PR image of Fluorescein sample: Suppression of emission with lifetime 5.80 ns by selectively imaging 5.59 ns emission	136
Figure 7.8b	Homodyne assisted PR image of Fluorescein sample: Suppression of emission with lifetime 5.59 ns by selectively imaging 5.80 ns emission	136
Figure 7.9a	DNA sample taken in circular capillary: Conventional digital image	140
Figure 7.9b	DNA sample taken in circular capillary: Homodyne assisted PR image	140
Figure 7.10a	DNA sample taken in circular capillary: Conventional digital image after 12th temperature cycling	142
Figure 7.10b	DNA sample taken in circular capillary: Homodyne assisted PR image after 12th temperature cycling	142
Figure 7.11a	DNA sample taken in circular capillary: Conventional digital image after 17th temperature cycling	142
Figure 7.11b	DNA sample taken in circular capillary: Homodyne assisted PR image after 17th temperature cycling	142
Figure A.1	Fingerprint development newspaper using Ninhydrin	150
Figure A.2	Fingerprint development on printing white paper using Ninhydrin	150
Figure A.3	Fingerprint development on coloured card by fuming	152
Figure A.4	Fingerprint development on polythene cover by fuming	152
Figure A.5	Fingerprint development on drinking bottle by fuming	152
Figure A.6	Fingerprint development on plastic bottle by fuming	152
Figure A.7	Fingerprint development by black magnetic powder	154
Figure A.8	Fingerprint development by silver powder on black surfaces	154
Figure A.9	Fingerprint development by white powder on coloured card	154

LIST OF FIGURES

Figure A.10	Blood contaminated print on non-porous surfaces-development by Amido black	155
Figure A.11	Fingerprint development on coloured adhesive surface by gentian violet	156
Figure A.12	Fluorescence detection of fingerprints when background emission is widely separated from that of fingerprint sample	157
Figure A.13	Fluorescence detection of prints when background emission is overlapping from that of fingerprint sample	157
Figure B.1	TR imaging set up for latent fingerprints	161
Figure B.2	Schematic diagram showing the principle of time-resolved imaging	162
Figure B.3	Normal ICCD image of fresh fingerprints	163
Figure B.4	TR image of fresh fingerprints	163
Figure B. 5	Normal ICCD Image of weak and fresh fingerprints	164
Figure B. 6	TR image of weak and fresh fingerprints	164
Figure B.7	Theoretical fluorescence decay of two fluorescence emissions having comparable emission intensity but different lifetime	165
Figure B.8	Theoretical fluorescence decay of two fluorescence having difference in emission intensity and lifetime	166
Figure B.9(a)	Conventional image of ultra-azure-blue powder treated finger prints on 'blue' fluorescing PPMA substrate	168
Figure B.9(b)	TR image of ultra-azure-blue powder treated fingerprints on 'blue' fluorescing PPMA substrate	168
Figure B.10(a)	Conventional image ultra azure-blue powder treated fingerprint on 'white' fluorescing PPMA substrate	169
Figure B.10(b)	TR image ultra azure-blue powder treated fingerprint on 'white' fluorescing PPMA substrate	169
Figure B.11(a)	Conventional image of ultra-white powder treated fingerprints on 'blue' fluorescing PPMA substrate	169
Figure B.11(b)	TR image of ultra-white powder treated fingerprints on 'blue' fluorescing PPMA substrate	169

LIST OF FIGURES

Figure B.12(a) Conventional image of ultra-white powder treated fingerprints on 'white' fluorescing PPMA substrate	170
Figure B.12(b) Conventional image of ultra-white powder treated fingerprints on 'white' fluorescing PPMA substrate	170

LIST OF TABLES

	Page. No
Table 2.1 Constituents of the fingerprint deposits	14
Table 3.1 Physical processes undergone by excited molecules	31
Table 6.1 Peak fluorescence emission wavelength of fingerprints treated with fluorescent powders	96
Table 6.2 Fluorescence lifetime of fingerprints treated with fluorescent powders	100
Table 6.3 Quantitative comparison of the fingerprint image quality obtained using homodyne assisted PR and conventional digital- imaging method	119
Table 7.1 Fluorescence lifetime of fluorescein sample at different concentrations	132

LIST OF SYMBOLS

Symbol	Meaning
A	Direct Current (DC) term of the intensity of the modulated laser excitation.
$A'(r_b)$	DC term of the background fluorescence signal.
$A'(r_s)$	DC term of the sample fluorescence signal.
B	DC term of the modulated gain.
$E(t)$	Exposure time function of a ICCD camera.
$F(r, t)$	Intensity of the fluorescence from the sample.
$G(t)$	Modulated gain signal of the detector.
$H(r, \phi, \phi_g)$	Homodyne mixing result of $F(r, t)$ and $G(t)$.
$H(r, \phi, \phi_g)_{avg}$	DC term in $H(r, \phi, \phi_g)$
$H_0(r, \phi, \phi_g)$	Homodyne signal after removing DC term from $H(r, \phi, \phi_g)$
$H_E(r, t, \phi_g)$	Heterodyne mixing result of $F(r, t)$ and $G(t)$.
$H_{E0}(r, \phi_g)$	Heterodyne signal after removing DC term from $H_E(r, t, \phi_g)$
$H_{01}(r, \phi_s, \phi_g)$	Phase-resolved fluorescence intensity from sample when background fluorescence is absent
$I(t)$	Intensity of the modulated laser beam.
m_b	Demodulation factor of the background fluorescence.
m_{ex}	Modulation depth of the modulated laser beam.
m_s	Demodulation factor of the sample fluorescence.
m_g	Modulation depth of the modulated gain.
$Q(\omega, \tau_s, \tau_b)$	Coefficient of modulation frequency on intensity of the extracted sample fluorescence.

LIST OF SYMBOLS

r	Any position on the sample.
r_b	Position where the background fluorescence exists.
r_s	Position where the sample fluorescence exists.
$S(r_s)$	Intensity of the extracted sample fluorescence.
t	Time.
T	Duration of the exposure time of the ICCD camera.
$\delta\omega$	Angular frequency difference between the modulated laser beam and the modulated gain in heterodyning.
ϕ	General term for phase shift
ϕ_g	Phase difference between $I(t)$ and $G(t)$ at $t = 0$
ϕ_b	Phase shift of the background fluorescence
ϕ_s	Phase shift of the sample fluorescence
ϕ_0	Initial value of ϕ_g
f	Modulation frequency
τ_b	Lifetime of the background fluorescence.
τ_s	Lifetime of the sample fluorescence.
ω	Angular frequency of the laser modulation
ω_1	Angular frequency of the ICCD modulation gain in heterodyning.

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND

Fluorescence spectroscopy and imaging has lot of applications in forensic science and biomedical areas. Sensitive detection techniques are needed when dealing with very weak fluorescence signals from the test samples. The never-ending quest of researchers for improved and sensitive fluorescence techniques for sensing and diagnostic purposes opened the door for frequency domain technique.

Frequency domain otherwise called phase-resolved (PR) spectroscopy generally targets the strong fluorescence emission from the test sample [1]. It also employed in specific biomedical applications where living cells are marked with strong fluorescent contrast agents [2-3]. Due to the high sensitivity and ability of PR technique to separate the close wavelength emissions based on their lifetime difference makes it a potential candidate in the imaging of fluorescent samples, which are deposited on strongly fluorescing backgrounds.

Most of the practical cases encounter the situation of fingerprints deposited on strongly fluorescing backgrounds and their separation offer challenging problems to fingerprint experts. The possibility of applying PR technique for latent fingerprint imaging is yet to be explored because the fluorescence emission intensity from samples in general will be very weak [4].

Test samples used in this study will be latent fingerprints and specific bio sample such as microlitre volume of DNA in capillary tube. A brief review of present methods employed in the area of latent fingerprint detection and bio imaging are described in the following sections by highlighting their limitations. The need for a better sensitive technique such as proposed PR technique is made evident in the following sections by highlighting the limitations of current techniques employed.

1.2 EXISTING FINGERPRINT DETECTION TECHNIQUES

Present fingerprint detection methods can be broadly classified in to conventional chemical based and fluorescence based detection methods [5]. Conventional chemical based methods employs the application of different chemical treatments to fingerprint samples, based on the substrate where it formed. All these conventional methods have their own limitations. The main drawbacks are that there is no generic approach in lifting the latent fingerprints. One has to undergo a series of systematic procedures and usage of chemicals, depending upon the substrate where the print is formed [5-13]. Moreover, these methods fails when deals with older fingerprints. Sometimes the contaminated prints (like oily prints, bloody prints etc) are often not retrieved by the conventional methods. The chemical and physical methods that are generally employed nowadays lack the quality of storing and later retrieval of the information.

Fluorescence based methods are based on emission from specific chemical agent treated fingerprint residue up on excitation with proper light source. Imaging of fingerprint fluorescence can be carried out by proper optical filtering and

photography [4]. But, optical filtering based detection methods totally fail when there is strong fluorescence emission from the background and also when emission from the fingerprint and that of background fall under the close wavelength range. Moreover, development of fingerprints on some 'difficult' surfaces is not possible by these methods [13].

In 1987, Menzel et al introduced time-resolved (TR) fluorescence imaging technique to latent fingerprint detection with the objective of suppressing unwanted background fluorescence [4,14-15]. Since it is necessary to have a fingerprint fluorescence lifetime that is longer than that of the background, the chemical treatment of latent fingerprints to achieve longer fingerprint fluorescence lifetime, typically employing a rare earth-based chemical, is a major process of time-resolved approach. In TR technique, rare earth based chemicals that are applied to the fingerprint samples helps to prolong their lifetimes into the millisecond range in order to separate it from background fluorescence, which is typically in nanosecond range [4,14-15].

However, the major limitations of this technique are that (i) it allows one to work only in the millisecond time range of fluorescence lifetime (ii) it demands the use of rare earth based chemicals to fingerprint samples to prolong the lifetime to millisecond range, and (iii) the fingerprint fluorescence lifetime should be longer than that of background fluorescence.

1.3 FLUORESCENCE BASED TECHNIQUES FOR BIO IMAGING

Characterisation of fluorescence emissions (endogenous or exogenous) from the cells often lead to conclusive results in the detection of cancerous cells and similar cell abnormalities [16-17]. When the fluorescence emission wavelengths are close, optical filtering or any such conventional methods will be ineffective to detect the desired emission.

Recently, the detection of cancerous cells through fluorescence technique took a sudden turn leading to the imaging of cells. The imaging of cell abnormalities has lots of advantages over conventional fluorescence spectroscopy method where one uses the spectral characteristics of different types of cell constituents [18-21]. But, all the conventional fluorescence methods for bio imaging have its own limitations. When the intensity of fluorescence signal from the cell is very weak, the sensitivity of these techniques comes under question. Imaging of biological cells often gives lot of additional information than that given by ordinary fluorescence spectroscopic methods.

In this context, frequency domain spectroscopic techniques offer a sensitive platform in the diagnostic arena for many such biomedical applications [2-3]. Till now, no work has been reported on the sensitivity of phase resolved technique when dealing with weak fluorescence emissions. Also, the ability of PR technique in separating multiple fluorescence emissions, which are close in wavelength scale but are separated in fluorescence lifetime scale, is yet to be explored. Reported literatures addressing frequency domain or PR spectroscopy were targeted at some specific

biomedical applications where living cells are marked with strong fluorescent contrast agents.

Detection and characterisation of real time Polymerase Chain Reaction (PCR) of DNA samples marked with specific fluorescent agents started to be explored recently [22-23]. In all such work, micro/milli litre volume of DNA samples in glass capillary is detected through sensitive spectrometer. But spectrometer based detection techniques become complicated when DNA samples are marked with multiple dyes, which are close in emission wavelengths. Also, the fluorescence emission intensity of such samples in general will be very weak before rapid cycle amplification of DNA [22-24] and it raises a question mark to the detection sensitivity. Moreover, minimising of the sample volume to make the experiment cost effective is always a dream of all biotechnology researchers. When mimimising the sample volume, spectrometer based detection systems will face challenges due to the weak fluorescence from the sample.

Above-mentioned limitations in latent fingerprint detection and bio imaging demands for a more generic and sensitive technique that can overcome most of the reported drawbacks. An imaging technique that can selectively detect weak fluorescence even when it is deposited on strongly fluorescing backgrounds is necessary to detect test samples like fingerprints and biological cells which may have weak fluorescence emission intensity. Keeping in mind all the limitations of existing techniques in latent fingerprint detection and bio imaging, the main objectives of this research work are summarised below.

1.4 OBJECTIVE

The main objectives to be achieved in this research work include:

- 1 Investigation in to the research and development of phase-resolved (PR) optical technique by incorporating homodyne and heterodyne signal processing concepts.
- 2 Application of developed PR technique for the detection of latent fingerprints and in bio imaging.

1.5 SCOPE

PR technique can be used to separate the close wavelength emissions based on their lifetime difference and hence make a potential technique for the detection of fluorescence emissions for various biochemical and biomedical applications. So far no work has been reported on the possibility of applying PR technique for fingerprint detection and bio imaging where the fluorescence emission intensity from samples in general is very weak. In order to achieve the desired objective, the proposed research has the main scopes as listed below.

- A Literature review on the basics of fingerprints, their detection methods and fluorescence techniques for bio imaging by highlighting their limitations.
- The attachment program at Scene of Crime Unit (SCU), Criminal Investigation Department (CID) of Singapore Police Force to carry out the detection of latent fingerprint samples on various substrates to confirm the drawbacks of conventional methods, which were reported in the literatures.

- Fluorescence lifetime study of fingerprint samples treated with various fluorescent powders and TR imaging with nanosecond resolution.
- Theoretical formulation and analysis of the PR optical technique by incorporating homodyne and heterodyne concept of signal processing for latent fingerprint detection and bioimaging. Also, Comparison of the relative performance between homodyne and heterodyne assisted phase-resolved optical imaging technique.
- Image quality evaluation for the fingerprint image obtained using PR technique and improvement in the sensitivity of imaging by incorporating ‘pi’ shift method along with homodyne assisted PR method.
- Detection and imaging of older fingerprints on various substrates and suppression of background substrate fluorescence for the better imaging of fingerprints deposited on strongly fluorescing substrate surfaces.
- Imaging experiments with fluorescein sample to show the potential application of PR technique in separating close wavelength emissions for biomedical applications. Also, imaging of DNA samples with better sensitivity taken in micro capillary tubes by the subsequent suppression of unwanted scattered and reflected light from the sample and environment.

1.6 ORGANISATION OF THE THESIS

Chapter 2 gives an overview of the basics of fingerprinting. It also describes the conventional methods employed for fingerprint detection highlighting the limitations.

Chapter 3 explains the basics of fluorescence phenomena followed by frequency domain spectroscopy and the current state of the art. It is followed by conventional

fluorescence based fingerprint detection methods by pointing out their limitations. This chapter also describes the fluorescence-based detection and imaging techniques for various biomedical applications followed by detection of rapid cycle DNA amplification.

Chapter 4 explains the theoretical formulation of homodyne and heterodyne assisted PR technique for the detection of fluorescent sample deposited on fluorescing and non-fluorescing backgrounds. Sensitivity improvement for the PR imaging by combining even-step-shift and pi-shift methods along with homodyne assisted PR technique is presented in the subsequent section. Comparison of the relative performance between homodyne and heterodyne assisted PR optical imaging technique is also described.

The proposed experimental setup and instrumentation details of the PR optical imaging system are explained in the chapter 5. It gives the experimental procedure for the homodyne and heterodyne assisted PR technique for imaging of the test samples.

Chapter 6 gives the experimental results of lifetime characterisation of fingerprint samples treated with various fluorescent powders. Later, it gives the experimental results and discussion of latent fingerprint imaging using the developed PR optical technique.

Chapter 7 gives the experimental results of the imaging of fluorescein chemical having same emission wavelength but differing in their lifetime values. It also

describes the improved sensitivity of PR imaging of DNA samples in micro capillary tubes by the subsequent suppression of unwanted scattered and reflected light from the sample and environment.

Chapter 8 gives the conclusions of the findings of this research work followed by the recommendations for future work.

CHAPTER TWO

LATENT FINGERPRINTS: AN OVERVIEW

2.1 INTRODUCTION

This chapter gives an overview of the basics of fingerprinting. A detailed review about types of fingerprints, historical backgrounds of fingerprinting and different fingerprint patterns will be discussed. This chapter will also discuss the existing conventional chemical treatment based methods for latent fingerprint detection by pointing out their limitations.

2.2 TYPES OF FINGERPRINTS

Physical evidence at the crime scene can be present in an infinite variety of forms, materials, dimensions, etc. and its value to the investigation can range from uncertain or of little importance to the identification of individual, an object, or a fact linked with the crime [25-26]. Few forms of evidence can permit the unquestionable identification of an individual (fingerprints, dental structure, genetic code) and possess all the necessary qualities for identification. Fingerprint acts as the most powerful tool for identifying people because the ridge patterns of every print are unique and immutable [27]. Hence, fingerprint detection plays a significant and important role in criminal investigation and forensic science.

The ridge patterns on the fingers are the most useful and generalised proof of identity. They are unique, immutable, universal, easy to classify and leave marks on

any object handled with bare hands [26-27]. There are mainly 3 types of fingerprints that come across in forensic science [28]. They are visible, plastic (impression prints) and latent. Ordinarily, a visible print does not require further treatment to be fully visualised. A plastic print is actually an indentation. This type of pattern is formed by pressing the friction ridges of the fingers onto a soft surface, such as wax, tar, soap, butter, or clay. Plastic prints generally do not require additional development to be fully visualised. They are usually photographed and sometimes may be cast.

As name suggests, latent prints are normally not visible. Some means of development is generally required for their visualisation [28-29]. Latent fingerprints enjoy unparalleled success in the scope of physical evidence than the other two types. Though the new technologies have altered methods for recovering and searching latent impressions, the basics of fingerprint comparison and identification have remained virtually unchanged since the beginning [26].

2.3 HISTORICAL BACKGROUND OF FINGERPRINTING

It is difficult to determine precisely when fingerprints were used for the first time as a means of personal identification. The history of fingerprinting dated back as early as 7,000 BC in ancient city of Jericho where the brick walls on the house were impressed with a pair of print of brick layer's thumb. It is one of the earliest documented fingerprinting reported today [26]. But the earliest trace of fingerprints being purposely impressed occurred much later in Mesopotamia and dated from 3,000 BC where authority asserts that a "digital pattern" was placed on each brick used in the construction of king's storehouse [15].

Thousands of years ago, the Chinese have recognised that the imprint of a fingerprint on a document was a unique signature [15]. A left thumb imprint is deeply embedded in a Chinese clay seal, dated before the third century BC, and on the reverse side is ancient Chinese script representing the name of the person who made the thumb imprint.

An English botanist, N.Grew, described the details of the sudoriferous pores, the papillary ridges and their patterns in 1684 [26]. He delivered a paper to the Royal society in London entitled “Philosophical Transaction”, in which he reported on the patterning of fingerprints and illustrated them with drawing of various patterns.

In the 18th century and early 19th century several works of anatomy based on the study of dermatoglyphics was reported. The word dermatoglyphics comes from two Greek words: derma means skin and glyphe means carve and refers to the friction ridge formations, which appear on the palms of the hands and soles of the feet. Later, Purkinje, in 1823, classified fingerprints patterns into 9 groups. Sir Francis Galton attempted to calculate the improbability of two persons having the same fingerprints and defined the basic patterns used in the present day method of fingerprint classification. He identified the minutiae in fingerprints and they have since been referred to as “Galton Details” or ridge characteristics [15-26].

Sir William Herschel, the administrator of Bengal, recorded handprints of the natives on contracts to prevent impersonation and refutation of signature. He started taking

fingerprints from each soldier as he found that he could not distinguish genuine claimants from the fake pensioner, which abruptly stopped the swindling [26].

Description of the historical background of fingerprinting will never be complete without mentioning the work of Sir Edward Henry, the successor of William Herschel. He was an inspector general of police in India, who studied the unique features of fingerprints and published a paper in 1899, dealing with fingerprint system, before the British association for the advancement of science. In 1901, Sir Henry was recalled to Scotland Yard where he introduced the famous and popular ‘Henry fingerprint system’, the first official adoption of fingerprint system in England and Wales. He devised a simple and viable classification system which he described in his book “Classification and uses of Fingerprints” in 1900 [15].

2.4. CONSTITUENTS OF THE FINGERPRINT DEPOSITS

When the finger touches a surface, perspiration along with body oils that may have been picked up by touching hairy portions of the body is transferred on to the surface, thereby leaving an impression of finger’s ridge pattern. Prints formed by this method are generally invisible to human eye and hence referred to as latent fingerprints [26]. The fingerprint is a complex mixture of natural secretions and contamination from the environment. Three types of glands are responsible for natural secretions from the body of the skin: the sudoriferous eccrine, apocrine and the sebaceous glands. The sudoriferous eccrine glands are distributed all over the body and produces sweat. These three glands have well defined functions and composition of their secretions as given in Table 2.1[26].

Table 2.1 Constituents of the fingerprint deposits.

SOURCES	CONSTITUENTS	
	Inorganic	Organic
Eccrine Glands	Chlorides Metal Ions (Na^+ , K^+ , Ca^{2+}) Sulfates Phosphates Ammonia Water (>98%)	Amino Acids Urea Uric Acid Lactic Acid Sugars Creatinine Choline
Apocrine Glands	Iron Water	Proteins Carbohydrates Sterols
Sebaceous Glands	--	Fatty Acids Glycerides Hydrocarbons Alcohols

The palms of hands and the soles of the feet produce only eccrine gland secretions whereas the apocrine glands are located in the groin, in the armpits and in the perianal regions. The sebaceous glands are found on the chest, back and on the forehead etc. These glands secrete oil (sebum), which serves to protect the skin and

hair against water, to act as lubricant, and also to help absorb fat (lipid) soluble substances [26]. The ridges of the hands are covered exclusively by eccrine glands and its secretions are present to some degree in every latent fingerprint. Contamination by sebaceous gland secretions is also very common whereas that from apocrine glands is rarer but may play an important role in certain crime investigations.

2.5 CLASSIFICATION OF FINGERPRINT PATTERNS [27]

A fingerprint has a general shape or pattern, which exist in many ridge characteristics. These features allow the classification of fingerprints into distinctive groups based on general similarities. In a way, this classification will facilitate the work of the fingerprint examiner in searching for an unidentified fingerprint from the fingerprint database.

2.5.1 Fixed points in fingerprints

“Cores” and “Deltas” are the names given to easily recognisable areas of a fingerprint, which are used as points of reference in the ridge system and it forms the central part of the fingerprint pattern. Generally during fingerprint matching the ridge details are counted from the central portion called ‘core’. It varies from pattern to pattern. Deltas are usually situated in the bottom half of the print with either on left side or on right side as shown in Figure 2.1.

Figure 2.1 'Delta' points on fingerprints.

2.5.2 Different fingerprint patterns

All fingerprints are divided into three major classes on the basis of their general patterns, namely. 'Loops', 'Whorls' and 'Arches'. It was reported that sixty to sixty five percent of the population has loops, thirty to thirty five percent has whorls and about five percent have arches.

a) Loops

A typical loop pattern, have one or more ridges entering from one side of the print pattern, re-curving and exiting from the same sides as shown in Figure 2.2. There is always at least one delta in all the loop pattern.

Figure 2.2 Loop fingerprint pattern.

b) Whorl

All whorls patterns will have type lines and a minimum of two deltas. They can be divided into four distinctive groups as shown in Figure 2.3.

Plain and central pocket loop whorls are identified by projecting a line between the left and right deltas. If the line crosses at least one recurving ridge in the pattern area, it is a plain whorl. If the line drawn between the left and right deltas does not cross at least one recurving ridge within the pattern area, it is a central pocket loop whorl. Double loop whorl consists of two separate loops each having a delta formation. Accidental whorl contains more than one pattern with the exception of the plain arch. They often have more than the two deltas, which are usually found in whorl patterns.

Figure 2.3 Whorl fingerprint pattern

c) Arches

Arches are the least common of the three general fingerprint patterns. It is classified into two distinct groups: plain arches and tented arches as shown in Figure 2.4. Arches are the simplest of all fingerprint patterns and are often referred to as the “pattern less” pattern as they contain no deltas, cores. In this fingerprint pattern, the ridges enter from one side and exit from the other with a rise in the centre.

Figure 2.4 Arch fingerprint pattern

2.6 FUNDAMENTAL PRINCIPLES OF FINGERPRINT IDENTIFICATION

Existence of fingerprint patterns as a unique feature of personal identification is based on some fundamental principles. They are listed below [27],

- A fingerprint is an individual characteristic: no two people's fingerprint have yet been found to possess identical ridge characteristic.
- A fingerprint pattern will remain unchanged during an individual's lifetime.
- Fingerprints have general ridge pattern and it allows them to be classified systematically.

2.7 CONVENTIONAL FINGERPRINT DETECTION METHODS

In the conventional methods of fingerprint detection, fingerprint experts use different chemicals, depending on the nature of surface on which print is formed. For different surfaces, different chemicals have to be applied to get good contrast between the print and the background [4,5-13]. In this method, fingerprint experts first classify the surface as porous, non-porous, contaminated etc on which the print is formed. Depending on the nature of surface they would then apply different chemicals to develop the latent fingerprints, details of which are given in the coming section.

2.7.1 Chemicals used for conventional fingerprint detection

a) Amido black

Amido Black is a dye that stains protein present in blood and other body fluids to give a blue-black product [5]. This protein stain can be used for enhancing fingerprints in blood in either a methanol or water based formulation to produce the blue-black fingerprints [30]. Amido black is not suitable for non-blood contaminated prints. The blue-black print developed by amido black also exhibits poor contrast when the background is of some dark-coloured surfaces. In addition, high background staining on porous surfaces can also obscure the developed prints. Despite this, Warrick reported good results in developing a bloody print on a cotton fabric with amido black used in conjunction with digital enhancement of the resulting developed print [31].

b) Gentian violet

Gentian violet (crystal violet) is a dye that stains the fatty constituents of sebaceous sweat producing an intense purple image. It is very effective for the development of latent fingerprints on the sticky surfaces of adhesive tapes. The nature of most adhesive tapes and the colour of gentian violet provide adequate contrast for visualisation of developed print. However, fingerprints on black or other dark adhesive surfaces treated with gentian violet will generally be invisible. They may be visualised by either transferring the dye image to a sheet of photographic paper or may be enhanced by fluorescence examination [5].

The gentian violet powder is dissolved to form a working solution. A study by Bramble *et al* revealed that weaker solutions produced a higher fluorescence yield from latent prints, but a similar result could also be obtained with concentrated solutions by subsequently washing the prints with an appropriate solvent [32]. Gentian violet has been recommended as a post superglue fuming treatment to enhance contrast [33]. Other methods of developing fingerprints on sticky surfaces such as the black powder method [34] and the “Sticky-side powder” [35] have arisen over the years, in which the latter has been reported to outlast gentian violet [36]. Similarly, exploiting the inefficiency of gentian violet on black tapes, white or ash grey powder [37] has been reported as likely replacements for gentian violet under such instances.

c) Iodine Fuming

Iodine vapour is physically absorbed by the latent print deposits and reacts with the unsaturated fats to form a brown image. This helps in developing prints of few days

old. The developed prints often fade unless fixed with α -naphthoflavone solution. This gives a dark blue image. Since Iodine vapour is highly toxic, extreme care must be taken in handling this. This chemical is not suitable for metals and dark surfaces [5].

d) DFO (1,8 Diazafluoren-9-one)

DFO is a very effective reagent for paper and some other porous surfaces such as paper, cardboard etc, but not on non-porous surfaces [5,7]. DFO reacts with amino acid to give a red-coloured product, which is highly fluorescent under suitable illumination. DFO dissolved in a mixture of 4 ml methanol and 2 ml acetic acid and then diluted to 100 ml with 1,1,2 trichloro trifluoro ethane. The porous sample is dipped in a freshly prepared DFO solution for around 5 sec, then allowing it to dry for 30-60 sec. Fingerprints were revealed by heating the paper at 100⁰C for 10 min. For this kind of developed print on papers, the optimum excitation wavelength is around 470 nm that will give the fluorescent emission at 570 nm [7]. This reagent is easy to apply and very effective provided that fluorescence is used to examine and photograph the fingerprints. This chemical is unsuitable when print is deposited on highly fluorescing substrates. Also, it cannot develop the fingerprints on substrates that are exposed to water.

e) Ninhydrin

Ninhydrin is a very effective reagent for paper and some other porous surfaces such as raw wood, plasterboard, some wall coverings etc. It reacts with amino acid and possibly some other components in fingerprint and produce a intermediate coloured

complex called Ruheumann's purple, which may vary from orange to purple depending on the donor of print and the development conditions. [5,6,38]. Later it is revealed that if zinc chloride is added along with the Ninhydrin, the complex formed will fluoresce in the dark yellow–orange colour under green excitation and will give good contrast with background [6].

f) Physical developer

It is a silver- based aqueous reagent that reacts with components of sebaceous sweats to form a grey silver deposit. This is mainly used on paper but can also be used on other porous materials like cardboard and raw wood. Since the reagent is sensitive to components in the latent fingerprint, which are not detected by ninhydrin and DFO, it may be used after the latter has been applied to produce extra numbers of useful fingerprints. It can develop fingerprints on paper, which are wet. The whole procedure of application of chemical and developing the print may take 45 minutes to 1 hour. However, physical developer is destructive and no other fingerprint development technique is effective after its application. Hence, it is recommended that it should be systematically applied at the end of any detection sequence on paper [5].

g) Regular Powders

There are different types of fingerprint developing powders that are available. The effectiveness of using powder is variable depending on the chemical and physical nature of the powder and care and expertise of the operator [5]. With fresh fingerprints, the aqueous component of the print contributes significantly to the adhesion of the powder. Powder can be used on any surface that is relatively smooth

and clean. The effectiveness with which the powder adheres to the ridges depends on the size and shape of the particles that compose the formulation. Small, fine particles adhere more easily than large, coarse ones [11]. This is not suitable for wet, very rough or dirty surface.

Powder dusting is effective on non-porous surfaces. There are different colours of regular powders available such as black, white and grey. In addition, they are either magnetic or non-magnetic in nature. The colour of powder chosen depends on the colour of the surface to which they will be applied. The application of non-magnetic powder to latent prints by brushing yields instantly apparent prints. The fingerprint powders that are commonly used include black, grey, silver, white powders and fluorescent powders such as red-wop, blitz red, green-wop, blitz green etc. Application of powder to fingerprint by brushing is a well-established technique but it has one disadvantage: contact of brush with the fingerprint has inevitable destructive effect so that high degree of caution is required while using this. Care should be taken to prevent the smudging of the imprint [11,39].

h) Radioactive sulphur dioxide

Sulphur dioxide gas (SO_2) dissolves in water present in latent fingerprints and there may be also some reactions with the fatty constituents. After an exhibit has been exposed to radioactive SO_2 , the presence of fingerprints can be detected and recorded by autoradiography. It can be used on surfaces like papers, adhesive surfaces but it is not so effective as Ninhydrin or Gentian Violet. This can be also

used to detect prints on some clean fabric but the result is not so good [5]. This method is not at all suitable for articles that have been wetted.

i) Small particle reagent

Small particle reagent (SPR) can be used successfully to develop latent fingerprints on wide range of surfaces [5,40]. It is especially advantageous on surface that are wet or covered with dust, such as the exterior of a vehicle. It is a suspension of fine molybdenum disulfide particles in detergent solution. It adheres to the fatty constituents of the latent fingerprints to form a grey deposit and is quick and simple process. This can also be used in non-porous surfaces like polythene bags.

j) Sudan black

Sudan Black is a dye, which stains fatty components of sebaceous sweat to produce a blue-black image. The formulation contains solid particles of dye as well as dye in solution. This is less sensitive compared to some other chemicals, but it is very effective when the surface is contaminated with oil or grease. Generally when a surface is found to be contaminated with oil or grease, fingerprint experts directly apply the Sudan black to enhance the print. Relatively insensitive to contaminated fingerprints and is very messy process to carry out [5].

k) Superglue

This method of latent fingerprint treatment involves the use of glues containing cyanoacrylate ester. The articles to be examined are placed into a closed container together with a few drops of the glue, which is subsequently vaporised. The

superglue vapour, ethyl-cyanoacrylate or methyl-cyanoacrylate, reacts with certain eccrine and sebaceous components in a latent fingerprint [5,8]. The vapour selectively polymerises on the fingerprint ridges to form white polymer via which the prints become visible. In a way, the formed polymer stabilises and protects the prints. The process can be used on a range of non-porous surfaces such as metals, glass and plastics.

The effectiveness of this process depends largely on processing conditions. Atmospheric pressure, normal room temperature and humidity of 80% are the optimal conditions for fingerprint development. Heating the glue to a temperature of 120°C is recommended for speeding up the evaporation. Due to the longer processing time associated with this vacuum technique, ways have been looked into to accelerate the treatment of prints within a vacuum, such as the introduction of heat inside a vacuum [41]. The high humidity is helpful in enhancing the polymerisation. This method is used for non-porous surfaces. It is also useful in developing the prints formed on vinyl and rubbers [5].

Glue-treated fingerprints provide a poor contrast with light coloured backgrounds. Under such instances, they can be effectively combined with dusting using fluorescent powders or staining using fluorescent dyes to distinguish the prints from the background [42]. Contrast enhancement of cyanoacrylate-treated latent fingerprints using biological stains and commercial fabric dyes [43] and by sublimating dyes [8] have also been reported.

1) Vacuum metal deposition

Vacuum metal deposition (VMD) utilises vacuum coating technology for the thermal evaporation of metals and deposition of thin metal films. A very thin, discontinuous, invisible coating of gold is deposited onto the surface of particles being examined and is followed by deposition of sufficient zinc metal film. Fingerprint appears as positive or negative images on a grey film of metallic zinc [5]. VMD is an established technique for the development of prints on non-porous surfaces [44]. It has advantages over cyanoacrylate fuming, especially in circumstances where prints are old, have been exposed to adverse environmental conditions or when prints are present on semi porous surfaces. This method is comparatively very expensive in comparison with other chemical treatment.

2.7.2 Limitations of chemicals based conventional fingerprint detection methods

The main limitation of fingerprint development by chemicals is that they cannot detect older fingerprints. The chemicals fail to develop the fingerprint ridge details when the print gets older. In order to get good results by chemical treatment, the substrate should have enough deposited fingerprint residues. However, when the prints gets older the residue reduces and that leads to the failure in developing such prints. In normal cases, even if the age of a print is over 2 months, it is very difficult to develop the print to extract all the ridge characteristics embedded in it.

Conventional methods using chemicals cannot be taken as a general method for all kinds of surfaces. One has to go through a series of systematic procedures in each

case and one particular chemical is only suitable for one type of substrate surfaces. Different surfaces have to be treated with different chemicals in systematic way to enhance the fingerprint visibility. If the contrast between the print and the background is poor upon first treatment, subsequent treatment with other chemicals will be futile. This is because of the fact that the targeted constituent of the print might have been already developed with first treatment. Hence, one can conclude that these methods are of ‘destructive’ type since no further treatment will be valid after the application of first one.

The work carried out at Scene of Crime Unit (SCU), Singapore Police force led to the familiarisation of most of the conventional methods of detecting latent fingerprints that mentioned above. The training and hands on experience at SCU provided a good platform to analyse and understand the existing conventional methods on fingerprint detection and their limitations. The details of these hands on training are presented in the appendix A.

2.8 SUMMARY

An overview of the basics of fingerprinting was given by discussing various fundamentals and different ridge patterns. Subsequently, limitations of the existing conventional methods for latent fingerprint detection were presented. These limitations demands for a generic detection scheme for latent fingerprint detection with better sensitivity.

CHAPTER THREE

FLUORESCENCE TECHNIQUES FOR FINGERPRINT DETECTION AND BIOIMAGING

3.1 INTRODUCTION

This chapter initially gives an overview of the basics of fluorescence phenomena and frequency domain fluorescence spectroscopy. Later, this chapter is divided into two sections, A and B. Section A will be describing the fluorescence based fingerprint detection techniques and their limitations. Section B gives an overview of the fluorescence-based detection and imaging techniques for various biomedical applications. It will also discuss on the limitations of current frequency domain spectroscopic technique for various biomedical applications.

3.2 FLUORESCENCE PHENOMENA

Fluorescence is the tool of choice for many years for investigating biophysical and biochemical applications [1,45,46-47]. An advantage of fluorescence spectroscopy (fluorometry) for bio-chemical applications is its high sensitivity, which is clearly superior to the sensitivity of conventional calorimetric techniques. Methods based on fluorescence are often one to two orders of magnitude (i.e. 10 to 100 times) more sensitive than calorimetric techniques [1].

When molecule absorbs energy, it will be in an excited energy state. Luminescence is the emission of electromagnetic radiation from electronically excited states. It has

two major sub divisions, i.e. fluorescence and phosphorescence [1]. This classification is based on the nature of ground and excited states involving in transitions. In singlet-excited state, the electron in the energetically excited state has opposite spin to that of the one at ground state. These two electrons are referred as in 'paired state'. In triplet state, these electrons are unpaired i.e. they have same spin orientation to each other. When an electron undergoes transition from electronically excited singlet state to an electronically relaxed ground state, no spin reversal occurs. It is in accordance with the quantum mechanics theory of electronic wave functions. But this spin reversal is needed in the case of triplet transitions.

In general, fluorescence is the emission of high wavelength photons due to the transition of singlet electron to lower energy level [1,45]. Such transitions are quantum mechanically allowed and frequency of such transitions is very high i.e. 10^8 sec^{-1} . This high emissive rates results in fluorescence lifetimes near 10^{-8} sec or 10 nanosec. Fluorescence lifetime is the average time a fluorophore will remain in excited state.

Phosphorescence is the emission of photons from excited molecules, in which transition between different states of multiplicity i.e. generally a triplet excited state returning to singlet ground state occurs. Such transitions are generally quantum mechanically not allowed; hence its frequency is much lesser compared to that of fluorescence. In this case the frequency of such transitions low i.e. 10^3 to 10^0 sec^{-1} . Hence, corresponding lifetime will be in milli to seconds range.

The Jablonski energy level diagram is shown in Figure 3.1[1], which illustrates the absorption and emission of light that occur in fluorescence and in phosphorescence.

Figure 3.1 Jablonski diagram for fluorescence [1].

The singlet ground, first, and second excited states are shown as S_0 , S_1 , and S_2 respectively. An electronic energy level is comprised of several vibrational energy sublevels and each vibrational energy level is comprised of rotational energy sublevels. Each of the above mentioned electronic energy states can exist in vibrational energy levels as depicted by 0, 1, 2, 3, etc. In this diagram solvent effects are not considered.

After the light absorption several processes usually occur as shown in Table 3.1. A fluorophore is usually excited to some higher vibrational levels of S_1 or S_2 . In few exceptions, molecules in condensed phase relax down rapidly to the lowest vibrational level of S_1 . This process is called internal conversion and generally

occurs in 10^{-12} sec. Since fluorescence lifetimes are typically near 10^{-8} sec, internal conversion is complete prior to emission [1].

Table 3.1 Physical processes undergone by excited molecules.

Physical Processes Undergone by Excited Molecules	
$S_0 + h\nu \longrightarrow S_1^V$	Excitation
$S_1^V \rightsquigarrow S_1 + \text{heat}$	Vibrational Relaxation
$S_1 \longrightarrow S_0 + h\nu$	Fluorescence
$S_1 \rightsquigarrow S_0 + \text{heat}$	Internal Conversion
$S_1 \rightsquigarrow T_1^V$	Intersystem Crossing
$T_1^V \rightsquigarrow T_1 + \text{heat}$	Vibrational Relaxation
$T_1 \longrightarrow S_0 + h\nu$	Phosphorescence
$T_1 \rightsquigarrow S_0 + \text{heat}$	Intersystem Crossing
$S_1 + A_{(S_0)} \longrightarrow S_0 + A_{(S_1)}$	Singlet-Singlet transfer (photosensitization)
$T_1 + A_{(S_0)} \longrightarrow S_0 + A_{(T_1)}$	Triplet-Triplet transfer (photosensitization)

V indicates vibrationally excited state
excited states higher than S_1 or T_1 are omitted

The efficiency of the photoluminescence emission process, or the quantum yield is at its highest when the incident light (excitation) matches with the absorption maximum of the fluorophore. The required wavelength for the incident light may be obtained by filtering out a broad band or by using some monochromatic source of light like laser at that wavelength. It is important that the incident light is chosen to correspond to the maximum absorption wavelength of the compound under investigation [1].

3.2.1 Fluorescence measuring techniques

Fluorescence measurements can be broadly classified into two types, steady state measurements and dynamic measurements. Steady state measurement is the most common type of measurement, where it is carried out by constant illumination and observation [1]. In this measurement scheme, sample is illuminated with a continuous beam of light with suitable power and intensity of the emission signal is recorded.

Dynamic measurements have lots of advantages over steady state measurements. Generally, the major part of the molecular information available from fluorescence is lost during steady state time-averaging process. If a molecule has two decay times, the steady state measurements can give only a weighted average of the two decays while the dynamic measurement can exactly give the two different lifetimes [1].

Dynamic measurement of a system is the general and sensitive way of characterising the fluorescent emission from the sample. This measurement technique has high sensitivity and broader applicability. Dynamic fluorescent measurements could be carried out in two ways. They are, time domain (time-resolved) and frequency domain (phase-resolved) techniques [1].

Time-resolved imaging technique takes the advantage of the large difference in lifetime of the specific luminescence and the non-specific background fluorescence. In this detection scheme pulsed excitation is used in combination with gated detection of the emission. Most of the non-specific background signals are caused by

naturally occurring fluorescence and therefore show lifetime in the order of 1-100ns [4,48]. The use of dyes with long lifetime luminescence (microseconds to milliseconds) along with the sample enables the separation of luminescence in the time domain. When applying time domain fluorescence microscopy, the lifetime of the dye is generally selected in such a way that the specific luminescence can be detected in the absence of unwanted background signals.

3.2.2 Basics of frequency domain spectroscopy

Frequency-domain or phase-resolved method allows the resolution of both the emission spectra and decay times for multi-exponential decays [1,49-51]. In this method, the sample is excited with intensity-modulated light; typically sine wave modulation is employed as shown in Figure 3.2 [1]. The intensity of incident wave is varied at a high angular frequency ($\omega = 2\pi f$, where f is the linear modulation frequency in Hertz) comparable with reciprocal of lifetime, τ of the sample.

Figure 3.2 Phase shifted fluorescence emission due to modulated excitation [1]

When a sample is excited in this manner, the emission will be also sinusoidal, but will be shifted by phase angle, ϕ . The modulation depth of the light source is defined, as $\frac{b}{a}$ similarly $\frac{B}{A}$ is the modulation depth of the emission, which follows the excitation [1].

The modulation factor, m , phase shift ϕ and angular modulation frequency ω are related through following equations as [1,52-55],

$$m = \frac{\left(\frac{B}{A}\right)}{\left(\frac{b}{a}\right)} = \left(1 + \omega^2 \tau^2\right)^{-\frac{1}{2}},$$

and

$$\tan \phi = \omega \tau.$$

Section A

3.3 FLUORESCENCE BASED FINGERPRINT DETECTION METHODS

Fluorescence based detection approach opened a new door in the area of latent fingerprints imaging. It proved to be a universal approach in most of the cases. This technique is based on the reaction of fingerprint residue with a specific chemical agent and will result in the fluorescence emission upon excitation with proper light source. Imaging of fingerprints by the subsequent suppression of background fluorescence can be carried out in any of the following methods.

3.3.1 Fluorescence examination and optical filtering

In some cases latent fingerprint will be visible only when it viewed through certain optical filters or goggles, after irradiation with powerful light sources [56-58]. This is due to inherent fluorescing nature of some contents of fingerprints and such prints can be viewed or photographed using proper cameras [4,59]. Generally high intensity excitation source is required to produce a luminescence visible to naked eye, as fluorescence intensity is proportional to excitation intensity.

Thus high power lasers became the excitation sources of choice for maximum sensitivity. Fingerprint residue absorbs primarily in the deep ultraviolet due to the presence of different organic constituents. [4,57]. There are fluorescent components in fingerprint residue that absorb in the blue-green and emit in the yellow-green. Hence inherent fluorescence emission from fingerprint sample can be obtained when it is excited using powerful lasers such as Argon-ion laser (having both UV and visible excitation option) [4,59]. Filtered lamps were also used but because their useful powers were only few hundred milliwatts, they sacrificed sensitivity. High sensitive camera or detection device should be used to detect the weak fluorescence emission.

Fluorescent powders are similar to regular powders except that the former fluoresce or phosphoresce upon exposure to ultraviolet light or visible light from laser and other light sources. They are useful for the visualisation of latent prints deposited on multi-coloured surfaces that would present a contrast problem if treated with regular

powders. The eventual selection of the most satisfactory powder will largely depend on the background colours and their luminescent properties.

Fluorescence examination followed by photography using proper filters is a useful method of visualising the print when the sample and background emit in different wavelengths. Once the best conditions are achieved for the illumination and viewing it can be photographed. The camera filtration is usually the same as that used for viewing when panchromatic films are used. In this method extreme care must be taken to avoid the photodecomposition of prints under the high intense light illumination [57-58]. The conventional method of fluorescence detection totally fails when the wavelengths of these two emissions are very close to each other and effective optical filtering is not possible [4].

3.3.2 Fingerprint detection using semiconductor nanocrystals

Photoluminescent semiconductor nanocrystals are also referred to as nanocrystallites, or quantum dots are formed from compounds such as cadmium sulphide (CdS). The salient virtues that distinguish the nanoparticles from other fluorescent labels are that their absorption and luminescence wavelengths can be tuned by varying the particle size, high photoluminescence efficiency, and encapsulation with flexibility of functionalisation with conjugating ligands for selective labeling of fingerprints [60]. Although this is a relatively new fingerprint treatment (first proposed by Menzel in 1999 [4]), it can be expected to be used for fingerprint detection in various ways namely incorporation into powder, by staining or chemical bonding to constituents of fingerprints [61-62].

One notable property of the nanocrystals is that its emission lifetime is in the range of hundreds of nanoseconds [63]. This makes it suitable as a reagent in time-resolved detection of latent fingerprint that is most applicable when the luminescence lifetime of the latent fingerprint falls roughly in the 10-1000 ns ranges. Lifetimes shorter than about 10 ns are too close to those of background fluorescence (roughly in the 1-3 ns range [63]) to be readily resolved.

3.3.3 Time-resolved imaging

As mentioned above, fingerprint experts often encounter prints deposited on fluorescing substrates and their separation becomes difficult with conventional methods of detection and enhancement. Menzel et al developed time-resolved (TR) technique with milli second resolution to overcome most of these limitations.

The objective of TR imaging is to image the luminescence profile of the latent fingerprint after successful suppression of fluorescence from the background, upon treating the prints with chemicals to prolong its lifetime from the order of nanoseconds to milliseconds. It is necessary for the fluorescence lifetime of the latent fingerprint to be significantly longer than that of the background in order for the time-resolved imaging to work [4, 14, 64-65]

TR imaging technique is based on the large difference in lifetime of emission from fingerprint and that of background. Large difference in lifetime is achieved by applying lanthanides (rare earth based chemicals) to fingerprints. Lanthanides are rare earth elements that include europium, terbium, etc. They generally come as +3

ions and chlorides, nitrates, acetates, etc of the lanthanides are readily available. The relatively new lanthanide-based fingerprint detection method has been used in both physical [66] and chemical [67] processes of fingerprint enhancement. One noteworthy characteristic of the lanthanide is its millisecond fluorescence lifetime. As the background fluorescence are generally short-lived (roughly in the 1-3.ns ranges [63], one can take advantage of this difference in lifetimes to suppress the background fluorescence.

Upon application of rare earth based chemicals to fingerprints, the fluorescence lifetime is prolonged to milli second range. If the fluorescence from the treated fingerprint is relatively long-lived, a well-chosen time delay between the actual excitation of the entire background and the observation of the continued fluorescence of the fingerprint will discriminate against an interfering fluorescent background. The delay must be sufficiently long to allow the background signal to decay, but short enough to allow for the recording only the fingerprint signal. Hence, this technique requires that the luminescence lifetime of the fingerprint to be longer than that of the background. Typically, the luminescence lifetime of background lies in the nanosecond order and it is desirable for the luminescence lifetime of fingerprint to be in the millisecond order, which the lanthanides are seen as potential candidates to achieve such conditions for the TR imaging.

Certain lanthanides, such as Eu^{3+} and Tb^{3+} emit (red and green, respectively) with lifetimes of millisecond order. They are thus in principle well suited for time-resolved imaging. Most of the studies have focused on Eu^{3+} , which emits in the red

under ultraviolet excitation [4,14,65]. This large stokes shift between excitation and emission has the virtue that in some instances background fluorescence can be suppressed by optical filtering alone provided background fluorescence is widely separated in wavelength scale with respect to this emission.

The introduction of image intensifiers opened a new path in the TR imaging techniques of fingerprints. The TR imaging prototype is as shown in Figure 3.3 and it utilises staining with dyes of lifetime in the range of 5×10^{-7} to 10^{-3} sec. The Continuous Wave (CW) laser beam is chopped by an electro optic modulator that operates at a rate of near megahertz [4]. The time resolved image appears on the phosphor screen of the image intensifier, and is visually inspected and then photographed. The system can overcome the limitation on the size of the article that could be examined. But it suffers the difficulty that the phosphor screens of microchannel plate (MCP) image intensifiers are small. Similarly visual examination and photography can be difficult to handle [4].

Figure 3.3 Time-resolved imaging prototype and image intensifier gating scheme [4]

The introduction of CCD cameras equipped with image intensifiers having gating option allowed the design of time resolved imaging system in a more fruitful way as depicted in Figure 3.4. The light chopper operates at about 500 Hz. The camera's MCP intensifier is synchronised with the light chopping, with computer controlled adjustable gate width, and gate delay. As shown in Figure 3.5, the gate delay is adjusted such that the gate width is only opened to slow decaying fingerprint fluorescence, which is in the order of milliseconds or microseconds.

When the excitation light source is suddenly turned off, the photoluminescence profile decays in an exponential way. This lifetime will be in milliseconds and that of background will be in nanoseconds [4]. After acquiring this condition in this time-resolved imaging, it is possible to turn on the imaging system after light is cut-off, with a delay such that the background fluorescence has already decayed whereas substantial fingerprint luminescence still remains. This procedure could be done

repetitively as shown in Figure 3.5. This type of scheme is referred to as gated imaging. If CCD camera is computer interfaced, then the image can be directly observed on the monitor in large size [4,65].

Figure 3.4 Time-resolved imaging system for latent fingerprint imaging-Block diagram [4].

Figure 3.5 Schematic diagram showing the principle of Time-Resolved imaging

3.3.4 Limitations of fluorescence based fingerprint detection techniques

In many cases, the prints developed on coloured surfaces have poor contrast and the ridge details may not be clear. Generally, fluorescence examinations of such prints are not possible as most of the surfaces itself fluoresce in the same wavelength

region of the treated fingerprints. The use of optical filters to suppress the unwanted background luminescence will not be effective when background luminescence is similar in colour to the fingerprint luminescence. On non-porous surfaces, after fixing the print with fuming, fluorescent examination fails when the background fluorescence intensity is high with respect to fingerprint fluorescence.

Photo luminescent semiconductor nanocrystals (quantum dots) used in fingerprint development by making use of the luminescence property has its own limitations. The nanocrystals for the enhancement of fingerprint luminescence cannot ultimately help in detecting the older prints of few months old when the emission is usually weak

As mentioned in earlier in section, Menzel et al introduced the time resolved imaging technique to overcome the limitations of optical filtering [4]. But it has its own limitations as well. The main limitations of TR imaging scheme is that it demands the need of fluorescence decay time of fingerprint sample should be longer than that of unwanted background emission. To achieve this requirement, the fingerprint sample is treated with some chemicals or dyes in order to bring the decay time the fingerprint fluorescence to millisecond range where background fluorescence still in nanosecond time range. For this purpose, rare earth based complex chemicals is usually employed [4,14,64].

TR imaging of fingerprints was carried out with nanosecond resolution as a part of this research work by making use of fluorescent powders that are easier to apply and use. The much improved nanosecond resolution in TR imaging is achieved through

sensitive signal processing and imaging scheme. The experimental results of this work are presented in appendix B.

Section B

3.4 FREQUENCY DOMAIN TECHNIQUE FOR BIOCHEMICAL AND BIOMEDICAL APPLICATIONS

Frequency domain spectroscopy has better sensitivity and hence offers an ideal platform in the detection of fluorescence from different cells and other biochemical samples for various diagnostic and characterising studies [2-3,68-71]. Frequency domain spectroscopy is applied successfully for the characterization of concentrated colloidal suspensions [72]. Later, this technique was further applied for the recovery of particle size distribution and volume fraction in opaque, multiple scattering suspensions of polystyrene and titanium dioxide samples [73-74].

Frequency domain spectroscopy study for scattering media involves monitoring the time-dependent propagation characteristics of multiply scattered light in random media [68]. Briefly, this technique depends on launching intensity modulated light into a multiply scattering medium at a single point source and detecting, at other discrete points some distance away from the incident light, the amplitude attenuation and the phase shift relative to the incident light. Upon modulating the incident light at various modulation frequencies, the measurements of phase-shift and amplitude attenuation at a point detector can be determined as a function of modulation frequency and the source-to-detector distance [68].

Frequency domain technique was successfully applied for the near infrared (NIR) fluorescence contrast enhanced imaging of cancer and other tissue abnormalities for clinical diagnostic purposes [2]. With frequency-domain measurement, the sensitivity and depth penetration of various fluorescence enhanced imaging studies can be surveyed with modulated excitation light at MHz range and fluorescence light is captured and processed by an intensity modulated charge coupled device camera (ICCD) system [2].

J. Lee et al developed ICCD based imaging system to perform the 3D fluorescence tomographic imaging in frequency domain using NIR fluorescent contrast enhancement agents [3]. This work was successful in achieving 3D fluorescence enhanced optical tomographic reconstructions from the experimental measurements of the time dependent light propagation on a relevant breast shaped tissue phantom.

Fluorescence spectral fingerprinting (identification) with total luminescence and synchronous excitation spectroscopy has been well described in recent years [54]. Phase-resolved spectroscopy was used for the spectral fingerprinting of crude oil samples in cyclohexane in aqueous solutions of sodium taurocholate micelles [75]. This was carried out by taking the phase resolved spectra at different modulation frequencies and compared later with steady state spectra to illustrate the effects of fluorescence lifetime selectivity. Similar studies have been carried out for various environmental samples such as fossil fuels [75]. Use of phase-resolved fluorescence spectroscopy at one or more modulation frequencies for multicomponent analysis,

using experimental conditions that were optimized for the particular components in the system, was reported [76-80].

Optical instrumentation using frequency domain spectroscopy for fluorescence lifetime measurements that make use of light emitting diodes was reported, [81-82], which explains the use of amplitude modulated near-ultraviolet light using an inexpensive, commercially available blue light-emitting diode. Similar Frequency domain setup was successfully developed using deuterium light source [83]. Unlike other modulated broadband light sources the deuterium lamp does not employ an external optical modulator. Therefore it does not require critical alignment and collimation and the light can be efficiently collected with a simple lens. These experimental setups find potential applications for nanosecond phase modulation and hence fluorescence lifetime measurements.

Zhiyi Zhang et al developed signal-processing scheme for the detection of fluorescence Lifetime using phase locked fluorescence detection [84]. Here, the measured lifetime is converted to a repetitive signal whose period is directly proportional to it, and thus a high measurement resolution can be achieved. But, this signal processing algorithm and procedure was not simple to implement and operate.

Venkatesh et al was successful in developing a frequency domain fluorometry set up by replacing the commercially available lock-in analyzer with an inexpensive phase detection circuit, which makes use of a simple voltage, controlled phase shifter and the PMT [85]. It also makes use of PIN photodiode for data collection and analysis.

This system makes use of a closed loop, phase sensitive lifetime detection scheme that can be used as an oxygen sensor.

Krishnan et al developed a multiphoton fluorescence lifetime imaging system using a streak camera [86]. This system allows rapid data acquisition and reliable and reproducible lifetime determinations. They also demonstrate the applicability of the system to Fluorescence lifetime imaging studies in cellular specimens including stained pollen grains and fibroblast cells expressing green florescent protein. This system using mode locked titanium sapphire laser seems to be bit complicated.

3.5 FLUORESCENCE DETECTION OF RAPID CYCLE DNA AMPLIFICATION

Rapid cycle DNA amplification and its monitoring using fluorescence technique is an interesting research topic. Several works has been reported on the detection of fluorescence intensity from DNA samples marked with specific fluorescent dyes [22-24, 87-94]. Rapid cycle DNA amplification can be monitored by three different fluorescence techniques. This was carried out by (i) the double strand specific SYBR dye, (ii) a decrease in fluorescein quenching by rhodamine after exo nuclease cleavage of a dual-labeled hydrolysis probe and (iii) resonance energy transfer of fluorescein to CY 5 dye by adjacent hybridization probes.

In DNA detection, different probes are labeled with different dyes that have unique emission spectra. Spectral data is collected with discrete optics or dispersed onto an array for detection [91]. Spectral overlapping between dyes is corrected by using

pure dye spectra to deconvolute the experimental data by matrix algebra. Single-labeled probes are easier to synthesise and purify than more complex probes with two or more dyes [91].

Systems based on the Real Time Polymerase Chain Reaction (RT-PCR) technique can detect two-fold amplification of DNA template while traditional PCR can only detect signal after 10-fold amplification [92-94]. This proves the sensitivity of RT-PCR based detection system. This RT-PCR machine has three discrete channels with photodiodes for detecting multiple fluorescence emitted from the PCR mix [92]. Narrow band pass filters and dichroic mirrors are used to separate different fluorescence wavelengths and to allow emission with the required wavelength to reach the corresponding channel. Since the emitting dye fluorescence has to pass through complicated optical path before it reaches the appropriate channel, the intensity is decayed to 40–50% of the fluorescence intensity detected by the previous channels. Therefore, it is impossible to have five different dye detection capabilities in one sample of PCR mix due to optical loss [95].

These limitations were overcome to an extent by using a grating based spectrometer system. D.S Lee et al was successful in developing a spectrometer-based system for the fluorescence sensing of DNA sample in microlitre volume taken in glass capillary [95]. It uses a Xenon light source and CCD linear array detector. DNA samples labeled with SYBR green 1 and the microlitre volume sample was taken in capillary of inner diameter 0.8mm and out diameter of 1.15mm. This will result in comparatively large volume of the test sample.

N. A. Friedman et al developed a thermal cycling method compatible with the sample taken in capillary tube. The desire to allow individual sample thermal profiles and real-time optical monitoring led to their experiments of DNA amplification study taken in glass capillary [96]. Such studies will reveal information about the surface effect of capillary in PCR experiments.

Generally in PCR study of DNA, it will be marked with some specific dye like SYBR along with the enzyme (polymerase). The enzyme used is very expensive and minimising its volume is always favorable. But, if its quantity is below an optimum amount will result in insufficient fluorescence intensity from the sample. So this will result in a tricky situation of optimising the cost and fluorescence intensity.

As mentioned above, only few literatures are addressing the problem of detecting microlitre DNA sample taken in capillary tube for PCR study. Moreover, when detecting the multiple DNA sample taken in capillary or biochip under same excitation wavelength will result in multiple emission from the sample. In order to collect each fluorescence emission, separate photodiode [92] based or PMT based detectors are necessary. In such scenario, the collection of multiple fluorescence emission is carried out by separating it through narrow band pass filters and dichroic mirrors before reaching the respective detectors. This demands for multiple detectors and may arise a question of detection sensitivity. It is obvious that the weak fluorescence emission from the sample after passing through such wavelength separating optics, will lead to insufficient intensity available to the detector.

In the case of fluorescence-based detection of microlitre DNA, samples will be excited with light from LED or laser. This will result in the presence of strong back-scattered light as well as unwanted background fluorescence from the sample environment. These emissions will limit the sensitivity of the detection when handling weak emission from microlitre volume of DNA sample. Hence, a technique that is capable of detecting even the fluorescence emissions from the test sample is always favorable. This will lead to a cost effective sample preparation by reducing its volume.

3.6 LIMITATIONS OF CURRENT PHASE-RESOLVED TECHNIQUE

As shown in previous sections, all the systems based on fluorescence detection, especially the frequency domain spectroscopy is targeting to detect the strong fluorescence emission from the sample. Till now, no work has been reported on the sensitivity of phase resolved technique when dealing with weak fluorescence emissions. Also, the ability of PR technique to separate fluorescence emissions, which are close in wavelength scale but are separated in fluorescence lifetime scale, is yet to be explored. Most of the literatures were addressing the development of frequency domain system for lifetime measurements. Frequency domain imaging systems for various biomedical and other sensing applications are yet to be explored.

Data analysis and signal processing of fluorescence signal, which are modulated at several tens of megahertz, is always a challenging problem in PR spectroscopy. Incorporation of homodyne and heterodyne technique of signal processing along with frequency domain fluorescence signal for the suppression of unwanted

fluorescence from the test sample is yet to be explored. It is often necessary to remove the DC offsets in the fluorescence emission. This will lead to a better sensitivity for frequency domain system, especially when dealing with very weak fluorescence emissions.

3.7 SUMMARY

An overview of the basics of fluorescence phenomena and frequency domain fluorescence spectroscopy was initially presented in this chapter. Later, limitations of the existing conventional and optical methods of latent fingerprint detection were presented. These limitations demands for a better and more generic detection scheme for fluorescence based latent fingerprint imaging system. In the later part of this chapter, a review of the fluorescence-based detection techniques and background for specific biomedical applications was presented by highlighting their limitations. Current state of the art in PR spectroscopy was explained by giving a direction for its improvement in latent fingerprint detection and bio imaging.

CHAPTER FOUR

THEORETICAL FORMULATION OF PHASE-RESOLVED OPTICAL TECHNIQUE

4.1 INTRODUCTION

The literature survey suggested the need for an improved fluorescence detection technique for latent fingerprint and bio imaging. In this context, a more sensitive phase-resolved optical technique for latent fingerprint detection and bio imaging is proposed. Theoretical formulation including the relevant digital and optical signal processing concepts for the phase resolved imaging is discussed in this chapter.

PR imaging theory described in this chapter addresses detection of fluorescent sample deposited on fluorescing and non-fluorescing backgrounds. The fluorescent sample can be latent fingerprint or test biosample, which are used in this study. This technique can be used for the selective imaging of a particular fluorescence emission by suppressing the other, which may lies in close wavelength and lifetime range.

The main objective of this theoretical formulation is to incorporate the homodyne and heterodyne concept of signal processing along with phase sensitive detection to ensure high signal to noise ratio when detecting weak fluorescence emissions, which are modulated at tens of mega Hertz. It also introduces novel concepts to remove the DC terms in fluorescence emission, which are free of phase information, to result in better sensitivity.

Initially, the complete theoretical formulations for the imaging of fluorescent sample, which is deposited on fluorescing substrates, using homodyne and heterodyne assisted phase resolved technique is derived and presented. In the later part, theoretical formulation for imaging of fluorescent sample deposited on non-fluorescing substrates was derived, which is a special case of the earlier one. A theoretical simulation study was carried out to show the effect of modulation frequency, which is dependent on the fluorescence lifetimes, to obtain a PR image with better contrast. Sensitivity improvement for the PR imaging by combining even-step-shift and pi-shift methods is presented in subsequent section. Comparison between the homodyne and heterodyne assisted PR technique is also presented.

4.2 IMAGING OF FLUORESCENT SAMPLE DEPOSITED ON FLUORESCING BACKGROUNDS

Consider the case of a fluorescent sample deposited on fluorescing background. In order to detect and image the fluorescent sample, suppression of background fluorescence is necessary. PR technique allows the resolution of both the emission spectra and decay times for single and multi-exponential decays. This method has high sensitivity and signal-to-noise ratio. In this method, the sample is excited with an intensity-modulated (sine wave) light. The modulated laser beam is allowed to fall on the sample surface. The resulting fluorescence emissions from the sample and the background will be phase shifted with respect to the excitation [1].

Let the fluorescent sample, which is deposited on a fluorescing background; be excited with a sinusoidally modulated laser beam at frequency ω [54],

$$I(t) = A[1 + m_{ex} \sin(\omega t)] \quad (4.1)$$

where, $I(t)$ is the total excitation intensity at time t and ω is the angular frequency that is equal to $2\pi f$, where f is the linear frequency. A is the DC component of excitation light and m_{ex} is the modulation depth. Modulation depth is given by the ratio of the AC modulated component to the steady state intensity of DC component.

The modulated laser intensity given by equation (4.1) can be represented as in Figure 4.1. As a result of the modulated excitation, the fluorescent sample and the background start to fluoresce. The resulting fluorescence signals are also modulated at the same frequency as that of the modulated laser beam, but are demodulated and phase shifted, ϕ , to an extent determined by their fluorescence lifetimes.

Figure 4.1 Graphical representation of modulated excitation and homodyne detector modulation.

The relation between the phase shifts and demodulation factors for single exponential decay is reported [1, 97-98]. In a similar way, for the fluorescent sample and the background fluorescence emissions, they can be given by the following two equations,

$$\begin{cases} \tan \phi_s = \omega \tau_s \\ m_s = [1 + (\omega \tau_s)^2]^{-1/2} \end{cases} \quad (4.2)$$

$$\begin{cases} \tan \phi_b = \omega \tau_b \\ m_b = [1 + (\omega \tau_b)^2]^{-1/2} \end{cases} \quad (4.3)$$

where, τ_s , τ_b are the lifetime values and ϕ_s , ϕ_b are the phase shift values for the fluorescence emission from the sample and that of background respectively. Similarly, m_s and m_b are the demodulation factor for a fluorescent sample and background fluorescence.

Hence, the fluorescence intensity at any position r of the sample can be expressed as,

$$F(r, t) = \begin{cases} A'(r_s)[1 + m_{ex}m_s \sin(\omega t - \phi_s)], & \text{when } r = r_s \\ A'(r_b)[1 + m_{ex}m_b \sin(\omega t - \phi_b)], & \text{when } r = r_b \end{cases} \quad (4.4)$$

where r_s and r_b represents the position of the fluorescent sample and background fluorescence respectively. The DC parts of emissions, $A'(r_s)$ and $A'(r_b)$ depends on parameters such as laser power, sample absorption, fluorescence quantum efficiency, etc [97]. The modulated fluorescence emissions from the fluorescent sample and background are denoted as shown in Figure 4.2.

Figure 4.2 Graphical representation of modulated fluorescence emission from the sample.

From the above equations, it is clear that the fluorescence emission from a fluorescent sample and background will be at same modulation frequency but have different phase shifts and demodulation factors, which is governed by their lifetime values. Hence, this offers a platform for phase-resolved detection of fluorescent sample to separate it from unwanted background fluorescence. As mentioned in previous section, homodyne and heterodyne technique can be used in the signal processing concept to have achieve easier processing of these high frequency signals and hence to increase the signal to noise ratio.

4.2.1 Extraction of sample fluorescence based on homodyne assisted phase-resolved method

Using homodyne technique, the fluorescence intensity signal $F(r,t)$ is mixed with another sine wave signal $G(t)$ at the same frequency. Where,

$$G(t) = B[1 + m_g \sin(\omega t - \phi_g)] \quad (4.5)$$

This signal can be represented as shown in Figure 4.1 and is represented as ‘homodyne detector modulation’. In the above equation, B represents the DC part, m_g is the modulation depth and ϕ_g is the phase difference between excitation modulation $I(t)$ and detector modulation $G(t)$ at $t=0$. By mixing the fluorescence intensity signal given by equation (4.4) with this sine wave signal at the same frequency results in DC terms that contains all the information of phase shifts and demodulation factors of the fluorescence emissions.

$$F(r, t) \times G(t) = \begin{cases} A'(r_s)B[1 + m_{ex}m_s \sin(\omega t - \phi_s)][1 + m_g \sin(\omega t - \phi_g)], & \text{when } r = r_s \\ A'(r_b)B[1 + m_{ex}m_b \sin(\omega t - \phi_b)][1 + m_g \sin(\omega t - \phi_g)], & \text{when } r = r_b \end{cases} \quad (4.6)$$

Generally, the imaging device such as Intensified Charge Coupled Device (ICCD) camera doesn't have high frequency response. Therefore, all high-frequency terms of the mixed signal will be averaged to zero. Gain modulated intensifiers and cameras can be used in such cases [97,99]. The resulting mixed signal, $H(r, \phi, \phi_g)$ after homodyning is given by,

$$H(r, \phi, \phi_g) = \begin{cases} A'(r_s)B \left[1 + \frac{1}{2} m_g m_{ex} m_s \cos(\phi_s - \phi_g) \right] \\ A'(r_b)B \left[1 + \frac{1}{2} m_g m_{ex} m_b \cos(\phi_b - \phi_g) \right] \end{cases} \quad (4.7)$$

Here, ϕ represents a general term to indicate the phase shift. This can be either ϕ_s or ϕ_b depending on the emission from the fluorescent sample or background. Equation (4.7) reveals that the homodyne signal is a function of ϕ_g and has DC terms. By

adjusting ϕ_g to be $\pi/2$ out-of-phase with the phase of background fluorescence, ϕ_b (e.g. $\phi_g = \phi_b \pm \pi/2$), only the second term in each of equation (4.7) comes to zero. However, due to the presence of DC part, the background fluorescence still remains. Therefore, DC part of the signal that do not have any phase and demodulation information must be filtered out initially to achieve effective phase-resolved detection.

4.2.1.1 Filtering out DC terms in homodyne signal

Consider that ϕ_0 is the initial phase setting. As shown in Figure 4.3 and referring to equation (4.7), the phase difference between excitation modulation and detector modulation is set at $\phi_g = \phi_0$, $\phi_g = \phi_0 + \frac{\pi}{2}$, $\phi_g = \phi_0 + \pi$ and $\phi_g = \phi_0 + \frac{3\pi}{2}$.

Figure 4.3 Graphical representation of even step phase shifting to cut off DC term of signal from fluorescence emission.

The initial ϕ_0 value is taken to be zero. Hence, the corresponding signals at each phase setting are given as follows,

$$H(r, \phi, \phi_g) = \begin{cases} A'(r_s)B[1 + \frac{1}{2}m_g m_{ex} m_s \cos(\phi_s)] \\ A'(r_b)B[1 + \frac{1}{2}m_g m_{ex} m_b \cos(\phi_b)] \end{cases} \quad \text{where, } \phi_g = 0 \quad (4.8a)$$

$$H(r, \phi, \phi_g) = \begin{cases} A'(r_s)B[1 + \frac{1}{2}m_g m_{ex} m_s \cos(\phi_s - \pi/2)] \\ A'(r_b)B[1 + \frac{1}{2}m_g m_{ex} m_b \cos(\phi_b - \pi/2)] \end{cases} \quad \text{where, } \phi_g = \pi/2 \quad (4.8b)$$

$$H(r, \phi, \phi_g) = \begin{cases} A'(r_s)B[1 + \frac{1}{2}m_g m_{ex} m_s \cos(\phi_s - \pi)] \\ A'(r_b)B[1 + \frac{1}{2}m_g m_{ex} m_b \cos(\phi_b - \pi)] \end{cases} \quad \text{where, } \phi_g = \pi \quad (4.8c)$$

$$H(r, \phi, \phi_g) = \begin{cases} A'(r_s)B[1 + \frac{1}{2}m_g m_{ex} m_s \cos(\phi_s - 3\pi/2)] \\ A'(r_b)B[1 + \frac{1}{2}m_g m_{ex} m_b \cos(\phi_b - 3\pi/2)] \end{cases} \quad \text{where, } \phi_g = 3\pi/2 \quad (4.8d)$$

Taking the outputs of these homodyne signals as above and followed by averaging will result in the DC term given by,

$$H(r, \phi, \phi_g)_{avg} = \begin{cases} A'(r_s)B, & \text{when } r = r_s \\ A'(r_b)B, & \text{when } r = r_b \end{cases} \quad (4.9)$$

By subtracting equation (4.9) from (4.7), only the term related to the phase of the fluorescence emissions will remain and it is given by,

$$H_O(r, \phi, \phi_g) = \begin{cases} \frac{1}{2}Bm_g m_{ex} A'(r_s) m_s \cos(\phi_s - \phi_g), & \text{when } r = r_s \\ \frac{1}{2}Bm_g m_{ex} A'(r_b) m_b \cos(\phi_b - \phi_g), & \text{when } r = r_b \end{cases} \quad (4.10)$$

Thus the DC part in the homodyne signal is filtered out effectively. It should be noted that this even-step phase shifting method is not only limited to 4-step but also other even steps such as 2-step, 6-step, etc can also be used. The phase-resolved

fluorescence signal represented by equation (4.10) can be schematically shown as in Figure 4.4.

Figure 4.4 Graphical representation of fluorescence emission from sample after removing the DC term.

4.2.1.2 Suppression of background fluorescence

The signal, which is represented by the equation (4.10) can be used for extraction of the sample fluorescence. Consider the situation of $\tau_s < \tau_b$ where time-resolved imaging is not applicable. By setting ϕ_g to be $\pi/2$ out-of-phase with the phase of the background fluorescence (i.e. $\phi_g = \phi_b - \pi/2$), the homodyne result of the background fluorescence goes to zero while that of the sample fluorescence remains.

$$H_O(r, \phi, \phi_g) = \begin{cases} \frac{1}{2} B m_g m_{ex} A'(r_s) m_s \sin(\phi_b - \phi_s), & \text{when } r = r_s \\ 0, & \text{when } r = r_b \end{cases} \quad (4.11a)$$

Similarly when $\tau_s > \tau_b$, the background fluorescence can be suppressed by setting

$$\phi_g = \phi_b + \pi/2.$$

$$H_O(r, \phi, \phi_g) = \begin{cases} \frac{1}{2} B m_g m_{ex} A'(r_s) m_s \sin(\phi_s - \phi_b), & \text{when } r = r_s \\ 0 & , \text{when } r = r_b \end{cases} \quad (4.11b)$$

Equations (4.11) shows that by using homodyne assisted phase-resolved method, the sample fluorescence can be extracted effectively irrespective of whether its lifetime is longer or shorter than that of the background. The phase-resolved fluorescence emission from a fluorescent sample after suppressing the background fluorescence is shown in the Figure 4.4, denoted as ‘background suppression result’.

4.2.2 Extraction of sample fluorescence based on heterodyne assisted phase-resolved method

In heterodyne assisted PR technique, the detector modulation signal is represented by

$$G_E(t) = B[1 + m_g \sin(\omega_1 t - \phi_g)] \quad (4.12)$$

where, $\omega_1 = \omega - \delta\omega$ ($\delta\omega \ll \omega$) and other terms carries the same meaning that is given in the case of equation (4.5). The modulated excitation light intensity, given by equation (1) [54] and the heterodyne detector modulation represented by equation (4.12) are schematically shown in Figure 4.5.

Figure 4.5 Graphical representation of modulated excitation and heterodyne detector modulation.

The modulated fluorescence emission from the sample can be schematically represented as given in Figure 4.2. By mixing the fluorescence intensity signal given by equation (4.4) with this sine wave at a frequency close to that of the modulated laser beam, which is given by equation (4.12), result in low frequency signals that contain all the information of phase shifts and demodulation factors can be obtained. As mentioned earlier the resulting high frequency terms will be averaged to zero. The resulting heterodyne signal is given by,

$$H_E(r, t, \phi_g) = \begin{cases} A'(r_s)B\{1 + \frac{1}{2}m_g m_{ex} m_s \cos[\delta\omega t - (\phi_s - \phi_g)]\}, & \text{when } r = r_s \\ A'(r_b)B\{1 + \frac{1}{2}m_g m_{ex} m_b \cos[\delta\omega t - (\phi_b - \phi_g)]\}, & \text{when } r = r_b \end{cases} \quad (4.13)$$

This time-varying signal at a much lower frequency $\delta\omega$ contains both DC and AC terms. This signal can be represented as shown in Figure 4.6.

Figure 4.6 Graphical representation of the time-varying fluorescence signal at heterodyne frequency, which includes both DC and AC terms.

Only the AC term contains the phase information and is useful for further phase-sensitive detection. Therefore, similarly to the homodyne method, the DC term in the heterodyne signal has to be eliminated.

4.2.2.1 Filtering out DC terms in heterodyne signal

Here, a method by which the exposure time of the ICCD camera is controlled to filter out the DC terms in the fluorescence emission. It is possible to describe the exposure time of the camera as function given by,

$$E(t) = \begin{cases} 1 & 0 \leq t \leq T \\ 0 & t > T \end{cases} \quad (4.14)$$

This can be represented as in Figure 4.7.

Figure 4.7 Graphical representation of the ICCD camera exposure time function

During the on ('I') period (or exposure time), the camera will do integration to the input signal. By setting $T = 2\pi/\delta\omega$, the integration result of the heterodyne signal can be given as

$$\int_0^{\frac{2\pi}{\delta\omega}} H_E(r, t, \phi_g) dt = \begin{cases} \frac{2\pi}{\delta\omega} BA'(r_s), & \text{when } r = r_s \\ \frac{2\pi}{\delta\omega} BA'(r_b), & \text{when } r = r_b \end{cases} \quad (4.15)$$

It can be noted that due to integration, the time-varying signal at frequency $\delta\omega$ in the equation (4.13) is averaged to zero. Hence, only the DC term of $H_E(r, t, \phi_g)$ remains after this signal integration. Again, by setting $T = \pi/\delta\omega$, the integration result of the heterodyne signal can be given as,

$$\int_0^{\frac{\pi}{\delta\omega}} H_E(r, t, \phi_g) dt = \begin{cases} \frac{\pi}{\delta\omega} BA'(r_s) + \frac{Bm_{ex}m_g}{\delta\omega} A'(r_s)m_s \sin(\phi_s - \phi_g), & \text{when } r = r_s \\ \frac{\pi}{\delta\omega} BA'(r_b) + \frac{Bm_{ex}m_g}{\delta\omega} A'(r_b)m_b \sin(\phi_b - \phi_g), & \text{when } r = r_b \end{cases} \quad (4.16)$$

In order to filter out DC terms, signal represented by equation (4.15) is divided by two and subtracted from equation (4.16), will result in the term that contains the phase difference of the fluorescence emissions. This signal is given by,

$$H_{E0}(r, \phi_g) = \begin{cases} \frac{1}{\delta\omega} Bm_g m_{ex} A'(r_s) m_s \sin(\phi_s - \phi_g), & \text{when } r = r_s \\ \frac{1}{\delta\omega} Bm_g m_{ex} A'(r_b) m_b \sin(\phi_b - \phi_g), & \text{when } r = r_b \end{cases} \quad (4.17)$$

Hence, by the proper camera exposure time control and integration of signal, the DC term in the heterodyne signal can be filtered out effectively. This signal is represented as shown in Figure 4.8.

Figure 4.8 Graphical representation of phase-resolved fluorescence emission from the sample after removing the DC term.

4.2.2.2 Suppression of background fluorescence

After filtering out the DC terms, equation (4.17) can be used for extraction of the sample fluorescence. When $\tau_s < \tau_b$, by setting ϕ_g to be π out-of-phase with the phase of the background fluorescence, i.e. $\phi_g = \phi_b + \pi$, the heterodyne result of the background fluorescence goes to zero while that of the sample fluorescence remains.

$$H_{E0}(r, \phi_g) = \begin{cases} \frac{1}{\delta\omega} Bm_g m_{ex} A'(r_s) m_s \sin(\phi_b - \phi_s), & \text{when } r = r_s \\ 0, & \text{when } r = r_b \end{cases} \quad (4.18a)$$

When $\tau_s > \tau_b$, the background fluorescence can be suppressed similarly by setting

$$\phi_g = \phi_b.$$

$$H_{E0}(r, \phi_g) = \begin{cases} \frac{1}{\delta\omega} Bm_g m_{ex} A'(r_s) m_s \sin(\phi_s - \phi_b), & \text{when } r = r_s \\ 0, & \text{when } r = r_b \end{cases} \quad (4.18b)$$

Equations (4.18) show that by using heterodyne assisted phase-resolved method, the sample fluorescence can be extracted effectively irrespective of whether its lifetime is longer or shorter than that of the background. The phase-resolved emission from the fluorescent sample after suppressing the unwanted background fluorescence is shown in Figure 4.8, denoted as ‘background suppression result’.

4.2.3 Imaging of fluorescent sample deposited on non-fluorescing backgrounds

In the case of a fluorescent sample deposited on non-fluorescing substrates, theoretical formulation for the imaging can be taken as the special case of the above-derived theory. Here, the background fluorescence will be zero. In order to detect weak fluorescent sample, high excitation power is always required. In such scenarios, PR method is significant in eliminating the reflected and scattered light from the substrates. Theoretically it can be achieved by filters, but when using high excitation power of the order of few hundreds of milli watts, the intensity of back reflected and scattered light from the non-fluorescing substrate will be very high. Hence, practically it is very difficult to achieve 100% filtering off scattered light

from the background by optical filters. Also, due to the high sensitivity, ICCD camera detects even the faintest scattered and reflected photons from the sample streaking through filters and resulting in poor image contrast in imaging

From equations (4.10) and (4.17) it is clear that if background fluorescence is zero, phase-resolved fluorescence intensity will turn out to be

$$H_{01}(r, \phi_s, \phi_g) = \begin{cases} \frac{1}{2} B m_g m_{ex} A'(r_s) m_s \cos(\phi_s - \phi_g), & \text{when } \textit{homodyne} \\ \frac{1}{\delta\omega} B m_g m_{ex} A'(r_s) m_s \sin(\phi_s - \phi_g), & \text{when } \textit{heterodyne} \end{cases} \quad (4.19)$$

or in another way, one can write

$$H_{01}(r, \phi_s, \phi_g) \propto \begin{cases} K m_s \cos(\phi_s - \phi_g), & \text{when } \textit{homodyne} \\ K' m_s \sin(\phi_s - \phi_g), & \text{when } \textit{heterodyne} \end{cases} \quad (4.20)$$

where K and K' together represents the constant terms in equation(4.19). The parameter, ϕ_g , is important and it can be set at any angle between 0^0 to 360^0 . This method can be applied to pick up fluorescence emission by virtue of the lifetime of the component.

At a particular modulation frequency ω , ϕ_g can be adjusted to be in phase or out of phase with ϕ_s at a given lifetime. In other words, fluorescence intensity of a particular component can be reduced to zero by setting ϕ_g to be 90^0 out of phase with the emission from that component with phase value ϕ_s in homodyne method or ϕ_g to be 180^0 out of phase with the emission having phase value ϕ_s in heterodyne.

Alternatively at a given ϕ_g , fluorescence components can be enhanced as a function of fluorescence lifetime by the use of modulation frequency as a parameter.

The back-scattered light can be easily cutoff by this method i.e. by adjusting the value of ϕ_g . For example, if one takes $\phi_g = 90^\circ$ relative to the emission in homodyne method, and the scattered light has theoretically zero lifetime, then from equation (4.20) it is obvious that scattered intensity is zero. Similar approach can also be applied in heterodyne method to eliminate excitation-scattered light from.

From equation (4.2),

$$m_s = [1 + (\omega \tau_s)^2]^{-1/2} = [1 + \tan^2 \phi_s]^{-1/2} = \cos \phi_s \quad (4.21)$$

The variation of m_s or $\cos \phi_s$ with respect to τ_s and ω is given in Figure 4.9. This graph clearly explains the dependence of modulation frequency and lifetime value on the m_s factor. It shows that at a particular lifetime value of the sample and at higher modulation frequencies, $\cos \phi_s$ also decreases or ϕ_s value increases. When working at lower frequencies for low fluorescence lifetime samples, components with high $\cos \phi_s$ value or lower ϕ_s value can be detected. Thus this graph clearly gives a direction to extract a particular lifetime component at different frequencies depending on the phase shift and lifetime. Lifetime of the sample can be calculated using time correlated single photon counting method or other lifetime measuring method. Once the lifetime of the emission is well studied, it allows working in the convenient frequency range that fixes the extraction of a component with a particular phase shift.

Figure 4.9 Graph showing the variation of linear frequency versus $\log(\tau)$ with respect to different m_s .

4.3 CHOICE OF MODULATION FREQUENCY AND OPTIMISATION OF FLUORESCENCE EMISSION

Equations (4.11) and (4.18) show that both homodyne and heterodyne assisted phase-resolved method yield a similar expression of the extracted sample fluorescence intensity,

$$S(r_s) \propto B m_g m_{ex} A'(r_s) m_s |\sin(\phi_b - \phi_s)| \quad (4.22)$$

i.e. $S(r_s)$, the intensity of the extracted sample fluorescence, is proportional to the parameters listed in equation (4.22). In addition, $S(r_s)$ is also inversely proportional to $\delta\omega$ when using heterodyne assisted phase-resolved method. For most of the fluorescent samples deposited on fluorescing backgrounds, intensity of sample

fluorescence in general will be very weak. Hence to improve the image contrast, increase in the intensity of the extracted sample fluorescence is always favorable. According to equation (4.22), it is possible to improve the intensity and hence image contrast by following ways.

- 1) When using heterodyne method, use of smaller frequency difference between excitation modulation and detector modulation will result in better image contrast.
- 2) A larger value of ' $Bm_g m_{ex}$ ' will contribute to a better image contrast, i.e. a higher gain of the detection device and a higher modulation depth of both the modulated laser beam and the modulated gain are beneficial.
- 3) A larger value of ' $A'(r_s)$ ' will also result in a better image contrast. As mentioned earlier, the value of $A'(r_s)$ depends on parameters such as laser power, a fluorescent sample absorption property, sample fluorescence quantum efficiency etc. Therefore, the image contrast can also be improved by employing a laser power as high as possible to achieve maximum fluorescence emission from sample. It should be noted that the fluorescence emission intensity always directly proportional the excitation intensity. Also, by applying chemical treatments, if any, to fluorescent sample that have higher efficiency will also favorable. This is significant when detecting very weak fluorescence from the sample.
- 4) It is also favorable to use optical geometry that helps in collecting maximum fluorescence emission from the sample. It is quite obvious that the imaging device

(ICCD camera) should place at a favorably close distance from the sample with its lens in full field of view of the fluorescent sample.

5) The image contrast is also proportional to the demodulation factor of the sample fluorescence and the phase difference between the fluorescent sample and the background fluorescence. However, these two parameters, which are functions of modulation angular frequency ω and lifetimes of the background and the sample fluorescence emissions, are not independent. Using ' Q ' to represent the product of these two parameters, i.e.

$$Q(\omega, \tau_s, \tau_b) = m_s |\sin(\phi_b - \phi_s)| = \frac{\omega}{\sqrt{1 + (\omega\tau_b)^2} [1 + (\omega\tau_s)^2]} |\tau_b - \tau_s| \quad (4.23)$$

Equation (4.23) is a coupled function. This implies that for certain background and sample fluorescence, there exists an optimum modulation frequency with which $Q(\omega, \tau_s, \tau_b)$ reaches it's maximum.

Figure 4.10 illustrates an example of theoretical result of equation (4.23) when τ_s is fixed at $8ns$ and τ_b has different values. This figure shows that the intensity of $Q(\omega, \tau_s, \tau_b)$ varies greatly with the modulation frequencies when the lifetimes of background and sample fluorescence are fixed. For example, $Q(\omega, \tau_s, \tau_b)$ has a maximum value of ~ 0.37 at a modulation frequency of $\sim 19MHz$ when $\tau_s = 8ns$ and $\tau_b = 2ns$. While at a modulation frequency of $\sim 70MHz$, this value is only ~ 0.15 . Therefore a much better result can be expected by using the optimum modulation frequency. Furthermore, this optimum modulation frequency also differs when the

lifetimes of the background fluorescence changes. For example, when $\tau_s = 8ns$ and $\tau_b = 6ns$, the optimum modulation frequency is ~ 15 MHz, and it comes to ~ 11 MHz when $\tau_s = 8ns$ and $\tau_b = 14ns$. It is also noted that in general it is always appreciable to work at lower modulation frequency for a given lifetime difference between the samples.

Figure 4.10 Theoretical result of optimum modulation frequency at different background lifetimes when $\tau_s = 8ns$.

Figure 4.11 illustrates another example of theoretical result of equation (4.23) when τ_s is fixed at a different value $4ns$. This figure again demonstrates that the optimum modulation frequency varies when either of the lifetimes changes. Figures 4.10 and 4.11 also reveal that a larger difference between the lifetime of the sample

fluorescence and that of the background is beneficial to get a stronger signal of the extracted sample fluorescence. When choosing chemical powders to enhance the fluorescent sample, the lifetime difference from the background is an important factor to be considered.

Figure 4.11 Theoretical result of optimum modulation frequency at different background lifetimes when $\tau_s = 4 \text{ ns}$.

4.4 SENSITIVITY IMPROVEMENT FOR PHASE-RESOLVED IMAGING BY COMBINING EVEN-STEP-SHIFT AND PI-SHIFT METHODS

Pi (π)-shift method has been used to obtain a higher contrast for lifetime-selective fluorescence imaging for different fluorophores [97]. With the use of π -shift method,

two images are taken at two detector phases that differ by 180° and the two resulting images are then subtracted. The two phases are selected so that the image pixel intensity for the unwanted fluorescence is the same for both phase settings. Due to the subtraction procedure, the equal pixel intensities obtained at both phase settings cancel out in the processed image.

However, when using π -shift method for a fluorescent sample imaging, difficulties exist in determining the two phases due to the unknown lifetimes of a fluorescent sample and background fluorescence. In this context, an even-step-shift method, which is developed for the homodyne assisted phase-resolved system and mentioned in section 4.2.1.1 is combined with π -shift method to determine the two phases required, which differ by π radians.

From equation (4.7), the fluorescence intensity from the sample after homodyning is given by

$$H(r, \phi, \phi_g) = \begin{cases} K_s [1 + C m_s \cos(\phi_s - \phi_g)], & \text{when } r = r_s \\ K_b [1 + C m_b \cos(\phi_b - \phi_g)], & \text{when } r = r_b \end{cases} \quad (4.24)$$

where, K_s , K_b and C replaces the constants like $A'(r_s)$, $A'(r_b)$, B , m_g , m_{ex} , which are related to excitation power, gain of ICCD, exposure time, sample absorption, fluorescence quantum efficiency, modulation depths, etc.

After removing the DC terms, which are free from phase and modulation information, the above equation can be modified according to equation (4.10) as

$$H_0(r, \phi, \phi_g) = \begin{cases} CK_s m_s \cos(\phi_s - \phi_g), & \text{when } r = r_s \\ CK_b m_b \cos(\phi_b - \phi_g), & \text{when } r = r_b \end{cases} \quad (4.25)$$

Equation (4.25) reveals that the image intensity is a function of ϕ_g only. According to equations (4.11a and 4.11b), by setting ϕ_g to be $\pi/2$ out-of-phase with ϕ_b , the background fluorescence suppression can be achieved. It is clear that the two phases used for π -shift method fall on the positions where the background fluorescence goes to zero, i.e. when $\phi_g = \phi_b \pm \pi/2$. By setting ϕ_g to be $\pi/2$ out-of-phase with the phase of the background fluorescence (e.g. the point of $\phi_g = \phi_b - \pi/2$), the image signal of the background fluorescence goes to zero while that of the sample fluorescence remains

$$H_1(r, \phi, \phi_g) = \begin{cases} CK_s m_s \sin(\phi_b - \phi_s), & \text{when } r = r_s \\ 0, & \text{when } r = r_b \end{cases} \quad (4.26)$$

Again from equation (4.25) it can be found that the background fluorescence also goes to zero at $\phi_g = \phi_b + \pi/2$ while the fluorescent sample image has a negative intensity. i.e.

$$H_2(r, \phi, \phi_g) = \begin{cases} -CK_s m_s \sin(\phi_b - \phi_s), & \text{when } r = r_s \\ 0, & \text{when } r = r_b \end{cases} \quad (4.27)$$

Thus it is clear that the two phases used for π -shift method fall on the positions where the background fluorescence goes to zero, i.e. when $\phi_g = \phi_b \pm \pi/2$. Then by

taking two images at these two phases and applying a digital image subtraction process, fluorescent sample image with improved intensity is resulted.

$$H_3(r, \phi, \phi_g) = \begin{cases} 2CK_s m_s \sin(\phi_b - \phi_s), & \text{when } r = r_s \\ 0 & , \text{when } r = r_b \end{cases} \quad (4.28)$$

Comparing equation (4.26) with equation (4.28), it is obvious that the fluorescent sample image intensity resulted here is twice the intensity obtained in the normal homodyne assisted PR imaging technique.

4.5 COMPARISON OF HOMODYNE AND HETERODYNE ASSISTED PR IMAGING TECHNIQUES

Equations given by (4.11) and (4.18) gives the phase-resolved fluorescence from fluorescent sample after suppressing the background emissions. It has to be noted that equations (4.11) and (4.18) have different units because, equation (4.11) is the photon signal before CCD is carrying out integration during exposure time. But Equation (4.18) is the image intensity after integration. Before comparing equations these two equations, equation (4.11) has to be multiplied by a factor, K , which will take care of exposure time and integration effect.

Hence these equations are given as

$$H_0(r, \phi, \phi_g) = \begin{cases} \frac{K}{2} B m_g m_{ex} A'(r_s) m_s \sin(\phi_b - \phi_s), & \text{when } r = r_s \\ 0 & , \text{when } r = r_b \end{cases} \quad \text{In homodyne}$$

assisted PR method (4.29)

and

$$H_{E0}(r, \phi_g) = \begin{cases} \frac{1}{\delta\omega} B m_g m_{ex} A'(r_s) m_s \sin(\phi_b \sim \phi_s), & \text{when } r = r_s \\ 0, & \text{when } r = r_b \end{cases} \quad \text{In heterodyne}$$

assisted PR method (4.30)

From equations (4.29) and (4.30) it is found that the sample fluorescence intensity after suppressing the background emission and filtering off the DC terms, depends on a factor ‘ $\frac{K}{2}$ ’ in homodyne assisted PR technique where as it is $\frac{1}{\delta\omega}$ in the case of heterodyning. It shows that to have higher intensity and hence better contrast for a fluorescent sample image $\delta\omega$ has to be as low as possible when applying heterodyne technique. It is to be noted that $\delta\omega = 2\pi\delta f$, where δf is the linear frequency difference between excitation and detection modulation.

Consider that for a given fluorescent sample, exposure time, K of 500ms and if δf is 1 Hz or δT of 1 sec, where T is the period. In homodyne assisted PR, the $\frac{K}{2}$ factor will be $\frac{0.5}{2}$ and $\frac{1}{\delta\omega}$ factor turns out to be $\frac{1}{2\pi}$ in heterodyne assisted PR method.

Comparing these two, it is obvious that intensity in homodyne assisted PR method is ~ 1.57 times higher than that of heterodyne assisted PR method at the optimised experimental conditions. It can be shown that For a given sample and under fixed settings, a value of δf as low as ~ 0.64 Hz in heterodyne technique will result in almost same intensity and hence contrast for a fluorescent sample image as in the case of homodyne method. But practical limitation of bringing down δf to such a low value always limits the image contrast in heterodyne assisted PR method.

It is also revealed from section 4.4 that the ‘pi’ shifts method to enhance the image contrast can only be applied to homodyne assisted PR method. Hence, it can be summarised that for a given sample and under fixed settings, it is always favorable to work with homodyne assisted PR method to achieve better contrast for a fluorescent sample image. This will be significant when the fluorescent sample is deposited on fluorescing substrates.

4.6 SUMMARY

Complete theoretical formulation for the PR optical imaging technique is presented. Homodyne and heterodyne concept of signal processing is incorporated with PR technique for easier processing of high frequency signals and hence to increase the signal to noise ratio. Sensitivity improvement for the PR imaging by combining even-step-shift and pi-shift methods is also formulated and presented. Later, relative merits of homodyne and heterodyne assisted PR technique in the case of PR imaging of fluorescent sample deposited on fluorescing substrate are also discussed. Finally, an image quality evaluation method for a fluorescent sample images obtained using PR technique is also presented and discussed.

CHAPTER FIVE

PHASE-RESOLVED OPTICAL IMAGING TECHNIQUE: INSTRUMENTATION DETAILS

5.1 INTRODUCTION

This chapter describes the instrumentation details of the phase-resolved optical technique for fluorescence imaging applications. Different system components and their features are highlighted. The experimental details of fluorescence lifetime measurement and study of excitation-emission spectra of fluorescent samples will be explained. Later, the experimental procedure of imaging the fluorescent sample by the suppression of unwanted background fluorescence is also explained.

5.2 PHASE-RESOLVED EXPERIMENTAL SETUP

Figure 5.1 shows the experimental set up of the proposed PR optical system. In this configuration an Argon-ion laser is used as the light source. The sample is excited by a sinusoidal modulated laser source. The laser beam is modulated by an electro-optic modulator. The modulated light is coupled into a single mode optical fiber or liquid light guide and it is incident on the sample surface. The fluorescence emission from the sample will be collected by the Intensified Charge Coupled Device (ICCD) camera, which is interfaced with a computer for signal processing. Details of the different system components and their features used in the proposed PR imaging are listed below.

Figure 5.1 Schematic diagram of Phase-resolved optical imaging set up

1: Argon laser, 2: EOM, 3: HRI, 4: ICCD camera, 5: Liquid light guide, 6: Computer, 7: Function generator 1, 8: Function generator 2, 9: Power amplifier of EOM.

5.2.1 Components of the PR optical system

The proposed PR optical system consists of optics unit, electronics unit and related software for signal processing and imaging. Optics unit consists of laser, electro-optic modulator and fiber, as well as the imaging system. The ICCD camera forms the important part of optical imaging system along with the necessary software. Electronics unit consist of function generators and low frequency oscillator for signal processing to achieve background fluorescence suppression. A program incorporating all the necessary signal processing routine is developed to automate the signal acquiring and processing.

(i) Laser source [100]

Coherent Innova 90 C Argon-ion laser is used in this setup. This Argon-ion laser is capable of lasing at different light operation modes such as single line visible, multiline visible and multiline UV. The single line visible mode of operation gives wavelength output ranging from 454 nm to 528 nm at different output power of few milliwatts to 2.5 W. The multiline visible mode of operation gives maximum output power of 6W. The multiline UV mode of operation is designed to give the output at 333 nm to 363 nm at few hundreds of milliwatt power. This laser needs to be cooled by water, so it can only be used in laboratory. For out door applications air-cooled modulated diode lasers can be used.

(ii) Intensified CCD camera (ICCD) [101]

Intensified CCD (Lavision, model: Pico Star HR) camera is the most important component in the proposed phase-resolved setup. The specifications of the ICCD camera are given in appendix C. The ICCD camera is controlled by an interfacing program.

The heart of the ICCD camera system is the camera head, which consists of an image intensifier that is lens coupled to the CCD sensor. The image intensifier mainly consists of there elements: photo cathode, micro channel plate (MCP) and phosphor. The image intensifier is operated by the High Rate Imager (HRI) controller. It provides all the necessary operation voltage for the image intensifier electronics. The image intensifier is used to amplify the weak fluorescence emission from the sample.

The image to be taken is focused on to photo cathode plane. The impinging photons generate electrons inside the photo cathode material. During the image intensifier gate time, a negative voltage is applied to photo cathode. Hence, only during this gating time, electrons are accelerated towards the MCP. It is a 1mm thin plate with an array of 10 microns holes that are grouped in a hexagonal structure. Between entrance and exit slit of MCP there is high voltage, so that the electrons hitting the walls of MCP will generate secondary electrons.

After leaving the MCP, the electron cloud is pulled by a voltage of about 6000 V on to phosphor. For each electron hitting the phosphor photons are generated in large numbers and imaged on to CCD by the lens system. The CCD collects the light emitted by phosphor and it acts as an integrating device. It holds the image information in a matrix of separated image elements.

(iii) Electro-optic modulator (EOM) [102]

Electro-optic modulator from Conoptics (Model 350-210) is used to modulate the excitation light. The electro-optic modulator uses potassium dihydrogen phosphate (KDP) crystal. The power amplifier is used as the power source to the modulator. The EO modulator is capable of operating in the 0 to 100 MHz range. The modulation is achieved by driving the EOM by a suitable signal from the function generator. The wavelength transmission range of the EOM is from 300-1000 nm.

(iv) Beam delivery systems

Laser light, which is modulated by the EO modulator, is coupled into single mode fiber or liquid light guide to illuminate the sample surface. The single mode optical fiber from OZ optics is used in the experiment [103]. The core diameter of the single mode fiber used for visible light operation is 3.5 microns and that for UV operation is 2 microns. In certain cases, in order to illuminate a large area of the sample, liquid light guide (Melles Griot) is also used.

(v) Optical accessories

Long pass optical filters (Melles Griot), which are used to cutoff the excitation-scattered light from the sample. A photodiode detector is used to check the response of EO modulator when driven by a signal from a function generator. Laser power meter (Ophir, Laserstar) is also used to measure the excitation laser power [104] for different fluorescent samples on different substrates.

(vi) Function generators [105]

To drive the EO modulator and HRI of ICCD camera, two identical single channel Agilent 80 MHz function/arbitrary waveform generator (Model 33250A) is used. This has an accuracy of 1micro Hz with stability of around ± 0.3 ppm at 18 to 28 degree temperature.

(vii) Low frequency oscillator

When working in heterodyne assisted PR technique, a trigger signal is needed to trigger the two-function generator and CCD external trigger. This ensures all units start working at same time without any delay. A low frequency oscillator, which is

built in house, is used for triggering the camera exposure control. It's giving a square wave out put in the frequency range 0.9 Hz to 2.4 Hz with a theoretical accuracy up to 1%.

5.3 SPECTROMETERS AND BROADBAND LIGHT SOURCE

5.3.1 Poly light source [106]

In order to determine the fluorescence emission wavelengths of fingerprint treated with different powders they are excited with a broadband light source followed by detecting the emission spectra. For this purpose, a poly light (board band light source, Omnichrome Spectrum 9000, Melles Griot) is used. This uses a Xenon arc lamp with specific filters at the emission side to tune the wavelength. The tunable mode feature in this light source allows for the tuning of the wavelength (300-700 nm) and bandwidth (up to 100 nm). A liquid light guide is used as the transmission cable in this case.

5.3.2 Fluorescence spectrometer [107]

The AVS-2000 fiber optic spectrometer (Avantes) is used in the study to record the fluorescence intensity as a function of wavelength. It has an entrance slit of 200 μm and a grating resolution of 600 lines/mm. The spectrometer covers a wavelength range from 200-850 nm. It uses a linear 2048-element charged-coupled device (CCD) array as the detector. The fluorescence signal collected using the fiber, which has a collimating lens, is directed, via an ADC-1000-USB interface to the Avasoft data acquisition software. Fluorescence spectrum displayed on the monitor of

computer, which is associated with the software. Figure 5.2 shows the schematic diagram of fluorescence spectrometer setup.

Figure 5.2 Schematic diagram of the experimental set up used for determination of fluorescence emission wavelengths.

5.3.3 Fluorescence lifetime analyser [108]

Before carrying out TR imaging of fingerprints (given in appendix B) with nanosecond resolution, experiments were done to determine the wavelength and intensity of fluorescence from treated fingerprint samples. This was done with the help of the fluorescence lifetime measurement setup, which is explained above.

Lifetime measurements of the samples were carried out using Time Correlated Single Photon Counting (TCSPC) spectrofluorometer ('Mini-Tau', Edinburgh Instruments).

It uses a microwatt power pulsed Light Emitting Diode (LED, Pico Quant, model PDL 800-B), with pico seconds pulse width. The detector of this instrument is a Photo Multiplier Tube (PMT, Hamamatsu). The light from the pulsed LED is coupled into an optical fiber, which has two ports. One of the ports acts as the excitation arm and the fluorescence signal collected using the same fiber is then fed to the second port, which acts as the detection port.

5.4 EXPERIMENTAL PROCEDURE

Knowledge of fluorescence lifetime of treated fingerprints and background fluorescence is highly significant when dealing with TR and PR imaging experiments. Hence, the measurement of the lifetime of fluorescence emission is always a must and it is carried out using the lifetime measurement system. Lifetime measurement using spectrofluorometer is explained in next section followed by the experimental procedure of imaging with homodyne and heterodyne assisted PR technique.

5.4.1 Lifetime measurement

Schematic diagram of the experimental set up used for lifetime measurement is given in Figure 5.3. The specimen sample is placed in the sample chamber and is covered with the lid to reduce the amount of ambient light during analysis. Initially, the time dependent fluorescent decay is recorded and displayed on the monitor of computer equipped with TCSPC software. Liquid type samples are taken in cuvette and placed inside the sample chamber of the system. But for other samples like treated fingerprints and different types of substrates, lifetime measurement was carried out

by keeping it in the sample holder, which is outside the instrument. In this case, excitation and collection of signal is carried out with the help of bifurcated optical fiber.

Figure 5.3 Schematic diagram of fluorescence lifetime measurement system.

Depending upon the intensity of fluorescence emission from the sample, different neutral density filters are used to limit the number of photons reaching the detector. Two LED's at wavelength 380 nm and 498 nm are used as the excitation source in the experiments. Samples that fluoresce under UV excitation are excited with 380 nm LED. Similarly for the sample, which shows emission up on excitation with blue green light, 498 nm LED was used as the excitation light source. In all experiments, 2.5 MHz repetition rate was chosen for the excitation light. Once the time dependent decay of the fluorescent sample was obtained, instrument response was measured using a highly scattering Ludox solution. These two decay plots were later subjected

to re-convolution tail fit and hence, lifetime data was obtained using the T 900 software associated with the instrument.

5.4.2 Phase-resolved imaging

Phase-resolved imaging was carried out with homodyne and heterodyne assisted signal processing technique. Experimental procedure and signal processing concepts are entirely different and hence they are described in separate section as given below.

5.4.2.1 Homodyne assisted phase-resolved imaging

The schematic diagram of the experimental setup is shown in Figure 5.4. Continuous wave Argon laser is modulated by an electro optic modulator. Laser is operating in the multiline UV mode with predominant laser line at 351 nm. The power amplifier is used as the power source to the modulator, which is modulated by a sinusoidal signal provided by the function generator 1. The maximum peak-to-peak voltage in this case will be 1 V. The modulated laser light is coupled into a liquid light guide that used to illuminate the sample. ICCD camera collects the resulting fluorescence emission from the sample. Function generator 2 is used as the signal source to modulate the gain of ICCD. It was carried out at by a signal of peak-to-peak voltage of 2 V at a defined frequency. These two function generators are synchronized for signal processing. A long pass optical filter (Melles Griot) connected to the camera lens is used to cut off the excitation-scattered light from the sample. The ICCD is connected with a camera interface board and controlled by the signal processing software through a computer.

Figure 5.4 Schematic diagram of homodyne assisted PR imaging set up

The two function generators provide sinusoidal outputs at the same frequency. The modulation frequency is adjusted according to the lifetime of the fluorescence emission from background and fingerprint. Due to homodyning, the fluorescence signals and gain signal meet at the intensifier of the ICCD and a frequency mixing process happens. Hence, CCD receives a signal with DC signal that contains terms with and with out phase information. Only the signal whose variation depends on the phase difference between the two-fluorescence signal and corresponding gain modulation is useful for further signal processing.

Once a signal satisfying the equation (4.7) is obtained, the even-step phase shifting method given by equation (4.8) is carried out to eliminate the DC component in the signal that is free from phase and modulation information. It starts by dividing a full phase cycle into an even number of steps equivalent to the number of phases required. The phase difference between the function generator 1 and the function generator 2 is then set to the number of phases required. This is carried out by

keeping the ϕ_g value to be at 90, 180, 270 and 360 degree and there will be an equal number of image signals from the ICCD camera. These image signals are stored in separate image buffers. Later, these images are averaged digitally and it will lead to the DC term given by equation (4.9). This DC term is removed from the fluorescence signal from the sample through digital subtraction process will result in signal satisfying the equation (4.10). Once the DC term is removed digitally, the background fluorescence suppression is effectively carried out by setting ϕ_g to be $\pi/2$ out of phase with ϕ_b , giving clear fingerprint image satisfying the equations (4.11a and 4.11b).

Through out the homodyne experiment, ICCD exposure time is kept to be 500 ms and low gain in HRI is used when testing strongly fluorescing samples. High Duty Cycle (HDC) slave mode is selected for ICCD camera operation. This means, if the repetition rate is 10 MHz, the maximum gate length is 50 ns.

5.4.2.2 Heterodyne assisted phase-resolved imaging

Schematic diagram of heterodyne assisted PR imaging system is shown in Figure 5.5. In this method, modulation of laser and gain of ICCD followed by capturing the fluorescence emission from the sample are carried out in the similar fashion as that of homodyne assisted PR imaging. But it is to be noted that these modulation are carried out at two different frequencies, which differ by a small value called heterodyne frequency.

CHAPTER FIVE

In order to eliminate the DC term in heterodyne fluorescence signal, which does not contain any phase information, a technique of controlling the camera exposure time is carried out. To control exposure time, camera was set to work in external trigger mode. The same trigger signal is required to trigger the two-function generators, which are working in external trigger mode. This way of triggering will ensure that all units start working at same time with out any delay. A low frequency oscillator, which is built in-house, is used for this purpose. It is capable of giving a square wave output in the frequency range 0.9 Hz to 2.4 Hz. The frequency of this triggering signal is kept to be at the heterodyne frequency ($\delta\omega$) between the modulated fluorescence emission and detector modulation.

The two function generators provide two outputs with close frequencies. Excitation modulation frequency is kept to be $\omega + \delta\omega$ and ICCD modulation to be at ω , i.e. both the laser and the gain of ICCD are modulated at frequency difference of $\delta\omega$. The whole experiment was carried out at heterodyne frequency, δf of 1 Hz. Hence, frequency mixing occurs between the fluorescence emissions and gain modulation signal when ICCD detects the signals from the sample. As a result of heterodyning, an image signal whose intensity varies at a low frequency is obtained from the ICCD.

Figure 5.5 Schematic diagram of heterodyne assisted PR imaging setup.

The two function generators were triggered externally by low frequency oscillator operating at 1 Hz. ‘External trigger set up’ and ‘Infinite burst’ modes are selected in the function generators while working with external trigger through the low frequency oscillator. When operating in external trigger mode, the ICCD will wait for the external trigger signal from the low frequency generator to start its defined ‘exposure time’, which serves as an important parameter in heterodyning to remove the DC term. During this defined exposure time, which acts as an “on” period of a square wave mentioned earlier, ICCD would carry out integration of the input intensity accordingly.

It is important to remove the DC term of the detected heterodyne signal before proceeding to phase-resolved fingerprint imaging. The variation of DC term of image

intensity can be obtained by setting the exposure time of ICCD to be at the period of heterodyne signal. Hence, according to equation (4.15) an image is captured at 1000 ms-exposure time and saved it in an image buffer. Later, as per the equation (4.16) the exposure time is set to be half of the period of heterodyne signal, i.e. at 500 ms and the image captured is stored in another buffer. First signal (DC signal) is digitally divided by 2 and subtracted it from the second signal will result in only AC term given by equation (4.17) that contains all phase shift and modulation information. After removal of DC term, as given by equation (4.18), background fluorescence suppression is carried out by setting ϕ_g to be π out of phase with ϕ_b or $\phi_g = \phi_b$ depending up on the lifetime values of the emissions. This will result in clearer image with better contrast.

Alignment of EOM was a critical factor in the experiment. Before starting the experiment it has to be ensured that modulator bias voltage and modulating voltage and frequency are properly set. By varying the bias voltage to the EOM, the modulated light from EOM is monitored through a photodiode and oscilloscope. If the modulation depth is poor and waveform is distorted, adjust the offset of the modulating voltage and bias voltage to result in a pure sinusoidal output for the excitation light.

In both homodyne and heterodyne assisted PR imaging, laser power is adjusted depending on the nature of substrate and intensity of emission from the sample. For weakly fluorescing sample, higher excitation power of about 100-200 mW is used. The maximum efficiency of modulator was found to be about 50%, i.e. for an input

power of 100 mW from laser will result in an about 50 mW from the modulator. Moreover, the efficiency of liquid light guide was found to be about 30% and that of single mode fiber was 70%. So ultimately only 15 mW and 35 mW of power will be available at sample surface from the liquid light guide and single mode fiber respectively when a 100 mW laser excitation is used.

5.5 SUMMARY

In this chapter, the proposed PR optical setup for the imaging of fluorescent sample is described. Various component of the set up is described by highlighting their salient features. Later, the experimental procedure for the imaging applications using PR technique is explained by emphasising the various signal processing concepts.

CHAPTER SIX

PHASE-RESOLVED IMAGING OF LATENT FINGERPRINTS

6.1 INTRODUCTION

This chapter describes the experimental results and discussion of the PR technique for imaging of latent fingerprints on different substrate surfaces. Initially lifetime characterisation results of fingerprints treated with different fluorescent powders are presented. Subsequently, PR imaging of latent fingerprints with homodyne and heterodyne signal processing procedures are presented. Sensitivity improvement for latent fingerprint imaging by combining even step phase shifting along with pi shifting method in homodyne assisted PR imaging is also discussed with experimental results. Finally, an illustrative comparison of homodyne and heterodyne signal processing assisted PR imaging techniques is presented with experimental data.

6.2 LIFETIME CHARACTERISATION OF FINGERPRINTS TREATED WITH FLUORESCENT POWDERS

Lifetime characterisation of fingerprint samples plays an important role in the time and frequency domain imaging techniques. Here, lifetime characterisation of fingerprint samples treated with different fluorescent powders was carried. Fingerprint samples deposited on non-fluorescing substrates such as polymethyl methacrylate (PMMA) sheet were used in the experiment. Fresh fingerprints from a

single person were deposited on each substrate. These prints are treated with different fluorescent powders. Non-fluorescing substrates were chosen to avoid the overlapping of background fluorescence with that of treated fingerprints. The powders being tested were Blitz-red, Blitz-green, Redwop, Greenwop, Ultra-yellow, Ultra-orange-yellow, Ultra-red, Ultra-gold, Ultra-green, Ultra-azure-blue and Ultra-white. All these powders are obtained from Medtech Forensics. Out of these powders, except Redwop and Greenwop all are magnetic in nature. Generally these types of powders are most effective to develop the prints on non-porous surfaces. Magnetic fluorescent powders were applied by means of a magnetic brush onto the latent fingerprint samples. While, other powders were applied using a normal brush. Care was taken to apply the powder almost uniformly onto fingerprint samples.

These samples were illuminated with light from a polilight source in the range of 350 to 700 nm and it was found that Ultra-azure-blue and Ultra-white powders show fluorescence when excited with UV-violet light. At the same time all other powders show fluorescence when excited with UV-blue light. The resulting fluorescence emissions are collected by a fluorescence spectrometer and their peak emission wavelength values are given in Table 6.1

Table 6.1 Peak fluorescence emission wavelength of fingerprints treated with fluorescent powders

Powders	Peak emission wavelengths (nm)
Blitz-Green	523
Greenwop	515
Redwop	597
Blitz-Red	599
Ultra-Yellow	510
Ultra-Orange-Yellow	585
Ultra-Red	592
Ultra-Gold	512
Ultra-Green	507
Ultra-Azure-Blue	450
Ultra-White	447

Lifetime measurements of the fingerprint samples were carried out with the help of a lifetime measurement spectrofluorometer ('Mini-Tau') as mentioned in section 5.3.3. LED at 380 nm (pulse width 300 ps) is used to excite the fingerprints treated with fluorescent powders such as Blitz-green, Greenwop, Ultra-gold, Ultra-green, Ultra-azure-blue and Ultra-white while, 498 nm (pulse width 600 ps) LED excitation for fingerprints treated with Blitz-red, Redwop, Ultra-yellow, Ultra-orange-yellow and Ultra-red powders.

Decay plot and lifetime value obtained for these samples are shown in Figures 6.1 to 6.11. The smallest lifetime value (in the sub nanosecond range) is attributed to contribution due to the scattered light coming from the sample.

Figure 6.1 Fluorescence decay plot for Blitz-green treated fingerprint sample

Figure 6.2 Fluorescence decay plot for Greenwop treated fingerprint sample.

Figure 6.3 Fluorescence decay plot for Redwop treated fingerprint sample.

Figure 6.4 Fluorescence decay plot for Blitz-red treated fingerprint sample.

Figure 6.5 Fluorescence decay plot for Ultra-yellow treated fingerprint sample.

Figure 6.6 Fluorescence decay plot for Ultra-orange-yellow treated fingerprint sample.

Figure 6.7 Fluorescence decay plot for Ultra-red treated fingerprint sample.

Figure 6.8 Fluorescence decay plot for Ultra-gold treated fingerprint sample.

Figure 6.9 Fluorescence decay plot for Ultra-green treated fingerprint sample.

Figure 6.10 Fluorescence decay plot for Ultra-azure-blue treated fingerprint sample.

Figure 6.11 Fluorescence decay plot for Ultra-white treated fingerprint sample.

The lifetime value obtained for fingerprints treated with various fluorescent powders are summarised in Table 6.2. The lifetime values obtained were all lie in nanosecond time range. Fingerprints treated with different fluorescent powders show different lifetimes and hence it allows a ‘signature’ characterisation of them. This helps in

identifying a fingerprint sample treated with a particular fluorescent powder by measuring its lifetime.

Table 6.2 Fluorescence lifetime of fingerprints treated with fluorescent powders.

Powders	Fluorescence lifetime value (ns)
Blitz-Green	8.96 ± 0.01
Greenwop	2.38 ± 0.01
Redwop	4.22 ± 0.02
Blitz-Red	3.85 ± 0.01
Ultra-Yellow	3.31 ± 0.02
Ultra-Orange- Yellow	3.58 ± 0.01
Ultra-Red	3.73 ± 0.03
Ultra-Gold	3.37 ± 0.03
Ultra-Green	2.60 ± 0.01
Ultra-Azure-Blue	3.44 ± 0.02
Ultra-White	3.36 ± 0.02

The observed nanosecond lifetime for the fingerprint samples treated with different fluorescent powders show that they can be used for time-resolved imaging when background lifetime is shorter than that of treated fingerprints. Significance of lifetime study in the context of TR imaging of fingerprints with nanosecond resolution is explained in Appendix B. These data are also useful in the PR imaging of latent fingerprints as explained in the following sections.

6.3 HOMODYNE ASSISTED PHASE-RESOLVED IMAGING

Fingerprints deposited on various substrates were tested using homodyne assisted PR imaging. These substrates include common and mostly interacting porous substrates such as fluorescing colour paper, magazine paper, smooth and shining papers etc. Experiments were also carried out for fingerprints deposited on nonporous material such as metal sheets, metal clamps, glass slides, transparency plastic sheets etc. A fingerprint deposited on difficult surfaces like currency notes was also examined. Simulated experiment was carried to show the superiority of PR technique over TR imaging when dealing with fingerprint fluorescence emission lifetime is shorter than that of background and also when their emission wavelength lie in close range.

Higher excitation power of few tens of milliwatt is used when the sample fluorescence emission intensity is very weak. This is significant when imaging the inherent fluorescence from un-treated fingerprint samples. Alternatively, gain voltage of the ICCD is increased to have higher intensity for the captured image. The image intensifier, which is controlled by the HRI, gives necessary voltage to the intensifier unit to amplify the weak fluorescence signals. The higher gain voltage to the image intensifier will result in better amplification of the weak fluorescence emission and hence in better intensity. Generally, intensifier is driven by a relatively low voltage of about 260-350 V when detecting strong fluorescence emission such as those treated fingerprints on fluorescing smooth paper. When imaging weak and old fingerprints deposited on non-fluorescing substrates, a higher gain of about 500 V is applied to the intensifier.

6.3.1 Imaging of fingerprints deposited on porous surfaces

(a) Simulated samples on fluorescing paper

In order to simulate a condition of having very close lifetime and emission wavelengths for fingerprint and background fluorescence, print deposited on highly fluorescing colour paper is used. In the first experiment, fingerprint deposited on fluorescing green paper (emission wavelength 516 nm and lifetime of 3.1 ns) is enhanced by Blitz-green fluorescent powder (emission wavelength 523 nm and lifetime of 8.96 ns). The fluorescence emission spectra of this sample at 476 nm excitation are shown in Figure 6.12. It was noted that background fluorescence intensity is much higher than that of fingerprint fluorescence. The back reflection of excitation light from the treated fingerprint sample was very high and hence the corresponding spectrum peak is not visible. This is due to the saturation of spectrometer sensitivity at this high emission intensity.

Figure 6. 12 Fluorescence emission spectra of Blitz-green treated fingerprint sample on fluorescing green paper at 476 nm excitation.

The sample was excited with 476 nm laser modulated at 25 MHz with an average power of 15 mW. Since the two fluorescence emissions are close in wavelength range, optical filtering method to suppress the background emission is not a fruitful option. As shown in Figure 6.13a, which is obtained by conventional digital imaging (normal optical filtering), shows the poor contrast of the fingerprint ridges. This poor contrast is due to the overlapping of the two fluorescence emissions. Moreover, it was noted that background fluorescence emission intensity is higher than that of fingerprint fluorescence.

Figure 6.13b, obtained using TR technique with nanosecond resolution, shows that the contrast of the ridge details has improved. However, total elimination of background fluorescence was not possible. This may be possible only when the fingerprint fluorescence lifetime is extended into millisecond range with the application of rare-earth chemicals. But this technique is not user friendly and imposes other challenges.

The advantage of the proposed PR technique over the above mentioned TR technique can be seen by comparing figures 6.13b and 6.13c. As shown in figure 6.13c, which is obtained using the PR technique, total background fluorescence suppression was possible and ridge contrast was improved by homodyne signal processing assisted phase-resolved technique. All the images presented are the result of the average of 10 images at the same phase or time delay settings. This will help to improve the image qualities.

Figure 6.13a Fingerprint deposited on fluorescing paper substrate: Conventional digital image.

Figure 6.13b Fingerprint deposited on fluorescing paper substrate: TR image.

Figure 6. 13c Fingerprint deposited on fluorescing paper substrate: Homodyne assisted PR image.

The phase between the two function generators was changed in a suitable way to suppress the unwanted background fluorescence. From this result it is obvious that PR technique can improve the contrast, resulting in better image over TR technique. This is significant when dealing with practical cases where background fluorescence and fingerprint fluorescence lie in close wavelength range.

In the second experiment, fingerprint deposited on smooth fluorescing paper (emission wavelength 516.5 nm and lifetime of 2.76 ns) is enhanced by the Greenwop fluorescent powder (emission wavelength 515 nm and lifetime of 2.38 ns). The fluorescence emission spectra of this sample at 476 nm excitation are shown in Figure 6.14. Here the background fluorescence emission intensity was also found to be higher than that of treated fingerprint fluorescence.

Figure 6.14 Fluorescence emission spectra of greenwop treated fingerprint sample on smooth fluorescing paper at 476 nm excitation.

The sample is excited using modulated (50 MHz) laser light operating at 476 nm and 20 mW power. The wavelengths of the two fluorescence emissions are too close to be separated using optical filters. The image of the fingerprint obtained by conventional imaging in the presence of strong background fluorescence is shown in figure 6.15a. It is clear that the contrast of the ridge details is poor. TR technique also

cannot be applied in this case to suppress the background emission, as the fingerprint lifetime is shorter than that of the background fluorescence. As shown in figure 6.15b, which is obtained using the PR technique, the contrast of the fingerprint is improved successfully by suppressing the strong background fluorescence.

Figure 6.15a Fingerprint deposited on fluorescing paper having emissions at very close lifetime and wavelength values: Conventional digital image.

Figure 6.15b Fingerprint deposited on fluorescing paper having emissions at very close lifetime and wavelength values: Homodyne assisted PR image.

As shown in earlier case, TR technique of imaging can be successful only when the lifetime of fingerprint fluorescence is longer than that of background. But in the proposed PR technique, irrespective of the lifetime difference, the suppression of strong background emission can be achieved. PR technique will work whether the fingerprint lifetime is shorter or longer than that of background emission. Another important point is that in PR technique the contrast of the fingerprint is better compared to image obtained using TR technique with nanosecond resolution. In all these experiment a long pass filter, which will transmit light above 500 nm is used to cutoff the excitation-scattered light from the sample.

(b) Fluorescing smooth papers and magazines

Imaging results of fingerprints deposited on smooth fluorescing colour magazine paper is shown in Figures 6.16a and 6.16b. As the fingerprint is fresh, usage of fluorescent powders to enhance the ridge details can be avoided. In order to excite the weak inherent fluorescence from the sample, 351 nm laser light from the argon laser, which is modulated at 20 MHz is used. A long pass optical filter which cutoff all the emissions below 400 nm is used to cut off the excitation light, which is scattered and reflected from the sample. Figures 6.16a show the fingerprint image by conventional imaging while; Figure 6.16b shows the PR image of the same fingerprint sample. Generally the inherent weak fluorescence from fingerprint sample will be in green-yellow region of visible spectrum.

Figure 6.16a Fingerprint deposited on fluorescing magazine paper: Conventional digital image.

Figure 6.16b Fingerprint deposited on fluorescing magazine paper: Homodyne assisted PR image.

It is obvious that the contrast of the fingerprints is very poor and hence ridge details are not clear by ordinary method of imaging. This is due to the strong fluorescence emission from background. But, it is evident that by PR imaging, the contrast is

improved and one can identify the ridge details clearly. This is due to the successful suppression of background fluorescence.

The prime significance of PR method is again demonstrated in the case of imaging of fingerprints deposited on fluorescing smooth calendar paper. This sample was provided by CID, of Singapore Police Force. Background fluorescence is strong and it has two fluorescing components. Hence, application of blitz green fluorescent powder enhances the fingerprint ridges. Figure 6.17 shows the decay plot and the lifetime values obtained for the fluorescing sample substrate.

Figure 6.17. Decay plot and lifetimes values of multi fluorescing calendar paper.

It was found that the background has two major fluorescence components with lifetime values 13.42 ns and 2.56 ns. The other smaller lifetime values are attributed to the contribution from the scattered light. As given in Figure 6.1, lifetime value of

fingerprint sample treated with Blitz-green powder was 8.96 ns, which is shorter than that of the major fluorescence component (13.42 ns) from the background.

Since the background fluorescence has a component with lifetime value longer than that of treated fingerprint, TR technique of imaging would not be a fruitful option. Figure 6.18a shows the conventional digital imaging result of the sample at 20 MHz modulation of laser at 351 nm excitation. As in earlier case, a 400 nm long pass optical filter is used to remove the excitation-scattered light. Due to the strong multiple fluorescence emissions from the background, fingerprint ridges are totally invisible. The dotted circle area shows the location of fingerprint in the image. Figure 6.18b shows the homodyne assisted PR imaging result of the same sample.

Figure 6.18a Fingerprint deposited on multi fluorescing calendar paper:
Conventional digital image.

Figure 6.18b Fingerprint deposited on multi fluorescing calendar paper:
Heterodyne assisted PR image.

As in earlier cases, it is clear that background fluorescence suppression was achieved successfully by imaging the fingerprint fluorescence. Contrast of the ridge details was improved the ridges can be identified clearly. This result validates the advantages of PR technique over the existing TR technique when fingerprints

deposited on fluorescing backgrounds, especially the fingerprint lifetime is shorter than that of background.

6.3.2 Imaging of fingerprints deposited on non-porous surfaces

The presence of strong excitation-scattered light makes the situation worse when imaging the fingerprints deposited on nonporous and shiny surfaces like metal sheets. Theoretically it is possible to cut off this excitation-scattered light with the help of long pass optical filters. But, when using high excitation power of the order of few tens of milli watts, the intensity of back reflected and scattered light from the non-fluorescing substrate will be high. Hence, practically it's very difficult to achieve 100% filtering off the scattered light from the background by optical filters. Also, due to the high sensitivity, ICCD camera detects even the faintest scattered and reflected photons from the sample streaking through filters and resulting in poor image contrast in conventional imaging.

(a) Non-fluorescing glass and metal surfaces

In criminal investigation, fingerprints deposited on glass surfaces often impose challenging problems. Dusting with powders may lead to good results. But when the print is weak dusting with powders is not a fruitful option. Here, detection of fingerprint deposited on glass and metal surfaces was examined without any chemicals. Samples were excited with 351 nm laser modulated at 25 MHz. As mentioned earlier, in non-porous surfaces such as glass and metal sheets, higher excitation power of about 100 mW is used to excite the weak inherent fluorescence from the sample. Figures 6.19-6.21 show the images of fingerprints deposited on

glass and aluminum clamp surfaces. It has to be noted that these prints are not treated with fluorescent chemicals for ridge contrast enhancement as in the previous case.

Figure 6.19. One-week-old Fingerprint deposited on glass substrate: Homodyne assisted PR image.

Figure 6.20. Two-week-old Fingerprint deposited on glass substrate: Homodyne assisted PR image.

Figure 6.21a One weak old fingerprint deposited on aluminum clamp substrate: Conventional digital image.

Figure 6.21b One weak old fingerprint deposited on aluminum clamp substrate: Homodyne assisted PR image.

It can be noted that Image quality and hence contrast of fingerprint image was improved by removing the excitation scattered light in PR imaging. These images shows that PR technique can be used to image the fingerprints deposited on non-porous and non-fluorescing substrates without any chemical.

6.3.3 Imaging of older fingerprints

When fingerprint gets older, the residue gets evaporated due to the influence of atmospheric conditions and hence only little residue is left behind. The chemical methods of lifting the prints often fail in such cases. In order to get good results by chemical treatment, the substrate must have enough residue left behind. Moreover, once a chemical is applied to such weak prints, further treatment with other chemicals is impossible because the targeted chemical constituent of fingerprint might have partially or fully reacted with the first chemical itself. Hence in most of the practical cases, conventional methods for old fingerprint detection are futile.

Figures 6.22-6.23 show the images of old fingerprints deposited on thin aluminum foil, which are failed to be detected to optimum level by any of the existing methods. Samples were excited at 351 nm laser light from the argon laser, which is modulated at 20 MHz. Figures 6.22a and 6.22b show the image of 18-month-old sweat fingerprint with conventional digital imaging method and the PR technique respectively. Figures 6.23a and 6.23b show the image of a 2-year-old fingerprint contaminated with oil with conventional digital imaging and PR technique respectively. Like in earlier cases, In order to obtain all these images, a long pass optical filter is used to cut off the excitation light, which is reflected and scattered from sample.

Figure 6.22a Sweat fingerprint deposited on aluminum substrate: Conventional digital image.

Figure 6.22b Sweat fingerprint deposited on aluminum substrate: Homodyne assisted PR image.

Figure 6.23a Oily fingerprint deposited on aluminum substrate: Conventional digital image.

Figure 6.23b Oily fingerprint deposited on aluminum substrate: Homodyne assisted PR image.

It's clear that the contrast of the fingerprints is poor and hence ridge details are not clear by conventional method of imaging. But, it is evident that by PR imaging, the contrast has improved and the ridge details can be clearly identified even though the prints are very old. Generally, detection of older fingerprints is not fully successful by any of the existing methods. The improved imaging result using the PR technique shows its significance when dealing with older prints without using any chemicals.

Figures 6.24a and 6.24b are the images of fingerprint on a fruit juice drink cover recorded with conventional digital method and PR technique respectively. Like in earlier case, this fingerprint is also of nearly 2 yrs old. Fingerprint residues on this substrate have almost dried off because of aging and dry storage conditions. Moreover, the substrate also shows strong fluorescence and hence literally no ridge details are visible using conventional method of imaging. Generally, such kinds of print will not help in fingerprint comparison, as it does not reveal the minimum ridge details required for the purpose. But, as shown in Figure 6.24b, using PR technique, the ridge details of the image are clearer after suppressing the background fluorescence and more ridges can be identified.

Figure 6.24a Fingerprint deposited on fluorescing fruit juice cover: Conventional digital image.

Figure 6.24b Fingerprint deposited on fluorescing fruit juice cover: Homodyne assisted PR image.

6.3.4 Imaging of fingerprints on ‘difficult’ surfaces

Fingerprint experts face difficulty in lifting fingerprints deposited on currency and similar ‘difficult surfaces’. Such kind of scenario often comes across in criminal investigations. Most of the currency will be nonporous and fluorescing up to an extent. Currency contains lot of writings and pictures, which often show multiple

fluorescence that makes the situation worse. Figures 6.25a show the image of a fingerprint deposited on a Singapore currency and imaged through ordinary digital imaging. Here the background also shows fluorescence with different lifetime of 2.85 ns and 3.23 etc. Ultra-azure-blue fluorescent powder was applied to enhance the fingerprint. Sample is excited with 351 nm laser modulated at 30 MHz. Circled area shows the location of fingerprint in the image. It is obvious that literally no ridge pattern is visible through ordinary imaging. But, Figure 6.25b shows the corresponding PR image. Fingerprint ridge details are clearer after suppressing the multiple background fluorescence from the currency.

Figure 6.25a Fingerprint deposited on currency: Conventional digital image.

Figure 6.25b Fingerprint deposited on currency: Homodyne assisted PR image.

In most of the cases, fingerprint deposited back to back on both sides of transparent substrates like thin and shiny plastic sheet and covers are also found difficult to develop. Due to the reflection of excitation light and overlapping of fluorescence emissions that coming from the top and bottom surfaces of transparency sheet makes the situation difficult to detect the fingerprints. Figure 6.26a-6.26d shows the images of fingerprint on such scenario. Since the surface is smooth and nonporous, fluorescent powders are applied to develop the prints. Fingerprint on one of the side

is treated with Blitz-green and other side with Ultra-azure-blue and excited with 351 nm laser light. Due to the overlapping of excitation-scattered light and fluorescence emissions from both sides, fingerprint ridge quality is poor. In order to minimise the effect of reflection and scattered light, sample is kept just above a black background and also a long pass optical filter is used. Fingerprint image on each side with normal and PR technique of imaging are given below.

Figure 6.26a Ultra-azure blue treated Fingerprint deposited on transparency sheet: Conventional digital image

Figure 6.26b Ultra-azure blue treated Fingerprint deposited on transparency sheet: Homodyne assisted PR image

Figure 6.26c Blitz-green treated Fingerprint deposited on transparency sheet: Conventional digital image.

Figure 6.26d Blitz-green treated Fingerprint deposited on transparency sheet: Homodyne assisted PR image.

It is quite obvious that ridge quality of the fingerprint patterns is improved considerably by suppressing the fluorescence emission from other side of transparency sheet. These results again show the supremacy of PR technique over any existing methods in detecting fingerprints on some ‘difficult surfaces’.

6.4 FINGERPRINT IMAGE QUALITY EVALUATION

In order to assess the quality of fingerprint image, an evaluation method that can give a quantified score for the image quality is proposed and presented here. For this, the sample pixels are obtained along two diagonal lines that are orthogonal to each other. A 200 X 200 square is chosen at the centre of image where ridge details of the print will be most concentrated. The absolute normalized intensity difference between the neighboring pixels is then added up and final score is obtained. Normalization is carried out using the highest intensity value in the sample pixel set. In most cases, the intensity variation along the diagonal lines is chosen for evaluation of the score.

Figure 6.27 shows the type of the diagonal sampling line that a fingerprint image will encounter. This region is represented by a pixel array as shown in Figure 6.28 that going diagonally will give us the following pixel data set,

$$[x_{11}, x_{22}, x_{33}, x_{44}, x_{55}, x_{66}, x_{77}, x_{88}] \quad (6.1)$$

x11	x12	x13	x14	x15	x16	x17	x18
x21	x22	x23	x24	x25	x26	x27	x28
x31	x32	x33	x34	x35	x36	x37	x38
x41	x42	x43	x44	x45	x46	x47	x48
x51	x52	x53	x54	x55	x56	x57	x58
x61	x62	x63	x64	x65	x66	x67	x68
x71	x72	x73	x74	x75	x76	x77	x78
x81	x82	x83	x84	x85	x86	x87	x88

Figure 6.27 Fingerprint image with the representative diagonal sampling line.

Figure 6.28 Representation of the pixel arrays that undergoing diagonal sampling.

Hence, the score that obtained from this sample region will be given by,

$$Score = \frac{[|x_{22}-x_{11}|+|x_{33}-x_{22}|+|x_{44}-x_{33}|+|x_{55}-x_{44}|+|x_{66}-x_{55}|+|x_{77}-x_{66}|+|x_{88}-x_{77}|]}{\max[x_{11}, x_{22}, x_{33}, x_{44}, x_{55}, x_{66}, x_{77}, x_{88}]}$$

6.2)

It is to be noted that the score obtained for the image will be higher if the numbers of ridges present are higher, or greater the variation in pixel intensity values in the neighborhood. This evaluation process is in general only suitable for image quality of fingerprints and their intensity variation of ridges.

Few sets of images are taken and their quantified score was found out. The scores that quantify the quality of the image obtained by the PR and conventional digital imaging/TR image is given in Table 6.3. The Figures corresponding to the each image are mentioned in the table.

It was evident from these experiments that the PR image consistently has a higher score than that for conventional image. It is to be noted that the score obtained for the image will be higher if the numbers of ridges present are higher, or greater the variation in pixel intensity values in the neighborhood. Hence, it is quantitatively demonstrated that the image quality of PR image is better than that of conventional or TR imaging.

Table 6.3 Quantitative comparison of the fingerprint image quality obtained using homodyne assisted PR and conventional digital imaging method.

Imaging Scheme		Score
Conventional/ TR technique	Figure 6.13b	12.4
	Figure 6.15a	8.3
	Figure 6.18a	9.7
	Figure 6.22a	16.1
	Figure 6.23a	14.7
	Figure 6.24a	6.5
PR Technique	Figure 6.13c	40.2
	Figure 6.15b	28.3
	Figure 6.18b	21.1
	Figure 6.22b	38.3
	Figure 6.23b	53.9
	Figure 6.24b	75.8

6.5 SENSITIVITY IMPROVEMENT FOR HOMODYNE ASSISTED PHASE-RESOLVED IMAGING

Generally, it is difficult to get an image with better contrast when fingerprint fluorescence is overshadowed by the strong background fluorescence. Also, in the case of fingerprint samples, the inherent fluorescence itself will be very weak. Hence, it is of great importance to improve the sensitivity of the PR fingerprint imaging system. Section 4.4 of this thesis discusses the theoretical formulation of combining $\text{Pi } (\pi)$ shift and even-step-shift method in homodyne assisted PR imaging for the sensitivity improvement for the fingerprint image.

To study the improvement in the sensitivity aspect, experiments were carried out on fingerprint samples deposited on two separate fluorescing colour paper. To distinguish, they are named as 'first' and 'second' sample. It was found that the inherent fingerprint fluorescence from sample is weak due to aging and hence to increase its emission intensity to an extent, prints are enhanced by Blitz-green fluorescent powder. Background fluorescence is much stronger in intensity than that of treated fingerprint under UV light excitation from the argon laser. Here, the laser is modulated at 20 MHz and the average power was about 60mW. Further increase in laser intensity will result in higher background fluorescence and ultimately reach the intensity saturation of the ICCD camera. In this scenario, increase in excitation power alone will not result in better emission intensity and hence improved contrast for fingerprint sample.

During the experiments, the DC offsets are removed first by using even-step phase shifting method. Then the phase difference between the modulated excitation and the modulated gain of the ICCD is adjusted until the background fluorescence goes to zero. The corresponding image gives the normal homodyne assisted phase-resolved image for the sample. Once the phase where the background fluorescence goes to zero is found, the two phases required for pi-shift method can be determined. By taking two images at these two phases, which differ by 180° , and applying the digital image subtraction process will result in the final fingerprint image.

Figure 6.29a is the fingerprint image of the first sample obtained by normal homodyne assisted PR imaging while Figure 6.29b is the image after applying pi

shifting along with homodyne PR imaging for the fingerprint on strongly fluorescing colour paper. For comparison, Figures 6.29a and 6.29b are displayed under the same intensity scale. It is clear that image contrast is poor for this fingerprint sample even after applying homodyne assisted PR technique. This is due to the aging of the fingerprint, which results in low fluorescence emission intensity. But, as shown in Figure 6.29b, image has higher intensity and hence better contrast compared to that of the image in Figure 6.29a. This highlights the significance of applying sensitivity improvement along with homodyne assisted PR technique when dealing with weak fluorescence emissions.

Figure 6.29a Fingerprint image of the first sample obtained by normal homodyne assisted PR imaging.

Figure 6.29b Fingerprint image of the first sample obtained by pi shift combined homodyne assisted PR imaging.

The significance of this method is again depicted in Figures 6.30a and 6.30b. These are images of Blitz-green treated fingerprint on second fluorescing colour paper, which is different from the first sample. The image of the fingerprint obtained by normal homodyne assisted PR imaging is shown in Figure 6.30a. It can be seen that the intensity and hence contrast of the ridge details is poor. As shown in Figure

6.30b, which is obtained by the pi shift combined homodyne assisted PR imaging technique, improved sensitivity was achieved for the fingerprint image. It is obvious that more ridge details are visible with the help of sensitivity improvement method.

Figure 6.30a Fingerprint image of the second sample obtained by normal homodyne assisted PR imaging.

Figure 6.30b Fingerprint image of the second sample obtained by pi shift combined homodyne assisted PR imaging.

Both the intensity and the contrast of the fingerprint image are also improved significantly. Theoretically, image contrast and hence sensitivity can be improved by two times after combining pi-shifting method along with homodyne assisted PR technique. The above experimental results confirm the improvement in sensitivity of homodyne assisted PR technique by incorporating the pi-shifting method into it.

6.6 HETERODYNE ASSISTED PHASE-RESOLVED IMAGING

Fingerprints deposited on porous and nonporous substrates are tested using heterodyne assisted PR imaging. These substrates include fluorescing colour paper, smooth and shining papers, nonporous material such as aluminum metal foils etc. The importance of the technique in suppressing the background fluorescence as well

as eliminating the excitation-scattered light was achieved with the help of heterodyne assisted PR technique to result in better fingerprint image quality. Samples earlier examined using homodyne assisted PR technique were used here again. This will help in a comparing the two techniques under fixed experimental settings.

The fingerprint sample is excited with a multi line UV light from the argon laser, which has a predominant laser line at 351 nm. The approximate power of the laser beam at the output end of the liquid light guide was varied from 30 mW-70 mW depending on the nature of substrate and fluorescence emission intensity.

Imaging results of fingerprints contaminated with oil and deposited on thin aluminum sheet is shown in Figure 6.31. It is to be noted that this fingerprint is of nearly 2 years old. Figure 6.31a shows the image of the print by conventional ICCD imaging with the help of optical filter. Fingerprint residues on this substrate have dried off to a great extent because of aging and dry storage conditions. Figure 6.31b shows the respective PR imaging result of the same sample. Figure 6.32a shows the image of blitz green fluorescent powder treated fingerprint on highly fluorescing colour paper under normal imaging. The emission from background and treated fingerprint differ by only about 7 nm. Figure 6.32b shows the respective heterodyne assisted PR imaging result of the same sample.

Experiments were again carried out on fingerprints deposited on fluorescing smooth calendar paper. Background fluorescence is very strong and it has two fluorescing components as given in section 6.3.1(b). Blitz-green fluorescent powder enhances the

fingerprint ridge patterns. Figure 6.33a shows the conventional imaging result, while Figure 6.33b gives the PR imaging result of the same sample.

Figure 6.31a Oily fingerprint deposited on aluminum substrate: Conventional digital image.

Figure 6.31b Oily fingerprint deposited on aluminum substrate: Heterodyne assisted PR image.

Figure 6.32a Fingerprint deposited on fluorescing paper substrate: Conventional digital image.

Figure 6.32b Fingerprint deposited on fluorescing paper substrate: Heterodyne assisted PR image.

Figure 6.33a Fingerprint deposited on multi fluorescing calendar paper:
Conventional digital image.

Figure 6.33b Fingerprint deposited on multi fluorescing calendar paper:
Heterodyne assisted PR image.

Comparison of set of figures in each case shows that suppression of background fluorescence and excitation scattered light from samples was achieved successfully by imaging the fingerprint fluorescence. Contrast of the ridge details was improved and one can identify the ridges clearly. Imaging of older and contaminated fingerprints on nonporous surfaces was also successfully carried out.

6.7 COMPARISON OF HOMODYNE AND HETERODYNE ASSISTED PR IMAGING TECHNIQUES

Theoretical comparison of homodyne and heterodyne assisted PR imaging given in section 4.5 revealed that for a given sample and under fixed settings, it is always favorable to work with homodyne assisted PR method to achieve better contrast for fingerprint image. This result will be significant when the fingerprint is deposited on fluorescing substrates. These experiments were carried out at the fixed settings such as 500 ms camera exposure time and 1 Hz heterodyne frequency. Theoretically under these conditions, contrast the image obtained by homodyne assisted PR technique is approximately 1.57 times better than that of heterodyne assisted PR technique.

Comparing the fingerprint images as given in Figures 6.23b with 6.31b, 6.13c with 6.32b, and 6.18b with 6.33b, which are homodyne and heterodyne assisted PR images, shows that homodyne assisted PR method is always superior to heterodyne assisted PR method and hence result in better quality and contrast for the fingerprint ridges. Moreover, experimental procedure to carry out heterodyne assisted PR method is complicated compared to homodyne assisted PR technique. The synchronisation of two function generators and the 'external trigger' of ICCD through a low frequency oscillator without any time delay is tricky and hence chance of experimental error is high. This will result in a time delay between the triggering of these units and result in difficulty to achieve the theoretical accuracy. In homodyne assisted PR method, ICCD camera is not required to work in external trigger mode and hence, makes the procedure easier to carry out. It was also noted that at longer duration of experiment, slight drift in the frequency occurs for the signal generated by the low frequency oscillator, which in turn may result in non-uniform integration of the fluorescence signal to achieve the phase resolved detection.

6.8 SUMMARY

This chapter presented the results and discussion of the homodyne and heterodyne assisted PR technique of imaging for latent fingerprints on different substrate surfaces. Fluorescence lifetime of fingerprints treated with various fluorescent powders was carried out. Significance of lifetime characterisation was successfully demonstrated in the case of PR imaging of latent fingerprints. A quantitative image

quality evaluation was carried to show the supremacy of PR technique over conventional techniques of imaging. Sensitivity improvement for latent fingerprint imaging by combining even step phase shift along with π shift method in homodyne assisted PR imaging was carried out and experimental results were presented. Finally, an illustrative comparison of homodyne and heterodyne signal processing assisted PR imaging techniques was also presented with experimental data.

CHAPTER SEVEN

PHASE-RESOLVED IMAGING FOR BIOMEDICAL APPLICATIONS

7.1 INTRODUCTION

This chapter gives the experimental results of the PR imaging of sample simulated with fluorescein chemicals having same emission wavelength but differing in their lifetime values. This finding would be useful in various biomedical and biochemical applications of fluorescence emissions, which are in close wavelength ranges. Later, imaging of DNA samples in micro capillary tubes is carried out with the help of PR technique. This technique provides better sensitivity for imaging due to the suppression of unwanted scattered and reflected light from the sample environment.

7.2 SEPARATION OF FLUORESCENCE EMISSIONS WITH SUB NANOSECOND RESOLUTION

Homodyne assisted PR imaging technique is proposed for the separation of close wavelength fluorescence emission wavelengths with sub nanosecond resolution. Experiments were carried out with simulated samples with fluorescein chemicals having same wavelength but with difference in their lifetime values. Suppression of either of the emissions by selective imaging of the other validates the superiority of the proposed technique.

7.2.1 Sample preparation

Fluorescein chemical is one of the commonly used exogenous fluorophore in many bioimaging applications. Fluorescein sample having different lifetime value but same emission wavelength was used in this experiment to show the feasibility of proposed PR imaging scheme in achieving sub nanosecond resolution for fluorescence lifetime. In order to simulate these conditions, Fluorescein sample at different concentrations but at fixed pH value was used as the test sample. This dependency of concentration on lifetime may attribute to quenching effect, which alters the non-radiative transition rate of fluorophore in the solutions. Fluorescein and Phosphate Buffered Saline (PBS) were purchased from Sigma-Aldrich and used without further purification. Fluorescein solutions at different concentrations were prepared by dissolving it in distilled water at a 0.01M PBS having a pH value of 7.4. Fluorescein samples at concentration of 10 ml/litre, 40 ml/litre, 60 ml/litre and 100 ml/litre were prepared. Pairs of these solutions were taken as test samples. Imaging of the fluorescence emission from any of the solution by the simultaneous suppression of other validates the significance of this technique.

7.2.2 Phase-resolved imaging of the sample

Prior to the PR imaging, the fluorescence emission spectrum of fluorescein sample excited at 402 nm (diode laser) was obtained using a spectrometer (Avantes) as shown in Figure 7.1. It was noted that sample is showing fluorescence emission at 514.75nm.

Figure 7.1 Fluorescence emission spectra of fluorescein sample at 402 nm excitation

Later, fluorescence lifetimes of each of the solutions were studied using ‘Mini-Tau’ spectrofluorometer. The light from the 498 nm pulsed LED (pulse width 600 ps) was used to excite the sample. The decay plots obtained for fluorescein samples at different concentrations are given in Figures 7.2-7.5. The corresponding lifetime values that are evident from the graph are given in Table 7.1. The lowest lifetime component in each case was attributed to the contribution from scattered light.

Figure 7.2 Fluorescence decay plot of fluorescein sample at 10 ml/lit concentration

Figure 7.3 Fluorescence decay plot of Fluorescein sample at 40 ml/lit concentration

Figure 7.4 Fluorescence decay plot of Fluorescein sample at 60 ml/lit concentration

Figure 7.5 Fluorescence decay plot of Fluorescein sample at 100 ml/lit concentration

This proves that by varying the concentration, sample at different lifetime but at same emission wavelength can be obtained. Hence, by varying the concentration, fluorscein samples with very close lifetime values can be obtained. It is well known that an excitation-collection at 90-degree-geometry could lead to inner filter effect

resulting in the wavelength shift of fluorescence emission [109]. To eliminate this effect, the illuminated region should be thin and close to detector. Therefore, the laser diode is positioned to excite only the surface layer of the solution. This consideration helps to mimic samples with same emission wavelengths but closely separated fluorescence lifetime values.

Table 7.1 Fluorescence lifetime of fluorescein sample at different concentrations

Fluorescein sample at concentrations (ml/lit)	Lifetime value (ns)
10	4.49
40	5.39
60	5.59
100	5.80

Schematic diagram of PR imaging set up is shown in Figure 4.4. Instead of argon laser, a compact current modulated laser diode module (LDM, 405.40/50.350, Omicron-Laser age) was used to excite the sample. It can be modulated up to 100 MHz delivering a maximum power of 100 mW at 402 nm [110]. Laser can be modulated externally with the help of 0-1V sine wave signal from the function generator. All the remaining system details are as given in section 4.2 of this thesis. A long pass optical filter (Melles Griot, transmission above 470 nm) connected to the camera lens is used to cut off the excitation-scattered light from the sample.

Initially, experiments were carried out by placing one drop of fluorescein solution having concentrations at 10 ml/lit and 100 ml/lit side by side on a glass slide. Laser modulated at 25 MHz at an average excitation power of 20 mW was used to excite the fluorescein sample. It was found that due to the difference in concentration, intensity of emission is also different. Sample with 100ml/lit shows higher emission intensity compared to that of sample at 10ml/lit. The lifetime difference between the samples in this case was 1.31 ns. Figure 7.6 shows the PR imaging results for this sample. The fluorescing spot on left hand side (when facing the paper) is due to the fluorescein sample at concentration 10ml/lit and the one on right hand side is due to 100ml/lit concentration solution.

Figure 7.6a Homodyne assisted PR image of fluorescein sample: Suppression of emission with lifetime 5.80 ns by selectively imaging 4.49 ns emission.

Figure 7.6b Homodyne assisted PR image of fluorescein sample: Suppression of emission with lifetime 4.49 ns by selectively imaging 5.80 ns emission.

Figure 7.6a shows that by homodyne assisted PR imaging, suppression of fluorescence component with lifetime 5.80 ns was achieved by selective imaging of lower lifetime component at 4.49 ns. Full suppression of larger lifetime component was not possible due to its higher emission intensity compared to the other. Figure 7.6b shows that suppression of fluorescein sample having lifetime 4.49 ns was successfully achieved by selective imaging of the longer lifetime component at 5.80 ns, which has higher emission intensity due to higher concentration. Hence, these results show the imaging of samples with sub nanosecond resolution using PR technique. Samples are getting dried off due to the high excitation power and hence showing drastic decrease in emission intensity.

In order to overcome this difficulty, samples at different concentrations were taken in two quartz cuvettes and positioned on the working area of imaging system. The fluorescence signals were then captured from the top of cuvettes by the ICCD camera. To eliminate the undesirable inner filter effect, the illuminated region should be thin and close to detector. Therefore, the laser excitation is adjusted in such way that it excites only the top surface layer of the solution. In this experiment, fluorescein samples having concentrations 40 ml/lit and 100 ml/lit were chosen. It was obvious that the lifetime difference between these emissions was only 0.41 ns.

Figure 7.7 shows the homodyne assisted PR imaging result of this sample. Fluorescence on top is due to fluorescein sample having lifetime 5.80 ns and the one on bottom is due to 5.39 ns. Figure 7.7a shows that total suppression of fluorescein sample fluorescence having lifetime 5.80 ns was successfully achieved by selectively

imaging the other component with lifetime 5.39 ns. Similarly, Figure 7.7b shows the total fluorescence suppression of sample having lifetime 5.39 ns by selective imaging the other component having lifetime 5.80 ns. The achieved time resolution of 0.41 ns in imaging is noteworthy since the fluorescein samples have difference in emission intensity due to concentration difference.

Figure 7.7a Homodyne assisted PR image of fluorescein sample: Suppression of emission with lifetime 5.80 ns by selectively imaging 5.39 ns emission

Figure 7.7b Homodyne assisted PR image of fluorescein sample: Suppression of emission with lifetime 5.39 ns by selectively imaging 5.80 ns emission

In the next experiment, a higher time resolution is achieved as shown in Figure 7.8. In this case, fluorescein sample at concentrations 60 ml/lit and 100 ml/lit having a lifetime of 5.59 ns and 5.80 ns respectively was used. The lifetime difference between the emissions was only 0.21 ns, which is the most sensitive PR imaging ever reported. Like in earlier case, fluorescence on top is due to sample having lifetime 5.80 ns and the one on bottom is due to 5.59 ns. Figure 7.8a shows that total

suppression of fluorescein sample fluorescence having lifetime 5.80 ns was successfully achieved by selectively imaging the other component having lifetime 5.59 ns. Again, Figure 7.8b shows that total suppression of fluorescein sample fluorescence having lifetime 5.59 ns by simultaneous imaging of 5.80 ns fluorescence component.

Figure 7.8a Homodyne assisted PR image of fluorescein sample: Suppression of emission with lifetime 5.80 ns by selectively imaging 5.59 ns emission.

Figure 7.8b Homodyne assisted PR image of fluorescein sample: Suppression of emission with lifetime 5.59 ns by selectively imaging 5.80 ns emission.

PR imaging of fluorescence components with such improved high resolution of 0.21 ns is reported here for the first time. The significant aspect to be noted is that this high resolution is achieved for fluorescence emissions having same wavelength and difference in emission intensity. Generally, when detecting endogenous or exogenous fluorescence emissions from biological cells, they can only be in close wavelength range but never will be at same wavelength. The high accuracy in lifetime selective

imaging reported here can find applications in the early detection of cell abnormalities.

This technique can even be applied in the early detection of cancer growth in living cells. It is understood that the typical lifetime difference for benign and cancerous tissues at initial stage of growth is around 0.4ns [111]. This shows that fluorescence property of normal cells will be different from that of abnormal ones. The difference in cell fluorescence property such as lifetime and emission wavelength will be very small compared to normal cells at the beginning of abnormality growth. The ability of PR technique to resolve and separate fluorescence emissions, which are close in wavelength and lifetime value, can be applied in such cases that will provide a tool for early detection of cell abnormalities.

7.3 DETECTION OF DNA SAMPLE FLUORESCENCE IN MICRO CAPILLARY TUBE

Detection of genomic DNA samples in capillary tube was carried out with high sensitivity using PR imaging technique. Detection of DNA sample tagged with specific fluorescent dyes often lead to conclusive results in the detection of some genomic disorders and mutation of DNA. It helps in identifying the damage of DNA through the irregularity in the DNA bases and their sequences.

7.3.1 Sample preparation

DNA samples (G304A, Promega) was mixed with SYBR green dye (Molecular Probes) and enzyme or otherwise called DNA polymerase (Platinum Taq, Catalogue

no 10966-018, Invitrogen) in an eppendorf (small mixing tube) at room temperature. DNA polymerase is responsible for the PCR of the sample and hence in amplification of fluorescence from the sample. These samples are transferred into micro capillary tubes with help of micropipette. Circular glass capillaries of 5cm long and having a radius of 0.27 mm was used in the experiment. Sample volume of as low as 5 microlitre and 10microlitre were tested in the experiments. A concentration of 0.1 ng/microlitre of DNA and a SYBR green dye at relative concentration of 1X was used. Enzyme concentration of 0.1 unit/microlitre was mixed with DNA sample tagged with SYBR green dye. One unit of platinum Taq DNA polymerase incorporates 10 nmol of deoxyribonucleotide into acid-precipitable material in 30 minute at 74 degree centigrade temperature.

7.3.2 Phase-resolved imaging of DNA fluorescence

Initially, DNA samples taken in a circular capillary was tested using PR imaging. One of the capillaries is filled with only DNA sample and other with DNA tagged with SYBR green dye. Ends of capillary tube were properly sealed after filling 5-microlitre volume of sample. The capillary tube with DNA attached to fluorescent dye should only show fluorescence effect. The other capillary tube filled with only DNA sample was used as the reference.

It was well understood that DNA samples tagged with SYBR green will show fluorescence at 510 nm up on blue laser excitation [112]. Hence, these two capillary tubes were placed side by side on a glass slide and excited with 488 nm laser from argon laser, which was modulated at 30 MHz at an average power of 4 mW. The

whole sample was covered with a small box to minimize the effect of ambient light. The resulting fluorescence emissions were then imaged using the ICCD camera, which is mounted vertically down as shown in Figure 4.1. Figure 7.9 shows the resulting images obtained by normal ICCD imaging and with PR technique. In both cases a long pass optical filter that transmit light above 500 nm was used to cutoff the excitation-scattered light from the sample environment.

Figure 7.9a shows that with normal digital imaging, the fluorescence emission from the capillary tube containing DNA and dye was not visible. This was due to the presence of strong excitation-scattered light, which in turn due to strong reflection from the shiny capillary and glass slide that overshadows the very weak fluorescence from the sample. Figure 7.9b shows the corresponding homodyne assisted PR image of the sample. It is quite obvious that fluorescence from the capillary tube was imaged successfully with better sensitivity. This was due to the suppression of strong excitation-scattered light and other unwanted fluorescence from the sample environment. The fluorescence from the sample is shown with dotted ellipse as in Figure 7.9b. The intensity of the fluorescence emission can be determined through the photon count, which is readily available through the software associated with the ICCD camera. Detection of emission intensity at a particular volume will characterise the DNA sample.

Figure 7.9a DNA sample taken in circular capillary: Conventional digital image.

Figure 7.9b DNA sample taken in circular capillary: Homodyne assisted PR image.

This result shows the high sensitivity of PR imaging technique when dealing with weak fluorescence from the test sample. Sample volume was as low as 5 microlitre and detection of fluorescence from such a low volume will give a direction for applying this technique in the detection of micro and nanolitre volume of DNA sample. This will provide cost effective fluorescence detection by minimising the use of expensive chemicals.

7.3.3 Detection of DNA amplification due to PCR

Preliminary study was carried out to detect the fluorescence amplification from DNA sample due to PCR with the help of PR technique. In this study, circular capillary tubes with the same dimension mentioned in earlier section were filled with DNA

samples. Sample volume of 10microlitre was used in this experiment. First capillary was filled with only genomic DNA, second one with DNA and SYBR green dye and the third capillary with DNA tagged with SYBR green dye and mixed with DNA Polymerase. This polymerase is responsible for the amplification of the fluorescence from DNA sample due to PCR. These capillary tubes were placed side by side on a glass slide and kept on a temperature controller that uses a Thermo Electric Controller (TEC) and hence temperature can be varied in cyclic way with maximum temperature of 95 degree and minimum temperature of 60 degree with constant temperature at 72 degree for 30 seconds. This will help in providing a step function like temperature profile. These temperature cycling is controlled through software.

Some of the initial results of DNA amplification due to PCR were presented in Figures 7.10-7.11 for 12th and 17th the temperature cycles. In all the figures the capillary marked 'a' is filled with only DNA, middle one (marked 'b') with DNA tagged with SYBR green dye along with DNA polyemrase and right capillary (marked 'c') with DNA tagged with SYBR green dye. Figure 7.10a and 7.11a shows the conventional digital image while 7.10b and 7.11b are the corresponding homodyne assisted PR image at two temperature cycling. There should not be any fluorescence from the capillary with only DNA sample. But, the bright spots seen in all the pictures for this capillary is actually due to the very strong reflection of excitation light from the edge of capillary tube. In the case of the 'b' and 'c' capillaries, this effect is negligible due to the presence of fluorescence from the sample.

Figure 7.10a DNA sample taken in circular capillary: Conventional digital image after 12th temperature cycling.

Figure 7.10b DNA sample taken in circular capillary: Homodyne assisted PR image after 12th temperature cycling.

Figure 7.11a DNA sample taken in circular capillary: Conventional digital image after 17th temperature cycling.

Figure 7.11b DNA sample taken in circular capillary: Homodyne assisted PR image after 17th temperature cycling.

Comparing figures 7.10b and 7.11b, it is clear that fluorescence intensity from the capillary, 'b' is high due to the amplification of fluorescence with PCR. It was understood that at the higher temperature cycle, the intensity will increase due to the PCR effect. The fluorescence from the sample was not visible through conventional imaging. Due to the suppression of unwanted excitation-scattered light and other fluorescence from the sample environment was possible by homodyne assisted PR technique. This will result in the more sensitive detection of DNA fluorescence amplification.

Detection of DNA amplification due to PCR with the help of PR technique is reported here for the first time. Such a detection technique provides a platform for the DNA fluorescence detection even for microlitre volume of the sample.

7.4 SUMMARY

Experimental results of the PR imaging of fluorescein chemicals having same emission wavelength but differing in their lifetime was reported with a resolution as low as 0.21 ns. This imaging technique has the potential applications in fluorescence lifetime imaging along with fluorescence microscopic research in biochemistry, biophysics, and cell physiology. Later, imaging of DNA samples in micro capillary tubes was carried out with the help of PR technique. Also, initial experiments were carried out to show the feasibility of applying PR techniques in detecting fluorescence due to DNA PCR amplification.

CHAPTER EIGHT

CONCLUSIONS AND RECOMENDATIONS

8.1 CONCLUSIONS

This chapter concludes the research work entitled ‘Investigation into phase-resolved optical techniques for latent fingerprint detection and bioimaging’ by highlighting the significant research findings as given below:

The attachment program at Scene of Crime Unit (SCU), Criminal Investigation Department (CID) of Singapore Police Force provided hands-on training to carry out the detection of latent fingerprint samples on various substrates. This helped to confirm the drawbacks of conventional methods, which was reported in the literatures. The attachment program helped to familirise with the chemicals and procedures that are currently in use to develop the fingerprints on various surfaces.

Theoretical formulation of PR imaging was carried out by incorporating the homodyne and heterodyne concept of signal processing. Theoretical formulation showed that separation and imaging of sample fluorescence can be achieved by suppression of background fluorescence, which may be in close wavelength range. Imaging of sample fluorescence can be achieved even if the fluorescence lifetime is longer or shorter than the lifetime of background fluorescence emission. Sensitivity improvement for the homodyne assisted PR imaging was carried out by incorporating the ‘pi’ shifting along with ‘even-step-phase shift’ method. Also, comparison of the relative performance

between homodyne and heterodyne assisted phase-resolved optical imaging technique was carried out.

PR imaging technique was successfully applied for the imaging of latent fingerprints deposited on various substrate surfaces. It was showed that PR technique can be used for the imaging of the latent fingerprints, which are deposited on strongly fluorescing backgrounds, even it's lifetime is longer than that of backgrounds. Imaging of old fingerprints was carried out with better contrast. Imaging of fingerprints deposited on some 'difficult' surfaces was also successfully carried out by PR technique. An Image quality evaluation for the fingerprint image obtained using PR technique was carried out. It quantitatively proves the quality of fingerprint image obtained by PR technique. The advantage of homodyne assisted PR imaging over heterodyne assisted PR imaging was both theoretically and experimentally demonstrated in the context of latent fingerprint imaging.

Developed PR optical imaging technique has many advantages over existing methods in the context of latent fingerprint detection and imaging. These are listed below:

- (i) It can image fingerprints on various surfaces and also detect few years old (up to 2 years) prints,
- (ii) This method can effectively be applied to suppress the unwanted fluorescence emissions from the background in almost all cases,
- (iii) Application of chemicals can be minimised for the detection of latent fingerprints,

- (iv) The developed PR technique is better in data storage capability as it is in digital form and later retrieval, which facilitate easy fingerprint comparison.

Time-resolved imaging of latent fingerprints was also carried out with nanosecond resolution. This technique is significant when dealing with practical cases having strong background fluorescence having nanosecond lifetime.

Experimental results of the PR imaging of fluorescein chemicals having same emission wavelength but differing in their lifetime was carried out with a resolution of as small as 0.21ns. This idea could be used in various biomedical and biochemical applications to selectively image a particular fluorescence emission from a mixture of emissions that lies in close wavelength and lifetime ranges.

Imaging of DNA samples in micro capillary tubes was successfully carried out with the help of PR technique. Detection of weak fluorescence from DNA sample with 5 microlitre volume proved the high sensitivity of PR technique when dealing with weak fluorescence emissions. This is especially significant for biochemical and biomedical applications where high sensitivity fluorescence detection is demanded. This technique can be used in the detection of micro and nanolitre volume of DNA sample. This will give cost effective fluorescence detection by minimizing the expensive chemicals. Also, initial experiments were carried out to show the feasibility of applying PR techniques in detecting DNA fluorescence due to PCR amplification.

8.2 RECOMMENDATIONS FOR FUTURE WORK

It was reported that fingerprint would show the inherent fluorescence when excited at deep UV wavelength. Limitation of getting modulated laser (up to 80 MHz) during the research work forced to use best available Argon laser with a 351nm. It is worth exploring the possibility of developing the system with deep UV wavelength of around 280 nm and can be modulated at tens of mega hertz frequencies.

EO modulator fails to give perfect sinusoidal output for the laser excitation at higher modulation frequencies. This distortion in waveform will result in poor depth of modulation. Higher depth of modulation is always recommended to have better imaging results when working with fluorescence emissions having close lifetime values. It is worth exploring the potential of using better modulation system to increase the sensitivity of the system.

The reduced contrast of the fingerprint image at higher modulation frequency compared to the contrast at lower frequency may also due to the lower frequency response of the photocathode of the intensifier of ICCD. It is worth exploring this problem, which can help to improve the sensitivity of the system when dealing with weak fluorescence emissions.

The developed PR imaging system fails to image the fingerprints deposited on white printing paper without the application of any chemical. This is due to the very large difference in fluorescence emission intensity. Practically paper fluorescence completely

overshadows the very weak inherent fluorescence from fingerprint sample. Hence, it is favorable to carry out a further investigation to improve the PR system in such scenarios.

In this research work a lifetime resolution of 0.21 ns was achieved when separating two fluorescence emissions, which lie in close wavelength range. This resolution can be further improved by using laser that gives sinusoidally modulated output at higher frequencies when dealing with samples having shorter lifetimes.

The difficulty of getting biosamples like cancerous cells limited the possibility of applying the PR technique on such real samples. It is understood that the fluorescence property of normal cells will be different from that of abnormal ones. The difference in cell fluorescence property such as lifetime and emission wavelength will be very small compared to normal cells at the beginning of abnormality growth. The possibility of applying PR technique in such real cases can be explored in future works. The ability of PR technique to resolve and separate fluorescence emissions, which are close in wavelength and lifetime values, can be applied in such cases. This will provide a tool for early detection of cell abnormalities.

Possibility of applying PR imaging technique to biochips containing multiple fluorescent wells can be explored. This will give a fruitful way of handling multiple fluorescence emissions and imaging of the desired emission by the subsequent suppression of other. This is worth exploring to detect different DNA samples under same excitation and imaging scheme.

APPENDIX A

CONVENTIONAL METHODS OF FINGERPRINT DEVELOPMENT

A.1 INTRODUCTION

The attachment program carried out at Scene of Crime Unit (SCU) of Criminal Investigation Department (CID), Singapore, provided hands-on training and an opportunity to understand the conventional methods employed in the latent fingerprint detection. The program also introduced all the currently available chemicals that are in use for the recovery of latent prints.

Fingerprint experts carry out systematic methods with the help of chemicals to develop the latent fingerprints depending upon the nature of the substrate on which they are formed. However, a well-trained expert will skip some of the steps and directly adopt the most effective methods by using their experiences and knowledge in the field. Fingerprint experts will first categorises the surface as porous, non-porous, adhesive (sticky), contaminated etc and then apply suitable chemicals to develop the prints.

A.2 FINGERPRINTS ON POROUS SURFACES

(a) Ninhydrin

Ninhydrin is the most common latent fingerprint-developing chemical applied to porous surfaces. Ninhydrin, in solution form, can be applied in three ways such as spraying, brushing or immersing the sample in the solution. After the application of

ninhydrin, the sample is dried in an oven to accelerate the development and visualisation of the print. Experiments were carried out on various surfaces such as newspapers and printing papers. The good prints developed were photographed using Polaroid camera as shown in Figures A.1-A.2.

Figure A.1 Fingerprint development newspaper using Ninhydrin.

Figure A.2 Fingerprint development on printing white paper using Ninhydrin.

This is a simple technique of developing prints on porous surfaces. It gives relatively good result with minimum effort. The print developed will be purple in colour. The contrast of the Ninhydrin-developed print can be improved by additional treatment of zinc chloride (ZnCl_2) solution to give an orange fluorescent complex compound. Since ninhydrin is not a harmful chemical it can be handled easily. However the main drawback of ninhydrin-developed fingerprint is that it cannot be applied to wet porous surfaces.

b) 1,8 Diazafluoren-9-one (DFO)

DFO is applied to the sample in the same way as ninhydrin i.e. by spraying, brushing or immersing in the solution. Generally the prints developed by DFO are highly fluorescent, and fluorescence examination is essential, since most prints developed

by DFO are not visible under normal lighting conditions. Experiments were carried out on different types of papers and latent prints developed were examined under different excitation wavelengths. The excitation wavelengths used ranging from 400 nm to 490 nm using a broadband light source. The prints developed using DFO could be photographed using a camera with proper filters.

A.3 FINGERPRINTS ON NONPOROUS SURFACES

a) Superglue Fuming

Superglue fuming is the first method that a fingerprint expert will apply to a nonporous surface. It was carried out in a conventional and portable pops 'n' fume enclosure. The primary purpose of fuming was to protect the prints and at the same time it also has the ability to develop prints partially. It was used mainly to keep prints on surfaces intact. If the contrast is poor, fluorescence examination can be carried out followed by fuming. The superglue was used along with an acid-alkaline mixture to generate the heat for fuming and the amount of glue applied is around 0.7 gm. The fuming duration was about 15- 20 minutes. Fingerprint samples on different surfaces were tried. This includes drinking bottles (Coca-Cola), plastic bottle, coloured card and plastic cover as shown in Figures A.3-A.6.

Figure A.3 Fingerprint development on coloured card by fuming

Figure A.4 Fingerprint development on polythene cover by fuming.

Figure A.5 Fingerprint development on drinking bottle by fuming.

Figure A.6 Fingerprint development on plastic bottle by fuming.

Fuming chamber

When large numbers of samples are to be fumed, fuming chamber is used. It is not portable, but is very useful to fume big and multiple samples at the same time. The recommended operating condition for 4gm glue fuming is at humidity of 80% and a temperature of 100°C.

Advantage and disadvantages of fuming

Fuming is a very effective method when large number of samples are involved. As the vapour is harmful, the operation must be carried out in a chamber fitted with extraction system. The use of fluorescent powders or dye is essential to reveal maximum number of fingerprints after fuming. Moreover fuming destroys other evidences such as DNA profiling and hairs.

In all the four developed samples, fingerprint enhancement was carried out by treating with various fingerprint powders. Though the print as a whole was visible, the contrast was not good especially when the background colour matches with that of developed print. In many cases the ridge details were blurred leading to failure in the identification of fingerprint details.

When the print gets older, the contrast of fingerprint image gets weaker and this makes it more difficult to reveal all the ridge characteristics on a fingerprint. The development of such prints is totally impossible by present conventional methods as illustrated in the case of print developed by DFO on plastic covers as shown in Figure A.3.

b) Powders

Different types of fingerprint powders were employed in fingerprint detection when the substrate is very smooth and non-porous as shown in Figures A.7 to A.9. The different fingerprint powders include magnetic, white, silver, black, red and green. These powders are used according to the background colour. These powders are

brushed onto the sample to develop the print. The following powders were used to develop the prints on the various substrates as mentioned earlier.

Drinking bottle and coloured card	White powder
Black plastic cover	Silver powder
Plastic bag	Black magnetic powder

Figure A.7 Fingerprint development by black magnetic powder

Figure A.8 Fingerprint development by silver powder on black surfaces.

Figure A.9 Fingerprint development by white powder on coloured card.

A.4 FINGERPRINTS ON BLOOD CONTAMINATED SURFACES

a) Amido Black

Amido black is the most appropriate chemical applied to surfaces contaminated with blood. This chemical is applied by running it over the sample in required quantity or immersing the sample in the solution for few minutes. After this, it is washed in running water to remove the excess chemical. The print developed on the telephone receiver is shown in Figure A.10.

Figure A.10 Blood contaminated print on non-porous surfaces-development by Amido black.

Advantage and disadvantages

Amido black is the unique chemical that can be effectively applied to blood contaminated prints. It is relatively insensitive to non-blood contaminated prints. The main draw back is that it is ineffective on blood-contaminated surfaces, which are dark in colours.

A.5 FINGERPRINTS ON ADHESIVE SURFACES

a) Gentian violet

Gentian violet is found to be very useful when the print is formed on adhesive surfaces like masking tape, scotch tape, insulation tape etc. Gentian violet solution is applied on to the sample to develop the prints and the excess chemicals are washed away in running water.

This chemical is very effective and simple to use for adhesive surfaces. It has an added advantage that the chemical is not toxic at all. But its main limitation is that it

cannot develop prints on coloured sticky surfaces such as black, blue, brown as shown in Figure A.11.

Figure A.11 Fingerprint development on coloured adhesive surface by gentian violet.

A.6 FLUORESCENCE EXAMINATION AND OPTICAL FILTERING

Fluorescence examination followed by photography using proper filters is a useful method of visualising the print when the sample and background emit in different wavelengths. Once the best conditions are achieved for the illumination and viewing, it can be photographed. In this method extreme care must be taken to avoid the photodecomposition of prints under the high intense light illumination. The conventional method of fluorescence detection totally fails when fluorescence emission from background and fingerprint occur in very close wavelength range and hence optical filtering is not effective. This can be shown through the following pictures where fingerprint deposited on a fluorescing magazine cover. It is obvious that from Figure A.12 that optical filtering was effective to eliminate background fluorescence and ridge details of the print can be obtained clearly when these two emissions are widely separated in wavelength scale. In the Figure A.13, it is clear that fingerprint ridge details are visible only in those regions where the background

emission is not interfering with the emission from the fingerprint. The circled area shows that Fluorescence detection of prints through optical filtering was not a fruitful option when background emission is overlapping from that of fingerprint sample.

Figure A.12 Fluorescence detection of fingerprints when background emission is widely separated from that of fingerprint sample.

Figure A.13 Fluorescence detection of prints when background emission is overlapping from that of fingerprint sample.

A.7 SUMMARY OF ATTACHMENT TRAINING AT SCU

The work carried out at SCU led to the familiarisation on some conventional methods of detecting latent fingerprints. In many cases the prints developed on porous surfaces like paper have poor contrast and the ridge details may not be clear. Fingerprint development by gentian violet resulted in poor contrast to ridge details. Hence, these experiments helped to confirm the limitations of existing finger print detection methods

APPENDIX B**TIME-RESOLVED IMAGING OF LATENT
FINGERPRINTS WITH NANOSECOND RESOLUTION****B.1 INTRODUCTION**

Menzel et al established time-resolved (TR) method of fluorescence study to overcome the problem of strong background fluorescence by making use of the fluorescence lifetime as an important parameter. Menzel had reported the use of rare-earth based chemicals, which is applied to the fingerprint samples so as to bring their lifetime into the millisecond range and hence allows the TR technique to separate them from background fluorescence, which is typically in nanosecond range. But the main limitation of this method is that it allows to work only in the millisecond time range of fluorescence lifetime and not in nanosecond range where sensitive detection is possible. Also it offers a broader chemical treatment procedure to fingerprint samples instead of the comparatively difficult to handle rare earth based chemicals as used by the previous researchers. In this context, TR imaging with nanosecond resolution is proposed for imaging of latent fingerprints.

B.2 SAMPLES TESTED

TR imaging of fingerprints were carried with two sets of samples. Initially experiments were carried out with two overlapping fingerprints treated with two fluorescent powders (Blitz green and Blitz red) having different lifetimes in nanosecond range. The two fluorescence emissions have large difference in their emission intensity. This is to prove the dependence of emission intensity in the TR

imaging when dealing with emissions having lifetime difference in nanosecond range. For this purpose a one-week-old print overlapping a fresh print and treated with two different fluorescent powders having lifetime in nanosecond range was used.

For the second sample, latent fingerprints deposited on fluorescing substrates were used for the time-resolved imaging with nanosecond resolution. The fluorescent powders used were ultra-azure-blue and ultra-white powders along with transparent PMMA substrates that show fluorescence in the violet-blue region of visible spectrum. Ultra-azure-blue and ultra-white powders also show fluorescence emissions in the same region of the spectrum. Fluorescence lifetime study of these substrates and powders were conducted prior to TR imaging experiments.

B.3 EXPERIMENTAL SETUP AND PROCEDURE

The experimental set up is shown in Figure B.1. Light from the argon-ion laser (Coherent, Innova 90 C) was square wave modulated at frequency 1 MHz with the help of an electro-optic modulator (EOM, Conoptics, model 350-210) through function generator 1 (Agilent, Model-33250A) that operates at 20% duty cycle. Laser is capable of operating in multiline UV and Single line visible mode. A similar square wave at same frequency and duty cycle was used to trigger the gating of the detecting device, an Intensified Charge Couple Device Camera, (ICCD, La Vision, PicoStar HR). The two function generators were synchronized so as to carry out gate delay of the ICCD. The total integration time of the ICCD is set to be 500ms and gate width can be varied from 0.2 ns to 1 ns. The modulated light from the EOM is

coupled into a liquid light guide and in turn to illuminate the sample surface. The resulting fluorescence signals from both the background and fingerprint sample were captured by the ICCD. The processing of these signals was carried out using the software associated with the camera.

The fluorescence intensity decays as given by the equation,

$$I = I_0 \exp\left(\frac{-t}{\tau}\right) \quad (1)$$

where, I is the intensity of the fluorescence emission at any time 't' and I_0 is the emission intensity at $t = 0$, while τ is its fluorescence lifetime.

Figure B.1 TR imaging set up for latent fingerprints

When the excitation light source is cut off, the photoluminescence profile decays in an exponential way. It is possible to turn on the imaging device system after the excitation light is cut off, with a delay such that the short lived fluorescence has already decayed where as long lived fluorescence still remains. As shown in Figure

B.2, during the “off” period of the modulated laser beam, the fluorescence intensity of the fingerprint and substrate would decay in accordance to the equation mentioned above. The ‘gating’ of the imaging device (ICCD) is carried out by properly adjusting the relative phase and thus the time delay between the two square waves (i.e. between the excitation modulation and camera modulation). This allows capturing of the long-lived fingerprint fluorescence after the short-lived background fluorescence is completely decayed

Figure B.2 Schematic diagram showing the principle of time-resolved imaging

B.4 RESULTS AND DISCUSSIONS

It is found that the fluorescence lifetime of fingerprints treated with blitz-green and blitz-red is 8.96ns and 3.85ns respectively. Figures B.3 and B.4 show the images of two overlapped fresh fingerprints treated with these two powders. These images were taken up on visible laser excitation at 476 nm. In these figures, the print on the left-hand side with solid-line circle is treated with fluorescent powder having a

comparatively longer lifetime, i.e. blitz-green, and the overlapped print on the right hand side with dashed-line circle, is treated with a shorter lifetime fluorescent powder, i.e. blitz-red. Figure B.3 shows that for fresh fingerprints treated with blitz-green and blitz-red in the presence of strong scattered light from background and in the absence of TR imaging, the contrast is very poor and one cannot identify the ridge details. These images were taken through normal imaging with the ICCD camera. Figure B.4 shows the significance of time resolved imaging of the latent fingerprint by suppressing the shorter lifetime fluorescence in the nanosecond time range. It shows that through proper gating, the scattered light can be cut off so as to clearly identify the ridge details of the print treated with fluorescent powder having longer lifetime. Suppression of fluorescence emission from the fingerprint treated with blitz-red is made possible by properly adjusting the gate delay and hence, selective imaging of longer lifetime fluorescence (i.e. fingerprint treated with blitz-green) in the nanosecond time range. This result shows a marked improvement from the reported data of time-resolved imaging of fingerprints where only milliseconds time resolution was possible.

Figure B.3 Normal ICCD image of fresh fingerprints.

Figure B.4 TR image of fresh fingerprints.

Figures B.5 and B.6 show the effect of relative fluorescence intensity on the TR imaging with nanosecond resolution for fingerprints. To achieve the difference in fluorescence intensity and different lifetime for fingerprints, two overlapping prints were deposited on non-fluorescing PMMA, in which one of the prints is of a week old print and another being a fresh print. In these figures, the week old print on the left-hand side with solid-line circle is treated with blitz-green powder and the overlapped fresh print on the right hand side with dashed-line circle is with blitz-red powder. The fluorescence emission intensity from blitz green treated one-week print will be less compared to the fresh one. Due to aging of the print the residues in fingerprint deposits might have dried off to an extent before reacting with the chemical powder. As mentioned in the previous case, it can be shown that by properly adjusting the gate delay the scattered light from the background can be subdued successfully. Figure B.6 shows that the contrast in the fingerprint for the longer lifetime fluorescence is better, despite the large intensity difference for fluorescence emissions.

Figure B. 5 Normal ICCD image of weak
and fresh fingerprints

Figure B. 6 TR image of weak and fresh
fingerprints

The dependence of relative intensity on the image contrast obtained from TR technique can be explained using the figures B.7 and B.8. These graphs were plotted based on the fundamental fluorescence decay equation (1),

In both figures, relative intensity of fluorescence emission is plotted on Y-axis in arbitrary unit (a.u) and time on X-axis. Figure B.7 shows the time dependent fluorescence decay of fingerprint samples treated with blitz-red and blitz-green powders with comparable intensity. It can be understood that since their intensity decay profile follow different paths, suppression of one emission is possible by adjusting the gate width of ICCD.

Figure B.7 Theoretical fluorescence decay of two fluorescence emissions having comparable emission intensity but different lifetime.

As shown in Figure B.8, the fluorescence intensity of longer lifetime decay (fingerprint treated with blitz-green) is much different from that of shorter lifetime fluorescence decay (fingerprint treated with blitz-red). In this experiment it was found that the fluorescence emission intensity of one-week-old fingerprint treated with blitz-green is almost half that of fresh print treated with blitz-red based on the relative photon counts. Due to the overlapping of intensity profile, the suppression of shorter lifetime component is not fully achievable in all range of lifetime values. It is obvious that higher the difference in the lifetime value for the decay in nanosecond range, greater the chance for suppressing the shorter lifetime component despite having higher decay intensity.

Figure B.8 Theoretical fluorescence decay of two fluorescence having difference in emission intensity and lifetime.

TR imaging of fingerprints deposited on fluorescing PMMA substrates was also carried out. Prior to the TR imaging study, lifetimes and emission wavelengths of the substrate and treated fingerprints were measured. For the time-resolved imaging, latent fingerprints deposited on 'blue' and 'white' fluorescing PMMA substrates were used. They are named so as under room light they appear in blue and white colour. These substrates show fluorescence in the violet-blue region of visible spectrum. The fluorescent powders used for TR imaging were ultra-azure-blue and ultra-white. The lifetimes of 'blue' and 'white' substrates were 1.00 and 1.40 ns respectively. Similarly, their fluorescence emission wavelengths were peaked at 426 and 432 nm. The lifetimes of the ultra-azure blue powder and ultra white powder were 3.44 and 3.36 ns respectively. This ensures the criterion of longer lifetime for the treated fingerprints in order for the TR imaging technique to be fruitful. Also, peak emission wavelengths of fluorescent powders were found to be 450 and 447 nm. It is noted that the largest lifetime difference between the treated fingerprint and the substrate is 2.44 ns and the smallest is just 1.96 ns.

It was found that the emission intensity of the treated fingerprint is lower than that of the fluorescence emission from the background in all cases and their peak emission wavelength is close to the wavelength of the background emission. Furthermore, the bandwidth of the background emission is very high and hence, fingerprint fluorescence emission is almost over shadowed by the background emission. In this scenario, the use of optical filters to suppress the background fluorescence is not a fruitful option. These circumstances thus highlight the importance of the use of TR

imaging in the detection of latent fingerprints where the conventional detection methods fail.

The fingerprint images obtained with and without the application of TR imaging technique are shown in Figures B.9 to B.12. The image of the fingerprint obtained with normal imaging (without TR technique) at strong background fluorescence shows that the contrast of the ridge details is poor. This is due to fingerprint fluorescence emission is almost over shadowed by the strong background emission. It shows that through proper gating, it was possible to cut off the fast decaying strong background fluorescence and hence a comparison between the pair of images in each case clearly shows that a better contrast is obtained for TR fingerprint image. Moreover, there is an improvement in the contrast of the fingerprint ridges as shown in the images, which is obtained using TR technique.

Figure B. 9(a) Conventional image of ultra-azure-blue powder treated fingerprints on 'blue' fluorescing PPMA substrate.

Figure B. 9(b) TR image of ultra-azure-blue powder treated fingerprints on 'blue' fluorescing PPMA substrate.

Figure B.10 (a) Conventional image ultra-azure-blue powder treated fingerprint on 'white' fluorescing PPMA substrate.

Figure B.10 (b) TR image ultra-azure-blue powder treated fingerprint on 'white' fluorescing PPMA substrate.

Figure B.11 (a) Conventional image of ultra-white powder treated fingerprints on 'blue' fluorescing PPMA substrate

Figure B.11 (b) TR image of ultra-white powder treated fingerprints on 'blue' fluorescing PPMA substrate

Figure B.12 (a) Conventional image of ultra-white powder treated fingerprints on 'white' fluorescing PPMA substrate

Figure B.12 (b) Conventional image of ultra-white powder treated fingerprints on 'white' fluorescing PPMA substrate.

B.5 SUMMARY

Time-resolved imaging of latent fingerprints was carried out with nanosecond resolution. The idea of imaging the latent fingerprints with nanosecond resolution is highly significant when dealing with practical cases having strong background fluorescence in nanosecond range. The reported literatures on TR method are only effective for cases with millisecond lifetime resolution. Hence, this work gives a new outlook to the TR imaging technique. It is shown that to achieve TR imaging in nanosecond range, the fingerprint fluorescence should have comparable or higher emission intensity with respect to the other emission component in the close nanosecond range besides having a longer lifetime.

APPENDIX C**SPECIFICATIONS OF THE ICCD CAMERA**

Items	Specification
Active image area	14,24,800 (1370 H x 1040 V)
Pixel size	6.45 x 6.45 microns
Sensitivity	70 counts/photoelectron @max gain
Spatial resolution	8-12 lp /mm
Spectral range	200-800 nm
Lens connector	Nikon-F-or C mount
Full well capacity	18,000 e
Cooling	2-stage Peltier with forced air regulated to 12 ⁰ C
Interface	Data transfer (FOL), control output, trigger input, gate disable

LIST OF PUBLICATIONS

PATENT FILED

1) "Method and apparatus for imaging latent fingerprints", Filed for Singapore patent (File number: 200307736-9, 29-12-2003).

INTERNATIONAL JOURNALS

1) **Dinish U.S**, Seah L.K, Murukeshan V.M, Ong L.S "Theoretical analysis of phase-resolved fluorescence emission from fingerprint samples" *Optics Communications*, Vol. 223 (1-3), 55-60 (2003).

2) Seah L.K, **Dinish U.S**, Ong S.K, Chao Z.X, Murukeshan V.M "Time-resolved imaging of latent fingerprints with nanosecond resolution" *Optics & Laser Technology*, 36(5), 371-376 (2004).

3) Chao Z.X, **Dinish U.S**, Seah L.K, Murukeshan V.M "Homodyne and heterodyne signal processing assisted phase resolved optical technique for latent fingerprint imaging: a theoretical study" *Journal of Modern Optics* 52(1), 119-129, (2005).

4) **Dinish U.S**, Chao Z.X, Murukeshan V.M, Seah L.K. "Homodyne assisted multi-step phase shifting in phase-resolved optical technique for latent fingerprint imaging" *Optical Engineering Letters* 43(12), 2381-2382, (2004).

5) **Dinish U.S**, Z.X Chao Z.X, A. Singh, L.K Seah, Murukeshan V.M "Formulation and implementation of phase-resolved optical technique for latent fingerprint imaging: Theoretical and experimental Analysis" *Applied Optics* 44(3), 297-304, (2005).

6) L.K Seah, **Dinish U.S**, W. Phang, Z.X Chao, Murukeshan V.M "Fluorescence optimisation and lifetime studies of fingerprints treated with magnetic powders" *Forensic Science International* (Accepted-in press).

LIST OF PUBLICATIONS

7) **Dinish U.S**, Z. X Chao, L.K Seah, L.S Ong, Murukeshan V.M “Nanosecond resolution in fingerprint imaging using optical technique”, *International Journal of Nanoscince* (Accepted).

8) L.K Seah, **Dinish U.S**, Z.X Chao, Murukeshan V.M “Phase-Resolved Optical Technique with Heterodyne Signal Processing for the Imaging of Latent Fingerprints: Theoretical and Experimental Analysis” *Journal of Modern Optics* (Under review).

9) Chao Z.X, Seah L.K, Murukeshan V.M, **Dinish U.S**, “Sensitivity Improvement for Phase-Resolved Fingerprint Imaging by Combining Even-Step-Shift and Pi-Shift Methods”, *Measurement Science & Technology* (Under review).

10) **Dinish U. S**, C.Y Fu. Z.X Chao, L.K Seah, V.M Murukeshan, B.K Ng, “Separation of Close Fluorescence Emissions for Biomedical Applications Using Frequency Domain Spectroscopy” *Biosensors & Bioelectronics* (Under review).

INTERNATIONAL CONFERENCES

1) **Dinish U.S**, Z. X Chao, L.K Seah, L.S Ong, Murukeshan V.M “Phase-resolved optical techniques for the detection and imaging of latent fingerprints”, Great Lake Photonics Symposium, 7-11 June 2004, Cleveland, Ohio, USA.

2) **Dinish U.S**, Z. X Chao, L.K Seah, L.S Ong, Murukeshan V.M “Nanosecond resolution in fingerprint imaging using optical technique”, Nanotech 2004, 13-17 July 2004, Singapore.

3) Z.X Chao, L.K Seah, **Dinish U.S**, Murukeshan V.M, L.S Ong “Improvement of latent fingerprint image contrast by combining even step-phase shift and Pi-shift methods in phase-resolved optical technique” ICO'04, Tokyo, Japan.

LIST OF PUBLICATIONS

- 4) **Dinish U.S**, Chao Z.X, Seah L.K, Murukeshan V.M, “Homodyne and heterodyne assisted frequency domain fluorescence technique for latent fingerprint imaging: A comparative study”, Photonics 2004, Dec 2004, Cochin, India.

REFERENCES

- 1) J.R. Lakowicz, (1999), Principles of fluorescence spectroscopy, 2nd Ed, Plenum publishers, New York.
- 2) J.P. Houston, A. B. Thompson, M. Gurfinke, E.M. Sevick Muraca, "Sensitivity and depth penetration of continuous wave versus frequency-domain photon migration near-infrared fluorescence contrast-enhanced imaging", Photochemistry and Photobiology, 77(4), 420–430, (2003).
- 3) J. Lee, E.M. Sevick Muraca, "3-Dimensional fluorescence enhanced optical tomography using referenced frequency-domain photon migration measurements at emission and excitation wavelengths," Journal of the Optical Society of America A, 19, 759-771, (2002).
- 4) E.R. Menzel, (1999), Fingerprint detection with lasers, 2nd Ed, Marcel Dekker, New York.
- 5) Manual of fingerprint development techniques, (1998), 2nd Ed, Home Office Scientific Research and Development Branch, London.
- 6) D.W Herod, E.R. Menzel, "Laser detection of latent fingerprints: ninhydrin followed by zinc chloride," Journal of Forensic Sciences. 27(3), 513-518, (1982).
- 7) C.A. Pounds, R. Grigg, T. Mongkolaussavaratana, "The use of DFO for the fluorescent detection of latent fingerprints on paper: A preliminary evaluation", Journal of Forensic Sciences, 35(1) 169-175, (1990).
- 8) S. Morimoto, A. Kaminogo, T. Hirano, " A new method to enhance visualisation of latent fingermarks by sublimating dyes, and its practical use with a combination of cyanoacrylate fuming", Forensic Science International, 97, 101-108, (1998).
- 9) E.R. Menzel, J.M Duff, "Laser detection of latent fingerprints-treatment with fluorescers," Journal of Forensic Sciences. 24(1), 96-100, (1979).

REFERENCES

- 10) E.R. Menzel, "Laser detection of latent fingerprints-treatment with phosphorescers," *Journal of Forensic Sciences*, 24(3), 582-585, (1979).
- 11) G.S. Sodhi, J. Kaur, J. "Powder method for detecting latent fingerprints: a review", *Forensic Science International*, 120, 172- 176, (2001).
- 12) Police Scientific Development Branch, (1990), *Fingerprint detection by fluorescence examination-a guide to operational implementation*. Home Office, Sandridge.
- 13) J.A. Burt, E.R. Menzel, "Laser detection of latent fingerprints: difficult surfaces," *Journal of Forensic Sciences*, 13(2), 364-370, (1985).
- 14) E.R. Menzel, "Recent advances in photoluminescence detection of fingerprints", *The Scientific World*, 1, 498-509, (2001).
- 15) H.C. Lee, R.E. Gaensslen, *Advances in fingerprint technology*, CRC press, London (1994).
- 16) S. Lam, C. MacAulay, J. Leriche, N. Ikeda, and Branko Palcic, "Fluorescence imaging of early lung cancer" in *Optical biopsy and fluorescence spectroscopy and imaging*, Proc. SPIE 2324, 2-8, (1995).
- 17) D. Erimberger, D. Zaak, H. Stepp, R. Knuchel, R. Baumgartner, P. Schneede, N. Schmeller, A. Hofstetter, "Auto fluorescence imaging to optimise 5-ALA- induced fluorescence endoscopy of bladder carcinoma" *Urology*, 58(3), 372-375, (2001).
- 18) D. Yova, V. Atalmazoglou, N. Kavantzias, and S. Loukas, "Development of a fluorescence-based imaging system for colon cancer diagnosis using two novel rhodamine derivatives", *Lasers in Medical Science*, 15, 140-147, (2000).

REFERENCES

- 19 R.R. Alfano, D.B. Tata, J. Cordero, P. Tomashefsky, F.W. Longo, and M.A. Alfano, "Laser-induced fluorescence spectroscopy from native cancerous and normal tissue", IEEE Journal of Quantum Electronics, 20(12), 1507-1511, (1984).
- 20 M. Zargi, "Autofluorescence imaging in the diagnosis of laryngeal cancer", Eur Arch Otorhinolaryngol, 257,17-23, (2000).
- 21) B.W. Pogue, "Instrumentation and design of a frequency-domain diffuse optical tomography imager for breast cancer detection", Optics Express, 1(13): 391-403, (1997).
- 22) C.T. Wittwer, G.C. Fillmore, D.J. Garling, "Minimizing the time required for DNA amplification by efficient heat transfer to small samples", Analytical Biochemistry, 186, 328-331, (1990).
- 23) C.T Wittwer, D.J Garling, " Rapid Cycle DNA amplification: Time and temperature optimization", Biotechniques, 10, 76-83, (1991).
- 24) L.J. Kricka, P. Wilding, " Mircochip PCR", Analytical and Bioanalytical Chemistry, 377, 820-825, (2003).
- 25) W.G. Eckert, (1997), Introduction to forensic sciences. 2nd ed, CRC Press, New York.
- 26) P. Margot and C. Lennard, (1994), Fingerprint Detection Techniques, 6th Ed, Lausanne: Institut de Police Scientifique et de Criminologie.
- 27) R. Saferstein, (1981), Criminalistics: an introduction to forensic science. 2nd ed, Prentice-Hall, New Jersey.
- 28) J.F. Cowger, (1983), Friction ridge skin: comparison and identification of fingerprints, Elsevier, New York.

REFERENCES

- 29) W.C. Smith, R.W. Kinney, and D.G. De Partee, "Latent fingerprints- a forensic approach", *Journal of Forensic Identification*, 43(6), 563-570, (1993).
- 30) G.S. Vaughn, and T.M. Prizeman, "Enhancement of fingerprints in blood- Part 1: the optimisation of amido black", *Journal of Forensic Identification*, 50(5), 470-480, (2000).
- 31) P. Warrick, "Identification of blood prints on fabric using Amido black and digital enhancement", *Journal of Forensic Identification*, 50(11), 20-29, (2000).
- 32) S. K. Bramble, A.A. Cantu, R.S. Ramotowski, J.S. Brennan, "Deep red to near infrared (NIR) fluorescence of gentian violet-treated latent prints", *Journal of Forensic Identification*, 50(1), 33-49, (2000).
- 33) W. Stitt, "New use for gentian violet", *Journal of Forensic Identification*. 47(3), 274-275, (1997).
- 34) N. Sneddon, "Black powder method to process duct tape", *Journal of Forensic Identification*, 49(4), 347-356, (1999).
- 35) D.S. Burns, "Sticky-side powder: the Japanese solution", *Journal of Forensic Identification*. 44(2), 133-138, (1994).
- 36) M.L. Gray, "Sticky-side powder versus gentian violet: The search for the superior method for processing the sticky side of adhesive tape", *Journal of Forensic Identification*, 46(3), 268-272, (1996).
- 37) B.L. Martin, "Developing latent prints on the adhesive surface of black electrical tape", *Journal of Forensic Identification*, 49(2): 127-129, (1999).

REFERENCES

- 38) J. Almong, A. Hirshfeld, A. Frank, H. Grant, Z. Harel and Y. Ittah, "5-methylthio ninhydrin and related compounds: A novel class of fluorogenic fingerprint reagents", *Journal of Forensic Sciences*, 37(3), 688-694, (1992).
- 39) "Chemical processing for latent fingerprints-Technical notes" (2000), Lightning Powder Company Inc, Salem, Oregon, U.S.A.
- 40) A. Frank, "Modified SPR for latent fingerprint development on wet, dark objects", *Journal of Forensic Identification*, 43 (3), 240-244, (1993).
- 41) D.P Grady, "Cyanoacrylate fuming: accelerating by heat within a vacuum", *Journal of Forensic Identification*, 49(4), 377-387, (1999).
- 42) E.R. Menzel, J.A Burt, T.W. Sinor, W.B. Tubach-Ley, K.J. Jordan, "Laser detection of latent fingerprints: treatment with glue containing cyanoacrylate ester", *Journal of Forensic Sciences*, 28(2), 307-317, (1983).
- 43) J.B. Kempton, W.F. Rowe, "Contrast enhancement of cyanoacrylate-developed latent fingerprints using biological stains and commercial fabric dyes", *Journal of Forensic Sciences*, 37(1), 99-105, (1992).
- 44) N. Jones, M. Stoilovic, C. Lennard, and C. Roux, "Vacuum metal deposition: factors affecting normal and reverse development of latent fingerprints on polyethylene substrates", *Forensic Science International*, 115: 73-88, (2001).
- 45) G.G. Guilbault, (1967), *Fluorescence: Theory, Instrumentation and Practice*, Edward Arnold Publishers Ltd, London.
- 46) A. Periasamy, P. Wodnicki, X. F. Wang, S. Kwon, G. W. Gordon, B. Herman, "Time-resolved fluorescence lifetime imaging microscopy using a picosecond pulsed tunable dye laser system", *Review of Scientific Instruments*, 67 (10), 3722-3731, (1996).

REFERENCES

- 47) Y. Chen, A. Periasamy, "Characterisation of two-photon excitation fluorescence lifetime imaging microscopy for protein localization", *Microscopic Research and Techniques*, 63, 72-80, (2004).
- 48) C.G. Morgan, A.C. Mitchell, and J.G Murray, " Prospects for confocal imaging based on nanosecond fluorescence decay time", *Journal of Microscopy*, 165 (1), 49-60, (1992).
- 49) K.W. Berndt, J.R. Lakowicz, "Detection and localisation of absorbing in scattering media using frequency- domain principles", *SPIE*, 1431, 149-160, (1991).
- 50) T. French, E. Gratton, J. Maier, " Frequency domain imaging of thick tissues using a CCD", *SPIE*, 1640, 254-261, (1992).
- 51) A.J. Ozinskas, H. Malak, J. Britz, R.B. Thompson, P.A. Koen, J.R. Lakowicz, "Homogenous model immunoassay of thyroxine by phase-modulation fluorescence spectroscopy", *SPIE*, 1885, 2-9, (1993).
- 52) J.A. Stienkamp, H.A. Crissman, "Resolution of heterogeneous fluorescence emission signals and decay lifetime measurement on fluorochrome-labelled cells by phase-sensitive FCM", *SPIE*, 1885, 278-289, (1993).
- 53) T.E French, D.P Stumbo, D.N Modlin, "Frequency domain light detection device", US patent, No: 6,317,207 B2, Nov 13, (2001).
- 54) L.B. McGown, D.W. Millican, "Multifrequency phase-resolution for total luminescence spectroscopy", *SPIE*, 909, 360-365, (1988).
- 55) J.R. Lakowicz, G. Laczko, I. Gryczynski, H. Szmajnski, W. Wiczak, M.L Johnson, "Frequency-domain Fluorescence spectroscopy; principles, biochemical applications and future developments", *Physical Chemistry*, 93, 316-327, (1989).

REFERENCES

- 56) S.A. Hardwick, (1990), Fingerprint Detection by Fluorescence Examination, Police of Scientific Development Branch, Home Office, U.K.
- 57) S.K. Bramble, K.E. Creer, W.G. Qiang, and B. Sherad, "Ultraviolet luminescence from latent fingerprints", Forensic Science International, 59, 3-14, (1993).
- 58) E.R. Menzel, "Comparison of argon-ion, copper-vapor, and frequency doubled Nd: YAG lasers for latent fingerprint development", Journal of Forensic Sciences, 30(2), 383-397, (1985).
- 59) B.E. Dalrymple, J.M. Duff, E.R. Menzel, "Inherent fingerprint luminescence-detection by laser", Journal of Forensic Sciences, 22(1): 106-115, (1977)
- 60) E.R. Menzel, M. Takatsu, R.H. Murdock, K.K. Bouldin, K.H. Cheng, "Photoluminescent CdS/dendrimer nanocomposites for fingerprint detection", Journal of Forensic Sciences, 45(4), 770-773, (2000).
- 61) K.K. Bouldin, E.R. Menzel, M. Takatsu, R.H. Murdock, "Diimide-enhanced fingerprint detection with photoluminescent CdS/dendrimer nanocomposites", Journal of Forensic Sciences, 45(6), 1239-1242, (2000).
- 62) E.R. Menzel, (2001). "Fingerprint development methods", US Patent No: 6306662 B1.
- 63) E.R. Menzel, S.M. Savoy, S.J. Ulvick, K.H. Cheng, R.H. Murdock, M.R. Sudduth, "Photoluminescent semiconductor nanocrystals for fingerprint detection", Journal of Forensic Sciences, 45(3), 545-55, (2000).
- 64) E.R. Menzel, "Ion-Laser Detection of fingerprints grows more powerful", Laser Focus World, 89-94, Nov (1989).

REFERENCES

- 65) R.H. Murdock, E.R. Menzel, "A computer interfaced time-resolved luminescence imaging system", *Journal of Forensic Sciences*, 38(3), 521-529, (1993).
- 66) E.R.A. Lock, W.D. Mazzella, P. Margot, "A new europium chelate as a fluorescent dye for cyanoacrylate pretreated fingerprints-EuTTAPhen: europium thenoylTri fluoroAcetone ortho-Phenanthroline", *Journal of Forensic Sciences*, 40(4), 654-658, (1995).
- 67) C.E. Allred, R.H. Murdock, E.R. Menzel, "New lipid-specific, rare earth-based chemical fingerprint detection method", *Journal of Forensic Identification*, 47(5), 542-556, (1997).
- 68) Z. Sun, Y. Huang E. M. Sevick-Muracaa, "Precise analysis of frequency domain photon migration measurement for characterization of concentrated colloidal suspensions" *Review of Scientific Instruments*, 73(2), 383-393, (2002).
- 69) A. Godavarty, D.J. Hawrysz, R. Roy, E. M. Sevick Muraca, "Influence of the refractive index-mismatch at the boundaries measured in fluorescence enhanced frequency-domain photon migration imaging" *Optics Express*, 10(15), 653-662 (2002).
- 70) R. Roy, E.M. Sevick Muraca, "Three-dimensional unconstrained and constrained image reconstruction techniques applied to fluorescence frequency-domain photon migration," *Applied Optics*, 40, 2206-2215, (2001).
- 71) A.B. Thompson, E.M. Sevick Muraca, "Near-infrared fluorescence contrast-enhanced imaging with intensified charge-coupled device homodyne detection: measurement precision and accuracy," *Journal of Biomedical Optics*, 8, 111-120, (2003).

REFERENCES

- 72) E.M. Sevick Muraca, J. Pierce, H. Jiang, J. Kao, "Photon migration measurement of latex size distribution in concentrated suspensions," *AICHE Journal*, 43, 655-664, (1997).
- 73) S.M. Richter, R.R. Shinde, G.V. Balgi, E.M. Sevick Muraca, "Particle sizing using frequency-domain photon migration," *Particle & Particle Systems Characterization*, 15, 9-18, (1998).
- 74) S.M. Richter, E.M. Sevick Muraca, "Characterization of concentrated colloidal suspensions using time-dependent photon migration measurements," *Colloids and Surfaces A*, 172: 163-173, (2000).
- 75) L.B. McGown, D.S. Kreiss, "Spectral fingerprinting using phase-resolved fluorescence spectroscopy", *SPIE*, 910, 73-80, (1988).
- 76) L.B. McGown, "Phase-resolved fluorimetric determination of two albumin-bound fluorescein species", *Analytica Chimica Acta*, 157, 327-332, (1984).
- 77) L.B. McGown, F.V. Bright, "Comparison of phase-resolved and steady state fluorimetric multicomponent determinations using wavelength selection", *Analytical Chemistry*, 56, 2195-2199, (1984).
- 78) L.B. McGown, F.V. Bright, "Simultaneous two component determinations using phase-resolved fluorescence spectroscopy", *Analytica Chimica Acta*, 169, 117-123, (1985).
- 79) F.V. Bright, L.B. McGown, "Four component determinations using phase-resolved fluorescence spectroscopy", *Analytical Chemistry*, 57, 55-59, (1985).
- 80) F.V. Bright, L.B. McGown, "Phase-resolved fluorimetric determination of four component systems using two modulation frequencies", *Analytical Chemistry*, 57, 2877-2880, (1985).

REFERENCES

- 81) J. Sipior, G.M. Carter J. R. Lakowicz, G. Rao, "Blue light-emitting diode demonstrated as an ultraviolet excitation source for nanosecond phase-modulation fluorescence lifetime measurements", *Review of Scientific Instruments*, 68 (7), 2666-2670, (1997).
- 82) T. Arakia and H. Misawa, "Light emitting diode-based nanosecond ultraviolet light source for fluorescence lifetime measurements", *Review of Scientific Instruments*, 66(12), 5469-5472, (1995).
- 83) C.G. Morgan, Y. Hua, A.C Mitchell, J.G. Murray, A.D. Boardman, "A compact frequency domain fluorometer with a directly modulated deuterium light source", *Review of Scientific Instruments*, 67 (1), 41-47, (1996).
- 84) Z. Zhang, K.T.V. Grattan, W. Andrew, "Phase-locked detection of fluorescence lifetime", *Review of Scientific Instruments*, 64 (9), 2531-2540, (1993).
- 85) V. Venkatesh, V. Srinivas, "A closed loop scheme for phase-sensitive fluorometry", *Review of Scientific Instruments*, 66(7), 3750-3754, (1995).
- 86) R.V. Krishnan, H. Saitoh, H. Terada, V.E. Centonze, B. Hermana, "Development of a multiphoton fluorescence lifetime imaging microscopy system using a streak camera", *Review of Scientific Instruments*, 74(5), 2714-2721, (2003).
- 87) J. Yguerabide, A. Ceballos, " Quantitative fluorescence method for continuous measurement of DNA hybridization kinetics using fluorescent intercalator", *Analytical Biochemistry*, 228, 208-220, (1995).
- 88) M. Sauer. B. Angerer, W. Ankenbauer, Z. Foldes-Papp , F. Gobel, K.-T. Han, R. Rigler, A. Schulz , J. Wolfrum, C. Zander, "Single molecule DNA sequencing in submicrometer channels: state of the art and future prospects", *Journal of Biotechnology*, 86, 181–201, (2001).

REFERENCES

- 89) R. Higuchi, G. Dollinger, P.S. Walsh, R. Griffith, "Simultaneous amplification and detection of specific DNA sequences", *Biotechnology*, 10(4), 413-417, (1992).
- 90) R. Higuchi, C. Fockler, G. Dollinger R. Watson, " Kinetic PCR analysis: real-time monitoring of DNA amplification reactions", *Biotechnology*, " 11(9), 1026-1030, (1993).
- 91) C.T. Wittwer, M.G Herrmann, C.N. Gundry, K.S.J. Elenitoba-Johnson, "Real time multiplex PCR", *Methods*, 25, 430-442, (2001).
- 92) Roche Molecular Biochemicals, Light Cycler Operator's Manual, Version 3.5, U.S.A
- 93) C.T. Wittwer, K.M. Ririe, R.V Andrew, D.A David, R.A Gundry, U.J. Balis, "The light cycler: a microvolume multisample with fluorimeter with rapid temperature control", *Bio Techniques*, 22, 176-181, (1997).
- 94) J. Peccoud, C. Jacob, "Theoretical uncertainty of measurement using quantitative polymerase chain reaction", *Biophysical Journal*, 71, 101-108, (1996).
- 95) D.S. Lee, M.H. Wu, U. Ramesh, C.W. Lin, T.M. Lee, P.H. Chen, "A novel real-time PCR machine spectrometer for fluorescence sensing in a microliter volume glass capillary", *Sensors and Actuators B*, 100(3), 409-418, (2004).
- 96) N.A. Friedman, D.R. Meldrum, "Capillary tube resistive thermal cycling", *Analytical Chemistry*, 70, 2997-3002, (1998).
- 97) J.R. Lakowicz, K.W Berndt , "Lifetime-selective fluorescence imaging using an rf phase-sensitive camera", *Review of Scientific Instruments*, 62 (7), 1727-1734, (1991).

REFERENCES

- 98) A.A Lamola (1971), Creation and detection of the excited state, Vol. 1, Part A, Marcel Dekker, New York.
- 99) A. Godavarty, M.J. Eppstein, C. Zhang, S. Theru, A.B. Thompson, M.Gurfinkel, E.M Sevick Muraca, “Fluorescence-enhanced optical imaging in large tissue volumes using a gain-modulated ICCD camera”, Physics in Medicine and Biology, 48, 1701-1720 (2003).
- 100) Manual of Coherent INNOVA 90C series ion laser, Coherent Corporation, U.S.A.
- 101) Manual of Lavision Pico Star HR camera System, Lavision Corporation, Germany.
- 102) Manual of Conoptics Laser Modulation System, Conoptics Inc, U.S.A
- 103) Manual and operating instructions for high power patch cords and connectors, OZ Optics Ltd, Canada.
- 104) Laserstar-Laser power/energy monitor, Ophir Optronics Ltd, Israel.
- 105) Manual of Agilent 33250 A: 80MHz Function/Arbitrary Waveform Generator, Agilent Technologies, U.S.A.
- 106) Operation Manual of Spectrum 9000, Melles Griot, U.S.A.
- 107) Avasoft Full Version 5.1 User’s Manual”, Avantes, Netherlands.
- 108) Minitau Miniature Fluorescence Lifetime Analyser – Operating Instructions, Edinburgh Instruments, U.K.

REFERENCES

109) B.C. MacDonald, S.J. Lvin, H. Patterson, "Correction of fluorescence inner filter effects and the partitioning of pyrene to dissolved organic carbon", *Analytica Chimica Acta*, 338, 155-162, (1997).

110) Manual of 404nm laser, Omicron, Germany.

111) A. Pradhan, B.B. Das, K.M. Yoo, R.R. Alfano, J.M.D. Cleary, R.M.D. Prudente, E.M.D. Celmer, "Time-resolved fluorescence of benign and malignant breast tissues", *SPIE*, 1599, 81-84, (1991).

112) "Fundamentals of Fluorometric Analysis",
http://home.fujifilm.com/products/science/si_appli/pdf/1.pdf