

THE UNIVERSITY OF MELBOURNE

DOCTORAL THESIS

Time-Resolved and Polarised Fluorescence Studies of Polymeric and Biological Systems

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School of Chemistry

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Declaration of Authorship

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- The thesis comprises only my original work towards the PhD except where indicated,
- Due acknowledgement has been made in the text to all other material used,
- The thesis is less than 100,000 words in length, exclusive of tables, figures, bibliographies and appendices.

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Publications

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- Time-resolved and Polarised Fluorescence Microspectroscopy and Imaging of Thin Films of Bio-Nano Materials, (Nano group, Sharif University of Technology, Dec 2017)(Invited talk)

Bertrand Russell, the great British philosopher and social critic, was asked a closing question: What would you tell a generation living 1,000 years from now about the life you've lived and the lessons you've learned. His answer is short, but pithy:

I should like to say two things, one intellectual and one moral:

The intellectual thing I should want to say to them is this: When you are studying any matter or considering any philosophy, ask yourself only what are the facts and what is the truth that the facts bear out. Never let yourself be diverted either by what you wish to believe or by what you think would have beneficent social effects if it were believed, but look only and solely at what are the facts. That is the intellectual thing that I should wish to say.

The moral thing I should wish to say to them is very simple. I should say: Love is wise, hatred is foolish. In this world, which is getting more and more closely interconnected, we have to learn to tolerate each other. We have to learn to put up with the fact that some people say things that we don't like. We can only live together in that way, and if we are to live together and not die together we must learn a kind of charity and a kind of tolerance which is absolutely vital to the continuation of human life on this planet.

THE UNIVERSITY OF MELBOURNE

Abstract

Faculty of Science

School of Chemistry

Doctor of Philosophy

**Time-Resolved and Polarised Fluorescence Studies of Polymeric and Biological
Systems**

by Hamid Soleimaninejad

Understanding the dynamics of energy migration for disordered complex systems such as polymeric solar cells is crucial for optimizing the charge transport process and obtaining efficient devices. Fluorescence anisotropy provides information on the mobility and orientation of the bio-molecules and has been used to investigate protein–protein interactions. Despite recent exploration and advances in techniques, the dynamics of fluorescence depolarisation in complex systems are not yet fully comprehended. This thesis reports a new strategy to map the fluorescence anisotropy and investigate the energy migration and macromolecular rotational diffusion in polymeric and biological systems. A fluorescence anisotropy imaging microscopy (FAIM) method combined with fluorescence lifetime imaging (FLIM), and evanescent wave time-resolved fluorescence anisotropy measurements (EW-TRAM) to explore the photophysics associated with morphological changes in a wide range of complex systems such as conjugated polymers, cells and soft bilayers.

First of all, the morphology and photophysics of aggregation in MEH-PPV conjugated polymer films cast by a controlled evaporative self-assembly (*Snake-Skin*) method have been investigated. FLIM and FAIM were used to monitor the photophysical changes and also depolarisation of emission, respectively. The tightly packed and coiled conformation regions in films were identified by shorter lifetime and lower emission anisotropy histograms. Also, the open chain conformation could be detected by observing high values of lifetime and anisotropy. In addition, a systematic visualization of the unique photophysical and fluorescence anisotropic features of polyfluorene coplanar conformation (β -conformation) are demonstrated. Inhomogeneous morphologies and fluorescence decay profiles at diverse micrometre-sized regions within all types of polyfluorene β -conformational spin-coated films have been distinguished. PFO and PODPF β -domains both had shorter emission lifetimes than those of the glassy conformation. In addition, β -conformational regions had larger fluorescence

anisotropy due to high chain orientation, while the low anisotropy in glassy conformational regions showed efficient energy migration from amorphous regions to β -conformation as a whole.

Moreover, TREWIFS and EW-TRAMs methods were utilised to study the interaction of melittin peptide with POPC and POPG vesicles on a silica interface. The emission decay time of NBD dye attached to the peptide was carefully monitored using the TCSPC method. Different fluorescence decay profiles were observed during depth profiling from interface in tens of nanometre increments. The fluorescence decay kinetics of all systems was found to be multi-exponential. Change of lifetime and rotational correlation time even in nanometre increments revealed the accuracy and power of the method. In addition, investigations demonstrate the ability of the method to detect changes in morphology of peptide-LUVs during interactions with interface.

Finally, a new method was developed to allow spatiotemporal visualization of the macromolecular crowding effect in cells. An amine-reactive aggregation-induced emission fluorogen (AIE) was used to label proteins in the cytoplasm. The change in the protein mobility as well as local viscosity was monitored by using FAIM and FLIM, respectively. The effect of osmotic stress on the cytoplasmic crowding in fixed Neuro-2A cells, treated with sorbitol at different concentrations was explored. Real time monitoring of cytoplasmic crowding effect in live cells showed the anisotropy histogram was shifted to higher values due to dense protein packing environments after sorbitol treatment. Total internal reflection fluorescence microscopy (TIRFM) was incorporated with FAIM to track morphological modifications during *Statin* drug treatments. FLIM results of CHO-K1 cells treated with different doses of statin drug exhibited minor changes in fluorescence lifetime histogram. Highly oriented F-actin filaments in cells (10 μ mol lovastatin treatment) displayed shorter mean lifetime and higher anisotropy in comparison with control cells. In addition, total internal reflection fluorescence anisotropy measurements (TIRFAM) exhibited the elevated fluorescence anisotropy in cells treated with 10 μ mol lovastatin compared to the cells treated with

5 μ mol lovastatin.

In conclusion, the results of this thesis showed how FAIM, FLIM and EW-TRAM can be employed as promising approaches toward the successful morphological investigation in polymeric and biological complex systems.

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List of Figures

1.1	Representation of the principle of Total Internal Reflection (TIR), where the exponentially decaying Evanescent Wave (a standing wave) penetrates into the medium with lower refractive index	10
1.2	Representation of the electrical field of TIR, where the exponentially decaying evanescent wave penetrates in the medium with lower refractive index. In this case the incident angle is far from the critical angle. The penetration depth is very shallow. The size of the studied area is $5(x - axis) \times 10(y - axis)$ micrometre. The intensity of electric field is depicted by colour coded scale bar at the right side	14
1.3	Representation of the total power of TIR, where the exponentially decaying evanescent wave penetrates in the medium with lower refractive index. In this case the incident angle is far from critical angle. The penetration depth is very limited and shallow. The size of studied area is $5(x - axis) \times 10(y - axis)$ micrometre. The intensity of electric field is depicted by colour coded scale bar at the right side.	15
1.4	Representation of the electrical field of TIR, where the exponentially decaying evanescent wave penetrates in the medium with lower refractive index. In this case the incident angle is close to the critical angle. The penetrated electrical field is clear. The size of studied area is $5(x - axis) \times 10(y - axis)$ micrometre. The intensity of electric field is depicted by colour coded scale bar at the right side	16

1.5	Representation of the total power of TIR, where the exponentially decaying evanescent wave propagates in the medium with lower refractive index. In this case the incident angle is close to the critical angle. The penetrated power is clear. The size of studied area is $5(x-axis) \times 10(y-axis)$ micrometre. The intensity of electric field is depicted by colour coded scale bar at the right side.	16
1.6	Representation of refracted electrical field , the electrical field is propagates into the medium with lower refractive index. In this case the incident angle is less than critical angle. The size of studied area is $5(x-axis) \times 10(y-axis)$ micrometre. The intensity of electric field is depicted by colour coded scale bar at the right side	17
1.7	Representation of refracted total power , the electrical field is propagates into the medium with lower refractive index. In this case the incident angle is less than critical angle. The size of studied area is $5(x-axis) \times 10(y-axis)$ micrometre. The intensity of electric field is depicted by colour coded scale bar at the right side	17
1.8	Comparison of steady-state and time-resolved fluorescence spectroscopy	20
1.9	Polarised emission from randomly oriented dipoles in the Cartesian coordinate system (i, j and k)	25
1.10	Schematic coordinate system of the electric field vectors of emission from a dipole aligned at an angle θ to the j -axis and an angle ϕ to the i axis (path of excitation) in bulk solution. The corresponding emission intensity is the square of the electric field amplitudes, where the anisotropy depends on the intensity projected onto both j and k axes . .	27

1.11	Schematic representation of the in-plane (ϕ) and out-of-plane (θ) depolarisation monitored through EW-TRAMS. In-plane anisotropy measures the depolarisation on the xy -plane and out-of-plane anisotropy measures the overall depolarisation projected onto the yz -plane.	31
1.12	Representation of fluorescence emission from a dipole aligned at an angle (ϕ) to the y -axis and angle (θ) to the z -axis. (s)-polarised propagating light incident on the interface that generates an evanescent wave polarised along the y -axis.	33
1.13	Out-of-plane anisotropy diagram of the emission electric field vectors from a dipole aligned at an angle θ to the z -axis. (p)-polarised propagating light incident on the interface and generates an evanescent wave polarised along the z -axis. The corresponding emission intensities illustrated on the z -axis and into the xy -plane (I_p).	36
1.14	Schematic presentation of the concept of FLIM and steady state imaging. The cell in this schematic has two regions that display the same fluorescence intensity but different decay times, $\tau_2 > \tau_1$	39
1.15	Pixel by pixel anisotropy calculation using intensity of different polarisations. This Matlab script is described in appendix 1.	42
2.1	Schematic representation of the home-built TCSPC optical arrangement. CD: cavity dumper, M1: partial reflector mirror (coverslip), DC: frequency-doubling crystal, PH: pin hole, M2: Mirror, PL: polariser, LN: lens, PS: polarisation scrambler, MC: monochromator, PMT: photomultiplier, CFD: constant fraction discriminator, TAC: time to amplitude converter, FFD: high speed photo diode, PC: personal computer.	46
2.2	Schematic representation of the first home-built EWIFS optical arrangement, used for time-resolved EWIFS and EW-TRAMS (64)	48

2.3	Schematic diagrams of first TREWIFS designs by Lensun et al and Colin Scholes. It achieved variable incidence angle through different means; (a) by the mounting the TREWIFS cell assembly on a rotation stage with a fixed excitation light path, (b) TREWIFS cell assembly fixed and the excitation light path altered by a coupled rotation – translation stage mounted mirror.	49
2.4	Schematic representation of the TREWIFS apparatus, using the two off-axis parabolic mirrors assembly to ensure variable incidence angle upon the active surface. CD: cavity dumper, M1: partially reflector mirror, DC: frequency-doubling crystal, PH: pin hole, CFD: constant fraction discriminator, TAC: time to amplitude converter, FFD: high speed photo diode, PC: personal computer.	51
2.5	The correction for the polarisation bias of the detection system (G factor). was determined to be very close to unity by both tail-matching the I_{VV} and I_{VH} profiles and the I_{HV}/I_{HH} ratio from the time-resolved fluorescence decays of Coumarin 153 in solution.	55
2.6	Schematic diagram of the TREWIFS/EW-TRAM apparatus. CD: cavity dumper, M1: partially reflector mirror (coverslip), DC: frequency-doubling crystal, PH: pin hole, M2: Mirror, SM1: Stepper motor No 1, SM2: Stepper motor No 2, SM3: Stepper motor No 3, SM1B: stepper motor No 1 control board, SM2B: stepper motor No 2 control board, SM3B: stepper motor No 3 control board, FR: Fresnel rhomb, BL: beam blocker, HP: hemi-cylindrical prism, SHL: sample holder, TOR: Teflon O-ring, MSI: microscope slide, FL: high pass filter, PL: polariser, LN: lens, DP: polarisation scrambler, MC: monochromator, PMT: photomultiplier, CFD: constant fraction discriminator, TAC: time to amplitude converter, MCA: multichannel analyser, FFD: high speed photo diode, PC: personal computer.	56

2.7	Schematic diagram of the total internal reflection fluorescence anisotropy imaging microscope developed in this work.	62
2.8	Fluorescence anisotropy imaging microscope developed in this work. . .	63
2.9	G-factor at total internal reflection of Auramine-O in ethanol for 60X objective.	65
2.10	Histogram of G-factor at total internal reflection of Auramine-O in ethanol for 60X objective.	65
3.1	Fluorescence lifetime images (top panels) for MEH-PPV thin films cast from various solvents chlorobenzene, toluene and chloroform and the fluorescence decay profiles of corresponding points (A,B and C) recovered from the lifetime images. The photophysical properties of the same polymer with the same casting condition are different by using three different solvents.	79
3.2	The fluorescence lifetime distribution histograms in the MEH-PPV films cast from chlorobenzene, toluene and chloroform. Films casted from chloroform have shorter distribution compared with films cast from the other solvents.	81
3.3	The steady state FAIM of MEH-PPV conjugated polymers casted from toluene and chloroform (left) and their corresponding anisotropy histogram (right).	83
3.4	General chemical structures of two generation polyfluorenes which could be induced to adopt a β -conformation. Schematic illustrations of dihedral angle and film orderliness in the amorphous and β -conformation film (133).	89

3.5	(a) UV-vis and (b) PL spectra of PFO amorphous and β -conformation films spincoated from $CHCl_3$ and DCE solutions, respectively, the inset showing the molecular structure of PFO and a photograph of PFO films with and without β -conformation under 365 nm UV lamp irradiation. (c) UV-vis and (d) PL spectra of PODPF films before and after annealing at 230°C, 275°C, 300°C, the inset showing the molecular structure of PODPF and a photograph of corresponding films under 365 nm UV lamp irradiation.	90
3.6	AFM images of PFO films spin-coated from (a) $CHCl_3$ and (b) DCE solution, respectively. The size of the AFM images is $5\ \mu\text{m} \times 5\ \mu\text{m}$. FLIM images of PFO (c) amorphous (α -) and (d) β -conformation film. FAIM images of PFO (e) amorphous and (f) β -conformation film. (g) Fluorescence lifetime distributions and (h) fluorescence anisotropy imaging distribution histograms of PFO amorphous and β -conformation films. Two film thickness are both about 100~105 nm.	93
3.7	Atomic force microscopy (AFM) images of PODPF films (a) before annealing and after annealing at (b) 230°C, (c) 275°C, and (d) 300°C. The size of the AFM images is $5\ \mu\text{m} \times 5\ \mu\text{m}$. The (a2-d2) FLIM and (a3-d3) FAIM images of the corresponding films. (e) Average fluorescence lifetime and (f) fluorescence anisotropy distribution histograms of PODPF films following annealing at different temperatures. All film thickness are about 105~110 nm.	96
4.1	Chemical structures of NBD labelled Melittin in two different terminals: (N and Lys-7).	103

- 4.2 The fluorescence decay kinetics and global analysis of the NBD-labelled melittin in buffer at the silica interface (top). Penetration depth vs contribution percentage (Bottom) for short, middle and long lifetime components. The global lifetimes are 0.554 (ns), 1.896 (ns) and 7.956 (ns) and the global goodness of fits χ^2 is 1.075. The weighted residuals for the fits are also shown. 107
- 4.3 The fluorescence decay kinetics and exponential component analysis of the NBD-labelled melittin in buffer at interface. Lifetime vs penetration depth (top) for short, middle and long lifetime components. The goodness of fits are χ^2 1.082, 0.920, 1.142 and 1.087 for bulk and penetration depths of 75 (nm), 64 (nm) and 60 (nm) respectively. The weighted residuals for the fits are also shown. 108
- 4.4 The fluorescence decay kinetics and global analysis of the NBD-labelled melittin interacting with POPC (top left) and POPC/POPG (top right) LUVs. Penetration depth vs contribution percentage (bottom) and residuals of fits POPC/POPG LUVs (middle left) and POPC LUVs (middle right) for short, middle and long lifetime components. The global lifetimes of POPC LUVs are 0.729 (ns), 2.796 (ns) and 7.495 (ns) and the global χ^2 for this type of vesicles is 1.144. The global lifetimes of POPC/POPG LUVs are 0.635 (ns), 2.470 (ns) and 7.665 (ns) and the global χ^2 for this type is 1.062. 112

4.5	The fluorescence decay kinetics and exponential component analysis of the NBD-labelled melittin interacting with POPC and POPC/POPG LUVs at the interface. Lifetime vs penetration depth (top) and residuals of fits POPC/POPG LUVs (bottom-left) and POPC LUVs (bottom-right) for short, middle and long lifetime components. The goodness of fits χ^2 for POPC/POPG vesicles are 1.006, 0.942, 1.110 and 1.165 for bulk and penetration depths of 75 (nm), 64 (nm) and 60 (nm) respectively. The goodness of fits χ^2 for POPC vesicles are 1.013, 1.004, 1.066 and 1.100 for bulk and penetration depths of 75 (nm), 64 (nm) and 60 (nm) respectively.	115
4.6	The average fluorescence lifetimes resulting from an exponential component analysis of the NBD-labelled melittin in buffer and also attached to POPC and POPC/POPG LUVs at the interface as a function of penetration depth.	116
4.7	Time resolved fluorescence anisotropy of NBD-labelled melittin interacting with POPC vesicles for bulk conditions (top-left) and the corresponding residuals (top-right), evanescent wave excitation with a penetration depth of 75 nm (bottom-left) and the corresponding residuals (bottom-right). EW-TRAM data in the bottom panels were obtained for both parallel (in-plane) and perpendicular (out-of-plane) of the interface of the hemi-cylindrical prism. The solid lines inside the dotted curves are the fits obtained using the above equations.	118
5.1	Design principle of using an amine-reactive dye TPE-Py-NCS to label proteins and monitor the micro-environment change in cells through FLIM and FAIM.	125
5.2	The original intensity images of cells divided into the separate polarisations via a polarisation beam splitter and acquired by an EMCCD camera (see apparatus schematic 2.7).	127

5.3	The polarised images after background subtraction (using ImageJ) from the original intensity image (figure 5.2).	127
5.4	The fluorescence anisotropy map corresponding to figure 5.3 calculated using Equation 2.10 via MatLab code (available in appendix 1).	128
5.5	Effect of osmotic stress on cytoplasmic crowding in fixed cells. (A–C) FAIM images and (E–G) FLIM images of fixed Neuro 2A cells treated with sorbitol at different concentrations for 30 min. (D) Fluorescence anisotropy distribution histograms from panels A–C. (H) Fluorescence lifetime distribution histograms from panels E–G. Control refers to cells without sorbitol treatment. [TPE-Py-NCS] = 10 mmol. Scale bar: 5 micro metre.	129
5.6	Real time monitoring of cytoplasmic crowding effect in live cells. (A–E) FAIM images of TPE-Py-NCS stained Neuro 2A cells taken at different time points after being exposed to 450 mmol sorbitol. (F) Fluorescence anisotropy distribution histograms from panels A–E. [TPE-Py-NCS]= 10 mmol. Scale bar: 5 micro metre. The corresponding total intensity of the anisotropy maps are depicted in figure 5.7.	131
5.7	Real time viscosity monitoring of cytoplasmic crowding effect in live cells. (A–E) intensity images of TPE-Py-NCS stained Neuro 2A cells taken at different time points after being exposed to 450 mmol sorbitol. [TPE-Py-NCS]= 10 mmol.	131

- 5.8 Organization of the actin cytoskeleton in fixed CHO-K1 cells treated with increasing concentrations of Lovastatin. The photon intensity images depicted in A,B and C panels (red: low, blue: high) and mean lifetime maps depicted in D,E and F panels are shown as color coded images on a scale of 800 - 4000 ps. The actin cytoskeleton was stained with NBD-pallacidin. Panels A & D show representative images for control cells, and panels B & E and C & F show images for cells treated with 5 μmol and 10 μmol Lovastatin, respectively. Increase of F-actin filaments can be observed upon treatment with increasing concentrations of Lovastatin. 138
- 5.9 Fluorescence lifetime distributions of control CHO-K1 cells (brown) and Lovastatin treated cells (green: 5 μmol and blue: 10 μmol) for short lifetime T_1 (top), longer lifetime T_2 (middle) and mean lifetime T_m (bottom). Highly oriented F-actin filaments in cells (in the presence of 10 μmol Lovastatin) exhibit shorter mean lifetime when compared to control cells with partially oriented filaments. Each curve is an average of three random spots of each condition and normalized to 1000. 139
- 5.10 Steady state total internal reflection fluorescence anisotropy imaging (blue: low, red: high) shown as color coded images of the actin cytoskeleton (stained with NBD-pallacidin) of fixed CHO-K1 cells excited by 465 nm CW laser and treated by 5 μmol Lovastatin drug. Polarized TIRFM image using parallel polarisers I_{ss} (A), in plane of interface anisotropy map (B) and out-of-plane anisotropy map (C). 142

5.11 The histogram distributions of the steady state fluorescence anisotropy imaging of control CHO-K1 cells (red lines) and Lovastatin treated cells (brown lines: 5 μ mol concentration and blue lines: 10 μ mol concentration) for bulk (left), in-plane of interface (middle) and out-of-plane of interface (right). F-actin cytoskeleton polymerisation correlates with the anisotropy histogram distribution. Highly oriented F-actin fibres in cells including 10 μ mol Lovastatin treatment experience higher values of bulk and in-plane anisotropy and lower values of out-of-plane anisotropy rather than partially oriented F-actin fibres in cells that include 5 μ mol Lovastatin concentration.	143
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List of Tables

3.1	The emission decay times, percentages, relative intensities and amplitude weighted-average lifetimes (calculated by equation 3.1) for three representative points in the lifetime maps corresponding to each of the three solvent-cast films.	80
3.2	Summary of optical spectra of PFO	91
3.3	Summary of optical spectra of PODPF	91
4.1	Detailed exponential components analysis (reconvolution) of the NBD-labelled melittin in buffer at interface	106
4.2	The results of fitting of fluorescence decays as a function of penetration depth by a global analysis (reconvolution) of the NBD-labelled melittin in buffer at the silica/sample interface	109
4.3	Detailed global analysis (reconvolution) of the NBD-labelled melittin in POPC (top) and POPC/POPG (bottom) LUVs at interface	113
4.4	Detailed exponential component analysis (reconvolution) of the NBD-labelled melittin in POPC (top) and POPC/POPG (bottom) LUVs at interface	114

*Dedicated to Zaynab & my parents: Mahmoud and
Hadigheh*

List of Abbreviations

TIR	Total-Internal Reflection
TIRF	Total-Internal Reflection Fluorescence
TIRFM	Total-Internal Reflection Fluorescence Microscopy
EWIFS	Evanescent-Wave Induced Fluorescence Spectroscopy
TREWIFS	Time- Resolved Evanescent-Wave Induced Fluorescence Spectroscopy
TRAM	Time- Resolved Anisotropy Measurements
EW-TRAM	Evanescent-Wave time- Resolved Anisotropy Measurements
FLIM	Fluorescence Lifetime Imaging Microscopy
FAIM	Fluorescence Anisotropy Imaging Microscopy
TCSPC	Time-Correlated Single Photon Counting
FWHM	Full Width at Half Maximum
WFM	Wide Field Microscopy
LSCM	Laser Scanning Confocal Microscopy
FCS	Fluorescence Correlation Spectroscopy
IRF	Instrument Response Function
MCA	Multi-Channel Analyser
MCP	Micro-Channel Plate
NA	Numerical Aperture
S/N	Signal/Noise

Contents

Declaration of Authorship	iii
Publications	v
Abstract	xi
Acknowledgements	xv
Contents	xxxvi
1 Introduction, Background and Theory	1
1.1 Introduction	1
1.1.1 Proem	1
1.1.2 Motivation	4
1.1.3 Aim and Scope	5
1.1.4 Overview of the Study	6
1.2 Total Internal Reflection	9
1.2.1 Evanescent Waves	12
1.2.2 Electric Field Amplitudes at Interface	13
1.3 Fluorescence Spectroscopy and Polarisation	18
1.3.1 Steady State and Time-Resolved Fluorescence Spectroscopy . . .	19
1.3.2 Why Time-Resolved Measurements?	21
1.3.3 Definition of the Lifetime and Decay Time	21
1.3.4 Evanescent Wave Time-Resolved Fluorescence Spectroscopy . .	22

1.4	Fluorescence Anisotropy	23
1.4.1	Time-Resolved Fluorescence Anisotropy	29
1.4.2	Time-Resolved Evanescent Wave Induced Fluorescence Anisotropy Measurements (EW-TRAMS)	30
1.4.3	In-Plane Anisotropy	30
1.4.4	Out-of-Plane Anisotropy	35
1.5	Fluorescence Lifetime Imaging Microscopy	38
1.5.1	Scanning Confocal Fluorescence Lifetime Imaging	39
1.6	Fluorescence Anisotropy Imaging Microscopy	40
1.7	Summary	41
2	Experimental Details	44
2.1	Introduction	44
2.2	Steady-State Absorbance and Fluorescence Spectra	44
2.3	Time-Resolved Fluorescence Spectroscopy	45
2.3.1	Time Correlated Single Photon Counting (TCSPC)	45
2.4	Time-Resolved Anisotropy Measurements (TRAM)	47
2.4.1	Equipment Development	47
2.4.2	Time-Resolved Anisotropy Measurements (TRAM) Apparatus	52
	G Factor and Equipment Bias Determination	54
	Data Analysis	58
	Discrete Lifetime Analysis	58
2.5	Fluorescence Lifetime Imaging (FLIM)	59
2.6	Fluorescence Anisotropy Imaging (FAIM) Apparatus	60
2.7	Materials and Sample Preparation	66
2.7.1	Polymer films	66
	PFO and PODPF Films	66
	MEH-PPVs	67

	Controlled Evaporative Self-Assembly Technique, <i>Snake – Skin</i>	
	Method	67
2.7.2	Bio-films	68
	Cleaning methods	68
	Large Unilamellar Vesicles (LUVs)	68
	F-actin labeling of CHO-K1 cells	69
	Aggregation-Induced Emission Samples (AIEs)	70
	Materials	70
	Cell culturing	71
	Cell Staining	71
2.8	Summary	72
3	Photophysics and Fluorescence Anisotropic Behavior of Conjugated Polymers	74
3.1	MEH-PPV Polymers	75
3.1.1	Introduction	75
3.1.2	FLIM of MEH-PPVs	78
3.1.3	FAIM of MEH-PPV films	82
3.1.4	Conclusion	84
3.2	PFO & PODPF Polymers	86
3.2.1	Introduction	86
3.2.2	FLIM and FAIM of PFO & PODPF systems	88
3.2.3	Conclusion	98
4	Time-Resolved Evanescent Wave-Induced Fluorescence Spectroscopy of POPC and POPG Membranes using NBD-Labelled Melittin	101
4.1	Introduction	103
4.2	TREWIFS of NBD-labelled melittin in buffer	106

4.3	TREWIFS of NBD-labelled melittin attached to POPC and POPC/POPG LUVs	110
4.4	TRAM of NBD-labelled melittin attached to POPC LUVs	117
4.5	Conclusion	120
5	Mapping the Fluorescence Anisotropy in Biological Systems	122
5.1	Measuring macromolecular crowding in cells through fluorescence anisotropy imaging	123
5.1.1	Introduction	123
5.1.2	FAIM of Osmotic Stress on Cytoplasmic Crowding in Cells	126
5.1.3	Conclusion	133
5.2	Actin Cytoskeletal Rearrangement by Cholesterol Lowering Statin Drugs	134
5.2.1	Introduction	134
5.2.2	Fluorescence Lifetime Imaging Microscopy (FLIM)	137
5.2.3	FAIM Results	140
5.3	Conclusion	144
6	Summary, Conclusion and Future Work	146
6.1	Introduction and Experimental Methods	147
6.2	EW-TRAM of LUVs	148
6.3	Mapping fluorescence anisotropy in polymeric and biological systems .	149
	Bibliography	152
A	MATLAB Code for Image Processing	186
B	Labview Code for Evanescent Wave Time-Resolved Anisotropy Measurements (EW-TRAM)	191

Chapter 1

Introduction, Background and Theory

1.1 Introduction

1.1.1 Proem

Understanding the dynamics of energy migration for disordered complex systems, such as polymeric solar devices, is crucial for optimizing the energy transport properties of the donors to maximize the migration-trapping strategy to acceptors. Fluorescence anisotropy is an avenue and experimental observable for monitoring energy migration in disordered chromophores in condensed systems. In the absence of rotational diffusion of chromophores, fluorescence depolarisation, or loss of initial fluorescence anisotropy, results from the randomisation of the initially photoselected emission transition dipole orientations through Forster-mediated energy migration. In soft matter biological systems the rotational diffusion of chromophores or dyes becomes the dominant cause of fluorescence depolarisation. Spatial mapping of the fluorescence anisotropy can provide valuable information on the mobility and orientation of the molecule and has been used to investigate protein–protein interactions, ligand–receptor binding processes, membrane mobility, and protein fibril formation.

Application and interpretation of spatial mapping of fluorescence anisotropy in both polymeric and biological systems are still challenging. There are several technical and theoretical issues such as lack of precise imaging methodology, correction factors

and quantitative data illustration and interpretation. Despite recent examinations and advances in techniques, the dynamics of fluorescence depolarisation in complex systems are not yet fully understood.

In the last three decades, advances in optics, photonics, electronics, mechanical automation and computational imaging tools have revolutionized microscopy and imaging techniques. Modern advanced microscopes have access to most of the spatial scales in polymeric and biological systems from individual molecules in nanometric dimensions to the whole system, and from single snapshots to time lapse measurements, the nanoseconds resolution to days. After developing new imaging methods, and as these new technologies appear, the scientific community faces a few new challenges to deliver and apply these methods to answer scientific questions.

Light microscopy in particular is a keystone of modern microscopy and imaging techniques with broad application in biological and polymeric systems. There is continuous rapid development at all levels of microscopy and imaging experiments such as sample labelling, noise, contrast, resolution, signal detection, image analysis and data processing. Commercial and automated microscopes are the results of combination of these technical advances.

Phase, polarisation and fluorescence are the bases for modern optical imaging and microscopy. Development of methods with high contrast in polymeric and biological specimens was one of the biggest challenges in microscopy and imaging. Zernike's phase contrast microscopy is one of the early methods that use phase shift for interference generation (1). A few decades later, Shinya Inoue used polarisation microscopy to increase contrast and explore the previously unseen details in spindle fibres (2). Furthermore, polarisation microscopy has the additional advantage of allowing the determination of the macromolecular structure complexes with the resolution on the tens of nanometre scale (3). Fluorescence microscopy made possible contrast generation through molecular specificity and created another avenue for collection of fluorescence from molecules in polymer matrix or cells. 3D imaging by optical sectioning

fluorescence microscopy provided the higher order structure probing in complex systems (4). The two methods, wide-field deconvolution microscopy (WFM) and laser scanning confocal microscopy (LSCM), improved the resolution in fluorescence microscopy to enable the visualisation of dynamic process and structures of biological and polymeric systems. Microscopy, when combined with digital detectors and sensitive cameras such as CCDs, is a quantitative technique able to be applied to a wide range of systems. In addition by using computational image processing, the invisible can become easily seen and quantifiable.

After introducing the WFM and LSCM, the rate of microscopy developments has accelerated significantly (5–7). Modern methods of microscopy and imaging have been introduced, such as laser scanning, multi-photon, light sheet (8), fluorescence correlation spectroscopy (FCS), fluorescence cross-correlation spectroscopy (FCCS) (9), and super-resolution techniques, (10) and are now in routine use in many research laboratories. The explosion of developing parallel techniques and imaging methods is still expanding at an increasing rate. These microscopy and imaging methods have their own advantages regarding the type of study. All these new imaging methods can be used as complementary or expanding tools for probing the structure, morphology and dynamics of biological and polymeric systems. To gain a clear understanding in biological or polymeric systems, a combination of these imaging methods will be required to completely investigate the area.

Each of the aforementioned techniques needs specific expertise and considerable research knowledge. In most of the cases they are results of close collaboration between physicists, chemists, biologists, engineers, software developers and mathematicians. These scientists express the power of collaborative teams through which they collect, process and assemble technology from many different fields into new tools for polymeric or biological discovery in light of microscopy.

1.1.2 Motivation

Beside the optical microscopy and imaging methods which were discussed above, there are numerous techniques able to investigate samples in a wide range of systems including polymers and bio-molecules. Techniques such as mass spectroscopy (11–13), slow-positron annihilation spectroscopy (14, 15), X-ray photoelectron spectroscopy (16, 17) and X-ray absorption fine structure spectroscopy (18, 19) are capable of providing invaluable information relating to conformation and morphology of such systems. There are some other characterisation methods that can be utilised to study of fluorescent systems in bulk solutions such as nuclear magnetic (20, 21) and electron spin resonance (22, 23). They also have been extended to study polymeric and biological systems. Using density profiles, both X-ray (24, 25) and neutron methods (26, 27) provide information about macromolecular conformation and morphology. These methods also suffer from some issues such as molecular damage caused by X-rays. In addition, deuterated water used for neutron studies is identified to change the hydrogen-bonding in protein networks (28, 29). Optical spectroscopy methods such as circular dichroism (30, 31) and photon correlation spectroscopy (32, 33) also provide information about macromolecular morphology.

Considering the wide applications of polymeric systems such as conjugated polymers, understanding of the photophysical characteristics of these systems has seen substantial progress in recent years. Several challenges still inhibit a clear understanding of the photophysical features of these systems. One of these challenges is the effect of aggregated inhomogeneities on the photophysical characteristics of a given polymeric system. It is necessary to understand the photophysical aspects of these systems, for example energy migration, when the morphology is not uniform. Recently, the nature of the relationship between morphology and energy migration has been under discussion (34). The concept of energy migration from the initial absorbed dipole to the closely neighbouring chains is linked to the details of depolarisation process or

anisotropy. The excitation of the initially excited chain may migrate to the neighbouring chains with different orientation and is the emission polarisation lost in each energy transfer step. One of the basic challenges in large scale manufacturing of photovoltaic devices is transforming nano/micro-metre morphological control from research labs to the metre-scale printed devices in factories. Keeping the entire morphology unique on the metre-scale is ideal but not easily accessible. The relationship between the morphology and its outcomes in photophysical properties of conjugated polymers plays an important role in producing more efficient devices.

One of the sensitive and reproducible techniques that provides a quantitative parameter is the fluorescence anisotropy approach. The anisotropy can be interpreted in terms of rigidity or fluidity of biological systems. There are numerous open questions in biological systems in which need to be addressed in terms of morphology. In addition, changes of the morphology in biological systems such as cells or bilayers induces a new environment. Nano/micro metre scale investigation of biological systems such as cells or lipid bilayers needs an accurate and quantitative approach. In this situation, a strategic approach to observe, understand and control the morphology is necessary. A reliable strategy that covers most of the challenges is addressed here.

1.1.3 Aim and Scope

This thesis aims to address a few chemistry problems and introduce a new strategy based on mapping the dynamics of fluorescence anisotropy in the context of the study of the morphology on photophysical properties of biological and polymeric systems. In particular, a concentration on dynamics of fluorescence depolarisation in inhomogeneous solid state polymer systems, which leads to the study of energy migration, and in crowded biological systems, which leads to the study of rotational correlation times of the dye.

Specific morphological features such as aggregation of polymeric chains, inhomogeneities and crowding of macromolecular systems will be investigated using imaging and spectroscopy. The main aim of these investigations is to observe the consequences of the specific morphology. The morphology properties which influence device efficiency in conjugated polymers or understanding protein interactions on crowding condition in cells are studied.

Different time-resolved and steady state techniques will be utilised. Time resolved methods such as time correlated single photon counting (TCSPC), evanescent wave time-resolved anisotropy measurements (EW-TRAM) and fluorescence lifetime imaging (FLIM) have been used. The concept of fluorescence anisotropy measurements will be expanded to 2D dimensions namely “fluorescence anisotropy imaging microscopy”(FAIM). Other complementary photophysical techniques will also be used including steady-state absorption and steady-state fluorescence spectroscopy.

This study aims, on a practical level, to introduce and clarify the possibilities and limitations of FAIM as a strategy to investigate the dynamics of fluorescence depolarisation within chemistry problems such as energy migration in polymeric and rotational diffusion in biological complex systems.

A deeper understanding of the dynamics of fluorescence anisotropy in biological or polymeric systems would introduce new research directions and provide valuable guidelines for the further development of the FAIM method.

1.1.4 Overview of the Study

This thesis is made up of six chapters and two appendixes with three main directions of research.

Chapter 1, discusses the introduction, background and theory of fluorescence spectroscopy and anisotropy in detail. Firstly, this chapter will discuss the physical background of total internal reflection (TIR) and the generation of evanescent waves at the

interface. The background of steady-state and time-resolved fluorescence spectroscopy and anisotropy techniques such as FLIM and FAIM are also reviewed.

Chapter 2, outlines the experimental details of the different spectroscopy and microscopy methods used that were introduced in chapter 1. Different spectroscopy and imaging methodologies are discussed with focus on the two primary experimental techniques, evanescent wave time resolved anisotropy measurements (EW-TRAM) and FAIM. These methods are also investigated in details, including their optical apparatus, advantages and limitations. In addition, the materials and methods which were used for sample preparation in this thesis are also discussed.

Chapter 3, investigates the optical photophysics and fluorescence anisotropic behavior of two families of conjugated polymers, *poly[2-methoxy-5-(20-ethyl-hexyloxy)-1,4-phenylenevinylene]* MEH-PPVs and *Poly(9,9-dioctylfluorene-2,7-diyl)* PFO/ *poly[4-(octyloxy)-9,9-diphenylfluorene-2,7-diyl]-co-[5-(octyloxy)-9,9-diphenylfluorene-2,7-diyl]* PODPFs, using FAIM and FLIM methods. Results indicate that combining FLIM and FAIM imaging methods is a powerful strategy to explore the photophysical properties of the morphology of the aggregated inhomogeneities in both types of polymers. The FAIM method supports the evidence of energy migration in the aggregated regions of MEH-PPV polymer films. In addition, the photophysical properties and depolarisation kinetics of amorphous and β -conformation forms of a polyfluorene system, prepared as a function of annealing temperature, are systematically investigated.

Chapter 4 explores the photophysical and morphological properties of the

1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine (POPC) and *1-hexadecanoyl-2-(9Zoctadecenoyl)-sn-glycero-3-phospho-(1'-rac-glycerol)* (POPG) membranes tagged via *(7-nitrobenz-2-oxa-1,3-diazol-4-yl* :

NBD) NBD-labelled Melittin, using TREWIFS and anisotropy methodology at a silica/membrane interface. Results indicate that this is a powerful method able to detect fluorescence lifetime changes and depth profiling with tens of nanometre resolution. Rotational correlation times and fluorescence lifetimes are sensitive to the micro-environment of the dye and can be used to describe the interior motion of vesicles in both bulk solution or at the interface. Two models of lifetime analysis, exponential decay and global analysis, are utilised to investigate the morphology of large unilamellar vesicles (LUVs). Both models confirm and show the same trend that the fluorescence lifetime of a dye in peptides or LUVs varies in terms of distance from the interface. This indicates the change of morphology or interaction pattern of these systems when the main buffer penetrate to the vesicles .

Chapter 5, discusses the use of mapping the dynamics of fluorescence depolarisation of two different biological systems including macromolecular crowding and actin-cytoskeleton rearrangements by cholesterol lowering mechanisms in cells. The FAIM approach is used for mapping the fluorescence anisotropy and FLIM method is used as a complementary technique. Results indicate that combined FLIM and FAIM imaging methods is a powerful methodology to observe the change of the morphology in both biological systems. Restriction of the intramolecular motion in the crowding effect can be detected by increases in the anisotropy in the depolarisation map. Real time monitoring of the cytoplasmic crowding effect of live cells is also possible with the same method. The FAIM approach is applied to total internal reflection fluorescence microscopy (TIRFM) to map the dynamics of fluorescence anisotropy in thin films of CHO-K1 cells. F-actin polymerisation is observed due to effect of high dosages of lovastatin drug treatment.

Finally, Chapter 6 contains the conclusions and addresses future research approaches and directions.

Appendix A lists the Matlab code for mapping the dynamics of fluorescence anisotropy which was used in chapters 3 and 5 of this thesis.

Appendix B lists the Labview code for generating evanescent wave time-resolved fluorescence anisotropy used in chapter 4 of this thesis.

1.2 Total Internal Reflection

When light is incident on the interface between two optically transparent media with different refractive indices, " n ", reflection and refraction are possible. The light may reflect and propagate back into the first medium, or it may "refract" into and propagate through the second medium (see figure 1.1) (35, 36). According to the law of reflection in equation 1.1, the light may be reflected with angle, θ_{ref} , which is equal to the incident angle θ_i :

$$\theta_i = \theta_{ref} \quad (1.1)$$

Otherwise, light transmitted into the second medium may be refracted at an angle, θ_r , determined by Snell's law, or the law of refraction in equation 1.2.

$$n_1 \sin \theta_i = n_2 \sin \theta_r \quad (1.2)$$

where n_1 and n_2 are the refractive indices of media 1 and 2, respectively, in figure 1.1.

It is useful to separate the electric fields on both sides of the interface into components that are orthogonal to each other and can be called "polarisations". Every wave can be represented as a superposition of two polarisations (S: electric field polarised perpendicular to the plane of the incidence (y-direction) and P: electric field polarised parallel to in the plane of incidence (x-z plane) in figure 1.1).

After solving Maxwell's equations of electromagnetic theory, the amplitude of the reflectiveness of the p and s components of the incident light are given by:

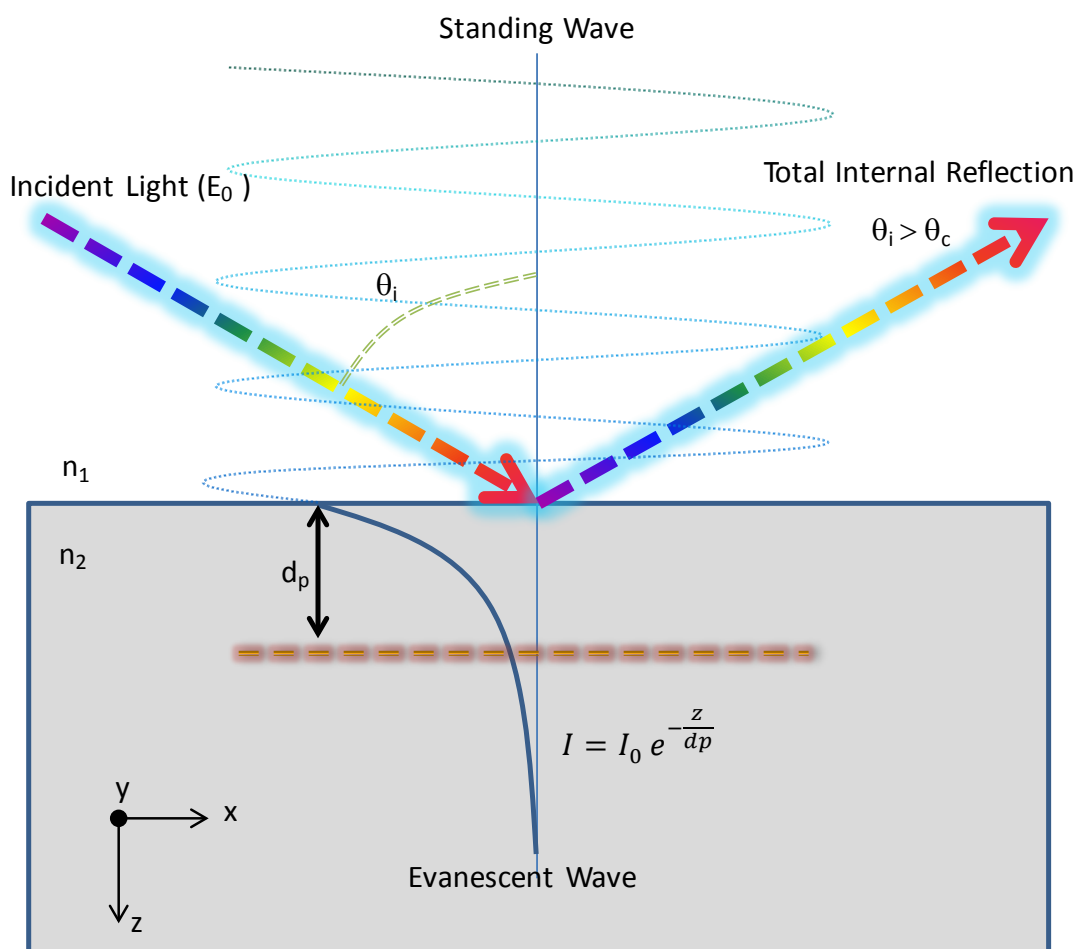


FIGURE 1.1: Representation of the principle of Total Internal Reflection (TIR), where the exponentially decaying Evanescent Wave (a standing wave) penetrates into the medium with lower refractive index .

$$r_p = \frac{n_2 \cos \theta_i - n_1 \cos \theta_r}{n_2 \cos \theta_i + n_1 \cos \theta_r} \quad (1.3)$$

$$r_s = \frac{n_1 \cos \theta_i - n_2 \cos \theta_r}{n_1 \cos \theta_i + n_2 \cos \theta_r} \quad (1.4)$$

where p and s represent the planes of polarisation of the incident light being parallel and perpendicular to the plane of incidence respectively.

Most of the work in chapter 4 of this thesis is based on events that occur when the refractive index of the second medium, n_2 , is lower than n_1 so light travels from an optically dense medium into the rarer medium. In this situation both reflection and refraction are possible but according to the Snell's equation 1.2 by increasing the incident angle, there is a limit, namely the "critical angle", the angle of incidence for which the angle of refraction is 90° . This critical angle can be derived from the refractive indices of the two media:

$$\theta_c = \arcsin n_{21} \quad (1.5)$$

and

$$n_{21} = \frac{n_2}{n_1} \quad (1.6)$$

When light intercepts the interface with an angle greater than the critical angle, the refracted angle become complex (37):

$$\cos \theta_r = i \sqrt{\frac{\sin^2 \theta_i}{n_{21}^2} - 1} \quad (1.7)$$

Substituting equation 1.7 in the Fresnel formulae (equations 1.3 and 1.4) results in them taking complex form:

$$r_p = \frac{n_{21}^2 \cos \theta_i - i \sqrt{\sin^2 \theta_i - n_{21}^2}}{n_{21}^2 \cos \theta_i + i \sqrt{\sin^2 \theta_i - n_{21}^2}} \quad (1.8)$$

$$r_s = \frac{\cos \theta_i - i\sqrt{\sin^2 \theta_i - n_{21}^2}}{\cos \theta_i + i\sqrt{\sin^2 \theta_i - n_{21}^2}} \quad (1.9)$$

In these modified Fresnel formulæ when the incident angle θ_i is greater than critical angle θ_c , $|r_p|=|r_s|=1$, indicating that n_{21} is a real number. Thus, for an angle of incidence greater than the critical angle, the interface acts as a perfect mirror and this phenomenon is known as Total Internal Reflection (TIR).

1.2.1 Evanescent Waves

In electromagnetics, the term "evanescent wave", is an oscillating electric and or magnetic field that does not propagate through next medium as an electromagnetic wave but whose energy is spatially concentrated in the vicinity of the source (oscillating charges and currents) at the interface. In total internal reflection phenomena, in terms of quantum mechanics, light is able to penetrate or tunnel into the rarer medium with limited distance from the interface (38). The standing wave inside the rarer (lower refractive index) medium is named the evanescent wave and shows the same frequency as the incident wave or laser light, while not propagating through the optically rarer medium. The electric field amplitude (E) of the wave decays exponentially away from the interface and is described by:

$$E = E_0 \exp\left(-\frac{z}{d_p}\right) \quad (1.10)$$

where z is the distance from the interfacial plane in figure 1.1 and E_0 is the electric field amplitude at the interface. The penetration depth d_p , can be defined as the distance at which the electric field amplitude falls to $\frac{1}{e}$ of its initial value at the interface and can be determined by:

$$d_p = \frac{\lambda}{2\pi\sqrt{n_1^2 \sin^2 \theta_i - n_2^2}} \quad (1.11)$$

where λ is the wavelength of the incident beam in denser medium. The penetration depth is often also defined by the electric field intensity:

$$I = I_0 \exp -\frac{z}{\Lambda} \quad (1.12)$$

where I and I_0 are intensities in the optically rarer medium at the interface. In this case, Λ is half the value of d_p , mentioned in equation 1.11. The penetration depth decreases with increasing angle of incidence and it has a maximum value of infinity at the critical angle.

1.2.2 Electric Field Amplitudes at Interface

In chapter 4 of this thesis, evanescent waves are used for excitation of fluorescence. For other chapters direct illumination is used as excitation. The evanescent wave's electric field components or polarisation are defined by three orthogonal orientations relative to the interfacial plane, x, y and z, as shown in figure 1.1. Considering both polarisation modes, (p) and (s), the evanescent wave electric field amplitude for each polarisation at the interface may be calculated from Maxwell's equations:

$$E_{0x} = \frac{2\sqrt{\sin^2 \theta_i - n_{21}^2} \cos \theta_i}{\sqrt{1 - n_{21}^2} \sqrt{[1 + n_{21}^2] \sin^2 \theta_i - n_{21}^2}} \quad (1.13)$$

$$E_{0y} = \frac{2 \cos \theta_i}{\sqrt{1 - n_{21}^2}} \quad (1.14)$$

$$E_{0z} = \frac{2 \cos \theta_i \sin \theta_i}{\sqrt{1 - n_{21}^2} \sqrt{[1 + n_{21}^2] \sin^2 \theta_i - n_{21}^2}} \quad (1.15)$$

where E_{0x} , E_{0y} and E_{0z} represent the electric field amplitudes at the interface along the coordinate system used in figure 1.1. The (p)-polarisation incident light, E_{0p} , generates an evanescent wave polarised along the x and z axes (Figure 1.1), which has the

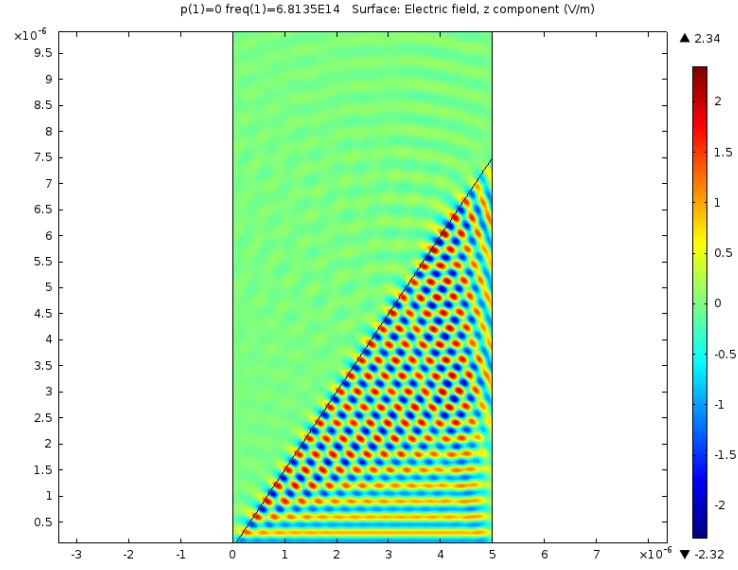


FIGURE 1.2: Representation of the electrical field of TIR, where the exponentially decaying evanescent wave penetrates in the medium with lower refractive index. In this case the incident angle is far from the critical angle. The penetration depth is very shallow. The size of the studied area is $5(x - axis) \times 10(y - axis)$ micrometre. The intensity of electric field is depicted by colour coded scale bar at the right side .

corresponding amplitude:

$$E_{0p} = \sqrt{|E_{0x}|^2 + |E_{0z}|^2} \quad (1.16)$$

On the other hand, (s)-polarisation incident light, E_{0s} , has an evanescent wave polarised along the y-axis , E_{0y} , in equation 1.14.

In this thesis a hemi-cylindrical silica prism with refractive index 1.466 was used as the high refractive index medium and water is used as rarer medium with refractive index 1.3374 . The dependence of the electric field amplitudes, E_{0z} , on the angle of incidence is shown in following figures. These are simulations of the silica-water interface using 440 nm laser calculated with the finite element method (FEM):

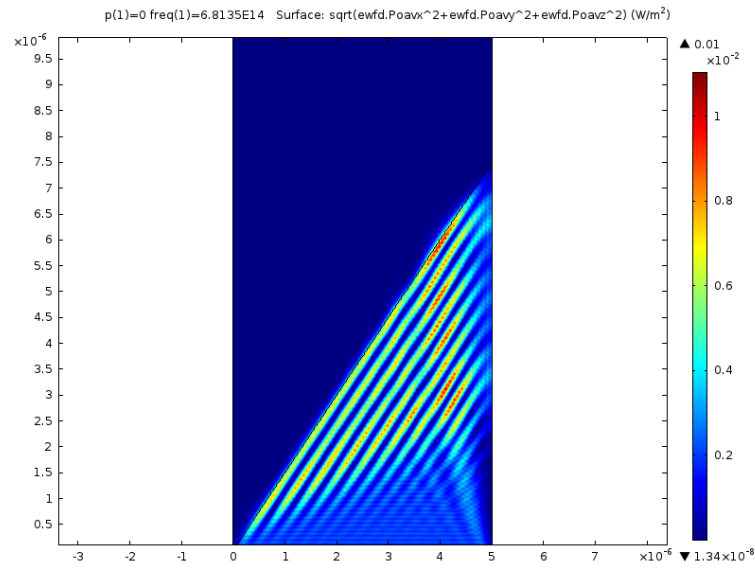


FIGURE 1.3: Representation of the total power of TIR, where the exponentially decaying evanescent wave penetrates in the medium with lower refractive index. In this case the incident angle is far from critical angle. The penetration depth is very limited and shallow. The size of studied area is $5(x - axis) \times 10(y - axis)$ micrometre. The intensity of electric field is depicted by colour coded scale bar at the right side.

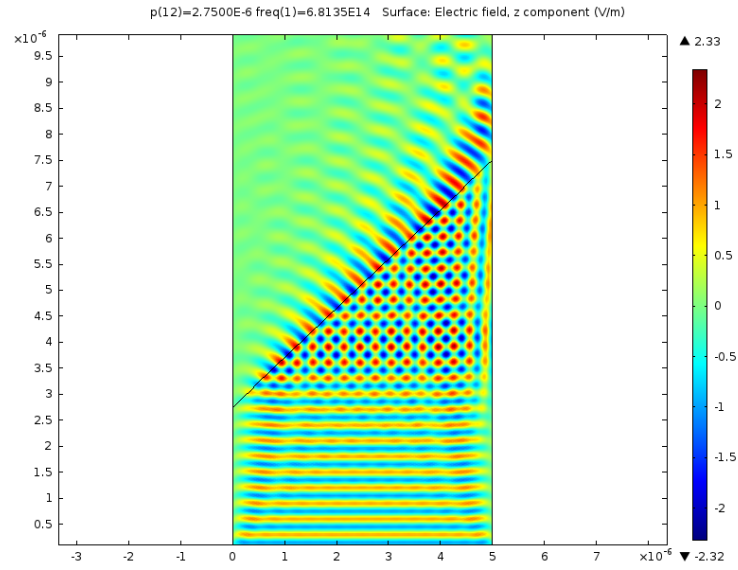


FIGURE 1.4: Representation of the electrical field of TIR, where the exponentially decaying evanescent wave penetrates in the medium with lower refractive index. In this case the incident angle is close to the critical angle. The penetrated electrical field is clear. The size of studied area is $5(x - axis) \times 10(y - axis)$ micrometre. The intensity of electric field is depicted by colour coded scale bar at the right side .

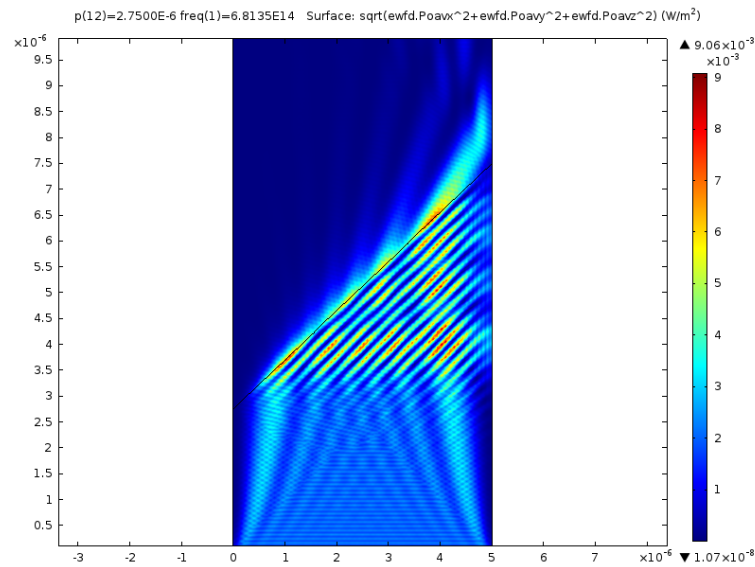


FIGURE 1.5: Representation of the total power of TIR, where the exponentially decaying evanescent wave propagates in the medium with lower refractive index. In this case the incident angle is close to the critical angle. The penetrated power is clear. The size of studied area is $5(x - axis) \times 10(y - axis)$ micrometre. The intensity of electric field is depicted by colour coded scale bar at the right side.

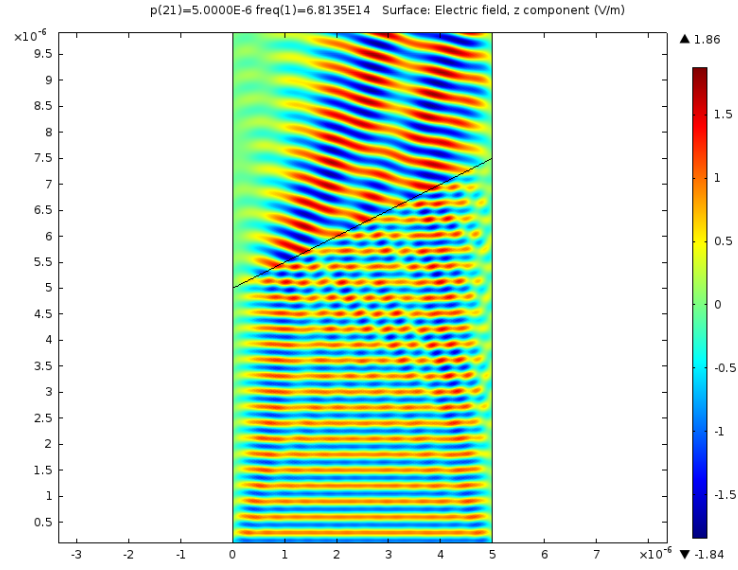


FIGURE 1.6: Representation of refracted electrical field , the electrical field is propagates into the medium with lower refractive index. In this case the incident angle is less than critical angle. The size of studied area is $5(x - axis) \times 10(y - axis)$ micrometre. The intensity of electric field is depicted by colour coded scale bar at the right side .

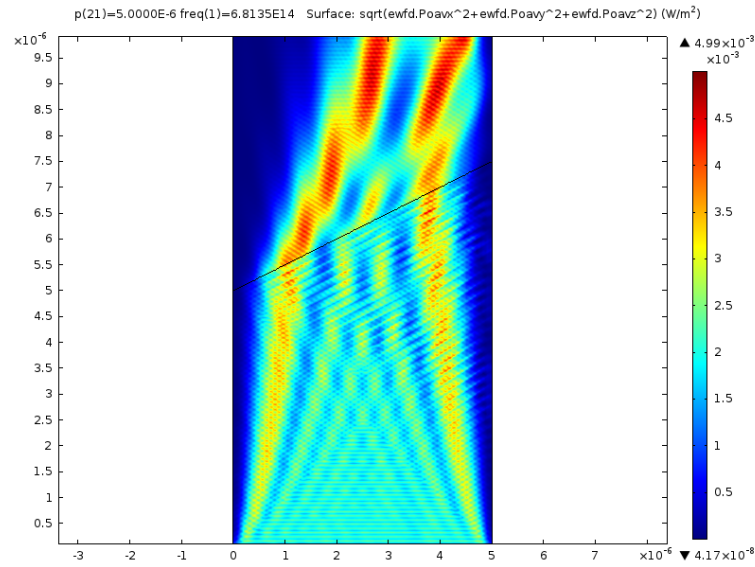


FIGURE 1.7: Representation of refracted total power , the electrical field is propagates into the medium with lower refractive index. In this case the incident angle is less than critical angle. The size of studied area is $5(x - axis) \times 10(y - axis)$ micrometre. The intensity of electric field is depicted by colour coded scale bar at the right side .

1.3 Fluorescence Spectroscopy and Polarisation

During the past few decades, fluorescence polarisation and anisotropy have been utilised in many different areas such as chemistry, biochemistry and biomedical fields. Since the mid-1980s, the availability of new instruments, polarisers and fluorescent probes, has increased the uptake of polarisation studies. The number of papers started around a dozen in the 1950s and has risen to over four thousands in the 2000s. Today's records count several tens of thousand of documents relating to this field, according to Google scholar metrics.

The term "polarised" came into the literature when Etienne-Louis Malus observed that the intensity of reflected light from windows of the Luxemburg Palace in Paris through Calcite was different when he rotated the crystal. He used this term to describe this aspect of light. David Brewster found that the polarisation of reflected light is related to the refractive index of the materials and the incident angle at their interfaces. He invented the "pile of plates" polariser. Regular polarisers, which are used in sunglasses and cameras, were developed by Edwin Land in 1929.

The term "fluorescence" was first used by Stokes in his famous 1853 manuscript (39). In 1920 the first polarised fluorescence emission has been observed by Weigert using quinine dye in aqueous solution (40). Vavilov and Levshin did an early extensive study of the new found phenomenon on 26 dyes in water / glycerol. They found the emission polarisation to be different in water and glycerol. Francis Perrin, son of the famous physicist Jean Perrin, produced a comprehensive review of the relationship between the fluorescence lifetimes of dyes, their rotation and their emission polarisation (41–43).

The emission of light from single (S) or triplet (T) states of any substance that occurs from electronically excited states to the ground state S_0 is called "Luminescence". The luminescence can be separated into the two categories namely "fluorescence" and "phosphorescence" depending on the nature of light emission from the excited state.

The emission rates of fluorescence are typically $10^8 s^{-1}$, so that a typical fluorescence lifetime is near 10 ns ($10 \times 10^{-9} s$). In this situation the lifetime of the fluorophore, τ , can be defined as the average time between its excitation and return to the ground state.

In the case of phosphorescence, the emission of light is red-shifted with respect to fluorescence, due to the lower triplet energy level compared to the singlet energy level and from the triplet excited state to the ground state. These transitions are forbidden and their rates are slow about ($10^3 - 10^0 s^{-1}$). So, the lifetimes of phosphorescence phenomena are usually in milliseconds to seconds ranges.

1.3.1 Steady State and Time-Resolved Fluorescence Spectroscopy

Fluorescence measurements can be performed under both steady-state and time-resolved conditions. Steady-state measurements are carried out by constant illumination and then the fluorescence emission spectrum is collected by a conventional, sensitive detector such as a photomultiplier tube (PMT). As mentioned above, the lifetimes of most samples are in the ns range. When light is incident on the sample, the steady state is reached almost immediately and most of the measurements are performed in this regime (figure 1.8).

The second type of fluorescence measurement is the regime to measure the intensity decay or anisotropy in the time domain. In this case the sample is excited using a pulsed light source (e.g. mode-locked laser, picosecond LED, flash lamp) with pulse widths shorter than the fluorescence decay time of the sample. The fluorescence decay profile is recorded with a fast detection system on the picosecond to nanosecond time scale. Understanding the difference between steady-state and time-resolved measurements is important. A steady-state collection is an average of the time-resolved fluorescence over the intensity decay of the sample.

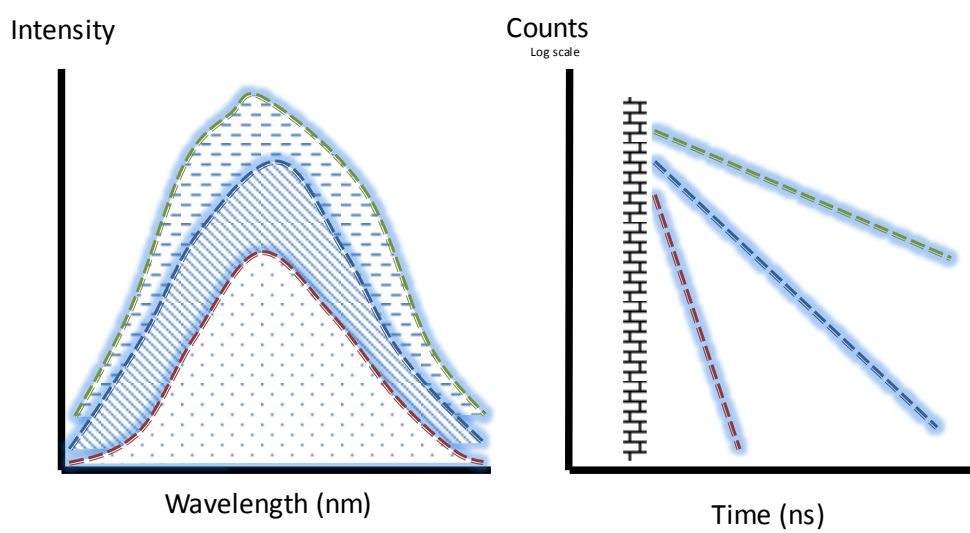


FIGURE 1.8: Comparison of steady-state and time-resolved fluorescence spectroscopy .

1.3.2 Why Time-Resolved Measurements?

Time-resolved fluorescence spectroscopy in the picosecond to nanosecond regime requires complex and expensive instrumentation and electronics. On the other hand, steady-state fluorescence measurements are relatively simple. A major advantage of using time-resolved fluorescence spectroscopy is that most of the molecular information is not lost during the time averaging process such as in steady state measurements. For instance, the fluorescence anisotropy decay of fluorescent macromolecules is generally complex and understanding the exact shape of the emission anisotropy decay provides valuable information about the macromolecular conformation and its rotational diffusion. Unfortunately, this kind of information is lost during averaging over the decay time. Some macromolecules are able to exist in more than a single conformation. Their time resolved fluorescence decay profile comprises multiple lifetimes. In this situation the steady-state measurements only provide a weight average of the lifetimes in the decay. Another advantage of using time-resolved fluorescence spectroscopy is in providing a clear understanding of resonance energy transfer between donor and acceptor. Time-resolved measurements can also identify whether quenching is static or dynamic in nature.

1.3.3 Definition of the Lifetime and Decay Time

It is important to have an understanding of the meaning of the fluorescence lifetime, τ , before any discussion on time resolved fluorescence measurements. Suppose a sample containing a fluorophore is excited with a sharp pulse of light. One possible photo-physical deactivation from the excited fluorophore is fluorescence by emitting a photon (44). The emitted fluorescence intensity, I_f , is dependent on the absorbance, A , the initial excitation intensity, I_0 , and the fluorophore fluorescence quantum efficiency, Φ_f (45):

$$I_f = \Phi_f I_0 [1 - 10^{-A}] \quad (1.17)$$

The fluorescence quantum efficiency, Φ_f , can be defined as the ratio of the fluorescence emission rate to sum of the rates of all possible relaxation mechanisms, both radiative and non-radiative. This quantum efficiency can be expressed in terms of the τ_f and the rate of relaxation pathways (46, 47):

$$\Phi_f = \frac{k_r}{k_r + k_{nr}} = k_r \tau_f \quad (1.18)$$

where k_r is the radiative deactivation rate coefficient (photon emission) and k_{nr} is defined as the non-radiative deactivation rate coefficient, where excitation energy may be transferred into the vibrational, rotational and translational motion of the surrounding molecules (phonon emission). Most of the fluorescence emission occurs due to transitions between the first excited electronic state, S_1 , and the ground state, S_0 , and the rate of depopulation of the first excited state is :

$$\frac{d[S_1]_t}{dt} = -[k_r + k_{nr}][S_1]_t = \frac{[S_1]_t}{\tau_f} \quad (1.19)$$

where $[S_1]_t$ is the first excited singlet state population at time t and then:

$$I_f(t) = I_{f_0} e^{-\frac{t}{\tau_f}} \quad (1.20)$$

where I_{f_0} is the fluorescence intensity at $t = 0$.

1.3.4 Evanescent Wave Time-Resolved Fluorescence Spectroscopy

Evanescent wave time-resolved fluorescence spectroscopy has been used in chapter 4 and so a description of this phenomenon is provided here.

Fluorophores at the interface of two materials of different refractive indices can be excited using evanescent waves and the intensity of the induced emission may be calculated by equation 1.17. The effect of the surface is important during evanescent wave induced fluorescence spectroscopy. One of the important parameters from equation 1.17 is the fluorescence quantum efficiency, $\Phi_f(z)$, which describes how the emission quantum yield varies with distance normal to the interface. $\Phi_f(z)$ is variable due to the effect of the interface and interactions with other adsorbed species (48). Therefore the fluorescence intensity variation with a change of evanescent wave penetration depth will give information about the quantum efficiency and the fluorophore's concentration profile. This can help unravel information about the localised microenvironments within the interfacial region (49). The change of quantum efficiency can occur due to the influence of other adsorbed species perturbing their microenvironments (50). To take account of this effect, the observed overall efficiency should be the weighted summation of the fluorescence lifetime, τ , and the radiative deactivation rates, k , of each of the n fluorophores.

$$\Phi_f = \sum_{n=1}^n a_i k_{ri} \tau_{fi} \quad (1.21)$$

where a_i is the fractional contribution of the i^{th} fluorophore to the total observed quantum efficiency.

1.4 Fluorescence Anisotropy

Fluorescence anisotropy measurements are widely utilised in biochemical and biophysical applications of fluorescence spectroscopy as they are a great tool to provide information on the size and shape of proteins or the rigidity or viscosity of various molecular micro-environments. Fluorescence anisotropy measurements have many

other applications and have been used to study the fluidity of membranes and protein–protein association. Fluorescence anisotropy, (r) , is defined as the change of initial emission polarisation and polarisation bias in emission from a series of fluorophores (51). The ground-state fluorophores in homogeneous solution are randomly oriented. When polarised light is incident on a sample of randomly oriented molecules, those fluorophores that have a component of their absorption transition moments oriented parallel to the electric vector of the incident light are preferentially excited. Therefore, an excited state population of partially oriented molecules is photoselected. There are a few processes that can cause the fluorescence emission to become depolarised. In this thesis two main depolarisation processes under discussion are rotational diffusion and energy migration. They will be discussed in detail in following chapters (chapter 3 for energy migration and chapter 4 and chapter 5 for rotational diffusion aspects of depolarisation).

A fluorophore emits in all directions, arbitrary in the Cartesian coordinate system in figure 1.9.

The anisotropy is then defined as the difference in intensity between orthogonal polarisations (k and j) emitted along the path of excitation i over the total emitted intensity (I_{total}) (44).

$$r = \frac{I_j - I_k}{I_i + I_j + I_k} = \frac{I_j - I_k}{I_{total}} \quad (1.22)$$

The radiation of energy around an oscillating dipole is spatially dependent. The emitted fluorescence is not propagated equally in all directions. For instance, the spatial probability of radiation from a dipole transition aligned with the i -axis is proportional to the detection angle, Ω , relative to the dipole's axis:

$$I = I_0 \sin^2 \Omega \quad (1.23)$$

Therefore, the radiation is also symmetrical around the j -axis. There is not any

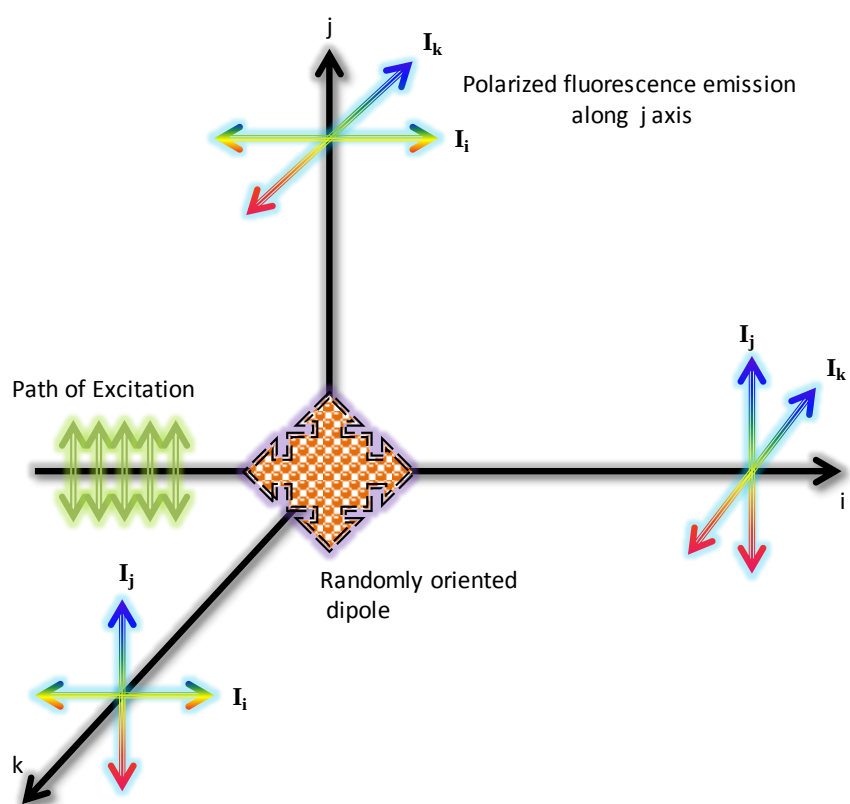


FIGURE 1.9: Polarised emission from randomly oriented dipoles in the Cartesian coordinate system (i , j and k).

energy emission radiated along the j -axis so no emission would be detected using a fluorescence detector placed along the this axis.

In bulk solution, there are many fluorophores with parallel absorption and emission transition moments that are orientated at angles θ relative to the j -axis and ϕ relative to the i -axis (figure 1.10). If these fluorophores are excited by light which is propagated along the i -axis and the plane of polarisation is parallel with the j -axis then the emission intensity is polarised symmetrically in the jk -plane i.e. I_j and I_k are equivalent, the anisotropy in this case becomes:

$$r = \frac{I_j - I_k}{I_j + 2I_k} \quad (1.24)$$

The anisotropy can be calculated from the emitted intensity along each axis and I is the square of the electric fields of the dipole projected onto each coordinate (figure 1.10):

$$I_j = \cos^2 \theta \quad (1.25)$$

$$I_k = \sin^2 \theta \sin^2 \phi \quad (1.26)$$

Consider randomly oriented fluorophores excited with polarised light. For excitation polarised along the i -axis all molecules at an angle ϕ from the i -axis are excited with equal probability because of symmetrically distribution. Hence, the $\sin^2 \phi$ component in the emitted intensity may be averaged over all possible angles of ϕ .

$$\langle \sin^2 \phi \rangle = \frac{\int_0^{2\pi} \sin^2 \phi d\phi}{\int_0^{2\pi} d\phi} = \frac{1}{2} \quad (1.27)$$

and therefore:

$$I_{\parallel} = I_j = \cos^2 \theta \quad (1.28)$$

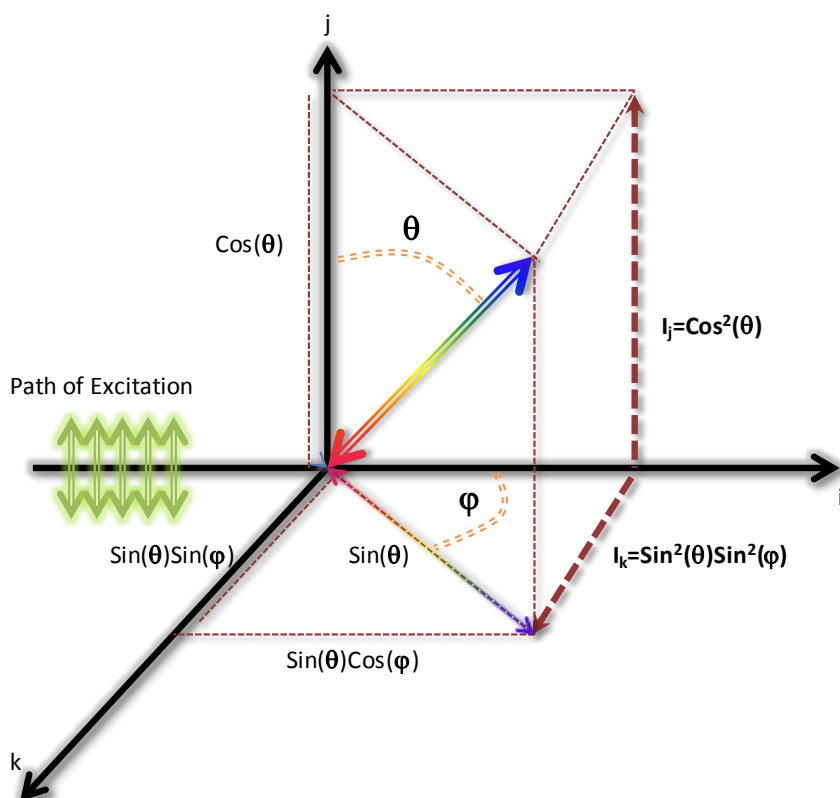


FIGURE 1.10: Schematic coordinate system of the electric field vectors of emission from a dipole aligned at an angle θ to the j -axis and an angle ϕ to the i axis (path of excitation) in bulk solution. The corresponding emission intensity is the square of the electric field amplitudes, where the anisotropy depends on the intensity projected onto both j and k axes .

$$I_{\perp} = I_k = \frac{1}{2} \sin^2 \theta \quad (1.29)$$

We are assuming a collection of fluorophores that are oriented relative to the j -axis having a probability, $f(\theta)$, (excitation photoselection) and then the emitted fluorescence intensities for this collection of molecules are:

$$I_{\parallel} = I_j = \int_0^{\frac{\pi}{2}} f(\theta) \cos^2 \theta d\theta = k \langle \cos^2 \theta \rangle \quad (1.30)$$

$$I_{\perp} = I_k = \frac{1}{2} \int_0^{\frac{\pi}{2}} f(\theta) \sin^2 \theta d\theta = \frac{k}{2} \langle \sin^2 \theta \rangle \quad (1.31)$$

where $f(\theta)d\theta$ is the probability that a fluorophore is oriented between θ and $\theta + d\theta$, and k is an instrumental constant.

Substituting equations 1.31 and 1.30 into equation 1.24 produces the anisotropy r , of a random configuration of fluorophores within bulk solution:

$$r = \frac{3 \langle \cos^2 \theta \rangle - 1}{2} \quad (1.32)$$

The photoselection phenomenon is when some fluorophores are excited with polarised light and results in an excited population of fluorophores that are partially oriented along the j -axis. The probability of absorption is proportional to $\cos^2 \theta$, and the probability for a molecule with angle θ in random orientation is proportional to $\int \sin \theta d\theta$. Therefore, the probability for a random numbers of fluorophores is a combination of photoselection and the magnitude of a molecule's transition moment at a specific angle:

$$f(\theta)d\theta = \cos^2 \theta \sin \theta d\theta \quad (1.33)$$

which, when substituted into equation 1.30, gives the average intensity of the fluorophore distribution polarised parallel to the j -axis.

$$\langle \cos^2 \theta \rangle = \frac{\int_0^{\pi/2} \cos^2 \theta \cos^2 \theta \sin \theta d\theta}{\int_0^{\pi/2} \cos^2 \theta \sin \theta d\theta} = \frac{3}{5} \quad (1.34)$$

According to Soleillet's rule for multiplication of depolarisation factors, the anisotropy is given by the product of the various depolarisation factors that occur in a sample:

$$r = \left(\frac{3 \langle \cos^2 \theta \rangle - 1}{2} \right) \left(\frac{3 \langle \cos^2 \delta \rangle - 1}{2} \right) \quad (1.35)$$

where δ is the displacement angle between absorbance and emission dipole and θ is an angle between the emission transition moments of a dipole relative to the j -axis. For the bulk solution this equation becomes

$$r_0 = 0.4 \left(\frac{3 \langle \cos^2 \delta \rangle - 1}{2} \right) \quad (1.36)$$

The fundamental anisotropy of the system has a value of 0.4 when there is no difference in the angle between the absorption and emission transition moments, i.e. the displacement angle $\delta = 0^\circ$. In contrast, the anisotropy has a minimum value of -0.2 when the absorption and emission transition moments are orthogonal, $\delta = 90^\circ$. When the difference between the transition moments is $\delta = 54.7^\circ$ it results in the fundamental anisotropy being zero; the so called "magic angle" conditions.

1.4.1 Time-Resolved Fluorescence Anisotropy

Molecular rotational diffusion (52) and electronic energy transfer (51) are two main phenomena that cause emission depolarisation, and time dependent anisotropy can be useful tool to investigate such processes. The time dependence of the recorded fluorescence anisotropy can be expressed as:

$$r(t) = \frac{I_j(t) - I_k(t)}{I_j(t) + 2I_k(t)} \quad (1.37)$$

1.4.2 Time-Resolved Evanescent Wave Induced Fluorescence Anisotropy Measurements (EW-TRAMS)

Time-resolved evanescent wave induced fluorescence anisotropy measurements (EW-TRAMS) method are used in chapter 4 and so the theory behind such measurements is given here.

By taking advantage of the three-dimensional polarisation nature of the evanescent waves, it is possible to have selective excitation of interfacial species oriented along either the y - (in-plane) or z - (out-of-plane) axes. Assume the configuration of a fluorophore at the interface is isotropic parallel to the interfacial plane. This assumption allows having two independent time-resolved fluorescence anisotropy measurements at the interface: in and out-of-the-plane of the interface anisotropies (see figure 1.11 (53–56)).

The in-plane anisotropy can be achieved by selectively exciting dipoles through the y -polarised evanescent wave and collecting the intensity of x and y -polarised emission ((p) and (s)-polarised emission). Out-of-plane anisotropy also can be achieved by exciting dipoles through a z -polarised evanescent wave and monitoring the x and y -polarised emission intensity in the same way as for in-plane anisotropy. These anisotropy measurements then probe the three-dimensional depolarisation behaviour projected onto the xy plane, i.e. in-plane anisotropy, and depolarisation relative to the z -axis independent of the xy -plane, i.e. out-of-plane anisotropy.

1.4.3 In-Plane Anisotropy

The in-plane anisotropy, $r_{(\phi)}$, evaluates depolarisation processes in the two-dimensional xy plane parallel to the interface. Using y -polarised evanescent wave excitation

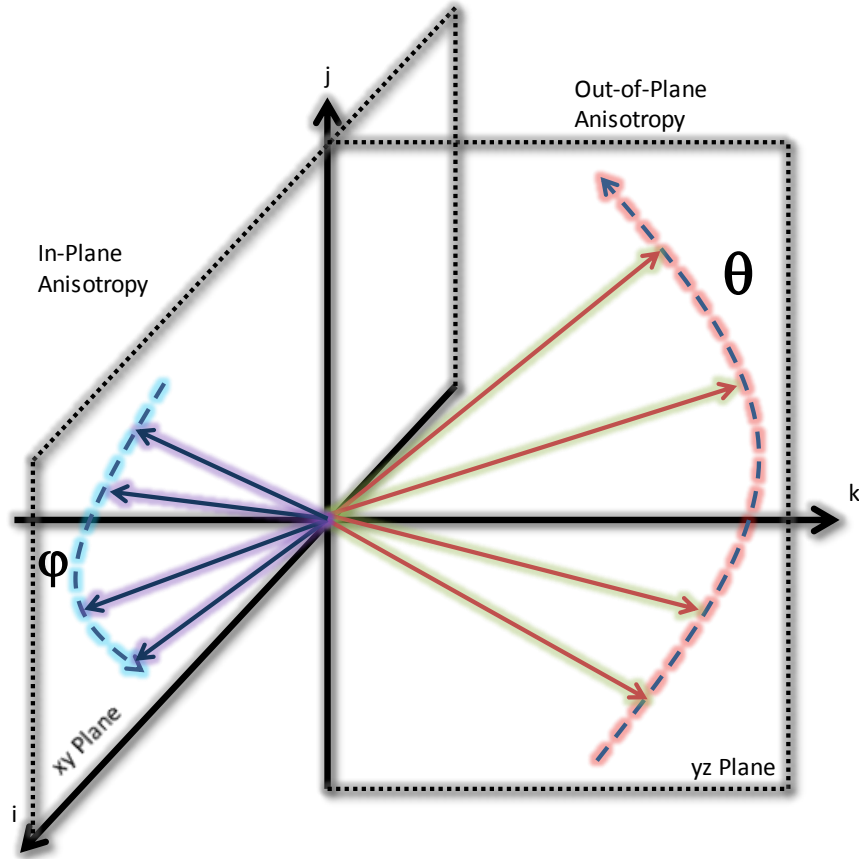


FIGURE 1.11: Schematic representation of the in-plane (ϕ) and out-of-plane (θ) depolarisation monitored through EW-TRAMS. In-plane anisotropy measures the depolarisation on the xy -plane and out-of-plane anisotropy measures the overall depolarisation projected onto the yz -plane.

and collecting emission normal to the interfacial plane no fluorescence will be detected from the z component. In this case the definition of the anisotropy (equation 1.22) becomes :

$$r = \frac{I_y - I_x}{I_x + I_y} \quad (1.38)$$

Consider a collection of fluorophores within the interfacial region figure 1.12 with an angle, (ϕ) , relative to the y -axis and an angle (θ) relative to the interfacial normal. Considering excitation from the y -polarised evanescent wave the emitted intensities are:

$$I_y = \cos^2 \phi \quad (1.39)$$

$$I_x = \sin^2 \phi \sin^2 \theta \quad (1.40)$$

Similar to the bulk case , we have symmetry in angle (θ) :

$$\langle \sin^2 \theta \rangle = \frac{\int_{-\frac{\pi}{2}}^{\frac{\pi}{2}} \sin^2 \theta d\theta}{\int_{-\frac{\pi}{2}}^{\frac{\pi}{2}} d\theta} = \frac{1}{2} \quad (1.41)$$

Therefore:

$$I_y = \int_0^{\frac{\pi}{2}} f(\phi) \cos^2 \phi d\phi = \langle \cos^2 \phi \rangle \quad (1.42)$$

$$I_x = \frac{1}{2} \int_0^{\frac{\pi}{2}} f(\phi) \sin^2 \phi d\phi = \frac{1}{2} \langle \sin^2 \phi \rangle \quad (1.43)$$

which, when substituted into the in-plane anisotropy definition (equation 1.38), gives:

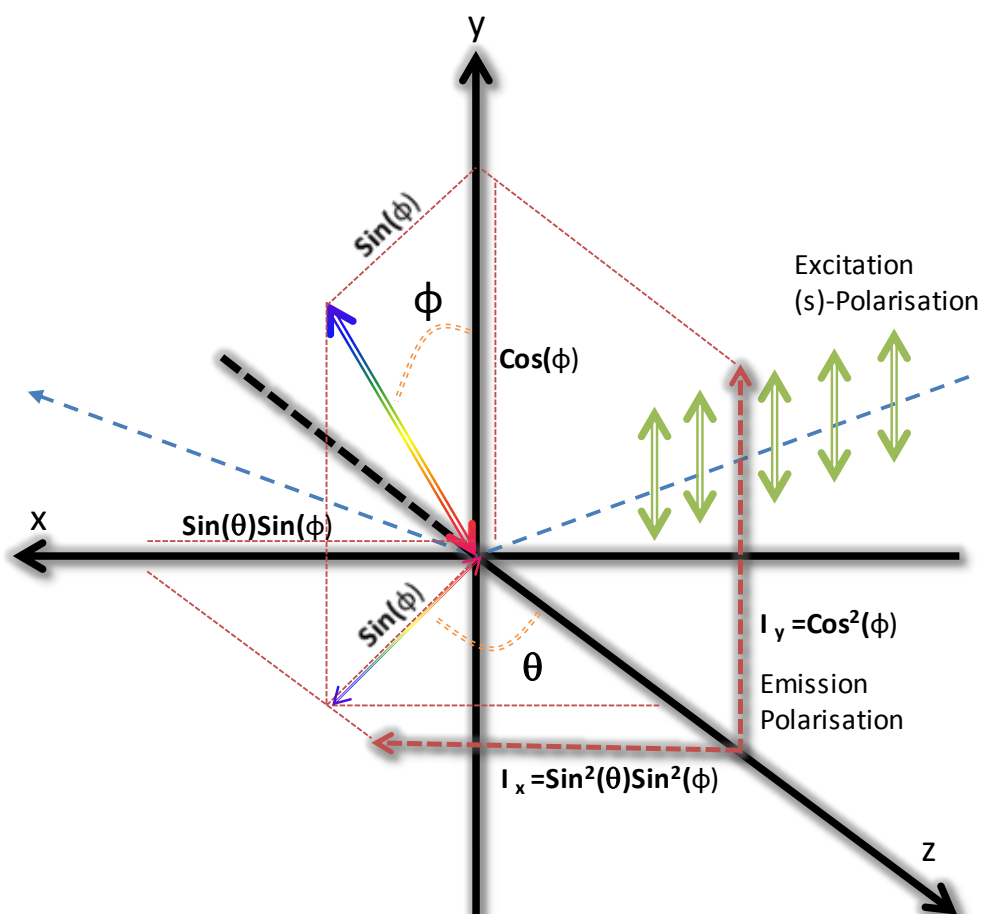


FIGURE 1.12: Representation of fluorescence emission from a dipole aligned at an angle (ϕ) to the y -axis and angle (θ) to the z -axis. (s)-polarised propagating light incident on the interface that generates an evanescent wave polarised along the y -axis.

$$r_\phi = \frac{3 \langle \cos^2 \phi \rangle - 1}{\langle \cos^2 \phi \rangle + 1} \quad (1.44)$$

The probability of a fluorophore being at an angle, ϕ , and, $\phi + d\phi$, to the y -axis provides:

$$f(\phi)d\phi = \cos^2 \phi \sin \phi d\phi \quad (1.45)$$

Therefore:

$$\langle \cos^2 \phi \rangle = \frac{\int_0^{\pi/2} \cos^2 \phi f(\phi) d\phi}{\int_0^{\pi/2} f(\phi) d\phi} = \frac{3}{5} \quad (1.46)$$

and the fundamental in-plane anisotropy can be determined using equation 1.44 to be 0.5. The fundamental anisotropy can therefore be defined as:

$$r_{\phi 0} = 0.5 \left(\frac{3 \langle \cos^2 \phi \rangle - 1}{\langle \cos^2 \phi \rangle + 1} \right) \quad (1.47)$$

In this equation the fundamental anisotropy for parallel absorption and emission dipoles is 0.5 and in the case of orthogonal absorption and emission transition dipoles $r_0 = -0.5$. The in-plane fundamental anisotropy value is zero at $\delta = 54.7$ degrees. For in-plane collection only the (s)-polarisation incident light is required and the collected emission polarisation can be defined in the (p) and (s) terminology and the in-plane anisotropy equation may be rewritten in terms of excitation and emission polarisation:

$$r = \frac{I_{ss} - I_{sp}}{I_{ss} + I_{sp}} \quad (1.48)$$

where I_{ij} is the collected intensity for j -polarisation with i -polarised incident light. For example I_{sp} is the observed intensity of (p)-polarised emission generated by (s)-polarised excitation light.

1.4.4 Out-of-Plane Anisotropy

For motions occurring orthogonal to the interface we consider out-of-plane emission anisotropy. The origin of the out-of-plane anisotropy is similar to the bulk solution case (equation 1.22) with the exception that transition moments penetrate into the rarer medium. Considering that the evanescent wave is polarised along the z -axis, I_j in equation 1.22 equals I_k . In such a case I_x and I_y are indistinct. To avoid any bias regarding x and y orientation, we consider the averaged intensity of both orientations using the term I_p :

$$I_p = \frac{I_x + I_y}{2} \quad (1.49)$$

and the out-of-plane anisotropy equals:

$$r_\theta = \frac{I_z - I_p}{I_z + 2I_p} \quad (1.50)$$

$$I_z = \langle \cos^2 \theta \rangle \quad (1.51)$$

$$I_p = \langle \sin^2 \theta \rangle \quad (1.52)$$

Substitution of equations 1.51 and 1.52 into equation 1.50 provides the fundamental out-of-the-plane anisotropy:

$$r_{\theta\theta} = \frac{3 \langle \cos^2 \theta \rangle - 1}{2} \quad (1.53)$$

again, photoselection and the probability of a fluorophore being at an angle θ may be written as:

$$f(\phi)d\phi = \cos^2 \theta \sin \theta d\theta \quad (1.54)$$

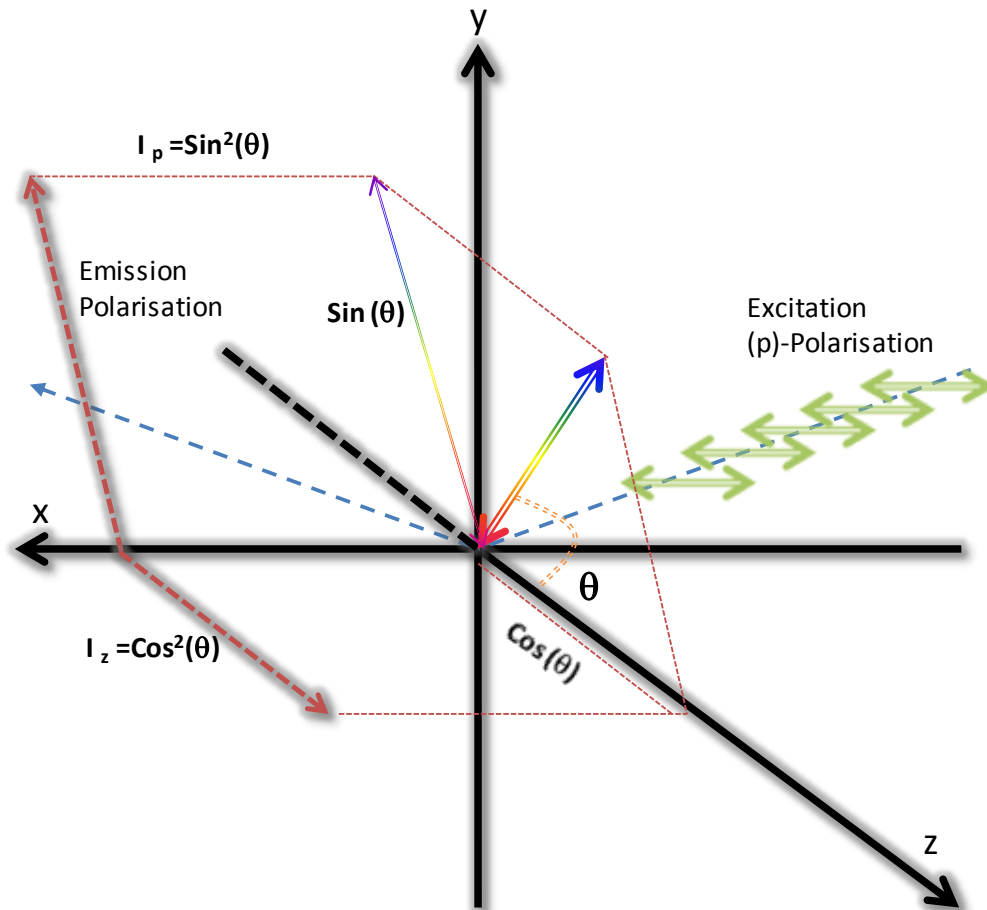


FIGURE 1.13: Out-of-plane anisotropy diagram of the emission electric field vectors from a dipole aligned at an angle θ to the z -axis. (p)-polarised propagating light incident on the interface and generates an evanescent wave polarised along the z -axis. The corresponding emission intensities illustrated on the z -axis and into the xy -plane (I_p).

Because the direct observation of (I_z) is not possible the projections of the transition moment amplitude into the plane of the interface are detected, i.e. (I_{zy}) or (I_{zx}) .

$$I_{zy} = \langle \sin^2 \theta \rangle = \frac{1}{2} \frac{\int_0^{\frac{\pi}{2}} \sin^2 \theta f(\theta) d\theta}{\int_0^{\frac{\pi}{2}} f(\theta) d\theta} = \frac{1}{5} \quad (1.55)$$

It is possible to determine (I_p) from the in-plane result independent of the out-of-plane measurement:

$$I_p = \langle \sin^2 \theta \rangle_\phi \quad (1.56)$$

and:

$$I_p = \langle \sin^2 \theta \rangle_\phi = \frac{\int_0^{\frac{\pi}{2}} \sin^2 \theta f(\theta) d\theta}{\int_0^{\frac{\pi}{2}} f(\theta) d\theta} = \frac{2}{5} \quad (1.57)$$

By replacing (I_{zy}) and (I_p) in the out-of-plane anisotropy definition (equation 1.50), the fundamental anisotropy can be calculated to be -0.2 , for a collection of randomly oriented fluorophores with parallel absorption and emission transition moments monitored normal to the interfacial plane.

Consider there is a displacement angle, δ , between the absorption and emission transition moments, then the fundamental anisotropy becomes:

$$r_{\theta 0} = -0.2 \left(\frac{3 \langle \cos^2 \delta \rangle - 1}{2} \right) \quad (1.58)$$

So the fundamental anisotropy is -0.2 when the absorption and emission transition moments are parallel. The observed anisotropy is 0.1 when there is an orthogonal displacement 90° between the two transition moments and at displacement of $\delta = 54.7^\circ$ the fundamental anisotropy is zero. To have a z -polarised evanescent wave it is necessary to excite the incident light using (p)-polarisation. Therefore, similar to the in-plane anisotropy equation 1.48, the out-of-plane anisotropy (equation 1.50) can be rewritten in the terms of both excitation and emission polarisation as:

$$r_{\theta}(t) = \frac{I_{ps}(t) - \frac{1}{2}(I_{ss}(t) + I_{sp}(t))}{I_{ss}(t) + I_{sp}(t) + I_{ps}(t)} \quad (1.59)$$

where I_{ij} is the observed intensity for j -polarisation when excitation is i -polarised.

1.5 Fluorescence Lifetime Imaging Microscopy

One of the most broadly used methods in the biological and biophysical sciences is fluorescence microscopy. In recent years, there has been rapid growth in the utilisation of fluorescence microscopy due to advances in several technologies including fluorescent probe chemistry, confocal optics, multiphoton excitation, detectors and computer power (44). Time-resolved fluorescence imaging, more commonly known as FLIM, is now a well-established alternative mode of fluorescence microscopy. One of the most powerful aspects of FLIM is its virtual independence of signal on fluorophore concentration. The fluorescence intensity of a fluorophore depends on its concentration but the lifetime of a fluorophore is generally independent of its concentration in the absence of aggregation or concentration quenching effects. The schematic concept of fluorescence-lifetime imaging is illustrated in figure 1.14. Assume that there is a cell with two distinct regions and both have the equal steady-state fluorescence intensities but the lifetime of the probe in the central region of the cell, τ_2 , is much longer than that in the outer region, τ_1 . For example, the presence of an ionic species such as calcium when binding the probe to a macromolecule or proteins, or other environmental factors can cause the longer lifetime in the central region. The quantum yields or concentrations of the probe could be the same or different in various parts of cell. The intensity image cannot distinguish between the different environments of the cells but the lifetime can easily discriminate between the distinct environments. The lifetime variations can be mapped over regions of the sample.

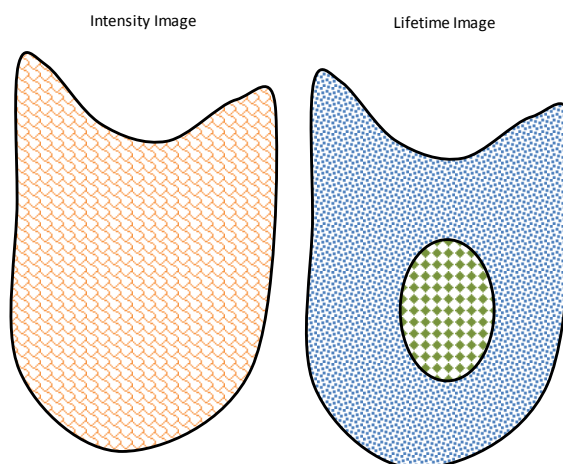


FIGURE 1.14: Schematic presentation of the concept of FLIM and steady state imaging. The cell in this schematic has two regions that display the same fluorescence intensity but different decay times, $\tau_2 > \tau_1$.

1.5.1 Scanning Confocal Fluorescence Lifetime Imaging

The scanning confocal fluorescence lifetime imaging method is widely used in chapter 3 and 5 and is described in detail here.

A lifetime image with reasonable spatial resolution needs measurement with 256×256 pixels or more individual lifetime measurements. Sometimes when higher spatial resolution is required, the FLIM collection takes significant time to achieve adequate signal. In addition, the lifetimes should be measured using a microscope. Mainly, there are two general approaches for FLIM: scanning or wide-field methods. The first method is used when the lifetime is measured at discrete locations and the latter method is achieved using a time-resolved imaging detector. The lifetime imaging approach was impractical using 1980s and early 1990s technology. In recent years, the situation has changed and now these measurements are practical. FLIM also can be performed using frequency-domain or time-domain methods. In this thesis all FLIM measurements have been performed using scanning confocal microscopy based on time-correlated single photon counting (TCSPC).

1.6 Fluorescence Anisotropy Imaging Microscopy

The fluorescence anisotropy imaging microscopy method is widely used in chapter 3 and 5.

High spatial resolution polarisation imaging can be achieved using specialised microscope devices. The concept of polarisation (fluorescence anisotropy) imaging is similar to that of the polarising fluorimeter and bulk anisotropy measurements. Simply, the sample is excited with a particular polarisation of light and the emission is resolved into components of emission polarised parallel and perpendicular to the excitation electric field. There are two general approaches for fluorescence anisotropy imaging. First, the polarisation component can be spatially separated using optics and measured simultaneously and in the second approach, the excitation or emission polariser can be rotated manually (57).

In fluorescence anisotropy imaging measurements, two fluorescence emission images are separated by a polarisation beam splitter from the same excitation region. One image has parallel emission polarisation relative to the excitation polarisation, I_{VV} , and the other has perpendicular emission polarisation relative to the excitation polarisation, I_{VH} , as schematic illustrated in figure 1.15. The fluorescence anisotropy is then calculated for each pixel to generate the whole anisotropy image.

In a similar way the steady state in-plane and out-of-plane anisotropy equations 1.38 and 1.48 can be used to calculate the anisotropy following evanescent wave excitation expressed as:

$$r_{in-plane} = \frac{I_{VV} - I_{VH}}{I_{VV} + I_{VH}} \quad (1.60)$$

and

$$r_{out-of-plane} = \frac{I_{HV} - \frac{1}{2}(I_{VV} + I_{VH})}{I_{VV} + I_{VH} + I_{HV}} \quad (1.61)$$

One of the early applications of fluorescence polarisation applied to an imaging system has been reported by Axelrod et al. (58) investigating the orientation of carbocyanine in erythrocyte ghosts membranes. The same group also applied TIRF (total internal reflection fluorescence) microscopy into polarisation imaging and extended it in theory (59). Bos et al. also studied the distribution of orientations of porphyrin-like molecules adsorbed to a surface and developed a theory of polarised TIRF microscopy (60).

1.7 Summary

In this Chapter the physical concept of TIR by simulating the electrical field of evanescent waves at the interface has been reviewed. The background of the fluorescence spectroscopy and polarisation was evaluated. In particular, EW-TRAM, FLIM and FAIM are outlined. The polarised fluorescence emissions from randomly oriented dipoles in the Cartesian coordinate system were used to calculate fluorescence anisotropy for both bulk and interface measurements. The in-plane and out-of-plane anisotropy at interface were derived. The pixel by pixel anisotropy calculation using image intensity of different polarisations and mapping the fluorescence anisotropy are explained.

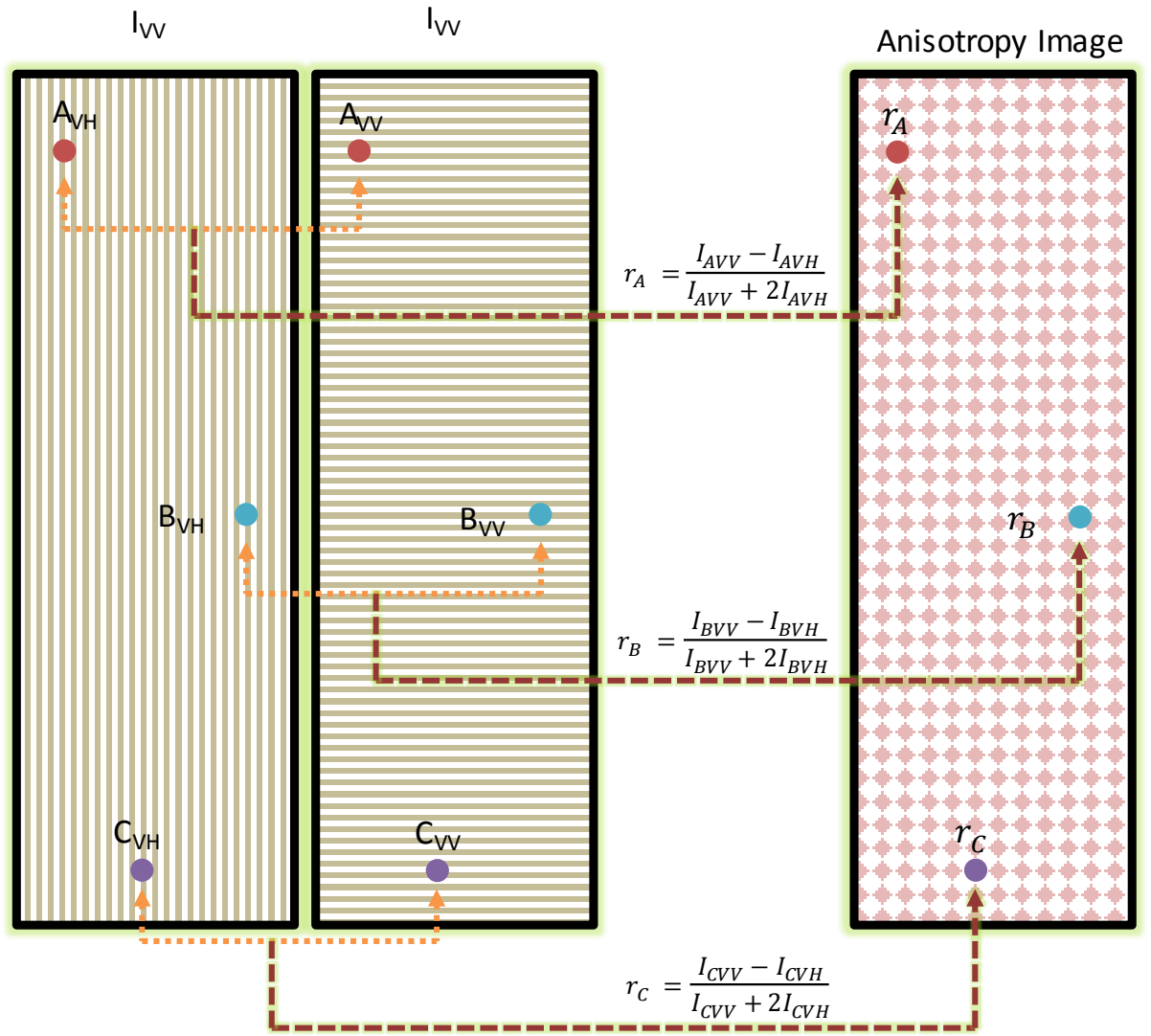


FIGURE 1.15: Pixel by pixel anisotropy calculation using intensity of different polarisations. This Matlab script is described in appendix 1.

Chapter 2

Experimental Details

2.1 Introduction

In this chapter, in addition to the sample preparation methods, technical details and procedures of experiments using the steady-state and time-resolved optical spectroscopy and imaging techniques are described. Particular emphasis is given to evanescent wave time-resolved fluorescence anisotropy measurements (EW-TRAM) and fluorescence anisotropy imaging microscopy (FAIM) methods.

2.2 Steady-State Absorbance and Fluorescence Spectra

The background protocols for both steady-state absorption and fluorescence spectroscopic techniques are nicely documented ([61](#)) and are not discussed further here. Steady-state absorption measurements (UV-VIS) for solutions or films were collected using a Varian Cary Bio 50 spectrometer against the appropriate solvent or film baseline. Steady-state fluorescence emission measurements of solutions/films were performed using a Varian Cary Eclipse fluorimeter (Agilent Technologies). The spectra of all films were recorded using a homemade mount in these devices to control the angle of the film relative to the excitation beam to avoid surface reflections into the detection system. 10 mm path length fluorescence quartz cuvettes were used for solutions.

All steady-state fluorescence spectra which are presented in this thesis are background subtracted and normalised to clarify the differences in figures.

2.3 Time-Resolved Fluorescence Spectroscopy

The TREWIFS, EW-TRAMS and FLIM methods depend on collecting the time- dependence of the fluorophore's excited state. In all the work reported in this thesis, time-resolved fluorescence measurements have been performed through the time-correlated single photon counting (TCSPC) (46, 62) methodology. All time-resolved techniques such as TREWIFS and EW-TRAM therefore use the same experimental apparatus, with appropriate modification for each type of experiment.

2.3.1 Time Correlated Single Photon Counting (TCSPC)

TCSPC is a method to study the radiative relaxation of fluorophores from their excited state to the ground state . The concept of TCSPC is based on the probability and frequency of distribution of single emitted photons in terms of time after excitation. This method can monitor the time period between excitation by short pulses of light from a pulsed light source such as a mode-locked laser or LED and the emitted fluorescence photon detected using specially configured detectors such as single photon PMTs. TCSPC is a powerful tool for determining fluorescence decay information on a nanosecond to microsecond timescale. The technique has been evaluated comprehensively (46, 62, 63) and only the relevant technical points are presented in here.

A schematic of the TCSPC experiments is illustrated in figure 2.1. Usually femto- or picosecond laser pulses are used as the excitation source in TCSPC experiments. The excitation source used here was a mode-locked and cavity dumped Ti:Sapphire laser (Coherent Mira 900F/APE PulseSwitch) pumped by a Coherent Verdi-10 *DPSS* – *Nd* :

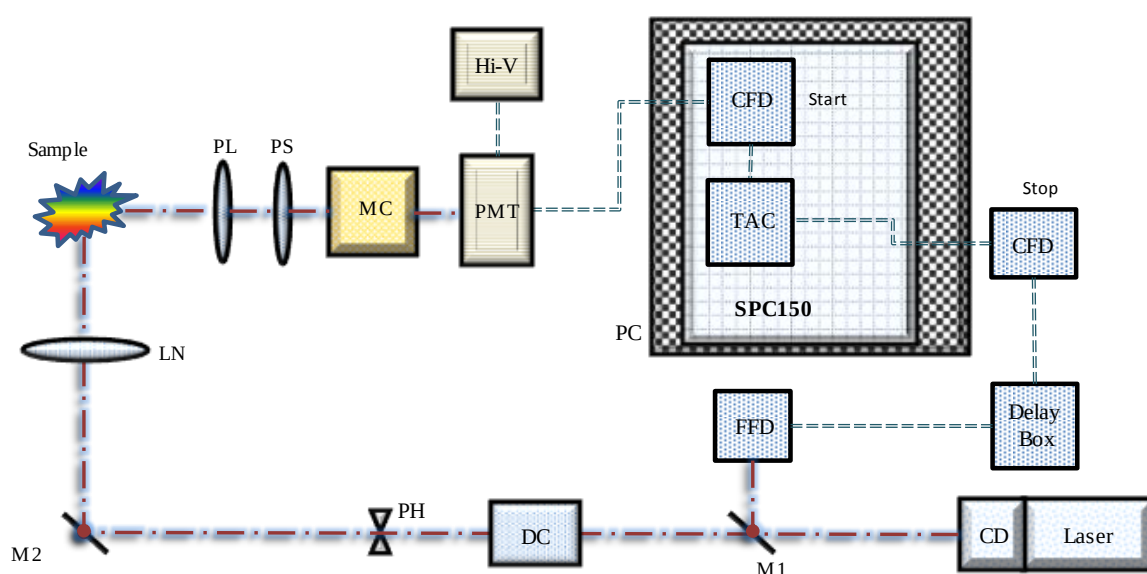


FIGURE 2.1: Schematic representation of the home-built TCSPC optical arrangement. CD: cavity dumper, M1: partial reflector mirror (coverslip), DC: frequency-doubling crystal, PH: pin hole, M2: Mirror, PL: polariser, LN: lens, PS: polarisation scrambler, MC: monochromator, PMT: photo-multiplier, CFD: constant fraction discriminator, TAC: time to amplitude converter, FFD: high speed photo diode, PC: personal computer.

YVO_4 laser. The laser output (880nm wavelength, 5.4MHz repetition rate) was frequency doubled to provide an excitation wavelength of 440 nm. Synchronisation of the laser pulses was achieved using a fast photodiode (Becker & Hickl, PHD-400-N) fed through a nanosecond delay box and constant fraction discriminator (Tennelec TC455) as the stop signal for the time-to-amplitude converter (TAC) in either a commercial card (SPC150 Becker & Hickl card). The emission was collected at right angles relative to the excitation, spectrally selected using a monochromator (Jobin Yvon H20) and detected using a micro-channel plate (ELDY, EM1 132/100). Emission polarisation was collected at the magic angle (54.7°) relative to that of the excitation to remove polarisation artifacts due to molecular rotation. The instrument response function (IRF) was determined experimentally by measuring the response of the detection system to excitation light from a non-fluorescent, weakly scattering solution of colloidal silica (Ludox) with the same conditions as the emission (except the wavelength was at the excitation rather than the emission wavelength).

2.4 Time-Resolved Anisotropy Measurements (TRAM)

2.4.1 Equipment Development

The TREWIFS apparatus was first assembled by Lensun et al (64) and then developed by Scholes et al (65) in the Ultrafast and Microspectroscopy Laboratories (UML), School of Chemistry, University of Melbourne. Initially, in the first setup, the change in the angle of incidence was controlled by using an xyz – rotation translation stage and the prism-sample assembly was fixed according to the schematic depicted in figures 2.2 and 2.3. The angle between excitation and detection is fixed in this configuration. This set-up was utilized in previous investigations and is described in detail elsewhere (64). For accurate depth profiling, it is necessary that fluorescence emission be detected normal to the interface for all incident angles. However the first setup Fig. 2.3, does

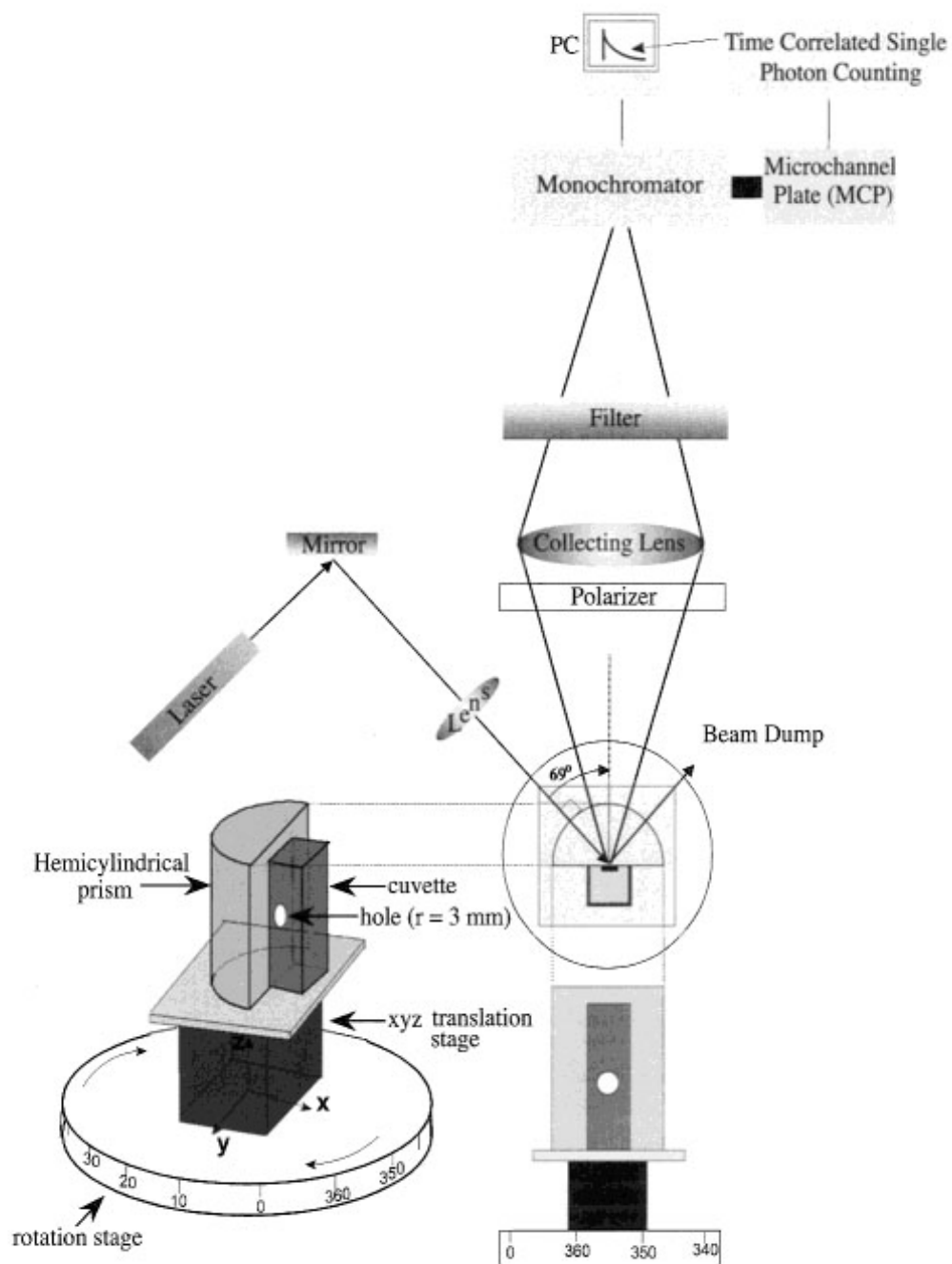


FIGURE 2.2: Schematic representation of the first home-built EWIFS optical arrangement, used for time-resolved EWIFS and EW-TRAMS (64).

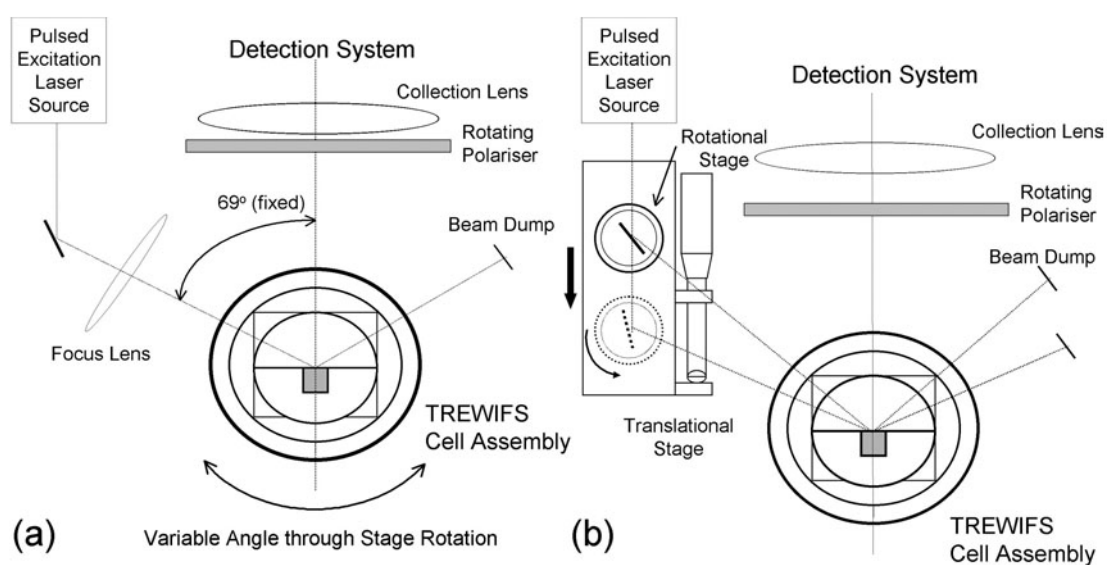


FIGURE 2.3: Schematic diagrams of first TREWIFS designs by Lensun et al and Colin Scholes. It achieved variable incidence angle through different means; (a) by the mounting the TREWIFS cell assembly on a rotation stage with a fixed excitation light path, (b) TREWIFS cell assembly fixed and the excitation light path altered by a coupled rotation – translation stage mounted mirror.

not meet this requirement. Wirth et al (66) have discussed instrumental error and artifacts caused by changing of the incident angle but not detecting emission normal to the interface.

To solve this problem, Colin Scholes developed another arrangement in which the angle between the interface and the detection axis was always 90° regardless of the angle of incidence. He tried three different approaches to obtain the final mirror design. First, an optical arrangement based on a report by Schneckenburger (67) was trialled which includes variable angles of incidence. In this configuration, a polarisation maintaining optical fibre was mounted on a rotating stage with the output directed to the interface of the prism. This method had a few problems such as low laser intensity due to losses in coupling light into the fibre and issues with adjustment of the light exiting the fibre. This configuration leads to low fluorescence signal to noise. The second approach was based on other EWIFS designs (55, 67), with a rotation mirror mounted on a translation stage (figure 2.3). This configuration provides variable angle of incidence at the interface. However, this approach suffered from a lack of positional stability of the excitation light on the interface with variable angle of incidence.

The third design used an elliptical mirror with a rotation mirror at one focal point and the prism/sample interface at the other. This method ensures the incidence angle and polarisation requirements, by using the mirror's foci relationship. In this configuration, it is possible to be confident that light reflected off the rotation mirror was always incident on the same spot on the hemicylindrical prism. However, only a small range of incident angles was achievable in this method. In addition, for each experiment the mirror's position was required to be changed and realigned. To solve this issue the elliptical mirror concept was separated into two optical components; namely the two off-axis parabolic mirror set-up. This change allows a greater range of angles of incidence to be achieved. This arrangement is depicted schematically in figure 2.4, which still suffered from many factors:

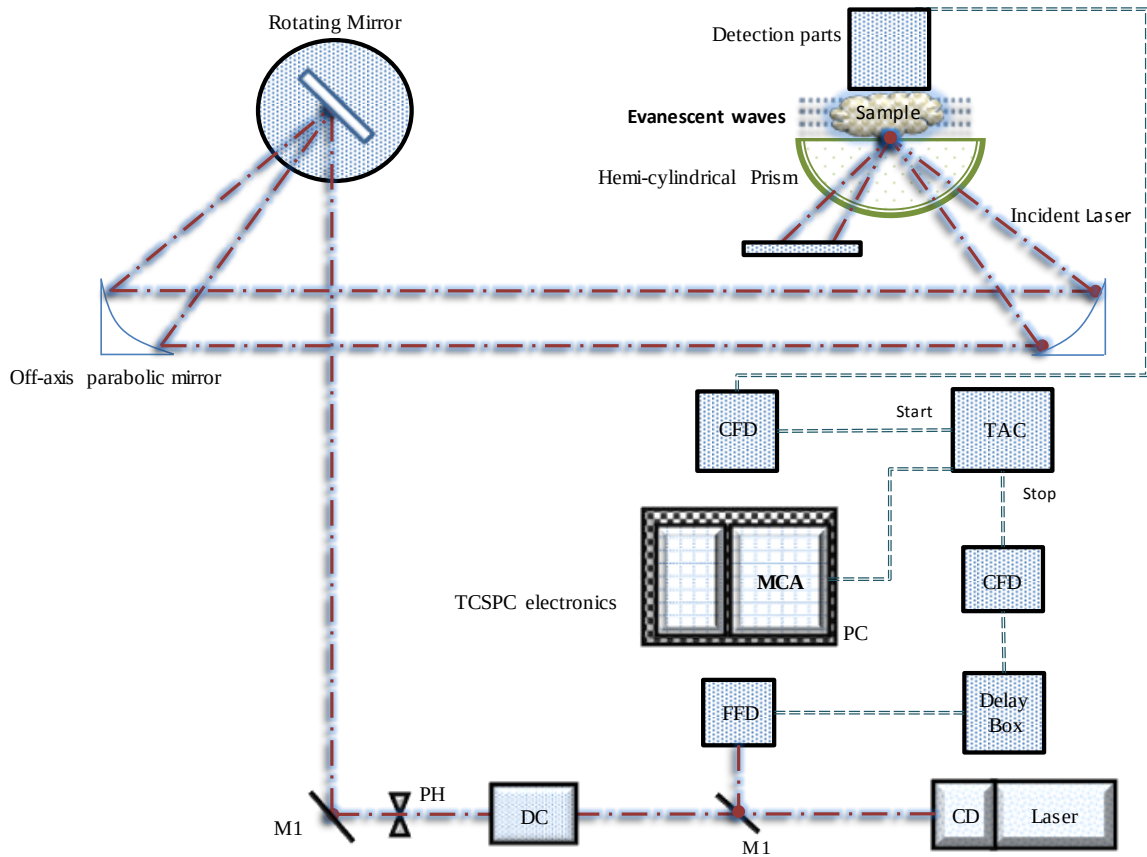


FIGURE 2.4: Schematic representation of the TREWIFS apparatus, using the two off-axis parabolic mirrors assembly to ensure variable incidence angle upon the active surface. CD: cavity dumper, M1: partially reflector mirror, DC: frequency-doubling crystal, PH: pin hole, CFD: constant fraction discriminator, TAC: time to amplitude converter, FFD: high speed photo diode, PC: personal computer.

- Optical alignment is difficult due to the use of two parabolic mirrors.
- Excitation polarisation is manually changed and experiments need to be performed sequentially.
- Large incident angles (low penetration depths) are not accessible because of limitations of the parabolic mirror.
- Right angle and conventional anisotropy measurement is impossible in this configuration.
- The rotating mirror is not motorized .

To solve these issues a new configuration was designed and implemented . The following section discusses the new apparatus and optical arrangement for time-resolved anisotropy measurements (TRAM).

2.4.2 Time-Resolved Anisotropy Measurements (TRAM) Apparatus

The apparatus used for the time-resolved evanescent wave-induced fluorescence measurements is shown schematically in figure 2.6. This is a modified version of our previously reported setup (68). The excitation source was a mode-locked and cavity dumped (CD) Titanium : Sapphire laser (Coherent Mira 900F/APE PulseSwitch) pumped by a Coherent Verdi-10 *DPSSNd : YVO₄* laser. The laser output (800nm-880 nm, 5.4 MHz repetition rate) was frequency doubled (DC) to provide the appropriate excitation wavelength. Most of the samples in this thesis were excited by wavelengths between 400nm-440nm. The individual fluorescence decay curves were collected using the time-correlated single photon counting technique as discussed above. Synchronisation of the laser pulses was achieved using a fast photodiode (FPD) (Becker & Hickl, PHD-400-N) fed through a nanosecond delay box and constant fraction discriminator (CFD) (Tennelec TC455) as the stop signal for the time-to-amplitude converter

(TAC) (Ortec model 457) operating in inverted TAC mode. The excitation beam passed through a silica double rhomb (FR) to select the input (vertical) excitation polarisation. For TREWIFS and TRAMs measurements a silica hemi-cylindrical prism (HP) was used to establish the total internal reflection geometry and thereby the evanescent field in the optical rarer medium (50, 64). The hemi-cylindrical prism was specially constructed (H. Groiss Ltd., Australia) of fused Supracil silica (refractive index 1.466), the height is 30 mm with a 20 mm radius of curvature, and polished by the manufacturer. The prism is held within a custom-built aluminium holder (SHL) that allows access to the flat optical face. A small Teflon O-ring (TOR) and microscope slide (MSI) were also used to sandwich and keep solutions in contact with the prism interface. For conventional (non-EW excitation) fluorescence anisotropy measurements of bulk solutions, a cuvette can be located on the sample holder instead of the hemi-cylindrical prism. The hemi-cylindrical prism, emission collection and detection optics were all located on an arm mounted on a large stepper motor driven rotation stage (SM3) (Deadal Mc1000) and controlled electronically (SM3B). This angular variation of the arm can generate different angles of incidence of the incident light at the interface, and therefore penetration depths, whilst still maintaining emission detection normal to the plane of the interface; a requirement for EW-TRAMs (wirth1991absorbate, 55, 69). A high pass optical filter (FL) was used to remove any scattered excitation light. The emission was collected at appropriate angles (90° relative to the excitation for bulk and normal to the planar interface at an angle of incidence of 75° - 85° for evanescent wave measurements, respectively), first passing through a polarisation analyser (PL) mounted on a stage that rotates both the polariser and collection lens (LN) to collect vertically and horizontally polarised emission. The analyser orientation was driven by a computer controlled stepper motor board (SM2B) (Phidgets 1067). The emission was then passed through a polarisation scrambler (DP) (ThorLabs DPU-25) and spectrally selected using a monochromator (MC) (Jobin Yvon, H20), and detected using a single

photon counting photomultiplier tube (PMT) (XP2020Q). The memory of the multi-channel analyser (MCA-p7882, Fastcom Tech) was divided into segments in which the various I_{ij} components (i and j correspond to the polarisation of the excitation and detection, respectively), generated following control of the stepper motors (SM1 & SM2), are saved during each acquisition sequence. Decay profiles were acquired by sequentially toggling between the respective polarisation orientations/memory locations to achieve equal collection conditions for each orientation. The correction for the polarisation bias of the detection system (G factor) was determined to be very close to unity by both tail-matching the I_{VV} and I_{VH} profiles and the I_{HV}/I_{HH} ratio from the time-resolved fluorescence decays of Coumarin 153 in solution (shown in figure 2.5). The G factor calculation will be discussed further in following sections.

Data analysis was carried out by nonlinear least-squares iterative reconvolution using commercial software (FAST, Edinburgh Instruments Ltd). The goodness of fit was evaluated by the reduced chi squared χ^2 value and the distribution of the weighted residuals. Exponential components analysis is used and applied for data interpretation. The data analysis will be discussed in details in following sections.

G Factor and Equipment Bias Determination

Polarisation bias within the detection assembly is an important factor that may change the accuracy of fluorescence anisotropy measurements (44) in either bulk solution or EW-TRAM measurements. This polarisation bias is particularly due to the monochromator, and can be corrected using a suitable correction factor (G factor) in the fluorescence anisotropy definition (equation 1.22) :

$$r(t) = \frac{I_{VV}(t) - G.I_{VH}(t)}{I_{VV}(t) + 2.G.I_{VH}(t)} \quad (2.1)$$

where I_{VV} and I_{VH} are the observed emission intensities polarised parallel and perpendicular relative to the vertically polarised excitation, respectively.

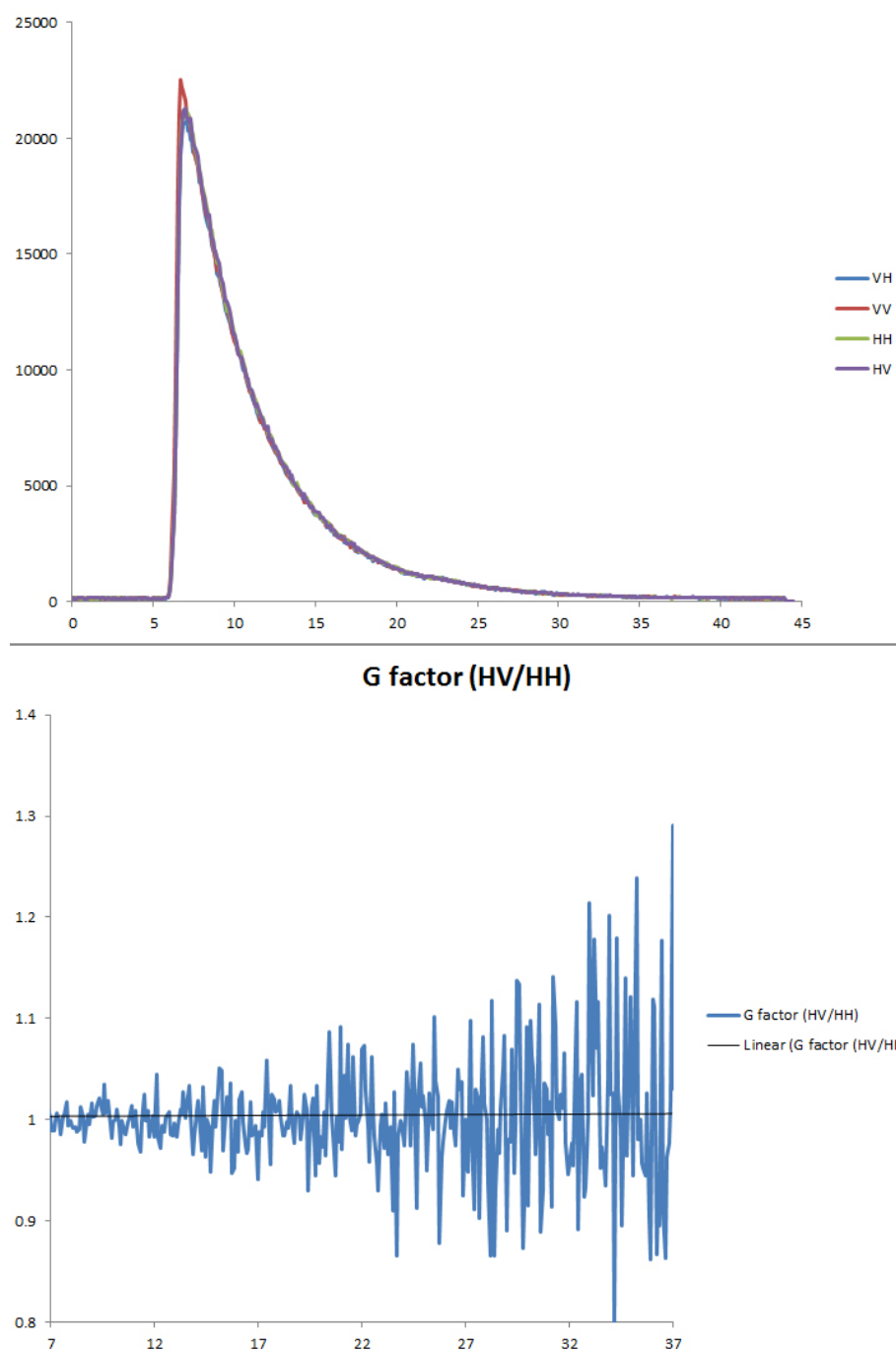
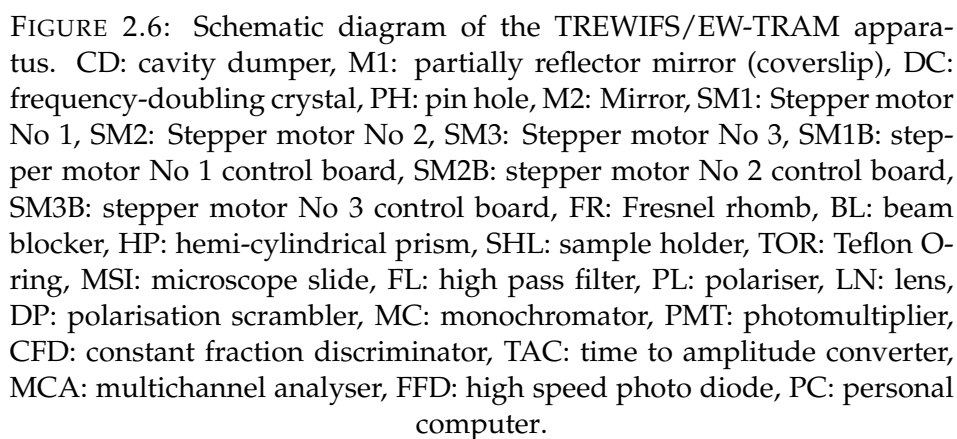


FIGURE 2.5: The correction for the polarisation bias of the detection system (G factor). was determined to be very close to unity by both tail-matching the I_{VV} and I_{VH} profiles and the I_{HV}/I_{HH} ratio from the time-resolved fluorescence decays of Coumarin 153 in solution.



In bulk solution configurations, the G factor is measured by exciting the sample using horizontally polarised light and subsequently measuring the horizontally and vertically polarised components of the emission intensity (I_{HH} and I_{HV}), each for the same period of time and other conditions. Since there is no difference between the number of photons coming towards the I_{HH} and I_{HV} channels from the sample, G is calculated as the ratio between the measured total intensities (counts) in each channel:

$$G_{bulk} = \frac{I_{HV}}{I_{HH}} \quad (2.2)$$

For evanescent wave induced fluorescence anisotropy measurements any polarisation bias of the detection assembly must also be considered. The equations of in plane anisotropy (equation 1.48) and out of plane anisotropy (equation 1.59) can be expressed, respectively, as:

$$r_{in} = \frac{I_{VV} - G.I_{VH}}{I_{VV} + G.I_{VH}} \quad (2.3)$$

$$r_{out} = \frac{I_{HV} - \frac{1}{2}(I_{VV} + G.I_{VH})}{I_{HV} + I_{VV} + G.I_{VH}} \quad (2.4)$$

The protocol for determining the G-factor is similar to bulk measurements. This is achieved by exciting with (H)-polarised light at the critical angle if the interfacial plane is assumed isotropic (i.e. no difference in motion between x and y orientations), then equal intensities for both (H) and (V) polarisations should be emitted and any detection bias is accounted for by the ratio:

$$G_{EW-TRAM} = \frac{I_{HV}}{I_{HH}} \quad (2.5)$$

Data Analysis

In this thesis several data analysis approaches have been used for extracting the decay coefficients of the excited states from the observed convoluted fluorescence decay profile all based on the non-linear least squares iterative reconvolution method (46). In this process, the recorded instrument response function (IRF) is convoluted with a trial fluorescence decay function that is based on initial guesses of all parameters that are believed to closely correspond to the solution. The resultant convoluted decay profile is compared with the recorded decay profile. Then, by minimizing the reduced χ^2 value (goodness of fit parameter), the trial decay is then iteratively modified in terms of all adjustable parameters to obtain a closer convoluted fit (46).

Discrete Lifetime Analysis

In the simplest case, the analysis of fluorescence decay profiles can include the use of a model with sum of a discrete number of exponential terms and then the iterative reconvolution procedure based on the Marquardt algorithm (70). To obtain the best fit to the experimental fluorescence decay profile, it is necessary to adjust parameters of the calculated convolution integral, $I_k(t)$:

$$I_k(t) = \int_0^t L(t') I(t - t') dt' = \int_0^t L(t' - \mu) \left(\sum_{i=1}^N \alpha_i \exp - \left(\frac{t - t'}{\tau_{fi}} \right) \right) dt' + \Delta \quad (2.6)$$

where the pre-exponential factors, (α_i) , and fluorescence lifetimes, τ_{fi} , are components of the fluorescence decay for a system with N discrete lifetime components. The Δ term is the baseline that accounts for the background noise (dark counts) of the detection system and μ is the shift term that accounts for the different time positions of the IRF and the fluorescence decay. The IRF is typically measured at a lower wavelength than the emission (scattered excitation), and this leads to an earlier arrival time of the

IRF photon. This is due to Einstein's explanation of the photoelectric effect, where the photon energy has to overcome the work function to liberate the photoelectron from the photocathode, and the energy difference between photon and work function is the kinetic energy of the photoelectron – this increases with decreasing wavelength. Due to this difference in energy, the transit time through the electron amplification process of the higher energy electrons is shorter than the photoelectrons generated from the low energy emission.

2.5 Fluorescence Lifetime Imaging (FLIM)

The excitation source for the FLIM measurements was either the 405 nm or 440 nm, frequency-doubled, mode-locked titanium sapphire laser (Coherent Mira 900f, 120 fs, 76 MHz) pumped by 9W from a DPSS laser (Coherent Verdi V10). In some cases this laser was cavity dumped to obtain 5.4 MHz, pumped by 4.5W from the V10. This excitation source was delivered to an inverted microscope (Olympus IX71) fitted with a 60X objective lens (Olympus PLANAPO water) via a single-mode optical fibre (S405 ThorLabs). The confocally isolated emission was detected with a single photon counting photomultiplier (Becker & Hickl PMC100) and acquired by a Becker & Hickl SPC-150 single photon counting card that was controlled using the SPCM TCSPC operating software. Intensity images are generated by summing all of the photons collected for each pixel individually using a separate off-line image analysis package "SPCImage" (Becker & Hickl, version 9.72). SPCImage applies a Levenberg-Marquardt nonlinear least squares iterative reconvolution routine to individually fit the fluorescence decay data from each pixel. Fluorescence lifetime images were generated by pseudo-colour mapping the average weighted fluorescence lifetime τ_m , of each pixel over the intensity image. We quote the weighted average lifetime τ_m , defined as:

$$\tau_m = \frac{\sum_{i=1}^j \tau_i \alpha_i}{\sum_{i=1}^j \alpha_i} \quad (2.7)$$

where j is the number of exponential terms in a sum-of-exponentials function required for an adequate fit with decay time τ_i and pre-exponential factor α_i .

2.6 Fluorescence Anisotropy Imaging (FAIM) Apparatus

A commercial TIRFM Nikon microscope was used for FAIM microscopy. It is possible to do fluorescence anisotropy imaging in both TIRFM and Epi illumination conditions. Both objective and prism-base configurations are available for TIRFM (71, 72). The objective lens method employs a high numerical aperture ($NA > 1.4$) microscope objective for illumination and collection, and the latter uses a high refractive index prism to create total internal reflection conditions and a separate objective to collect the emission. According to the application of polarisation microscopy several developed microscopes are available commercially (71–74).

During this work a polarised emission microscope was developed using an objective-lens based scheme. All images in these experiments were acquired using this microscope schematically depicted in figure 2.7. The excitation source was a CW laser (465 nm Blue DPSS BL473T6 Series Laser, Shanghai Laser & Optics Century Co). The linearly polarised excitation beam was coupled to a polarisation-maintaining optical fibre (PM-S405-XP, 400 - 680 nm range, NA: 0.12, ThorLabs Co) via an objective lens (5X, NA: 0.12) on a fibre launch unit. The optical fibre was connected to the fibre optic input port of the Nikon TIRF microscope (TIRF2 system TE 2000). The linearly polarised excitation can be rotated 90° utilising a liquid crystal retarder (ϕ 10 mm, ARC 350 - 700 nm, ThorLabs Co) which is controlled via a liquid crystal controller (LCC25, ThorLabs Co). Two linear polarisations for excitation can be created by changing the

liquid crystal RMS voltage via this controller. A high numerical aperture TIRF objective (Nikon Apo 60X, NA: 1.45) is equipped to focus incident light into the specimen. The incident angle at the TIR condition is also variable using beam position adjustment in the microscope. Index matching oil is applied between the objective lens and cover-slip. The sample's fluorescence was collected with the aforementioned objective-lens and directed to the exit port of the microscope. An OptoSplit II (Cairn Research) device includes a 50:50 polarising beam splitter cube (UVFS, 400-700 nm, ThorLabs Co) which was used to separate the S and P polarisation components of the emission beam. An EMCCD camera (Photon MAX camera 1024, Princeton Instruments) is connected to the exit port of the OptoSplit II and able to take simultaneous S and P polarisation images on two halves of the detector array. Four image combinations comprising two the polarisations for excitation and two for emission were saved on the acquisition PC. The exposure time for each image depends on the sample. It is typically of order hundreds of milliseconds for the polymer systems reported in chapter 3 and 1-4 seconds for biological systems discussed in chapter 5.

I_{ij} can be defined as the image intensity under excitation i and emission j polarisation, which incorporates the four components $I_{ss}, I_{sp}, I_{pp}, I_{ps}$ by considering two types of polarisation (P , polarised normal to the cover glass plane) and (S , polarised in the cover glass plane) I_{ss} is the observed emission polarised parallel with the S -polarised excitation, I_{sp} is the observed emission polarised perpendicular relative to the S -polarised excitation, I_{pp} is the observed emission polarised parallel with the P -polarised excitation and I_{ps} is the observed emission polarised perpendicular relative to the P -polarised excitation. The steady state fluorescence anisotropies (Equations 2.8, 2.9 and 2.10) are defined in terms of using these combinations: (54, 55)

$$r_{in} = \frac{(I_{ss} - B) - G \cdot (I_{sp} - B)}{(I_{ss} - B) + G \cdot (I_{sp} - B)} \quad (2.8)$$

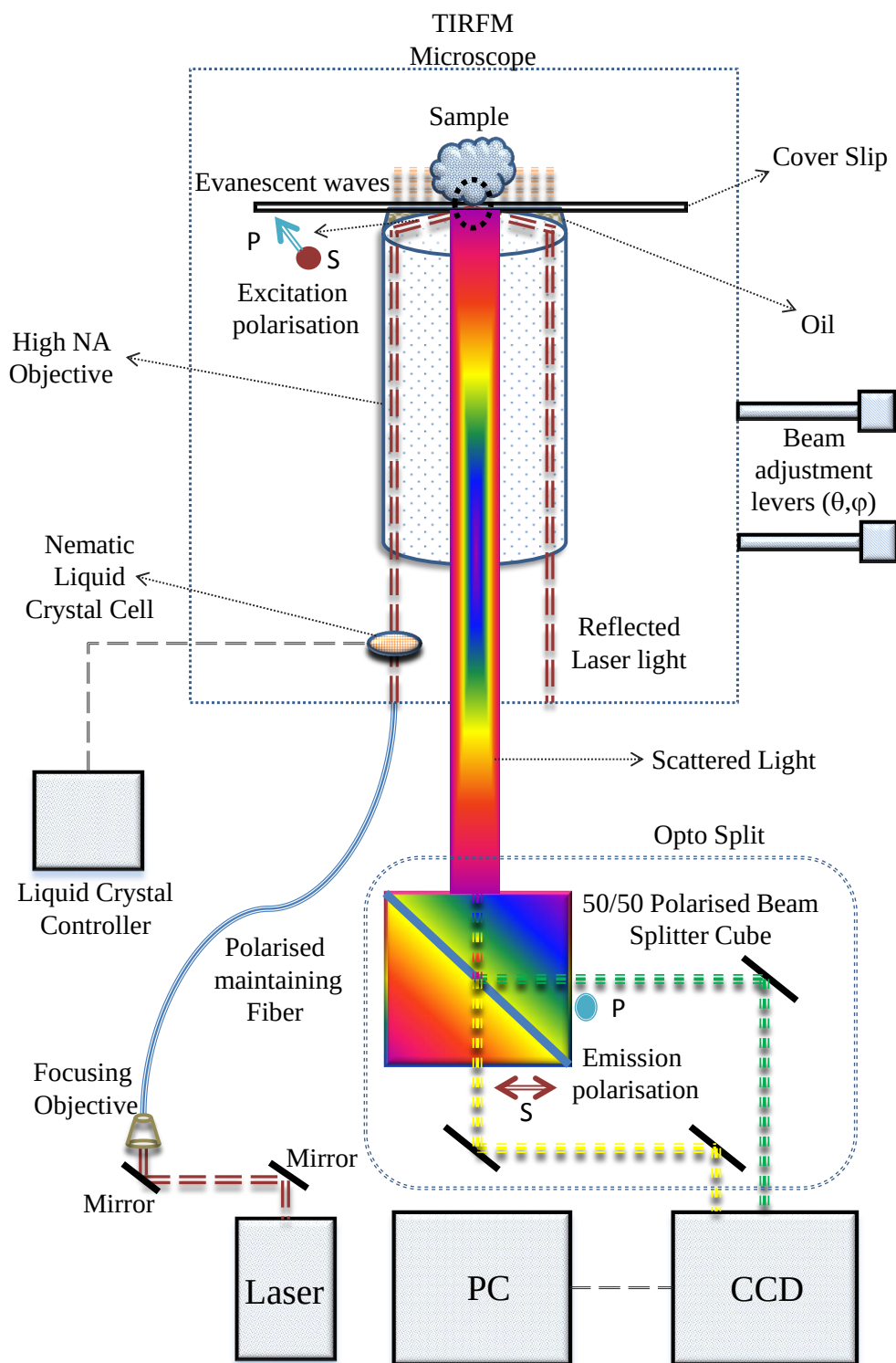


FIGURE 2.7: Schematic diagram of the total internal reflection fluorescence anisotropy imaging microscope developed in this work.

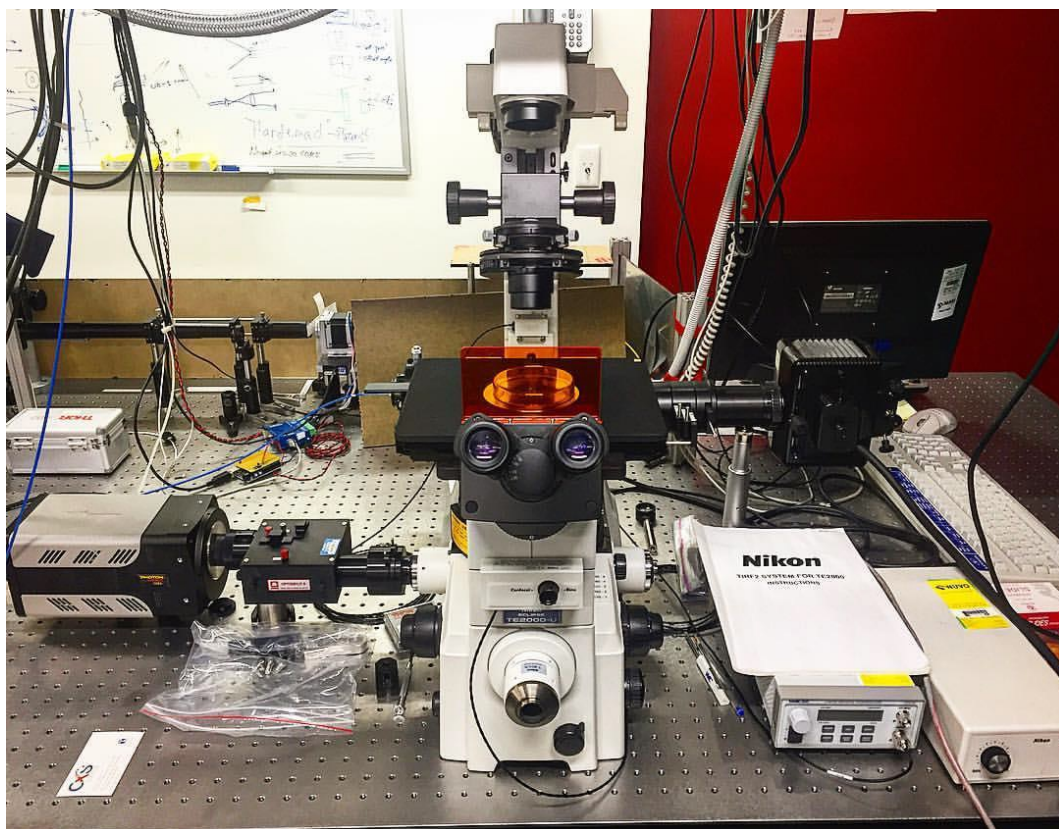


FIGURE 2.8: Fluorescence anisotropy imaging microscope developed in this work.

$$r_{out} = \frac{(I_{ps} - B) - \frac{1}{2}((I_{ss} - B) + G \cdot (I_{sp} - B))}{(I_{ps} - B) + (I_{ss} - B) + G \cdot (I_{sp} - B)} \quad (2.9)$$

$$r_{Bulk} = \frac{(I_{ss} - B) - G \cdot (I_{sp} - B)}{(I_{ss} - B) + 2 \cdot G \cdot (I_{sp} - B)} \quad (2.10)$$

where r_{Bulk} is regular anisotropy, r_{in} corresponds to emission depolarisation of an emission dipole positioned at the interface in the plane of the interface and r_{out} corresponds to emission depolarisation of an emission dipole positioned out of the plane of the interface. The B is the background, G is determined by observing a system that emits equal intensity in both polarisation combinations and derived by equation 2.11:

$$G = \frac{I_{ps}}{I_{pp}} \quad (2.11)$$

Calculating the G -factor at total internal reflection conditions is complicated by the effects of the interface on the isotropic rotational behaviour of a fluorophore in close proximity to an interface. At this point there is no clear strategy to calculate the G -factor or correction factor under total internal reflection conditions and so in this work it was generally, assumed to be unity.(75)

For the FAIM measurements the G -factor needs to be determined as a function of position within the image, i.e. on a pixel-by-pixel basis in figure 2.9. This was achieved using a dilute solution of Auramine-O in ethanol. The G -factor varies between 0.925 and 1.042 over the collection area. Although, we were expecting that G -factor varies as function of radius, we have observed random distribution of G -factor across the G -factor's image. A mean G -factor 0.982, is selected and the histogram of the spatial G -factor is illustrated in figure 2.10. All data analyses and when generating in-plane and out-of-plane anisotropy maps have been done using ImageJ 1.49 V (NIH, Bethesda, USA) or Matlab code.



FIGURE 2.9: G-factor at total internal reflection of Auramine-O in ethanol for 60X objective.

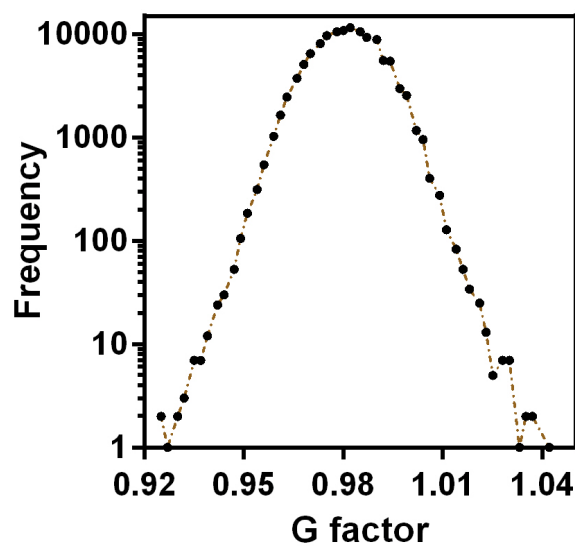


FIGURE 2.10: Histogram of G-factor at total internal reflection of Auramine-O in ethanol for 60X objective.

2.7 Materials and Sample Preparation

2.7.1 Polymer films

PFO and PODPF Films

All the solvents and reagents were purchased from commercial suppliers and used without further purification unless otherwise noted. All products were purified by flash column chromatography using Kanto Silica Gel (Kanto Chemical Co., Inc., Xuhui District, Shanghai, China) 60N (40–63/μm). Spectrochemical-grade solvents were used for optical measurements. *Palladium(II)acetate*, *tetra(triphenylphosphine)palladium*, *1,1'-bis(diphenylphosphino)ferrocene(dppf)*, *2,7-dibromo-9-fluorenone*, *2-bromo-9-fluorenone*, *bromobenzene*, *1-bromo-4-(octyloxy)benzene*, and *2,2'-bithiophene* were obtained from Aldrich Chemical Co. Borontrifluoride etherate, potassium carbonate, magnesium sulfate, chloroform and toluene were purchased from Sinopharm Chemical Reagent Co, Ltd (Nanjing, Jiangsu, China) and were used without further purification. Dichloromethane was dried using anhydrous sodium at room temperature. THF and toluene were dried over sodium using benzophenone as an indicator and distilled under dry nitrogen atmosphere immediately prior to use.

Poly(9,9-dioctylfluorene-2,7-diyl) (PFO, average $M_n = 62.1$ kDa, PDI = 1.7) and *poly[4-(octyloxy)-9,9-diphenylfluorene-2,7-diyl-co-[5-(octyloxy)-9,9-diphenylfluorene-2,7-diyl]]* (PODPF, average $M_n = 42.7$ kDa, PDI = 1.6) were synthesised in which diphenyl moieties lie at the 9-position of the fluorene group and the alkyl side chains substituted at the 4-position of the fluorene, according to literature procedure⁽⁷⁶⁾. The preparation of samples is credited to Ms. Meng-Na Yu and other members from Prof. Linghai Xie's group in the Centre for Molecular Systems and Organic Devices (CMSOD), Nanjing University, China.

In order to prepare substrates for spincoating quartz strip substrates with dimensions of 2.5 cm × 2.5 cm × 0.1 cm were cleaned by sonicating sequentially in NaOH

(1M), distilled water, acetone, isopropanol and dichloromethane. Substrates were kept in isopropanol until further use, and dried with nitrogen and then subjected to UV/ozone treatment for 30 minutes prior to coating.

Pristine amorphous films were prepared by spin-coating on quartz substrates from chloroform solution ($10 \frac{mg}{mL}$) at a spin rate of 1200 rpm for 30 s. The PODPF films with increasing contents of β -conformation were obtained by adjusting the annealing temperature from 230°C, 275°C and 300°C in nitrogen, respectively, and the annealing time was 10 min.

MEH-PPVs

The *poly[2-methoxy-5-(2-ethyl-hexyloxy)-1,4-phenylenevinylene]* MEH-PPV polymer samples were purchased from Sigma Aldrich, with molecular weight (M_w) of $2600 \frac{kg}{mol}$ and used without further purification. The powder was dissolved in a solution of 3% (by weight) Poly(Methyl Methacrylate) (PMMA, $M_w = 350 \frac{kg}{mol}$, Aldrich) in chloroform, chlorobenzene or toluene to a concentration of $0.1 \frac{mg}{ml}$ to minimize MEH-PPV aggregation during thin film preparation. No filtration of the solutions prior to film casting was performed.

Controlled Evaporative Self-Assembly Technique, *Snake – Skin Method*

The "snake-skin" method has been used, which is described in detail elsewhere, (77) to cast MEH-PPV polymer films from three different solvents onto the coverslips. Briefly, a drop of MEH-PPV/PMMA mixture in the appropriate solvent (Toluene, Chlorobenzene and Chloroform) was placed onto a 0.17 mm thick glass coverslip and a spherical lens mounted on an upright microscope was dipped into the drop. The spherical lens was withdrawn in micrometre increments and allowed to dry at each position over a few seconds and then for about 30 minutes producing a capillary-held

solution. The solution only evaporates at the edge of the capillary. Evaporation causes formation of two-dimensional "snake-skin" structures.

2.7.2 Bio-films

Cleaning methods

General glassware was cleaned immediately prior to use by first sonicating for one hour in 10% Dextran surfactant solution, rinsing with copious amounts of Milli-Q water followed by soaking for 4 hours in concentrated ($\sim 5M$) sodium hydroxide solution then finally rinsing with copious amounts of Milli-Q water. The hemi-cylindrical prism that is the substrate for the LUVs and the reflective surface for the evanescent wave experiments was cleaned by rinsing with copious amounts of Milli-Q water followed by soaking for up to 2 hours in a warm ammonical peroxide solution (1:1 hydrogen peroxide and Milli-Q water with a few millilitres of concentrated ammonia) to oxidise residual organic contaminants noncorrosively. It was then immediately rinsed with copious amounts of Milli Q water just prior to each experiment.

Large Unilamellar Vesicles (LUVs)

The preparation of LUVs studied in chapter 4 is described below, and is credited to Ms. Sreetama Pal from the Centre for Cellular and Molecular Biology (CCMB), Hyderabad, India. Cholesterol and 3-(N-morpholino) propanesulfonic acid (MOPS) were obtained from Sigma Chemical (St. Louis, MO). 1 - *hexadecanoyl* - 2 - -(9Z - *octadecenoyl*) - *sn* - *glycero* - 3 - *phosphocholine* (POPC) and 1 - *hexadecanoyl* - 2 - (9Z*octadecenoyl*) - *sn* - *glycero* - 3 - *phospho* - (1' - *rac* - *glycerol*) (sodium salt) (POPG) were purchased from Avanti Polar Lipids (Alabaster, AL). The synthetic NBD labelled melittin analogues were custom synthesized by Mimotopes (Clayton, Australia). Lipids were checked for purity by thin layer chromatography on silica gel precoated plates (Sigma) in chloroform/methanol/ water (65:35:5, v/v/v) and were

found to give only one spot in all cases with a phosphate-sensitive spray and on subsequent charring (78). All other chemicals used were of the highest purity available. The solvents used were of spectroscopic grade. Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout.

All experiments were performed using large unilamellar vesicles (LUVs) of 100 nm diameter. The first case contains POPC (320 μ M) / NBD-labelled melittin (1.6 μ M) with Lipid/Peptide ratio of 200. The second case contains POPC 70%-POPG 30% (320 μ M) / NBD-labelled melittin (1.6 μ M) with Lipid/Peptide ratio of 200. LUVs of 100 nm diameter were prepared by the extrusion technique using an Avestin Liposofast Extruder (Ottawa, Ontario, Canada). (79) Briefly, the multilamellar vesicles were freeze-thawed five times using liquid nitrogen to ensure solute equilibration between trapped and bulk solutions, and then extruded through polycarbonate filters (pore diameter of 100 nm) mounted in the extruder fitted with Hamilton syringes (Hamilton, Reno, NV). The samples were subjected to 11 passes through polycarbonate filters to give the final LUV suspension. The samples were incubated in the dark for 12 h at room temperature (23° C) for equilibration before making measurements. All experiments were done at room temperature 23° C.

F-actin labeling of CHO-K1 cells

The preparation of samples studied in chapter 5 section 2 is described below, and is credited to Mr. Parijat Sarkar from Centre for Cellular and Molecular Biology (CCMB), Hyderabad, India. $CaCl_2$, $EDTA$, gentamycin sulfate, $M\beta CD$, $MgCl_2$, penicillin, sodium bicarbonate, streptomycin, and Tris were obtained from Sigma Chemical Co. (St. Louis, MO). DMEM/F-12 [Dulbecco's modified Eagle's medium/nutrient mixture F-12 (Ham) (1:1)] and fetal calf serum (FCS) were from Gibco/Life Technologies (Grand Island, NY). Lovastatin was obtained from Calbiochem (San Diego, CA). NBD-Phalloidin was purchased from Molecular Probes (Eugene, OR). All other chemicals

used were of the highest available purity. Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout.

Chinese Hamster Ovary (CHO-K1) cells were maintained in D-MEM/F-12 (1:1) supplemented with 2.4 g L^{-1} of sodium bicarbonate, 10% fetal calf serum, $60 \text{ }\mu\text{g mL}^{-1}$ penicillin, $50 \text{ }\mu\text{g mL}^{-1}$ streptomycin, $50 \text{ }\mu\text{g mL}^{-1}$ gentamycin sulfate, and $200 \text{ }\mu\text{g mL}^{-1}$ geneticin in a humidified atmosphere with 5% CO_2 at 37°C . The stock solution of lovastatin was prepared as described previously (80) and added to cells grown for 24 hours (final concentration of statin $5 \text{ }\mu\text{mol}$ - $10 \text{ }\mu\text{mol}$ and incubated in 10% serum for 48 hours. Control cells were grown under similar conditions without lovastatin treatment.

F-actin labeling in CHO-K1 cells was carried out as described previously. (81) Briefly, cells were plated at a density of 1×10^4 on glass coverslips and grown in DMEM-F12 (1:1) medium for 24 hours followed by incubation in the presence of lovastatin for 48 hours. Cells were then washed with buffer A (PBS containing 1 mmol CaCl_2 and 0.5 mmol MgCl_2), and fixed with 3.5% (v/v) formaldehyde for 10 min. Permeabilization of cells was carried out in buffer A with 0.5% Triton X-100 (v/v) for 6 min. Cells were washed, stained with NBD-Pallacidin (82) (from Invitrogen Detection Technologies) for 1 hour, and mounted.

Aggregation-Induced Emission Samples (AIEs)

Materials

The preparation of these samples is credited to Dr. Yuning Hong and Ms. Moore Chen from the University of Melbourne.

OptiMem, Dulbecco's modified Eagles medium (DMEM), fetal bovine serum (FCS), glutamine, penicillin, streptomycin and ER Tracker red were purchased from Thermo Fisher. Sorbitol and all other reagents used in this study were purchased from Sigma-Aldrich. TPE-Py-NCS was synthesized as described in Ref (83). The $\text{TPE} - \text{Py} -$

NCS, was used as molecular rotor to label cytosolic proteins in Neuro-2A cells. The isothiocyanate (NCS) moiety can react with amine groups and thus covalently label the proteins under physiological conditions. The high brightness of *TPE – Py – NCS* in the aggregated state enables us to utilize it as a contrasting agent for cell imaging.

Cell culturing

Neuro 2A and HeLa cell-lines (from lab cultures originally obtained from ATCC) were used in this study and tested and cleared for mycoplasma. Neuro-2A cells were maintained in OptiMem (Life Technologies) supplemented with 10% FCS, 1 mmol glutamine, $100 \frac{U}{ml}$ penicillin and $100 \frac{\mu g}{ml}$ streptomycin in a humidified incubator with 5% atmospheric CO_2 . For microscopy experiments 9×10^4 Neuro-2A cells were plated on 8-well μ -slides (Ibidi). HeLa cells were maintained in DMEM supplemented with 1 mmol glutamine, $100 \frac{U}{ml}$ penicillin/streptomycin and 10% $\frac{W}{v}$ FCS. Cells were cultured at $37^\circ C$ in a humidified incubator with 5% atmospheric CO_2 . For microscopy experiments 7×10^4 HeLa cells were plated on 8-well μ -slides (Ibidi).

Cell Staining

TPE-Py-NCS was dissolved in DMSO as 1 or 2 mmol stock solution. The stock solution was kept at $4^\circ C$ in the dark. Plated cells were rinsed with PBS and then treated with freshly diluted TPE-Py-NCS (1-10 μ mol in PBS) for 30 min at $37^\circ C$. For live cell imaging, TPEPy-NCS was removed and cells were rinsed with PBS prior to the image acquisition. For fixed cell imaging, after the removal of TPE-Py-NCS, the cells were fixed in solution with 4 % $\frac{w}{v}$ paraformaldehyde for imaging. For co-staining experiment, ER-Tracker Red (ThermoFisher) was used according to manufacturer's instructions with 1 μ mol staining concentration. Cells were fixed afterwards.

2.8 Summary

In this chapter the experimental details corresponding to the techniques outlined in chapter 1, were presented. Steady state and time resolved fluorescence spectroscopy, the chronological equipment development of evanescent wave time resolved fluorescence spectroscopy and anisotropy have been reviewed. The development of a new and upgraded EW-TRAM apparatus is demonstrated. The new apparatus configuration of FAIM is explained in details in section 2.6. The polarised 2D mapping method is designed and then fabricated in terms of polarisation correction factors. A novel G factor calculation method is represented. In this approach, the polarisation bias (G factor) is spatially mapped. The most frequent G factor was 0.98 which is very close to unity. Sample preparation and chemical procedures of complex materials which have been used in this thesis are also reviewed in detail.

Chapter 3

Photophysics and Fluorescence Anisotropic Behavior of Conjugated Polymers

In this chapter the photophysical and fluorescence anisotropic behavior and morphological inhomogeneities such as aggregated chains of two types of light emitting conjugated polymer films (MEH-PPVs and PFO/PODPF) will be discussed in detail. A strategy based on FAIM to study energy migration in conjugated polymers is also introduced.

Briefly, in the first section of this chapter, FAIM and FLIM are combined and utilized to investigate the morphology of aggregated inhomogeneities in thin films of the conjugated polymer, *poly[2-methoxy-5-(20-ethyl-hexyloxy)-1,4-phenylene-vinylene]* (MEH-PPV) in three solvents, cast by a controlled evaporative self-assembly (*snake-skin*) method. Photophysical changes and depolarisation of initially excited dipoles caused by energy migration, can be monitored using FLIM and FAIM, respectively. Highly aggregated areas with short emission lifetimes in FLIM images correspond to highly depolarised emission regions in FAIM images attributed to energy migration. The tightly packed and coiled conformation regions can be identified by lower values of emission lifetime and anisotropy values in FLIM and FAIM images, respectively.

The open chain conformation can be detected by observing high values of emission lifetime and anisotropy.

In the second section of this chapter, a visualization of the unique photophysical and fluorescence anisotropic characteristics of polyfluorene coplanar conformation (β -conformation) using FLIM and FAIM measurements, is presented. Inhomogeneous morphologies and fluorescence decay profiles at various micrometer-sized regions within all types of polyfluorene β - conformational spin-coated films have been observed. *Poly(9,9-dioctylfluorene-2,7-diyl)* (PFO) and *poly[4-(octyloxy)-9,9-diphenylfluorene-2,7-diyl]-co-[5-(octyloxy)-9,9-diphenylfluorene-2,7-diyl]* (PODPF) β -domains both have shorter lifetime than those of the glassy conformation for the longer effective conjugated length and rigid chain structures. In addition, β -conformational regions have larger fluorescence anisotropy corresponding to regions of low molecular rotational motion and high chain orientation, while the low anisotropy in glassy conformational regions shows more molecular rotational freedom of the chain and efficient energy migration from amorphous regions to β -conformation as a whole. In this regard, FLIM and FAIM measurements provide an effective platform to explore the fundamental photophysical process of conformational transitions in conjugated polymers.

3.1 MEH-PPV Polymers

3.1.1 Introduction

The advantageous features of conjugated polymers, such as their electrical and photophysical properties, durability and ease of production, make these materials desirable for applications in various areas such as polymeric solar cells (PSCs) and light emitting diodes (LEDs) (84–89). The efficiency of conjugated polymer-based solid state devices (LEDs & PSCs) can be optimised through investigation of the morphological,

intrachain and interchain (34) interactions and exciton transfer/transport behaviour of these materials in thin films (90–92). Aggregation is a common cause of inhomogeneity in conjugated polymer films and can have a deleterious effect on solid state device performance. Large scale printing and other methods of coating of conjugated polymer films to substrates, such as for solar panel applications, induces inhomogeneities ranging in size from nanometres to several micrometres. Various methods exist with which to form thin polymer films and the morphology that materials adopt during film formation can be strongly dependent on the casting method used and variety of experimental parameters, including the type of solvent, miscibility of materials or any environmental conditions (temperature, casting time, cooling rate etc).

There are many different printing methods for casting polymer films. Conventional spin-coating or drop casting typically produce homogeneous or semi-homogeneous films from some hundreds of nanometres to many micrometres thick, and the different forces and rates involved in the drying process can induce different levels of molecular homogeneity. Off-centre spin coating reportedly produces a level of molecular alignment and homogeneity (93). An alternative, controlled evaporative method, in which a curved surface is withdrawn slowly from a drop of polymer solution, produces a morphology that resembles the pattern of a snake skin (77) but the homogeneity within this structure has not previously been studied from the aspect of photophysical properties of aggregation after film formation.

Steady-state and time-resolved fluorescence anisotropy measurements are useful for studying intra- and inter-molecular energy migration in conjugated polymers (34, 94, 95) and in concentrated conditions of smaller molecules such as dyes in organic solar concentrators (96). Since the rotational diffusion of chromophores of the fluorophore is highly inhibited in solid state films, energy migration becomes the dominant cause of fluorescence depolarisation from the initially photoselected emission transition dipoles.

High-resolution optical microscopy coupled with spectroscopy methods can be

used to probe the spatial and morphological properties of thin films to provide useful information relating to the mechanism and photophysical characteristics of charge transfer/transport as a function of location in polymer films. These micro-spectroscopic techniques can be extended to map the extent of emission depolarisation over regions of a film.

A few studies have been published investigating the emission polarisation and morphology of conjugated polymer chains which are aligned or oriented along a specific direction and were prepared using electro-spinning (97), nano-imprinting (98), liquid crystalline-self-organization (99), Langmuir-Blodgett technique (100, 101), stretching drawing (102) and evaporative assembly methods (77). Due to rapid energy transfer of excitons in conjugated polymers, the observed fluorescence emission may come from excited state of a completely different chromophore (90, 103) to the one that was originally photoexcited. During this energy transfer process, the emission becomes depolarised. The rate and chance of depolarisation are highly correlated to the morphology.

In this part of this chapter, the inhomogeneities in the morphology and fluorescence dynamics of thin films of the light-emitting conjugated polymer, MEH-PPV are investigated with submicrometer spatial resolution using FAIM which reports the depolarisation/energy migration mechanism, and then picosecond temporal scales using FLIM. Dramatic changes in the morphology and photophysical properties of MEH-PPV polymer films, extending to the tens of microns scale, have been observed using FAIM and FLIM microspectroscopy measurements under certain casting conditions (snake-skin method) in three different solvents.

In this part of the thesis how aggregated spatial inhomogeneities due to aggregation can affect the photophysical properties of a specific morphology will be discussed. In addition, the uniformity of the cast films and how this uniformity plays an important role in photophysical investigation in conjugated polymer systems and solar cell devices is discussed.

To gain better understanding of several types of aggregation, we have combined FLIM and FAIM. The aggregated inhomogeneous spots have lower fluorescence lifetime and lower values of anisotropy. These spots are assumed to correspond to the tightly packed and coiled conformation. The other possible morphology is an open chain conformation. In the former case, the conjugated polymers in these areas have higher lifetime and higher fluorescence anisotropy.

3.1.2 FLIM of MEH-PPVs

Conventional fluorescence imaging techniques only map the emission intensity as a function of location in samples such as polymer films, but there are many factors such as chromophore concentration, sample roughness and surface properties of the film that can influence the fluorescence intensity during collection. On the other hand, FLIM is less susceptible to the aforementioned effects, and can provide significant additional information beyond simply intensity. In the FLIM method, variations in the fluorescence decay characteristics are mapped, rather than intensity, as a function of location. Due to the sensitivity of fluorescence decay characteristics to the local environment of the fluorophore, such measurements can indicate changes in the micro-environments of the chromophore.

Time-resolved scanning confocal fluorescence imaging measurements have been carried out on MEH-PPV polymer films cast from various solvents; chlorobenzene, toluene and chloroform, by an evaporative assembly or colloquially named "Snake-Skin" method (77). For each solvent, the fluorescence lifetime map and fluorescence decay profiles corresponding to three representative locations on the map are depicted in figure 3.1. The effect of the specific solvent on morphology and fluorescence decay behaviour of the MEH-PPV polymer films is clear. In addition, the change of fluorescence decay kinetics corresponding to the presence of aggregation on the micrometre

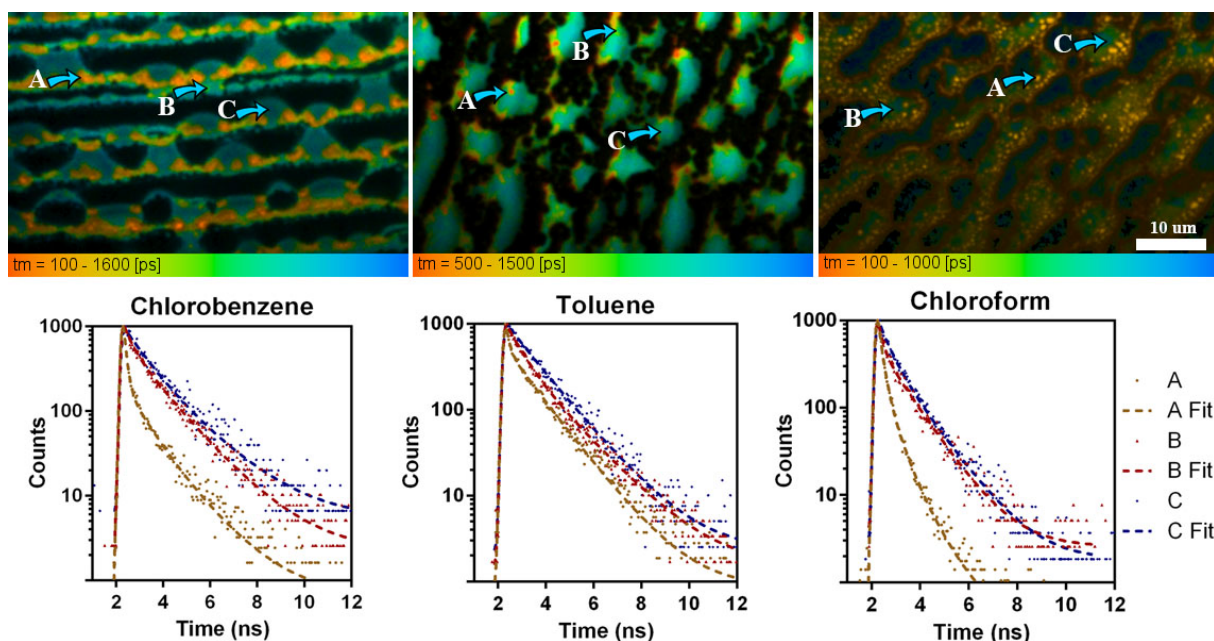


FIGURE 3.1: Fluorescence lifetime images (top panels) for MEH-PPV thin films cast from various solvents chlorobenzene, toluene and chloroform and the fluorescence decay profiles of corresponding points (A,B and C) recovered from the lifetime images. The photophysical properties of the same polymer with the same casting condition are different by using three different solvents.

scale are also observable. Three types of regions corresponding to aggregates, and non-aggregated or uniformly coated, and uncoated are clearly depicted in figure 3.1. The colour coded lifetime image of each point from the aggregated or non-aggregated areas and their corresponding fluorescence decay profiles are also presented. The fluorescence decays are multi exponential. The decay times, the percentage of relative intensities and the amplitude weighted-average emission lifetimes (calculated by equation 3.1) for three representative points in the lifetime map in each of the three solvent-cast films are summarised in Table 3.1 .

$$T_M = f_1 * \tau_1 + f_2 * \tau_2 + f_3 * \tau_3 \quad (3.1)$$

Fitted decay time (ps) and relative intensities (%)								
Film solvent	Location	f_1	τ_1 (ps)	f_2	τ_2 (ps)	f_3	τ_3 (ps)	T_m (ps)
Chlorobenzene	A	88	117	8	792	4	1525	227.32
	B	43	315	48	1186	9	2008	885.45
	C	64	866	3	1780	33	1801	1201.97
Toluene	A	63	131	26	1071	11	1463	521.92
	B	58	605	8	1403	34	1550	990.14
	C	42	983	10	1282	48	1494	1258.18
Chloroform	A	87	150	20	397	2	884	214.08
	B	62	177	35	1017	3	1427	508.5
	C	50	404	10	649	40	1177	737.7

TABLE 3.1: The emission decay times, percentages, relative intensities and amplitude weighted-average lifetimes (calculated by equation 3.1) for three representative points in the lifetime maps corresponding to each of the three solvent-cast films.

In figure 3.1, the regions depicted by the small dot shape (A) are assumed to correspond to the aggregated areas with the tightly packed and coiled polymer conformation because of the shortest fluorescence emission decay profile compared with the other more homogenous regions including the non-aggregated areas (B) and (C). Previously we have studied the effect of concentration on the photophysical properties of conjugated polymers (104). Increasing the concentration reduces the lifetime which indicates the presence of emissive excited state aggregates in concentrated samples. The B and C areas may correspond to the open chain conformation with higher longer emission and higher anisotropy values. The anisotropy values will be discussed in the following section. Similar fluorescence decay behaviour for films cast from chlorobenzene and toluene have been observed, whereas in the case of chlorobenzene, the average lifetimes T_M for three representative points are lower than for the toluene case. Our observed decay times are similar to the lifetimes typically reported in the literature for conjugated polymers like MEH-PPV solutions and films using ensemble averaged measurements such as regular TCSPC (105–107). Nanosecond components that were ascribed to relaxed intrachain excitons have been reported previously (102).

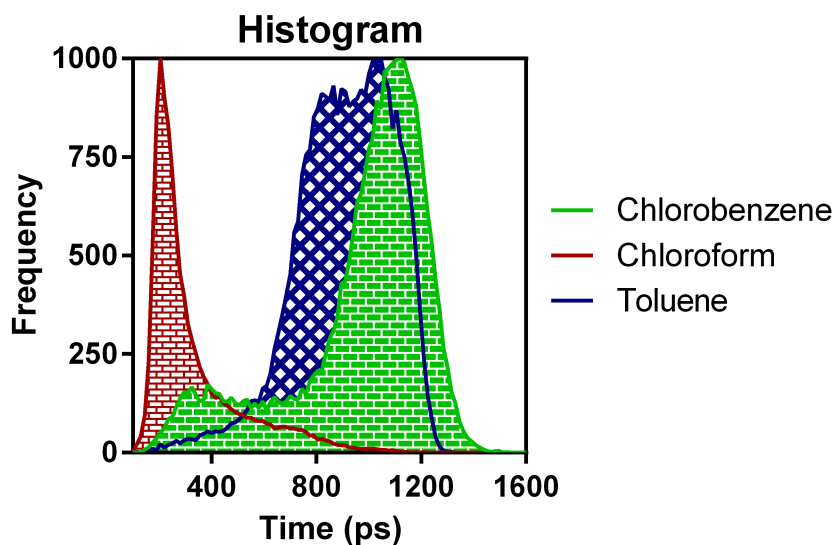


FIGURE 3.2: The fluorescence lifetime distribution histograms in the MEH-PPV films cast from chlorobenzene, toluene and chloroform. Films casted from chloroform have shorter distribution compared with films cast from the other solvents.

Time-resolved fluorescence measurements have been performed to investigate the dynamics of MEH-PPV polymers in aggregation spots using a *core – shell* model (108). Based on that work, the longer-lived fluorescence decay component is assigned to the emission from singlet states resulting from transfer from non-emissive excited states to intrachain excited states (109). These results show that inhomogeneities can be found on micrometre scales relying on chain structure and packing formation of MEH-PPV conjugated polymers and that this is dependent on the properties of the solvent from which the film is cast. This suggests that the areas including points B and C are uniformly evaporated in the snake-skin method and include open chain conformation with longer emission lifetimes.

The spatial photophysical inhomogeneities observed through the aggregated spots is caused by the $\pi - \pi$ electronic interaction both within and between polymer chains (110). In figure 3.2 the fluorescence lifetimes of the MEH-PPV films cast from various solvents are depicted. The lifetime histograms are generated from the representative

regions of the films. The distributions were calculated based on the results of 2-4 samples for polymers cast from each solvent. The lifetime distribution of the chloroform-cast films shows higher frequencies of the shorter lifetimes compared to the toluene and chlorobenzene cases. The films cast from toluene have the broadest distributions and the films cast from chloroform have the narrowest lifetime distribution. This indicates the conformation of the polymer in the aggregated spots in the films cast from chloroform is different to films cast from toluene. We have investigated the effect of concentration on time-resolved fluorescence behaviour of other conjugated polymers which showed an decrease in the fluorescence decay time as the concentration increased. (104). This suggests that the fluorescence decay behaviour shown in figure 3.2 is consistent with the high local concentration expected within these highly aggregated forms of the polymer.

To provide more information and clarification about energy migration in MEH-PPV conjugated polymer films, these polymers were investigated using fluorescence anisotropy imaging microscopy in the following section.

3.1.3 FAIM of MEH-PPV films

The steady state FAIM of MEH-PPV conjugated polymers cast using the "snake-skin" method from three solvents have been performed using the polarised microscope constructed. The anisotropy distribution of films cast from toluene is more broad than for the films cast from chloroform as seen in figure 3.3. This difference is attributed to the prevalence of tightly packed and coiled conformations in the polymer chains. The chloroform-cast film has a higher frequency of polymer chains adopting a tightly packed or coiled conformation, which is characterised by completely depolarised emission, as seen in figure 3.3. The anisotropy distribution plots are the most informative way of reporting the FAIM data in a similar way that lifetime distributions represent

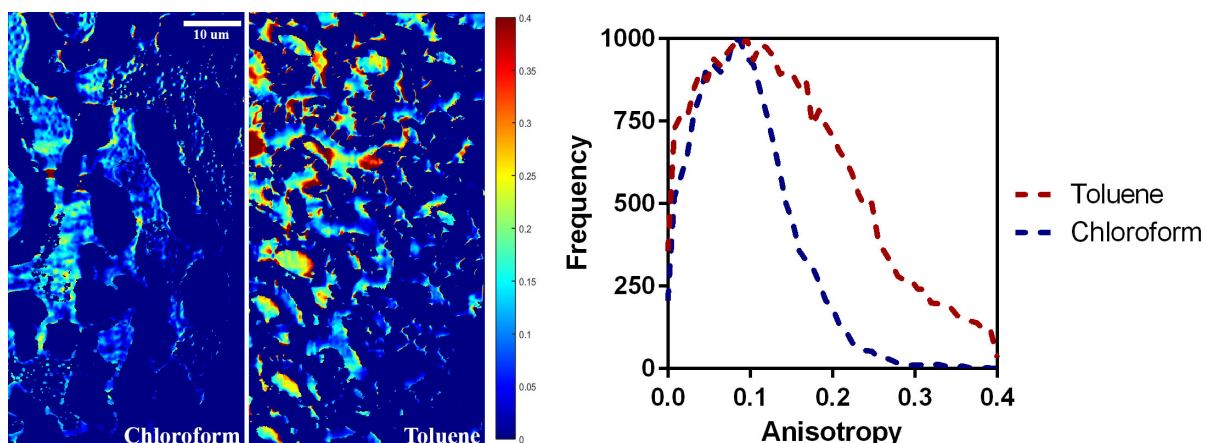


FIGURE 3.3: The steady state FAIM of MEH-PPV conjugated polymers casted from toluene and chloroform (left) and their corresponding anisotropy histogram (right).

the information in FLIM images. The FAIM histogram of the films cast from chloroform range in anisotropy values between 0-0.3 compared to 0-0.4 for the films cast from toluene. However, both histograms have the same peak at 0.1 anisotropy units.

It is necessary to consider that the fluorescence lifetime distribution of films cast from toluene and chloroform in figure 3.2 show similar trends to the fluorescence anisotropy histograms of the corresponding solvents in figure 3.3. Films casted from toluene has a broader distribution of anisotropy and lifetime compared to the films cast from chloroform. Yin et al. (111) discussed the exciton migration process which the original exciton polarisation is lost, and the emission becomes depolarised. They believe that "in the solid states, the inhomogeneous $\pi - \pi$ stacking and chain packing lead to aggregated states, in which the exciton polarisation in the initially excited chain

¹F. Perrin related the observed polarisation to the excited state lifetime and the rotational diffusion of a fluorophore using following equation:

$$\frac{r_0}{r} = 1 + \frac{\tau}{\tau_c}$$

In the Perrin equation, if lifetime τ changes, the steady state anisotropy will also change, even if τ_c is constant. Simultaneous measurements of anisotropy and lifetime will help provide clear visualisation of the cause and extent of the emission depolarising process. This can be achieved with scanning confocal measurements, but this mode is incompatible with TIRFM. Wide field lifetime imaging combined with steady state anisotropy imaging can be an option for these measurements but requires an intensified CCD camera or other widefield FLIM device, which were unavailable during this work. New widefield FLIM devices are becoming more readily available and this would make a valuable addition to extend the methods discussed in this thesis.

may migrate to the neighboring chains with different orientation and is gradually lost the initial polarisation"(111). Taking advantage of this concept, the FAIM represents a unique method to map the depolarised spots and is a useful tool to identify chain packing or π - π stacking in the morphology of conjugated films. Lower values of anisotropy are depicted by blue colour in figure 3.3. Apparently the film cast from chloroform is generally more depolarised compared to the films cast from toluene, which suggests more exciton migration. The lower values of anisotropy and lifetime corresponds to the tightly packed and coiled conformation. Both FLIM and FAIM studies of MEH-PPV cast from chloroform may indicate there are more events of intrachain energy migration in comparison with the other solvents.

Vogelsang et al. (112) discussed the effect of “poor” or “good” solvents on aggregation of MEH-PPV/PMMA mixture. To probe the energy migration process in large-scale ordered morphologies, they have developed a solvent vapour annealing (SVA) technique to control the average size and morphological order of aggregates. They applied single molecule experiments on conjugated polymer films prepared using the SVA method as a function of good versus poor solvent ratio. They found that there is a correlation between the size of the aggregated conjugated polymers cast from SVA method with from solvent mixtures of varying good/bad solvent ratio. This is in agreement with the findings shown in figure 3.3, for films cast from chloroform (good solvent), the emission from which is mostly depolarised due to enhanced energy migration occurring as a result of morphological changes within the larger bigger size of aggregat compared to films cast from the other solvents.

3.1.4 Conclusion

The study in this section illustrates the power of FLIM combined with FAIM to investigate the degree of aggregation in MEH-PPV/PMMA films cast from a range of solvents using a controlled evaporative self-assembly (*Snake – Skin*) method. FLIM

provides information about photophysical properties of inhomogenities or aggregated spots and the latter provides evidence of energy migration in conjugated polymer films. The spatiotemporal spectral information acquired showed variation the degree of aggregation such as highly aggregated, short-lived species in the FLIM image. These spots correspond to the tightly packed and coiled conformation. The “single-chain like” regions emitt with approximately nanosecond emission decay components. The spatial FAIM images provided the complementary information about highly aggregated regions; blue FAIM images exhibit largely depolarised regions, and more uniform regions with partially polarised anisotropy spots. The overall morphology, life-time in FLIM images and anisotropy in FAIM images were sensitive to the solvent from which the film was cast. FAIM is able to map depolarised areas in conjugated polymer films where high degree of energy migration is exhibited.

3.2 PFO & PODPF Polymers

3.2.1 Introduction

As discussed, conjugated polymers are materials that have demonstrated use for emerging optoelectronic applications (113–115). Consequently, programmable chain conformation is a tool that can be used to control the optical and electronic properties of conjugated polymers, such as their absorption, emission and conductivity, especial allowing for their anisotropic behaviour because of the intrinsic directional p -orbital overlap and π -electron delocalization along the conjugated backbone (116, 117). In addition, conjugated polymers are weakly bonded macromolecules with numerous degrees of conformational freedom with various angles between adjacent conjugated repeat units, resulting in different chain arrangements varying from completely amorphous, semi-ordered to crystalline structures (117–122). The capacity to control the degree of chain order and molecular conformation is a key component to exploit the anisotropic properties of these materials. Previously, the planar conformation adopted by some conjugated polymers has been investigated as an alternative to achieve molecular alignment and regular molecular crystalline structure in solid state systems (123–125). These rigid coplanar structures in the solid state can also increase the effective conjugation length and suppress the nonradiative decay and excited polaron formation with improved photon absorption and emission properties (34, 126, 127).

This part of this chapter explores the effect of planar conformational transitions and chain oriented arrangement in a conjugated polymer on the photophysical and anisotropic properties. Among all light-emitting conjugated polymers, polyfluorenes (PFs) exhibit fascinating conformation-induced polymorphic behaviours, involving mainly two kinds of chain conformations, namely the glassy and β -conformation states. These enable the conformation-dependent photophysical properties of conjugated polymers to be investigated in detail (113, 121, 128–131). The coplanar β -conformation, which

is endowed with longer effective conjugated length, effective intra/inter- molecular charge transport, high density molecular stacking and highly anisotropic properties, is a promising way to form ordered and oriented structures that can result in improved carrier mobility, polarised emission, excellent spectral stability and enhanced device efficiency (76, 132–135). On the other hand, the coexistence of diverse chain conformations or multi-phases in the film states likely induces inhomogeneous morphologies, which significantly affects the dynamics of electron/hole transport and device performance (118, 136–138). The nature of these spatial areas or connections in various conformations, phases or aggregates is poorly understood and should be the subject of further study. In spite of extensive investigations, the precise conformational behaviour, photophysical processes and anisotropic properties of polyfluorenes have remained elusive. Moreover, analysis of the time-resolved emission behavior of the diverse conformations, especially in terms of excitonic behaviour, have been discussed in many literature articles but the interpretation of these observations is still controversial (126, 139–143). Furthermore, the interplay between the diverse conformations adopted and the anisotropic properties in conjugated polymers is far from being clearly articulated. One difficulty originates from these conformations' intermediate location on the oriented-disorder scale in these inhomogeneous films. As discussed above, high-resolution optical spatiotemporal microscopy methods are a useful tool to provide this spatial information (110). Combining ultrafast spectroscopic visualization methods with high spatial resolution optical techniques makes it possible to map the dynamics of the photoinduced charge transfer/transport processes as a function of location in the film. This part of this chapter, systematically explores the fluorescence dynamics and anisotropic properties of polyfluorenes exhibiting diverse conformation (glassy (α -, amorphous) and β -conformation) behaviours in inhomogeneous self-doped films (133) on submicrometre spatial and picosecond temporal scales using FLIM and FAIM

measurements. The results show that the fluorescence decay characteristics of the diverse conformations adopted can be determined via FLIM. The use of FAIM in polyfluorene films with and without β -conformation is also explored here to monitor their change upon the conformation transition.

3.2.2 FLIM and FAIM of PFO & PODPF systems

In recent years, numerous efforts have been made to investigate the effects of molecular characteristics, such as molecular weight (144), alkyl substituent (145) and copolymer structure (146, 147) on the formation of the β -conformation in polyfluorenes. A mechanism for the β -conformation formation has been proposed, where the interactions between the side chains provide the chemical energy to overcome the activation energy barrier and planarize the polymer backbone, leading to the extended conjugation length that characterizes the β -conformation (133, 145, 148). Therefore, the prerequisite to achieve efficient adoption of the β -conformation is the van der Waals (VDW) forces of alkyl side chains must be strong enough to overcome the steric repulsion and planarize the polymer backbone (145). The delicate balance would be easily destroyed by the introduction of molecular segments at higher copolymerized fractions. Previous research has revealed that two types of polyfluorenes could induce the formation of the β -conformation, namely polydialkylfluorene (PDAF) (first generation) and polydiarylfluorenes (second generation) derivatives (figure 3.4) (76, 146, 147, 149, 150). An optimal chain length for the formation of β -conformation in polyfluorene derivatives is regarded as an octyl side chain on the polyfluorene groups through which it is easier to form the required interdigitation packing than any other length and substituted style (145, 150).

In addition, most literature examples show that the low-content β -conformation of PDAFs is only retained after the introduction of copolymerized units, chiral groups, cross linking networks, and/or surfactant Zwitterion groups (146, 147, 151, 152).

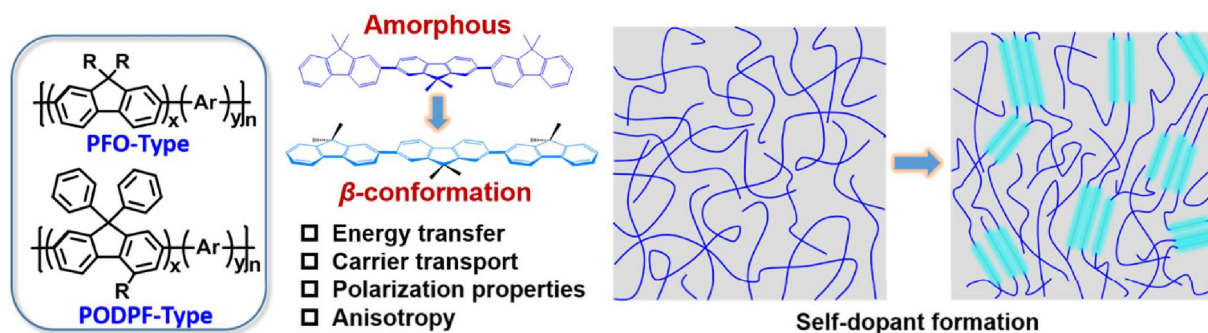


FIGURE 3.4: General chemical structures of two generation polyfluorenes which could be induced to adopt a β -conformation. Schematic illustrations of dihedral angle and film orderliness in the amorphous and β -conformation film (133).

In general, this coplanar conformation in conjugated polymers induces longer effective conjugated lengths, which may induce an extra shoulder in the absorption spectra and red-shifted PL spectra (138, 153). Therefore, the polyfluorenes' β -conformation is very characteristic in optical spectroscopy. For example, a new absorption shoulder peak at 430~445 nm, below the $\pi - \pi^*$ transition is attributed to the polyfluorene β -conformation. The β -conformation of PFO also produces marked changes in the emission spectrum, where the structured and red-shifted emission of the β -conformation dominates, even for very small copolymer fractions. The domination of the β -conformation emission comes about through efficient Förster transfer from the "host" α -conformational chain to the β -conformation inclusions (or guests) in the amorphous matrix (121, 133, 154, 155) which induced self-dopant film formation (figure 3.4). According to the literature time-resolved energy transfer measurements show this transfer to be a very efficient process (~ 5 ps) in PFO type materials (126). β -Conformation emission is characterized by a spectrum of very well-defined sharp vibronic replicas with almost no Stokes shift. Indeed, the spectrum is very similar to that of a ladder polymer (156, 157).

As shown in figure 3.5 (a), the absorption spectrum of the PFO film spin-coated

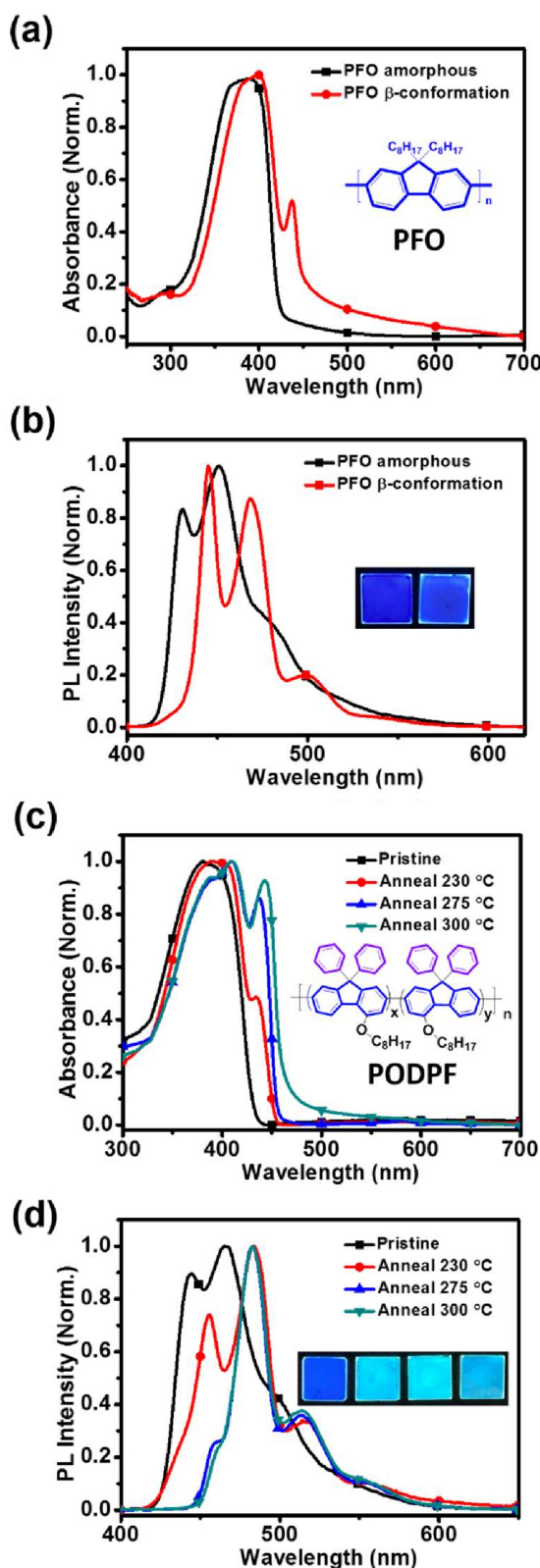


FIGURE 3.5: (a) UV-vis and (b) PL spectra of PFO amorphous and β -conformation films spincoated from CHCl_3 and DCE solutions, respectively, the inset showing the molecular structure of PFO and a photograph of PFO films with and without β -conformation under 365 nm UV lamp irradiation. (c) UV-vis and (d) PL spectra of PODPF films before and after annealing at 230°C, 275°C, 300°C, the inset showing the molecular structure of PODPF and a photograph of corresponding films under 365 nm UV lamp irradiation.

TABLE 3.2: Summary of optical spectra of PFO

PFO	UV-vis (nm)	PL (nm)
Amorphous	382	430, 450, 482
β -Conformation	394, 437	445, 468, 499

TABLE 3.3: Summary of optical spectra of PODPF

PODPF	UV-vis(nm)	PL (nm)
Pristine	385	445, 466, 499
Anneal 230°C	392, 434	458, 483, 513, 555
Anneal 275°C	388, 409, 443	459, 483, 514, 556
Anneal 300°C	388, 409, 443	460, 483, 514, 556

from $CHCl_3$ solution shows a maximum at 382 nm, and the corresponding emission peaks appear at 430, 450 and 482 nm, suggesting the amorphous state. The β -conformation of PFO is planar with an extended effective conjugation length, which can be probed by the characteristic shoulder in the normalized absorption spectrum at 437 nm in addition to the main peak at 394 nm (figure 3.5 (a))(158, 159). The PL spectrum exhibits three emission bands with peaks at 445, 468, and 499 nm, corresponding to the 0-0, 0-1 and 0-2 vibrational transitions of the β -conformation, respectively, which is ascribed to efficient energy transfer from the amorphous region to β -domains (figure 3.5 (b)). The inset in figure 3.5 (b) shows the the deep blue colour of the film turning to sky blue after conformational transition under UV lamp irradiation at 365 nm. The detailed optical properties are listed in tables 3.2 and 3.3.

PODPF in the β -conformation could be also obtained via post treatment of a pristine amorphous film after thermal annealing at temperatures above 220°C. As shown in figures 3.5 (c) and 3.5 (d), for the pristine film, the maximum absorption peak is at 385 nm, and the PL spectrum exhibits three emission bands at 445, 466, and 499 nm, indicative of the amorphous state. However, dramatic differences appeared after thermal annealing. An absorption shoulder peak appears at 434 nm following annealing the pristine film at 230°C, which was similar to the characteristic absorption peak of the PFO β -conformation. With increasing annealing temperature, the intensity of the

shoulder peak increased, indicating an enhanced content of β -conformation. A new peak at 409 nm in the absorption spectra of films annealed at 275°C and 300°C indicates the presence of strong chain aggregation resulting from chain rearrangement under thermal activation, where the α -chains transferred into the planar conformation. The absorption spectrum of the film annealed at 300° exhibits a prominent additional peak at 443 nm, and the intensity in the wavelength range of 457 to 550 nm increased compared to the other three profiles, which demonstrates that ordered crystalline structure in the film leads to strong self-absorption and light scattering. In terms of emission spectra, the films after annealing treatment exhibit four emission bands at 457, 483, 514, and 556 nm (figure 3.5 (d)). Similar to this work (133), excitation wavelength-independent PL spectra of PODPF and PFO also confirmed the self-dopant formation in β -conformation film. The inset of figures 3.5 (d) illustrates that the film changes from blue to green under UV lamp (365 nm) irradiation indicating that the chain coplanar conformation of polyfluorene can be obtained in both the PFO spin-coated film from DCE solution and in PODPF thermally annealed films.

The atomic force microscopy images of PFO films spin-coated from $CHCl_3$ and DCE solutions, (figures 3.6 (a) and 3.6 (b), respectively) illustrate the change in morphology between the α - and β -conformation films. The α -conformation film is uniform and smooth, which is consistent with previously reported amorphous films (135, 155). Significant differences between the two films are also observed in their FLIM (figures 3.6 (c) and 3.6 (d)) and the corresponding average fluorescence lifetime histograms (figure 3.6 (g)). The FLIM image of the film cast from $CHCl_3$ is homogeneous without any indication of large aggregates and domains. The average fluorescence lifetime distribution of the amorphous film was reasonably narrow peaking at around 370 ps. These emission decay times are in general agreement with those typically reported for PFO solutions and films recorded in large area illumination (ensemble averaged) measurements. In contrast, the film cast from DCE solution exhibited a root mean square (RMS) roughness (2.15 nm) in the AFM image (figure 3.6 (b)), significantly higher than

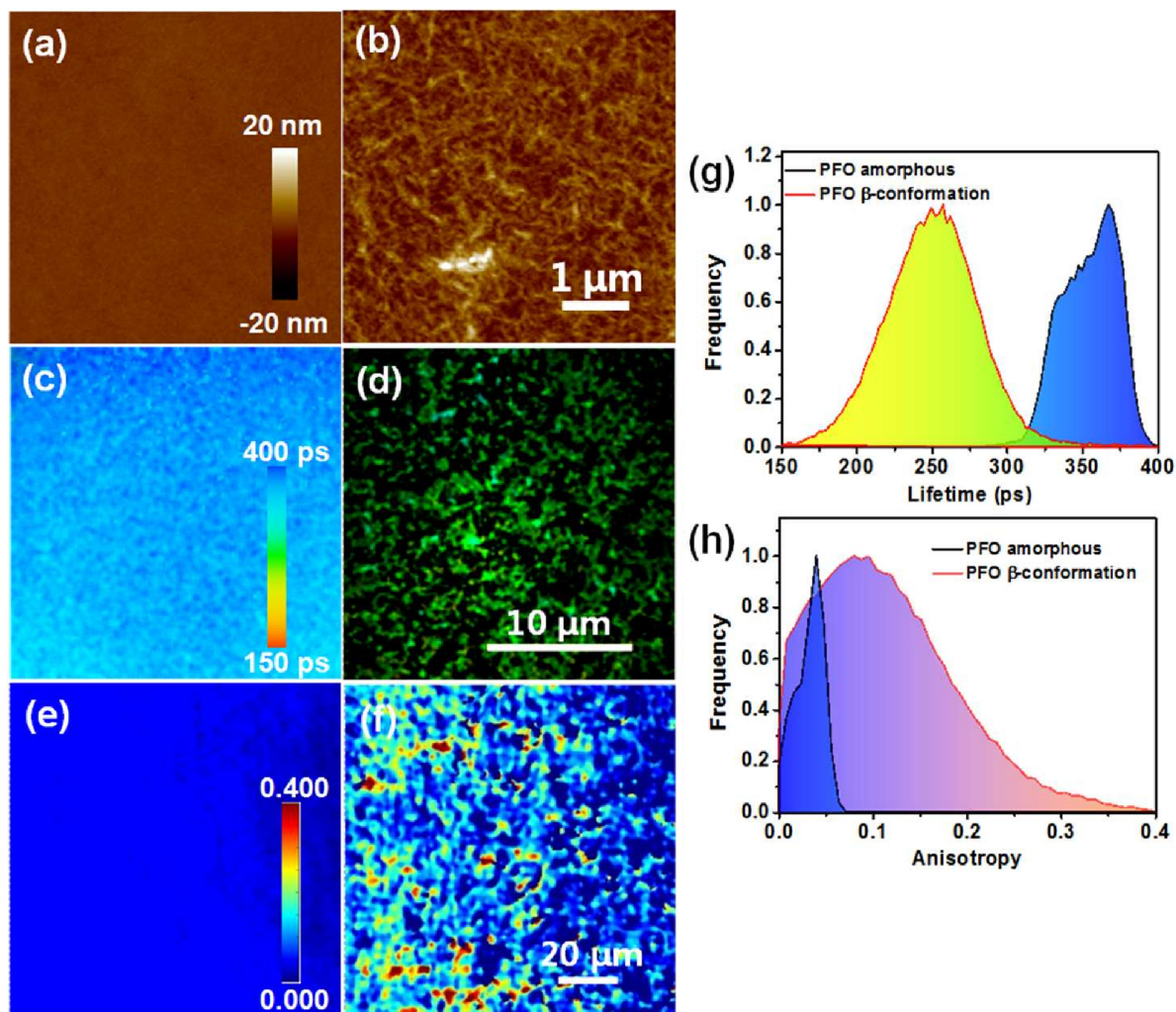


FIGURE 3.6: AFM images of PFO films spin-coated from (a) CHCl_3 and (b) DCE solution, respectively. The size of the AFM images is $5\text{ }\mu\text{m} \times 5\text{ }\mu\text{m}$. FLIM images of PFO (c) amorphous (α -) and (d) β -conformation film. FAIM images of PFO (e) amorphous and (f) β -conformation film. (g) Fluorescence lifetime distributions and (h) fluorescence anisotropy imaging distribution histograms of PFO amorphous and β -conformation films. Two film thickness are both about 100~105 nm.

that of the amorphous sample, corresponding to marked topographical structure. The increased roughness was ascribed to the chains' aggregation in a solvent (DCE) of moderate quality for this material (155). The high β -conformation content is further represented by an increase in the inhomogeneity of the lifetime map on micro- and submicro-metre scales in the FLIM image (figure 3.6 (d)) in keeping with the results of the AFM analysis (135, 155), and a significant change in the corresponding average fluorescence lifetime histogram (figure 3.6 (g)) that shifts to shorter (150~350 ps, with peak at about 253 ps) times and becomes more uniformly distributed. The chain planarisation therefore leads to a marked decrease in the fluorescence lifetime, and slightly decreased emission quantum yield.

Films comprising molecular aggregates or crystalline structures can be viewed as a 'self-doped' system (133), where a fraction of chains with a lower energy gap are intimately dispersed within a 'matrix' of more amorphous polymer chains with a larger energy gap. According to this work (126) the excitons photo-generated in the amorphous matrix of PFO film surrounding the β -conformation chains can transfer to the lower energy β -conformation chains on very rapid (shorter than ~ 5 ps) time scales, which is shorter than the temporal resolution of our detector and the TCSPC method used.

Energy migration can, however, be visualized by FAIM which reports on the difference between fluorescence intensities emitted parallel and perpendicular to the plane of polarisation of the excitation light that result from emission depolarising processes occurring within the excited-state lifetime (57, 160). In the absence of rotational diffusion of chromophores, fluorescence depolarisation can result from the randomization of the orientation of the emission transition dipole, relative to the absorption transition dipoles of the initially photoselected chromophore, following steps of Förster-mediated energy migration (96, 104).

The FAIM images of the amorphous and β -conformation films (figures 3.6 (e) and

3.6 (f), respectively) illustrate a marked difference in the degree of emission depolarisation that is quantified in the fluorescence anisotropy histograms calculated on a pixel-by-pixel basis (figure 3.6 (h)). The uniformity of the FAIM image and the reasonably narrow corresponding anisotropy distribution of the amorphous film (deep blue) reflect the homogeneous morphology seen in both the other microscopy modes. In comparison, the FAIM image of the PFO β -conformation film (figure 3.6 (f)) shows some pixels of high anisotropy values (red), and many exhibiting quite low anisotropy values (dark blue), corresponding to high and low degrees of emission depolarisation, respectively. The fluorescence anisotropy distribution (figure 3.6 (h)) is correspondingly broader and centered at higher anisotropy values than that of the amorphous film. These observations reflect the heterogeneous environment within the β -conformation film, which comprises aggregated domains, with short emission decay times and variable emission depolarisation attributed to energy transfer/migration from the amorphous state to the β -conformation. According to PFO absorption spectra in figure 3.5 (a), the fraction of β -conformation in this PFO film is estimated roughly by subtracting the contribution from the amorphous state in the absorption spectrum and the calculation result is about 39.5% , which is largely in agreement with the results of the FLIM and FAIM images. To our knowledge, this is the first direct visualization method allowing the self-dopant behaviour in PFO β -conformation films.

In order to confirm our assumption above, we also systematically explored the photophysical properties of a second generation β -polyfluorene, PODPF, in various conformations using the same methods. The β -conformation can often be obtained in films following post-treatment, such as solvent and thermal annealing. Owing to the chemical oxidation, chain aggregation and morphological instability (lower glass and crystallization transition temperature, $T_g = 65^\circ\text{C}$, $T_c = 120^\circ\text{C}$), the PFO β -conformation films cannot be widely applied in optoelectrical devices. In contrast, the present PODPF material shows intrinsically high chemical stability in air, so the fraction of β -conformation

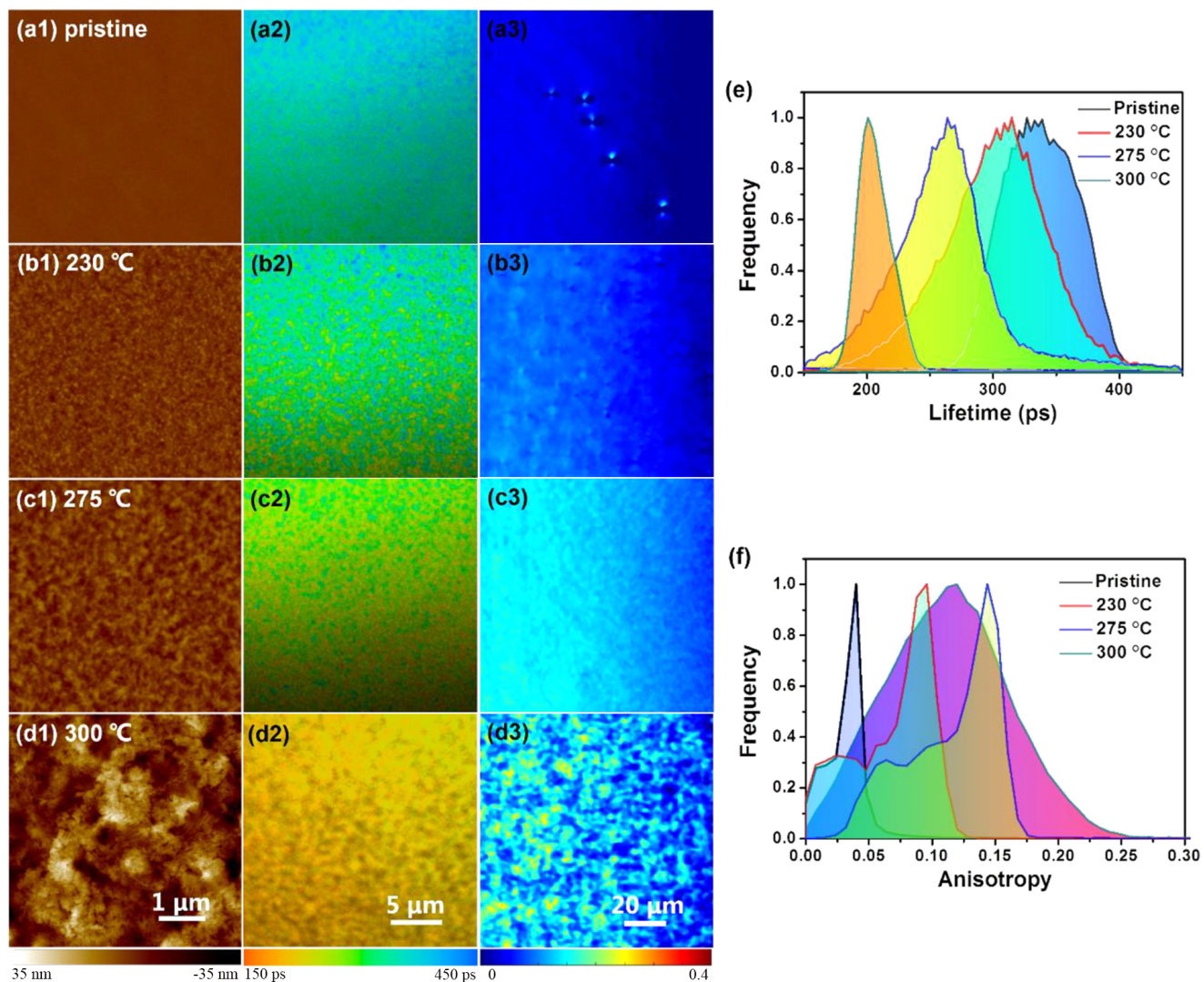


FIGURE 3.7: Atomic force microscopy (AFM) images of PODPF films (a) before annealing and after annealing at (b) 230°C, (c) 275°C, and (d) 300°C. The size of the AFM images is 5 μm × 5 μm. The (a2-d2) FLIM and (a3-d3) FAIM images of the corresponding films. (e) Average fluorescence lifetime and (f) fluorescence anisotropy distribution histograms of PODPF films following annealing at different temperatures. All film thickness are about 105~110 nm.

in the film states can be precisely tuned via controlling the thermal annealing temperature. Firstly, a homogeneous morphology is observed in the AFM image of the pristine amorphous film (figure 3.7 (a1))(132, 150), suggesting excellent film forming stability and homogeneous morphology similar to the PFO pristine film. This is confirmed by the FLIM image and corresponding average fluorescence lifetime distribution (figure 3.7 (e)). The lifetimes of the amorphous film range from 250~420 ps with a peak of about 335 ps, similar to the behavior of the PFO amorphous film spin-coated from $CHCl_3$ solution discussed above. By annealing the pristine film at 230°C for 10 min, the roughness increased to 1.79 nm and several small “granular” domains appeared with a size of tens of nanometres (~20 nm, figure 3.7 (b1)). As the annealing temperature is increased, more and larger granular (~100-800 nm) structures appeared (figure 3.7 (c1) and 3.7 (d1)), while simultaneously more β -conformation (~45.8%), according to the absorption spectra in figure 3.5 (c), was obtained. The increasingly rough topography is attributed to chain aggregation, which was confirmed by the stronger absorption peak at 409 nm, and the aggregation induced conformational transition(150). In fact, the absorption shoulder of this film is about 443 nm, red shifted about 6~9 nm compared with those of the pristine film thermal annealed at 230 or 275 °C. The FLIM images (figures 3.7 (a2)-3.7 (d2) and 3.7 (e)) illustrate that as larger domains form with increasing annealing temperature, the average fluorescence decay time becomes significantly shorter. The tendency of emission decay times to decrease in PODPF as the β -conformation forms is similar to PFO. It is noteworthy that the film morphology obtained following thermal annealing at 300°C is significantly different from the other three samples. Such a high temperature is close to the melting point (310°C) of PODPF, which activates the chain motion most dramatically, causing the maximum aggregation or crystallization of the chains. The morphology adopted following annealing at this temperature also corresponded to the shortest and narrowest decay time distribution. The fluorescence anisotropy distribution plots (figure 3.7 (f)) calculated from the FAIM images (figures 3.7 (a3)-3.7 (d3)) indicate that the emission is markedly more

polarised and oriented after annealing at the lower two temperatures compared to the pristine film. The unique behaviour of the film annealed at 300°C compared to the lower temperatures is also evident from the FAIM analysis, with the anisotropy distribution centered at an intermediate anisotropy value and being far broader than the other distributions. The low fluorescence anisotropy exhibited by the pristine film is consistent with completely random chain packing inferred from the AFM and FLIM images. After thermal annealing at 230 and 275°C, the increase in the peak anisotropy value is indicative of increased order (crystallinity) within the film resulting in a higher degree of emission within one plane of polarisation. The higher degree of order is consistent with the increased absorption at 409 nm (figure 3.5 (c))(150) attributed to the increased chain crystallisation than those of the PODPF film with a lower fraction β -conformation and PFO β -conformation spin-coated from DCE solution. However, these fluorescence anisotropy maps and their anisotropy distribution plots show that some level of disorder remains within these films, indicated by the shoulder in the histograms to lower values of fluorescence anisotropy. The broad anisotropy distribution of the sample containing the highest level of PODPF β -conformation (thermal annealed at 300°C) reflects a heterogeneous environment comprising mixed amorphous and β -conformation regions (amorphous, crystalline and other phase) in the film.

3.2.3 Conclusion

In conclusion, we systematically investigated the photophysical and fluorescence anisotropic behavior of polyfluorene in amorphous and β -conformation states, prepared either as a function of solvent or annealing temperature, respectively. Variations in the level of β -conformational polyfluorenes influence the absorption and emission spectra, emission decay properties, morphology and fluorescence anisotropy of the film. The excitons of the β -conformation decay faster than those in the amorphous state. The fluorescence anisotropy of conjugated polymers can report on the level

of order within the film, which can be varied by controlling chain conformations via solvent or thermal methods. Such simple approaches will find broad applications in nanostructured, polymeric optoelectronic thin film systems.

Chapter 4

Time-Resolved Evanescent

Wave-Induced Fluorescence

Spectroscopy of POPC and POPG

Membranes using NBD-Labelled

Melittin

One increasing issue facing the treatment of infectious diseases is cell death by antimicrobial agents such as antibiotics and antivirals. The development of antimicrobial resistance, which is the ability of microorganisms to prevent cell death by these agents are so important. In order to probe the interactions between antimicrobial agents and membranes, one approach is to assemble a supported lipid bilayer on a solid substrate, and probe these interactions using a method that can provide a level of surface-specificity, and that is capable of detecting and probing conformational changes and dynamics. TREWIFS provides a convenient avenue to study these soft bilayer systems near an interface. For example, when soft bilayers interact with an interface their structure and/or morphology may change. As a result of this interaction, the penetration of solvent through the bilayers may vary.

Using fluorescent dye molecules tagged to an antimicrobial peptide it is possible to report these changes in terms of fluorescence lifetimes. To have micrometre or nanometre resolution different techniques of microscopy or nanoscopy are required. In this chapter a simple optical apparatus capable of probing within 100 nanometres of an interface has been used to investigate bilayers and the effect of peptides on these systems.

In this chapter, TREWIFS and EW-TRAMs methods have been utilised to study the interaction of melittin peptide with 1 - *hexadecanoyl* - 2 - (9*Z* - *octadecenoyl*) - *sn* - *glycero* - 3 - *phosphocholine* (POPC) and 1 - *hexadecanoyl* - 2 - (9*Z* *octadecenoyl*) - *sn* - *glycero* - 3 - *phospho* - (1' - *rac* - *glycerol*) (POPG) vesicles on a silica interface. The fluorescence decay of a dye attached to the peptide is carefully investigated using TCSPC. Different decay times have been observed during scanning the interface. These changes of lifetime even over just tens of nanometres reveals the accuracy and power of the method used in this chapter. In addition, these results demonstrate the ability of this method and apparatus to detect the change in morphology of peptide-LUVs during interaction with the interface.

The peptide was labelled at position lysis-7 with the NBD (7 - *nitrobenz* - 2 - *oxa* - 1,3 - *diazol* - 4 - *yl*) group. The fluorescence decay kinetics of all systems were found to be multiexponential with a short-lived component, τ_1 , interpreted as reporting on peptide-solvent interactions and longer-lived components, τ_2 and τ_3 , assigned as reflecting peptide-peptide interactions. The two vesicle types exhibited different fluorescence decay kinetics at the interface. Both lifetimes are sensitive to the microenvironment of the probe. The short-lived component, τ_1 , increased upon increased interactions with the surface in comparison with bulk solution in POPC vesicles. However, in POPC/POPG vesicles, the short-lived component decreased from the bulk to the evanescent wave region. The average fluorescence lifetimes, τ_M , of the studied systems decreased at the interface. EW-TRAM studies of POPC vesicles displayed hindered rotational motion of the probe both in-plane and out-of-plane of the interface.

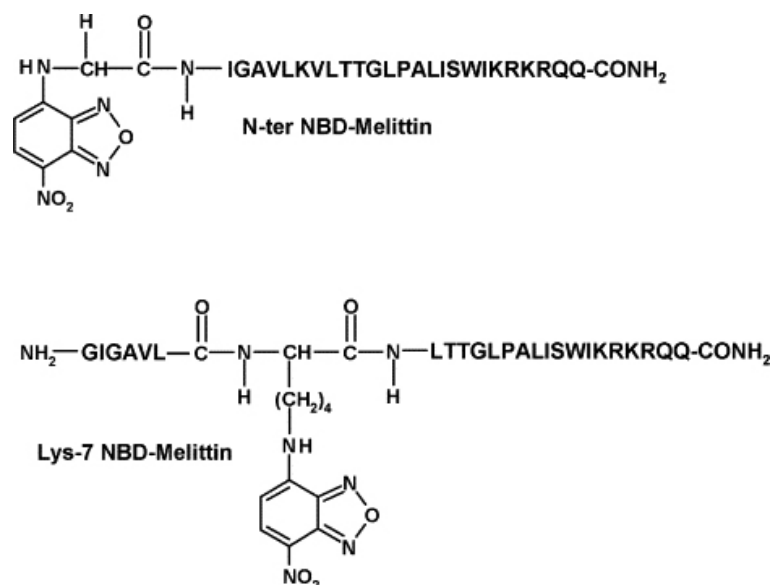


FIGURE 4.1: Chemical structures of NBD labelled Melittin in two different terminals: (N and Lys-7).

4.1 Introduction

Some of the well-studied antimicrobial peptides are short-chain, α -helical peptides such as melittin (161), magainins (162), protegrins (163), cecropins (164) and alame-thicin (165). Melittin is a cationic and hemolytic peptide (161, 166) extracted from the principal toxic component in the venom of the European honey bee *Apis – mellifera*. The antimicrobial peptides, include melittin (161, 167) have great applications in the treatment of tumours and cancers (168). This antimicrobial activity and application are linked to Melittin's linear composition of 26 amino acid residues:

$(NH_2-GIGAVLKVLTTGLPALISWIKRKRQQ-CONH_2)$ comprising predom-inantly hydrophobic (residues 1-20) and hydrophilic (residues 21-26) groups (161). Wa-ter solubility and membrane associations are also possible due to a sequence of amino acids, coupled with the amphiphilic nature of this peptide (169–173). These character-istics have led to the use of melittin as an appropriate peptide to study lipid–protein interactions in membranes. Employing high peptide/lipid ratios causes osmolysis, or the breakdown of the lipid bilayer into micelles, leading to the cytolytic activity of

melittin (173, 174). Melittin can be found in various orientations, positions and association status within membrane/lipid systems (166, 175). Several models have been proposed to describe the mechanism of these interactions (176). Although there is a significant body of literature on lipid-peptide interactions, there is no exact interpretation about characteristics that affect this phenomenon particularly when probed at an interface.

Several techniques such as solid-state nuclear magnetic resonance spectroscopy (177) x-ray diffraction (178) neutron diffraction (179) and oriented circular dichroism (OCD) spectroscopy (180, 181) have been exploited to probe lipid-peptide interactions to provide information about the mobility, location, orientation and conformation of melittin peptide and its association with the membranes. In addition, techniques such as fluorescence quenching, (182, 183) red-edge excitation shift spectroscopy (REES), (184) and time-resolved fluorescence anisotropy measurements (TRAMs) (185, 186) provide complementary information. In bulk solution systems, there are numerous lipid-peptide interactions which makes the study more complex. (187)

Spectroscopic techniques that use the total internal reflection phenomenon can be applied to investigate samples within close proximity to an interface and thereby provide a path by which semi-ordered systems such as supported bilayers can be investigated. Evanescent wave-induced fluorescence spectroscopy is one the techniques (188, 189) with some advantages over other methods. Evanescent waves created by total internal reflection can photo-excite surface-bound or semi-adsorbed fluorophores in close proximity (~ 100 nm) to the interface (71). Evanescent wave-induced fluorescence spectroscopy (EWIFS) and its time-resolved equivalent (TREWIFS) are surface sensitive methods that can provide precise information about the microenvironment and interfacial characteristics of many species present at the interface ranging from small dye molecules to macromolecules such as proteins, polymers, enzymes, and DNA (190). Fluorescence decay dynamics are sensitive to the polarity of the microenvironment and can be used to report on the range of environments within the evanescent

region (48, 191). TRAM performed under evanescent wave excitation can illustrate the extent and dynamics of emission depolarisation processes occurring both parallel and perpendicular to the plane of surface. This includes the mobility of fluorescent probes or fluorescently labelled species in both planes (56, 64, 192).

A widely used fluorophore in the biochemical and biophysical sciences, particularly studies of membranes, is NBD (193). NBD derivatives exhibit polarity spectral sensitivity in the visible range making them useful probes for a variety of spectroscopic and microscopic techniques (194–196). Their fluorescence decay characteristics are also sensitive to the environmental polarity (196). For this reason, NBD labelled lipids are in common use in time-resolved fluorescence spectroscopic studies (197).

In this chapter, TREWIFS and EW-TRAMs methods are applied to the study of lipid-peptide interactions. Two sets of membranes; POPC and (POPC 70%/POPG 30% - anionic), have been used as large unilamellar vesicles (LUVs). The emission from the environment-sensitive NBD group covalently labelled to the amino terminal Lys-7 of melittin is used to probe these interactions. Fluorescence-based studies of NBD-labelled melittin and membranes have been reviewed previously (78, 175). Fluorescence decay times of NBD-labelled melittin act as reporters of the conformation and dynamics of the peptide interacting with membranes of different lipids. We illustrate the presence and effect of anionic and zwitterionic LUVs on the fluorescence decay properties of the NBD group, and suggest that conformational change of the lipid-peptide system occurs at the interface. In addition, fluorescence anisotropy measurements have been carried out for these LUVs, in both bulk solution and within the evanescent region, to show the mobility and rotational diffusion of vesicles. These two sets of interpretations provide insight into lipid-peptide interaction and morphological changes at the interface.

Fluorescence decay characteristics of NBD exponential components analysis							
Penetration Depth	f_1	τ_1 (ns)	f_2	τ_2 (ns)	f_3	τ_3 (ns)	χ^2
60 nm	44.782	0.596	28.696	2.146	26.522	8.501	1.087
64 nm	38.731	0.446	41.692	1.343	19.577	6.348	1.142
75 nm	24.842	0.366	50.522	0.911	24.637	3.515	0.920
Bulk	7.527	0.238	65.187	1.152	27.286	2.084	1.082

TABLE 4.1: Detailed exponential components analysis (reconvolution) of the NBD-labelled melittin in buffer at interface

4.2 TREWIFS of NBD-labelled melittin in buffer

The evanescent field is a standing wave that decays in field strength exponentially with distance from the interface. As discussed in chapter 2 in equation 1.12 the penetration depth, Λ , is defined as the distance into the rarer medium that the field strength has decreased to half its value at the interface. In hydrophobic media such as a membrane, NBD has long a fluorescence lifetime, (198, 199) however, the lifetime of NBD is significantly reduced in the presence of water ($\sim 1\text{ns}$) (194, 195) and this is attributed to hydrogen bonding interactions between the fluorophore and the solvent (196) which leads to an increase in the rate of nonradiative decay (200). This feature of the NBD group makes it useful in numerous biological applications. For example, careful analysis of NBD fluorescence lifetimes revealed precise information relating to a mechanism of membrane insertion for cholesterol-dependent cytolysins (201).

The environmental and morphological sensitivity of NBD-labelled melittin in MOPS buffer, pH: 7.2 without any LUVs is illustrated by employing TREWIFS as a function of incident angle and therefore, Λ . The fluorescence decay profiles of this system are shown in Figures 4.2 and 4.3 along with the corresponding fits using both global and sums-of-exponential fitting analyses.

The fluorescence decay profiles are well described by tri-exponential decay functions and are noticeably sensitive to penetration depth. The tri-exponential behaviour may be interpreted in terms of the NBD fluorophores experiencing three dominant

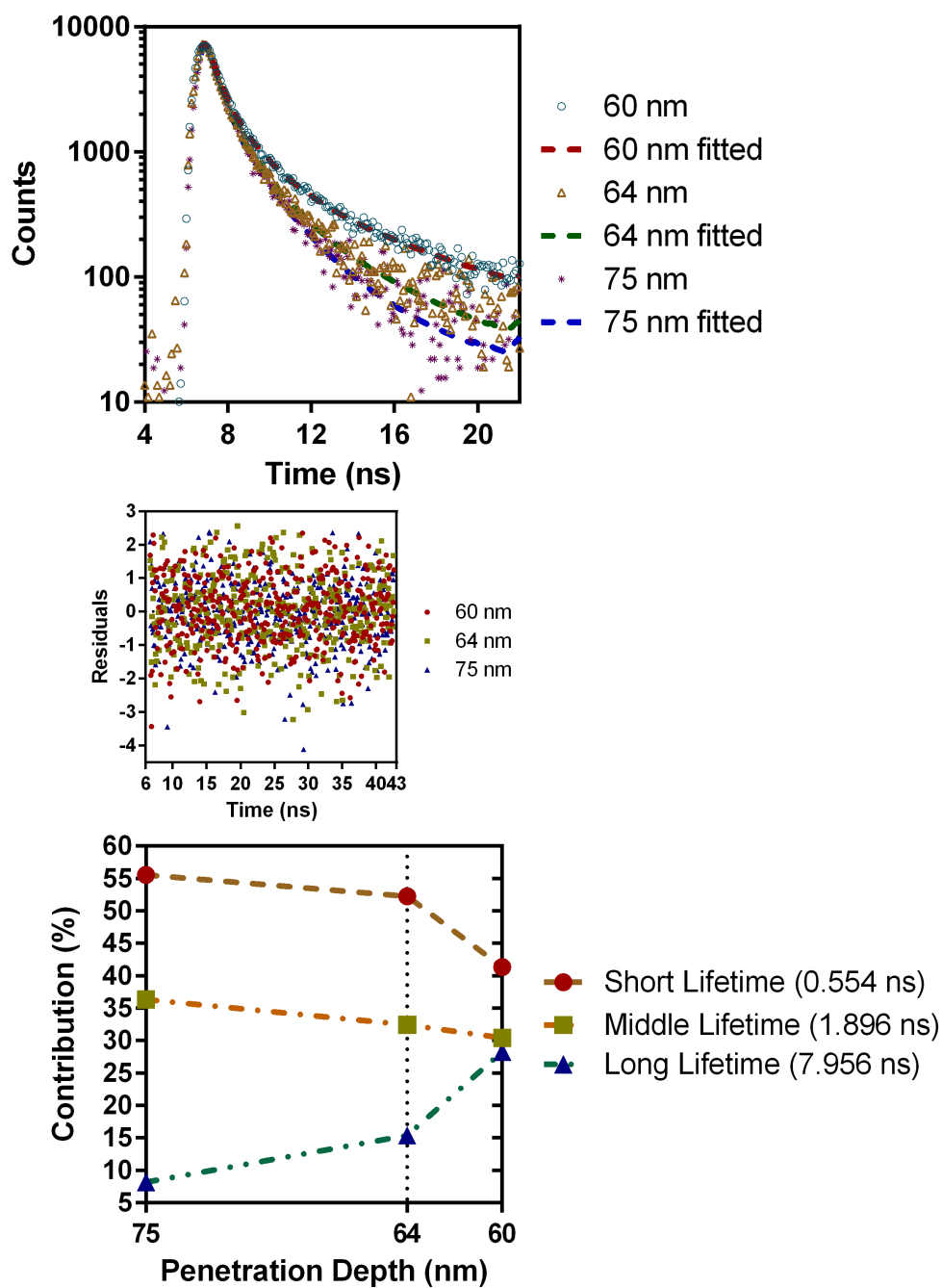


FIGURE 4.2: The fluorescence decay kinetics and global analysis of the NBD-labelled melittin in buffer at the silica interface (top). Penetration depth vs contribution percentage (Bottom) for short, middle and long lifetime components. The global lifetimes are 0.554 (ns), 1.896 (ns) and 7.956 (ns) and the global goodness of fits χ^2 is 1.075. The weighted residuals for the fits are also shown.

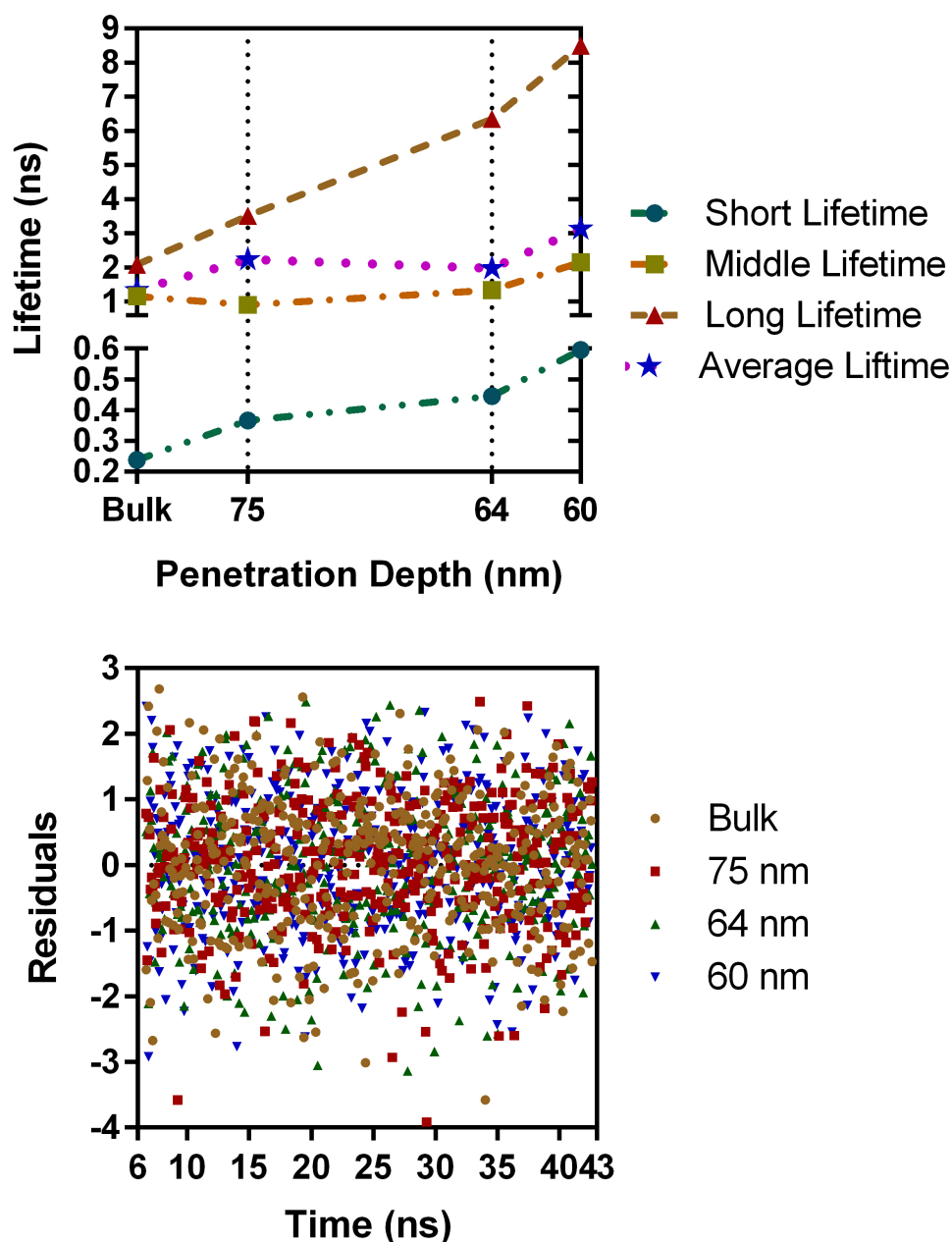


FIGURE 4.3: The fluorescence decay kinetics and exponential component analysis of the NBD-labelled melittin in buffer at interface. Lifetime vs penetration depth (top) for short, middle and long lifetime components. The goodness of fits are χ^2 1.082, 0.920, 1.142 and 1.087 for bulk and penetration depths of 75 (nm), 64 (nm) and 60 (nm) respectively. The weighted residuals for the fits are also shown.

Fluorescence decay characteristics of NBD Global Analysis (Reconvolution)								
Penetration Depth	f_1	τ_1 (ns)	f_2	τ_2 (ns)	f_3	τ_3 (ns)	χ^2	χ^2_{global}
60 nm	41.31	0.554	30.43	1.896	28.26	7.956	1.096	1.075
64 nm	52.21	linked	32.43	linked	15.36	linked	1.168	
75 nm	55.51		36.31		8.1733		0.960	

TABLE 4.2: The results of fitting of fluorescence decays as a function of penetration depth by a global analysis (reconvolution) of the NBD-labelled melittin in buffer at the silica/sample interface

environments, represented by τ_1 , τ_2 and τ_3 respectively, near the interface. All three lifetime values of the NBD label at the Lys-7 position along the peptide chain are reporters of the local environment experienced by the peptide. Any change of these lifetimes can be related to changes in the conformation of the peptide. The percentage contribution of the short component in the global analysis reduces from 55.51% to 41.31% as the penetration depth is reduced and the long component increases from 8.17% to 28.26% as the penetration depth is reduced. Under this interpretation, this suggests that within penetration depths of 64-60 nm, peptide chains are close to each other because of the presence of the surface and it seems that the peptide chains experience more interaction compared to when probed further into the solution phase (e.g. penetration depth $\sim$ 75 nm). It seems that close to the interface there is a colony of peptides which interact with others and solvent cannot penetrate significantly to that region. On the other hand, far from the interface solvent can penetrate between peptides and as result a reduction of lifetime is observed. Although the exact nature of each lifetime component is difficult to define here, we speculate that the short component is due to peptide-solvent interactions and respectively, the middle and longer components relate to peptide-peptide interactions. Far from the interface the peptide chains are more dispersed and solvated as seen by the contribution of the short lifetime (figure 4.2). Close to the interface, the middle and longer lifetimes, and therefore peptide-peptide interactions, become more dominant than further from the interface. In addition, the electrostatic charge interactions between the cationic peptide and the

anionic silica surface might be expected to enhance this. This interaction causes free peptides to be retained close to the silica surface, in other words, the density of peptides is higher at the interface. Fluorophores can experience different environments further from the interface compared to close to the interface and hence, their lifetimes can vary. Close to interface (e.g. penetration depth ~ 60 nm), all three lifetimes of the dye have higher values rather than the solution phase (e.g. penetration depth ~ 75 nm) in figure 4.3. The long lifetime from the exponential component analysis of NBD increases from 2.084 (ns) to 8.501 (ns) as the penetration depth is reduced. The detailed fluorescence decay characteristics (lifetimes) of NBD global analysis and exponential components analysis (Reconvolution) are illustrated in tables 4.1 and 4.2. Both global analysis and exponential component analysis are well aligned to interpret that peptides interactions is high at shallower penetration depths.

4.3 TREWIFS of NBD-labelled melittin attached to POPC and POPC/POPG LUVs

The TREWIFS technique was also used to evaluate the fluorescence decay kinetics for NBD-labelled melittin incorporated into POPC and POPC/POPG LUVs. The fluorescence decay profiles were measured while buffer was present at the surface of the silica hemi-cylindrical prism. The fluorescence decay profiles recorded at each penetration depth were acquired by changing the incidence angle of the laser excitation source (see figure 2.6, in chapter 2, and equation 1.12). Both the radiative and non-radiative rate constants of a fluorophore can be influenced by the presence of a dielectric medium such as interface. Adsorption of a fluorophore to an interface is commonly accompanied by a decrease or increase in the fluorescence quantum yield. In the literature, there are some reports of fluorophores such as malachite green (202)

which have a very low fluorescence quantum yield in bulk aqueous solution but exhibit significantly enhanced emission when adsorbed at an aqueous/quartz interface). Conversely, tetramethyl rhodamine (203) demonstrates almost complete quenching of its emission when adsorbed to a silica surface. The changes in NBD emission characterised in the previous section, suggest that variations in the fluorescence decay behaviour of this fluorophore can be used to extract information concerning the local environment that the probe experiences.

The fluorescence decay profiles of NBD-melittin attached to POPC or POPC/POPG are tri-exponential as observed previously for NBD-melittin in buffer without LUVs (figure 4.2 & 4.3). However, the relative contributions of these components are changed dramatically when the labelled melittin interacts with LUVs (figure 4.4). The fluorescence decay kinetics and global analysis of the NBD-labelled melittin in POPC and POPC/POPG LUVs are depicted in figure 4.4. There is a jump for all vesicles from bulk solution to the evanescent region in the contribution of the components following global analysis. The contribution of the short lifetime component for both LUVs reduces from bulk to EW region. These changes are interpreted in terms of interactions between the LUVs and the surface causing a new nano-environment to be experienced by dye. For a penetration depth between 75 nm to 64 nm there is not too much variation and the nano-environment appears to be quite homogenous.

The fluorescence decay kinetics and exponential component analysis of the NBD-labelled melittin incorporated with POPC and POPC/POPG LUVs at the interface are illustrated in figure 4.5. As previously observed in the global analysis, there is a considerable change in the lifetimes in going from bulk solution to the evanescent region (penetration depth 75 nm). The short emission decay time of the POPC/POPG vesicles lower than the bulk at large penetration depth (~ 75 nm) but increases as the penetration depth is reduced. The intermediate and long lifetime components are essentially invariant with penetration depth ~ 75 nm to 60 nm like global analysis in figure 4.5. However, there is a small rise for lifetimes close to the interface from (e.g.

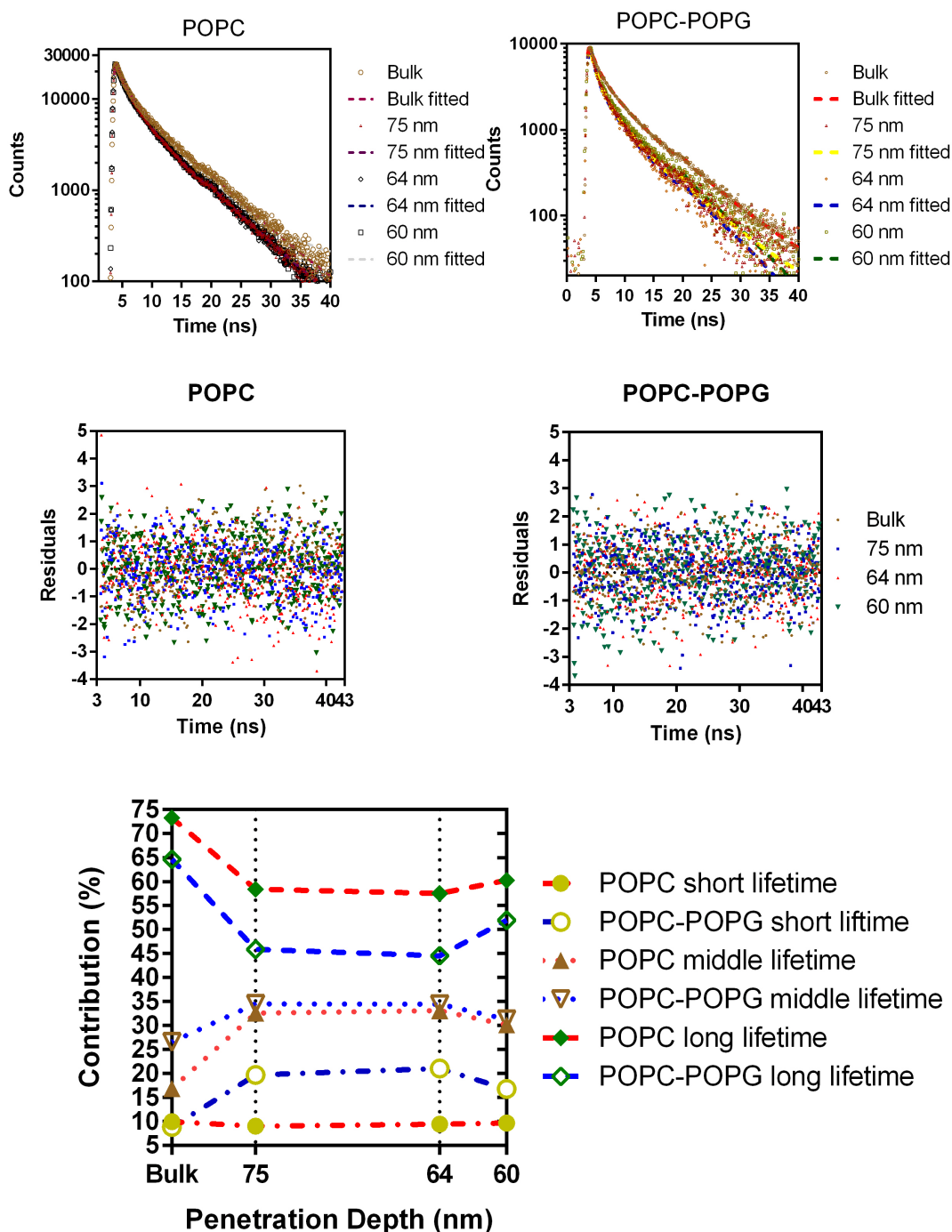


FIGURE 4.4: The fluorescence decay kinetics and global analysis of the NBD-labelled melittin interacting with POPC (top left) and POPC/POPG (top right) LUVs. Penetration depth vs contribution percentage (bottom) and residuals of fits POPC/POPG LUVs (middle left) and POPC LUVs (middle right) for short, middle and long lifetime components. The global lifetimes of POPC LUVs are 0.729 (ns), 2.796 (ns) and 7.495 (ns) and the global χ^2 for this type of vesicles is 1.144. The global lifetimes of POPC/POPG LUVs are 0.635 (ns), 2.470 (ns) and 7.665 (ns) and the global χ^2 for this type is 1.062.

Fluorescence decay characteristics of POPC Global Analysis (Reconvolution)								
Penetration Depth	f_1	τ_1 (ns)	f_2	τ_2 (ns)	f_3	τ_3 (ns)	χ^2	χ^2_{global}
60 nm	9.637	0.729	30.10	2.796	60.26	7.495	1.113	1.144
64 nm	9.393	linked	33.10	linked	57.51	linked	1.222	
75 nm	9.022		32.59		58.39		1.055	
Bulk	9.921		16.81		73.26		1.188	
POPC/POPG Global Analysis (Reconvolution)								
60 nm	16.78	0.635	31.27	2.470	51.95	7.660	1.214	1.062
64 nm	20.99	linked	34.45	linked	44.55	linked	1.113	
75 nm	19.65		34.47		45.87		0.941	
Bulk	8.801		26.56		64.63		0.980	

TABLE 4.3: Detailed global analysis (reconvolution) of the NBD-labelled melittin in POPC (top) and POPC/POPG (bottom) LUVs at interface

penetration depth ~ 60 nm) to ~ 64 nm depth.

The average lifetimes from the exponential component analysis of the NBD-labelled melittin in buffer and also interacting with POPC and POPC/POPG LUVs at the interface as a function of penetration depth are illustrated in figure 4.6. The average lifetime of both LUVs decreases as the penetration depth is reduced. However, the average lifetime of NBD labelled melittin in buffer increases as the penetration depth is reduced. The average lifetime of NBD in bulk solution is 1.33 ns and 1.41 ns in buffer. This suggests that the nano-environment for the bulk solution is very similar to that at a penetration depth close to ~ 75 nm. In contrast, close to the interface (e.g. penetration depth ~ 64 nm to 60 nm) the nano-environment experienced by the fluorescent probe is somewhat different in comparison with far from the surface (penetration depth ~ 75 nm). The decrease in the average fluorescence lifetimes at the interface may be partly due to the high refractive index. The radiative rate constant is a function of the refractive index according to the Strickler-Berg formula, such that an increasing refractive index increases the radiative rate constant, and consequently shortens the fluorescence lifetime. Other effects near an interface can also influence the fluorescence lifetime of

POPC Exponential Components Analysis (Reconvolution)							
Penetration Depth	f_1	τ_1 (ns)	f_2	τ_2 (ns)	f_3	τ_3 (ns)	χ^2
60 nm	44.782	0.569	28.696	2.146	26.522	8.501	1.087
64 nm	38.731	0.446	41.692	1.343	19.577	6.348	1.142
75 nm	24.842	0.366	50.522	0.911	24.637	3.515	0.920
Bulk	7.527	0.238	65.187	1.152	27.286	2.084	1.082
POPC/POPG Exponential Components Analysis (Reconvolution)							
60 nm	18.306	0.709	31.206	2.564	50.488	7.846	1.165
64 nm	21.995	0.644	30.672	2.434	47.333	6.992	1.110
75 nm	18.779	0.603	39.007	2.641	42.214	8.769	0.942
Bulk	10.332	0.702	31.413	2.954	58.255	8.326	1.006

TABLE 4.4: Detailed exponential component analysis (reconvolution) of the NBD-labelled melittin in POPC (top) and POPC/POPG (bottom) LUVs at interface

chromophores - energy and/or electron transfer between the substrate and the chromophore are well known to affect these. According to global and exponential component analyses and the results of average lifetimes, three dominant environments are apparent. These environments can be classified as bulk, far from the interface (e.g. penetration depth ~ 75 nm to 64 nm) and close to the interface (e.g. penetration depth ~ 64 nm to 60 nm). The detailed fluorescence decay characteristics (lifetimes) of NBD in POPC or POPC/POPG LUVs Global Analysis and Exponential Components Analysis (Reconvolution) are summarised in table 4.3.

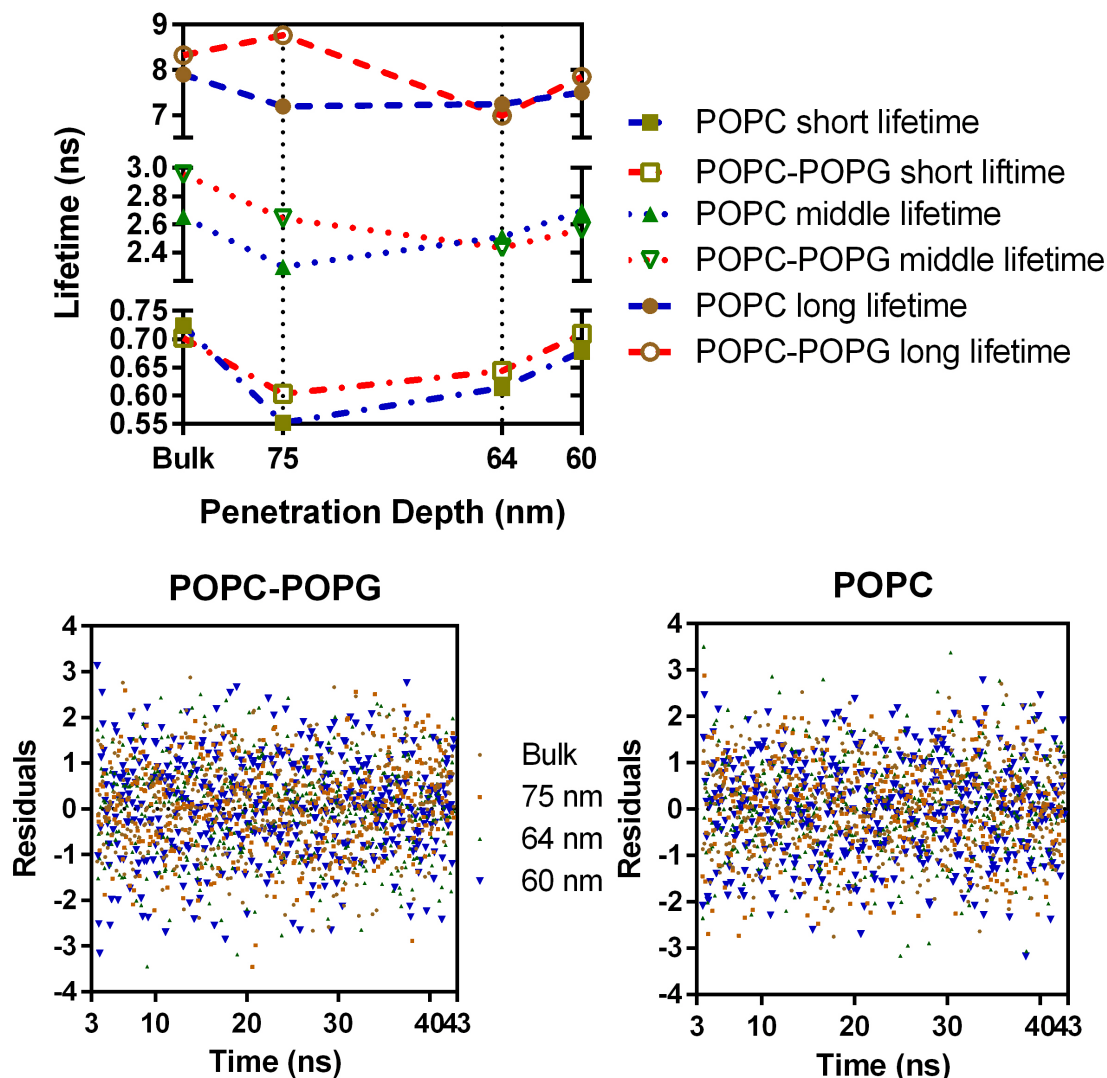


FIGURE 4.5: The fluorescence decay kinetics and exponential component analysis of the NBD-labelled melittin interacting with POPC and POPC/POPG LUVs at the interface. Lifetime vs penetration depth (top) and residuals of fits POPC/POPG LUVs (bottom-left) and POPC LUVs (bottom-right) for short, middle and long lifetime components. The goodness of fits χ^2 for POPC/POPG vesicles are 1.006, 0.942, 1.110 and 1.165 for bulk and penetration depths of 75 (nm), 64 (nm) and 60 (nm) respectively. The goodness of fits χ^2 for POPC vesicles are 1.013, 1.004, 1.066 and 1.100 for bulk and penetration depths of 75 (nm), 64 (nm) and 60 (nm) respectively.

Average lifetime VS Penetration Depth

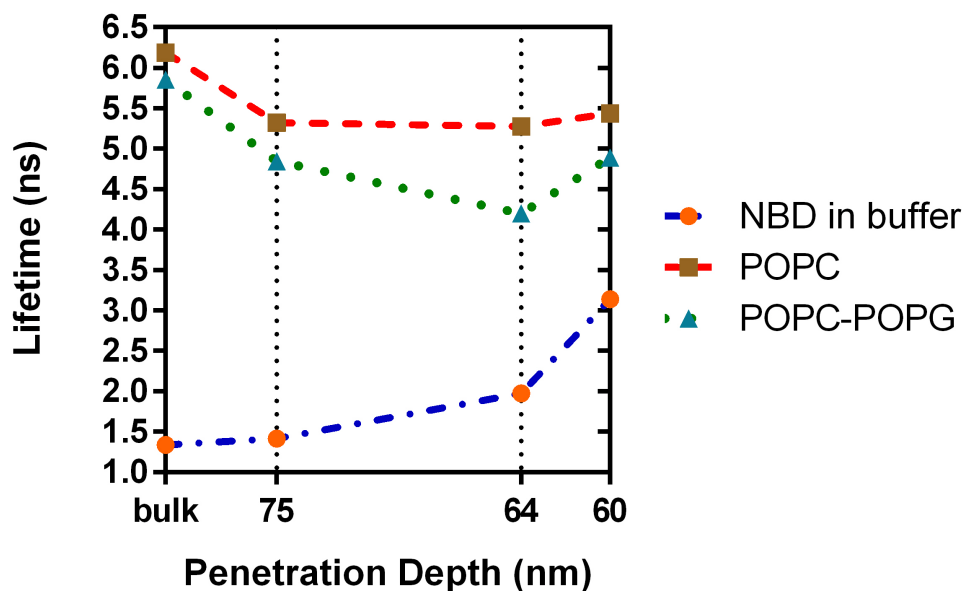


FIGURE 4.6: The average fluorescence lifetimes resulting from an exponential component analysis of the NBD-labelled melittin in buffer and also attached to POPC and POPC/POPG LUVs at the interface as a function of penetration depth.

4.4 TRAM of NBD-labelled melittin attached to POPC

LUVs

EW-TRAMs (56, 64, 192) have the unique ability to probe and differentiate fluorescence depolarisation processes occurring in planes parallel and perpendicular to that of the interface, and in the current case, provide information about a fluorescence probe, such as NBD, labelled to melittin. Time resolved fluorescence depolarisation kinetics of NBD-labelled melittin attached to POPC vesicles under bulk and evanescent wave conditions with a penetration depth of 75 nm is depicted in figure 4.7. It is necessary to distinguish between EW-TRAM and regular TRAM approaches. TRAM studies of LUV-peptides provide information on all orientations of the absorbing chromophore but EW-TRAMs studies are surface sensitive and the anisotropy of the system shows preferentiality for the two distinct planes as discussed in chapter 2.

Here, the data have been fit using an "associative" anisotropy model in which each fluorescence decay time is linked to a specific rotational component. As previously observed, three fluorescence lifetimes, τ_1 , τ_2 and τ_3 , are required to adequately describe the time-resolved emission of the NBD-labelled melittin interacting with the POPC vesicles when in the presence of the surface or under bulk solution conditions (figure 4.2 & 4.3). These lifetimes were different in the bulk compared to the evanescent wave region. In the associative model, the anisotropy decay can be represented by Equation 4.1 :

$$r(t) = \sum_x^n r_{0x} \beta_x e^{\frac{-t}{\tau_{cx}}} + r_\infty \quad (4.1)$$

In this system we assume three fluorescence lifetimes and three modes of rotational motion. In this scenario equation 4.1 can be written as:

$$r(t) = r_{01} \beta_1 e^{\frac{-t}{\tau_{c1}}} + r_{02} \beta_2 e^{\frac{-t}{\tau_{c2}}} + r_{03} \beta_3 e^{\frac{-t}{\tau_{c3}}} + r_\infty \quad (4.2)$$

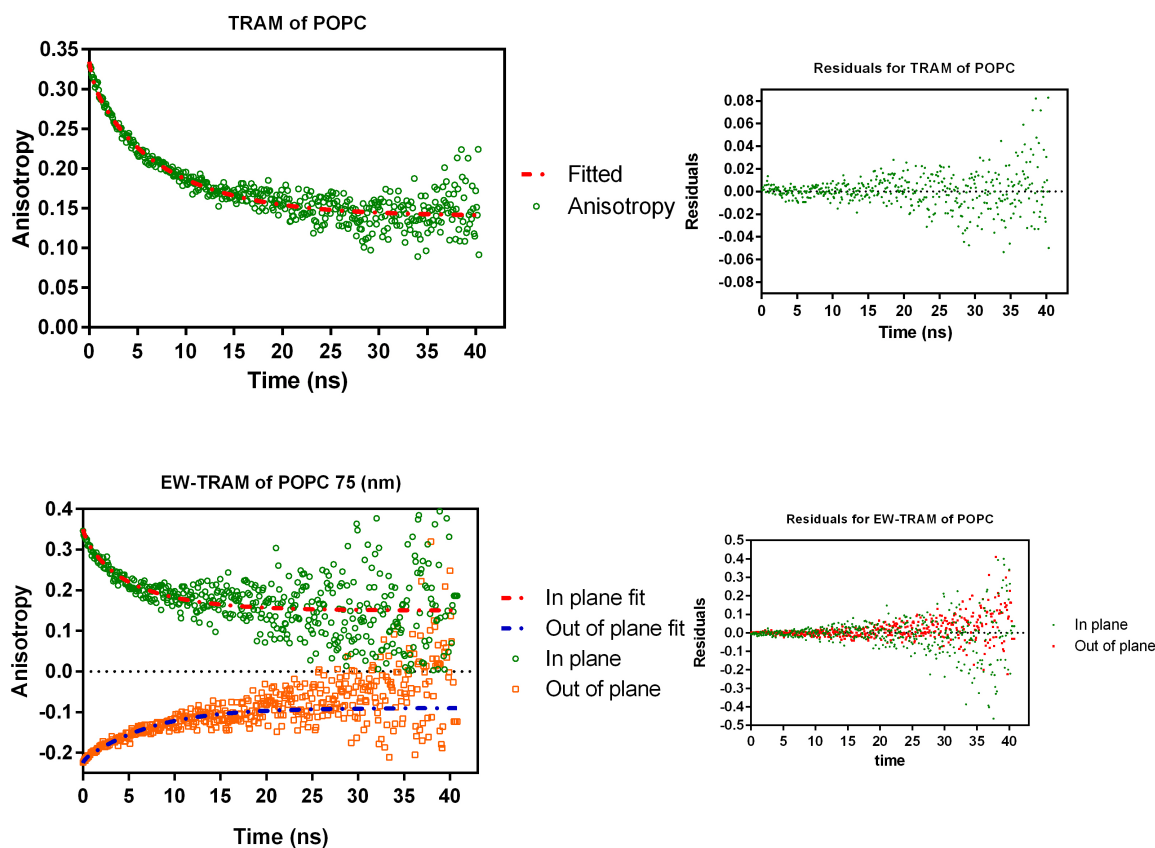


FIGURE 4.7: Time resolved fluorescence anisotropy of NBD-labelled melittin interacting with POPC vesicles for bulk conditions (top-left) and the corresponding residuals (top-right), evanescent wave excitation with a penetration depth of 75 nm (bottom-left) and the corresponding residuals (bottom-right). EW-TRAM data in the bottom panels were obtained for both parallel (in-plane) and perpendicular (out-of-plane) of the interface of the hemi-cylindrical prism. The solid lines inside the dotted curves are the fits obtained using the above equations.

where r_{0x} is the anisotropy at time $t=0$ and ϕ_{cx} is the rotational correlation time, of fluorophore j in microdomain x with fluorescence lifetime τ_j . r_∞ accounts for hindered rotation as in the nonassociative model. β_x is the time-dependent fractional contribution of fluorophores in microdomain x with lifetime τ_{jx} to the net anisotropy:

$$\beta_x(t) = \frac{\sum_{j=1}^n \alpha_{xj} e^{\frac{-t}{\tau_{jx}}}}{\sum_{x=1}^n \sum_{j=1}^n \alpha_{xj} e^{\frac{-t}{\tau_{jx}}}} \quad (4.3)$$

where α_{xj} is the amplitude of the contribution of fluorescence lifetime of fluorophore j in microdomain x .

Many studies of the fluorescence anisotropy of peptide's motion in LUVs have been reported (185, 186, 204, 205). Typical reported rotational correlation times are about 0.5-5 ns according to the location of the attachment to the peptide helix and the size of the LUV. The emission depolarisation process in the bulk solution differs from the evanescent wave case. They have the same limiting anisotropy of ~ 0.15 although their rotational correlation times are different. The rotational correlation times acquired from the tri-exponential and associative fits for TRAM of NBD-labelled melittin attached to POPC vesicles in bulk solution (red line in figure 4.7 top) are 0.8 ns, 6 ns and 8.5 ns. The rotational correlation times for this system from global analysis (blue-red lines in figure 4.7 bottom) corresponding to processes occurring in and out of the plane of the interface are 0.6 ns, 4 ns and 6.5 ns. NBD-labelled melittin in the presence of POPC under bulk conditions exhibits a longer rotational correlation time for both components compared to near the interface. As discussed previously, the short fluorescence decay time decreases in going from the bulk case to evanescent wave conditions, as shown in figure 4.5 top. A related change is also observable in the rotational correlation times. These results suggest that the short lived fluorescence decay component, which reports peptide-solvent interactions, is quite sensitive to the presence of the interface. It is related to the surface of the vesicles where the hydrophilic part of LUVs is in contact

with the prism interface. It seems that this short component reports surface interaction. The longer rotational correlation time component is also variable. These changes support the morphological modification and buffer penetration into the LUVs during interaction with the interface.

4.5 Conclusion

TREWIFS and EW-TRAMs have been shown capable of producing information about LUVs-peptide interactions by observation of fluorescence lifetimes and depolarisation of a fluorescence probe interacting with a peptide chain. These non-destructive methods can be utilised on a wide variety of sensitive samples such as vesicles without any concerns such as photo-bleaching. They are also able to provide nanometre resolution in depth profiling of systems. We found that the fluorescence lifetime behaviour is sensitive to the microenvironment (e.g. solvent) the fluorophore experiences and can be utilized as a reporter as a function of penetration depths from an interface. Rotational correlation times are also sensitive to the solvent micro-environment and can be used to describe the interior motion on vesicles in both bulk solution or within the evanescent region.

Chapter 5

Mapping the Fluorescence Anisotropy in Biological Systems

In chapter 3 it was demonstrated that the fluorescence anisotropy in polymeric systems can be mapped on microscopic levels, providing detail regarding variability of emission depolarising processes, specifically energy migration. This method can be utilized in investigations of other sample types such as complex biological systems. In this chapter the fluorescence depolarisation behaviour, attributed to rotational motion, of two different biological systems is mapped. These studies include macromolecular crowding and actin-cytoskeleton rearrangements by cholesterol lowering mechanisms in cells. The FAIM method is used for mapping the emission anisotropy and the FLIM method is used as a complementary technique to provide detail on the local environment of the fluorophore. The combined FLIM and FAIM imaging methods is a powerful methodology to observe the changes in the morphologies of biological systems. Restriction of the intramolecular motion due to the crowding effect can be detected by an increase in the emission anisotropy in the depolarisation map. Real time monitoring of cytoplasmic crowding effects is also possible in live cells with the same method. FAIM will be applied to TIRFM to map the fluorescence anisotropy with nanometre depth resolution in thin films of CHO-K1 cells. F-actin polymerisation is observed due to the effect of high doses of Lovastatin drug treatment.

5.1 Measuring macromolecular crowding in cells through fluorescence anisotropy imaging

In this part of this chapter, spatiotemporal visualization is employed to explore the macromolecular crowding effect in cells. An amine-reactive, aggregation-induced emission fluorogen is used as "molecular rotor" to label proteins in the cytoplasm and the change in the protein mobility as well as local viscosity can be monitored by using fluorescence anisotropy imaging and fluorescence lifetime imaging, respectively.

5.1.1 Introduction

Cells are densely packed with bio-macromolecules, in particular proteins (206, 207). The effective concentration of total proteins inside the cell is estimated to be in the range of 200–300 gL^{-1} (207). At such high concentrations, proteins, as well as other biopolymers (e.g. RNA), occupy a significant portion of the volume in the interior of the cells, resulting in a crowded environment.

The macromolecular crowding, also known as the excluded volume effect, influences the diffusion rates, protein conformation, protein folding and aggregation, catalytic efficiency of enzymes, intracellular signalling, and many other intracellular processes (208–213). The crowding effect has been routinely studied in vitro with the addition of artificial macromolecular crowding agents such as polyethylene glycol, Ficoll 70, and dextran in the solution (207, 214). However, whether such efforts can reflect the physiological conditions in cells remains inconclusive (214, 215). Tools that can measure the crowding effect, especially in the heterogeneous and dynamic environment in the cellular milieu, are thus highly desirable.

The dynamics of the crowding effect in a cell is often presumed from the diffusion coefficient of a protein which can be estimated using fluorescence recovery after photobleaching (FRAP) or fluorescence correlation spectroscopy (FCS) techniques (216–218).

These techniques are useful for measuring the dynamic processes of intracellular proteins on the millisecond or longer time scale. The diffusion process is not only affected by the excluded volume but also the viscosity and the interaction with other species in the cellular environment (219). More recently, a genetically encoded protein sensor and a microinjected polymer sensor, both based on fluorescence resonance energy transfer (FRET), have been reported to quantify the crowding effect with spatiotemporal resolution in cells (215, 220). Changes induced on the cell's environment can cause the protein or the polymer to fold more compactly and thereby increase the crowding forces, which can result in an increase in the FRET efficiency between the donor and the acceptor labelled on each end of the sensor. Small molecule probes, on the other hand, are usually cell permeable, easy to handle, and thus applicable in a broad range of biological systems (221). However, no small molecule has yet been reported for measuring the macromolecular crowding environment in cells.

As discussed in chapter 2 fluorescence anisotropy or polarisation reports on the rotational motion of the entire dye molecule within the excited-state lifetime (57). Fluorescence anisotropy provides information on the mobility and orientation of the molecule and has been used to investigate protein–protein interactions, ligand–receptor binding processes, membrane mobility, and protein fibril formation (57, 222, 223). In this part of this chapter, the use of FAIM to map the fluorescence anisotropy in cells and monitor their change upon the increase of the macromolecular crowding effect will be discussed.

An amine-reactive dye, TPE-Py-NCS, was chosen as a type of "molecular rotor" to label cytosolic proteins (Fig. 5.1). The isothiocyanate (NCS) moiety can react with amine groups and thus covalently label the proteins under physiological conditions (83). Tetraphenylethene (TPE) is well-known AIEgen. In TPE, four phenyl rings are linked to a central ethene rod through single bonds. These phenyl rings have great freedom to rotate or twist against the ethene stator. The isolated TPE molecules in

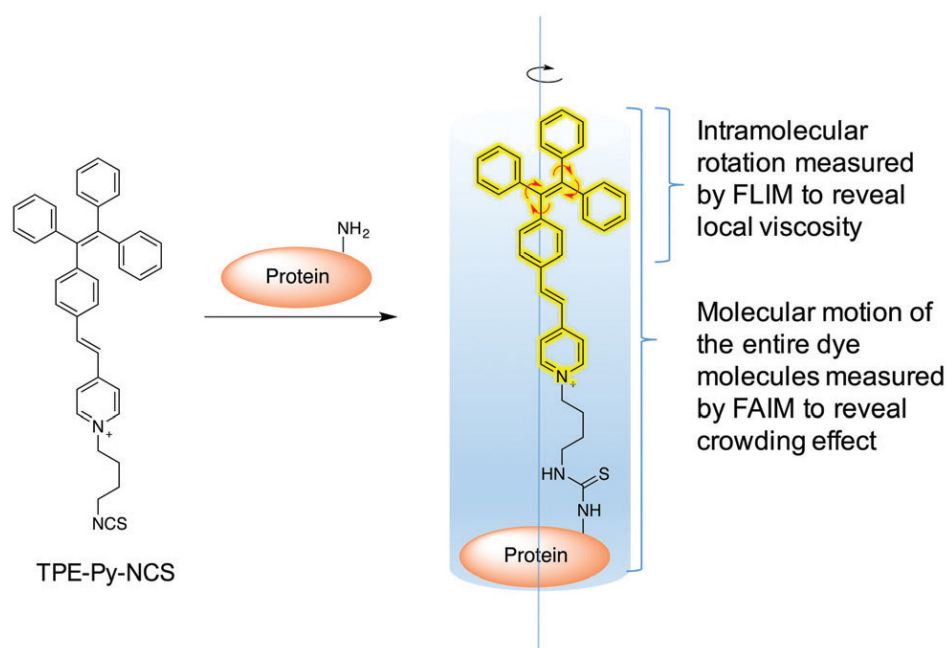


FIGURE 5.1: Design principle of using an amine-reactive dye TPE-Py-NCS to label proteins and monitor the micro-environment change in cells through FLIM and FAIM.

a dilute solution can undergo active intramolecular rotations, which serves as a relaxation channel for the excited states to non radiatively decay to the ground state. In aggregate state, the intramolecular rotations are restricted due to the involved physical constraint, which blocks the radiationless relaxation channel and opens the radiative decay pathway. A series of control experiments have been reported in literature (224, 225) to externally and internally modulate the intramolecular rotations. External control experiments such as increasing solvent viscosity, decreasing solution temperature, and pressurising solid films. TPE emits more efficiently in more viscous solvent, at lower temperature, and under higher pressure, which verifies that the restriction of intramolecular rotation process is indeed a cause for the AIE effect here. In addition, theoretical calculations and simulations on the low-frequency intramolecular motions also support the restriction of intramolecular rotation mechanism (226, 227).

The change in the crowding condition would impact the rotational freedom of the

dye molecules as a whole detected by changes in the fluorescence anisotropy. A consequence of the tetraphenylethene (TPE) core is that TPE-Py-NCS possesses aggregation-induced emission (AIE) properties due to the non-radiative energy loss mechanisms provided by the rotating phenyl groups (83). The phenyl rings on the propeller shaped TPE core can undergo intramolecular rotational motions that reduce fluorescence efficiency through non-radiative deactivation of the excited state (228–231). Such intramolecular motions are highly dependent on the viscosity of the environment and steric constraints. The fluorescence lifetime of such molecules has also been demonstrated to be sensitive to the change in intracellular viscosity proportionally to the emission quantum yield (232). Therefore, changes in intracellular viscosity under crowding conditions can be monitored effectively via FLIM.

5.1.2 FAIM of Osmotic Stress on Cytoplasmic Crowding in Cells

The extensive protein network labelled by TPE-Py-NCS was used as the sensor to quantitatively measure the macromolecular crowding effect in cells induced by the change in osmotic pressure. Fluorescence anisotropy imaging was performed under a wide-field fluorescence microscope with a polarisation splitter to facilitate the simultaneous collection of both the horizontally and vertically polarised emission light from each sample spot on the same CCD camera. This apparatus described in figure 2.7 (233).

Because the dye does not enter the nucleus, a threshold was set to remove signals from the unstained area before the intrinsic background noise of the detector was subtracted from the whole image. The polarised images before and after background subtraction are illustrated in figures 5.2 and 5.3. The pseudo-red colour indicates regions with larger fluorescence anisotropy (low molecular rotational motion) while the pseudo-blue colour indicates low anisotropy regions corresponding to more rotational

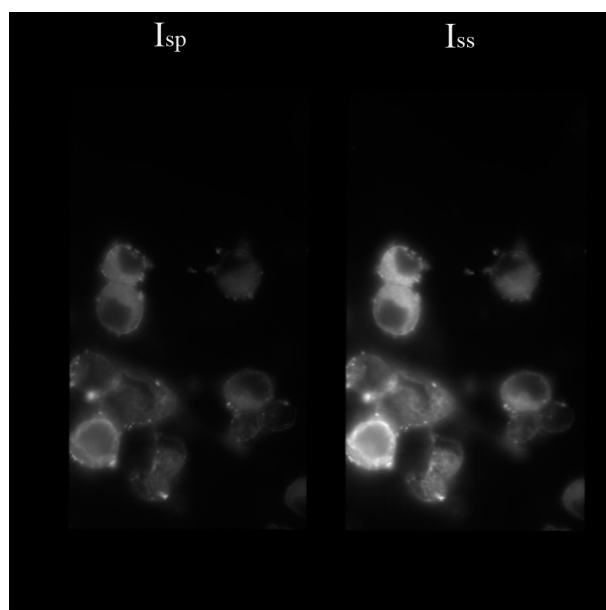


FIGURE 5.2: The original intensity images of cells divided into the separate polarisations via a polarisation beam splitter and acquired by an EM-CCD camera (see apparatus schematic 2.7).

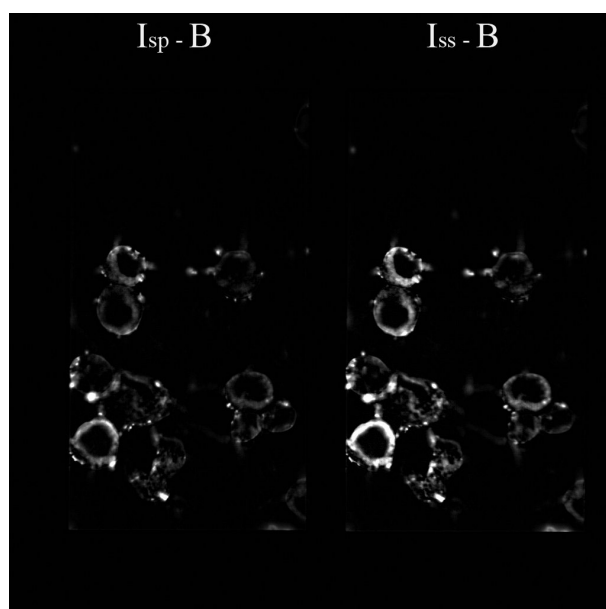


FIGURE 5.3: The polarised images after background subtraction (using ImageJ) from the original intensity image (figure 5.2).

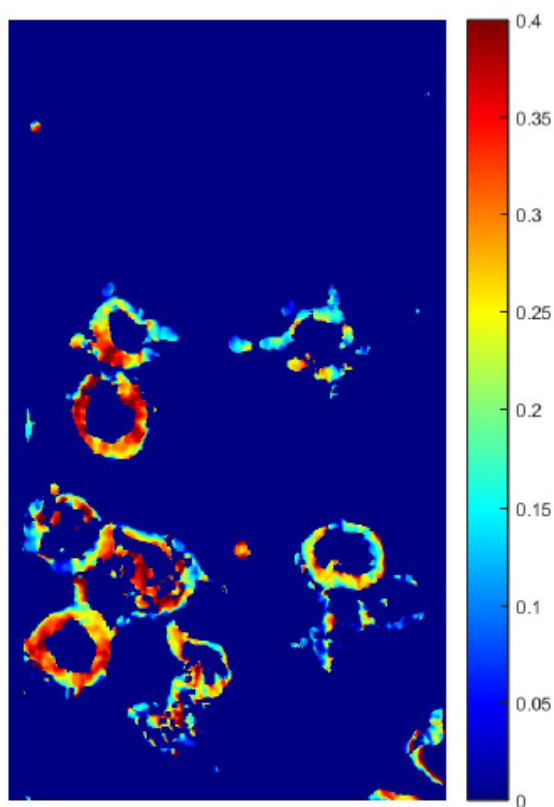


FIGURE 5.4: The fluorescence anisotropy map corresponding to figure 5.3 calculated using Equation 2.10 via MatLab code (available in appendix 1).

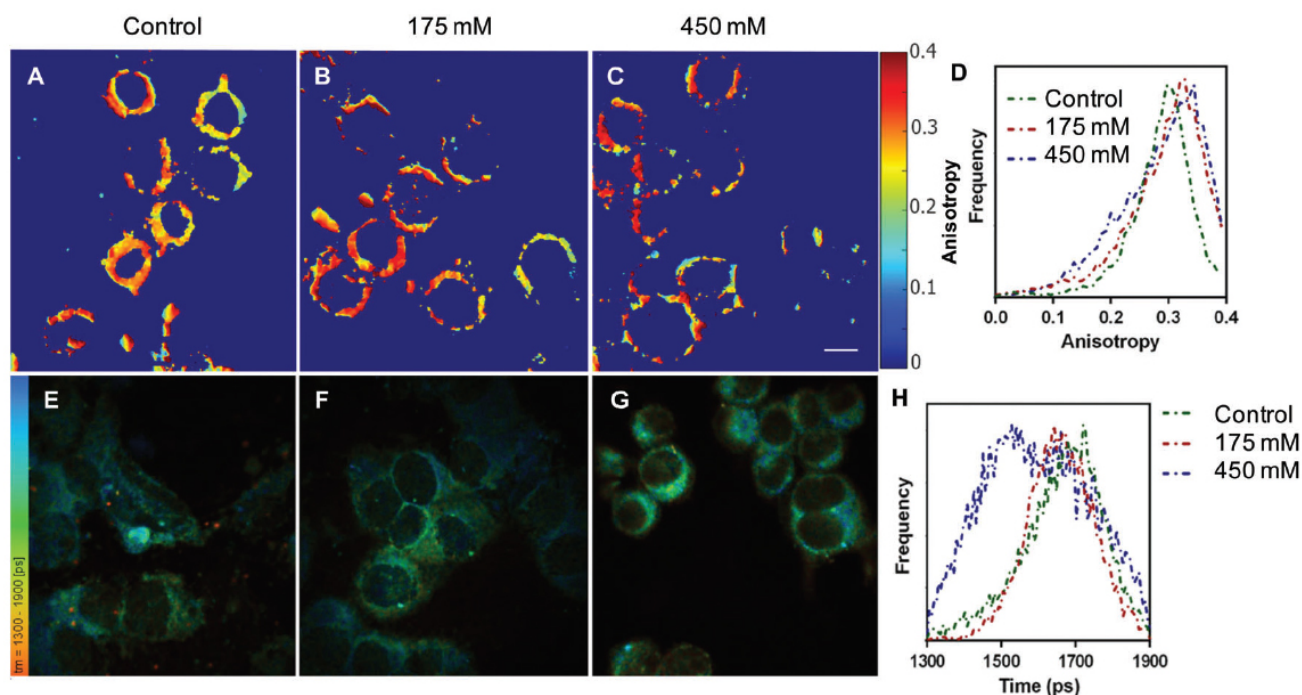


FIGURE 5.5: Effect of osmotic stress on cytoplasmic crowding in fixed cells. (A–C) FAIM images and (E–G) FLIM images of fixed Neuro 2A cells treated with sorbitol at different concentrations for 30 min. (D) Fluorescence anisotropy distribution histograms from panels A–C. (H) Fluorescence lifetime distribution histograms from panels E–G. Control refers to cells without sorbitol treatment. [TPE-Py-NCS] = 10 mmol. Scale bar: 5 micro metre.

freedom of the fluorophore molecules as a whole (figure 5.4). The large blue areas are zeroed using the threshold level.

Sorbitol is a sugar alcohol with a sweet taste which the human body metabolizes slowly. It can be obtained by reduction of glucose, which changes the aldehyde group to a hydroxyl group. Sorbitol was used as an osmotic stressor to increase the crowding effect in cells (220, 234). Figure 5.5(A–C) shows fluorescence anisotropy maps from Neuro-2A cells without and with the treatment of increasing concentration of sorbitol. The cells were first stained with the TPE-Py-NCS dye and treated with different concentrations of sorbitol for 30 minutes before fixation. The distributions of the anisotropy value collected from each pixel in the images are shown in figure 5.5(D)

for comparison. The broad distribution of the anisotropy of TPE-Py-NCS reflects the heterogeneous environment in cells. With sorbitol treatment, the anisotropy distribution became broader with larger populations exhibiting higher anisotropy values. The anisotropy value corresponding to the largest population changes from approximately 0.29 for the control to 0.35 after the cells were exposed to 450 mM sorbitol. An increasing population with lower anisotropy (0.1–0.2) was also observed with the increase in sorbitol concentration. This observation suggests that the dye molecules labelled on the proteins experienced at least two distinct environments upon the osmotic upshift.

To gain more information on the micro-environment the dye molecules experience, time-domain FLIM was used to monitor the changes in the emission decay behaviour of the fluorophore under the same treatment (see figure. 5.5 (E-G)). To focus on the intracellular region, the extracellular unlabelled areas were masked and the fluorescence decay of the fluorophore from the cell interior was analysed with the variation ranging from 1.3 to 1.9 ns (Figure. 5.5 (H)). The average fluorescence lifetime of the majority of the TPE-Py-NCS molecules from the untreated cells was around 1.67 ns. The lifetime was slightly shorter (~ 1.65 ns) when the cells were exposed to 175 mmol sorbitol. A large population exhibiting a noticeably shorter average fluorescence lifetime ~ 1.5 ns emerged when the sorbitol concentration was increased to 450 mmol, while the distribution of the longer-lived species remained at ~ 1.66 ns. The constant lifetime of the long-lived species indicated that the local viscosity of the protein network did not change significantly under osmotic stress. The emergence of the short-lived population with 450 mmol sorbitol, which was consistent with the FAIM data, implies that further increasing the osmotic pressure causes some of the dye molecules to become more loosely bound to the protein network.

To further examine the impact of osmotic stress on the macromolecular crowding effect, we performed in situ measurements of the live cells upon sorbitol treatment. 450 mmol sorbitol was added to the TPE-Py-NCS labelled Neuro 2A cells and FAIM images were recorded at different time intervals (figure. 5.6 (A-E)). The first image

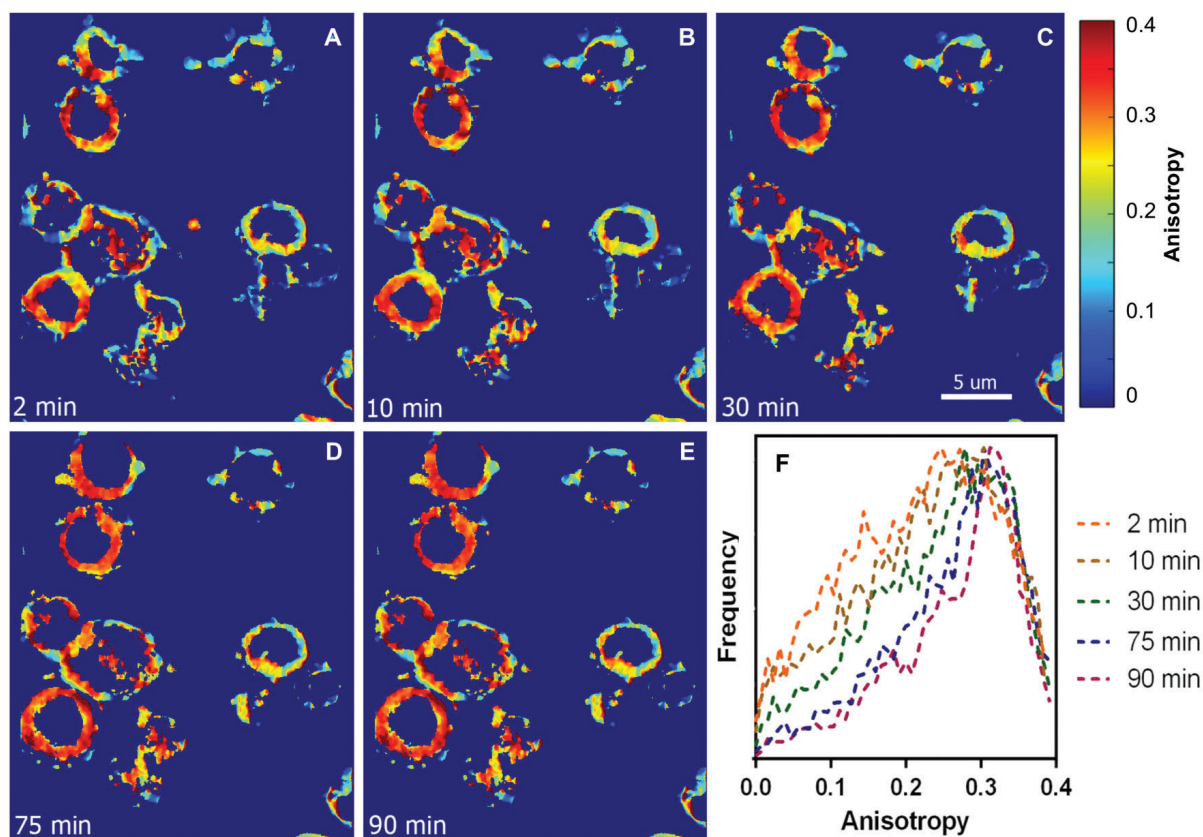


FIGURE 5.6: Real time monitoring of cytoplasmic crowding effect in live cells. (A–E) FAIM images of TPE-Py-NCS stained Neuro 2A cells taken at different time points after being exposed to 450 mmol sorbitol. (F) Fluorescence anisotropy distribution histograms from panels A–E. [TPE-Py-NCS] = 10 mmol. Scale bar: 5 micro metre. The corresponding total intensity of the anisotropy maps are depicted in figure 5.7.

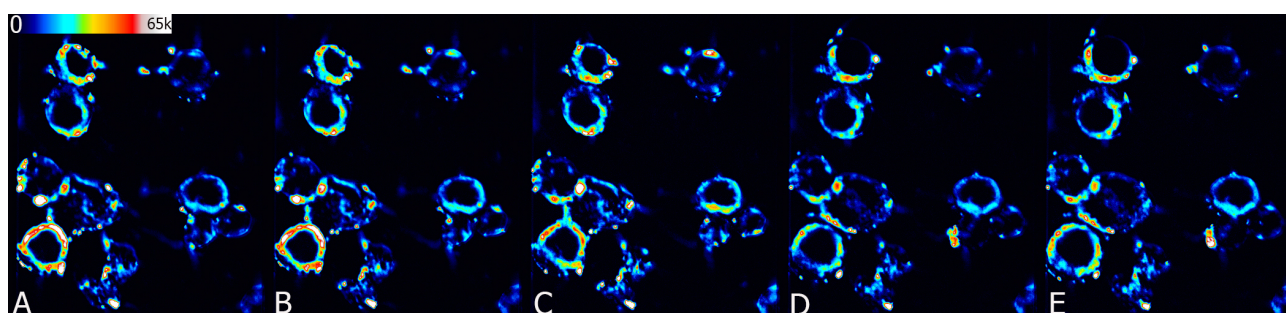


FIGURE 5.7: Real time viscosity monitoring of cytoplasmic crowding effect in live cells. (A–E) intensity images of TPE-Py-NCS stained Neuro 2A cells taken at different time points after being exposed to 450 mmol sorbitol. [TPE-Py-NCS] = 10 mmol.

was captured 2 min after sorbitol incubation. It is clear, the images based on the polarised fluorescence intensity, that the morphology of the protein network, as well as that of the plasma membrane, change significantly on timescales longer than 30 min, after which the plasma membrane started swelling and was separated from the labelled protein network. Meanwhile, the nucleus membrane also swelled but no obvious change can be observed from the labelled protein network. The anisotropy of these labelled regions in the cytoplasm increased during the incubation with sorbitol, as seen from the expanded pseudo red colour region in the images (figure.5.6 (A–E)). The histogram clearly displays the increase in anisotropy and the decrease in the population with low anisotropy during the incubation process (figure. 5.6 (F)). Real time viscosity monitoring of the cytoplasmic crowding effect in live cells is also possible using the total intensity of the fluorescent probe (see figure 5.7). As discussed before, the emission intensity of AIEs is sensitive to the viscosity of the medium and they usually exhibit high intensity in more viscous mediums.

The exposure to an osmotic stressor induces changes in membrane morphology and thus alters the cell volume (235). However, how such changes impact the intracellular environment remains unclear. The combination of different techniques provides comprehensive information to interpret the intracellular environment.

In this part of this chapter, a TPE-Py-NCS dye based on a "molecular rotor" motif was used to label the cytoplasmic protein network as the sensor to detect the changes induced by the macromolecular crowding effect through the use of FAIM, FLIM, and conventional fluorescence imaging. These results show that treatment with sorbitol at sub-molar concentrations indeed enhanced the crowding effect in cells, as indicated by the upshift of the anisotropy distribution in cells. Increasing the sorbitol concentration forces the proteins to pack more tightly and thus leads to less free space for the entire dye molecules labelled on proteins to rotate. Such circumstances can alter the viscosity of the microenvironment, as the lifetime of the long-lived emission of the dye changed, although not dramatically. A portion of dye molecules was observed, perhaps on the

surface of the dense protein network, that experienced more rotational freedom when the cells were exposed to 450 mmol sorbitol. This can be attributed to the change in cell volume caused by the swelling of the plasma membrane under such treatment.

5.1.3 Conclusion

In this section a new method based on fluorescence anisotropy imaging of the cells stained with suitable dyes was used to measure the macromolecular crowding effect in live cells. The incorporation of the dyes that undergo restriction of their intramolecular motions of the aryl groups in the molecule also allows us to map the intracellular viscosity under the same conditions. Further studies using these techniques to assess the intracellular environment under disease conditions are ongoing in our laboratory. Such a simple approach will find broad application not only in cell imaging but also in nanostructured, polymeric, and biomaterial thin film systems.

5.2 Actin Cytoskeletal Rearrangement by Cholesterol Lowering Statin Drugs

5.2.1 Introduction

Cholesterol is a crucial constituent of eukaryotic cell membranes and a dominant lipid component of mammalian cells (236, 237). Cholesterol has an important effect on extensive membrane characteristics including permeability, elasticity (238) stiffness, fluidity (239, 240) and molecular transport processes (241, 242). The cytoskeleton, which is a complex network of interlinking filaments and tubules that extend throughout the cytoplasm, from the nucleus to the plasma membrane, plays an important role in these processes. It is necessary to address the question of how cholesterol participates in the interactions between the membrane and the cytoskeleton. Although the function of the actin cytoskeleton in cellular processes such as trafficking (243) and motility (244) has been widely studied, reorganisation of the actin cytoskeleton upon modulation of membrane cholesterol needs further investigation. It has been shown that cholesterol depletion can cause distortion of cells in a study by Byfield et al. by evaluation of membrane deformation length (245). Membrane proteins settle in environments with high cholesterol concentration, which need actin anchors to continue their correct operation, according to a study by Lillemeier et al. (246).

The actin cytoskeleton controls the operation of lipid rafts (247) and these localized lipid raft proteins can control the cytoskeleton (248). During this complex process, rafts keep the cell polarity, (249) and alignment of cell shape and size constant (250). Kwik et al. (251) is demonstrated that cholesterol depletion in the membrane can decrease protein mobility which was caused by changes in the actin network in the cytoskeleton, but beyond the membrane, the whole cell shape globally can be modified in light of actin change.

The effect of cholesterol-lowering drugs, such as Lovastatin, on cells and diseases

is still an active area of investigation (252, 253). The statins class of drugs is one of the best selling globally and in clinical history. Statin drugs such as simvastatin, pravastatin and Lovastatin are extensively applied as medical treatments for stroke, hypercholesterolemia and coronary artery diseases (254, 255). Beyond heart diseases, statin is suitable in many other illnesses such as multiple sclerosis and macular degeneration (256, 257). Lovastatin competitively inhibits HMG-CoA reductase (3-hydroxy-3-methyl-glutaryl-coenzyme), a reductase, and blocks the conversion of HMG-CoA to mevalonate and the precursors of cholesterol and other isoprenoids (258, 259). Polyisoprenylation is required for membrane association and proper functioning of Rho GTPases that are involved in actin cytoskeleton organization and dynamics (260, 261).

The most popular phallotoxin of the *Amanita phalloides* mushroom is pallacidin (262). Wieland et al. (262, 263) has shown that pallacidin is appropriate to visualize cellular actin and specifically binding to F-actin and G-actin oligomers. (264) Due to this and the visible properties of the NBD fluorophore, NBD-pallacidin is widely used to stain plant and animal cellular actin for emission studies (263).

Fluorescence microscopy is an effective technique to investigate molecular interactions which is fundamental for cellular functions (265, 266). FLIM has many applications in cell biology such as environmental sensing. Because of the non-invasive aspect of this method, it is popular (267, 268). Compared to the other non-invasive methods, FLIM provides information about environments that fluorophores experience immediately without cell manipulation.

TIRFM is applied on various sub-research areas such as chemistry, bio-chemistry and cell biology, (71, 269) and more specifically for applications such as granule tracking, (270) cell-substrate contact and interactions, (188, 271) single-molecule fluorescence (272) and the study of membranes (273). TIRFM is also able to be combined with numerous modes of study such as polarisation, (58, 274, 275) fluorescence resonance energy transfer FRET (276) and time-resolved emission studies (67, 277).

As discussed in chapter 3, FAIM is a novel technique that combines anisotropy

measurements with imaging. One of the applications of FAIM is for the study of protein interactions in cells (278, 279). There are surprisingly few fluorescence anisotropy studies on TIRFM condition (280, 281). Fluorescence anisotropy measurements at an interface are useful to study fluorescence depolarising process of molecules. This approach enables selective excitation of fluorophores that have a component of their absorption transition moment aligned with the polarisation of the exciting electric field. Subsequently, the fluorescence emission from these fluorophores is polarised to an extent dependent on the angular displacement between the absorbance and emission transition moments within the molecule. Studying the steady state nature of this fluorescence depolarisation provides a wealth of information about the orientation of the molecules in the biological systems.

In order to monitor the changes in the actin cytoskeleton upon cholesterol depletion in a quantitative manner, steady state fluorescence anisotropy imaging has been used here. Anisotropy measurements using the polarised TIRFM system described above are able to report on microenvironments and F-actin polyisoprenylation dynamics at an interface. High resolution anisotropy maps can be generated by manipulating polarised emission images. Such maps can provide information about the micro-rigidity of filaments in the presence of statin drugs.

In this section the role of membrane cholesterol in the regulation of linear polymer microfilaments (F-actin) in Chinese Hamster Ovary (CHO) cells is investigated and comparisons are made between the emission anisotropy maps of different concentrations of statin, interrogating behaviour in and out-of-the plane of interface. In addition to fluorescence anisotropy imaging, FLIM images can provide emission decay time distributions of NBD-pallacidin at various concentrations of statin drug, which in conjunction with FAIM results, may provide clear understanding of polyisoprenylation (polymerisation) of actin cytoskeleton.

5.2.2 Fluorescence Lifetime Imaging Microscopy (FLIM)

In order to monitor the role of membrane cholesterol on the underlying actin cytoskeleton, the CHO-K1 cells were treated with Lovastatin, a commonly used statin which lowers cholesterol content by inhibiting HMG-CoA reductase. The HMG-CoA reductase is the key enzyme in cholesterol biosynthesis that catalyzes the rate-limiting step in the biosynthetic pathway. Figure 5.8 shows the effect of increasing concentrations of Lovastatin on the organisation of the actin cytoskeleton in CHO-K1 cells. Figure 5.8 shows scanning confocal time-resolved fluorescence images of the actin cytoskeleton (stained with NBD-phalloidin) of CHO-K1 cells. The photon intensity images (red: low, blue: high) and corresponding mean lifetime maps are depicted as color coded images on a scale of 800 - 4000 ps. Treatment of CHO-K1 cells with Lovastatin results in polymerisation of F-actin, and the consequent increase of F-actin filaments and formation of F-actin fibre can be observed under these conditions (see Figure 5.8, panels A-C). The fixed cells without Lovastatin in panel A largely maintain their original shape after the addition of 5 μmol Lovastatin. Highly oriented F-actin fibres are observed in cells treated with 10 μmol Lovastatin (see figure 5.8C).

The fluorescence lifetime of the fluorophore used is sensitive to the environment it experiences (282). The fluorescence lifetime distribution image can provide locational and environmental information about cellular structure (283). The sensitivity of the fluorescence lifetime of NBD-phalloidin fluorophore in CHO-K1 cells treated by Lovastatin is explored by time-resolved fluorescence imaging. The spacial lifetime distribution of the fluorophore, color-coded for emission lifetime on a scale of 800 - 4000 ps, is depicted in figure 5.8. These lifetime maps suggest that the fluorophore experiences a partially homogeneous environment inside the cell when bound to the actin cytoskeleton. Although, the actin cytoskeleton undergoes significant structural changes, the lifetime distribution monitored utilizing NBD-phalloidin does not indicate substantial change.

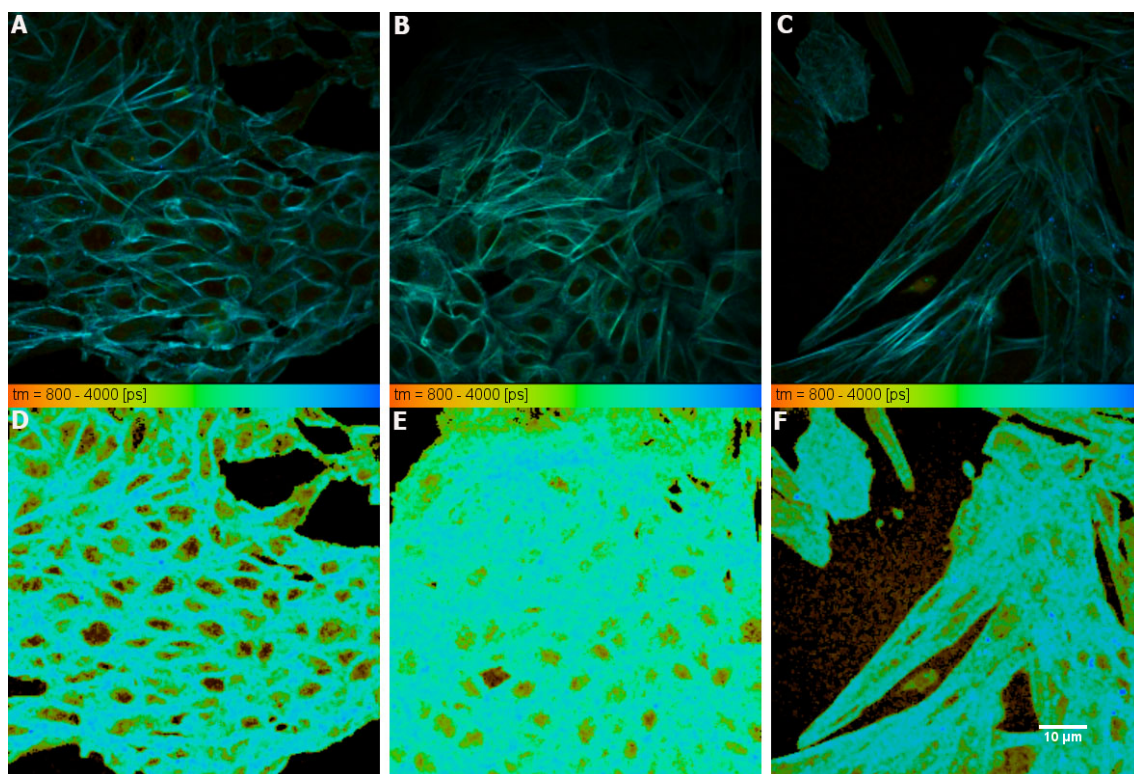


FIGURE 5.8: Organization of the actin cytoskeleton in fixed CHO-K1 cells treated with increasing concentrations of Lovastatin. The photon intensity images depicted in A,B and C panels (red: low, blue: high) and mean life-time maps depicted in D,E and F panels are shown as color coded images on a scale of 800 - 4000 ps. The actin cytoskeleton was stained with NBD-pallacidin. Panels A & D show representative images for control cells, and panels B & E and C & F show images for cells treated with 5 μmol and 10 μmol Lovastatin, respectively. Increase of F-actin filaments can be observed upon treatment with increasing concentrations of Lovastatin.

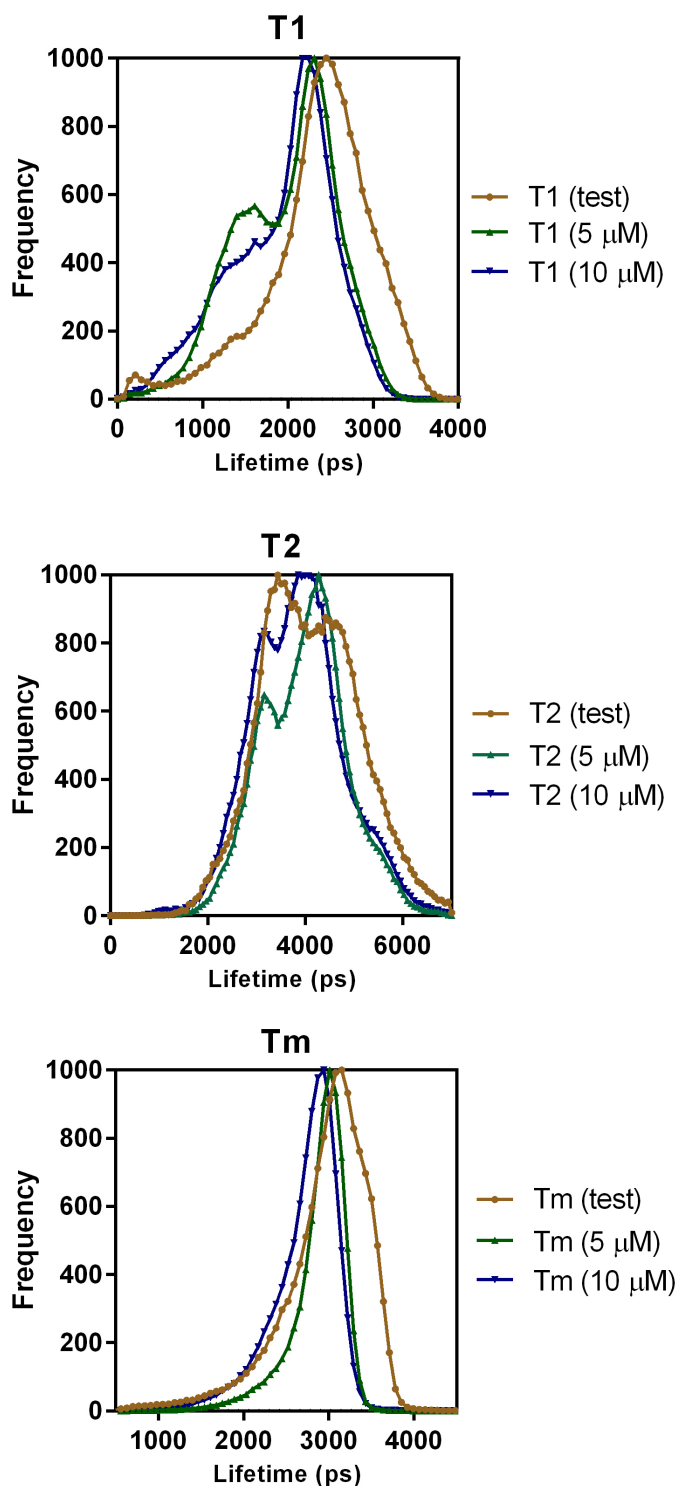


FIGURE 5.9: Fluorescence lifetime distributions of control CHO-K1 cells (brown) and Lovastatin treated cells (green: 5 μ mol and blue: 10 μ mol) for short lifetime T_1 (top), longer lifetime T_2 (middle) and mean lifetime T_m (bottom). Highly oriented F-actin filaments in cells (in the presence of 10 μ mol Lovastatin) exhibit shorter mean lifetime when compared to control cells with partially oriented filaments. Each curve is an average of three random spots of each condition and normalized to 1000.

The lifetime changes during F-actin fibre formation as a consequence of different concentrations of Lovastatin, as illustrated by the lifetime distributions of several samples (see figure 5.9). NBD-pallacidin excited with a 440 nm laser has two fluorescence decay components namely, T_1 , the short lifetime and T_2 , the longer lifetime. T_m is the mean lifetime calculated from the values of T_1 and T_2 and their fitting amplitudes. The distribution of the T_1 in control cells (without any Lovastatin treatment) has higher values in comparison with Lovastatin-treated cells (compare T_1 in control image and T_1 (5 μmol), T_1 (10 μmol) in figure 5.9 top). The mode of the T_1 distribution for the control cells is 2450 ps. Lovastatin treated cells have lower modes of their respective distributions at 2310 ps and 2170 ps for T_1 (5 μmol) and T_1 (10 μmol), respectively. The distribution of the T_2 component (figure 5.9 middle), shows two peaks (control) in the distribution and the shape of this distribution is clearly different to those of the Lovastatin treated cells. The T_2 (5 μmol) peak shifted about 840 ps and T_2 (10 μmol) peak shifted about 420 ps from the T_2 (test) towards longer decay times. The distributions of the mean lifetimes have peaks at 3150 ps for T_m (control), 3010 ps for T_m (5 μmol) and 2940 ps for T_m (10 μmol). These lifetime shifts of the Lovastatin treated cells from control cells are due to alteration of the actin cytoskeleton environment in close proximity to the fluorophore. A decrease in mean lifetime was observed upon higher polymerisation of the F-actin cytoskeleton. These shifts are not drastic, however, they correlate as concentration to Lovastatin in cells.

5.2.3 FAIM Results

Recently, FAIM has gained some popularity for some investigations in cell imaging (278, 279). In the literature there are a limited number of reports using FAIM coupled with TIRF microscopy (280, 281). Applying imaging to fluorescence anisotropy measurements can provide more information and detail than intensity-based images

alone. This approach provides higher spatial resolution about the anisotropy of microenvironments. Study of the photophysical properties and surface fluctuations are also feasible using polarisation imaging measurements. The combination of polarised-TIRFM with anisotropy measurements has a great potential to provide information on processes occurring within tens of nanometres of an interface and with micrometre lateral resolution.

The F-actin polymerisation as consequence of Lovastatin treatment in CHO-K1 cells was depicted in figure 5.8. Fluorescence lifetime imaging microscopy can provide information about the lifetime distribution of the fluorophore NBD-phalloidin, however, in this case the lifetimes did not change significantly as a function of Lovastatin concentration. In this situation using another method to study polymerisation can provide complementary information. In addition, F-actin polymerisation in close proximity to the cell membrane would be best studied by a more surface specific method such as TIRFM.

Figure 5.10 shows steady state total internal reflection fluorescence anisotropy images of the actin cytoskeleton (stained with NBD-phalloidin) of CHO-K1 cells treated by 5 μmol Lovastatin. The polarised TIRFM image using parallel polarisers, I_{ss} , is depicted in figure 5.10 A. The corresponding in-plane anisotropy map calculated using equation 2.8 is illustrated in figure 5.10 B. The out- of-plane anisotropy calculated using equation 2.9 is depicted in figure 5.10 C. As previously observed in figure 5.8 B, the cytoskeleton of the CHO-K1 cells treated by 5 μmol Lovastatin have some order of F-actin polymerisation similar to what is seen from the steady state total internal reflection fluorescence anisotropy imaging of the same concentration (figure 5.10 A). The anisotropy image of the thin layer of the film of actin cytoskeleton in close proximity to the membrane excited by evanescent waves strongly depends on the spatial morphology and distribution of the fluorophore at the interface. Linear regions of high intensity, assumed to be polymer sequences of F-actin formed as a result of statin treatment (figure 5.10A), have high NBD concentration and show a high fluorescence intensity.

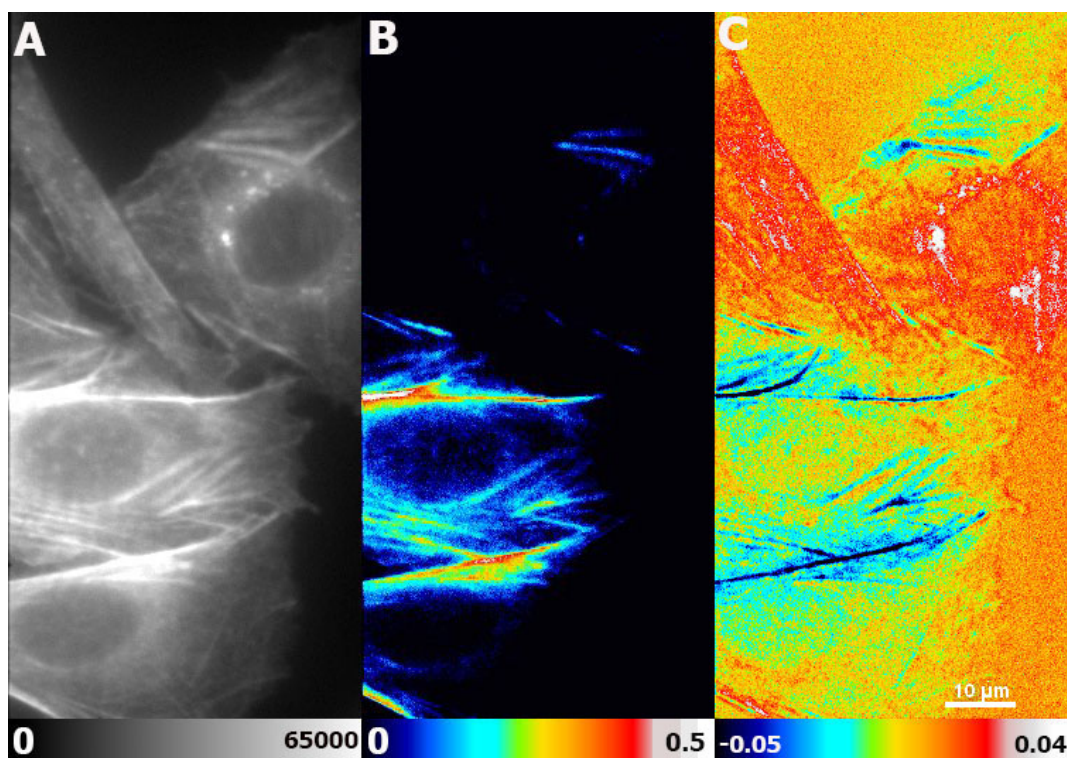


FIGURE 5.10: Steady state total internal reflection fluorescence anisotropy imaging (blue: low, red: high) shown as color coded images of the actin cytoskeleton (stained with NBD-pallacidin) of fixed CHO-K1 cells excited by 465 nm CW laser and treated by 5 μmol Lovastatin drug. Polarized TIRFM image using parallel polarisers I_{ss} (A), in plane of interface anisotropy map (B) and out-of-plane anisotropy map (C).

In addition, they have considerable values in their corresponding anisotropy images; white and red spots for in-plane signal (figure 5.10 B) and blue spots for out-of-plane signal (figure 5.10 C). F-actin polymers experience a high order of rigidity indicated by the limiting values for the anisotropy of 0.4 for in plane and -0.05 for out-of-plane signals.

To provide a clear observation of the changes induced during F-actin polymer formation in consequence of different concentrations of Lovastatin (5 μmol and 10 μmol), steady state, total internal reflection anisotropy measurements of the in-plane and out-of-plane anisotropy were performed. The resultant histograms calculated from several samples have been analysed and are reported in figure 5.11. The in-plane anisotropy (calculated via equation 2.8) for control cells has values that range between 0 and 0.5

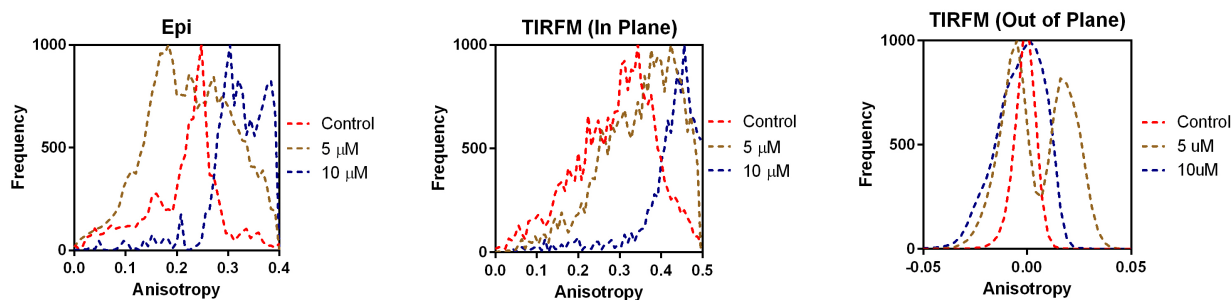


FIGURE 5.11: The histogram distributions of the steady state fluorescence anisotropy imaging of control CHO-K1 cells (red lines) and Lovastatin treated cells (brown lines: 5 μmol concentration and blue lines: 10 μmol concentration) for bulk (left), in-plane of interface (middle) and out-of-plane of interface (right). F-actin cytoskeleton polymerisation correlates with the anisotropy histogram distribution. Highly oriented F-actin fibres in cells including 10 μmol Lovastatin treatment experience higher values of bulk and in-plane anisotropy and lower values of out-of-plane anisotropy rather than partially oriented F-actin fibres in cells that include 5 μmol Lovastatin concentration.

with a peak at 0.35. The values for cells treated with 5 μmol and 10 μmol Lovastatin are also between 0 and 0.5 but a shift to higher anisotropy values is observable. The in-plane anisotropy histogram of control samples without treatments have completely different shape compared with the drug treated samples in figure 5.11 middle. Their anisotropy values significantly increased after Lovastatin treatment. As previously discussed, cells treated with 5 μmol and 10 μmol Lovastatin have a high order of F-actin polymerisation which corresponds to the higher values of anisotropy in these cells. The range of out-of-plane anisotropy is also dependent on drug concentration see figure 5.11 right. The out-of-plane anisotropy of the control cells are always distributed close to zero in comparison with drug treated cells. In addition, as expected, drug treated cells have lower out-of-plane anisotropy values in relation with Lovastatin concentration. These shifts to higher or lower values are due to the order induced by F-actin polymerisation. These values are also compared with corresponding regular bulk anisotropy using equation 2.10 in figure 5.11 left.

5.3 Conclusion

TIRFM is a non-destructive imaging method capable of interrogating the behaviour of fluorescent species in close proximity to an interface and has many applications in diverse research areas. TIRFM can be combined with emission anisotropy measurements to obtain unique information about molecular conformation, morphology and photo-physics of dye-cell interactions at an interface. Although FLIM is a robust method to provide lifetime information of microenvironments experienced by a fluorophore, it has some limitations in some cases for interpretation of exact physical phenomenon occurring in cell membranes such as F-actin polymerisation in the cytoskeleton. The FLIM images illustrated only a small variation of lifetime distribution as a consequence of drug treatments in CHO-K1 cells. For complementary physical interpretation, steady state fluorescence anisotropy imaging is shown to provide valuable supporting information. Fluorescence anisotropy images of CHO-K1 cells exhibited a significant change of fluorescence anisotropy for both out-of-plane and in-plane processes after cell treatment using 5 μmol and 10 μmol Lovastatin. These anisotropy results suggest that great order of F-actin polymerisation is observed due to the effect of high dose Lovastatin drug treatment.

Chapter 6

Summary, Conclusion and Future Work

The fundamental aim of the research in this thesis was investigate the morphology in biological and polymeric systems using the strategy of measuring time-resolved fluorescence behaviour and mapping the fluorescence anisotropy following epi- and evanescent wave based imaging and spectroscopic techniques. The inception of the tactics of FAIM, TR-EWIFS and EW-TRAM through both a theoretical and experimental discussion highlighted their capability to provide specific information and address several chemical and morphological problems in a wide range of complex systems.

The establishment of the FAIM method to map the fluorescence anisotropy variations proved to be one of the major outcomes of this project. That is, the capacity of this technique to scrutinize the photophysical and morphological properties of a wide range of systems including biological soft matter and conjugated polymers.

Chapter 1 discussed the recent literature with a review of recent developments in fluorescence imaging and spectroscopy near an interface. Emphasis was given to the developments of the methodology using fluorescence anisotropy in both imaging and thin film spectroscopy. After the theoretical review of the different concepts in chapter 1, their experimental details are outlined in chapter 2. Mapping fluorescence anisotropy in polymeric and biological systems was reviewed in chapters 3 and 5, respectively. The EW-TRAM and TREWIFS methods are utilised in chapter 4 to study the morphological changes in different types of LUVs near an interface.

6.1 Introduction and Experimental Methods

Chapter 1 reviewed the physical concept of total internal reflection (TIR) by simulating the electrical field of evanescent waves at the interface. The 2D electrical field calculations simulate the real evanescent waves generated and the variation of the penetration depths in experiments that were performed. The background of the fluorescence spectroscopy and polarisation was evaluated in detail. In particular, the evanescent wave time-resolved fluorescence spectroscopy and anisotropy measurements are outlined. The polarised fluorescence emissions from randomly oriented dipoles in the Cartesian coordinate system were used to calculate the fluorescence anisotropy for both bulk and interface measurements. The in-plane and out-of-plane anisotropy expressions were derived to probe photophysical processes at an interface. The pixel by pixel anisotropy calculation using image intensity of different polarisations and mapping the fluorescence anisotropy was one of considerable consequence of this research. Intensity images were captured by an EMCCD camera attached to the FAIM microscope. The images were background subtracted by ImageJ open source software. After background subtractions, the images with distinct polarisations were separated to the individual pixels by MATLAB code. The anisotropy values were calculated for all corresponding pixels and then mapped into the 2D image with the same size as the initial measurement.

In chapter 2, the experimental details of the theoretical concepts which were discussed in chapter 1, were presented. After a brief review on steady state and time resolved fluorescence spectroscopy, the chronological equipment development of evanescent wave time resolved fluorescence spectroscopy and anisotropy have been reviewed. One of the other outcomes of this thesis is the development of a new and upgraded evanescent wave time-resolved anisotropy measurements (EW-TRAM) apparatus. In the current configuration the limitations of the former (EW-TRAM) apparatus have

been overcome. There were previously a few restrictions such as limited incident angles and therefore penetration depths, poor collection efficiency and unstable mirrors. The new apparatus configuration for fluorescence anisotropy imaging microscopy (FAIM) is explained in details in chapter 2 section 2.6. This arrangement is capable of 2D mapping of the fluorescence anisotropy in a wide range of systems. 2D mapping of the fluorescence anisotropy has the potential to create a new chapter in optical imaging. Traditional anisotropy measurements are carried out by averaging the depolarised events on large scale and numbers of molecules. Most polymer films and biological systems like cells are not uniform in terms of their morphology. This polarised 2D mapping method is designed and then fabricated in terms of polarisation correction factors. A novel G factor calculation method is represented. In this approach, the polarisation bias (G factor) is spatially mapped. The most frequent G factor was 0.98. The applications of both methods are represented in the results chapters.

6.2 EW-TRAM of LUVs

In chapter 4 the photophysical properties of two types of LUVs (POPC and POPG) near the silica interface were explored using TREWIFS and EW-TRAMs. NBD dye labelled the lysis-7 terminal of the melittin peptide. The fluorescence lifetime of the fluorophore is sensitive to the micro-environment which it experiences. The morphological change of LUVs during interaction with the interface has been observed in terms of lifetime/rotational correlation time vs penetration depth. Nanometre depth profiling of bilayers is feasible by using a simple optical setup. The fluorescence lifetimes and rotational correlation times of LUVs were obtained with three exponential components: short-lived component τ_1 , interpreted as reporting on peptide-solvent interactions and two longer-lived components τ_2 and τ_3 assigned as reflecting peptide-peptide interactions.

The global analyses of the fluorescence decay profiles of peptides without LUVs indicate significant change of contribution percentage in terms of penetration depths. These meaningful fluctuations in lifetimes specify a trend in change of microenvironment as penetration depth reduces. The exponential component analysis assists the outcomes of the global analysis. In the former analysis, both lifetimes τ_1 and τ_3 increase with decreasing penetration depth. These findings provide evidence to support that the presence of solvent is reduced and peptide-peptide interactions are enhanced close to the interface.

The fluorescence decay kinetics and global analysis of the NBD-labelled melittin in POPC and POPG LUVs are more complex at the interface. The results demonstrate that the short lifetime diminished noticeably from the bulk to the surface measurements for both LUVs. The exponential component analysis showed that the lifetimes decrease significantly at close to the surface.

6.3 Mapping fluorescence anisotropy in polymeric and biological systems

Chapter 3 investigated the photophysical and fluorescence anisotropic behaviour of two types of conjugated polymer films (MEH-PPVs and PFO/PODPF). Different photophysical responses in terms of location in polymer films were observed.

The morphology of aggregated inhomogeneities in thin films of the conjugated polymer (MEH-PPV) in three solvents, cast by a controlled evaporative self-assembly (*snake-skin*) method was evaluated. The film morphology is dependent on the solvent which was used to cast the film as reported by the anisotropy and lifetime maps. The films cast from chloroform showed the shortest lifetime distribution and more depolarised anisotropy map. Both methods support the formation of aggregated inhomogeneities with high degree of energy migration.

The photophysics of polyfluorene β - conformational films (PFO/PODPPF) have been observed under different conditions. Variations in the level of β -conformational polyfluorenes influence the absorption and emission spectra, emission decay properties, morphology and energy migration of the film. β -conformational regions in both films have larger fluorescence anisotropy and shorter lifetimes than those of the glassy conformation. The investigation of chain conformation via thermal methods become feasible using 2D mapping of the fluorescence anisotropy.

In chapter 5 the photophysical and fluorescence anisotropic behavior of two types of biological systems have been investigated including macromolecular crowding in cells and actin cytoskeleton rearrangement as a side effect of statin, a cholesterol lowering drug.

A new strategy was outlined where mapping the fluorescence anisotropy coupled with lifetime imaging allows spatiotemporal visualization of the macromolecular crowding effect in cells. The protein mobility, morphology and local viscosity can be tracked using fluorescence anisotropy imaging and fluorescence lifetime imaging, respectively. In addition, real time monitoring of cytoplasmic crowding effect in live Neuro-2A cells is possible using this strategy.

TIRFM incorporated with fluorescence anisotropy measurements can record the morphological transformation during drug treatments. FLIM of CHO-K1 cells treated with different doses of statin drug show minor changes in the fluorescence lifetime histogram, although the reorganization of cell morphology is clear. F-actin cytoskeleton polymerization correlates with lifetime distribution. Highly oriented F-actin filaments in cells (in the presence of 10 μmol lovastatin) exhibit shorter mean lifetime when compared to control cells with partially oriented filaments at lower concentration. Total internal reflection fluorescence anisotropy measurements reproduced high magnitude of fluorescence anisotropy in cells treated by 10 μmol lovastatin compared to the cells treated by 5 μmol lovastatin drug.

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Appendix A

MATLAB Code for Image Processing

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%%
clc
clear
close all

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[fname dname] = uigetfile('.tif', 'Choose raw image', '');
temp = imread([dname fname]);
rawImage = uint16(temp);

ind_fname = strfind(fname, '.');
fname = fname(1:ind_fname-1);

mkdir(strcat(dname, '/', fname, '_subBG', '_results'));
% rmdir(strcat(dname, '/', fname(1:ind_fname-1), '_results'), 's');

dname = strcat(dname, '/', fname, '_subBG', '_results');

temp_rawImage = uint16(double(rawImage)/max(max(double(rawImage)))*2^16);

leftI = imcrop(rawImage, [89 130 419 743]); % [89 130 419 743]
imwrite(leftI, strcat(dname, '/', fname, '_left.tif'), 'tif');

rightI = imcrop(rawImage, [555 130 419 743]); % [555 130 419 743]
imwrite(rightI, strcat(dname, '/', fname, '_right.tif'), 'tif');

backgroundI = imcrop(rawImage, [4 4 50 50]);
bg_threshold = mean(mean(backgroundI));

figure(1), clf;
montage(cat(4, leftI, rightI), 'size', [1 2], 'DisplayRange', [200 5000]);
text(size(leftI, 2)/2, 0, 'Left image', ...
     'HorizontalAlignment', 'center', 'VerticalAlignment', 'bottom');
text(size(leftI, 2)+size(rightI, 2)/2, 0, 'Right image', ...
     'HorizontalAlignment', 'center', 'VerticalAlignment', 'bottom');

temp_leftI = uint16(double(leftI)/max(max(double(leftI)))*2^16);
temp_rightI = uint16(double(rightI)/max(max(double(rightI)))*2^16);

% temp_leftI = imcrop(temp_rawImage, [206 524 228 228]);
% temp_rightI = imcrop(temp_rawImage, [672 524 228 228]);

imwrite(temp_leftI, strcat(dname, '/', fname, 'left_image.tif'), 'tif');
imwrite(temp_rightI, strcat(dname, '/', fname, 'right_image.tif'), 'tif');

figure(2), clf;
Composite = cat(3, temp_rightI, temp_leftI, temp_leftI);

```

```

imshow(Composite), title('Color composite (Red = right; Cyan = left)');

%%

cpselect(temp_leftI,temp_rightI);
%%
input_points = movingPoints;
base_points = fixedPoints;

leftI_points_corr = cpcorr(input_points,base_points,leftI,rightI); %optimism
the points
% leftI_points_corr = input_points;           % just for test

fTlinear = cp2tform(leftI_points_corr,base_points,'similarity');
info = imfinfo(strcat(dname,'/',fname,'_left.tif'));

leftI_corr = imtransform(leftI,fTlinear,'XData',[1 info.Width], 'YData',[1
info.Height]);

%% Background subtraction
figure(10); clf;
hist(reshape(double(leftI_corr),1,numel(leftI_corr)),1000);

figure(11); clf;
hist(reshape(double(rightI),1,numel(leftI_corr)),1000);

% bg_threshold_left = 1725;
% bg_threshold_right = 1725;

%%
leftI_corr = leftI_corr - bg_threshold;
leftI_corr(find(leftI_corr<0)) = 0;

rightI = rightI - bg_threshold;
rightI(find(rightI<0)) = 0;

figure(10); clf;
hist(reshape(double(leftI_corr),1,numel(leftI_corr)),1000);

figure(11); clf;
hist(reshape(double(rightI),1,numel(leftI_corr)),1000);

%%

g_factor = 0.98;

% aniI = -(double(leftI_corr) - double(rightI))./(double(leftI_corr) +
double(rightI));
temp_Intensity = double(leftI_corr) + double(rightI);

```

```

[index_m index_n index_v] = find(double(temp_Intensity) < 350);

aniI = -(g_factor*double(leftI_corr) -
double(rightI))./(1*g_factor*double(leftI_corr) + double(rightI));

% filter function
aniI = medfilt2(aniI, [5 5]);

for i = 1:length(index_m)

    aniI(index_m(i),index_n(i)) = 0;

end

anifilename = strcat(dname,'/',fname,'ani_results.csv');
% save anifilename -ASCII '-tabs' aniI;

dlmwrite(anifilename,aniI,'delimiter','(',')

% saveas(aniI, strcat(dname,fname,'_aniResult.csv'),'csv');

temp_aniI = aniI - min(min(aniI));

temp_aniI = floor(temp_aniI/max(max(temp_aniI))*2^16);

figure(4)
imshow(uint16(temp_aniI))
%%

temp_leftI = uint16(double(leftI_corr)/max(max(double(leftI_corr)))*2^16);
temp_rightI = uint16(double(rightI)/max(max(double(rightI)))*2^16);

% figure(5), clf;
Composite_corr = cat(3,temp_rightI,temp_leftI,temp_leftI);
% imshow(Composite_corr), title('Color composite (Right = red, Left =
cyan)');

% figure(5), clf;
% montage(cat(4,Composite,Composite_corr),'size',[1 2]);
% text(size(leftI,2)/2,0,'Original',...
%      'HorizontalAlignment','center','VerticalAlignment','bottom');
% text(size(leftI,2)+size(rightI,2)/2,0,'Transformed',...
%      'HorizontalAlignment','center','VerticalAlignment','bottom');
% title('Color composite (Right = red, Left = cyan)');

figure(5), clf;
title('Comparison of transformed images (Red = right; Cyan = left)');

subplot(1,2,1);
imshow(Composite);

```

```
title('Original');

subplot(1,2,2);
imshow(Composite_corr);
title('Transformed');

%%
bg_anii = anii;
bg_anii(find(isnan(anii)))=0;
bg_anii(find(anii==1))=0;

% anii = bg_anii;

figure(6);
imshow(bg_anii, [-0.2 0.4])
colormap(jet)
colorbar
saveas(figure(6),strcat(dname,'/',fname,'_aniiResult.tif'),'tif');
%%
anii_value_bin = -0.2:0.008:0.4;

anii = bg_anii;

anii(find(anii <= -0.2)) =NaN;
anii(find(anii > 0.4)) =NaN;

figure(7);

hist(reshape(anii,1,numel(anii)),anii_value_bin);
[an_counts an_value] = hist(reshape(anii,1,numel(anii)),anii_value_bin);
an_counts_portion = an_counts/sum(sum(an_counts));
% plot(an_value,an_counts_portion)

dlmwrite(strcat(dname,'/',fname,'anii_hist.csv'), [an_value',an_counts']);

dlmwrite(strcat(dname,'/',fname,'anii_hist_portion.csv'),
[an_value',an_counts']);
```

Appendix B

Labview Code for Evanescent Wave Time-Resolved Anisotropy Measurements (EW-TRAM)

