Study of Dynamics in Biological Cells via Advanced Microscopy Techniques

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

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DECLARATION

I, Alireza Lajevardipour, hereby declare that this research thesis entitled:
“Study of Dynamics in Biological Cells via Advanced Microscopy Techniques”
and presented herein contains original work conducted by the author. This thesis
has not been submitted previously, in whole or in part, as the requirement for any
academic award or degree, at Swinburne University of Technology or any other
institution.

Alireza Lajevardipour

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# TABLE OF CONTENTS

ACKNOWLEDGEMENTS .................................................. ii

LIST OF FIGURES ........................................................ vi

LIST OF TABLES ............................................................ viii

CHAPTER

I. Introduction .............................................................. 1

1.1 Proem ................................................................. 1

1.2 Fluorescence lifetime imaging microscopy ................. 3

1.3 Principle of frequency-domain FLIM ........................... 3

1.3.1 Data analysis of lifetime images .......................... 9

1.4 Phasor Plot ......................................................... 10

1.4.1 General properties of phasor plot ......................... 15

1.5 Solvent relaxation dynamics ...................................... 19

1.6 FLIM instrumentation .............................................. 23

1.7 Fluorescence correlation spectroscopy ....................... 26

1.8 Image correlation microscopy ................................... 30

1.9 Photobleaching ICS ............................................... 32

1.10 Current status of topics .......................................... 33

1.11 Aims of the thesis ................................................ 34

1.12 Synopsis of the thesis ............................................. 35

II. Sub-microscopic translational motions of fluorescent particles determined by phasor-FLIM* ............................................. 37

2.1 Preamble .............................................................. 37

2.2 Materials and methods ............................................ 41

2.2.1 Numerical simulations ........................................ 41

2.2.2 Fluorescence lifetime imaging microscopy ............ 44

2.3 Results and discussion ............................................ 45

2.3.1 Frequency-domain FLIM at steady-state ............... 45
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.3.2 Frequency-domain FLIM including single particle fluctuations</td>
<td>47</td>
</tr>
<tr>
<td>2.3.3 Phasor-FLIM during motion of a single large Gaussian particle</td>
<td>49</td>
</tr>
<tr>
<td>2.3.4 Phasor-FLIM for an ensemble of particle motions</td>
<td>51</td>
</tr>
<tr>
<td>2.3.5 Effect of noise and motion on phasor plots</td>
<td>52</td>
</tr>
<tr>
<td>2.3.6 Effect of noise and motion on phasor plots</td>
<td>55</td>
</tr>
<tr>
<td>2.4 Scope of the method</td>
<td>59</td>
</tr>
<tr>
<td>2.5 Conclusions</td>
<td>60</td>
</tr>
<tr>
<td>III. Solvent relaxation dynamics in Golgi and plasma membranes of living HeLa cells</td>
<td>62</td>
</tr>
<tr>
<td>3.1 Preamble</td>
<td>62</td>
</tr>
<tr>
<td>3.2 Materials and methods</td>
<td>65</td>
</tr>
<tr>
<td>3.2.1 Stain preparation</td>
<td>65</td>
</tr>
<tr>
<td>3.2.2 Cell culture and treatments</td>
<td>65</td>
</tr>
<tr>
<td>3.2.3 Solution experiments</td>
<td>66</td>
</tr>
<tr>
<td>3.2.4 Fluorescence lifetime imaging microscopy</td>
<td>66</td>
</tr>
<tr>
<td>3.2.5 Confocal laser scanning microscopy</td>
<td>66</td>
</tr>
<tr>
<td>3.3 Results and discussion</td>
<td>67</td>
</tr>
<tr>
<td>3.3.1 Solvent relaxation in viscous solvents</td>
<td>67</td>
</tr>
<tr>
<td>3.3.2 Solvent relaxation in the Golgi and plasma membranes of living cells</td>
<td>75</td>
</tr>
<tr>
<td>3.4 Conclusion</td>
<td>82</td>
</tr>
<tr>
<td>IV. Measuring Receptor Clustering by Photobleaching Image Correlation Spectroscopy</td>
<td>84</td>
</tr>
<tr>
<td>4.1 Preamble</td>
<td>84</td>
</tr>
<tr>
<td>4.2 Materials and methods</td>
<td>86</td>
</tr>
<tr>
<td>4.2.1 Cell culture and treatments</td>
<td>86</td>
</tr>
<tr>
<td>4.2.2 Confocal microscopy</td>
<td>86</td>
</tr>
<tr>
<td>4.2.3 Step-by-step analysing procedure</td>
<td>86</td>
</tr>
<tr>
<td>4.2.4 Calculating spatial autocorrelation of images</td>
<td>88</td>
</tr>
<tr>
<td>4.2.5 General theory of pbICS</td>
<td>89</td>
</tr>
<tr>
<td>4.3 pbICS explicit aggregation distribution</td>
<td>93</td>
</tr>
<tr>
<td>4.4 Results and discussion</td>
<td>95</td>
</tr>
<tr>
<td>4.5 Advantages and disadvantages and of pbICS</td>
<td>105</td>
</tr>
<tr>
<td>4.6 Conclusions</td>
<td>105</td>
</tr>
<tr>
<td>V. Précis and Conclusion</td>
<td>107</td>
</tr>
<tr>
<td>5.1 Future work</td>
<td>108</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure

1.1 The cartoon of a cell .................................................. 2
1.2 The lifetime resolved techniques diagram .......................... 4
1.3 The concept of frequency domain FLIM ............................ 5
1.4 The reconstruction of emission wave ............................... 9
1.5 Illustrating of a phasor plot ......................................... 12
1.6 Phasor diagram .......................................................... 13
1.7 Phasor plots with different frequencies ............................ 14
1.8 Phasor plots with connecting line between two points ............ 17
1.9 Finding lifetimes of species via two experimental lifetimes of $\tau$ and $\tau'$ .................................................. 18
1.10 Resolving overlapped phasors ....................................... 19
1.11 Jablonski diagram ..................................................... 20
1.12 Jablonski diagram of solvent relaxation .......................... 21
1.13 Spectrum shift ......................................................... 22
1.14 Schematic of emission spectra in time-domain and frequency-domain .................................................. 23
1.15 FLIM setup .............................................................. 24
1.16 Illustration of FLIM setup ............................................. 25
1.17 Principal of Confocal Microscopy .................................... 27
1.18 FCS family tree ......................................................... 28
1.19 Photobleaching process ............................................... 32
1.20 Photobleaching process .............................................. 33
2.1 Distortion in phase and modulation detection-1 .................... 40
2.2 The phasor of moving Gaussian particle ............................ 50
2.3 The phasor of 100 moving beads ..................................... 52
2.4 Plot of the variance in phasor plot .................................. 53
2.5 Effect of noise on phasor plots ...................................... 54
2.6 Phasor plots of experimental FLIM measurements ............... 56
2.7 The phasor plot variance vs. MSD ................................... 58
3.1 Phase and mod lifetimes of NBD-X in Glycerol water mixture .... 68
3.2 Phase and mod lifetimes of NBD-X in Glycerol water mixture .... 69
3.3 Phasor plot of NBD-X in Glycerol with different water percentage .... 71
3.4 The log-log relation between viscosity and $T_2$ ..................... 73
3.5 The plot of solvent relaxation time vs. viscosity .................... 74
3.6 The confocal image of Hela cells stained with $C_6$-NBD-Ceramide .... 75
3.7 Lifetime images of a stained HeLa cell in 530 nm ..................................... 77
3.8 Lifetime images of a stained HeLa cell in 600 nm ..................................... 78
3.9 Phasor plot of FLIM experiments on membranes of HeLa cell various wavelengths .......................................................... 79
3.10 Phasor plot of FLIM experiments on membranes of HeLa cell .............. 80
3.11 Column graph of derived lifetimes for 10 individual cells ................. 81
4.1 Confocal microscopy image of CHO cells with GFP-tagged EGF receptor before and after photobleaching .................................................. 96
4.2 Magnified image of one ROI and its autocorrelation image ................. 96
4.3 Intensity profile of images in photobleaching period ............................ 97
4.4 Fitting autocorrelation data to find \( \langle j \rangle \) ........................................ 98
4.5 Fitting autocorrelation data to find \( c_{js} \) ........................................ 98
4.6 Molar fraction vs EGF concentration .................................................. 101
4.7 Averaged \( j \) values versus \( CD(p = 1) \langle j \rangle \) for no EGF stimulation ... 102
4.8 Averaged \( j \) values versus \( CD(p = 1) \langle j \rangle \) with EGF stimulation .... 103
B.1 Phasor plot of NBD-X in Glycerol with different water percentage .... 132
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Microscopy methods to measure diffusion constant</td>
<td>38</td>
</tr>
<tr>
<td>2.2</td>
<td>Computed phasor components</td>
<td>48</td>
</tr>
<tr>
<td>2.3</td>
<td>Results of moving beads</td>
<td>57</td>
</tr>
<tr>
<td>4.1</td>
<td>Averaged $j$ values obtained by fitting equation 4.25 on results of</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>experiments with CHO cells transfected with GFP-tagged EGF receptor and</td>
<td></td>
</tr>
<tr>
<td></td>
<td>treated with EGF</td>
<td></td>
</tr>
<tr>
<td>4.2</td>
<td>Tabulated results of experiments with CHO cells transfected with</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>GFP-tagged EGF receptor and treated with EGF</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER I

Introduction

1.1 Proem

Cells as building blocks of life are the vital units of life. The functioning of cellular systems is largely under the control of complex molecular mechanisms, all of which involve proteins, lipids, carbohydrates and nucleic acids. A clear understanding of the macromolecular interactions and cellular events will lead us to an ultimate treatment of many human diseases.

Dynamics are indispensable for protein function. Proteins must be at the right place and at the right time to function properly. Translational movement, rotation, reorientation, conformation, vibration and binding are different aspects of protein dynamics in cells [1–11].

Recent breakthroughs in photonics such as microscopy, fluorophore design and molecular tools have paradigmatically shifted biophysical experiments to study dynamics of biological structures noninvasively [12]. Imaging methods provide the key advantage that quantitative information on the location, dynamics [2], interaction strengths [13] and stoichiometries [14] of protein-protein interactions can be measured in single living cells. Fluorescent microscopy is a sensitive method that can detect even single molecules.

Fluorescently-tagged biomolecules offers a valuable alternative to biochemical
methods. Genetically encoded tags i.e. Green Fluorescent Protein (GFP) or tagged antibodies are targeted specifically to the molecule of interest for accurately revealing subcellular events [15, 16]. They provide us with dynamic information about local environment and quantity of molecules of interest.

Proteins are involved in different cellular pathways in the dynamic cellular environment. The desire to capture protein interactions in living cells in spatial and temporal nano-scale has fostered the development of highly advanced imaging methods.

Despite many developments in biological science, there is a need to develop new basic technologies that can probe the internal structure and dynamics of living cells. Biochemical methods often suffer from the lack of quantitative information on interaction strength, dynamics and cellular spatial context, Fluorescence lifetime imaging microscopy (FLIM) is well-suited for characterising molecular interaction processes. It allows assessment of the reactivity of a fluorophore at the molecular
level throughout a biological structure.

1.2 Fluorescence lifetime imaging microscopy

Fluorescence lifetime imaging microscopy or FLIM is a technique to measure excited state lifetimes of fluorophores. FLIM produces digital images in which each pixel value represents the fluorescence lifetime ($\tau$) \[17\]. It is possible to use FLIM to obtain lifetime information from living cells. The fluorescent lifetime of a fluorophore can be influenced by environmental factors such as ion concentration, pH value, hydrophobic properties, oxygen concentration, molecular binding, and molecular interaction by energy transfer, however it is independent of operational parameters like dye concentration, photobleaching, light scattering and excitation light intensity. Therefore fluorescence lifetime imaging can be used as an indicator for environmental conditions to detect cellular events.

Two lifetime resolved techniques, time-domain and frequency-domain, are most widely used to measure lifetimes (Figure 1.2). In the time-domain method, a short pulse of light uses to excite fluorophores then the lifetime can be extracted from a decay profile by fitting a proper decay function over acquired data. In the frequency-domain technique, modulated light can be applied for excitation and then the lifetime can be revealed by phase and modulation changes in the harmonic response of the sample. Time-domain and frequency-domain techniques are theoretically equivalent, but the principles of the instruments are completely different. The most widely used technique in the time domain is the time-correlated single-photon counting technique \[18\].

1.3 Principle of frequency-domain FLIM

The principles of fluorescent lifetime measurements are illustrated in Figure 1.2. In the simple case of time-domain FLIM, we have a simple $\delta$ excitation function
Figure 1.2: The lifetime resolved techniques. The excitation waves are in left side and related responses are in right side.

to excite the fluorescent sample and then a single exponential decay response, \( I(t) \), with a time constant, \( \tau \), called the excited-state lifetime. Generally excitation and response functions are complicated functions. The fluorescence emission response of sample, \( R(t) \), is always a convoluted product of excitation function \( E(t) \) and the intensity decay \( I(t) \).

\[
R(t) = E(t) \otimes I(t) = \int_{-\infty}^{t} E(t') I(t - t') \, dt'
\]

(1.1)

In the simple case, when \( E(t) \) is a \( \delta(t) \) function then \( R(t) \) equals to \( I(t) \). In the time-domain FLIM, obtaining response parameters requires a deconvolution of the fluorescence response.

For frequency-domain FLIM, the excitation light intensity is modulated at a
single frequency typically in the wide range of $1 - 200$ MHz. The fluorescence response is delayed due to the finite fluorescence lifetime of the fluorophore then the fluorescence emission is demodulated and phase shifted with respect to the excitation light (see Figure 1.3).

Generally for a repetitive excitation pulse, $E(t) \approx e^{i\omega t}$, by using equation (1.1), it can be shown that the periodic response, $me^{-i\varphi}$, is the Fourier transform of $I(t)$ (Figure 1.2):

$$m e^{-i\varphi} = \frac{\int_{0}^{\infty} I(t)e^{-i\omega t}dt}{\int_{0}^{\infty} I(t)dt}$$  \hspace{1cm} (1.2)

By analogy with $m e^{-i\varphi} = m \cos \varphi + m \sin \varphi i = A + B i$, we can introduce the
sine and cosine transforms $A$ and $B$ of the response (equations \(1.3a\)–\(1.3b\)):

\[
A = \frac{\int_{0}^{\infty} I(t) \cos(\omega t) \, dt}{\int_{0}^{\infty} I(t) \, dt} \quad (1.3a)
\]
\[
B = \frac{\int_{0}^{\infty} I(t) \sin(\omega t) \, dt}{\int_{0}^{\infty} I(t) \, dt} \quad (1.3b)
\]

Then phase shift and modulation depth can be directly calculated as:

\[
\varphi = \tan^{-1} \left( \frac{B}{A} \right) \quad (1.4a)
\]
\[
m = \sqrt{A^2 + B^2} \quad (1.4b)
\]

In the special case of single exponential decay, we have

\[
I(t) = \alpha e^{-t/\tau} \quad (1.5)
\]

where $\tau$ is the decay time constant and $\alpha$ is the amplitude. Then we can obtain

\[
A = m \cos \varphi = \frac{1}{1 + (\omega \tau)^2} \quad (1.6a)
\]
\[
B = m \sin \varphi = \frac{\omega \tau}{1 + (\omega \tau)^2} \quad (1.6b)
\]

and from them, the phase shift and modulation depth are

\[
\varphi = \tan^{-1} (\omega \tau) \quad (1.7a)
\]
\[
m = \frac{1}{\sqrt{1 + (\omega \tau)^2}} \quad (1.7b)
\]
Generally for a multi-exponential decay, the response is

\[ I(t) = \sum_{i=1}^{n} \alpha_i e^{-t/\tau_i} \]  

(1.8)

where \( \alpha_i \) is the fractional intensity of component \( i \). Using Eqs. \ref{1.3a} and \ref{1.3b}, the sine and cosine Fourier transforms, A and B, are given by

\[ A = \sum_{i=1}^{n} \frac{\alpha_i \tau_i}{1 + \omega^2 \tau_i^2} \]  

(1.9a)

\[ B = \sum_{i=1}^{n} \frac{\alpha_i \omega \tau_i^2}{1 + \omega^2 \tau_i^2} \]  

(1.9b)

In frequency-domain FLIM, we use a sinusoidal excitation \( E(t) \) with modulation frequency of \( \omega \):

\[ E(t) = E_1 + E_2 \cos (\omega t) \]  

(1.10)

or equivalently with defining modulation depth of \( m_0 \) and average intensity of \( E_0 \) we can write:

\[ E(t) = E_0 \left( 1 + m_0 \cos (\omega t - \varphi_0) \right) \]  

(1.11)

By using such modulated excitation wave, the fluorescence response will also be sinusoidally modulated at the same frequency, but with two key differences related to the fluorescence lifetime; the response will come with different modulation factor of \( m \) (0 < \( m \) < 1), and different phase shift of \( \varphi \) (0 < \( \varphi \) <= \( \pi/2 \) radians):

\[ R(t) = R_0 \left( 1 + m m_0 \cos (\omega t - \varphi_0 - \varphi) \right) \]  

(1.12)
The phase shift, $\varphi$, and the modulation, $m$, can be found via Fourier analysis:

\[ A = \frac{2 \int_{0}^{\frac{2\pi}{\omega}} R(t) \cos(\omega t) \, dt}{\int_{0}^{\frac{2\pi}{\omega}} R(t) \, dt} \]  \hspace{1cm} (1.13a) \]

\[ B = \frac{2 \int_{0}^{\frac{2\pi}{\omega}} R(t) \sin(\omega t) \, dt}{\int_{0}^{\frac{2\pi}{\omega}} R(t) \, dt} \]  \hspace{1cm} (1.13b) \]

and then equations (1.4a)–(1.4b). Given single exponential decay (equations (1.7a)–(1.7b)), the lifetime can be obtained from the phase shift and the modulation depth independently:

\[ \tau_{\varphi} = \frac{1}{\omega} \tan \varphi, \]  \hspace{1cm} (1.14a) \]

\[ \tau_{m} = \frac{1}{\omega} \sqrt{\frac{1 - m^2}{m^2}} \]  \hspace{1cm} (1.14b) \]

The $\tau_{\varphi}$ and $\tau_{m}$ are defined as the lifetime determined from the phase and the modulation, respectively. They should be equal in the case of single exponential decay, however they can be different due to complex decay kinetics, noise or other physical process.

In wide-field FLIM, there is a gain-modulated image intensifier coupled to CCD camera while in confocal systems modulated PMTs are used. In the case of homodyne detection (detector is gain-modulated at identical frequency ($\omega$) to excitation), a steady state signal is used to take several phase images by varying the phase of the detector gain-modulation. With data points provided by several phase images, one period of the waveform can be reconstructed (see Figure 1.4).
1.3.1 Data analysis of lifetime images

Using periodic excitation light leads us to handle data via Fourier analysis of the acquired images. In homodyne detection, we acquire $K$ images by shifting phase of the detector gain-modulation for $2\pi/K$ radians, then we can analysis the value of...
each image pixel by introducing discrete Fourier analysis:

\[
F_{i,j}^{\sin} = \left( \frac{2}{K} \right) \sum_{k=1}^{K} \sin \left( \frac{2\pi k}{K} \right) D_{i,j}^k
\]

(1.15a)

\[
F_{i,j}^{\cos} = \left( \frac{2}{K} \right) \sum_{k=1}^{K} \cos \left( \frac{2\pi k}{K} \right) D_{i,j}^k
\]

(1.15b)

\[
F_{i,j}^{dc} = \left( \frac{1}{K} \right) \sum_{k=1}^{K} D_{i,j}^k
\]

(1.15c)

\(F\)s represent Fourier components, \(D_{i,j}^k\) is the image obtained at the detector phase shift of \(2\pi k/K\) and \((i,j)\) indices display image pixels. To compare with what we had so far, we can write:

\[
A_{i,j} = \frac{F_{i,j}^{\cos}}{F_{i,j}^{dc}}
\]

(1.16a)

\[
B_{i,j} = \frac{F_{i,j}^{\sin}}{F_{i,j}^{dc}}
\]

(1.16b)

By using equations (1.4a) and (1.4b), the phase image (\(\varphi\)) and modulation image (\(m\)) can be obtained by:

\[
\varphi_{i,j} = \tan^{-1} \left( \frac{F_{i,j}^{\sin}}{F_{i,j}^{\cos}} \right),
\]

(1.17a)

\[
m_{i,j} = \frac{1}{F_{i,j}^{dc}} \sqrt{\left( F_{i,j}^{\cos} \right)^2 + \left( F_{i,j}^{\sin} \right)^2}
\]

(1.17b)

1.4 Phasor Plot

A typical detector CCD includes more than a million pixels. In any lifetime imaging, the user needs to read a set of \((m, \varphi)\) from each CCD pixel. So implementing a graphical tool to interpret this huge lifetime data is a vital key. Phasor plot, polar plot or AB plot [19–21], can assist us in interpreting lifetime distributions. It is a simple, graphical and rapid technique to demonstrate the phase and modu-
lation data of a lifetime measurement. Further than just a simple demonstration, phasor plot can even be used to analysis lifetime information. The phasor plot is same as the Cole-Cole plot that was already used to analysis dielectric relaxation experiments [19] [22].

In lifetime imaging, the lifetime data are extracted by Fourier analysis [21] [23] [24]. Referring to equations (1.6a) and (1.6b), we had

\[ A = m \cos \varphi \]  \hspace{1cm} (1.18a)

\[ B = m \sin \varphi \]  \hspace{1cm} (1.18b)

where \( \varphi \) and \( m \), the phase and modulation, are function of fluorescence lifetime \( \tau \) and modulation frequency \( \omega \). In AB plot, \( m \) and \( \varphi \) represent the distance from the origin and the angle from the A-axis, respectively. Figure 1.5 shows a simulated lifetime in the phasor plot. The semicircle is a universal curve which indicates the position of lifetime components with single exponential decay. The phasor plot presentation of the measured phase and modulation allows us to extract valuable information about the lifetime distribution through a convenient visualised graph. The phasor approach simplifies interpreting FLIM images, drops exponential analysis and provide us with a graphical sight of fluorescence decay process occurring at each pixel [25].
Figure 1.5: Each set of \((m, \varphi)\) represents a point in Phasor, Polar or AB plot. Any point on the universal semicircle (green line) represents lifetime of single exponential decay where \(\tau_\varphi = \tau_m\) or equivalently \(m = \cos \varphi\). The inside of semicircle (blue area) is the location of real lifetime data where \(\tau_m > \tau_\varphi\).

In Figure 1.5 the location \((m \cos \varphi, m \sin \varphi) = (1, 0)\), the right-bottom corner, represents zero lifetime \(\tau_\varphi = \tau_m = 0\) while \((0, 0)\) at left-bottom corner represents infinite lifetime. The direction of increasing lifetime is counter-clockwise to the left from 0 to \(\infty\).

Each phasor point emanates from differences between excitation wave (Equation (1.11)) and detected emission wave (Equation (1.12)). Figure 1.6 demonstrates this relation explicitly.
Figure 1.6:
In top panel there are three graphs. Left is a phase diagram to show excitation and emission phasors (blue and red arrow, respectively), Middle displays excitation and emission (blue and red color, respectively). Right plot is a phasor plot that demonstrates related point for such excitation and emission waves. Since there is no big difference between excitation wave and emission wave ($\varphi = 14^\circ, m = 0.97$) then the point in phasor plot is on fast lifetime region. Middle panel is same as top one but differences between excitation and emission are bigger ($\varphi = 45^\circ, m = 0.707$). Bottom panel is for even bigger differences ($\varphi = 78.7^\circ, m = 0.196$). With $\omega = 2\pi \times 39.7887 \text{MHz}$ phasor plots from top to bottom panels represent $\tau$ equals 1 ns, 4 ns and 10 ns, respectively.
Figure 1.7 depicts $\omega$ dependency of $\varphi$ and $m$. It shows that the locations of points in phasor plot are depend on the modulation frequency as expressed in equation 1.14. Phasor plots display the intensity-weighted average of lifetime components, then in a two component system all combinations are on the line between them. For example, in a two component system with lifetime of 1 ns and 8 ns all intensity combination of them fall on their connecting line.

Figure 1.7: Four phasor plots demonstrate same lifetimes for different modulation frequencies ($\omega$). As you can see, the position of a specific lifetime varies according to modulation frequency. In $f = 39.7887\, MHz$ the middle of semicircle, (0.5, 0.5), represents $\tau = 4.0\, ns$. In a sample with two components of e.g. 1 ns and 8 ns, all other different ratios are on the their connecting line. The slim black line displays connecting line between phasor points with lifetimes of 1 ns and 8 ns. Blue line shows the location of typical ($m, \varphi$), here $\tau = 4\, ns$. 
1.4.1 General properties of phasor plot

Phasor plot is a plot in classical polar coordinate system, thus it can inherit all properties of polar coordinate system [25]. In this system each point is represented by a set of \((m, \varphi)\) where \(m\) is the magnitude of distance of the point to the origin and \(\varphi\) represents the counter clock-wise angle of connecting line with the fixed x axis. The pair of magnitude and angle reflects vector notation where polar points sum rather like vectors than scale points. In other words, to add two points in polar coordinate, one need to calculate their components then add them properly to obtain the result.

1.4.1.1 Distribution of two lifetimes in phasor plot

The lifetime of any single exponential decay will lie on the universal semicircle. However in a real physical system with more than one lifetime, there will be a distribution of points in phasor plot depending on the fraction of each species as Equation (1.9) shows.

Given the multi-exponential decay profile of \(I(t) = \alpha_1 e^{-t/\tau_1} + \alpha_2 e^{-t/\tau_2}\) with two lifetimes of \(\tau_1\) and \(\tau_2\), the Equation (1.9) reduces to:

\[
A = \left( \frac{\alpha_1 \tau_1}{\sum_{i=1}^{2} \alpha_i \tau_i} \right) \frac{1}{1 + \omega^2 \tau_1^2} + \left( \frac{\alpha_2 \tau_2}{\sum_{i=1}^{2} \alpha_i \tau_i} \right) \frac{1}{1 + \omega^2 \tau_2^2} \quad (1.19a)
\]

\[
B = \left( \frac{\alpha_1 \tau_1}{\sum_{i=1}^{2} \alpha_i \tau_i} \right) \frac{\omega \tau_1}{1 + \omega^2 \tau_1^2} + \left( \frac{\alpha_2 \tau_2}{\sum_{i=1}^{2} \alpha_i \tau_i} \right) \frac{\omega \tau_2}{1 + \omega^2 \tau_2^2} \quad (1.19b)
\]

or equivalently with employing Equation (1.6), they can be written as:

\[
A = k_1 \ m_1 \ \cos \varphi_1 + k_2 \ m_2 \ \cos \varphi_2 \quad (1.20a)
\]

\[
B = k_1 \ m_1 \ \sin \varphi_1 + k_2 \ m_2 \ \sin \varphi_2 \quad (1.20b)
\]
where \( k_j = \left( \frac{\alpha_j \tau_j}{\sum \alpha_i \tau_i} \right) \) is fraction of fluorescent species. It can be shown that any distribution of these two lifetimes can lie on connecting line between them in phasor plot as in Figure 1.7, the connecting line between 1 ns and 8 ns has been shown. To prove this consequential property, the general equation of a line in polar coordinate is utilised:

\[
m = \frac{1}{\rho \sin \varphi + \sigma \cos \varphi}
\]  

(1.21)

It can be morphed into

\[
\rho m \sin \varphi + \sigma m \cos \varphi = 1
\]

(1.22)
or \( \rho B + \sigma A = 1 \). Substituting equations (1.20) and using this fact that \((m_1, \varphi_1)\) and \((m_2, \varphi_2)\) are on the connecting line reduce the proof to the condition of \( k_1 + k_2 = 1 \) that is already satisfied by using normalising factor of \( \sum_j \alpha_j \tau_j \).

The equation of the connecting line passes through two known points of \((m_1, \varphi_1)\) and \((m_2, \varphi_2)\) comes from the equation of a straight line in polar coordinate (Equation (1.21)) with constants as:

\[
\rho = \frac{m_2 \cos \varphi_2 - m_1 \cos \varphi_1}{m_1 m_2 \sin(\varphi_1 - \varphi_2)}
\]

(1.23a)

\[
\sigma = \frac{-m_2 \sin \varphi_2 - m_1 \sin \varphi_1}{m_1 m_2 \sin(\varphi_1 - \varphi_2)}
\]

(1.23b)

If two points \((m_1, \varphi_1)\) and \((m_2, \varphi_2)\) lie on semi-circle \((m_1 = \cos \varphi_1 \text{ and } m_2 = \cos \varphi_2)\), the above equation will reduce to:

\[
\rho = \tan \varphi_1 + \tan \varphi_2
\]

(1.24a)

\[
\sigma = 1 - \tan \varphi_1 \tan \varphi_2
\]

(1.24b)
Figure 1.8: Phasor plots with connecting line between two points. In this plot the relation between $k_1$ and $k_2$ is 3 : 2.

It is same as (by using $\tan \varphi = \omega \tau$):

$$\rho = \omega (\tau_1 + \tau_2) \quad (1.25a)$$

$$\sigma = 1 - \omega^2 \tau_1 \tau_2 \quad (1.25b)$$

In Figure 1.8 $\tau$ is an experimental lifetime come from an ensemble of two species with lifetimes of $\tau_1$ and $\tau_2$. Phasors of all the possible linear weighting combination of two species with fractional weighting of ($k_1$ and $k_2$) lie on the connecting line between their individual phasors ($\tau_1$ and $\tau_2$).

1.4.1.2 Finding lifetimes from distribution in phasor plot

In Figure 1.9 we have two ensembles of two species with different distributions (mean lifetimes of $\tau$ and $\tau'$). We are able to obtain lifetime of each species ($\tau_1$ and $\tau_2$) via finding intersection of connecting line with universal semi-circle. This approach can be used to subtract probable background autofluorescent lifetime [26].
Figure 1.9: Finding single exponential lifetimes of contributing species ($\tau_1$ and $\tau_2$) from two experimental lifetimes ($\tau$ and $\tau'$) related to two different distributions.

Then we calculate intersections of the known line, $m = 1/(\rho \sin \varphi + \sigma \cos \varphi)$, with semi-circle ($m = \cos \varphi$) from $\tan \varphi_{1,2} = 1/2(\rho \pm \sqrt{\rho^2 + 4\sigma - 4})$ or equivalently from:

\[
m_1 \cos \varphi_1 = \frac{1}{1 + \left(\frac{1}{2}(\rho + \sqrt{\rho^2 + 4\sigma - 4})\right)^2} \tag{1.26a}
\]
\[
m_1 \sin \varphi_1 = \frac{1/2(\rho + \sqrt{\rho^2 + 4\sigma - 4})}{1 + \left(\frac{1}{2}(\rho + \sqrt{\rho^2 + 4\sigma - 4})\right)^2} \tag{1.26b}
\]
\[
m_2 \cos \varphi_2 = \frac{1}{1 + \left(\frac{1}{2}(\rho - \sqrt{\rho^2 + 4\sigma - 4})\right)^2} \tag{1.26c}
\]
\[
m_2 \sin \varphi_2 = \frac{1/2(\rho - \sqrt{\rho^2 + 4\sigma - 4})}{1 + \left(\frac{1}{2}(\rho - \sqrt{\rho^2 + 4\sigma - 4})\right)^2} \tag{1.26d}
\]
1.4.1.3 Resolving lifetimes by phasor plot

As Figure 1.10 indicates the exact location of a lifetime on phasor plot is related to modulation frequency ($\omega$). With exploiting this postulate, one can resolve two overlapped phasors of different distributions (the left graph in Figure 1.10). If there are two different distributions but occupy same place in phasor plot in a same modulation frequency, it is possible to resolve them by employing higher modulation frequency, as the right graph in Figure 1.10 displays.

![Figure 1.10](image)

**Figure 1.10:**

*Left graph:* There are two distribution of lifetimes (blue and orange lines) while measured lifetimes ($\tau$ and $\tau'$) are overlapped. The modulation frequency is 30 MHz.

*Right graph:* There are same distributions in this graph but modulation frequency has changed to 60 MHz. Therefore the overlapped lifetimes are resolved.

1.5 Solvent relaxation dynamics

Dye molecules in solvents can get energy for electronic excitation through some process like molecular collisions or optical excitations. Inter-molecular interactions, however, dissipates deposited energy soon. Also there are intra-molecular interactions can dissipate electronic excitation in a specific molecule, processes like vibrational relaxation. Figure 1.11 shows a Jablonski diagram that demonstrates possible processes in a molecule. In the Jablonski diagram the singlet states are labelled $S_0$
Figure 1.11: Jablonski diagram for possible photo-processes in a molecule. VR stands for vibrational relaxation. IC is for internal conversion and ISC means intersystem crossing.

for ground state and $S_1$, $S_2$ for excited states. The triplet states are labelled $T_1$ and $T_2$.

An absorption results in excitation of an electron from $S_0$ to one sublevels of $S_1$ while a rapid vibrational relaxation makes it to fall to the bottom of the $S_1$ state. The excited electron in $S_1$ can be transferred to other states via fluorescence, internal conversion, or intersystem crossing.

By emitting a photon, the electron can transfer to ground state. This process of fluorescence emission has a lifetime on the average of a few nanoseconds. Intersystem crossing is a process that causes singlet to triplet conversion for excited states. Transferring the electron from triplet state to ground state is accompanied by an emission called phosphorescence with typical lifetime of milliseconds to seconds. The excited electron in $S_1$ can also transfer directly to vibrational levels of the ground state via nonradiative process called internal conversion.
In polar solvent, the electronic excitation from \( S_0 \) to say \( S_1 \) can be associated with changing dipole moment of the molecule. Since the excitation is an ultrafast process, there is no enough time for surrounding molecules to reorient concurrently so the system of excited molecule and its surrounding molecules transitions to a non-equilibrium system after excitation (see Figure 1.12). This non-equilibrium state is called Franck-Condon state \( (S_{FC}^1) \). To minimise the energy of system, surrounding molecules start to reorient with respect to excited molecule to reach a relaxed state. The new equilibrium state is called relaxed excited state \( (S_{Re}^1) \). The minimising energy or relaxation results in a red-shift in the emission spectrum.

The red-shift spectrum cover emission energies from maximum frequency of Franck-Condon state, \( \nu(t = 0) \), to minimum frequency of relaxed state, \( \nu(\infty) \).

For a given dipole moment change, more polar solvent gives bigger spectral shift \( \Delta\nu = \nu(0) - \nu(\infty) \). Also the time-dependent spectral shift is based on solvent relaxation kinetics that is sensitive to the mobility of the solvent. A spectral correlation function can be defined as:

\[
S_\nu(t) = \frac{\nu(t) - \nu(\infty)}{\Delta\nu}
\]

(1.27)

where the solvent relaxation time, \( T_S \), can be obtain from:
For a homogeneous solvent with single exponential decay we can have:

$$\nu(t) = \Delta \nu e^{-t/T_S} + \nu(\infty)$$  \hspace{1cm} (1.29)$$

then the steady state spectrum can be written as

$$\nu_S = \frac{1}{\int_0^\infty e^{-t/\tau} dt} \int_0^\infty \nu(t) e^{-t/\tau} dt$$  \hspace{1cm} (1.30a)$$

$$= \frac{\Delta \nu}{1 + \tau/T_S} + \nu(\infty)$$  \hspace{1cm} (1.30b)$$

with rewriting the latest equation as $\tau = \left( \frac{\Delta \nu}{\nu_S - \nu(\infty)} - 1 \right) T_S$ it is obvious that bigger red-shift means more relaxed system with less emission energy (see Figure 1.13). The dependency of red-shift on dipole moment and mobility of environment causes the relaxation time to be greatly affected by polarity and viscosity of solvent.
To reveal relaxation event, one can measure blue and red sides of the emission spectrum. Differences between these two sides in both time-domain and frequency-domain fluorometry techniques can point at the solvent relaxation (see Figure 1.14). As it is clear from time-domain graph, the intensity decay measured on the blue side is more rapid than the decay of red side, because the blue side decays owing to shift in spectrum and loss of population while red side initially raises in intensity owing to the red shift and then decays due to loss of excited state population. In frequency domain, there is a phase lag between red and blue emissions due to solvent relaxation. This combination causes an inversion between phase lifetime and modulation lifetime. In the phasor plot, the relaxation process results in a separation between phasors of blue and red sides of emission wave [27] and in some cases causes the red shifted phasor to move outside the universal semi-circle.

1.6 FLIM instrumentation

Figure 1.15 demonstrates our FLIM setup that consists of a inverted wide-field fluorescence microscope (Nikon Ti) with Lambert instruments LIFA attachment includes modulation signal generator, modulated intensified CCD camera (LI²CAM),
Figure 1.15: Our FLIM setup includes widefield fluorescence microscope (Nikon Ti) and Lambert instruments LIFA attachment. Top photo displays the system from right side point view where the bottom one shows left side.
Figure 1.16: The illustration of our FLIM setup. 1- The wide-field inverted fluorescent microscopy (Nikon Eclipse Ti). 2- Multi-LED light source. 3- TIRF/epi-fluorescent illumination port. 4- Modulated solid-state laser. 5- Signal generator. 6- Optical fibre. 7- Modulated intensified CCD camera. 8- Hyper-spectral imaging system.

multi-LED modulated lightsource and a PC equipped with LI-FLIM software. Also a GOOCH & HOUSEGO HyperSpectral Imaging System (HSi-400) was fitted on the optical path before the CCD camera. The system can measure lifetimes in the range of 0 to 300 ns with 30 ps time resolution. The wavelength range is 400 nm to 800 nm.

The Multi-LED unit combines 3 LEDs with different wavelengths (448 nm, 474 nm and 529 nm) aligned on a single optical path. The unit is connected to the standard epi-fluorescence illumination port of the microscope (see Figure 1.16). The selection of excitation wavelength can be done via the LI-FLIM software without requiring any realignment of the light source. The system also capable to switch and use modulated diode lasers as excitation light source instead of LED unit especially for TIRF microscopy.

The signal generator is used to generate and control the sinusoidal modulation
signals for the light sources (LED or Laser) and the intensified camera. The frequency range is 1 to 120 MHz with resolution of 1 KHz. It also contains the high voltage power supply generating the DC voltages for the image intensifier.

The intensified camera contains an image intensifier that is fibre-optically coupled to the CCD camera for maximum gain and speed. The intensifier is composed of photocathode, anode and Multi Channel Plate (MCP) where photons convert to electron at photocathode and accelerate and multiply in the MCP, then electron clouds convert back to photons at anode.

The HSi-400 Hyper-Spectral Imaging system includes an acousto-optic tunable filter (AOTF). The AOTF is a high speed, high throughput and random-access optical filter with an adjustable optical bandpass. The AOTF technology provides variable bandwidth resolution as less as 1.5 nm. The HSi consists of an automated 11-position filter wheel that includes a factory installed filter set compatible with most standard fluorescence dyes. It also includes a bypass mode to get the AOTF out of the optical path. This piece of equipment in our FLIM set-up enable us to adjust detecting emission window. We use the AOTF to detect blue and red sides of emission wave separately for measuring solvent relaxation, as it will be described later in Chapter 4.

1.7 Fluorescence correlation spectroscopy

Fluorescence correlation spectroscopy (FCS) was originally developed to study the kinetics of chemical reactions in equilibrium. FCS measures fluctuation of the fluorescence intensity. FCS and its derivative methods are the mainstream to study cellular dynamics, particularly after introducing confocal microscopy (Figure 1.17) [28]. Figure 1.18 shows a hierarchical diagram of FCS family.

The FCS method can be used to measure the number of molecules. This information can be extracted from the autocorrelation function at zero time-lag. This
method can be also employed to measure and the diffusion of fluorescent from the decay of the autocorrelation function with time [29]. In Figure 1.18 there are FCS-based techniques that enable us to extract information about number of particles, measuring aggregation and dynamics of fluorescent molecules.

We can categorised FCS-based family to two classes, methods that measure dynamics in single point (FCS, FRET-FCS, FCCS) or via scanning (sFCS, RICS, TICS, STICS, ICCS) and methods that measure aggregation in single point (PCH, dcPCH, FIDA) or via imaging (SpIDA, ICS, N&B, pbICS).

In the dynamics measurement class, FRET-FCS assesses the conformational dynamics of biomolecules, but it can separate kinetic information from the diffusion

contributions [30]. FRET measures fluorescence of a second probe that gets excited via energy transfer. The system must be contained of two types of probes where they can get together close enough to ensure transferring energy. The FRET signal is weak, but has the advantage to detect a local reaction.

Also in this class, Scanning FCS (sFCS) is a method to move the measurement
volume across the focused area of a sample in a pre-defined way. It provides precise
diffusion coefficient in the presence of weak photobleaching \cite{31} and also it can
measure aggregation in slow diffusion systems like cell membranes \cite{32}. RICS (raster
image correlation spectroscopy) quantifies molecular diffusion \cite{33}.

On the other hand, in the aggregation measurement class, FIDA (Fluorescence
Intensity Distribution Analysis) and PCH (Photon-Counting Histogram) measure
particle brightness of a species in the observation volume \cite{34,35}. While methods
based on autocorrelation function can distinguish species via their size and tempo-
ral fluctuations, PCH differentiates them based on their brightness and concentra-
tion \cite{36}. Also in this class, N&B (number and brightness) measures aggregation
and number of mobile molecules \cite{33,37}. N&B analysing method provides a map of
N (apparent number of molecules) and B (apparent molecular brightness) for each
pixel in the image by detecting intensity fluctuations during accusation time. The
average number of particles, $\langle N \rangle$ is obtained from the ratio of the square of the
average intensity to the variance while the average brightness is acquired from the
ratio of the variance to the average intensity. Since the brightness value of immo-
ble components is 1, changing the illumination power can separate the mobile from
immobile fraction in a plot of the ratio variance/intensity versus intensity.

In addition, SpIDA (Spatial Intensity Distribution Analysis) is an analysis tech-
nique based on fluorescence imaging \cite{38,40} to measure oligomerization states and
fluorescent particle densities. SpIDA was based upon PCH technique and utilised
to measure monomer-dimer distribution of receptor molecules.

Another method in the aggregation measurement class is ICS method that will
be explained in the following chapter.
1.8 Image correlation microscopy

Image correlation spectroscopy (ICS) is an important member of FCS family. It is based on analysing the fluorescence intensity fluctuations of the microscopy image series by means of correlation functions. With ICS technique, it is possible to determine the average number of fluorescent entities. The fluorescence fluctuation data are sampled from multiple points in space on the acquisition timescale. The autocorrelation analysis reveals the average number of fluorescent particles (protein monomers or protein clusters) from the amplitude of the autocorrelation function \( G(0) \propto 1/N \) for a given image area. One can calculate the relative degree of aggregation \( DA \) of proteins as:

\[
DA = G(0) \langle I \rangle \propto \frac{N_p}{N_c}
\]  

(1.31)

where \( G(0) \) represents the autocorrelation function in time lag equals zero, \( \langle I \rangle \) is the average intensity, \( N_p \) and \( N_c \) represent the number of proteins and the total number of clusters in the focal volume, respectively. Changes in protein distribution result in changes in the number of fluorescent particles then fluctuation in the correlation function accordingly. Measuring this fluctuation allows us to determine the kinetics of protein aggregation in real time on living cells.

The temporal autocorrelation function of a signal, \( I(t) \), is the correlation of the signal at time \( t \) with itself at time \( t + \tau \), or equivalently it is the correlation of the variation of the signal, \( \delta I(t) \):

\[
G(\tau) = \frac{\langle I(t)I(t + \tau) \rangle}{\langle I(t) \rangle^2} = \frac{\langle \delta I(t)\delta I(t + \tau) \rangle}{\langle I(t) \rangle^2} + 1
\]

(1.32)

where \( \delta I(t) = I(t) - \langle I(t) \rangle \). It has been proved that \( G(\tau) = 1 \) for an uncorrelated signal, while \( G(\tau) \) can be \( > 1 \) and \( < 1 \) for correlated and anticorrelated signals, respectively. Dividing factor of \( \langle I(t) \rangle^2 \) normalises the correlation function and elimi-
inates intensity change caused by fluctuation of laser power or quantum yield of dye.

The Wiener-Khintchen theorem \[41\] enables us to use the power spectrum of a signal, \( FT\{I(t)\} \cdot FT^\dagger\{I(t + \tau)\} \), to calculate the correlation function of the signal as:

\[
G(\tau) \langle I(t) \rangle^2 = \langle I(t)I(t + \tau) \rangle = FT^{-1}\{FT\{I(t)\} \cdot FT^\dagger\{I(t + \tau)\}\}
\]

(1.33)

where \( FT \) and \( FT^{-1} \) represent Fourier and inverse Fourier transformations, respectively and \( FT^\dagger \) displays the complex conjugate of \( FT \).

To calculate the correlation function of a series of images stored in a stack, one can use ImageJ (NIH, Bethesda). The “FD Math” procedure in ImageJ employs the Fast Fourier Transformation (FFT) to convert images to frequency domain, calculates the power spectrum image, then converts it back to spatial domain by inverse FFT. Based on the Wiener-Khintchen theorem, the result is the correlation of images.

In an ergodic ensemble, the ensemble average of an stochastic process is equivalent to the spatial or temporal average. It empowers us to behave spatial and temporal averages correspondingly as \( \langle I(t) \rangle = \langle I(x) \rangle \). The 2D spatial autocorrelation for an image is:

\[
G(\eta, \sigma) = \langle I(x, y)I(x + \eta, y + \sigma) \rangle
\]

(1.34)

In ICS method, a series of images is used to calculate autocorrelation images that provide us with \( G(0) \) in spatial domain.
1.9 Photobleaching ICS

Photobleaching ICS (pbICS) refers to a newly developed method \[42\] for determining the aggregate distribution of immobile molecules. pbICS is based on varying the proportion of tagged particles through gradual photobleaching of fluorophores. In this technique a time-series of confocal laser scanning images is taken and recorded during a photobleaching process. Using ICS, the average cluster density is calculated for each image in the time-series and related to the fluorescent fraction remaining. The relationship between cluster density and fractional fluorescence remaining is linear for monomers and a nonlinear function for clusters with two or more tagged subunits. This method does not require fluorescence brightness standards.

Figure 1.19: The cartoon of photobleaching process of fluorescently tagged particles. 

*Left panel:* Shows some particles with green fluorescent tags. *Middle panel:* depicts 50% of tags bleached after 10 bleaching steps. *Right panel:* all green tags photobleached after 20 bleaching steps.

Figure 1.19 illustrates three steps of photobleaching process for some fluorescently tagged molecules where green tags are bleached in middle image by 50% and totally bleached in right image. One can define fraction of remained fluorescent tags \((p)\) as:

\[
p = \frac{\text{The mean intensity of the bleached image}}{\text{The mean intensity of the original image}} \tag{1.35}
\]
Figure 1.20: The corresponding autocorrelation images of three different photo-bleaching steps in Figure 1.19 from left to right, respectively.

where it is 1, 0.5 and 0 for left, middle and right panel in Figure 1.19 respectively.

Figure 1.20 shows autocorrelation images of three bleaching steps shown in Figure 1.19. Each autocorrelation image can provide us with a $G(0)$ as:

$$G(0) = \frac{\langle \text{The maximum intensity of the autocorrelation image} \rangle}{(\text{The mean intensity of the bleached image})^2}$$ (1.36)

As one can note, the maximum intensity in autocorrelation images decreases when fluorescent tags are bleached. This implies that the maximum intensity of autocorrelation image is proportion to fraction remaining fluorescent tags. In other words, the probability of finding non-bleached tags (fraction remaining) connects to $G(0)$

$$G(0) \left( \sim \frac{1}{N} \right) \propto \Phi(p)$$ (1.37)

where $N$ is number of fluorescent entity and $\Phi(p)$ is a probability function of $p$. The pbICS method will be fully explained in Chapter IV.

1.10 Current status of topics

FLIM can be used to characterise phenomena that alter lifetime of fluorophors through various processes. To date we can exploit FLIM microscopy to reveal different physical or chemical events such as quenching [43–49], binding [50–52], PH [53, 54], energy transfer [55], aggregation [56], viscosity [57, 58], proximity to
nanoparticles [59][62], localising nanoparticles [63][64]. However, there are still some other phenomena which can be measured by FLIM experiment like diffusion of fluorophores, solvent relaxation time in membrane and stoichiometry of cellular interactions.

To give a better understanding of diffusion in living cells, the influence of particle movement on FLIM measurement during image acquisition can be exploited. A theoretical description of this effect with supporting experimental results can provide us with a new application.

Solvent or dipolar relaxation is a fundamental process that occurs in the condensed phase. Chemical as well as biological reactions can be influenced by the rate of dipolar relaxations. From a cell biology perspective, one would like to have an experimental measure of these processes within the complex environment of the living cell. Although solvent relaxation has been a favourite topic in scientific milieu for many years, there has not been any study to use lifetime imaging microscopy to measure solvent relaxation time in cell yet.

Image Correlation Spectroscopy (ICS) is another modern microscopy method that can be exploited to analysis time-series of images taken by confocal laser scanning or wide-field microscopes. It can provides valuable information on aggregation of fluorescently labelled macromolecules.

1.11 Aims of the thesis

The general goal of this thesis is to gain a detailed insight into dynamics of living cells. Outlined projects to satisfy the goal include:

(i) To develop a method to measure diffusion coefficient and lifetime of fluorescent particles.

(ii) To develop a method to image dipolar dynamics of environment in living cells.
(iii) To develop a method to measure complex interactions between molecules in fixed cells.

1.12 Synopsis of the thesis

To unravel the often complex entanglement of dynamics in living cells, microscopy mechanisms such as the effects of lateral motion on phasor plot and measuring solvent relaxation must be well understood. The present works also aims to ascertain a detailed description of characterising clustering state of surface receptors by means of photobleaching Image Correlation Spectroscopy method. To start using FLIM and ICS methods, fundamental concepts of methods and their key parameters should be discussed. These concepts will be explained in the Chapter I along with properties of phasor plot and concepts of solvent relaxation dynamics. The employed instrumentation in the research will be mentioned in this chapter too.

As already mentioned, effects of translational movement of fluorescent beads on the phasor plot will be addressed in details. This will be addressed in Chapter II with the view point of connecting variance in phasor plot to mean square displacement of moving beads. Solvent relaxation dynamics is the next issue to address. Chapter III is dedicated to measuring solvent relaxation time in plasma membrane of living cells. The practical framework to reveal solvent relaxation time will be also addressed in this chapter. The last but not least issue is the measure of aggregation of EGF receptors in surface of CHO cells in absence and presence of EGF stimulation. Chapter IV will be fully addressed the theory of implemented photobleaching-ICS method and experimental results. Thus, the general organisation of the thesis is as follows:

Chapter I

The proem of the thesis plus Introduction to the topics along with an explanatory description of FLIM and ICS methods. Detailed properties of phasor plot
and concepts of solvent relaxation in addition to instrumentation are other contents of the chapter. The final sub-section in current chapter before this synopsis is a brief summary of our current understanding.

Chapter II

A novel method is developed to measure translational diffusion coefficient of fluorescent particles. The experimental study with simulations were used to connect lifetime distribution to mean square displacement of stochastically moving beads. Also the scope of the method will be explained.

Chapter III

A novel approach is developed to measure relaxation dynamics in living cells. Effects of solvent relaxation on phasor plot are addressed. Solvent relaxation time in Golgi membrane is reported for very first time.

Chapter IV

The pbICS method is implemented for first time to measure aggregation of EGF receptors on surface of CHO cells. Both theoretical development of method and experimental procedure are reported.

Chapter V

Précis of the major conclusion of the work plus future work.
CHAPTER II

Sub-microscopic translational motions of fluorescent particles determined by phasor-FLIM*

2.1 Preamble

Macromolecular interactions and motions are believed to play central roles in biology. Also many cellular and subcellular events depend critically on the rheology of cells. There are many attempts to characterise cell mechanics via particle tracking methods where fluorescent beads are used to study cytoskeletal dynamics and rheology of live cells [65–70]. There are different microscopy methods that one can use to measure diffusion coefficient of moving molecules inside of biological cells. Table 2.1 shows such techniques.

FLIM or fluorescence lifetime imaging microscopy [78] is traditionally a useful tool for spatially-mapping fluorescence lifetimes and interactions (through FRET [79]). In the vast majority of cases, lateral motions are either frozen (e.g. fixed cells), averaged on the timescale of the measurement (e.g. solutions), or observed but considered as an artefact [80]. The purpose of this chapter is to demonstrate that lateral motions of particles can be determined through careful analysis of FLIM experiments. The basic idea is illustrated in Figure 2.1. In frequency-domain analysis of FLIM the intrinsic time-resolved fluorescence is characterized by the properties of a

* This chapter has been published in the Journal of Fluorescence (2013) 23:671-679
Table 2.1: Various techniques to study diffusion of moving particles.

<table>
<thead>
<tr>
<th>Method</th>
<th>Advantages</th>
<th>Comments</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCS</td>
<td>well-known and widely used technique.</td>
<td>requires knowing geometry of the measurement volume.</td>
<td>[71][72]</td>
</tr>
<tr>
<td>Scanning FCS</td>
<td>There is no need to know the exact size of the measurement volume.</td>
<td>needs careful calibration of the scan radius.</td>
<td>[31]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>has problem with photobleaching.</td>
<td></td>
</tr>
<tr>
<td>Spatial FCCS</td>
<td>reveals more precise diffusion constant than FCS.</td>
<td>requires knowing the optical geometry of the detection.</td>
<td>[73]</td>
</tr>
<tr>
<td></td>
<td>discriminates diffusion from other processes that bias the intensity fluctuations.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RICS</td>
<td>can be used to measure fast cellular diffusions.</td>
<td>has problems with photobleaching.</td>
<td>[74]</td>
</tr>
<tr>
<td>FRAP</td>
<td>highly efficient to quantify mobility of lipids on a cell membrane.</td>
<td>requires a lot of experiments with different bleaching areas.</td>
<td>[75][76]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>has problems with 3D and anisotropic diffusions.</td>
<td></td>
</tr>
<tr>
<td>Single-Particle Tracking (SPT)</td>
<td>great to measure 2D and 3D diffusions. reveals random, confined and active diffusions.</td>
<td>requires a lot of datasets for reliable results. causes some level of photodamage.</td>
<td>[77]</td>
</tr>
<tr>
<td>Present work</td>
<td>The method is able to measure diffusion and lifetime simultaneously.</td>
<td>It is based on the phasor plot calculation.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>There is no need to track particles directly.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

sinusoidal function (phase and modulation values) at a given modulation frequency (frequency-domain instrumentation) or laser repetition frequency (time-domain in-
strumentation). In FLIM with moving particles, occupancy fluctuations of fluorescent species perturb this normal sinusoidal intensity profile causing a deviation in the “expected” phase and modulation values. This perturbation can be visualized most conveniently in phasor space [20, 21, 25, 81] as a blooming or spreading of phasors.
Figure 2.1: Qualitative concept of FLIM with moving particles. *Left column;* A. Projected fluorescent particles in a CCD pixel at different times with no change in number of particles. Blue line; modulated excitation, Green line; detected emission (sine wave). B. The CCD pixel at different times with extreme change in particle number. Blue line; modulated excitation, Green line; detected emission. Note distorted sine wave detected due to large particle number fluctuation. C. The CCD pixel at different times with moderate change in particle number. Blue line; modulated excitation, Green line; detected emission. Note slightly distorted sine-wave detected due to moderate particle number fluctuation. *Right column;* Phasor plots for FLIM depend on particle occupancy. A. Phasor plot for frozen single particle (no movement). B. Phasor plot for occupancy sequence (1,0,0,0), and C. fluctuations about an average occupancy leading to phasor plot broadening.
The organisation of this chapter is as follow: First, using a simple “back of the envelope” analytic theory that determine how extreme particle fluctuations can perturb phasor positions for a single particle. Second, numerical simulations with Gaussian convoluted particle are employed to demonstrate motion-related phasor broadening. Third, it is explicitly shown how the variance in the phasor plot is related to the extent of particle motion. Fourth, The effect of noise is examined. Finally, an experimental demonstration of the phasor broadening effect is provided with fluorescent beads where it is shown that the outcome supports provided work.

The analysis and results presented on the one hand reveal how lateral motions can influence lifetime measurements. When these motions are significant enough to cause broadening of apparent lifetime distributions then information about these motions can be extracted. On the other hand the analysis provides a complementary way of measuring intensity fluctuations and average lifetimes in the one experiment.

2.2 Materials and methods

2.2.1 Numerical simulations

The complete numerical simulation can be divided in the two sub-simulations; simulation of beads movement and simulation of FLIM experiment.

To simulate Brownian motion, a FORTRAN code was developed to solve the Langevin equation with velocity Verlet algorithm \[82\]. Simulated particles were moved stochastically on the surface of an image grid with size of 1392 × 1040 pixels (size of a regular CCD) but 2 by 2 binning where each pixel has a binned size of 12.9µm × 12.9µm. To place initial position, particles were distributed randomly on the image grid with random initial velocities.

We simulated three different ensembles, 100 point-like particles, a single Gaussian bead with size 21 by 21 pixels, and two Gaussian beads (each with size 21 by 21 pixels).
To ensure the simulations conformed to Brownian behavior, and to provide a measure of extent of motion, we computed the mean square displacement (MSD) as follows:

$$
\langle \Delta r^2 \rangle = \left\langle \frac{1}{N} \sum_{l=1}^{N} (x_l (k) - x_l (0))^2 + (y_l (k) - y_l (0))^2 \right\rangle \tag{2.1}
$$

Where \( x, y \) are spatial coordinates of particle \( l \) in image number of \( k \). \( N \) represents the total number of particles. As expected for normal diffusive motion, the MSD values scaled linearly with time.

To simulate the FLIM experiment, we assumed sinusoidal behavior of the detected intensity as a function of phase delay between fluorescence and detector. Moreover, for simplicity we assumed single exponential decay of the fluorescence with fixed phase (\( \varphi \)) and modulation (\( m = \cos \varphi \)). The total intensity as a function of image number and pixel location is then given by the equation,

$$
I_{i,j} (k) = n_{i,j} (k) \left[ 1 + \cos \varphi \cos \left( \frac{2\pi}{10} k - \varphi \right) \right] \tag{2.2}
$$

Where \( i \) and \( j \) are pixel indices and \( n_{i,j} (k) \) is the number of particles in a pixel element (e.g. 0, 1, 2, ...) in point-like particles ensemble and is the element of Gaussian matrix for single Gaussian ensemble, both at image number \( k \) (derived from the Brownian motion simulations). To extract the phase and modulation of the detected signal we used Fourier analysis techniques as described in detail elsewhere.

By inspection of equation (2.2) it is clear that if \( n_{i,j} (k) \) is a constant in a particular pixel for all \( k \) then the phase of the detected signal will be \( \varphi \) and the modulation will be \( m = \cos \varphi \). We put \( \varphi = \pi /6 \) in our simulation then as shown in Figure 2.1A, the simple output is \( (m \cos \varphi, m \sin \varphi) = (0.75, 0.43) \). However, if \( n_{i,j} (k) \) is not constant for all \( k \)s, i.e. particles move in or out of the pixel, then the detected phase and modulation will not be equal to \( \varphi \) and \( \cos \varphi \), respectively.

In the latter case the phasor plots contain several points related to different detected phase and modulation. The mean value of the phasor \( (\langle m \cos \varphi \rangle, \langle m \sin \varphi \rangle) \)
was determined by;

\[
\langle m \cos \varphi \rangle = \frac{1}{n} \sum (m \cos \varphi)_i
\]

\[
\langle m \sin \varphi \rangle = \frac{1}{n} \sum (m \sin \varphi)_i
\]

Where \(\sum\) represents the sum of \(n\) data points.

The variance was computed using the equation

\[
Var = \sqrt{\langle R^2 \rangle - \langle R \rangle^2}
\]

Where \(R\) is the distance between the mean phasor value and a given point on the phasor plot, \(R^2\) is the square of the distance, \(\langle R^2 \rangle\) is the average squared-distance for all points and \(\langle R \rangle^2\) is the average distance for all points-squared.

To stimulate noise into our simulation, we exploit multiplication of random numbers to detected intensity that could have an effect only on specific percentage of detected intensity of a bead as shown in equation (2.5).

\[
I_{i,j}(k) = n_{i,j}(k) \left[ 1 + \cos \varphi \cos \left( \frac{2\pi}{10} k - \varphi \right) \right] (1 - p + p \xi_n)
\]

Where \(p\) is fraction of detected intensity that fluctuates, \(\xi_n\) is a random number in the range of 0 to 1 for each \(n_{i,j}(k)\).

In simulation of particle motions, we can adjust parameters of Langevin equation to produce different ensembles with various MSDs. Then we use these ensembles in simulation of FLIM experiment. At the end, we relate variance in phasor plot caused by movement of beads to MSD of movements.

Based on the nature of random walk, ensembles with the same MSD may produce slightly different variance on phasor plot depend on history of movements (e.g. initial random position of beads and probable passing border of pixel). It can cause an error
in relating diffusion coefficient to variance so that we needed to take this variance error into account.

### 2.2.2 Fluorescence lifetime imaging microscopy

To confirm our simulation results experimentally, we performed FLIM measurements on moving fluorescent beads. Coverslips were washed with methanol and surfaces were then treated with 1× phosphate buffered saline (PBS) solutions plus 5% bovine serum albumin (BSA). This protein treatment pacified the surface and reduced the likelihood of bead adsorption during experimentation.

10μL of 2.5 micron fluorescent beads (100% intensity, In-Speck, Invitrogen) in 200μL water and 1× PBS plus 5% BSA was placed between two coverslips. A Nikon microscope with Lambert instruments LIFA (The Netherlands) FLIM attachment was employed to measure FLIM images of the moving beads (see Chapter I for details) [83]. The beads were excited with sinusoidally-modulated (40 MHz) 470 nm light focused through a 100X, 1.4NA oil objective and the emission was observed through a 515LP filter. Ten phase steps were recorded at four different exposure times (2ms, 4ms, 5ms and 10ms) using software provided by the manufacturer. Rhodamine 6G in distilled water (lifetime = 4.1 ns) was used as a reference [80]. Lambert LI-FLIM software was used for all analysis of experimental data.

The phasor plots were exported using the Lambert LI-FLIM software. The mean value of the phasor and its variance were computed using equations 2.3 and 2.4, respectively. The lifetimes of the beads were \( \tau_\phi = 4 \text{ns} \) and \( \tau_m = 4 \text{ns} \) (determined from an immobilized sample).
2.3 Results and discussion

2.3.1 Frequency-domain FLIM at steady-state

FLIM measures the lifetime of fluorescence on a pixel-by-pixel basis [80, 84]. In the frequency-domain method the intensity at a single pixel follows a sinusoidal profile as a function of phase delay (homo-dyne) or time (heterodyne detection). Analysis of the function, using Fourier techniques delivers the phase and modulation of the detected signal. Detailed presentations of the theory are given elsewhere [78, 84–86], for homodyne method it can be found in Chapter I of this thesis too.

Let us first consider conventional FLIM where the concentration of species is assumed to be either fixed or at steady-state during acquisition. For simplicity we consider a simple 4-phase homodyne experiment as this is analytically tractable. Beginning with the detected signal as a function of the image number \( k \) and comparing with equation 1.12 in Chapter I, we can write;

\[
I_k(\phi) = DC + AC \cos \left( \frac{2\pi}{4} k - \phi \right) \quad (2.6)
\]

Where \( DC \) is unmodulated signal component, \( AC \) is the amplitude of the modulation signal component, and \( \phi \) is a phase difference between the excitation and emission. If we measure the intensities at detector phase positions 0, \( \pi/2 \), \( \pi \), \( 3\pi/2 \) (corresponding to \( k = 0, 1, 2, 3 \)) then the resulting intensities will appear as;

\[
\begin{align*}
I_0(\phi) &= DC + AC \cos \phi, \\
I_1(\phi) &= DC + AC \sin \phi, \\
I_2(\phi) &= DC - AC \cos \phi, \\
I_3(\phi) &= DC - AC \sin \phi \\
\end{align*} \quad (2.7)
\]

Using Fourier techniques, the phase and modulation of the detected signal can be
determined from the cosine and sine transforms (equation 2.8 and 2.9).

\[ F_{\text{sin}} = \frac{1}{2} \sum_{k=0}^{3} \sin \left( \frac{2\pi k}{4} \right) I_k(\varphi) = (I_1 - I_3)/2 = AC \sin \varphi, \]

\[ F_{\text{cos}} = \frac{1}{2} \sum_{k=0}^{3} \cos \left( \frac{2\pi k}{4} \right) I_k(\varphi) = (I_0 - I_2)/2 = AC \cos \varphi, \]

\[ F_{\text{DC}} = \frac{1}{4} \sum_{k=0}^{3} I_k(\varphi) = DC \quad (2.8) \]

The phase and modulation are given by;

\[ \varphi = \arctan \left( \frac{F_{\text{sin}}}{F_{\text{cos}}} \right) = \varphi, \]

\[ m = \left( \frac{1}{F_{\text{DC}}} \right) \sqrt{(F_{\text{sin}})^2 + (F_{\text{cos}})^2} = AC/DC \quad (2.9) \]

By inspection of equation 2.7, the components of the phasor can be represented in terms of intensities and are given by the equations;

\[ m \cos \varphi = \frac{AC \cos \varphi}{DC} = \frac{2}{I_0 + I_1 + I_2 + I_3} (I_0 - I_2), \]

\[ m \sin \varphi = \frac{AC \sin \varphi}{DC} = \frac{2}{I_0 + I_1 + I_2 + I_3} (I_1 - I_3) \quad (2.10) \]

Note that we do not explicitly mention the modulation frequency in these equations since in the homodyne method one records essentially a steady-state signal at each phase setting (k) on the detector. The modulation frequency appears intrinsically in the relationship between the phase, modulation and the fluorescence lifetime (for example, for a single component lifetime system, \( \tan \varphi = \omega \tau \) where \( \tau \) is lifetime, \( \omega \) is the modulation frequency and \( \varphi \) is the phase).
2.3.2 Frequency-domain FLIM including single particle fluctuations

Let us now consider the effect of particle fluctuations on the phase and modulation values or more precisely the phasor components. We will consider the most rudimentary type of particle fluctuation- the particle is either inside the observation volume at a given time and has an intensity value \( I(\phi) \) (see equation 2.2) at the observed detector phase position or is not inside the observation volume at that time (or phase position) and has an intensity value of zero. Table 2.2 lists the possible binary combinations of particle occupancies and corresponding modulation and phase values (represented by the phasor components) for the 4-phase example using equation 2.10. It is clear that if the particle is present in the observation volume during all phase recordings then the phase and modulation measured will be the true phase and modulation expected. However, if the particle occupancy is not constant during all 4 phase recordings then the phase and modulation values no longer represent the true lifetime of the fluorophore. A very convenient depiction of these effects is with the polar plot (phasor plot or AB-plot) which is a plot of \( x = m \cos \phi \) as a function of \( y = m \sin \phi \), see Figure 2.1. For the non-fluctuation case, the fluorescence appears as a single point on the phasor diagram, as expected. (Parenthetically we recall that for all time-resolved fluorescence decays, even of multicomponent fluorescence with non-negative amplitudes the phasor positions are located within the semi-circle \([19]\)). However it is seen that inclusion of fluctuations can cause an expansion in the possible values on the phasor plot. The maximal excursion appears to occur at values of \((2,0),(0,2),(0,-2),(-2,0)\) (Table 2.2) where the particle appears only once during 4-phase acquisitions. Simulations with other numbers of phase steps indicate that the magnitude of the phasors during motion also do not exceed 2 (see also example for ten phase steps). This is not a simulation effect but rather comes from the nature of the equations. Note that the actual phasor position provides a unique 2D map of the particle occupancy sequence (or more precisely the
We only consider single particle occupancies here at this point but investigate other types of occupancies later using simulations.

Table 2.2: Computed phasor components as a function of particle occupancy for an idealized four-phase FLIM experiment.

<table>
<thead>
<tr>
<th>Occupancy</th>
<th>Intensity</th>
<th>x</th>
<th>y</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 1, 1, 1</td>
<td>$I_0, I_1, I_2, I_3$</td>
<td>$m \cos \varphi$</td>
<td>$m \sin \varphi$</td>
</tr>
<tr>
<td>0, 1, 1, 1</td>
<td>0, $I_1, I_2, I_3$</td>
<td>$\frac{2m \cos \varphi - 2}{3 - m \cos \varphi}$</td>
<td>$\frac{4m \sin \varphi}{3 - m \cos \varphi}$</td>
</tr>
<tr>
<td>1, 0, 1, 1</td>
<td>$I_0, 0, I_2, I_3$</td>
<td>$\frac{4m \cos \varphi}{3 - m \sin \varphi}$</td>
<td>$\frac{2m \sin \varphi - 2}{3 - m \sin \varphi}$</td>
</tr>
<tr>
<td>1, 1, 0, 1</td>
<td>$I_0, I_1, 0, I_3$</td>
<td>$\frac{2m \sin \varphi + 2}{3 + m \cos \varphi}$</td>
<td>$\frac{4m \sin \varphi}{3 + m \cos \varphi}$</td>
</tr>
<tr>
<td>1, 1, 1, 0</td>
<td>$I_0, I_1, I_2, 0$</td>
<td>$\frac{4m \cos \varphi}{3 + m \sin \varphi}$</td>
<td>$\frac{2m \sin \varphi + 2}{3 + m \sin \varphi}$</td>
</tr>
<tr>
<td>0, 0, 1, 1</td>
<td>0, 0, $I_2, I_3$</td>
<td>$\frac{2m \cos \varphi - 2}{2 - m \cos \varphi - m \sin \varphi}$</td>
<td>$\frac{2m \sin \varphi - 2}{2 - m \cos \varphi - m \sin \varphi}$</td>
</tr>
<tr>
<td>1, 0, 1, 0</td>
<td>$I_0, 0, I_2, 0$</td>
<td>$2m \cos \varphi$</td>
<td>0</td>
</tr>
<tr>
<td>1, 1, 0, 0</td>
<td>$I_0, I_1, 0, 0$</td>
<td>$\frac{2m \cos \varphi + 2}{2 + m \cos \varphi + m \sin \varphi}$</td>
<td>$\frac{2m \sin \varphi + 2}{2 + m \cos \varphi + m \sin \varphi}$</td>
</tr>
<tr>
<td>1, 0, 0, 1</td>
<td>$I_0, 0, 0, I_3$</td>
<td>$\frac{2m \cos \varphi + 2}{2 + m \cos \varphi - m \sin \varphi}$</td>
<td>$\frac{2m \sin \varphi - 2}{2 + m \cos \varphi - m \sin \varphi}$</td>
</tr>
<tr>
<td>0, 1, 1, 0</td>
<td>0, $I_1, I_2, 0$</td>
<td>$\frac{2m \cos \varphi - 2}{2 - m \cos \varphi + m \sin \varphi}$</td>
<td>$\frac{2m \sin \varphi + 2}{2 - m \cos \varphi + m \sin \varphi}$</td>
</tr>
<tr>
<td>0, 1, 0, 1</td>
<td>0, $I_1, 0, I_3$</td>
<td>$0$</td>
<td>$2m \sin \varphi$</td>
</tr>
<tr>
<td>1, 0, 0, 0</td>
<td>$I_0, 0, 0, 0$</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>0, 1, 0, 0</td>
<td>0, $I_1, 0, 0$</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>0, 0, 1, 0</td>
<td>0, 0, $I_2, 0$</td>
<td>$-2$</td>
<td>0</td>
</tr>
<tr>
<td>0, 0, 0, 1</td>
<td>0, 0, 0, $I_3$</td>
<td>0</td>
<td>$-2$</td>
</tr>
</tbody>
</table>
Now consider a collection of observation volumes in an imaging experiment. In an imaging arrangement with an array detector such as a CCD camera, each volume element can be considered a pixel or set of pixels. If we consider very fast movement of a single particle from one region to the next to the next, etc will produce a \((1,0,0,0)\) occupancy sequence in one region, a \((0,1,0,0)\) occupancy in the next, \((0,0,1,0)\) and so on. The resulting single phasor for one stationary particle (no movement) will become 4 phasors for a rapidly moving particle. We refer to the change in a single phasor to multiple phasors as phasor broadening.

### 2.3.3 Phasor-FLIM during motion of a single large Gaussian particle

The aforementioned example is somewhat idealized because the particle fluctuations are considered as binary events. We simulated a FLIM experiment with a moving Gaussian particle of a size that occupies many area elements (21 by 21 pixels). The phasor of the particle without movement is shown in Figure 2.2A. The calculated phasor plot with increasing extents of stochastic particle movement (over 10 phase steps) is shown in Figures 2.2B-2.2F for comparison. As the extent of motion is increased the number of points outside the semi-circle on the phasor plot increases. At maximal movement simulated, the phasor diagram contains 10 points located at a radius of 2 on the phasor plot. This is analogous to the 4-phase example.
Figure 2.2: Phasor plots for FLIM during movement of a single particle. Simulation of the motion of single Gaussian particle projected to 21 by 21 CCD pixels each 6.45 $\mu m$ by 6.45 $\mu m$. A. Phasor plot for Gaussian particle with no motion. B. Phasor plot for small amount of Brownian motion where projected MSD on CCD is $5.63 \times 10^{-5} \text{mm}^2$ at the $10^{th}$ image. In this case the variance in phasor plot is 0.59. C. Phasor plot for larger extent of motion (with projected MSD on CCD of $1.69 \times 10^{-4}\text{mm}^2$ at the $10^{th}$ image). The variance in phasor plot is 0.76. D. (MSD, Variance) = $(2.72 \times 10^{-3}\text{mm}^2, 1.07)$ E. (MSD, Variance) = $(3.29 \times 10^{-2}\text{mm}^2, 1.82)$ F. (MSD, Variance) = $(2.28\text{mm}^2, 2)$. 

50
2.3.4 Phasor-FLIM for an ensemble of particle motions

For random (essentially Brownian) motion a particle ensemble will undergo different types of occupancy fluctuations (e.g. 0,1,2,0,3 etc). It is useful to simulate these effects and to calculate the resulting phasor diagram. For this purpose we simulated the effects of having a large fixed number of Brownian particles of point-like size and determined the influence of extent of motion on the phasor plot (see Figure 2.3). Figure 2.4 plots the mean-squared displacement as a function of time from the simulations. Note the linearity of the MSD versus time plot is consistent with random motion. Figure 2.3 displays corresponding phasor plots for different extents of motion. Qualitatively it is clear that with no motion all particles have identical phasor position while with some random motion some of the particles have phasor positions that are significantly perturbed with respect to the expected position. Increasing the extent of motion increases the number and excursion of points away from the expected phasor position. Plotting the variance in the phasor plot as a function of time (Figure 2.4) and MSD (Figure 2.4) it is clear that the blooming of the phasor plot, reflected in the increase in variance, increases with increasing particle movement up to a certain point where the variance is independent of extent of motion. At this point the phasor positions have reached their extreme positions.
Figure 2.3: FLIM simulation of collection of point particle motions. Six different ensembles of 100 beads with stochastic movement were used in the simulation. A. Phasor plot of 100 beads with no motion. B. Phasor plot with 100 beads with some Brownian motion with MSD \(= 9.9 \times 10^{-7} \text{ mm}^2\) (variance in phasor plot is 0.55) C. Phasor plot with increased particle motion (MSD, Variance)\(= (1.43 \times 10^{-5} \text{ mm}^2, 1.37)\) D. Phasor plot (MSD, Variance)\(= (5.72 \times 10^{-5} \text{ mm}^2, 1.61)\) E. Phasor plot (MSD, Variance)\(= (1.36 \times 10^{-2} \text{ mm}^2, 1.99)\) F. Phasor plot (MSD, Variance)\(= (7.4 \times 10^{-1} \text{ mm}^2, 2)\).

### 2.3.5 Effect of noise and motion on phasor plots

In real FLIM images, even of homogeneous solutions, the phasor plots have some finite width due to noise in the system (for an excellent discussion of noise
Figure 2.4: A. Plot of the variance in phasor plot as a function of time compared with the MSD as a function of time for three different diffusion coefficients where square symbol represents MSD and triangle represents variance. Red represents particles diffusing with $D = 4.95 \times 10^{-4} \text{mm}^2\text{s}^{-1}$, Blue represents simulation for particles with diffusion constant $D = 1.0 \times 10^{-3} \text{mm}^2\text{s}^{-1}$ and Green represents $D = 1.51 \times 10^{-3} \text{mm}^2\text{s}^{-1}$. The plot was generated from simulation of 100 point-like beads. B. Same plot as A but for two single Gaussian particles.

refer to [87]). As an indication the measured phase and modulation lifetimes have typical standard deviations of about 0.1 ns under optimal measurement conditions. Clearly, even noisier FLIM images result when photon noise increases in the case of weak emission or for rapid FLIM acquisition. We have simulated the effect of adding noise on the width of resulting phasor plots for no particle movement and for varying degrees of particle motion. As expected, adding noise increases the width of phasor plots even without any particle motion (see Figure 2.5). However noise has a less dramatic effect on the total phasor broadening for particles undergoing motion. This is illustrated in the inset of Figure 2.5A. For example when the variance in the phasor plot due to particle motion is large, adding noise does not appreciably increase the total variance. The motion-effect on the variance is much larger than the noise effect.
Figure 2.5: Effect of noise on phasor plots for FLIM. A. Plot of the variance in phasor plot as a function of noise (red line, main figure) for static beads. Inset; Plot of variance versus noise for different extents of particle motion where green, blue, brown, black and pink lines represent ensembles used with different MSDs. B. Phasor plot of static beads without noise. C. Static beads with 100% noise.

In practice photon noise will be always present in the detector and this noise will depend on the features of the system as well as the exposure time. In general increasing the exposure time will decrease the photon noise due to signal integration.
If the exposure time is faster than the transit time of a particle through the observation volume then increasing the exposure time will increase the chance of motion and increase the variance in the phasor plot. On the other hand if the exposure time is already too long—much longer than the transit time for particle movement through the observation volume—then increasing the exposure time will simply increase the averaging of signals. Therefore in practice any increase in the variance of the phasor plot with increased exposure time must be due to motion effects and not noise.

2.3.6 Effect of noise and motion on phasor plots

To provide a concrete experimental example, we measured frequency-domain FLIM images of 2.5 micron diameter fluorescent beads. We used a commercially-available FLIM set-up under wide-field excitation/detection conditions using ten phase steps. To vary the probability of motion during acquisition we varied the exposure time per phase image. Figure 2.6 displays phasor plots of the fluorescent beads as a function of exposure time. Figure 2.6A, 2.6B, 2.6C and 2.6D respectively show that increasing the exposure time increases the broadening in the phasor plots. For fast exposures of 2 ms, the phasor plot resembles a fluorophore in a nearly homogenous environment (Figure 2.6A). At the other extreme, (Figure 2.6C) a 10 ms second exposure per image causes a significant broadening in the phasor plot. Based on shot noise considerations alone, an increase in exposure time from 2 ms to 10 ms should increase the signal to noise ratio by a factor of \( \sqrt{5} \). This would be expected to decrease the width of the phasor plot in the absence of particle motion. Therefore the observed increased broadening in the phasor plot cannot be due to increases in signal to noise ratio from increasing the exposure time. A more likely explanation is that the phasor broadening is due to particle motions.

Table 2.3 collates the measured phasor plot variances from the FLIM experiment as a function of exposure time. To relate the variance to a diffusion coefficient we first prepared 45000 ensembles of two stochastically moving particles with different
Figure 2.6: Experimental FLIM measurement of fluorescent beads. A. Phasor plot for an exposure time of 2 ms per image (phasor plot variance is 0.2438) B. Phasor plot (exposure time of 4 ms per image and phasor variance of 0.3104) C. Phasor plot (exposure time of 5 ms per image and phasor variance is 0.3256) D. Phasor plot (exposure time of 10 ms per image and phasor variance of 0.3968).

MSDs. Then we used the ensembles in simulation of FLIM experiment to compute phasor plot variances (see Figure 2.7). This plot reveals a non-linear, sigmoidal-like dependence of the phasor plot variance upon the extent of motion. In other words, when the particles do not diffuse significantly from the observation volume, the corresponding variance in the phasor plot is negligible, however when particle diffuse away from the initial positions the resulting intensity fluctuations increase.
Table 2.3: Motion of fluorescent beads determined by phasor-FLIM.

<table>
<thead>
<tr>
<th>Variance ± Std</th>
<th>Total elapsed time (ms)</th>
<th>MSD ($mm^2 \times 10^{-5}$) ± Std</th>
<th>D ($mm^2 s^{-1} \times 10^{-8}$) ± Std</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.24375 ± 0.00001</td>
<td>20</td>
<td>2.0 ± 0.8</td>
<td>2.6 ± 1.0</td>
</tr>
<tr>
<td>0.3104 ± 0.0001</td>
<td>40</td>
<td>4.0 ± 1.0</td>
<td>2.5 ± 0.8</td>
</tr>
<tr>
<td>0.3356 ± 0.0002</td>
<td>50</td>
<td>5.0 ± 1.0</td>
<td>2.3 ± 0.7</td>
</tr>
<tr>
<td>0.397 ± 0.002</td>
<td>100</td>
<td>9.0 ± 2.0</td>
<td>2.2 ± 0.6</td>
</tr>
</tbody>
</table>

The variance.

We used our fitted curve relating variance to MSD to determine the MSD and diffusion coefficient ($D = MSD/(4 \times \text{time})$), as shown in Table 2.3. By inspection it is clear that the MSD increases with exposure time, as expected. The computed apparent diffusion coefficient, D, ranges from $2.2 - 2.6 \times 10^{-8} \ mm^2/s$ and does not appear to vary greatly with exposure time. This suggests that the motion sampled is Brownian-like.

There are two different source of error in our derivation of the diffusion coefficient. The first source of error is essentially sampling error. From computer simulations we found that for a given set of conditions there was a variation in the computed MSD and phasor plot variance. This error gives rise to the broad line in Figure 2.7B. The second source of error is in the experimental determination of the variance in the phasor plot. We determined this second error experimentally by replicate measurement of the polar plot variance of a rhodamine 6G solution under different conditions that is much less than the former error by the factor of 10.

The experimental result can be compared with theoretical calculation based on the Stokes-Einstein relationship. For beads with radius of 1.25µm in a solution with viscosity $2.6 \times 10^{-3} N \ s \ m^{-2}$ (PBS viscosity) and at 19 °C, the diffusion coefficient
A. Plot of the phasor plot variance as a function of MSD from simulations of two moving Gaussian particles. Data was obtained from simulations of three different diffusion coefficients; red symbols ($D = 1.0 \times 10^{-3} \text{ mm}^2\text{s}^{-1}$), blue symbols ($D = 4.65 \times 10^{-3} \text{ mm}^2\text{s}^{-1}$), and green symbols ($D = 9.94 \times 10^{-3} \text{ mm}^2\text{s}^{-1}$). B. Plot of the phasor plot variance as a function of the logarithm of MSD. Points are taken from A and solid line is a fit to the data. Fitted curve function is $y = \left(c_1 + c_2 \sqrt{x} + c_3 x\right) / \left(1 + c_4 \sqrt{x} + c_5 x\right)$ where $c_1 = -0.0039$; $c_2 = 76.1$; $c_3 = 11780$; $c_4 = 106.9$; $c_5 = 559.6$. 

Figure 2.7:
is calculated to be $6.4 \times 10^{-8} \ mm^2 s^{-1}$. This is within an order of magnitude agreement with the experimental result of $2.2 - 2.6 \times 10^{-8} \ mm^2 s^{-1}$. This is reasonable agreement given that the apparent diffusion coefficients are derived from 10 images of two particles and we have ignored possible influences of BSA and cover-slips on our theoretical estimates.

2.4 Scope of the method

The simulations and experiments were designed to examine the effect of particle motions of fluorescence lifetime imaging microscopy experiments. We deliberately used conditions that would produce the largest effects i.e. single particles and beads. Other conditions (micromolar concentrations) may produce too small a fluctuation to be visible using the phasor approach. This aspect needs to be tested further. One might ask what is new about this approach since methods for determining diffusion coefficients are already well developed. The first difference is that the fluorescence lifetime of the particles can also be determined using this experimental approach. For example, by averaging the data into one pixel the lifetime of the moving particles can be determined. The second difference, is that the position of a point on the phasor plot encodes information about the particle occupancy or intensity fluctuation in that pixel (or group of pixels). For example, if Table 2.2 we showed that each type of particle occupancy encodes a particular point on the phasor plot.

Reader should be aware that any process that changes the intensity during acquisition will cause a broadening of the phasor plot, in other words changes in noise, diffusion or changes in particle number or changes in the brightness of the particles. Noise can be separated from the other effects by doing experiments with a fixed acquisition time but varying the time-delay between image acquisitions. Because the camera is exposed to the same number of photons in each case the level of noise
is expected to be the same. However if by changing the delay between images other changes in intensity can be observed.

2.5 Conclusions

In the absence of motion a FLIM experiment represents the lifetime of a sample. We have examined the effect of particle motions on FLIM experiments with a particular focus on motions that produce large intensity fluctuations on the timescale of image acquisition. Motions are conveniently revealed on the phasor diagram. The position of a given pixel on the phasor plot reflects both the intrinsic lifetime of the fluorescent particle and the occupancy history of the particle in that pixel. For population of pixels, the variance of the phasor values is shown to be related to the extent of motion during acquisition. It is shown that the values of the phasor during motion are constrained to lie within a circle with radius 2 in the phasor plot.

This method possess some novel advantages that allow FLIM users to study dynamical parameters while they examine lifetime. There are some other advantage as follow:

• There is no need to track particle to determine MSD.

• Shown that the MSD of a particle in an image can be obtained directly from a lifetime image if image acquisition is comparable to timescale of motion.

• Particles and tracers can be used to measure diffusion coefficients and mechanical properties of cells.

• Lifetimes can be used to measure environments and interactions.

• Being able to measure both motion and lifetime in the same experiment.

Use of beads as tracers for cell mechanics has the advantage that changes in particle number or particle brightness are not expected to contribute significantly
to the phasor broadening. Further experimentation and developments are required to adapt this method for molecules in cells.
CHAPTER III

Solvent relaxation dynamics in Golgi and plasma membranes of living HeLa cells

3.1 Preamble

In the previous chapter FLIM was used to measure the dynamics of a fluorescent particle moving through a solvent. In this chapter we deal with a different type of motion- the motion of the solvent molecules.

Solvent or dipolar relaxation is a fundamental process that occurs in the condensed phase. Chemical as well as biological reactions can be influenced by the rate of dipolar relaxations. Examples include charge transfer reactions through to protein and membrane dynamics. From a cell biology perspective, one would like to have an experimental measure of these processes within the complex environment of the living cell.

In the last few decades, studies addressing solvent relaxation in membranes have come closer into focus. The solvent relaxation method is able to detect viscosity and polarity changes in the local environment of a probe in three membrane domains; the external interface, the headgroup region and the hydrocarbon backbone region. The solvent relaxation times are different depending to the located region of probes. In bulk aqueous solution the relaxation time is in order of few pico seconds. Near the external interface of membrane, the solvent relaxation time is in the range of
sub-nano seconds. In the headgroup region of a bilayer the relaxation time is within
the range of nano seconds and in the hydrophobic backbone it is in the order of
several nano seconds [88, 89].

There are variety of fluorescent approaches to measure solvent relaxation time.
For instance, Time-Resolved Emission Spectra (TRES) which is based on measurement
of emission spectrum as a function of time to characterise solvent relaxation quantitatively in pure solvents or in membranes [88, 90]. Also for a large dipole
moment difference between ground and excited states where dipolar relaxation time
is comparable to the fluorescent lifetime, one can exploit Red-Edge Excitation Spectroscopy (REES) [91]. REES is a steady-state fluorimetry method that is based on a
shift in the wavelength of the maximum fluorescent emission of polar fluorophores to
higher wavelength by shifting the excitation wavelength toward the red-edge of the
absorption band. In other words, the emission spectrum from a fluorescent probe
will undergo a detectable red-shift from the initially excited Franck-Condon state
to the solvated or relaxed state. This spectral relaxation can be followed in time
using time-resolved fluorescence spectroscopy [89, 92]. Alternatively, the presence
of a red-shifted steady-state emission spectrum upon excitation into the red-edge of
the absorption (REES) is also a signature of dipolar relaxation on the fluorescence
time-scale [93]. REES can work perfectly in notionally restricted media like very
viscous media, condensed phases, biomembrane or liquid-crystalline phase [94–98].

In frequency-domain, the presence of solvent relaxation as an excited-state re-
action results in that the phase shift exceeds modulation depth where the quantity
m/cosϕ gets bigger than 1. It can lead to a conversion between phase and modula-
tion lifetimes where in the red side of emission, we have τϕ > τm [99].

Lifetime measurement has considerably evolved our current picture of solvent
relaxation process in biomembranes and in general the structure and function of
biomembranes [27, 100–106]. NBD, Prodan, Laurdan and Patman have played the
main roles in this road as fluorophors. NBD (as a relatively polar fluorophore)
can be attached to the polar headgroup of phospholipids to examine the relaxation dynamics on the level of the glycerol and the headgroup regions of the bilayer \[107\]–\[109\]. Laurdan and Patman can be used to study relaxation dynamics of the glycerol region \[110\]–\[111\].

NBD group have a rich history to use as labels of lipids to study cell membrane \[93\]–\[105\]–\[112\]–\[115\]. They have been widely used as fluorescent tags in studies of membrane because of their resemblance to native lipids of biological membrane. NBD group are weakly fluorescent in aqueous solvents while in aprotic medium their emission wavelength is in visible range with high degree of sensitivity to local environment particularly to polar environment. The NBD is mildly polar then phospholipid with an NBD-labeled head group tend to locate the lipid-water interface region of membranes rather than the hydrophobic interior \[113\]–\[115\].

While sphingolipids are vital parts of the plasma membrane of cells, ceramides are the biological building blocks of sphingolipids. Metabolism of ceramides typically occurs in Golgi and endoplasmic reticulum membranes. NBD ceramide is widely used as vital stain for Golgi apparatus \[116\]. On the other hand, there are other derivatives of NBD to use for studying other type of membranes, for example NBD phospholipid has been used to characterise lipid domains in giant unilamellar vesicles via FLIM \[105\].

We use a Golgi-specific membrane probe, NBD-ceramide, which preferentially partitions into the membranes of the Golgi apparatus. Fluorescence lifetime imaging microscopy with tunable emission wavelength detection is our experimental approach to determine the spatial distribution of the NBD-ceramide probe and its associated excited-state dynamics in single living HeLa cells. Surprisingly we reveal that the dipolar solvation dynamics occurs on a sub-nanosecond time scale in the Golgi membranes as distinct from the plasma membrane where the corresponding dynamics is nanoseconds. We suggest that this altered dynamics is linked to the function of the Golgi and may provide a biological marker for diseases linked to
In this chapter, we report three outcomes of our research to measure solvent relaxation. First, we develop a simple new method to measure solvent relaxation time by means of frequency domain FLIM. Second, we test the new method with commercially available fluorescent probe, NBD-X, in glycerol-water mixtures. Third, we apply the new method to measure the dipolar dynamics in the membranes of living cells.

### 3.2 Materials and methods

#### 3.2.1 Stain preparation

$C_6$-NBD Ceramide (N-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl) amino]hexanoyl]-D-erythro-sphingosine) (Avanti polar lipids; Alabaster; AL) was used as a fluorescent lipid. 10 µL of chloroform solution of the fluorescent lipid was dried. Then it was dissolved in 1 mL DMEM to give a concentration of 5.75 µM $C_6$-NBD Ceramide. The solution was vortexed and kept at 4°C before use.

#### 3.2.2 Cell culture and treatments

HeLa Cells were cultured in flask for 2 days in DMEM (+ HEPES +5–10% FCS+1 : 100 Glutamate in 10% CO$_2$) at 37°C in CO$_2$ incubator. After splitting, cells were freshly plated onto chambered coverglass (Lab-Tek II; Thermo Fisher Scientific; Rochester; NY) and were incubated for 1 day. Cells were rinsed with phosphate buffered saline (PBS) twice. They were incubated again for 30 minutes with 1mL DMEM containing 40µL of prepared stain solution result in a final concentration of 0.7µM $C_6$-NBD Ceramide. Cells were washed with DMEM twice then with adding 1mL DMEM, they got ready for imaging.
3.2.3 Solution experiments

The solvent relaxation of NBD-X (6-((N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoic acid) (AnaSpec Inc; Fremont; CA) was measured in glycerol. NBD-X was dissolved in glycerol as a fluorescent dye in a stock concentration of 31.25 µM. To measure the effect of water, stocks of NBD-X in glycerol solution were prepared in glass containers with different volume percentage of added water (0%, 3%, 10%, 20% and 30% water).

3.2.4 Fluorescence lifetime imaging microscopy

A Nikon microscope (Model Ti, Nikon, Japan) with Lambert instruments LIFA (The Netherlands) FLIM attachment was employed to measure FLIM images of solutions and HeLa cells (see Chapter I for details). Samples were excited with sinusoidally- modulated (35 MHz) 474 nm light focused through a 100X, 1.4NA oil objective and the emission was observed through a hyper-spectral imaging system (His-400; Gooch & Housego; Orlando; FL) set at 530 ± 20.2 nm and 600 ± 20.8 nm, respectively. Twelve phase steps were recorded in pseudo-random order by using software provided by the manufacturer. Rhodamine 6G in distilled water (lifetime=4.1 ns) was used as a reference [80]. Lambert LI-FLIM software was used for all analysis of experimental data. The phasor plots were exported using the Lambert LI-FLIM software. To measure lifetime of solutions in a glass container, 40X NA 0.7 air objective was employed. The detected emission wavelengths were in the range from 520 nm to 620 nm in a span of 10 nm.

3.2.5 Confocal laser scanning microscopy

An Olympus FV1000 scanning laser confocal microscope was used to confirm the Golgi localisation of the C₆-NBD Ceramide dye in HeLa cells. Excitation of the dye was provided with the 488 nm laser line focused through a 100X oil objective.
Dye fluorescence was detected through a band-pass filter in the wavelength range 500 – 530 nm.

### 3.3 Results and discussion

#### 3.3.1 Solvent relaxation in viscous solvents

To evaluate the behaviour of NBD in a model solvent system we measured the excited-state decay behaviour of NBD-X dissolved in glycerol solvent. Figure 3.3.1 depicts the phase lifetime and modulation lifetime of NBD-X as a function of emission wavelength. Of particular note is the change in relative magnitude of the phase lifetime compared with the modulation lifetime as the detection wavelength is shifted from the blue region to the red region of the emission spectrum. Thus at wavelengths less than 560 nm we have $\tau_\phi < \tau_m$, whereas at wavelengths greater than 560 nm, $\tau_\phi > \tau_m$. As shown by Lakowicz and et. al. [101, 117, 118], this behaviour is a characteristic of solvent relaxation.
Figure 3.1: Lifetime data for NBD-X in glycerol as a function of emission wavelength. Lifetimes were derived from the measured phase (in black) and the measured modulation (in red) at a frequency of 35 MHz. Note the change in relative magnitude of $\tau_\phi$ and $\tau_m$ at wavelengths $> 560$ nm.

Figure 3.2 represents the data of Figure 3.3.1 in the form of a phasor plot where $x = m \cos \varphi$, $y = m \sin \varphi$ and m is modulation and p is phase. Data recorded from the red-part of the emission spectrum exhibited an increased phase compared to the blue part of the spectrum, which again is consistent with a solvent relaxation process (i.e. a phase delay in the emission from blue to red)[27]. Interestingly the data obtained from several wavelengths is well-approximated by a straight-line in phasor-space which suggests a two-state model is resolvable to describe the excited-state dynamics in this system. Because of this linearity we were also permitted to use data from two wavelengths to extract the relevant decay dynamics.

To provide a quantitative feel for the rates of solvent dipolar relaxation processes, we analysed the data according to an approximate model for the relaxation processes. This model considers a time-dependent spectral shift in terms of detected emissions
Figure 3.2: Phasor plot of NBD-X in glycerol for different detection wavelengths. Data corresponds to 11 FLIM measurements at detection wavelengths ranging from 520 nm to 620 nm with a shift of 10 nm.

at 530 nm and 600 nm. The Generalized Polarization (GP) is given by,

\[ GP = \frac{I_{530\,nm} - I_{600\,nm}}{I_{530\,nm} + I_{600\,nm}} \]  

(3.1)

During dipolar relaxation the GP will change from a value corresponding to the Franck-Condon spectrum (GP\(_0\)) to the GP value of the relaxed spectrum (GP\(_\infty\)). Assuming an exponential time course for the relaxation process with solvent-correlation time \( T_s \), the time-dependent GP function is given by

\[ GP(t) = (GP_0 - GP_\infty) e^{-\frac{t}{T_s}} + GP_\infty \]  

(3.2)

Assuming \( I_{total}(t) = (I_{530\,nm} + I_{600\,nm})(t) = I_0 e^{-t/T} \), one can find \( I_{530\,nm} \) and
with some manipulation, we can obtain

\[
I_{530\text{ nm}}(t) = \frac{1}{2} (1 + GP(t)) I_0 e^{-t/T} + \frac{1}{2} (GP_0 - GP_\infty) I_0 e^{-t/(\frac{1}{T_1} + \frac{1}{T_2})}
\]

\[
I_{600\text{ nm}}(t) = \frac{1}{2} (1 - GP(t)) I_0 e^{-t/T} - \frac{1}{2} (GP_0 - GP_\infty) I_0 e^{-t/(\frac{1}{T_1} + \frac{1}{T_2})}
\] (3.4)

These equations resemble double exponential decay \((\alpha e^{-t/T_2} + \beta e^{-t/T_1})\). The required dipolar relaxation time, Ts, is then given by the relation;

\[
\frac{1}{T_s} = \frac{1}{T_1} - \frac{1}{T_2}
\] (3.5)

Ts as well as the normal excited-state lifetime can be determined from this double exponential global analysis of FLIM data collected at 530 nm and 600 nm. As discussed in Chapter I of this thesis, the global analysis yields two time-constants, \(T_1\) and \(T_2\) that characterise the emission \([21, 119]\). This is analogous to polarisation decay experiments \([119]\).

Figure 3.3 represents phasor plots for NBD-X in glycerol-water solutions of differing composition recorded at 530 nm and 600 nm. Indicated \(T_1\) and \(T_2\) in this figure come from intersections of each extrapolated lines with universal semi-circle. We can use each set of measured lifetimes (related to blue and red sides of emission) to fit an extrapolated line in polar plot via \(m \sin \varphi = u + v m \cos \varphi\) where \(u\) (offset)
Figure 3.3: Phasor plot for NBD-X in 100% glycerol (red line), 97% glycerol/3% water (black line), 90% glycerol/10% water (blue line) and 80% glycerol/20% water (brown line). In each set there are two phasor points located outside and inside of semi-circle that correspond to detection wavelengths of 600 nm and 530 nm, respectively. Also there are two intersections between each fitted lines with guiding semi-circle. $T_1$ and $T_2$ for red line are displayed in the graph.

and $v$ (slope) come from [21]:

$$u = \frac{(m \sin \varphi)_r - (m \sin \varphi)_b}{(m \cos \varphi)_r - (m \cos \varphi)_b}$$

$$v = (m \sin \varphi)_r - u (m \cos \varphi)_r$$

where $b$ and $r$ indices represent blue (530 nm) and red (600 nm), respectively. $T_1$ and $T_2$ are intersections of the extrapolated line with semi-circle ($m = \cos \varphi$). They can be directly calculated from:

$$T_{1,2} = \frac{1 \pm \sqrt{1 - 4u(u + v)}}{2\omega u}$$

It is clear that adding water decreases the value of $T_2$, decreases the value of
and decreases the value of $T_s$. This is in accordance with the expected increase in polarity (affecting $T_2$) and decreased viscosity of glycerol (affecting $T_2$ and $T_s$) with increasing water fraction. For the 80% glycerol/20% water solution, the linear extrapolation yields a negative value of $T_1$ that may be related to some complex lifetime that is inside of semi-circle (not rely on semi-circle). Such a relaxation will not resolved with this proposed model that requires $T_1$ and $T_2$. A negative $T_1$ could result from associative solvation dynamics wherein a long lifetime-long correlation time probe fluorescence mixes with the fluorescence from a short lifetime-short correlation probe. Further work is needed to address this issue more carefully. It is important to note that solvation dynamics are quite complex and available estimates suggest that at least two correlation times are required to account for the solvation process. Our analysis assumes only one relaxation time. However, the derived relaxation time is a weighted average of the more complex process.

In a theoretical treatment, the Förster-Hoffman equation traditionally describes the relationship between fluorescence quantum yield ($\Phi_f$) and viscosity ($\eta$) as:

$$\Phi_f = z \eta^\alpha$$

where $z$ and $\alpha$ are constant. This equation can be simply adjusted to reveal dependency of fluorescent lifetime on viscosity [120]:

$$\tau_f = \frac{z}{k_d} \eta^\alpha$$

$$\log \tau_f = \log \left( \frac{z}{k_d} \right) + \alpha \log \eta$$

where $k_d$ is the radiative decay rate.

Figure 3.4 depicts the log-log relation between viscosity and $T_2$ for NBD-X in different glycerol water mixture. The percentage of water varies from 0 to 30 percent. It is clear from the graph that $T_2$ behaves like a real fluorescent lifetime with
The log-log plot of $T_2$ vs. viscosity for NBD-X in different glycerol water mixture. Varied colours show different water percentages, where red, green, blue, purple and black dots represent 0, 3, 10, 20 and 30 percent added water, respectively.

great sensitivity to viscosity. Viscosities of glycerol with different added water come from [121].

Figure 3.5 displays relation between $T_s$ and viscosity of NBD-X in glycerol water mixtures for three different water percentages, 0, 3 and 10%. It also shows a fitted curve on data that reveals a power relation between relaxation time and viscosity like equation 3.9.

As Figure 3.5 indicates, the solvent relaxation time for pure glycerol is $1.88 \pm 0.04 \, ns$. This value is in good agreement with other reports of $T_s = 1.98 \, ns$ (theoretical approach) from [122], $T_s = 1.8 \, ns$ (NMR experiment) from [123] and $T_s = 1.1 \, ns$
Figure 3.5: The plot of solvent relaxation time vs. viscosity for NBD-X in different glycerol water mixture in 20°C. Varied colours show different water percentages, where red, green and blue dots represent 0, 3 and 10 percent added water, respectively.

The plotted data follows the equation $y = 0.01x^{0.7239}$ with $R^2 = 0.999$. (frequency response of dielectric method) from [124], all in 20°C. As it has been previously reported, the solvent relaxation time of 40% glycerol/ 60% water is around 50 ps in the temperature of 20°C [125]. However the extrapolation of fitted line in Figure 3.5 can provide us with relaxation time of $T_s = 26$ ps for the same mixture with viscosity of $\eta = 3.72 \text{ cP}$ in 20°C that is in the same order of magnitude of the reported experimental relaxation time.

Taken together these experiments in solution demonstrate that our instrumentation and analysis procedures are capable to measure solvent relaxation time. We next turn to the application of these approaches to determining the solvent relax-
ation behaviour of Golgi membranes and plasma membranes in living cells. For this purpose we use NBD-ceramide, which has been shown previously to be a specific stain for the membrane of Golgi [126].

3.3.2 Solvent relaxation in the Golgi and plasma membranes of living cells

Figure 3.6 represents a typical confocal laser scanning image of a HeLa cell stained with NBD-ceramide. The image resembles previously published images of the probe localised to the Golgi membrane [126].

Figure 3.6: Confocal image of Hela cell stained with \(C_6\)-NBD-Ceramide (\(\lambda_{ex} = 488\ nm\)). 4% formaldehyde solution was used in fixation procedure.

Figure 3.7 depicts fluorescence intensity and lifetime images of a typical HeLa cell stained with NBD-ceramide obtained with our wide-field FLIM set-up. In this figure, lifetimes of three regions; Golgi membrane, excluded Golgi and outer cell
membrane are differentiated.

As can be seen in Figure 3.7A, most of the probe is located in internal structures (resembling Golgi) and excluded from the nucleus. There is weaker fluorescence from the plasma membrane, which can be excluded by adjusting intensity threshold. These observations are consistent with previously reported [126]. Figure 3.7B and C represent fluorescence lifetime images of the Golgi-stained membranes and Figure 3.7D and E non-Golgi membranes. When detected at 530 nm, the NBD-ceramide probe has a lifetime of about $7 \pm 0.2\, \text{ns}$ and largely independent of spatial location as determined from the phase or modulation of the emission. On the other hand, Figure 3.8 shows fluorescence intensity and lifetime images of the same HeLa cell stained with NBD-ceramide, but for $\lambda_\text{ex} = 470\, \text{nm}$ and $\lambda_\text{em} = 600 \pm 20.8\, \text{nm}$.

In Figure 3.8, differences in fluorescence lifetimes are more evident for the data collected at 600 nm. In both the Golgi and non-Golgi membranes the phase lifetime is significantly larger than the modulation lifetime ($\Delta 0.6\,\text{ns}$ for Golgi; $\Delta 1.0\,\text{ns}$ for non-Golgi). This is evidence for dipolar relaxation in the membranes of the HeLa cell in both the Golgi and in non-Golgi regions.
Figure 3.7: Fluorescence intensity and lifetime images of a HeLa cell stained with NBD-ceramide ($\lambda_{ex} = 470\text{nm}$, $\lambda_{em} = 530 \pm 20.2\text{nm}$). A. Intensity image of cell. Images in the left column represent modulation lifetime images where in the right column show phase lifetime images. Golgi membrane: B. $\tau_m = 6.99$; C. $\tau_\varphi = 6.79$. Cell excluded Golgi: D. $\tau_m = 6.94$; E. $\tau_\varphi = 7.04\text{ ns}$. Outer membranes: F. $\tau_m = 6.97$; G. $\tau_\varphi = 7.1\text{ ns}$. 
Figure 3.8: Fluorescence intensity and lifetime images of a HeLa cell stained with NBD-ceramide ($\lambda_{ex} = 470nm$, $\lambda_{em} = 600 \pm 20.8nm$). A. Intensity image of cell. Images in the left column represent modulation lifetime images where in the right column show phase lifetime images. Golgi membrane: B. $\tau_m = 7.08$; C. $\tau_\phi = 7.66$. Cell excluded Golgi: D. $\tau_m = 7.12$; E. $\tau_\phi = 8.12\text{ ns}$. Outer membranes: F. $\tau_m = 7.2$; G. $\tau_\phi = 8.13\text{ ns}$. 
Figure 3.9 represents FLIM measurement of Golgi membrane with three different detection wavelengths. Similar to Figure 3.2 data yielded from different wavelengths is well-approximated by a line which enable us to apply a two-state model for describing the relaxation dynamics.

Figure 3.9: Phasor plot representation of FLIM experiments of Golgi membrane with different detection wavelengths. Points correspond to three FLIM measurements at detection wavelengths of 530nm, 570nm and 590nm with colours of blue, orange and red, respectively.

Figure 3.10 and Figure 3.11 provide a representation of this analysis related to Figure 3.7 and Figure 3.8. In Figure 3.10 we have plotted the phasor clouds corresponding to the pixel-by-pixel data from the cells recorded at 530 nm and 600 nm. The average values of these clouds are shown by the red-dots in the main plot in Figure 3.10.
Figure 3.10: Phasor (or polar or AB) plot representation of FLIM experiments. The inset diagrams and associated phasor clouds represent lifetime information from individual cells at pixel resolution. The averages of the phasor clouds (the region-averaged phasors) collected from the blue side of the emission and the red part of the emission are depicted by the blue and red dots, respectively. A linear extrapolation is denoted by the black line with the two component lifetimes, $T_1$ and $T_2$ indicated by the positions of intersection of the line with the semi-circle. Note the position of one of the phasors is outside the semi-circle indicative of dipolar relaxation.

Values of $T_1$, $T_2$ and $T_s$ from 10 individual cells are summarised in Figure 3.11. $T_1$ is significantly smaller in the Golgi membranes than in the non-Golgi regions but $T_2$ are similar in all detected membranes. As a consequence $T_s$ is much smaller (0.83 ns) than in non-Golgi regions (3.46 ns) and outer membranes ($T_s = 2.94$ ns). This suggests that dipolar relaxation in the membrane interface of Golgi is less
restricted than non-Golgi membranes.

![Figure 3.11: Derived lifetimes ($T_1$, $T_2$) and dipolar solvent relaxation time ($T_s$) from 10 individual cells. Left columns denote Golgi membranes while middle columns indicate non-Golgi membranes and right columns show outer membranes. Magnitudes of the columns are related to the average value while the error bars represent one standard deviation. Note the significantly faster relaxation in the Golgi membrane ($T_s = 0.83 \text{ ns}$) compared to the non-Golgi and outer membranes ($T_s = 3.46 \text{ ns}$ and $T_s = 2.94 \text{ ns}$ respectively).]

This result is at first glance quite surprising. The Golgi apparatus provides an important role in modifying proteins ready for transport to other parts of the cell. Thus the membrane interface is expected to be crowded with proteins and enzymes. On this basis alone a more crowded environment should have a higher viscosity and correspondingly slower dipolar relaxation.

The reason for faster solvent relaxation may lie in the membrane composition of the Golgi which is in turn related to its biogenic function. The cis-Golgi is composed of thin bilayers with loose lipid packing, and neutral cytoplasmic charge. Loose lipid packing may contribute to faster overall dynamics. The plasma membrane, in contrast, contains thick bilayers with tight lipid packing related to its barrier function. This might partially explain why the solvent relaxation times are different.
for these two membranes.

There have been few studies on modelled membranes. For example, Laurdan was used to determine the relaxation time (0.66 ns) of phospholipid vesicles (DPPC) at the phase transition temperature [127]. In other study, Prodan was used to measure relaxation times in artificial unilamellar vesicles composed of HOPCS, AOPC and POPC lipids. The reported relaxation times were 2.1 ns, 2.3 ns and 3.0 ns, respectively [102].

Golfetto and et. al. [27] used phasor approach to show emission shift form blue to green in study of effects of cholesterol contents on membrane fluidity by dipolar relaxation of Laurdan, however they did not report the relaxation time. NBD lifetime in membrane has been reported as around 7 ns where it depends on both excitation and emission wavelengths. However, NBD lifetime decreases abruptly in presence of water to around 1 ns [95, 128]. The reported 7 ns for lifetime of NBD in membrane is in good agreement with our result. As we showed, dependency of NBD lifetime to excitation and emission wavelengths can be related to the relaxation time. Also Sasmal and et. al. [129] measured solvent relaxation time in cytoplasm and nucleus of CHO cells. They exhibited an average relaxation time of 1.3 ns for the cytoplasm and 0.78 s for the nucleus. Our finding of 0.83 ns for relaxation time of Golgi membrane is in the same order of magnitude with reported relaxation times for cytoplasm and nucleus.

3.4 Conclusion

In this chapter, a new method was developed to quantify dipolar solvent relaxation dynamics in an image. The method utilized a commercial frequency-domain FLIM set-up with tunable emission detection. Theory was developed to show that an average excited-state lifetime and an average solvent correlation time could be obtained from measurements with one modulation frequency and two detection wave-
lengths. Measuring only two wavelengths from blue and red side of emission is advantage of this method where it along with using Multi Channel Plate intensifier make the method a highly time and photon efficient.

The method was validated with a commonly-used fluorophore and solvents of known composition. Surprisingly good agreement (i.e. to within 20%) was found between the new method and other methods for detection of solvent relaxation in the nanosecond time range. A power relationship was noted between the measured solvent relaxation time and the viscosity of a series of glycerol-water solutions. For first time, the solvent relaxation time in Golgi membrane of living cell was measured. It was reported that the solvent correlation times in the plasma membranes were $2.94 \, ns$ in contrast to the much shorter correlation times of $0.83 \, ns$ in the Golgi membranes.
CHAPTER IV

Measuring Receptor Clustering by
Photobleaching Image Correlation Spectroscopy*

4.1 Preamble

Clustering of cell-surface macromolecules is thought to be an important biological control mechanism. For example, activation of receptors that control cell growth is generally thought to proceed through a ligand-mediated monomer to dimer transition. Aggregation of protein molecules is also considered to be important for diseases such as Alzheimer’s disease where loss of brain function is linked to neuronal cell death which in turn is linked to the formation of insoluble protein aggregates. Determining the exact nature of the oligomeric states of protein molecules on a cell surface is a difficult but important biophysical problem.

The signalling network plays a central role in development of different diseases like cancer [130, 131], Alzheimer’s disease [132] and heart disease [133]. The signalling network comprise cell surface receptors and binding ligands. Receptors include the Epidermal Growth Factor receptor, EGF receptor, that also called HER1 or ErbB1 along with other ErbB family members (ErbB2, ErbB3 and ErbB4). Binding of ligand to a receptor initiates oligomerisation that is crucial for activation of receptors and signalling. The ErbB family has been a main target to study cancer

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and to improve cancer treatment [134]. Modern microscopy methods developed experimental techniques to measure different aspects of signalling e.g. oligomerisation of EGF receptor family members on the cell surface [83].

Researchers have established a number of powerful methods for determining oligomeric states of macromolecules. Many of these methods are based upon fluctuation spectroscopy reviewed in Chapter 1. While these methods have a number of advantages and disadvantages they all require the use of a fluorescence standard to calibrate the oligomeric state being measured. Moreover, these prior methods commonly extract either an average oligomeric state or at most resolve one or two states.

In this chapter, a novel method called pbICS (photobleaching image correlation spectroscopy) is described. pbICS enable us to determine the complex aggregation distributions of molecules without the need of a brightness standard. The method uses the fact that a cluster containing many fluorescent labels will have a greater survival probability against photobleaching than a monomer labelled with one fluorophore.

The chapter is organised as follows. In Section 4.2.5 the general theory of photobleaching image correlation spectroscopy are introduced. The theory includes the analytical derivation of the full expression that relates theory to experiment for any oligomeric state distribution. In Section 4.3 explicit expressions are presented for calculating oligomeric distributions of interest. In Section 4.4 results are discussed when the method are applied to specifically determine the clustering of an important biomedical receptor molecule (the EGF receptor) on the surface of CHO cells.
4.2 Materials and methods

4.2.1 Cell culture and treatments

Chinese Hampster Ovary (CHO) cells transfected with GFP-tagged EGF receptor and treated with different concentrations of EGF were kindly provided by Prof. Marisa Martin-Fernandez. These cells were used as received in a fixed and mounted state.

4.2.2 Confocal microscopy

An Olympus FV1000 confocal microscope was employed to carry out photobleaching process and imaging. The fluorescent tags were photobleached with 488 nm laser light focused through a 100X, NA:1.35 oil objective and FITC emission filters in 20 steps. Each image (640×640 pixels) were scanned in horizontal scanning mode with the speed of 2.0 µ s/pixel and zoom level of 2. The 40mW Ar ion laser was adjusted to 25% output power.

Stacks were imported to ImageJ (National Institute of Health, Bethesda, MD) to calculate autocorrelation images by means of “FD Math...” command under Fast Fourier Transform (FFT) process. The process provide us with a list of mean intensity value of images and a list of maximum intensity of autocorrelation images. Then extracted lists of numerical data were exported to MS Excel and then to Mathematica® to compute cluster density and to fit a non-linear curve. A step-by-step procedure is described later.

4.2.3 Step-by-step analysing procedure

1. Import an image stack (includes 20 images) to ImageJ, select regions of interests (ROIs) (normally 10 ROIs per image stack each 32×32 pixels).

2. Separate ROIs and calculate mean intensity values for each image in the ROI
stack via Image → Stacks → Plot Z-axis Profile. There will be 20 intensity values for each ROI.

3. Run “FD Math...” command from: Process → FFT → FD Math... In FD Math window, there are four options to set. Operation option must be set to ‘Correlation’; ‘Do inverse transformation’ need to be had a check mark; Image1 and Image2 drop boxes should be set for a same image in image stack to obtain corresponding autocorrelation image. To yield autocorrelation images of all images in the stack this step must be repeated 20 times.

4. Run Measure comment (Analyze → Measure) to find maximum intensity for each autocorrelation image in the ROI stack. There will be 20 Max gray intensity values for each ROI stack.

5. Save mean intensity values and Max gray intensity values for each ROI in a csv file.

6. Import csv file to Microsoft Excel and adjust mean intensity values by subtracting noise value and normalise them through dividing all values by the mean intensity of first image. These processed values are the fraction of fluorescence remained ($p$).

7. Divide the Max gray intensity values by square of the mean intensity values and ROI area ($32 \times 32$) then subtract 1. This quantity is $g(0, 0)$ (as equation 4.4).

8. Calculate Cluster Density (CD) through $\frac{1}{g(0,0)}$ then export the list of CD and $p$ as an xls file.

9. Import data to Mathematica® to fit a non-linear curve (via equation 4.21) to determine molar fractions ($c_1 - c_2 - c_4 - c_6 - c_8$). The parameter of “Estimated Variance” can reveal the quality of fit.
To execute this long procedure for huge number of ROI stacks (∼ 500) required macro coding for ImageJ and Excell. Nonetheless plenty of time is still required to select proper ROIs, run macros and analysis data.

4.2.4 Calculating spatial autocorrelation of images

In an ergodic ensemble, the ensemble average of an stochastic process is equivalent to the spatial or temporal average. It empowers us to perform spatial and temporal averages correspondingly as \( \langle I(t) \rangle = \langle I(x) \rangle \). To apply this hypothesis in image analysis, the image should be homogeneous. For example, if the image contains a cell on a background, a homogeneous ROI needs to be selected to crop the background [135].

As Petersen et al. [136] showed, we can write 2D spatial autocorrelation for an image as:

\[
G(\eta, \sigma) = \langle I(x, y) I(x + \eta, y + \sigma) \rangle \\
G(\eta, \sigma) = \frac{1}{MN} \sum_{i=1}^{M} \sum_{j=1}^{N} I(i, j) I(i + \eta, j + \sigma)
\] (4.1a)

where \( M \) and \( N \) are the number of discrete points (pixels), \( \eta \) and \( \sigma \) represent spatial-lags for \( x \) and \( y \), respectively. It is pragmatically important to express the autocorrelation of fluctuation function, \( \delta I(x, y) = I(x, y) - \langle I(x, y) \rangle \).

\[
g(\eta, \sigma) = \frac{\langle \delta I(x, y) \delta I(x + \eta, y + \sigma) \rangle}{\langle I(x, y) \rangle^2} \\
g(\eta, \sigma) = \frac{G(\eta, \sigma)}{\langle I(x, y) \rangle^2} - 1
\] (4.2a)

where \( \langle I(x, y) \rangle^2 \) is normalising factor.

Practically, no one used the above-mentioned equation to calculate autocorrelation of an image. Instead, one can exploit fast Fourier transformation along with the
Weiner-Khinchin theorem to obtain the autocorrelation of the image more efficiently as mentioned in Chapter I.

\[ g(0, 0) = \frac{\text{FT}^{-1}\{|\text{FT}\{I(x, y)\}|^2\}}{\langle I(x, y) \rangle^2} - 1 \]  

(4.3)

where \( \text{FT} \) represents Fourier transformation and \( |\text{FT}\{I(x, y)\}|^2 \) is the power spectrum of the image. In practice, the maximum gray-value (zero-lag) of autocorrelation image can be used to calculate \( g(0, 0) \) as

\[ g(0, 0) = \frac{\text{The maximum gray-value of the autocorrelation image}}{MN \times (\text{The normalised mean intensity of the image})^2} - 1 \]  

(4.4)

4.2.5 General theory of pbICS

In pbICS method, the probability of finding non-bleached tags connects to cluster density of oligomer. To execute this idea we require to compute autocorrelation of images then theoretically the inverse of autocorrelation is proportional to the cluster density.

Following statistical mechanics derivation for the fractional fluctuation of a random process in an ensemble \cite{137} yields that the fractional fluctuation is proportional to the inverse of particles in the observation volume:

\[ \frac{\langle I - \langle I \rangle \rangle^2}{\langle I \rangle^2} = \frac{1}{\langle N \rangle} \]  

(4.5)

where we have \( \delta I = I - \langle I \rangle \)

\[ g(0, 0) = \frac{\langle \delta I \rangle^2}{\langle I \rangle^2} = \frac{1}{\langle N \rangle} \]  

(4.6)

then the autocorrelation function at zero-lags can provide us with the density of fluorescent molecules.

On the other hand, Elson and Magda \cite{138} previously showed that there is direct
connection between the detected intensity resulting from fluorescent fluctuation and
the number of molecules of different species in illuminated volume.

\[ I(t) = g \sum_j \epsilon_j Q_j \int L(\mathbf{r}) C_j(\mathbf{r}, t) d^3 \mathbf{r} \]  \hspace{1cm} (4.7)

where \( I(t) \) is detected intensity, \( L(\mathbf{r}) \) displays laser intensity at point \( \mathbf{r} \), \( C_j(\mathbf{r}, t) \) demonstrates the concentration of the \( j \)th fluorescent components at position \( \mathbf{r} \) and time \( t \), \( \epsilon_j \) and \( Q_j \) are molar extinction coefficient and fluorescent quantum yield of the \( j \)th component, respectively and \( g \) is an instrumental factor. Concentration fluctuation results in fluctuations in detected intensity:

\[ \delta I(t) = g \sum_j \epsilon_j Q_j \int L(\mathbf{r}) \delta C_j(\mathbf{r}, t) d^3 \mathbf{r} \]  \hspace{1cm} (4.8)

with some manipulation described by Elson and Magda \[138\], one can reach to:

\[ g(0, 0) = \frac{\langle \delta I(t)^2 \rangle}{\langle I(t) \rangle^2} = \frac{\sum_j (\epsilon_j Q_j)^2 \langle C_j \rangle}{V \left( \sum_j \epsilon_j Q_j \langle C_j \rangle \right)^2} \]  \hspace{1cm} (4.9)

where \( \langle C_j \rangle \) is the mean concentration of the \( j \)th species. Therefore the detected intensity fluctuation is proportional to the concentration of fluorescent molecules. Assuming that the molar extinction coefficient of a cluster linearly related to the molar extinction coefficient of its constituent monomers (i.e. \( \epsilon_j = n_j \epsilon \)) and that quantum yield is unaffected by aggregation \( (Q_j = Q) \) \[32\], then one can write \[42\]:

\[ g(0, 0) = \frac{\sum_j n_j^2 c_j}{V \left( \sum_j n_j c_j \right)^2} \]  \hspace{1cm} (4.10)

where \( n_j \) is the number of monomers in the \( j \)th species and \( c_j \) is the mean concen-
tration ($= \langle C_j \rangle$)

Since the photobleaching is a random process then the number of non-photobleached monomers in a specific species can fluctuate through sample, then we should use average number of monomers as:

$$g(0, 0) = \frac{\sum_j \langle n_j^2 \rangle c_j}{V \left( \sum_j \langle n_j \rangle c_j \right)^2} \quad (4.11)$$

Finding average number of non-bleached monomers for $j$th species is connected to the historical problem in statistical mechanics [139]. The problem is similar to the classical problem of boxes containing white and black balls. Consider $j$ boxes, each containing $P$ white balls and $Q$ black balls. We need to find the probability, $w_j(i)$, of finding $i$ white balls by drawing one ball from each of the $j$ boxes.

The probability of drawing a white white ball from a box is $p = P/(P + Q)$, where it is $q = Q/(P + Q) = 1 - p$ for a black ball. Generally, the probability of drawing $i$ white balls from $i$ specified boxes and $j - i$ black balls from other $j - i$ boxes is $p^i q^{j-i}$. But there are $j!/(j-i)! i!$ ways to select $i$ boxes from a total number of $j$ boxes. Newton found the answer for the problem as

$$w_j(i) = \frac{j!}{(j-i)! i!} p^i q^{j-i} \quad (4.12)$$

since $p+q = 1$ and $(a+b)^n = \sum_{k=0}^{n} \frac{n!}{(n-k)! k!} a^k b^{n-k}$ (binomial expansion) then $\sum_{i=0}^{j} w_j(i) = (p + q)^j = 1$. It is means that $w_j(i)$ works as a probability density and we can use it to find mean value of variable as $\langle i \rangle = \sum_{i=0}^{j} i w_j(i)$.

We consider the relation

$$(px + q)^j = \sum_{i=0}^{j} w_j(i)x^i \quad (4.13)$$
where \( x \) is an arbitrary variable. First and second derivatives with respect to \( x \) give

\[
jp(px + q)^{j-1} = \sum_{i=0}^{j} iw_j(i)x^{i-1} \quad (4.14a)
\]

\[
(j - 1)p^2(px + q)^{j-2} = \sum_{i=0}^{j} i(i - 1)w_j(i)x^{i-2} \quad (4.14b)
\]

If we let \( x = 1 \) in these equations, the right hand sides give mean value of variables

\[
jp = \sum_{i=0}^{j} iw_j(i) = \langle i \rangle \quad (4.15a)
\]

\[
j(j - 1)p^2 = \sum_{i=0}^{j} i(i - 1)w_j(i) = \langle i^2 \rangle - \langle i \rangle \quad (4.15b)
\]

In our case, after photobleaching starts the number of non-bleached monomers will vary

\[
\langle n_j \rangle = \langle i \rangle = \sum_{i=0}^{j} w_j(i) i \quad (4.16a)
\]

\[
\langle n_j^2 \rangle = \langle i^2 \rangle = \sum_{i=0}^{j} w_j(i) i^2 \quad (4.16b)
\]

where \( n_j \) for any \( j \)-mers with \( i \) non-bleached monomers equals \( ni \) with \( n = 1 \) for a monomer. By applying equation 4.15 one can obtain

\[
\langle n_j \rangle = jp, \quad (4.17a)
\]

\[
\langle n_j^2 \rangle = (jp)^2 + j p(1 - p) \quad (4.17b)
\]
then the equation 4.11 can be rewritten as

\[ g(0, 0) = \frac{\sum_j [(j p)^2 + j p(1 - p)] c_j}{V \left( \sum_j j p c_j \right)^2} \]  (4.18)

By taking \( \langle N \rangle / V \) as cluster density, \( CD \), and using equation 4.6 and 4.18 we will have

\[ CD(p) = \frac{\left( \sum_j j c_j \right)^2}{\sum_j [j c_j + j(j - 1) c_j p]} \]  (4.19)

That is the cluster density of a mixture of homogeneous j-mers as a function of fraction of remained fluorescence \( p \) (= [mean intensity value of an image after bleach] / [mean intensity value of the image before photobleaching]). Ciccotosto and et al. [42] also introduced an explicit equation for a single j-mer that can be obtained by dropping summations in equation 4.19

\[ CD_j(p) = \frac{j c_j p}{1 + (j - 1)p}, \quad j = 1, 2, ... \]  (4.20)

### 4.3 pbICS explicit aggregation distribution

By using equation 4.19, we can obtain expressions for complex aggregated distributions involving mixture of j-mers. For monomers with concentration of \( c_1 \), dimers...
with concentration of $c_2$ and so on, we have

\[ CD(p)_{\{1\}} = c_1 p \]

\[ CD(p)_{\{1,2\}} = \frac{(c_1 + 2c_2)^2 p}{(c_1 + 2c_2 + 2c_2 p)} \]

\[ CD(p)_{\{1,2,4\}} = \frac{(c_1 + 2c_2 + 4c_4)^2 p}{(c_1 + 2c_2 + 4c_4 + (2c_2 + 12c_4) p)} \]

\[ CD(p)_{\{1,2,4,6\}} = \frac{(c_1 + 2c_2 + 4c_4 + 6c_6 + 8c_8)^2 p}{(c_1 + 2c_2 + 4c_4 + 6c_6 + 8c_8 + (2c_2 + 12c_4 + 30c_6 + 56c_8) p)} \]

where $CD(p)_{\{1\}}$ is the cluster density of monomers with the mean concentration of $c_1$, $CD(p)_{\{1,2,4,6,8\}}$ demonstrates the cluster density of a mixture of 5 oligomers, monomers—dimers—tetramers—hexamers—octamers, with mean concentrations of $c_1 - c_2 - c_4 - c_6 - c_8$ respectively. $p$ displays fraction of fluorescence remained. As expected, density of monomer decays linearly with fraction of molecules remaining, but density of oligomers decay in rational form of $CD(p) = \frac{\alpha^2 p}{\alpha + \beta p}$ where $\alpha$ and $\beta$ are constants that depend on $c_1 ... c_8$.

To fit the photobleaching data to above model, we used Non-linear model fitting procedure in Mathematica® with the following initial condition for 5 oligomers:

\[ CD_{\text{max}}(p = 1) = \frac{(c_1 + 2c_2 + 4c_4 + 6c_6 + 8c_8)^2}{c_1 + 4c_2 + 16c_4 + 36c_6 + 64c_8} \]

(4.22)

Also one can use the Equation (4.20) to find average $j$ parameter by fitting autocorrelation data and using the initial condition of $CD_{\text{max}}(p = 1) = c_j$ as shown in Figure 4.4. Generally the initial conditions can be derived form

\[ CD_{\text{max}}(p = 1) = \frac{\left( \sum_j j c_j \right)^2}{\sum_j j^2 c_j} \]

(4.23)

Then the relation between $\langle j \rangle$ extracted from this method and $c_j$s can be formulated
by

\[
\langle j \rangle = \frac{\sum j^2c_j}{\sum_j j^2c_j} = \frac{\sum j^2c_j}{\sum_j j^2c_j} \frac{\sum_j j^2c_j}{CD_{\text{max}}} \quad (4.24)
\]

Equivalently we can change the Equation (4.20) to

\[
\frac{CD(p)}{CD_{\text{max}}} = \frac{\langle j \rangle p}{1 + (\langle j \rangle - 1)p} \quad (4.25)
\]

### 4.4 Results and discussion

To determine the oligomeric state of the EGF receptor, pbICS was employed using confocal microscopy. Figure 4.1 demonstrates the confocal image of CHO cells expressing GFP-tagged EGF receptors taken at the basolateral side of the cell membrane. The right panel in Figure 4.1 displays the same image after 12 photobleaching steps. Four 32 × 32 pixels regions of interests (ROIs) have been sketched and numbered in the image.

In Figure 4.2 the left-hand side image displays the magnified ROI#2 located in Figure 4.1 showing clusters of fluorescent GFP-tagged EGF receptors. The right-hand side image is the zoomed autocorrelation image of ROI#2. The selected square in the autocorrelation image covers three-quarters of its area.
Figure 4.1: Left: Image of CHO cells transfected with GFP-tagged EGF receptor and treated with 1µM affibody. The image comprises 4 labelled ROIs with size of 32 × 32 pixels, Right: The same image after 12 photobleaching steps.

Figure 4.2: Left: Extracted and magnified image of ROI#2 from the first slide in Figure 4.1. Right: The corresponding autocorrelation image of ROI#2 (for first slide in the image stack. There are 20 autocorrelation images per stack for each ROI). The drawn square ROI will use to compute the intensity profile. The autocorrelation image is equivalent to $G(0) = FT^{-1}\{FT\{I(t)\} FT^\dagger\{I(t)\}\}$

In Figure 4.3, the top panel depicts mean intensity value of images versus image numbers in the image stack. The plot is an intensity profile that shows an exponential decay with time as the fluorescent EGF receptors are gradually photobleached.
The bottom panel demonstrates a 2D projection of averaged intensities of selected pixels in the autocorrelation image. The cluster density is inversely proportion to amplitude of the autocorrelation function.

![Graph 1](image1.png)

![Graph 2](image2.png)

**Figure 4.3:** Computed graphs from images in Figure 4.2. *Top graph:* Mean intensity profile of ROI\#2 versus slide number in the image stack. *Bottom graph:* The profile plot of the autocorrelation image of the first slide in the stack. The profile plot comes from columnar averaged intensities of selected pixels of the autocorrelation image.

Figure 4.4 depicts a plot of Cluster Density (CD) versus fraction of remaining fluorescence (p). By the fitting the data to Equation (4.25), one can obtain an averaged $\langle j \rangle$ value that indicates average oligomeric state or brightness. For example $\langle j \rangle = 1$ indicates monomeric state.

Alternatively, one can fit data points of Cluster Density (CD) versus fraction of fluorescence remaining (p) to a multiple oligomeric state distribution model (using...
Figure 4.4: Cluster Density (CD) as a function of fraction remaining (p) for EGF receptor-GFP in CHO cells. Experimental points are presented by the dots and fit to averaged $\langle j \rangle$ represented by solid line. The equation (4.25) is used to fit results to find averaged $\langle j \rangle$. With the same results depicted in Figure 4.5, we can acquire $\langle j \rangle = 2.963$ as an averaged brightness.

Figure 4.5: The graph depicts cluster density of GFP-tagged EGF receptor versus fluorescence fractional remaining. Fitting parameters can be acquired in this instance as $\{c_1, c_2, c_4, c_6, c_8\} = \{0.47, 0.28, 0.22, 0.03, 0.0001\}$.
Equation (4.21) and Equation (4.23)). This analysis provides us with the molar concentration of discrete oligomeric forms (monomer- dimer- tetramer- hexamer- octomer).

Figure 4.5 provides us with molar fractions \( \{c_1, c_2, c_4, c_6, c_8\} \) while Figure 4.4 supplies us with \( \langle j \rangle \) value. It is interesting that with using Equation (4.24), one can relate molar fractions in Figure 4.5 with \( \langle j \rangle \) value in Figure 4.4.

Table 4.1: Averaged \( j \) values obtained by fitting equation 4.25 on results of experiments with CHO cells transfected with GFP-tagged EGF receptor and treated with EGF. The first column shows EGF concentrations while the last one indicates the number of measured cells. \( \pm \) represents the standard error.

<table>
<thead>
<tr>
<th>EGF</th>
<th>( \langle j \rangle )</th>
<th># of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 nM</td>
<td>1.9 ± 0.1</td>
<td>46</td>
</tr>
<tr>
<td>1 nM</td>
<td>2.3 ± 0.2</td>
<td>15</td>
</tr>
<tr>
<td>100 nM</td>
<td>1.9 ± 0.1</td>
<td>10</td>
</tr>
<tr>
<td>400 nM</td>
<td>1.9 ± 0.4</td>
<td>31</td>
</tr>
<tr>
<td>1 ( \mu )M</td>
<td>4.2 ± 0.4</td>
<td>10</td>
</tr>
<tr>
<td>2 ( \mu )M</td>
<td>3.7 ± 0.4</td>
<td>24</td>
</tr>
</tbody>
</table>

Table 4.1 displays averaged \( j \) values of EGFR with different EGF concentrations. In the control cells with no EGF, the average brightness was 1.9 (range: 1.15 to 3.45), which indicates presence of dimers and a smaller contribution of monomers. Addition of EGF in the concentration range from 1-400 nM did not significantly alter the average brightness values as these remained in the range 1.9-2.3. However, addition of 1-2 \( \mu \)M EGF produced a significant increase in average brightness to about 4 (range: 1.5 to 8.0). In the context of a homogeneous model of aggregation this implies an EGF-mediated dimer to tetramer transition. The number of examined cells is in the last column. Due to the range of 1.5 to 8.0 for averaged
brightness, we used model of 5 oligomers from monomer to octomer.

However, it is not possible to infer the detailed contribution of different oligomer in aggregation state from only the averaged j value. Alternatively, Table 4.2 reports molar fraction of aggregation forms of EGF receptor in the same experiment.

Table 4.2: Tabulated results of experiments with CHO cells transfected with GFP-tagged EGF receptor and treated with EGF. The first column shows EGF concentrations while other columns display molar fraction of oligomeric distribution. ± represents the standard error.

<table>
<thead>
<tr>
<th>EGF</th>
<th>Monomer</th>
<th>Dimer</th>
<th>Tetramer</th>
<th>Hexamer</th>
<th>Octomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 nM</td>
<td>0.67 ± 0.02</td>
<td>0.28 ± 0.02</td>
<td>0.04 ± 0.01</td>
<td>0.01 ± 0.01</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>1 nM</td>
<td>0.56 ± 0.02</td>
<td>0.34 ± 0.02</td>
<td>0.08 ± 0.02</td>
<td>0.01 ± 0.01</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>100 nM</td>
<td>0.58 ± 0.03</td>
<td>0.36 ± 0.02</td>
<td>0.07 ± 0.03</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>400 nM</td>
<td>0.59 ± 0.02</td>
<td>0.36 ± 0.03</td>
<td>0.05 ± 0.03</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>1 µM</td>
<td>0.54 ± 0.03</td>
<td>0.21 ± 0.04</td>
<td>0.11 ± 0.03</td>
<td>0.06 ± 0.02</td>
<td>0.07 ± 0.05</td>
</tr>
<tr>
<td>2 µM</td>
<td>0.47 ± 0.01</td>
<td>0.25 ± 0.02</td>
<td>0.17 ± 0.02</td>
<td>0.07 ± 0.02</td>
<td>0.04 ± 0.02</td>
</tr>
</tbody>
</table>

Figure 4.6 displays results of CHO cell experiments with different EGF concentrations (detailed results in Table 4.2). One can see that in absence of EGF, there are 67% monomers and 28% preformed dimers. Also other preformed oligomers contributed to the aggregation state (total 6% mole percent). Addition of 1nM EGF decreased the EGF receptor monomer contribution (by 11%), increased the
dimer contribution (by 6%) and increased tetramer formation (by 4%). Although the level of monomers were invariant over the EGF concentration range between 1nM and 400nM, the dimer contribution plateaued at 100-400 nM at the expense of decreasing contribution of higher oligomers. Interestingly, micomolar concentrations of EGF, caused a significant drop in both monomer and dimer contributions and a concomitant increase in higher-order oligomers.

![Figure 4.6](image_url)

Figure 4.6: The plot of molar fraction for CHO cells transfected with GFP-tagged EGF receptor. The horizontal axis demonstrates different treating concentration of EGF.

Our analysis thus far indicates that EGF receptor aggregation depends somewhat upon EGF. An important question is how EGF receptor oligomeric state depends on EGF receptor density (or expression level in the membrane). The pbICS analysis can be utilised to relate molecular number to molecular brightness, much in the spirit of the Number and Brightness technique, but derived from different quantities. The product of the cluster density and the brightness or $CD \langle j \rangle$ provides a measure of the average number of molecules in a region of interest or average molecular density, while the $\langle j \rangle$ provides a measure of the average oligomeric state. A plot of $\langle j \rangle$ as a function of $CD \langle j \rangle$, therefore provides a convenient measure of how receptor...
aggregation depends upon receptor density. Figure 4.7 displays the average $j$ value versus the average receptor density for EGF receptor in CHO cells for two different population regimes. As one can see, there were upward trends in both cases that indicate higher $\langle j \rangle$ is occurred for higher population. It is in agreement of finding preformed higher oligomers in crowded environment.

Figure 4.8 shows the same situation but for different EGF concentrations. Comparing these two plots reveals that higher EGF concentration stimulates higher oligomers.

The traditional model of EGF receptor activation indicates that in the absence of ligand, EGF receptors is monomeric while in the presence of EGF stimulation, receptors form active dimers. Purified EGF receptors of A-431 cells showed that in absence of EGF binding, 70-80% of receptors are in a monomeric form [140]. It is noteworthy to notice that in the experiment they removed receptors from their native environment.

There are a few studies on EGF receptors of CHO cells to compare our results. Saffarian and et.al. [141] employed a modified version of fluorescence-intensity dis-
Figure 4.8: The plot depicts averaged $j$ values versus $CD(p = 1) \langle j \rangle$ for experiment of CHO cells transfected with GFP-tagged EGF receptor treated with EGF. Left: EGF concentration: 100nM. Right: EGF concentration: 2µM.

The distribution analysis with quantal brightnesses to assess oligomeric state of EGF receptor tagged with eGFP expressed in living CHO cells. They showed that depletion or loading cellular cholesterol change complex equilibrium between aggregation state of the EGF receptors involving monomers, dimers and high order oligomers. Where lack of cellular cholesterol enhanced clustering, loading cholesterol inversely decreased EGF receptor clustering. They found that in a control experiment (no EGF) that 70% of EGF receptors were monomeric, 20% were dimeric and less than 10% EGF receptors were in higher order oligomers. Whilst noting that our experiments were performed on live cells after chemical fixation, our analysis (67%, 28% and 6% for monomer, dimer and higher oligomer, respectively) compared very well with oligomeric distribution derived from Saffarian et al.

Nagy and et.al. [142] used number and brightness analysis technique to show that in the absence of ligand stimulation, EGF receptors are mainly monomeric in cells containing 50,000 EGF receptors per cell. Whilst in cells containing 500,000 EGF receptors per cells there were about 30% preformed dimers and 70% monomers, as inferred from average brightness values. Our finding is in good agreement with their results while our reported results in Table 4.2 shows 67% monomers and 28%
dimers in absence of EGF stimulation. As they reported, adding EGF stimulation can induce larger cluster of the receptors both in over populated system (600,000 receptor per cell) and in physiological conditions (50,000 per cell). They inferred that stimulation of cells with EGF lead to an increase in the cluster size of EGF receptors. This is in good agreement with our finding too.

Liu and et. al. [143] exploited single wavelength fluorescence cross-correlation spectroscopy (SW-FCCS) to quantify dimer fraction of EGF receptors on the cell surface. They found 60% of EGF receptors are preformed dimer on the surface of CHO cells, however they did not refer to any preformed higher oligomer in their results. They indicated that dimer percentages were not affected by the receptor expression levels, but our result (Figure 4.7) shows that the percentage of oligomerisation depends on the expression levels. This dependence is compatible with previously observed homo-dimerisation of EGF receptors by Moriki and et. al. [144]. They showed that over-expressed level causes spontaneous preformed dimers in the absence of EGF stimulation. While the dimerisation of the receptors at lower expression levels was dependent on the presence of bound EGF.

In a very recent paper, Zanetti-Domingues and et. al. (paper in press) developed a new method named fluorophore localisation imaging with photobleaching (FLImP) to determine the separation of two fluorophors. They used single molecule microscopy to reach a resolution of 7nm. This method can be used to study oligomerisation of cell surface receptors. They reported that EGF receptors can form inactive oligomers on the surface of cell membrane without any requirement for intracellular domain. Their observation of extended higher-order oligomers in the absence of stimulation is in good agreement with the higher-order oligomers observed in our work.
4.5 Aadvantages and disadvantages and of pbICS

pbICS is a new technique that like other methods has essential pros and cons. The pbICS works with measuring variation of the proportion of labelled molecules during gradual photobleaching. It inherits some properties of ICS methods, for example the resolution of images depends on the point-spread function of the microscope and the spatial scale of spatial correlations. Although other methods like N&B and spIDA methods are not dependent on the spatial correlation, they exploit amplitudes of temporal image fluctuations at the resolution of single pixels (N&B analysis) or the histogram of fluorescence intensities in an image (spIDA method) to obtain aggregation. The pbICS, instead, provides complementary information about spatially correlated interactions that have relevance at the subcellular and supramolecular length-scales. The key advantage of the pbICS method is that unlike M&B method, no brightness standards are needed to obtain quantitative information about the average degree of aggregation or aggregation distributions. Also separate preparations of different labelled samples are not needed since photobleaching automatically generates a label distribution that can be analysed for the autocorrelation analysis.

On the other hand, pbICS could be toxic to living cells and its process can perturb biochemical and physiological function of living cells. Also motions of macromolecules in living cells will invalidate the assumptions in the pbICS theory. For these reasons, such method is recommended for fixed cells. Moreover, poor image contrast (signal to noise) can significantly distort pbICS curves.

4.6 Conclusions

The pbICS method connects the probability of finding non-bleached monomers to cluster density of oligomers. To execute this idea, one requires to compute the autocorrelation of images. The inverse of autocorrelation is theoretically proportional
to the cluster density.

Where the current methods of studying complex oligomeric states of membrane proteins require brightness standards as a major shortcoming of the field and give average information about oligomeric status of the sample, the pbICS method detects molar fraction of different oligomers in detail without requesting prior auxiliary brightness information.

In this chapter, we demonstrated a mathematical formalism for pbICS image processing that was required to employ the pbICS method for examining the oligomeric state of cell surface receptors. Also we examined oligomeric states of GFP-tagged EGF receptor expressed in CHO cells with different concentrations of EGF stimulation. Our results revealed that EGF receptor oligomerisation is a complex process which is influenced by receptor density and EGF concentration. In the absence of EGF, dimerisation and higher-order oligomerisation can occur with increasing receptor density. However, EGF addition also increased receptor clustering over a concentration range from nanomolar to micromolar. Moreover, we have revealed significant cell-by-cell heterogeneity which may partially account for conflicting results reported from different laboratories.

To summarise, the pbICS results suggest that the classical monomer-dimer model is not completely general and requires modification to account for other oligomeric forms.
CHAPTER V

Précis and Conclusion

To briefly recapitulate, the primary goal of the work presented in this thesis was to yield a more detailed and thorough understanding of the mechanisms underlying dynamics of living cells. The motivation for such a study being that to reliably measure dynamical parameters by means of photophysical responses through different microscopy methods. Such aim was required to enhance current microscopy techniques or to develop new methods. The fundamental complicated dynamics of living cell needs to be more thoroughly elucidated where developed microscopy methods can be employed to shed light on this vital subject. After a brief proem and current understanding, a detailed introduction in Chapter I was opening of the thesis, but the core of thesis began from next chapter.

In Chapter II a new method was developed to relate frequency-domain FLIM measurements to diffusion coefficient of moving particles. Motion during acquisition was shown to alter the calculated fluorescence lifetime distribution owing to loss of phase coherence of the detected fluorescence. This relation enabled calculation of diffusion coefficient from the lifetime image.

In Chapter III was concerned with motional dynamics of solvent. A new method was developed to exploit FLIM for measuring solvent relaxation time in living cells. Theory was developed to show that an average excited-state lifetime and an average solvent correlation time could be obtained from measurements with one modulation.
frequency and two detection wavelengths. The method was validated with a common fluorophore, NBD, in water-glycerol mixtures. Solvent correlation times in the membranes of living cells were reported for the very first time.

Solvent correlation times in the membranes of living cells were reported for the very first time. Using a probe that preferentially partitions into the membranes of the Golgi apparatus were we able to record the dynamics in the Golgi and plasma membrane of living HeLa cells. The solvent correlation times in the plasma membranes were \( 2.94 \text{ ns} \) in contrast to the much shorter correlation times of \( 0.83 \text{ ns} \) in the Golgi membranes. These results underscore the utility of imaging solvent dynamics, especially in complex structures such as living cells.

In Chapter IV the pbICS method was thoroughly elucidated to quantify oligomer state of membrane receptors. The relevant aggregation model was introduced to compute mole fraction of each oligomeric state a mixture of 5 oligomers, \( \text{monomers} - \text{dimers} - \text{tetramers} - \text{hexamers} - \text{octamers} \). Fixed samples of CHO cells with GFP-tagged EGF receptors treated with 6 different EGF concentration from zero to \( 2 \mu M \) were used to measure their oligomeric states. The average brightness of each conditions and their detailed cluster densities were reported. The pbICS results indicate that the classical monomer-dimer model is not completely general and requires modification to account for other oligomeric forms.

## 5.1 Future work

There are infinity ideas and thoughts to enhance and develop introduced methods. However some are important modification for future study. For example it is possible to go further and equip FLIM to measure diffusion coefficient of particles with uncorrelated stochastic movement and with different lifetimes. In the current simulation, we studied a single big Gaussian bead with stochastic motion, however there is a question what if there are two big Gaussian beads with different lifetimes.
and uncorrelated stochastic movements. Another topic should be worked on is enhancing the introduced method to measure sub-pixel movement as a super-resolution method. Since the projected image of a big Gaussian bead on CCD can occupy some pixels, there is possibility to detect sub-pixel movement during acquisition time by study its effect on phasor plot.

Moreover, the established method to measure solvent relaxation time, is ready to measure the effects of drug or changing cell components like cholesterol on solvent relaxation time of plasma membrane especially Golgi membrane of living cells. Further work is needed to understand dipolar solvent dynamics in the membranes in living cells. A future goal is to understand the factors influencing solvent dynamics in complex environments and whether solvent relaxation can be used as a biosensor for detection of organelle-affected diseases.

Also for a further study, it is a good idea to study effects of antibody or affibody on aggregation state of EGF receptors by means of introduced pbICS method. In our presented work, we studied the effect of EGF stimulation on oligomeric state of fixed CHO cells, however it is possible to exploit the method to investigate effects of other effective macromolecules e.g. antibodies or affibodies on the cell signalling pathway.

Addressed topics in the thesis serves to illustrate the complex interplay of the dynamics in living cells. It is hoped that the studies outlined in this thesis will provide a firmer foundation for improving the understanding of some aspects of living cells.
APPENDICES
APPENDIX A

Source Codes

A.1 Stochastic motion

As mentioned in Chapter I, we developed the following FORTRAN 90 code to simulate 2D Brownian motion of fluorescent beads.

! Simulation of stochastically moving Beads Brownian Motion
!-----------------------------------------------------------------------

INCLUDE 'mkl_vsl.fi'

MODULE PARAMETERS
USE MKL_VSL_TYPE

USE MKL_VSL

REAL*8, ALLOCATABLE:: X(:),Y(:),Z(:)
REAL*8, ALLOCATABLE:: VX(:),VY(:),VZ(:)
REAL*8, ALLOCATABLE:: FX(:),FY(:),FZ(:)
INTEGER,ALLOCATABLE:: IP(:)
REAL*8, ALLOCATABLE:: X0(:),Y0(:)
REAL*8, ALLOCATABLE:: DXI(:),DYI(:)
REAL BOXX, BOXY, BOXZ, OLDV, KAPA, KB
REAL SIGMA, Epsilon, Gama, Xesi, KBT, Dif
REAL TEMP, DT, TIME
REAL RCUT
REAL MEAN, STANDARDV
REAL h, hh, RR, R2, R2X, R2Y
REAL time, begin, time_end, DURATION, TIME

INTEGER NMD
INTEGER NALL
INTEGER NMAX
INTEGER ISAVE, IPRINT
INTEGER ITN, hStep
INTEGER NSTEP, NIMAGE
INTEGER SEED, STATUS

PARAMETER (PI = 3.141592)

CHARACTER TITLE*80, ELA*2, ELB*2, TEXT*3

INTEGER (kind=4) errcode
INTEGER brng, method, seeddX, seeddY, seeddZ
REAL(kind=8) RSeedX, RSeedY, RSeedZ
TYPE (VSL_STREAM_STATE) :: streamX, streamY, streamZ

END MODULE PARAMETERS

!***********************************************************************

PROGRAM MD_SIMULATION_AT_SWINBURNE
USE PARAMETERS

! USE IMSL
USE iFPOR
USE MKL_VSL_TYPE
USE MKL_VSL

! IMPLICIT NONE
CHARACTER (50) FILENAME
CALL CPU_TIME(time_begin)
OPEN(99, FILE=`VMD.XYZ`, STATUS=`UNKNOWN`)  
OPEN(101, FILE=`ltmsd.DAT`, STATUS=`UNKNOWN`)

CALL CONTROL_DATA()
Dif = 1.5*10.0**(1)
hh = NMD/500

DO hStep = 0, 399
   IF (hStep.LT.9) THEN
      WRITE(FILENAME1, `(I1)`) hStep+1
      FILENAME = `VMD00'/FILENAME1 //`.DAT'
   ELSEIF (hStep.LT.99) THEN
      WRITE(FILENAME2, `(I2)`) hStep+1
      FILENAME = `VMD0'/FILENAME2 //`.DAT'
   ELSE
      WRITE(FILENAME3, `(I3)`) hStep+1
      FILENAME = `VMD'/FILENAME3 //`.DAT'
   ENDIF
   WRITE(6,*) FILENAME
ENDDO
OPEN(910+hStep, FILE=FILENAME, STATUS=`UNKNOWN`)
! Dif = hh * (hStep+1)
NMD = hh + hh * hStep
CALL INCRD()
CALL LANGVEN_RUN()
!write(2233,*) Dif
CALL CPU_TIME(time_end)
DURATION_TIME = time_end - time_begin
WRITE(6,*) hStep, DURATION_TIME

113
CALL CPU_TIME(time_end)

DURATION_TIME = time_end - time_begin

WRITE(6,*) DURATION_TIME/3600.0
STOP

END ! Main

!*****************************************************************************
!
SUBROUTINE INCRD()

USE PARAMETERS
USE MKL_VSL_TYPE
USE MKL_VSL
IMPLICIT NONE
INTEGER II , JJ

REAL RANDOMXX(1) , RANDOMYY(1)

CALL RANDOMNUMBER(RSeedX)
CALL RANDOMNUMBER(RSeedY)
! CALL RANDOMNUMBER(RSeedZ)

seeddX = AINT(357 * RSeedX * 1.0E6)
seeddY = AINT(357 * RSeedY * 1.0E6)
! seeddZ = AINT(357 * RSeedZ * 1.0E3)

errcode=vslnewstream(streamX , brng , seeddX)
errcode=vslnewstream(streamY , brng , seeddY)
! errcode=vslnewstream(streamZ , brng , seeddZ)

DO JJ=1, NALL
    errcode = vsrnggaussian(method , streamX , 1 , RANDOMXX , &
                            MEAN , STANDARDV)
    errcode = vsrnggaussian(method , streamY , 1 , RANDOMYY , &
                            MEAN , STANDARDV)

END SUBROUTINE INCRD
! To avoid putting particle close to border, divide \texttt{BOXX} by 4.0
\begin{align*}
  X(JJ) &= (2.0 \times \texttt{RANDOMXX}(1) - 1.0) \times \texttt{BOXX} / 4.0 \\
  Y(JJ) &= (2.0 \times \texttt{RANDOMYY}(1) - 1.0) \times \texttt{BOXY} / 4.0 \\
  Z(JJ) &= 0.0 \\
  X0(JJ) &= X(JJ) \\
  Y0(JJ) &= Y(JJ)
\end{align*}

\texttt{ENDDO}

\begin{verbatim}
  DO JJ=1, NALL
    errcode = \texttt{vsrnggaussian} (method, \texttt{streamX}, 1, \texttt{RANDOMXX}, \texttt{MEAN, STANDARDV})
    errcode = \texttt{vsrnggaussian} (method, \texttt{streamY}, 1, \texttt{RANDOMYY}, \texttt{MEAN, STANDARDV})
    VX(JJ) = \texttt{RANDOMXX}(1) \times \texttt{SQRT}(2 \times \texttt{Gama} \times \texttt{Dif} \times \texttt{h}) \times 0.1
    VY(JJ) = \texttt{RANDOMYY}(1) \times \texttt{SQRT}(2 \times \texttt{Gama} \times \texttt{Dif} \times \texttt{h}) \times 0.1
    VZ(JJ) = 0.0
  \texttt{ENDDO}

  WRITE (910+hStep,145) NALL
  WRITE (910+hStep,143) \texttt{BOXX,BOXY,BOXZ}
  WRITE (910+hStep,146) NIMAGE

  143 FORMAT(3F10.3)
  145 FORMAT(I6)
  146 FORMAT(I6)
  RETURN
\end{verbatim}

\texttt{END}

\begin{verbatim}
! ******************************************************
! ******************************************************
\textbf{SUBROUTINE } \texttt{LANGVEN, RUN()}\textbf{ }
\end{verbatim}

\begin{verbatim}
  USE \texttt{PARAMETERS}
  \textbf{USE RNOR_INT}
\end{verbatim}
DO ITN=1, NMD
   IF (MOD(ITN+9,10) .EQ. 0) THEN
      CALL IMPRSN()
   ENDIF

   ! Monitor

   ! IF ((ITN .EQ. 1) .OR. (MOD(ITN,IPRINT) .EQ. 0)) THEN
   !      WRITE(6,4271) ITN, SQRT(R2/(ITN*NALL))
   !   ENDIF

   ! FOR VISUALIZATION XYZ file format

   ! IF ((ITN .EQ. 1) .OR. (MOD(ITN,ISAVE) .EQ. 0)) THEN
   !      WRITE (99,333) NALL
   !      WRITE (99,427) ITN
   !      WRITE (99,428) ( 'C', X(I), Y(I), Z(I), I = 1, NALL)
   !   ENDIF

   ! FOR Results

   IF ((MOD(ITN,ITN*NMD) .EQ. 0)) THEN ! (ITN .GT. ISAVE) .AND.
      WRITE (910+hStep,427) NIMAGE*ITN/NMD
      WRITE (910+hStep,429) (X(I), Y(I), Z(I), I = 1, NALL)
      ! WRITE (101,*) NIMAGE*ITN/NMD, SQRT(R2/(NALL))
      R2 = 0.0
   ENDIF

   IF ((MOD(ITN,NMD) .EQ. 0)) THEN
      WRITE (101,431) ITN, R2/(NALL), SQRT(R2/(NALL))
      ! WRITE (901,432) ITN, SQRT(R2X/(NALL)), SQRT(R2Y/(NALL))

   INTEGER I, N, J
!WRITE (1010+hStep, 431) ITN, SQRT(R2/NALL)
R2 = 0.0
WRITE(6, 4271) ITN, R2/NALL
ENDIF
CALL FORCES()
CALL X, Y()
END DO !ITN
333 FORMAT(I12)
427 FORMAT(I12)
4271 FORMAT(I12, E18.4)
428 FORMAT(A4, 3E25.8)
429 FORMAT(2E30.16, E20.3)
431 FORMAT(I16, E22.6, E22.6)
432 FORMAT(I16, 2E20.6)
RETURN
END

*******************************************************************************
*******************************************************************************
SUBROUTINE FORCES()
USE PARAMETERS
IMPLICIT NONE
INTEGER II
DO II = 1, NALL
FX(II) = 0.0
FY(II) = 0.0
ENDDO
RETURN
END

*******************************************************************************
*******************************************************************************
SUBROUTINE X, Y()
USE PARAMETERS
USE MKL, VSL, TYPE

USE MKL, VSL

IMPLICIT NONE

REAL RandX(1), RandY(1)
REAL*8 XI, YI
INTEGER II

DO II = 1, NALL
! CALL RNNOR (1, RANDX)
! CALL SSCAL (1, STANDARDV, RANDX, 1)
! CALL SADD (1, MEAN, RANDX, 1)
errcode = vsrnggaussian (method, streamX, 1, RandX, &
               MEAN, STANDARDV)
errcode = vsrnggaussian (method, streamY, 1, RandY, &
               MEAN, STANDARDV)
XI = X(II)
VX(II) = VX(II) - Gama * h * VX(II) + SQRT(2 * Gama * Dif * h) * RandX(1)
X(II) = X(II) + h * VX(II)
DXI(II) = DXI(II) + X(II) - XI

YI = Y(II)
VY(II) = VY(II) - Gama * h * VY(II) + SQRT(2 * Gama * Dif * h) * RandY(1)
Y(II) = Y(II) + h * VY(II)
DYI(II) = DYI(II) + Y(II) - YI

Z(II) = 0.0
! R2 = R2 + (X(II) - XI)**2 + (Y(II) - YI)**2
! R2X = R2X + (X(II) - XI)**2
! R2Y = R2Y + (Y(II) - YI)**2
IF ((MOD(NIMAGE*ITN, NMD).EQ.0)) THEN
   R2 = R2 + DXI(II)**2 + DYI(II)**2
   DXI(II) = 0.0
   DYI(II) = 0.0
! The subroutine that enforces periodic boundary conditions

SUBROUTINE IMPRSN()

USE PARAMETERS

IMPLICIT NONE

INTEGER I

DO I = 1, NALL

X(I) = X(I) - BOXX * ANINT(X(I) / BOXX)
Y(I) = Y(I) - BOXY * ANINT(Y(I) / BOXY)

! Z(I) = Z(I) - BOXZ * ANINT(Z(I) / BOXZ)

ENDDO

RETURN

END

SUBROUTINE CONTROL(); DATA();

USE PARAMETERS

USE MKL
USE VSL

IMPLICIT NONE

! NMD=Number of Iteration points
! NSTEP= Iteration number THAT DONE
! NALL= Total number of atoms
! NMAX= maximum number of atoms
! IP=Flag for atomic colour
! ISAVE=Flag for saving the Configuration file
! IPRINT=Flag for printing Intermediate results
! IEQULV= Flag for volume equilibrium
! IEQULT= Flag for temperature equilibration
! DT=value of every time step
! TAU=

! ----------------------------------Control Data----------------------------------
NMD = 2000000
NIMAGE = 10
BOXX = 8.9784 !mm CCD horizontal size
BOXY = 6.708 !mm CCD vertical size
BOXZ = 0.0
NALL = 100 !100 !1000
ALLOCATE (X(NALL),Y(NALL),Z(NALL),IP(NALL))
ALLOCATE (VX(NALL),VY(NALL),VZ(NALL))
ALLOCATE (FX(NALL),FY(NALL),FZ(NALL))
ALLOCATE (X0(NALL),Y0(NALL))
ALLOCATE (DXI(NALL),DYI(NALL))
ISAVE=1000
IPRINT=1000

! IEQULV=0
! KAPA = 1.0E-19 !JOULE
! BOXX = 40 !MICROMETER
! BOXY=40 !// //
! SIGMA0 = 5E-21 !JOULE/MICROMETER^2
! ETA = 10^-3 !JOULE SECOND/METER^3
TIME = 9.13E-13 !SECOND
DT=5.0E-16 !SECOND
h=dt/time
h=1.0E-07 ! h=1.0E-09 fast= 1.0E-4 slow= 1.0E-6
! Dif = 258.6
Gama = 1.0E4

! KBT = 25.84 !meV
! Sigma = 3.4 !Angstrom
! Epsilon = 10.23 !meV
KB = 1.38E-23 ! J/K or (m2.kg/s^2.K)

! TEMP = 297 ! K

! KBT = KB * TEMP

! Eta = 9.135E-4 ! Water viscosity at T=297K

! Ro = 0.5E-6 ! Meter, Beads’ radius

! Gama = 10.0 ! SlowMove_Gama=10000 FastMove_Gama=1000

! Gama = 6*PI*Eta*Ro

! Xesi = KBT / Epsilon

! Xesi = 0.5

! Ta = 1.6E-11

! h = 10.0E-9/Ta

MEAN = 0.0

STANDARDV = 1.0

brng = VSL_BRNG_MCG59

method = VSL_METHOD_SGAUSSIAN_BOXMULLER

CALL RANDOM_SEED()

END

!******************************************************************************
A.2 FLIM computation

The following code computes lifetime of fluorescent beads while they are moving.

![FORTRAN 90 source code:]

![Lifetime calculation of moving fluorescent beads]

```
MODULE PARAMETERS

REAL*8, ALLOCATABLE:: X(:,,:),Y(:,,:),Z(:,,:), Modul(:,:)
REAL*8, ALLOCATABLE:: DPhi(:,:)
REAL*8, ALLOCATABLE:: Fcos(:,,:), Fsin(:,,:), Fdc(:,:)
REAL*8, ALLOCATABLE:: Mcos(:,,:), Msin(:,:)
REAL*8, ALLOCATABLE:: NewMcos(:,,:), NewMsin(:,:)
REAL*8, ALLOCATABLE:: INTENSITY(:,,:), INTENSITY0(:,:)
INTEGER, ALLOCATABLE:: IG(:,:)
REAL*8 BOXX, BOXY, BOXZ
REAL time,begins, time_end, DURATION_TIME
REAL*8 PI
REAL*8 Kall
REAL*8 Prsnt
INTEGER NALL, hStep, rndcoef
INTEGER ISAVE, IPRINT
INTEGER NSNAPSHOT
INTEGER NSTEPS,NIMAGE
INTEGER Xpixel, Ypixel, Zpixel
INTEGER NXgrid, NYgrid
INTEGER Ix, Jy
PARAMETER (PI=3.141592)
CHARACTER TITLE*80,ELA*2,ELB*2,TEXT*3

END MODULE PARAMETERS

!******************************************************************************
!******************************************************************************

PROGRAM LifeTime_SIMULATION_AT_SWINBURNE
```
USE PARAMETERS

! USE IMSL

USE IFPORT

IMPLICIT NONE

CHARACTER (100) FILENAME

CHARACTER (1) FILENAME1

CHARACTER (2) FILENAME2

CALL CPU_TIME(time_begin)

OPEN(150, FILE='01 Variances .DAT', STATUS='UNKNOWN')

OPEN(2900, FILE='02 McosMsin01 .DAT', STATUS='UNKNOWN')

CALL CONTROL_DATA()

CALL INCRD()

hstep = 0

DO rndcoef = 0, 100, 2 ! 98

IF (hStep.LT.9) THEN
    WRITE(FILENAME1, '(I1)') hStep+1
    FILENAME = 'VMD0' //FILENAME1 // '.DAT'
ELSE
    WRITE(FILENAME2, '(I2)') hStep+1
    FILENAME = 'VMD' //FILENAME2 // '.DAT'
ENDIF

WRITE(6,*) FILENAME

OPEN(910+hStep, FILE=FILENAME, STATUS='OLD')

CALL INCRD_read()

CALL CALCULATION()

CLOSE(910+hStep)

CLOSE(2900+hStep)

CLOSE(1910+hStep)
CALL CPU_TIME(time_end)
DURATION_TIME = (time_end - time_begin)/60.0

WRITE(6,*) DURATION_TIME, hStep

ENDDO
STOP

END ! Main

!********************************************************************
!********************************************************************

SUBROUTINE INCRD()

USE PARAMETERS

IMPLICIT NONE

INTEGER II, JJ

OPEN(910, FILE='VMD01.DAT', STATUS='OLD')

READ(910,*) NALL
READ(910,*) BOXX, BOXY, BOXZ
READ(910,*) NSTEPS

NIMAGE = NSTEPS

Kall = Real(NIMAGE)

ALLOCATE (X(NIMAGE,NALL), Y(NIMAGE,NALL), Z(NIMAGE,NALL))
ALLOCATE (INTENSITY(NIMAGE, NXgrid, NYgrid))
ALLOCATE (INTENSITY0(NIMAGE, NXgrid, NYgrid))
ALLOCATE (Modul(NXgrid, NYgrid), DPhi(NXgrid, NYgrid))
ALLOCATE (Fsin(NXgrid, NYgrid), Fcos(NXgrid, NYgrid))
ALLOCATE (Fdc(NXgrid, NYgrid))
ALLOCATE (Msin(NXgrid, NYgrid), Mcos(NXgrid, NYgrid))
ALLOCATE (NewMsin(NXgrid, NYgrid), NewMcos(NXgrid, NYgrid))

CLOSE(910)
RETURN
END

!********************************************************************
SUBROUTINE INCRD
   USE PARAMETERS
   IMPLICIT NONE
   INTEGER II , JJ

   READ(910+hStep,*) NALL
   READ(910+hStep,*) BOXX,BOXY,BOXZ
   READ(910+hStep,*) NSTEPS

   NIMAGE= NSTEPS
   Kall= Real(NIMAGE)
   DO JJ=1,NIMAGE
      READ(910+hStep,*) NSNAPSHOT
      DO II=1, NALL
         READ(910+hStep,*) X(JJ,II),Y(JJ,II),Z(JJ,II)
      ENDDO
   ENDDO
   RETURN
END

SUBROUTINE CALCULATION()
   USE PARAMETERS
   USE iFPORT
   IMPLICIT NONE
   INTEGER I,J,M,N,K,W,P,Q
   REAL*8 GridD, GridU, GridL, GridR, U
   REAL*8 XI, YI, RR2, Variance2, Vari
   REAL*8 R2cntr, Rcntr2, X1cntr, Y1cntr
   REAL*8 McosAV, MsinAV, McosAV2, MsinAV2
   REAL*8 McosCntr, MsinCntr
   REAL*8 Dist, LongDist, Reff
   END

REAL*8 CalPrsnt, RTemp, Counter
REAL Rndm, SD
INTEGER DivU, ZeroCont, FlagP

DO N=1,NIMAGE
     DO J=1, NYgrid
          DO I=1, NXgrid
               INTENSITY(N, I, J) = 0.0
               INTENSITY0(N, I, J) = 0.0
          ENDDO
     ENDDO
     CALL CPU_TIME(SD)
     CALL SEED(INT(SD*1357931))
ENDDO

DO N=1,NIMAGE
     DO M=1, NALL
          GridD = -BOXY/2.0
          DO J=1, NYgrid
               GridU = -BOXY/2.0 + real(J)* BOXY/real(NYgrid)
               GridL = -BOXY/2.0
          ENDDO
          DO I=1, NXgrid
               GridR = -BOXX/2.0 + real(I)* BOXX/real(NXgrid)
          IF ((X(N,M) .LT. GridR) .AND. (X(N,M) .GE. GridL)) THEN
               IF ((Y(N,M) .LT. GridU) .AND. (Y(N,M) .GE. GridD)) THEN
                    CALL RANDOM(Rndm)
                    !Rndm=1.0
                    INTENSITY(N, I, J) = INTENSITY(N, I, J) &
                    + (1.0 - rndcoef/100.0) &
                    + (rndcoef/100.0) * Rndm
               ENDIF
          ENDIF
     ENDDO

 126
GridL = GridR
ENDDO
GridD = GridU
ENDDO
ENDDO
ENDDO !N

DO W=1, 1 !100 !200
U = real(W)* PI/6.0 ! 0.005

DO J=1, NYgrid
  DO I=1, NXgrid
    Fsin(I,J) = 0.0
    Fcos(I,J) = 0.0
    Fdc(I,J) = 0.0
  ENDDO
ENDDO

DO J=1, NYgrid
  DO I=1, NXgrid
    DO K=1, NIMAGE
      INTENSITY0(K, I, J) = INTENSITY(K, I, J)
      * (1.0 + 1.0*DCOS(U) * DCOS(2.0*PI*(K-1)/Kall-U))
    !write(6,*)) K, I, J, INTENSITY0(K, I, J)
  ENDDO
ENDDO
ENDDO
ENDDO

DO J=1, NYgrid
  DO I=1, NXgrid
    DO K=1, NIMAGE
      Fsin(I,J) = Fsin(I,J) + (2.0/Kall) &
      * DSIN(2.0*PI*(K-1)/Kall) * INTENSITY0(K, I, J)
$F_{cos}(I,J) = F_{cos}(I,J) + (2.0/K_{all}) \times \cos(2.0 \times \pi \times (K-1)/K_{all}) \times \text{INTENSITY0}(K,I,J)$

$F_{dc}(I,J) = F_{dc}(I,J) + (1.0/K_{all}) \times \text{INTENSITY0}(K,I,J)$

ENDDO

ENDDO

ZeroCont = 0

McosAV = 0.0

MsinAV = 0.0

DO J=1, NYgrid
   DO I =1, NXgrid
      IF ( Fdc(I,J) .EQ. 0 ) THEN
         Modul(I,J) = 0.0
         DPhi(I,J) = 0.0
         Mcos(I,J) = 0.0
         Msin(I,J) = 0.0
         ZeroCont = ZeroCont +1
         WRITE(6,*ZeroCont
      ELSE
         Mcos(I,J) = (1.0/Fdc(I,J)) \times F_{cos}(I,J)
         Msin(I,J) = (1.0/Fdc(I,J)) \times F_{sin}(I,J)
         Modul(I,J) = (1.0/Fdc(I,J)) \times \sqrt{F_{sin}(I,J) \times F_{sin}(I,J) + F_{cos}(I,J) \times F_{cos}(I,J)}
         DPhi(I,J) = \text{ATAN}(F_{sin}(I,J)/F_{cos}(I,J))
         McosAV = McosAV + Mcos(I,J)
         MsinAV = MsinAV + Msin(I,J)
         ! McosAV2 = McosAV2 + Mcos(I,J)**2
         ! MsinAV2 = MsinAV2 + Msin(I,J)**2
      ENDIF
      IF ( Fdc(I,J) .NE. 0 ) WRITE(2900+hStep,112) Mcos(I,J), Msin(I,J)
      ! WRITE(116,111) DPhi(I,J), Modul(I,J)
   END DO
END DO
ENDDO

McosCntr = McosAV/real(NXgrid * NYgrid - ZeroCont)
MsinCntr = MsinAV/real(NXgrid * NYgrid - ZeroCont)

! WRITE(1900+hStep,115) McosCntr, MsinCntr
ENDDO !W

!--- Calculating Longest distand and variance from new center ---
LongDist = 0.0
XI = 0.0
YI = 0.0
RR2 = 0.0
DO J=1, NYgrid
   DO I=1, NXgrid
      IF ( Fdc(I,J).NE.0 ) THEN
         NewMcos(I,J) = Mcos(I,J) - McosCntr
         NewMsin(I,J) = Msin(I,J) - MsinCntr
         XI = XI + NewMcos(I,J)
         YI = YI + NewMsin(I,J)
         RR2 = RR2 + NewMcos(I,J)**2 + NewMsin(I,J)**2
         ! Dist = NewMocos(I,J)**2 + NewMsin(I,J)**2
         ! IF ( Dist.GT. LongDist ) LongDist = Dist
      ELSE
         NewMcos(I,J) = 10000.0
         NewMsin(I,J) = 10000.0
      ENDIF
   ENDDO
ENDDO

! write(6,*) RR2

XIcntr = XI / real(NXgrid * NYgrid - ZeroCont)
YIcntr = YI / real(NXgrid * NYgrid - ZeroCont)
Rcntr2 = XIcntr**2 + YIcntr**2

R2cntr = RR2 / real(NXgrid * NYgrid - ZeroCont)

Variance2 = R2cntr - Rcntr2

! write(6,*) R2cntr, Rcntr2, Variance2

Vari = DSQRT(Variance2)

! WRITE(1900+hStep,120) Vari

WRITE(150,120) Vari

111 FORMAT(2F12.6)
112 FORMAT(2F12.5)
113 FORMAT(3F12.5)
114 FORMAT(F10.2,F12.5)
115 FORMAT(F16.6,F16.6)
116 FORMAT(F10.5)
117 FORMAT(F10.2,F12.2,F12.2, F12.2)
119 FORMAT(F16.6,F15.3)
120 FORMAT(E25.15)

RETURN
END

!*******************************************************************************************
!*******************************************************************************************
SUBROUTINE CONTROL_DATA()

USE PARAMETERS
IMPLICIT NONE
NXgrid = 1392
NYgrid = 1040
Prsnt = 100.0 ! %90
!NALL = 1   !%90
!NIMAGE = 10
!read(*,*) NALL
END

!*******************************************************************************************
APPENDIX B

Associative Relaxation

As it was discussed in chapter 3.3.1, the solvent relaxation of NBD-X measured in different glycerol-water mixtures appeared to display anomalous behavior at higher water percentages (20% or more water). Figure B.1 represents the same graph with added phasors of NBD-X fluorescence in 30% water /70% glycerol.

The graph shows that increasing water percentage results in increasing the slope of fitted line. As one can see for the range of different water percentages, 0%, 3%, 10%, 20% and 30%, slopes can vary as 20.96°, 27.08°, 39.45°, 54.39° and 56.81°, respectively. Consequentially, for the 20% and 30% water /glycerol mixtures, the fitted lines cross the semi-circle at the x-axis. This then indicates a negative solvent relaxation time process, which at first sight is unphysical.

To search for a possible explanation, we draw on the analogous situation in time-resolved anisotropy. It is well-known that for an associative system where short-lifetime short-rotational correlation time fluorescence is mixed with long-lifetime long-rotational correlation time fluorescence, the time-resolved anisotropy undergoes a dip and rise behaviour with increasing time. In the frequency-domain this can give rise to apparent negative rotational correlation times.

In analogy with time-resolved anisotropy, one can understand the negative solvation time situation as an associative solvation dynamics process where a long
Figure B.1: Phasor plot for NBD-X in 100% glycerol (red line), 97% glycerol/3% water (black line), 90% glycerol/10% water (blue line), 80% glycerol/20% water (brown line) and 70% glycerol/30% water (yellow line). In each set there are two phasor points located outside and inside of semi-circle that correspond to detection wavelengths of 600 nm and 530 nm, respectively.

lifetime-long solvent correlation time fluorescence mixes with the fluorescence from a short lifetime-short solvent correlation time probe. The long solvation time-long lifetime may represent the NBD-X probe surrounded by glycerol molecules which relax slowly, where the short lifetime-short solvation time may represent the NBD-X probe surrounded by fast relaxing water molecules.

It is known that both the excited state lifetime of NBD and also the average dielectric relaxation time of the glycerol solvent increase with increasing water content. Although the glycerol-water mixture is formally well-mixed macroscopically, there is a probability that at the moment of light absorption that different NBD molecules will have different numbers of glycerol and water molecules in the solvation shell. The report of microscopically Immiscible dimethysulfoxide-glycerol binary solvent determined using solvent relaxation leads credence to this concept [145].
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144


