FLUORESCENCE LIFETIME-RESOLVED IMAGING MICROSCOPY STUDIES: QUANTITATIVE IMAGE ANALYSIS, SPECTRAL-FLIM, AND PHOTOSYNTHESIS

BY

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DISSERTATION

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Abstract

Development of instrumentation and image analysis methods for the fluorescence lifetime-resolved imaging microscopy (FLIM) were carried out in this thesis. With the new instrument setup and imaging processing algorithms, valuable information has been provided for studying in vivo photosynthesis activities.

Chapter 1 introduces the physics of fluorescence lifetimes and different techniques of FLIM measurements. It also provides practical considerations for successfully acquiring fluorescence lifetime imaging data, especially with in vivo samples. Chapter 2 discusses the image analysis algorithms essential for quantitative analysis of high through-put FLIM data. The advantages of the polar plot, a model-free analysis for fluorescence lifetime data, will be described in detail. The denoising procedure, variance-stabilizing-transform translation-invariant Harr wavelet, and the multi-scale edge detection algorithm, wavelet transform, were applied to improve the precision of lifetime resolution in the images, and to select features at the desired spatial frequency, respectively. K-means cluster analysis was used to analyze a polymer brush microfluid device and the chlorophyll (Chl) a fluorescence transient of Chlamydomonas reinhardtii cells. It will be shown that additional information is provided by the cluster analysis, which would otherwise be hidden in the large quantity of data. Chapter 3 discusses the instrumentation and image analysis methods for the spectrally-resolved FLIM (Spectral-FLIM), which was developed to overcome problems of separating lifetime components in complex environments, especially for fluorescence signals from fluorophores with very low intensity (< 2% of the background). Spectral-FLIM also allows a more detailed and accurate analysis of Förster resonance energy transfer (FRET) measurements. It will be shown in Chapter 3 that a single measurement of Spectral-FLIM can resolve three fluorescence signals (donor undergoing FRET to the acceptor, acceptor excited by FRET, and directly excited acceptor) on the polar plot.

Chapter 4 describes FLIM applications in photosynthesis studies. FLIM data of Chlamydomonas reinhardtii could distinguish the Chl a signals undergoing two different non-photochemical quenching pathways, energy-dependent quenching and state transitions. Spectral-FLIM
measurements of avocado leaves (*Persea americana* Mill.) during the slow phase of Chl a fluorescence transient were also carried out. The data show that the red- and far-red regions of the spectrum have different kinetics and lifetimes, which might suggest that there are two separate binding sites for xanthophyll molecules. Chapter 5 describes experiments to study the lutein-epoxide and violaxanthin cycles operating in parallel in avocado leaves. A good correlation of the fluorescence intensity and Chlorophyll a lifetimes is shown by FLIM measurements, indicating two different quenching states; therefore, the FLIM measurements provide evidence that both cycles are energy-dependent quenching pathways.
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1 Introduction to Fluorescence Lifetime-resolved Imaging Microscopy

1.1 Overview

Fluorescence lifetimes contain vital information critical for a detailed analysis of fluorescence signals (Becker et al., 2004, Hanson et al., 2002, Grant et al., 2005, Holub et al., 2000, Gadella, 2009, Periasamy & Clegg, 2009, Mizeret et al., 1999, Krishnan et al., 2003). Lifetimes are sensitive to the local environment of fluorophores and they are often the most reliable way to measure the efficiency of Förster resonance energy transfer (FRET). Barring aggregation, binding to - or interaction with - other molecular components, lifetimes are independent of fluorophore concentrations. Lifetime measurements are especially advantageous when studying complex cellular physiological and biological morphology. For this reason, fluorescence lifetime-resolved imaging (FLI; when specifically referring to microscopy, this is called FLIM) has become popular for in vivo experiments (Gadella, 2009, Periasamy & Clegg, 2009). This chapter first introduces the basic physics of fluorescence lifetimes and the techniques to carry out FLIM measurements, and then discusses some practical concerns for successfully acquiring fluorescence lifetime-resolved imaging data with in vivo samples.

1.2 Fluorescence Lifetimes

Figure 1.1 shows the Perrin-Jablonski diagram of a fluorophore (Noomnarm & Clegg, 2009). When a fluorophore is excited from the ground state $S_0$ to a higher energy electronic state, $S_1$ or $S_2$, it can return to the ground state by emitting a photon, or the excited molecule can pass through several alternative pathways of de-excitation; e.g. dynamic quenching, FRET, internal conversion (vibrational relaxation), intersystem crossing to and from the triplet state $T_1$ and $T_2$, and photochemical reactions (e.g., in photosynthesis, the light energy absorbed by chlorophyll is transformed via chemical reactions for generating food from CO$_2$). The average rate of decay
from an ensemble of excited molecules can be measured conveniently by detecting the time course of fluorescence decay. In the case of a single lifetime component, the decay is a decreasing exponential (Fig. 1.2 (a)) and this defines the fluorescence lifetime $\tau$. The fluorescence response $F_0(t)$ of a single component excited by a very short light pulse can be expressed as Eq. 1.1:

$$F_0(t) = F_0 e^{-t/\tau}$$

(1.1)

where $F_0$ is the amplitude of the exponential fluorescence decay, which is related to the fluorophore concentration $C_0$. The measured rate of decay, $1/\tau$ of Eq. 1.1, is equal to the sum of all rates of all pathways for exiting the excited state. The kinetic rate constant of the natural radiative pathway, $k_f$, is one term of the sum of rate constants. The natural rate of radiation is the rate of de-excitation provided that the only pathway for de-excitation is radiative (that is, the fluorescence pathway). Therefore, the total rate of de-excitation is a sum of all the rates, measuring fluorescence lifetimes provides information of other pathways, e.g. FRET.

Figure 1.1. The Perrin–Jablonski diagram of a fluorophore, showing the possible excitation and de-excitation pathways and the corresponding for transition time (in second). $S_0, S_1,$ and $S_2$ are electronic singlet states with different energy level, and $T_1$ and $T_2$ are electronic triplet states of different energy level. See text for details; the figure is from (Noomnarm & Clegg, 2009).
1.3 Fluorescence Lifetime Imaging Microscopy

There are several configurations of FLIM instrumentation (Gadella, 2009, Periasamy & Clegg, 2009), and each of them has its own strength for in vivo measurements. FLIM systems can be categorized according to the two major methods of data acquisition: time-domain and the frequency-domain techniques (Figure 1.2). They are discussed in the following paragraphs.

1.3.1 Time-domain FLIM

In the time-domain the fluorescence molecule is excited by a very short light pulse, which is usually applied repetitively, and the decay from the excited electronic state to the ground state is recorded directly in time (Eq. 1.1 and Fig.1.2 (a)). There are two categories of time-domain instruments (Periasamy & Clegg, 2009): time-gated and photon counting methods. In the time-gated method, the fluorescence signal is integrated over certain time intervals during the fluorescence decay (Pieter De et al., 2007). The integrated intensity of different time gates at each delay is analyzed to determine the pre-exponential factors and the time constants of each lifetime component. The number of gates is chosen depending on the number of fluorophores (with different lifetimes) to be resolved in the sample, which determines the total amount of time
for data acquisition. The photon counting method records the number of photons detected at
different times after the excitation (Becker et al., 2007); the number of photons detected at the
different times is used to reconstruct the decay profile. Photon counting methods feature high
lifetime resolution, relatively long times are required to acquire a complete lifetime image with
high spatial resolution – i.e. many pixels. The acquisition time, can be up to several minutes, and
the time for data analysis is also relatively long.

1.3.2 Frequency-domain FLIM

For frequency-domain FLIM, the excitation light is modulated repetitively at 10-200 MHz
frequencies. The modulated excitation light and the fluorescence response do not have to be
pure sinusoids. Any repetitive wave form can be expanded in a Fourier series, and each sinusoid
component can be analyzed separately from all other frequency components contributing to the
Fourier expansion. The following discussion refers to the fundamental frequency component,
which has the same frequency as the repetitive wave form (higher frequency components are
treated in the same manner). The changes in the modulation depth and the phase delay of the
signal, which are related to the fluorescence lifetime, are recorded and analyzed for the
fundamental repetition frequency (Schneider & Clegg, 1997); the lifetime information is derived
from the differences of the modulation depth and phase of the fluorescence signal relative to the
excitation waveform (Fig.1.2 (b)). The advantage of the frequency-domain method is the rapid
data acquisition, which is important for measuring molecular kinetics. The other advantage is
that modulation and phase data can be analyzed without further modelistic assumptions by
means of a “polar plot” (Redford & Clegg, 2005a, Chen et al., 2009b); see chapter 2. This
means prior knowledge of the lifetime model is not required in order to resolve the lifetimes.

Figure 1.3 shows the instrument setup of a frequency-domain homodyne FLIM system
constructed in our lab. Homodyne FLIM is a full-field, rapid data acquisition system. As shown
in the figure, the light source intensity is modulated repetitively by a Pockels cell, which is an
electro-optic modulator; in our case the Pockels cell is operated at a repetition frequency of 10-
200 MHz. Assuming a pure sinusoidal repetitive excitation, the modulated excitation light can be
expressed in complex notation (Euler's formula, $e^{i\theta} = \cos(\theta) + i\sin(\theta)$, where $i = \sqrt{-1}$) as
The oscillating light in Eq. 1.2 has a modulation radial frequency \( \omega \) (\( \omega = 2\pi f \), where \( f \) is the repetition frequency) with an initial phase value for the excitation \( \varphi_E \), a time-independent averaged DC intensity \( E_0 \), and a modulation depth (AC/DC) of \( M_E \). As mentioned above, the repetitive modulation of the excitation light (and thereby also that of the fluorescence intensity) does not have to be a pure sinusoid. The data are analyzed with a digital Fourier decomposition of the recorded signal at each pixel, and the component of the Fourier analysis of interest is usually the fundamental Fourier component (that is, the frequency component at the repetition frequency in Eq. 1.2). It is also possible to analyze the higher Fourier components (Periasamy & Clegg, 2009, Chandler et al., 2006). Each Fourier component can be analyzed separately, also on the polar plot (see next chapter for a description of the polar plot). The experiments shown in this thesis were carried out using essentially sinusoidal excitation at the fundamental frequency. Therefore, for simplicity, excitation modulation Eq. 1.2, the fluorescence emission, Eq. 1.3, and the resulting homodyne signal, Eq. 1.4, are all shown as pure sinusoids.

The fluorescence response to a repetitive excitation wave form is the convolution of the light (Eq.1.2) and the fluorescence decay process for a short excitation pulse (Eq.1.1) (Valeur, 2002, Lakowicz, 2006), which becomes for sinusoidal excitation:

\[
I(t) = QE_0(\sum_i F_i) \left[ 1 + M_E e^{i(\omega t + \varphi_E)} \sum_i \left( \frac{F_i}{\sum_i F_i} \frac{1}{\sqrt{1+(\omega \tau_{0,i})^2}} e^{-i \tan^{-1} \omega \tau_{0,i}}} \right) \right]. \tag{1.3}
\]

\( F_i \) denotes the steady-state fluorescence intensity contributed from fluorophore i. The value of \( F_i \) includes the fluorophore concentration \( C_{0,i} \), the fluorescence lifetime \( \tau_{0,i} \), and the fluorescence radiative rate constant \( k_{f,i} \) \( (F_i = C_{0,i} \tau_{0,i} k_{f,i}). \) \( \left( \frac{F_i}{\sum_i F_i} \right) \) is the fractional intensity of fluorophore i. Q accounts for instrument factors of the FLIM system. Eq.1.3 shows that for every frequency the fluorescence lifetime of each separate fluorophore is embedded in the corresponding modulation depth \( (M_i = \frac{1}{\sqrt{1+(\omega \tau_{0,i})^2}}) \) and the phase \( (\varphi_i = \tan^{-1} \omega \tau_{0,i}). \)

In our lab, a homodyning technique is used to measure this high frequency signal (Schneider & Clegg, 1997):
The fluorescence image is focused onto an image intensifier (Fig. 1.3). The intensifier is modulated at the same frequency as the excitation light, with gain $G_0$, modulation depth $M_G$, and phase shift $\varphi_G$. The recorded signal is a time-independent DC value that depends on the phase shift $\varphi_E + \varphi_G$, as well as the phase shift related to the exponential decay of all components, $\varphi_i$. The two modulation wave forms (Eq. 1.3 and Eq. 1.4) must be phase-locked, meaning that the repetitive frequencies must be identical, and their relative phases (phase difference between them) must remain constant during the measurement. The phase ($\varphi_G$) of the voltage amplification of the image intensifier (relative to the excitation) is shifted in steps from 0 to $2\pi$ to reconstruct a complete period of the sinusoidal function of Eq. 1.4.

Knowing $M_E \cdot M_G$, $\varphi_E$, and $\varphi_G$, the modulation and phase of the sample fluorescence can be determined. A minimum of three data points at different phases is necessary for determining the phase and modulation depth of a sinusoidal function, because there are three unknowns: DC, AC, and the phase. In FLIM experiments shown in this thesis, 8 phase shifts over $2\pi$ are usually acquired for a period of the sine wave, which from our experience is a good compromise between the data acquisition speed and the accuracy for sine reconstruction. Fluorophores of known lifetime are used to calibrate the instrument parameters ($M_E \cdot M_G$, $\varphi_E$, etc.). In this way, the modulation depth (AC/DC) and phase from the fluorescence sample are corrected for the instrument parameters in order to determine the true phase ($\varphi_i$) and modulation ($M_i$) parameters of the fluorescence signal.

### 1.4 Instrument considerations

It is always better to diminish noise contributions during data acquisition as much as possible. Many noise sources can be minimized by careful consideration before acquiring FLIM data. However, some sources of noise are unavoidable and are intrinsic to the measurement and instrument, and the use of a denoising algorithm improves the lifetime accuracy and image quality of FLIM. The discussions on the types of errors in FLIM measurement can also be found
in the literature (Spring & Clegg, 2009, vandeVen et al., 2005). Important considerations regarding the instrument are summarized in the following sub-sections.

![Instrument Setup Diagram]

Figure 1.3. The instrument setup of a full-field frequency-domain FLIM. The light source is first modulated at 10-100 MHz by a Pockels cell, then the excitation light is directed to the sample. Part of the excitation signal is monitored by a reference photomultiplier tube (PMT). The fluorescence emission of the sample is then demodulated by an image intensifier, which is modulated at the same MHz frequency as the Pockels cell but with a series of different phase shifts. Then the image is received by the charge-coupled device (CCD). With add-on components, such as a spinning disc module or a spectrograph, FLIM is capable of achieving confocality or resolving spectral information. The frequency synthesizer and phase shifter control the modulation/demodulation frequency and the relative phase between the excitation and modulation of the intensifier.

1.4.1 Laser and Pockels cell stability

A reference photomultiplier tube (Fig. 1.3, reference PMT) records a fraction of the modulated excitation light passing through the Pockels cell. Because both the laser and Pockels cell can undergo thermal drift over time with temperature fluctuations, the reference PMT signal is used to calibrate the instrument continuously in real time. We also use the homodyne technique to acquire the PMT signal. The modulation depth and phase shift from the reference PMT signal (which represents the excitation light parameters) are initially acquired with a FLIM sample of a known lifetime standard in order to calibrate the excitation parameters. By knowing $M_l$ and $\varphi_l$ of the standard, we can determine the $M_E \cdot M_G$ and $\varphi_E + \varphi_G$ of Eq. 1.4. The identical measurement
is then made on the sample, and the actual phase and modulation can be determined by using the standard calibration. This corrects for delays due to the speed of light as well as changes in modulation depth as the light passes through the microscope. In addition, since the PMT and the FLIM data are acquired simultaneously, fluctuations in light intensity are also continuously corrected. Random noise is reduced by averaging the homodyne data acquired over an extended time, and also by taking several consecutive average measurements.

### 1.4.2 Modulation and demodulation frequency

The modulation and phase of the fluorescence signal is a function of both the sample’s lifetime and the modulation frequency. Therefore, for every lifetime value, there exists an optimal frequency that provides the most reliable modulation and phase values, or in other words the best lifetime resolution, Eq. 1.5 (Redford & Clegg, 2005a).

\[
\omega_{\text{optimal}}^2 = \frac{1+\sqrt{3}}{2\tau^2},
\]  

(1.5)

\(\omega_{\text{optimal}}\) represents the optimal angular frequency \((\omega_{\text{optimal}} = 2\pi f_{\text{optimal}})\) for a sample with lifetime \(\tau\). For example, 100 MHz is the optimal modulation frequency for a 1.86 ns lifetime, and 40 MHz gives the best lifetime resolution for a 4.66 ns lifetime fluorophore. While the modulation frequency sets the lifetime resolution, the highest frequency the instrument can reliably operate depends on the electronic components. For example, the phase shifter and Pockels cell are both capacitive, and have the response times that work best in certain frequency ranges. The typical response times of the phase shifter or Pockels cell are less than 100 ps, which is more than enough for most fluorescence lifetime measurements. But measuring lifetimes below 50 picoseconds requires faster electronics; although, because we operate in the frequency mode, faster measurements can still be made with reduced signal-to-noise ratios.

### 1.4.3 Excitation light intensity

Often, higher excitation intensities result in better signal-to-noise ratios. But especially for biomedical samples, care must be taken to avoid photodamage and photobleaching. Sometimes
underlying reaction mechanisms that are triggered by light affect the fluorescence response; and the reactions often respond differently to different levels of light intensity. Photosynthesis is an example of these effects. In the case of photosynthesis, different fluorescence quenching pathways are induced by different levels of light intensities. This is related to the mechanisms by which plants and algae balance their photophysical response between the light-harvesting reaction and protection mechanisms against light damage (Papageorgiou & Govindjee, 2004).

This will be discussed in the following chapters. A fluorophore’s concentration is another parameter that influences the signal intensity and thereby the signal-to-noise ratio. When the signal is derived from intrinsic fluorescence, the concentration of the fluorophore is beyond the experimenter’s control. In such cases, the exposure time is the major variable parameter to obtain a satisfactory and reliable signal-to-noise ratio.

For full-field imaging, the laser beam is expanded to illuminate the full observed field of the sample. The laser beam has a Gaussian intensity profile, and the light intensity of even an expanded beam is not identical over the entire image. To avoid as much as possible the induction of different reaction activities mentioned in the previous paragraph (e.g. during photosynthesis) at different locations of the image, an expander with diffuser is recommended. That is, one should try to keep the excitation light intensity the same throughout the image. Then, at least, any changes in time are similar throughout the image for identical fluorophore environments in the image. Note that, barring intermolecular interactions and the sort of light-triggered reactions discussed above, the fluorescence lifetime is generally independent of the fluorophore concentration and excitation light intensity. This is also the case when there are multiple fluorophores or multiple deactivation pathways. However, if particular deactivation pathways respond differently to different levels of excitation, the laser intensity can be a factor influencing the measured fluorescence response.

### 1.4.4 Optics alignment

Some components are especially sensitive to the alignment of the optics. For photomultiplier tubes, the angle and the area that photons impinge on the photocathode can influence the efficiency of the photoelectron collection onto the first dynode, and thereby influence the PMT
output (this effect depends on the type of tube). For the Pockels cell, which uses the birefringence property of the crystal to modulate the light intensity, a correct alignment is necessary to produce the deepest modulation depth and the highest light intensity. To align the optics, the laser beam must be measured at different points along the optical path. Mounts with four degrees of freedom are desirable. For accurate Pockels cell alignment, one can also check for Lissajous figures using a diffusing element (Dartigalongue & Hache, 2003). By placing a light diffuser in front of the Pockels cell, which produces a beam with the wave-vector of the laser light oriented in all directions, an isogyro pattern can be seen by placing a white screen right after the Pockels cell (image not shown here, see Fig. 4 in (Dartigalongue & Hache, 2003)). If the Pockels cell is well-aligned, when the diffuser is not in the beam, then the outgoing laser beam will be in the center of the isogyro pattern when a diffuser was in the beam. The isogyro pattern is a simple and accurate way to align a Pockels cell.

1.4.5 Fluorescence lifetime standard

As mentioned above, frequency-domain FLIM requires a fluorescence lifetime standard to calibrate the instrument parameters. Common fluorophore lifetimes can be found in several references (Lakowicz, 2006, Boens et al., 2007). Fluorescein is one of the most common lifetime standards, due to of its high quantum yield and the excitation and emission wavelengths are convenient and similar to many dyes. Because the measured lifetime of the sample depends on how well the instrument is calibrated, any error in the measurement of the lifetime standard will degrade the accuracy of the sample measurement. Therefore, it is important to characterize and calibrate the instrument carefully with a known and robust reference fluorophore. A second or even third lifetime standard can be used to iteratively cross-calibrate the instrument. Also, a lifetime standard that has a similar lifetime value as the sample is preferable, as they are in the same optimal modulation frequency range. For many applications, it is not necessary that the reference fluorophore have a single lifetime; however, if an accurate measurement of the lifetime is desired, one should know the values of the lifetime components. This is because one is calibrating directly the modulation depth and the phase delay, see Eq. 1.4. It is only important to be able to determine the modulation and phase parameters of the excitation light. And it is most
important that the standard be chemically stable and that the phase and modulation characteristics be highly reproducible.

1.5 References


2 Quantitative Analysis of High-Content Frequency-Domain Fluorescence Lifetime Images

2.1 Introduction

Fluorescence lifetime imaging microscopy (FLIM) has been developed in the last two decades (Periasamy & Clegg, 2009, Gadella, 2009, Becker, 2005). The instrumentation has matured and enables fast data acquisition up to video rate (Requejo-Isidro et al., 2004, Holub et al., 2000, Redford & Clegg, 2005b). FLIM has been applied to record the fast kinetics of lifetimes in living cells (Holub et al., 2007, Matsubara et al., 2010), high throughput screening for cells and tissue (Esposito et al., 2007), and automatic detection of lifetime morphology (Spring & Clegg, 2009, Esposito et al., 2008, Buranachai et al., 2008). In order to extract quantitative information from large FLIM data sets, we have developed image processing methods for cluster analysis in the frequency-domain fluorescence lifetime images. It will be shown that the morphology and characteristics of each clustered lifetime elucidates the differences between them, and therefore helps to identify the mechanism of the lifetime variation within or between FLIM images. Since homodyne frequency-domain FLIM essentially takes fluorescence intensity images at different phase shifts ((Chen et al., 2009a); also see Chapter 1), many conventional and sophisticated imaging analysis algorithms are readily applicable. It is important for image processing of FLIM data to maintain energy conservation, because the intensity information at different phase images will be used to reconstruct lifetimes. In this context, conserving energy refers to the conservation of the total intensity over all spatial frequencies. It should also be taken into account that the characteristics of FLIM detectors are often different from those used for steady-state fluorescence images.

FLIM provides much useful information about the sample, including: fluorescence lifetimes, spatial morphologies of different fluorescence components, steady-state fluorescence intensities, etc.; therefore, there are several approaches for carrying out cluster analysis in FLIM. The choice is dictated by the sample properties. The values of the lifetime-resolved parameters can be
clustered, and the clustering results can be used to identify image morphologies belonging to particular lifetime components; spectroscopic properties (e.g. intensity) can be partitioned and related to the fluorescence lifetime and morphology; or the morphological features can be clustered in order to examine the correlation between physiological structures and lifetime distributions.

In this chapter, K-means clustering is applied to group the lifetime data for a polymer brush microfluid device on the polar plot (shown later in Fig. 2.8 and 2.10). K-means clustering is also used to distinguish *Chlamydomonas reinhardtii* cells having different intensity time traces, and then the resulting clusters are correlated with their lifetime values (shown later in Fig. 2.13 and Fig. 2.15). There are several image analysis procedures essential for robust cluster analysis in FLIM images, and they will be introduced in this chapter as well.

Lifetime data is presented by a modal-free data analysis method called the polar plot (Holub et al., 2007, Hanley & Clayton, 2005, Redford & Clegg, 2005a, Colyer *et al.*, 2008, Digman *et al.*, 2008, Buranachai *et al.*, 2008, Chen et al., 2009b); this method is especially suitable for comparing large data sets and complicated cellular dynamics. Polar plots offer an immediate insightful visualization of FLIM results. Prior knowledge of a lifetime modal is not required, and recursive fitting processes are bypassed. Because the locations of the data points on the polar plot are related to underlying lifetime values, it can be used to cluster lifetime pools even without performing model-based iterative regression of the FLIM data to determine lifetime values. Details on how to interpret data *via* polar plots will be discussed in this chapter.

Another image analysis procedure we have found to be valuable for clustering FLIM data is a denoising algorithm called variance-stabilizing-transform translation-invariant Harr wavelet (VST-TI Haar). TI-Haar was developed by Willet and Nowak (Willet & Nowak, 2003, Willett & Nowak, 2004) for photon-limited medical images having Poisson or signal-independent Gaussian noise. Because the detectors used for our FLIM instrumentation have different signal-to-noise characteristics, the TI-Haar algorithm can only be applied to denoise FLIM images after applying a variance-stabilizing transform (Spring & Clegg, 2009). Image denoising is very helpful when complex morphologies or lifetime heterogeneities are encountered. VST-TI Haar denoising provides an important asset for interpreting FLIM data.
The third algorithm, wavelet transform, is applied for multi-resolution edge detection in FLIM images. Wavelets are used to select features or objects at the spatial scale of interest, and reject backgrounds with different spatial scales. The morphology of the objects is used to separate fluorescence signals in overlapping locations, which is useful when they cannot be discriminated by simply thresholding the intensity. Another important property of the wavelet transform is that it maintains completeness and energy conservation of the images (Mallat, 2008), in addition to emphasizing the edges of objects. As will be shown in this chapter, the FLIM images reconstructed from the desired wavelet subbands retain the correct lifetime information. Therefore, morphology identification by wavelets can be effectively applied for FLIM clustering analysis.

2.2 Materials and Methods

2.2.1 Modal-free representation of data: polar plot

In a polar plot analysis, the modulation and phase of the fluorescence are used to construct a modal-free plot. Model-free means the plot requires only the measured modulation depth and the phase of the fluorescence signal. Figure 2.1(a) shows how to make a polar plot from the modulation and phase of the sample data. The measured values of $M_{\text{tot}}$ and $\varphi_{\text{tot}}$ are used to define new coordinates as in Eqs. 2.1 and 2.2 (Holub et al., 2007, Hanley & Clayton, 2005, Redford & Clegg, 2005a, Colyer et al., 2008, Digman et al., 2008, Buranachai et al., 2008, Chen & Clegg, 2009, Chen et al., 2010):

\[
x_{\text{tot}} = M_{\text{tot}} \cos(\varphi_{\text{tot}}) = \sum_i \left( \frac{F_i}{\sum F_i} \right) M_i \cos(\varphi_i) \tag{2.1}
\]

\[
y_{\text{tot}} = M_{\text{tot}} \sin(\varphi_{\text{tot}}) = \sum_i \left( \frac{F_i}{\sum F_i} \right) M_i \sin(\varphi_i) \tag{2.2}
\]

where $F_i$ denotes the steady-state fluorescence intensity contribution from fluorophore i, and $\left( \frac{F_i}{\sum F_i} \right)$ is the fractional intensity of fluorophore i. For emphasis, we have written the subscript “tot” in Eqs. 2.1 and 2.2 in order to indicate that the signal is the overall measured signal. Eqs. 2.1 and 2.2 show how $M_{\text{tot}}$ and $\varphi_{\text{tot}}$ are related theoretically to the corresponding parameters of...
individual fluorescence components in the case of multiple lifetime components. When there is a single lifetime, the (x,y) points will fall on a universal semicircle centered at (x,y)=(0.5,0), with a radius of 0.5. When the measured fluorescence signal is due to several lifetime components, the (x,y) positions on the polar plot will lie inside of the semicircle, and the locations of the (x,y) points are determined by the weighted intensity fractions of the contributing individual lifetime components (see Fig. 2.1(b) and the following discussion). As x and y values are also functions of the modulation frequency, data points corresponding to the same lifetime measured at different modulation frequencies have different locations on the polar plot. Polar plots are useful for interpreting complex cellular dynamics; the following discussion presents more details.

![Polar plot representation of simulated lifetime data at 100 MHz.](image)

Figure 2.1. Polar plot representation of simulated lifetime data at 100 MHz. The measured modulation $M_{tot}$ and phase $\varphi_{tot}$ are used to define the axes of the new axis system of polar plot, which has $x_{tot} = M_{tot} \cos (\varphi_{tot})$ and $y_{tot} = M_{tot} \sin (\varphi_{tot})$. (a) On the polar plot, single lifetime data falls on the universal semicircle, like the example of $\tau = 1$ ns. (b) Multiple lifetimes data locate inside of the semicircle. For a fluorescence system that has only 3 ns and 1 ns components, the point on the polar plot of a measurement lies on a straight line (dotted line) connecting these two constituent lifetimes. The location depends on the relative contribution from each lifetime components.

### 2.2.1.1 Data points on the semicircle

In Figure 2.1, it is shown that the locations on a polar plot of simulated single-lifetime data of a 100 MHz measurement lie on the universal semicircle. Longer lifetimes are located more counter-clockwise on the semicircle (greater phase), and shorter lifetimes are more clockwise. That is, for a single frequency of modulation, the phase angle increases and the modulation depth decreases as single lifetimes become longer. If there is light scattering in the recorded data, which is like having a fluorescence lifetime of 0 ns, the corresponding polar plot scattering component is at the most clockwise point: (x,y)=(1,0). It was mentioned in Chapter 1 that 100
MHz gives the best lifetime resolution lifetimes of 1.86 ns. The reason for this is easy to see from the distribution of points on the polar plot. Lifetime values ranging from 1 ns to 2 ns have the best resolution of the modulation and phase when modulating at the frequency of 100 MHz.

### 2.2.1.2 Data points inside of the semicircle

If there are multiple lifetime components, the points on the polar plot using the measured modulation depth and phase will fall inside of the semicircle. As shown in Fig. 2.1(b), if the signal is composed of 3 ns and 1 ns, then the polar plot points lie on a straight line connecting the points corresponding to the two single lifetimes positions, which lie on the semicircle (Periasamy & Clegg, 2009, Redford & Clegg, 2005a, Colyer et al., 2008, Chen & Clegg, 2009). The relative distances on the straight line from the measured point to the corresponding single-lifetime points on the semi circle are proportional to the intensity contributions of these single-lifetime components. If the 3 ns component contributes a higher intensity, then the point is closer to 3 ns, and vice versa; that is, the intensity contribution of each component is proportional to the fractional distance on the opposite side of the straight line from the measured point. When measuring FLIM during active cellular dynamics that interconvert between two fluorescence components, the measured points on the polar plot progress along the straight line between two lifetimes, as in Fig. 2.1(b). However, sometimes the interpretation is not immediately clear. Such a change in the intensity fractions between two separate lifetime components could be due to: 1) molecules from the pool of one lifetime component exchanging with molecules from the other lifetime pool, which means the total number of active fluorophores remains the same (just the fraction in each lifetime pool change), or 2) one of the lifetime components is photobleaching at a faster rate than the other. In the latter case, the total number of fluorophores is decreasing. Because the fluorescence intensity and the lifetimes are measured together in FLIM, one can calculate the number of molecules of each component and resolve this issue. The individual lifetimes can be determined from the intersection of the extension of the straight line. Thus, the molecular mechanism responsible for the phase and modulation changes can often easily be determined. A derivation of the relation between the lifetime data and fluorescence intensities, which allows the calculation of the concentrations of the different lifetime components, is given in Chapter 5 and in the literature (Holub et al., 2007, Matsubara et al., 2010).
2.2.1.3 **Data points outside of the semicircle**

Assuming that the instrument is calibrated carefully, there are generally two reasons if the polar plot data fall outside of the semicircle: either 1) Förster resonant energy transfer (FRET) is taking place, and one is observing (at least partially) the fluorescence of the acceptor (or some other product of an excited state reaction) (Chen et al., 2009b) or 2) photobleaching is happening during illumination (Redford & Clegg, 2005a, Malachowski et al., 2007). To tell which one is the real cause, one can simply check the phase data to see whether the average of the recorded sinusoidally modulated signal is decaying with time.

If the fluorescence signal comes solely from the acceptor of a FRET pair, the modulation $M^A_e$ and phase $\phi^A_e$ (after the initial analysis and corrections) are given as (Chen et al., 2009b, Lakowicz & Balter, 1982, Hanley, 2009):

\[
M^A_e = M^A_0 M^D \\
\phi^A_e = \phi^A_0 + \phi^D
\]  

(2.3)  
(2.4)

Here $M^D$ and $\phi^D$ are the modulation and phase of the donor in the presence of FRET, and $M^A_0$ and $\phi^A_0$ are the modulation and phase of the acceptor alone (that is, what would be measured if the acceptor were directly excited by excitation light). As calculated in Chapter 3, Eqs. 2.3 and 2.4 show that the polar plot points, if just the acceptor fluorescence is detected, lie outside the semi circle. According to Eqs. 2.3 and 2.4, if the acceptor lifetime in the absence of FRET is known, the donor $M^D$ and $\phi^D$ in the presence of FRET can be calculated and the FRET efficiency determined.

In the case of a decaying sinusoidal signal (for instance, with very rapid photolysis taking place during the data acquisition), one can fit the data with Chebyshev polynomials to evaluate the true modulation, phase, and decay constant (for instance, the decay rate of the fluorescence intensity due to photolysis) (Malachowski et al., 2007). In the Chebyshev algorithm, the data set is operated on by a bounded Chebyshev transform to obtain Chebyshev polynomial coefficients. Then algebraic equations involving recursion relations of the Chebyshev coefficients are solved algebraically to derive the parameters of the corresponding fluorescence parameters (phases, modulation values and the time constant of the photolysis decay). The method has the advantage
of avoiding recursive iterative fitting procedures, which would require starting parameters and is very time-consuming. Because the Chebyshev method is a single pass procedure, and the parameters are determined by numerical analysis of an algebraic equation, the Chebyshev fitting procedure is rapid (orders of magnitude faster than iterative fitting) and suitable for \textit{in vivo} FLIM, especially when there is large amount of data (e.g. a high number of pixels).

2.2.2 \textbf{Denoising FLIM images: VST-TI Haar algorithm}

Denoising algorithms have been shown to be very promising for FLIM image analysis (Spring & Clegg, 2009, Chen et al., 2010, Chang & Mycek, 2010a, Chang & Mycek, 2010b). The intrinsic and unavoidable noise of the FLIM measurement is due mainly to photon noise and contributions from the intensifier and CCD. If one knows the characteristics of the noise, one can apply an appropriate denoising algorithm in order to improve the construction of the lifetime-resolved image with greater precision. For frequency-domain FLIM, every phase-shifted image is denoised before calculating the modulation and phase values. TI-Haar is a multiscale image estimation method for data with Poisson and Gaussian noise. The algorithm has several important properties that make it useful for FLIM data. This multi-scale estimate can remove the noise without blurring the edges and boundaries in the image, and the image can be reconstructed with high accuracy. The algorithm developed by R. M. Willet and R. D. Nowak (Willett & Nowak, 2003, Willett & Nowak, 2004) also has the advantages of reduced computational time.

2.2.2.1 \textbf{Variance-Stabilizing Transform}

As photon noise is Poissonian, the variance-vs-mean characteristic from just a CCD is a linear curve, and has a slope of 1. But with an image intensifier used in FLIM, new noise is introduced into the image. As shown in Figure 2.2, the recorded signal has larger variance that cannot be characterized simply as Poissonian. The noise distribution of the intensified CCD (ICCD) has a signal-dependent Gaussian distribution (this is not just the Gaussian corresponding to the limit at high number of photons of the original Poissonian noise; there are additional effects introduced by the intensifier (Spring & Clegg, 2009)). In order to use TI-Haar denoising of the signal-independent Gaussian noise, a Variance-Stabilizing Transform has to be first applied (Prucnal &
Saleh, 1981). From the variance-vs-mean characteristic curve, the variance \( \sigma(x)^2 \) can be expressed as a function of the signal intensity \( x \),

\[
\sigma(x)^2 = f(x),
\]

and then normalized to 1:

\[
y(x) = VST(x) = \int \frac{dx}{\sqrt{f(x)}}.
\]

After applying TI-Haar denoising, the data must undergo an inverse VST to recover the actual denoised image, with the true signal-to-noise dependence.

![Figure 2.2. The mean-variance curves of the ICCD at 100 MHz and with gain as 760 volts and 660 volts. The variance of the signal is larger than 1, which clearly shows the ICCD noise is not Poissonian.](image)

**2.2.3 Multiscale edge detection: wavelet transform**

Convolution filters (Fig. 2.3(a)) are commonly used in edge detections, in which the Gaussian smooth filter is used to get rid of high frequency components, and x- and y-derivative filters emphasize the edge transitions in the images. At the proper scale, convolution filters are capable of detection objects of interest (Meijering & van Cappellen, 2007, Adiga et al., 2006). But FLIM requires the edge detection algorithm that has multiresolution compatibility for lifetime calculations, which means the image represented by lower resolution functions should be contained within those by higher resolution ones, and the signal reconstructed from all scales should converge to the original signal. Wavelet transform is therefore applied for object detection for FLIM image (Mallat, 2008). It includes the wavelet- and scaling functions in the analysis:
and are integers. The dilation step $s_0$ is usually chosen as 2, which corresponds to the dyadic sampling in the frequency axis. As both equations are localized functions of certain dimension, they represent signals by translation and dilation. In comparison to Fourier transforms, the wavelet function is confined both in frequency and space; therefore, it is more appropriate for edge detection. The scaling function is defined as the summation of wavelet functions at scales larger than one (Mallat, 2008), in order to cover the lower frequency part of signal left by a wavelet function. For the 2D wavelets, the functions include a scaling function and three wavelet functions in the horizontal, vertical, and diagonal directions, as shown in Fig. 2.3(b).

The wavelet- and scaling function can be considered as a band-pass and low-pass filter for a signal:

$$f_j(x) = \sum_k \lambda_j(k) \varphi(2^j x - k) + \sum_k \gamma_j(k) \psi(2^j x - k).$$

Eq. 2.9 states that a signal $f(x)$ at scale $j$ can be decomposed into the lower frequency approximation and higher frequency details. $\lambda_j$ and $\gamma_j$ are the coefficients for the scaling and wavelet functions, acquired by the inner product of the signal to the corresponding function. In Figure 2.4, the signal is iteratively decomposed into an approximation and details by the wavelet transform. As wavelets satisfy the multi-resolution compatibility property, features can be selected from the appropriate scale without loss of information (like the A2-A6 in Fig. 2.4).

In the *Chlamydomonas* experiments (shown later in Fig. 2.13), wavelets are applied to distinguish the cells from the inhomogeneous background and the high frequency noise. Afterwards, intensity thresholding is possible to recognize all the boundaries in the image. Then image opening by a disc of diameter of 8 pixels is applied to choose the objects of proper size (Gonzalez *et al.*, 2004).
Figure 2.3. Different methods for edge detection in imaging analysis. (a) Convolution filter includes a Gaussian smoothing filter to get rid of high frequency components (top), y-derivative (middle) and x-derivative (bottom) filters for edge detection. (b) Haar wavelet bases for 2D image analysis. It uses a scaling function as a low-pass filter, and wavelet functions in x-, y-, and diagonal directions as a band-pass filter.

Figure 2.4. Wavelet transform of a row of the *Chlamydomonas reinhardtii* fluorescence images in Figure 2.13. At different filter stage, the signal at previous scale is splitted by the wavelet- and scaling functions. The wavelet function can be considered as a band pass filter; the center frequency of the wavelet base at the first scale is shown. The objects of interest can be picked out at the proper scale range, as the A2-A6 figure includes the image features at the scales of 2 to 5.
2.2.4 Clustering components in fluorescence lifetime images: K-means and B-spline

K-means clustering using Euclidean distance is applied in this chapter:

$$\arg \min_{\mu_1, \ldots, \mu_k} \frac{1}{n} \sum_{i=1}^{k} \sum_{j=1}^{n} |x_{ij} - \mu_i|^2.$$  \hspace{1cm} (2.10)

After defining the number of clusters $k$, K-means finds the location of centroids of each cluster $\mu_i$, and then assigns data points $x_{ij}$ to the closest centroid. The clustering result is when the total distance from the data points to their cluster center is the minimum. Therefore, the definition of the distance measurement decides the location of cluster centroids. Euclidean distance is a linear measurement (Gentleman et al., 2005), and each variable is equally weighted in the calculation.

For the time-course intensity data, least-squared cubic B-spline approximation is applied to summarize the intensity curves, and then the spline coefficients are used for K-means clustering. B-spline is used to reduce the data size for large amount of measurements, which speeds up the clustering calculation and reduces the possibility of computational errors (Abraham et al., 2003, Bar-Joseph et al., 2002, Luan & Li, 2003, Tarpey, 2007). The calculation can be expressed according to the following equations:

$$S(x, \beta) = \sum_{i=1}^{K+d+1} \beta_i B_i(x)$$  \hspace{1cm} (2.11)

$$\beta = \arg \min_{\beta} \frac{1}{m} \sum_{j=1}^{m} |y_j - S(x_j, \beta)|^2 = (B^TB)^{-1}B^Ty.$$  \hspace{1cm} (2.12)

A spline $S(x, \beta)$ is defined according to the spline basis function $B(x)$ and the spline coefficient $\beta$. The total number of parameters $K + d + 1$ depends on the $K$ knots in the data region (the number of knots is the number of sections that the original data is divided into, where each section is approximated by a spline function), and the $d$ degree of spline function. The least-squared approximation of the curve $y(x)$ by a B-spline is as shown in Eq. 2.12. Since all the curves for clustering analysis will be approximated by the same basis functions $B(x)$, and those functions are localized in $x$, the representation by coefficient $\beta$ is meaningful for the data set (Abraham et al., 2003). In Figure 2.14 (shown later), after the wavelet analysis and object selection, 71 objects are marked and each of them has 30 continuous temporal data points. With
approximation to a cubic spline of one polynomial piece, the 30 intensity data points can be represented by the 4 coefficients of cubic B-spline basis functions.

### 2.2.5 Sample preparation

The polymer brushes microfluid device was designed to study the molecular diffusion in the polymer layer (Tu et al., 2004, Heitzman et al., 2004). They were made by Nihan Yonet-Tanyeri in the material science and engineering department at UIUC. The silane initiators of the poly(oligoethylene glycol) acrylate (POEGA) brushes were imprinted on the coverslip, and the brushes formed by atom transfer radical polymerization. The brush pattern was covered by the polydimethylsiloxane (PDMS), which was thermally bonded to the coverslip (Figure 2.5). The polymer brush pattern includes dots 30 µm in diameter, and lines 30 µm wide and 1 cm long; both were 100 µm in height. The dye solution was flowed into the polymer brush regions from the holes punched through the PDMS; the incubation time for the dye to diffuse was about 3~10 days. In the FLIM measurements, 1 mM of 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS) in the pH 10 buffer was used. The laser excitation wavelength was 440 nm and was modulated at 40 MHz. A 40X objective was used. The CCD exposure time was adjusted to acquire satisfactory signal-to-noise ratios. In order to account for the light scattering from PDMS, a FLIM measurement of the PDMS region far away from the dye injection holes was taken. This was considered as the background signal, and subtracted from all the FLIM images.

![Figure 2.5. Polymer brush microfluid device. (a) The polymer is patterned on the coverslip, and then covered by PDMS. (b) The fluorescence intensity image of the polymer brushes, showing the dye diffusion pattern in the polymer brushes and the PDMS regions. (Image courtesy of Nihan Yonet-Tanyeri, UIUC.)](image)
Wild-type *Chlamydomonas reinhardtii* cells grown in a liquid media (12.0 mM Tris; 17.4 mM acetate; 7.0 mM NH$_4$Cl, 0.4 mM MgSO$_4$, 0.3 mM CaCl$_2$, 1 mM phosphate buffer, 1 ml/L Hunter’s trace metal elements; adjusted to pH 7 with 1 ml/L glacial acetic acid) were deposited onto a nitrocellulose filter paper, which has pore size of 1.2 μm (Holub et al., 2007, Chen et al., 2010). The cells were excited with a 488 nm laser and the fluorescence emission of chlorophyll a between 670~725 nm was measured. The modulation frequency of excitation was 100 MHz, and a 40X objective lens was used in the FLIM microscope. After focusing the image, the cells were dark adapted for 5 minutes before 30 continuous FLIM measurements were taken. The total experiment took about 240 seconds and the complete fluorescence transient was recorded. During the 30 FLIM experiments, the laser light illuminated the cells. In all the FLIM data, the CCD dark current was subtracted before further image analysis.

### 2.3 Results

**2.3.1 Wavelet analysis and TI-Haar denoising of simulated FLIM data**

A frequency-domain FLIM data set was simulated to demonstrate wavelet analysis and TI-Haar denoising (Chen et al., 2009a). The modulation and demodulation frequency was set at 100 MHz. The fluorescence intensity image of the simulation is shown in Fig. 2.6 (a). The background has a 0.5 ns lifetime with Gaussian intensity distribution in addition to a white readout noise. On top of the background, there are two larger ellipses with a 3 ns lifetime and ten smaller ellipses with a 2 ns lifetime. Inside of the larger ellipses, there are round objects each has a lifetime of 1 ns. Poisson noise was added to the simulated data. Fig. 2.6 (b) is the polar plot analysis of every pixel from Fig. 2.6 (a), which shows visually that there are four different distributions of lifetime pools. Except for the lifetime pool corresponding to the background-only area, all three other pools have more than one lifetime component. Fig. 2.6 (c) and Fig. 2.6 (e) are the fluorescence intensity images of Fig. 2.6 (a) after performing wavelet multi-resolution analysis. The wavelet scale is determined by the spatial frequency of interest for the different objects in the original image. Fig. 2.6 (c) is the third-scale details image, which highlights the edges of objects and produces only the intensity differences at the edges. All the different wavelet scales of detail are analyzed separately; then the values of the third scale of details at
every pixel and for every phase are selected for the digital Fourier analysis (see Chapter 1). The result is plotted on the polar plot of Fig. 2.6 (d), which gives directly the lifetime values of the three separate components. Note here that no prior model assumption is made prior to the wavelet and polar plot analysis. Fig. 2.6 (e) is the fluorescence intensity image after subtracting the first two scales of detail and the 10th-scale approximation image. In other words, the high-frequency noise and the low-frequency background are eliminated from the data, and the polar plot representation in Fig. 2.6 (f) shows that the three lifetime pools have no background contribution and the distributions on the polar plot are more compact due to noise removal. The gray scale bar of the image indicates the fluorescence intensity (in arbitrary units) and the gray scale bar for the polar plot represents the number of pixels with that value.
Figure 2.6. Wavelet analysis of and the corresponding polar plot representation. (a) Fluorescence intensity of the simulated FLIM data. (b) Polar plot analysis of all the pixels in (a). (c) Fluorescence intensity at the third-scale wavelet detail of (a), which detects the edges of all the objects. (d) Polar plot representation of (c) gives the exact lifetime values of the objects. (e) Fluorescence intensity image after subtracting the two highest scales of detail and the 10th-scale of approximation image. (f) Polar plot of (e) shows the result after noise and background removal. The gray scale bar of the image indicates the fluorescence intensity (A.U.) and the one for the polar plot gives the values of pixel numbers.

In Fig. 2.7 the TI-Haar algorithm is used to remove the Poisson noise from the raw image. Compared with Fig. 2.6 (b), the polar plot of the image after denoising (Fig. 2.7 (b)) has data points located at almost single values. Thus, with the proper choice of a denoising algorithm, one can apply a wavelet analysis after denoising for better results (also shown later in Fig 2.8, 2.12, and 2.13).
2.3.2 Clustering lifetimes based on the polar plot: polymer brushed microfluid device

The FLIM measurements of the polymer brushes are shown in Figure 2.8. The intensity column of Fig. 2.8 shows the steady-state fluorescence intensity of 5 different parts of the microfluid device. Because the fluorescence dye reaches different parts of the device through diffusion, the amount of dye in those parts of the image depend on their distance from the dye injection hole. In the polymer brush region, the fluorescence intensity is usually higher than the PDMS only area. In parts (b) - (d) of the microfluid device the pattern of the polymer brushes is not distinctly observable, because these locations are farther from the dye injection hole. In the images in the phase column, it shows the phase value, which is related to the fluorescence lifetime ($\phi = \tan^{-1} \omega \tau$). The polymer brushes and PDMS-only regions are clearly distinguished according to their phase values, even in the parts where the amount of dye is too small to show a difference in intensity. The FLIM results demonstrate that the fluorescence lifetime of HPTS in the PDMS region is shorter, which means the fluorophore has lower quantum yields in PDMS than in polymer brushes. That is, the intensity difference is not merely due to the amount of dye in them.

The lifetime values at different areas of the image can be calculated one by one, as shown in Figure 2.9. In this case, because we are interested in the properties of the fluorophore in many different regions, the clustering analysis is applied to help interpret the large number of data. In the polar plot column of Figure 2.8, every pixel of the denoised FLIM image is presented on the polar plot. Three major lifetime pools are revealed. All the lifetime data are then analyzed by K-means clustering (Figure 2.10). Three clusters are assigned in the calculation, and the centroid and boundary of each cluster are indicated in Fig. 2.10 (b). The lifetime clustering result is
applied to the images in the K-means column of Fig. 2.8. The pixels belonging to the cluster of the longest lifetime pool are shown in white, the pixels of the lifetime cluster that has lifetime values in between are in gray, and the pixels from the shortest lifetime cluster are in blue. The results show that the polymer brush- and PDMS-only regions belong to different lifetime clusters, as also shown by the phase images. The advantage of using the results of the K-means analysis over just using the phase images is that the location of the gray lifetime cluster provides additional information on its origin. The gray pixels were located around the polymer brushes in images of (a) and (e); therefore, it is reasonable to conjecture that there should a small gap between the interface of the polymer brushes and the PDMS, and the dye is in close contact of both materials at that location. Because the PDMS quenches the fluorescence dynamically, the lifetime values of HTPS at the interface between the two materials are between the lifetimes in each material. Based on this observation, the gray pixels in image (c) and (d), which are not in proximity to polymer brushes, could also be interpreted as being in the small gap between the PDMS and the glass. Since not all the fluorophore molecules in those regions are in close contact with PDMS, the detected lifetime would correspond to the intermediate value. Therefore, it is evident that the clustering analysis is able to make out the correlation between the fluorophore locations and their lifetimes; such information is crucial to understand the fluorescence characteristics and the physical structures of the samples.
Figure 2.8. Fluorescence lifetime measurement of HPTS diffusion in different parts ((a)~(e)) of a polymer brush microfluid device. The excitation wavelength was 440 nm and modulated frequency was 40 MHz. A 40X objective was used. The intensity column shows the steady state fluorescence intensity images, with colormap indicating the intensity in A.U.; the phase column shows the images of the phase value, with the colormap in degrees; the polar plot column is the lifetime representation of every pixel from the corresponding de-noised FLIM data, and the colormap is the number of pixels; the last column, K-means, are lifetime images after cluster analysis. The K-means cluster result is shown in Fig.2.10. The white color represents the cluster of the longest lifetime, the gray area has the intermediate lifetime value, and the blue color has the shortest lifetime. See text for details.

Figure 2.9. The polar plot analysis of selected regions from the intensity column in Fig. 2.8. The results of least-squares fitting to single lifetime of each region are also shown.
Figure 2.10. K-means clustering of the polymer brush device FLIM data based on the polar plot. The 3D (Fig. 2.10 (a)) and 2D (Fig. 2.10 (b)) polar plot of all the pixels from Fig. 2.9 (a)–(e). The z-axis and color code represent the pixel count. The black circles and black lines in (b) indicate the cluster centroids and boundaries.

2.3.3 Clustering lifetimes based on the intensity transient: the slow phase of chlorophyll a fluorescence transient of Chlamydomonas reinhardtii

The image processing procedures for clustering the slow phase of chlorophyll a fluorescence transient is shown in Figure 2.11. The usefulness of image denoising is first demonstrated in Figure 2.12. This figure represents a FLIM data set taken during the fluorescence transient (Papageorgiou & Govindjee, 2004, Lazár, 1999). Fig. 2.12 (a) is the fluorescence intensity image of the cells. The gray-scale bar indicates the fluorescence intensity in arbitrary units. After VST-TI Harr denoising, the fluorescence lifetime analysis at every pixel from Fig. 2.12 (a) is shown on the 3D polar plot (Fig. 2.12 (b)). The z-axis represents the number of pixels corresponding to a particular point on the polar plot. It is clearly seen that the cells’ lifetimes are not from a single component. Besides a major population, some pixels show longer lifetimes. Figs. 2.12 (c) and (d) are polar plots in 2D and 3D before denoising of the same data as analyzed in Figs. 2.12 (a) and (b) (after denoising). The centroid of the lifetime pool remains the same after the denoising; however the width of the lifetime distribution is noticeably tighter. Therefore, denoising the image can considerably reduce the spread of the lifetime data. Only noise components due to random variance of the measurement are removed; the variance due to distributions of lifetimes is not affected. This is especially relevant in cases when it is important to study the morphology of the sample and the statistics of lifetime heterogeneity, as will be shown in Fig. 2.13.
Figure 2.11. Flow chart of the quantitative analyses of the fluorescence lifetime images shown later in Fig. 2.13. See text for details.

(a) Denoising FLIM
(b) Wavelet transform of the images at the selected spatial frequency
(c) Intensity thresholding for boundary recognition
(d) Morphological opening by a structuring element of selected shape and size
(e) ~ (h) K-means clustering of the intensity transient curves

Figure 2.12. VST-TI Harr denoising of FLIM data from *Chlamydomonas reinhardti* cells. Data from one of 30 FLIM measurements during the slow phase of chlorophyl a fluorescence transient is shown here. *Chlamydomonas* cells were deposited on a nitrocellulose filter paper (with pore size 1.2 μm) for FLIM measurements. The cells were excited by a continuous 488 nm laser and imaged by a 40X objective lens. The modulation frequency was 100 MHz.

(a) The steady-state fluorescence intensity image of the *Chlamydomonas* cells from the FLIM data. The gray-scale bar is the intensity in arbitrary unit (A. U.). (b) The histogram of VST-TI Harr denoised FLIM data on the polar plot. The gray-scale bar and the z-axis represent the pixel counts. The data is composed of a major lifetime pool, and a minor fraction of signal at longer lifetime value. (c) and (d) are the polar plot of the raw data (before denoising) in 2D and 3D respectively. Compared with the denoised data in (b), the longer lifetime pool is not as noticable in (c) and (d).
Fig. 2.13 (a) is the fluorescence intensity image from one of the 30 FLIM measurements. It is denoised by VST-TI Haar analysis, as shown in Fig. 2.12. The denoised image is processed by the wavelet transformation, and the features between scales 3-6 of the wavelet transform procedure are selected for the boundary identification. In this scale range, the high spatial frequency features and the inhomogenous low spatial frequency intensity distribution can be ignored; that is, by this analysis the boundaries are robustly identified. The results of the boundary selection are shown in Fig. 2.13 (c). Then, after the subsequent image opening operation (Gonzalez et al., 2004, Meijering & van Cappellen, 2007) the boundaries are left with proper area, as shown in 2.13 (d). The 71 fluorescence intensity time traces of the objects that were recognized by the above operations (wavelets, intensity thresholding, and image opening) were then used for cluster analysis. The cluster algorithm was instructed to find five clusters, and the results are shown in Fig. 2.13 (e)-(h). The data are color-coded to designate their cluster assignment. The clusters having intensity values are colored from high to low as: yellow, red, cyan, blue, and pink (Fig. 2.13 (f)). In Fig. 2.13 (e), the fluorescence intensity clustering of cells approximately correlates with the intensity distribution of the excitation illumination (shown in Fig. 2.14; the illumination power was recorded using a fluorescein solution.). In the 2D and 3D polar plots in Fig. 2.13 (g) and (h), the lifetimes correspond well to the fluorescence intensity; that is, longer lifetimes correspond to higher fluorescence intensities, and *vice versa*.
Figure 2.13. Quantitative analysis of FLIM data during the slow phase of chlorophyll a fluorescence transient. (a) Fluorescence intensity image of *Chlamydomonas* cells after VST-TI Harr denoising, also see Fig. 2.12. (b) Wavelet analysis of the image chosen at scale 3–6. (c) Intensity thresholding for boundary recognition. (d) Using image opening to get rid of objects that are too small. 71 objects are left. (e) Applying K-means clustering to the fluorescence transient. 5 groups of cells are colored according to the cluster they belong to. (f) The time trace of the steady-state fluorescence intensity. (g) and (h) are the polar plot representation of the clustered time-course FLIM data of all 71 cells in 2D and 3D, also shown later in Fig. 2.15. The color of the clusters with intensity from high to low is: yellow, red, cyan, blue, pink.

Figure 2.14. Gaussian intensity profile of the laser illumination in *Chlamydomonas* FLIM experiments. Measured from a fluorescein solution.
The cluster results indicate several interesting findings. For the two clusters within the middle fluorescence intensity range (cyan and blue), the Chalmys were mostly at locations with middle power illumination (Fig. 2.15). Those cells can be further divided into two subgroups (both the cyan and the blue cells are each divided into two subgroups): (1) one group was illuminated by higher power excitation (those are in the right of the image), and (2) the other group is located at positions of the image with slightly lower power illumination. For the higher power illumination subgroup (1), the *Chlamydomonas* cells had shorter lifetimes and lower intensities. On the contrary, the other subgroup of *Chlamydomonas* cells (2) gave longer lifetimes and higher intensities. The difference between the two subgroups can be explained by the activation of non-photochemical quenching (NPQ) pathways, which alters the chlorophyll a fluorescence intensity and lifetime as a response to different light intensity.

While blue and cyan clusters are controlled by photo protection mechanisms, the other three clusters cannot be considered solely as NPQ reactions. The Chalmys of the yellow and red clusters received the most excitation light, and yet their fluorescence lifetimes are the longest (around 1 ns) and the fluorescence intensity is the highest. The *Chlamydomonas* cells from the pink cluster all have shorter lifetimes and lower fluorescence intensities, in spite of the expectation that low intensity light is not expected to have activated any (or much) NPQ reaction. For those clusters of cells, the lifetimes and fluorescence intensities are positively correlated with the power of illumination; therefore, NPQ is not the dominant pathway for them.

Through cluster analysis, the cellular response to excitation light has been categorized according to the relation between the illumination intensity and the lifetime values. This provides valuable information concerning the delicate balance between the different cellular light-response pathways.
2.4 Discussion

The results with polymer brushes and *Chlamydomonas* demonstrate the advantages of cluster analysis for large sets of FLIM data. Additional information can be extracted from the correlation between clustered lifetimes and morphology within the image. With the help of wavelet multiresolution detection, objects of interest are chosen for cluster analysis. This gives a quantitative view of the lifetime and fluorescence intensity heterogeneity in the sample. In both examples of polymer brushes and Chalmys, new information is provided from the clustering analysis, which is otherwise hidden in the large quantity of data.

The advantage of using wavelets for edge detection in FLIM images can be seen from Fig. 2.16. Convolution filters are linear operations. Because of this linearity present in convolution filters, the different spatial frequencies will still overlap in the final image. In order to select cleanly the different spatial frequencies for the FLIM calculation, the complete separation of neighboring spatial frequencies is required. This is available from the wavelet transformation. Fig. 2.16 (a)
shows an intensity image of the simulated FLIM data from Fig. 2.6. After x- and y-derivative filters are applied, the edges of the image are highlighted. But the features of the image with different spatial frequencies are still present in the image, and they are amplified by the convolution as well, producing the noisy signals in Fig. 2.16 (b).

Figure 2.16. Convolution filters for edge detection and the corresponding polar plot results. (a) After applying x-derivative and y-derivative convolution filters, the intensity gradient image highlights the edges. (b) The polar plot representation of all the pixels from (a). As signals at different spatial frequency are still included in the image, the result looks noisier than Fig. 2.6 (d).

The transformation of the lifetime data onto the polar plot enables the cluster analysis for multidimensional FLIM data. As demonstrated in this chapter, not only can the data points on the polar plot be clustered, but curves after parameterization by the B-spline approximation, can also be clustered. Therefore, higher dimensional FLIM data (e.g. spectrally-resolved FLIM or time-lapse FLIM) can also be summarized by a 3D B-spline on the polar plot, and then analyzed quantitatively by cluster analysis as well.

2.5 Acknowledgements

I thank Chittanon Buranachai for his initial work on applying wavelet for FLIM image analysis, and Bryan Spring for developing VST-TI Haar denoising procedure. I also thank my collaborator, Nihan Yonet-Tanyeri from Professor Paul V. Braun’s group, for making the polymer brush devices and useful discussions.
2.6 References


3 Spectral resolution in conjunction with polar plots improves the accuracy and reliability of FLIM measurements and estimates of Förster resonance energy transfer efficiency

3.1 Overview
A spectrograph with continuous wavelength resolution has been integrated into a frequency-domain fluorescence lifetime-resolved imaging microscope (FLIM). The spectral information assists in the separation of multiple lifetime components, and helps resolve signal cross-talking that can impede an accurate analysis of multiple lifetime processes. This broadens the number of dyes that can be measured simultaneously in a FLIM measurement. Spectrally-resolved FLIM (Spectral-FLIM) also provides a means to measure more accurately the lifetime of a dim fluorescence component (as low as 2% of the total intensity) in the presence of another fluorescence component with a much higher intensity. With Spectral-FLIM more reliable separation of the donor and acceptor fluorescence signals are possible for Förster resonance energy transfer (FRET) measurements; this allows more accurate determinations of both donor and acceptor lifetimes. With the polar plot analysis of Spectral-FLIM data, the spectral dispersion of the acceptor signal can be used to derive the donor lifetime - and thereby the FRET efficiency - without iterative fitting. The lifetime relation between the donor and acceptor is also used to separate the FRET pair from the donor alone signal. This method can be applied further to quantify the signals from separate FRET pairs, and provide information on the dynamics of the FRET pair between different states.

3.2 Introduction
Fluorescence lifetime-resolved imaging microscopy (FLIM) has become popular for in vivo measurements of living cells (Spriet et al., 2007), histopathology samples (Eliceiri et al., 2003), and tissue morphologies (Hanson et al., 2002, De Beule et al., 2007). Both intrinsic and extrinsic
fluorophores provide useful information for studying biological problems. However, a challenge frequently encountered with *in vivo* lifetime measurements is how to separate lifetime components in a complex environment. For example, when several fluorophores are measured simultaneously, spectral overlap makes it difficult to separate and determine the separate lifetimes. In a similar manner, fluorescence from intrinsic cellular components can influence the reliability of the analysis of specific fluorescence components and lessen the accuracy of lifetime calculations. The fluorescence signal of interest is often low compared to the background, either because the specific fluorescence intensity is low, or because the excitation intensity must be minimized to avoid photodamage of the sample. Spectral-FLIM with high wavelength resolving power (518 wavelength channels) has been developed to acquire quantitative data for *in vivo* measurements, and to overcome the problems of separating lifetime components, especially for the dimly fluorescent samples. By incorporating a spectrograph into the FLIM setup, individual lifetime components are more reliably and accurately resolved, and the fluorescence signal from fluorophores with very low intensity (<2% of the total intensity) are more easily identified and detected.

Spectral-FLIM also allows a more detailed and accurate analysis of Förster resonance energy transfer (FRET) measurements. Linear unmixing can cleanly separate the fluorescence spectrum of the donor and acceptor. In addition, using Spectral-FLIM a novel analysis method has been developed using a polar plot representation for Spectral-FLIM FRET measurements (Chen et al., 2009b). In FRET, acceptor fluorescence is excited by the excited donor, which is decaying in a time-dependent manner. As such, the time response of the acceptor fluorescence is different than if it were excited directly by a light pulse. The actual form of the acceptor time-dependent fluorescence signal depends on the excited lifetime of the donor and acceptor, as well as the efficiency of energy transfer. The directly excited acceptor will have the lifetime corresponding to a sample in the absence of donor (provided that there is no reverse energy transfer from the acceptor to the donor). It will be shown in this chapter that a single measurement of Spectral-FLIM can be used to resolve the three fluorescence signals (donor undergoing FRET to the acceptor, acceptor excited by FRET, and directly excited acceptor) on the polar plot. This analysis method makes it possible to resolve the signal interference between donor and acceptor by a single measurement, especially the interference by directly-excited acceptor, providing more reliable data to determine FRET efficiency. Spectral FLIM can also differentiate signals.
from the donor and acceptor with and without FRET. The method can also be applied in cases of very different intensity contributions. The data analysis methods presented in this chapter are demonstrated for our frequency-domain FLIM. But these methods can also be applied to time-domain fluorescence lifetime measurements by using Fourier techniques to transform time-domain data to the frequency domain, from which the modulation depth and phase can then be presented on the polar plot (Chen et al., 2009b).

3.3 Materials and Methods

3.3.1 Fluorescence lifetimes of FRET pairs

The fundamental fluorescence responses (using a very short excitation pulse) of the donor and acceptor molecules undergoing FRET are (Birks, 1968, Birks, 1970, Chen et al., 2009b):

\[ I_D(t) = D_0^*k_f^D e^{-t(k_0^D + k_e)} = D_0^*k_f^D e^{-t\tau_D} = D_0^*k_f^D e^{-t/\tau_D} \]  

(3.1)

\[ I_A(t) = AD_0^* \frac{k_f^A k_e}{k_f^D - k_0^A} (e^{-t\tau_A^D} - e^{-t\tau_D}) = AD_0^* \frac{k_f^A k_e}{k_f^D - k_0^A} (e^{-t/\tau_A^D} - e^{-t/\tau_D}) . \]  

(3.2)

In equations 3.1 and 3.2, \( A \) and \( D_0^* \) are the concentrations of the unexcited acceptor and excited donor molecules. \( k_f^D \) and \( k_f^A \) are the natural radiative rate constants of the fluorescence emission decay for donor and acceptor (this rate applies only to the fluorescence pathway, independent of any other pathway of de-excitation, and is the same in all situations, including that in the presence of FRET). \( k_0^D \) and \( k_0^A \) are the sum of the rate parameters of the donor and acceptor in the absence of FRET. \( k_e \) is the rate constant of the energy transfer from the donor to the acceptor, and \( k^D \) is the total energy decay rates of the donor in the presence of FRET (\( k^D = k_0^D + k_e \)). The donor lifetime in the presence of FRET, \( \tau_D \), is reduced from \( \tau_0^D \) (the donor lifetime in the absence of FRET). And the acceptor fluorescence response is not an exponentially decay process, because the excitation of the acceptor is from the energy transfer from the donor, and this induces a time delay to the fluorescence emission of the acceptor (shown in the inset of Fig.3.1 (a)).
In the frequency-domain FLIM measurement, the sinusoidally modulated excitation can be expressed as

$$E(t) = E_0 (1 + M_e e^{j(\omega t + \varphi_E)}), \quad (3.3)$$

in which $\omega$ is the modulation radial frequency ($\omega = 2\pi f$, where $f$ is repetition frequency), $\varphi_E$ is the initial phase value of the excitation, $E_0$ is a time-independent averaged DC intensity, and $M_e$ is the modulation depth. The emission from the donor and acceptor become the convolution of the sinusoidal excitation with the fluorescence responses, as in Eqs.3.1 and 3.2:

$$I_D(t) = QE_0 D_0 \tau^D_k \frac{k^D \kappa_a}{k^D - k^A} \left[1 + M_e e^{j(\omega t + \varphi_E)} \frac{1}{1 + j\omega \tau^D} \right]$$

(3.4)

$$I_A(t) = QE_0 A_D^e \frac{k^D \kappa_a}{k^D - k^A} (\tau^A_0 - \tau^D) \left[1 + M_e e^{j(\omega t + \varphi_E)} \left(\frac{\tau^A_0}{\tau^A_0 - \tau^D} - \frac{1}{1 + j\omega \tau^D} \right) - \frac{\tau^D}{1 + j\omega \tau^D} \right]. \quad (3.5)$$

The donor signal in Eq.3.4 is the typical fluorescence response of a single lifetime, as expected. The acceptor signal is now a linear combination of the acceptor lifetime in the absence of FRET, and the donor signal in the presence of FRET. From Eq.3.5, the acceptor modulation $M_e^A$ and phase $\varphi_e^A$ can be expressed as (Lakowicz & Balter, 1982, Chen et al., 2009b):

$$M_e^A = \frac{1}{\sqrt{(1 + (\omega \tau^A_0)^2)(1 + (\omega \tau^D)^2)}} = M_0^A M^D \quad (3.6)$$

$$\varphi_e^A = \tan^{-1}\left(\frac{\omega(\tau^A_0 + \tau^D)}{1 - \omega^2 \tau^A_0 \tau^D}\right) = \varphi^A_0 + \varphi^D \quad (3.7)$$

where the subscript $e$ denotes that the fluorescence signal of the acceptor is due to energy transfer. Eq.3.6 shows that the acceptor modulation with FRET equals the multiplication of the acceptor modulation without FRET (direct excitation) and the donor modulation with FRET. In Eq.3.7, the phase of the acceptor with FRET is the sum of the acceptor phase without FRET (direct excitation) and donor phase with FRET. The acceptor signal on the polar plot will be located at:

$$x_e^A = M_e^A \cos(\varphi_e^A) = \frac{1 - \omega^2 \tau^A_0 \tau^D}{(1 + (\omega \tau^A_0)^2)(1 + (\omega \tau^D)^2)} \quad (3.8)$$
Eqs.3.1~3.2 \& 3.4~3.9 are simulated in Fig.3.1 to show the relation between the donor and the acceptor signals. In the simulation, the modulation frequency is set at 40 MHz, and the original lifetimes (without FRET) of the donor and acceptor fluorophores are 3 ns and 4 ns, respectively. Several parameters (\(D_0^\alpha, A, k_f^D, k_f^A, Q, \) and \(E_0\)) are set as 1 for the clarity of the plot. Fig.3.1(a) shows that as the distance of the FRET pair decreases, the donor lifetime (green circles) becomes shorter and moves counterclockwise along the semicircle. The corresponding acceptor signal (red squares) is also moving from the left towards the right, until it reaches the universal semicircle at the original acceptor lifetime location. When the two signals are measured in the same channel, so that the fluorescence of the two signals overlaps (blue squares), the data points move from being closer to the donor and become closer to the acceptor. This is because as the FRET efficiency grows the intensity contribution from the donor decreases and the contribution from the acceptor increases. The inset of Fig.3.1(a) plots Eqs.3.1 and 3.2, where the FRET distance between the fluorophores is set at the Förster radius, and other parameters are the same as just mentioned.

Using the polar plot analysis, and by measuring the FLIM signal of only the acceptor fluorescence, it is possible to determine immediately the lifetimes of the acceptor in the absence of FRET and the donor lifetime with FRET without any recursive fitting. This is because on the polar plot, the lifetimes of the quenched donor lifetime (with FRET), the original acceptor lifetime (excited directly by light), and acceptor with FRET lie on a straight line with slope

\[
\frac{\omega^2 \tau_A^D \tau_D - 1}{\omega (\tau_A^D + \tau_D)}
\]

; see equations 3.8 and 3.9 (Chen et al., 2009b). This slope is the negative reciprocal of the tangent of the acceptor phase \(\varphi_e^A\) (Eq.3.7). In other words, with a single measurement of the acceptor signal in the presence of FRET, the slope is known from the measurement of \(\varphi_e^A\) and all three lifetimes can be identified immediately on the polar plot.

Another advantage of measuring the acceptor signal is that the excitation of the acceptor by FRET and by direct excitation can be distinguished using polar plots. This is because the resulting signal is still on the straight line with slope

\[
\frac{\omega^2 \tau_A^D \tau_D - 1}{\omega (\tau_A^D + \tau_D)}
\]

, and the position on the line is decided by the intensity contribution from each lifetime component. By measuring at least two
data points along the straight line, the slope \( \frac{\omega^2 \tau_0^A t^D - 1}{\omega (\tau_0^A + t^D)} \) can be calculated to resolve the lifetimes.

The simulation in Fig. 3.1(b) gives further explanation of this analysis method. As shown in this figure, at the Förster radius, the donor lifetime is quenched from 3 ns to 1.5 ns, and the acceptor-alone signal is located outside of the universal semicircle. The straight line connecting these two data points also passes through the lifetime position of the original acceptor lifetime (4 ns), as just discussed. If the value of \( \tau_0^A \) is known, this provides a check on the analysis. The signals with any intensity combination of these three lifetimes (donor with FRET, acceptor with FRET, and acceptor in the absence of FRET) all lie on this line. The blue squares in Fig. 3.1(b) are signals composed of both the donor and acceptor with FRET, where the relative intensity contribution from the two components has been varied from 0.2~0.8. Such a variation of the intensity fractions is observed from FLIM measurements at different emission wavelengths. Therefore, spectral-FLIM measurements reveal intrinsically the straight line connecting the three lifetimes; this is the subject of the next section.

Figure 3.1. Simulation of the fluorescence signal from a FRET pair. The donor alone has the lifetime of 3 ns and the acceptor alone has the lifetime of 4 ns. (a) The polar plot as the FRET efficiency increases; with increasing FRET efficiency the donor-alone and the acceptor-alone signals move clockwise. As FRET increases, the fractional intensity from each component also changes, and the signal comprised of both donor and acceptor fluorescence moves from the donor towards the acceptor. The inset shows the fluorescence responses of the donor and the acceptor at the Förster radius. (b) The signal from the FRET pair at the Förster radius, having different fractional intensities contributed from the two components. See text for details.
### 3.3.2 Spectral-FLIM

As each fluorophore has its own unique spectrum, the intensity contribution of each lifetime component varies with wavelength. The polar plot data are distributed between different lifetime pools according to the intensity contribution of each component at the different wavelengths. In our Spectral-FLIM system, a Czerny-Turner spectrograph (Andor Technology plc., Belfast, Northern Ireland) disperses the fluorescence signal emitted by the sample before it reaches the intensifier and the CCD (Fig.3.2, (Chen & Clegg, 2009). The spatial information is displayed along one axis on the CCD image, and the wavelength information is acquired on the other axis (Fig.3.3). The entrance slit width of the spectrograph sets both the wavelength resolution and the spatial resolution in the direction the perpendicular to the slit.

![Figure 3.2. Instrumentation setup of the Spectral-FLIM.](image-url)
Figure 3.3. The spectrograph adds the wavelength dimension to the FLIM data. The fluorescence signal is dispersed by the spectrograph before it is imaged by the ICCD, and signals with different amount of phase shifts on the image intensifier are recorded for lifetime calculations.

3.3.3 Spectrograph considerations and calibration

Gratings are often used in spectrographs for their good resolving power in the visible, which is a measure of their ability to separate adjacent wavelengths. There are some concerns about choosing a grating instead of prism as a dispersion device. One is the overlap of wavelengths from different diffraction orders, which introduces inaccuracy in the spectrum (http://gratings.newport.com/handbook/handbook.asp). But in fact, for the fluorescence emission measurement, the higher order signal usually does not appear within recording wavelength range. For example, when a fluorophore is excited at 440 nm, the emission spectra range is from about 450 to 800nm. The higher order signal within this wavelength range starts to appear only at wavelengths longer than approximately 900nm. Therefore, the superposition of different orders is seldom a problem. Another consideration is the imaging property of grating systems. Gratings have inherent artifacts such as astigmatism, background stray light, spectral aberration, etc. (http://gratings.newport.com/handbook/handbook.asp, Singh, 1999). In the full-field FLIM, those optical aberrations influence the lifetime resolution. Czerny-Turner spectrographs are one of the most common setups used for full-field imaging (Simon et al., 1986, Rosfjord et al., 2000). This setup has two concave mirrors in addition to a plane grating, so that the dispersed spectrum is focused and linearly dispersed on the CCD of our FLIM.
Spectral-FLIM was checked for astigmatism and spectral aberration, which means the focusing property of the tangential plane and capital plane, and the focusing property at different wavelengths (diffraction angle). A resolution mask, which has specific known patterns with various dimensions, was used to examine the focusing property. The dispersion characteristic of the spectrograph was also calibrated. The Mercury lamp, which has hyperfine structures and several peaks over the visible light range, was used to identify the wavelength location on the wavelength axis of the image. Figure 3.4 shows the Spectral-FLIM image of the mercury spectrum. The linear dispersion relation and the reciprocal dispersion were then calculated. For the purpose of many Spectral-FLIM measurements, it is not required to acquire an absolute true spectrum in order to distinguish fluorescence lifetime components; the calibration of the instrument must simply be repeatable. But in some cases, the absolute spectrum is better for identifying the fluorescent molecules in the sample, such as for data of autofluorescence of tissues (Chorvat & Chorvatova, 2006, De Beule et al., 2007). Several optics and electronics components in the instrument have wavelength dependent sensitivities. For example, the dichroic mirror, grating, image intensifier and the CCD are all wavelength dependent. The wavelength sensitivity of the instrument should be calculated if true spectra are desired; but this is not necessary in the experiments shown here.

Figure 3.4. Using the spectrum of Mercury to calibrate the spectrograph. (a) is the partial spectrum of Mercury, which is calculated from the Spectral-FLIM image of a mercury lamp in (b).
3.3.4 Linear unmixing

Linear unmixing is applied in the Spectral-FLIM data to separate lifetime pools. It is a method commonly used to resolve spectral imaging in many fields, such as steady state fluorescence microscopy (Zimmermann, 2005, Zimmermann et al., 2003) and infrared remote sensing (Chang, 2007). With knowledge of the spectrum of each fluorescent species, the measured spectrum is unscrambled in order to determine the intensity contribution of each component (which is related to the concentration). This method can be readily applied to separate lifetime components of our Spectral-FLIM data, because frequency-domain homodyne FLIM measurements are essentially fluorescence intensity images corresponding to different phase shifts of the amplification of the intensifier. Spectral-FLIM data are analyzed by the following equations:

\[
g_{m \times p} = S_{m \times n} \cdot f_{n \times p} \tag{3.10}
\]

\[
f = (S^T S)^{-1} S^T g \tag{3.11}
\]

In Eq.3.10, the subscript \(m\) is the number of wavelength channels of the detector, \(p\) is the number of phase shifts applied to the intensifier, and \(n\) is the number of fluorophores to be decomposed. \(g_{m \times p}\) is the measured data from the Spectral-FLIM (Fig.3.5), which is a matrix containing information on the spectrum in rows and phase shifts in columns. \(S_{m \times n}\) has the spectral information on the \(n\) constituents, which is acquired from individual measurements of the dyes by the Spectral-FLIM. \(f_{n \times p}\) is a matrix of the intensity contribution from each dye at different phase shift. The only required condition for Eq.3.10 is that the number of wavelength channels should be equal or larger than the number of dyes to be decomposed (Zimmermann, 2005, Zimmermann et al., 2003, Kraus et al., 2007). Eq.3.11 represents the least-squared fit of the intensity contribution matrix \(f\). The fractional contributions of the constituents at different phases of the homodyne measurement can then be used to calculate the modulation depth and phase of each component. Using Spectral-FLIM, more accurate lifetime values can be achieved even for samples with low intensity values or low signal-to-noise ratios. This is because the least-squared fit of “\(f\)” minimizes the deviation from the true spectrum. For example, our Spectral-FLIM setup uses 518 pixels to resolve a fluorescence spectrum. Random noise in those pixels is decreased significantly after fitting the spectrum.
The constituent spectra in the S matrix could be, for example, the combined spectra of a donor and acceptor pair undergoing FRET. That is, the spectra used in the S matrix do not have to correspond to single fluorophores. Just as fluorophores with different lifetimes have different intensity contributions at certain phase shifts of the homodyne signal, the combined spectra used in the S matrix would be the spectra of the fluorophores that would be observed at the same phase shift of the intensifier as the measured data:

$$\mathbf{g}_{m \times 1}^{p} = \mathbf{S}_{m \times n}^{p} \cdot \mathbf{f}_{n \times 1}^{p} \quad .$$ (3.12)

The superscript p denotes the p\textsuperscript{th} phase shift of the spectral and the intensity contributions. The intensity contributions \( \mathbf{f}_{n \times 1}^{p} \) would then be the same for every p, if the spectrum and lifetime information in \( \mathbf{S}_{m \times n}^{p} \) matrix is correct. Therefore, this method uses the lifetime dependence of fluorophores at different states to construct the constituent spectrum, and then has the advantages of linear unmixing that can distinguish signals from states with different intensity contributions.

![Figure 3.5](image.png)

Figure 3.5. The \( \mathbf{g}_{m \times p} \) matrix measured by the Spectral-FLIM. Linear unmixing is applied to differentiate between the constituents at each phase for lifetime calculations. The data are measured from Fluorescein and Rhodamine B in 0.1 N NaOH.

### 3.3.5 Data analysis and sample preparation

The details of our FLIM instrument are shown in Fig.3.2. The data displayed in the figures are all simulated or measured at 40 MHz repetition frequency, except Fig.3.13 and 3.14, which are
measured at 100 MHz. The excitation wavelength is 440 nm, and the intensity of the laser and the exposure times were adjusted to achieve a good signal level. A dark frame (which contains the CCD dark current and the read-out noise) was subtracted from every Spectral-FLIM image before any further analysis. The entrance slit width of the spectrograph was 500 µm, which corresponds to a full width at half maximum (FWHM) on the CCD of 13 pixels. The dispersion of the Spectral-FLIM is calibrated by taking the spectrum of a mercury lamp (Chen et al., 2010), which gives 2.7 pixels/nm. All the spectra shown here are the raw data recorded by the intensifier and CCD, which means they are not corrected for the wavelength sensitivity of the intensifier and the grating efficiency of the spectrograph. As Spectral-FLIM provides the spatial information along one dimension, which is not required for the dye solutions measured here, the spectrum and lifetime data were averaged over 500 pixels (the spatial resolution) at the same wavelength. This averaging process decreases random noise and provides visually clearer representations of our analysis methods, but it can be seen in Fig.3.15 and in the following discussion that averaging is not required for the analysis method proposed here. The only denoising process of the spatial dispersion is a 5-point moving average filter to smooth the spectrum. The spectra of the constituent dyes are measured under the same experimental setup as the samples. Because fluorescence molecules are particularly sensitive to the local environment, small shifts in the spectrum are often present (Valeur, 2002). To correct for this, the spectrum of each constituent dye is allowed to shift before each linear unmixing calculation. The amount of the spectrum shift is determined by minimizing the residuals between the raw spectrum and the least squared fitting results; the shift is always less than 9 pixels (or 3.3 nm).

On the polar plot, two different analyses are applied to calculate the lifetimes of the Spectral-FLIM data. One is the least-squared fitting of the lifetime data at wavelengths from 500~650 nm to find the two constituent lifetime pools. The calculated values are labeled on the polar plots as “Spectral-FLIM” (see Figs.3.7, 3.9, 3.11~3.12, and 3.14~3.16). The other one is linear unmixing to separate the spectra first, and then calculate the modulation and phase from the intensity contribution matrix f. These values are labeled as “Linear Unmixing” on the figures. The advantages of each method will be compared in the following discussion.

The fluorescent dyes and solvents are indicated in the figure legends. The DNA samples were purchased from Integrated DNA Technologies (Integrated DNA Technologies, Inc., Coralville,
IA, USA). The donor (6-carboxy-fluorescein, FAM) and acceptor (6-carboxy-hexachlorofluorescein, HEX) are labeled at the 5’ end of the DNA. 10 µM of single-stranded (ss) or double-stranded (ds) DNA were measured in the phosphate buffer (8 mM Na₂HPO₄, 2 mM NaH₂PO₄, and 100 mM NaCl) at room temperature. The sequences of the DNA are as follows: HEX-5’-ACGCCGCA-3’, FAM-5’-TGCGCGT-3’, HEX-5’ACGTATATTATATGCA-3’, and FAM-5’-TGCATATAATACGT-3’.

3.4 Results

3.4.1 Fluorescence lifetime standards
Several xanthene dyes measured by Spectral-FLIM are plotted together in Fig.3.6 (see figure legend for detailed information). The lifetime-resolved data are shown in a polar plot in Fig.3.6(a); the color bar shows the steady state fluorescence intensities. The peaks and valleys of the spectra can be seen from the color-coded intensity values. The same color coding for the intensities at different wavelengths are used for the points on the polar plot for each fluorophore. The calculated lifetimes and the solvents used are indicated on the figure. The calculated lifetimes of these dyes are all within 0.05 ns or less from literature values (Boens et al., 2007). Fig.3.6(b) are the normalized fluorescence spectra of the dyes (these spectra are acquired with the FLIM instrument). The spectral information is acquired simultaneously with the lifetime data.
Figure 3.6. Spectral-FLIM measurements of fluorescence lifetimes and emission spectra of several commonly used dyes: Fluorescein (FL), Rhodamine B (RhB), Eosin Y (EosinY), and Erythrosine B (EryB). The spectrum is acquired simultaneously as the lifetime. (a) The lifetimes of the dyes presented on the polar plot. The color bar represents the steady state fluorescence intensity in arbitrary unit (A.U.). The lifetime values calculated by least-squared fit are indicated on the figure, and the solvents used are shown in the parentheses. (b) The normalized fluorescence spectra of the dyes from (a).

3.4.2 Multiple dyes

Figs.3.7~3.10 show Spectral-FLIM data measured with two or three dyes in solution (0.1N NaOH). The lifetimes of Fluorescein, Rhodamine B, and Eosin Y in 0.1N NaOH are 4.3 ns, 1.64 ns, and 1.08 ns, respectively (data not shown). Fig.3.7(a) shows the lifetime data of wavelengths between 500~650 nm. The color bar represents the steady-state fluorescence intensity and two peaks are clearly seen in the figure. While one of the peaks belongs to Fluorescein and intersects the semicircle at a point corresponding to 4.22 ns, the other intersection with the semicircle is from Rhodamine B and corresponds to 1.59 ns. The lifetime data at the wavelengths between the two peaks, which are from overlapping spectra of the two dyes, are on a straight line connecting the two peaks. They provide an immediate visualization of the two constituent lifetimes without any further calculation. The two composition lifetimes extracted from the least-squared fit of Spectral-FLIM signal are shown as small black circles on the figure, and the linear unmixing calculation results are the larger circles. The two results are very close to each other.

Figs.3.7(b)~(d) show data from one of the 8 phases in the Spectral-FLIM data acquisition. Fig.3.7(b) is the image, with wavelength information dispersed along the x-axis and the spatial data displayed on the y-axis. Fig.3.7(c) shows the spectrum analyzed from Fig.3.7(b), together with the decomposed spectra of the constituent dyes. Fig.3.7(d) shows the residuals of the raw
spectrum subtracted from the best fit of the linear unmixing. Fig.3.8 is a measurement with three dyes. The data on the polar plot is inside of a triangle defined by the three points on the semicircle defined by the corresponding lifetimes of the separate dyes. As one moves through the spectrum (increasing wavelengths) the measured points on the polar plot move from the Fluorescein lifetime, towards the Eosin Y lifetime, and ultimately tend toward the Rhodamine B lifetime. Because linear unmixing can separate signals from different dyes robustly, lifetimes and intensity fractions can be resolved more efficiently and accurately by the method used here.

Figure 3.7. Spectral-FLIM measurements of Fluorescein and Rhodamine B in 0.1N NaOH. (a) Polar plot representation of the lifetime data at different wavelengths. The constituent lifetimes calculated by the least-squared fitting (small black circles) or analyzed by the linear unmixing (large black circles) are shown on the figures. (b) The Spectral-FLIM data at one of the phase shift on the ICCD. (c) The spectrum calculated from (b) and the linear unmixing results. (d) Residuals of the best fit in (c).

Figure 3.8. Spectral-FLIM measurements of Fluorescein, Rhodamine B, and Eosin Y in 0.1N NaOH. (a) Polar plot representation of the lifetime data at different wavelengths. The constituent lifetimes calculated by the least-squared fitting (small black circles) or analyzed by the linear unmixing (large black circles) are shown on the figures. (b) The Spectral-FLIM data at one of the phase shift on the ICCD. (c) The spectrum calculated from (b) and the linear unmixing results. (d) Residuals of the best fit in (c).
Figure 3.9 shows the measurement of Fluorescein and Rhodamine B with very different fractional intensity contributions. The fluorescence intensity of Rhodamine B is less than 2% of the intensity of Fluorescein; therefore, the presence of Rhodamine B can barely be observed from the lifetime-resolved data on the polar plot (Fig. 3.9(a)), or from the spectral data before decomposition (Fig. 3.9(b) and 3.9(c)). Because the signal from Rhodamine B contributes much less than Fluorescein, most data points are located close to the Fluorescein lifetime on the polar plot. Even at longer wavelengths, where the intensity contribution of such a low concentration of Rhodamine B is still much lower than Fluorescein, the effective lifetime data on the polar plot is still approximately that of the Fluorescein lifetime. Nevertheless, the least-squared fitting of the Spectral-FLIM data is still influenced by the low intensity level and the low signal-to-noise ratio, and the predicted lifetime of Rhodamine B is 1.55 ns. Thus, even at these low Rhodamine B signals, the linear unmixing procedure results in reasonably accurate results. The fluorescence spectrum is successfully decomposed by linear unmixing to find the intensity contribution of each fluorophore, and the obtained spectrally dispersed and separated intensity values are used to calculate the lifetime values. The inset of Fig. 3.9(c) is a close-up of the fitting result for Rhodamine B. Thus, provided that the level of signal-to-noise suffices, the spectra can still be differentiated by the linear unmixing method, in spite of the low intensity level. And the modulation and phase values calculated from linear unmixing are distributed about the expected values.
Figure 3.9. Spectral-FLIM measurements of Fluorescein and Rhodamine B in 0.1N NaOH. (a) Polar plot representation of the lifetime data at different wavelengths. The constituent lifetimes calculated by the least-squared fitting (small black circles) or analyzed by the linear unmixing (large black circles) are shown on the figures. (b) The Spectral-FLIM data at one of the phase shift on the ICCD. The inset shows the contribution from Rhodamine B. (c) The spectrum calculated from (b) and the linear unmixing results. (d) Residuals of the best fit in (c).

3.4.3 FRET pairs on the DNA duplexes

The lifetimes of ssDNA labeled with fluorescence dyes are shown in Fig.3.10, and the Spectral-FLIM measurements of doubly labeled dsDNA with different lengths are in Fig.3.11 and Fig.3.12. The lifetimes of FAM and HEX bound to the oligos are both around 3.8 ns (Fig.3.10); therefore, the lifetime trajectory of a FRET excited acceptor, having 3.8 ns lifetime when excited directly by light, is simulated on the polar plot as a reference point (squares on Fig.3.11(a) and Fig.3.12(a)). On Fig.3.11(a) and 3.12(a), the acceptor signals are outside of the polar plot, indicating the presence of FRET between donors and acceptors. The Spectral-FLIM data are analyzed to determine the two intersections on the semicircle of the polar plot; these intersections are the points representing the donor lifetime in the presence of FRET and the acceptor lifetimes in the absence of FRET. The straight line calculated from least-squared fitting of the Spectral-FLIM data is also plotted on the figures. It connects the calculated points corresponding to the lifetimes of the donor (with FRET) and the acceptor (both with and without FRET). The calculated lifetime of the directly-excited acceptor is longer than 3.8 ns from both data (values shown on the figure). This might be because the original acceptor lifetime is not exactly on the universal semicircle. As the Spectral-FLIM data pass the original lifetime position, it intersects the semicircle at a different location. The donor lifetime resulting from the least-squared fitting of the Spectral-FLIM is found to be 2.15 ns and 0.89 ns for the 16-mer and 8-mer dsDNA,
respectively. By using 3.8 ns as the original lifetime of donor, the FRET efficiency of the oligos are 0.43 for 16-mer, and 0.76 for 8-mer. The corresponding theoretical values of FRET efficiency from a helix DNA model are 0.23 and 0.70 (Förster radius is set as 50 Å; the vertical distance between donor and acceptor if attached to the same base pair is set as 3 Å, and all the other parameters are the same as in the literature (Clegg et al., 1993)). Therefore, the lifetime data agree with the theoretical model. The linear unmixing calculation (large circles) for the donor is not on the semicircle in both cases, which is different from the position of quenched donor lifetime determined by Spectral-FLIM fitting. This is due to the presence of extra single-stranded donor (this is confirmed by absorption spectrum measurements). Therefore, the measured point on the polar plot includes both signals with and without FRET and is located between the two intersection points on the polar plot semicircle. The acceptor positions from linear unmixing are also not on the acceptor reference curve. This is due to direct excitation of the acceptor. The absorption spectra of FAM and HEX overlap at 440 nm (the wavelength where the sample is excited) so that direct excitation of HEX is possible. Thus, provided that linear unmixing is able to separate donor and acceptor signals, the lifetimes of fluorophores can be observed and analyzed in detail, without worrying that interference between fluorophores might lead to wrong interpretations.

Figure 3.10. The fluorescence lifetimes of FAM and HEX bound at the 5’ end of ssDNA. The green circle is FAM bound on the 8-mer ssDNA, the pink circle is HEX bound on the 8-mer ssDNA (which has the same lifetime as the FAM on the 8-mer ssDNA), the blue circle is FAM on the 16-mer DNA, and the red circle is HEX on the 16-mer ssDNA.
Figure 3.11. Spectral-FLIM measurement of FAM and HEX labeled on the 16-mer dsDNA. The signal trajectory of a FRET excited acceptor (squares), having original lifetime of 3.8 ns, is plotted as a reference. (a) The lifetime data at different wavelength is plotted on the polar plot. The least-squared fitting gives the lifetimes value of the donor with FRET and the acceptor without FRET (small black circles). The straight line connects the predicted donor lifetime (with FRET) and acceptor lifetimes (both with and without FRET). The linear unmixing calculations of each component are shown as large black circles. (b) The Spectral-FLIM data from the ICCD. (c) The spectrum calculated from (b) and the linear unmixing results. (d) Residuals of the best fit in (c).

Figure 3.12. Spectral-FLIM measurement of FAM and HEX labeled on the 8-mer dsDNA. The signal trajectory of a FRET excited acceptor (squares), having original lifetime of 3.8 ns, is plotted as a reference. (a) The lifetime data at different wavelength is plotted on the polar plot. The least-squared fitting gives the lifetimes value of the donor with FRET and the acceptor without FRET (small black circles). The straight line connects the predicted donor lifetime (with FRET) and acceptor lifetimes (both with and without FRET). The linear unmixing calculations of each component are shown as large black circles. (b) The Spectral-FLIM data from the ICCD. (c) The spectrum calculated from (b) and the linear unmixing results. (d) Residuals of the best fit in (c).
3.4.4 Separating signal-stranded and double-stranded DNA

Figs. 3.13 and 3.14 show measurements from samples having both ssDNA (labeled with FAM) and dsDNA (doubly labeled by FAM and HEX), but with different amounts of each oligo. In Fig. 3.13, linear unmixing separates the lifetime signals of FAM and HEX (shown as the large black circles). The fluorescence intensity of the Fluorescein emission is mostly from the FAM-labeled ssDNA; therefore the point on the polar plot is around the position corresponding to 3.8 ns. The signal of HEX is only from dsDNA, and its location on the polar plot also shows that it is excited mostly through FRET. Because the Spectral-FLIM signal is mostly from the non-FRET donor lifetime, the least-squared fitting doesn’t give a good result. Therefore, the calculation of the FRET pair lifetimes from Fig. 3.11 is plotted in Fig. 3.13 as a reference.

Figure 3.13. Spectral-FLIM can separate ssDNA and dsDNA signals. The doubly-labeled 16-mer DNA (FAM and HEX) and the singly-labeled 16-mer DNA (FAM) are both present in the samples. The modulation frequency is 100MHz here. The linear unmixing (large circles) shows the signal of HEX from dsDNA, and the signal of FAM from both dsDNA and ssDNA.

The sample used for data in Fig. 3.14 contains more dsDNA then ssDNA. There are three lifetime components in the sample: donor without FRET, donor with FRET, and acceptor with FRET. The donor and acceptor spectra alone are not sufficient to distinguish these three lifetime species; therefore, the spectra of donor alone and donor plus acceptor undergoing FRET are used as the constituent spectra in linear unmixing (Eq. 3.12). The spectrum contributed by the FRET pair is subtracted from the total signal at every phase shift, which gives the singly-labeled donor signals. The intensity contribution of the FRET pair ($I_{n\times1}^p$) is the same at every phase shift, which
confirms that the right amount of signal is subtracted. Then the three lifetimes can be calculated from linear unmixing, shown as the large circles. The least-squared fitting result also gives the lifetimes of the donor with FRET and acceptor without FRET, which are close to the calculation of Fig.3.11.

Figure 3.14. Spectral-FLIM can separate ssDNA and dsDNA signals. The doubly-labeled 16-mer DNA (FAM and HEX) and the singly-labeled 16-mer DNA (FAM) are both present in the samples. The modulation frequency is 100MHz here. The FRET pair signal of dsDNA is subtracted from the total signal, therefore the signal of FAM from ssDNA is left. The linear unmixing (large circles) give the lifetimes of the donor with FRET, donor without FRET, and acceptor with FRET.

3.5 Discussion

The linear unmixing method can differentiate two lifetime component, where the intensity of one component is less than 2% of the other one; this was measured using only half the dynamic range of our 12-bit CCD (assuming maximum modulation depth of the Pockels cell and the fluorophore lifetime). The accuracy of the lifetimes determined by Spectral-FLIM depends on the accuracy of the linear unmixing calculation; uncertainties in the modulation and phase values will propagate through the $f$ matrix calculation. And the goodness of spectrum separation is influenced by the signal to noise ratio (SNR) of the detector. The intensifier and CCD used in our measurements have together a signal-dependent Gaussian noise (Spring & Clegg, 2009). For example, the noise of the instrument at 40MHz has a characteristic variance of:

$$
\sigma^2 = 3.25\mu + 1.2 \times 10^{-4}\mu^2 
$$

(3.13)
The SNR is defined as $\mu/\sigma$. At every pixel, the SNR of 1200 A.U. fluorescence intensity is about 18.8, and the SNR of 200 A.U. fluorescence intensity is about 7.8. Thus in Fig.3.15(a), in which signals from one of the spatial axes is presented, the HEX data points have a narrower distribution than the lower intensity signals of FAM. Whereas averaging pixels along the spatial dimension can increase the SNR, there is obviously a tradeoff between the spatial resolution along the slit direction and the lowest signal that can be detected. In the calculations in this chapter, a 5-point average filter was applied to smooth high frequency noise. Because frequency-domain FLIM measurements are essentially steady state images taken at different phase shifts, full advantage can be taken of denoising algorithms to further improve both the wavelength and spatial resolution (Spring & Clegg, 2009).

![Image](image.png)

Figure 3.15. Same data as Fig.3.12, except signal from one pixel along the spatial axis is shown and analyzed on the polar plot. Signal from the HEX has higher intensity and better signal to noise ratio than the FAM, therefore has narrower distribution. See text for discussion.

In addition to the SNR at every single pixel, the accuracy of lifetime determinations also depends on instrument stability during data acquisition. With the settings of our instrument, the stability of a measurement is controlled mainly by the light source and the DC and RF gains of the Pockels cell and the intensifier. Any instability of the instrument results in uncertainties in the measured modulation and phase; the positions of lifetime data on the polar plot have a deviation about 0.5% to 5%, the value of which depends on the modulation frequency and the laser used. Another factor influencing error propagation on the polar plot is the modulation frequency, because the coordinate of the polar plot has a nonlinear relation with respect to the lifetime or modulation frequency. There exists an optimal modulation frequency for every lifetime
\( \omega_{\text{optimal}}^2 = \frac{1 + \sqrt{3}}{2 \tau^2} \), see (Redford & Clegg, 2005a), which reduces errors in the polar plot analysis.

The high wavelength resolving power of our Spectral-FLIM enables correction of the spectral shifts of fluorophores, which is important for low intensity signals to be separated. The spectra are often shifted in different environments (Valeur, 2002); therefore, shifting the spectrum will influence the accuracy of the linear unmixing calculation, which affects the corresponding lifetime calculation. Thus, often by allowing a small shift (in this chapter, less than 3-4 nm) between the spectra of the sample and the spectra used in the constituent matrix, the calculated intensity contribution by linear unmixing is more accurate. The effect of binning every 20 wavelength pixels before any spectrum calculation is shown in Fig. 3.16. Each spectrum channel then has bandwidth of 7.4 nm. Even though 25 wavelength channels should be sufficient to separate known constituent spectra and binning wavelength channels increases the SNR, a lower wavelength resolving power is not as capable to account for small spectral shifts. This lowers the accuracy of the lifetime calculation for a low intensity signals. The high wavelength resolving power of our Spectral-FLIM setup can detect small spectral shifts or spectral changes in intensity. Therefore, using the spectrum-dependent fluorescence signal in conjunction with FLIM, we have the sensitivity to measure, for example, changes in the fluorescence properties of charge-sensitive dyes caused by changes in cell membrane potentials (Zal et al., 2006) or depth-dependent spectral shifts of autofluorescence, or of fluorescence dyes, in tissues (Zavattini et al., 2006, Schwarz et al., 2008).
Figure 3.16. Same data as shown in Figure 8, but binning every 20 pixels along the wavelength dispersion direction before linear unmixing and lifetime calculations. Even though 25 wavelength channels are sufficient to separate constituent spectra, the wavelength resolving power becomes lower to account for small shifts of the spectrum, which is more crucial to the calculation of lower intensity signals. The lifetime calculation of Rhodamine B is therefore less accurate here.

3.6 Acknowledgements

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3.7 References


4 FLIM Applications in Photosynthesis

4.1 Introduction

In photosynthesis, chlorophyll (Chl) molecules absorb light, and subsequently transfer the energy to the reaction centers of the photosystems. Fluorescence is one of the de-excitation pathways from the excited Chl a, and is an excellent way to follow photosynthetic reactions (Lichtenthaler, 1988, Papageorgiou & Govindjee, 2004). Photochemical quenching also plays an important role in the protecting plants from reactive oxygen species. There are several non-photochemical quenching (NPQ) pathways in chloroplasts that can modulate the flow of energy from the chlorophyll a to the reaction center (Muller et al., 2001). Each of the NPQ relaxation kinetics has distinct fluorescence features and time scales; therefore, the change in chlorophyll a lifetime and intensity can help identify the NPQ pathways and then be used to study their functions and regulations. One of the NPQ pathways is energy-dependent quenching, qE. It is triggered by a decrease in the lumen pH (Gilmore et al., 1995), which is an indication that the absorbed light energy exceeds the capacity of the reaction center. The different xanthophyll molecules bind to the photosystem II (PS II) antenna and accept energy from excited Chl molecules, and the energy is then dissipated thermally. Changes in the lumen pH trigger the activity of enzymes that interconvert the xanthophylls between forms with different efficiencies to accept energy from the excited chlorophylls. When energy from the excited chlorophylls can be transferred to xanthophylls, the fluorescence lifetime of the chlorophyll molecules is shortened, and concomitantly the intensity also decreases. Another NPQ pathway is the process of state transitions, qT. qT refers to the movement of light harvesting complex proteins between the two photosystems, which balances the energy absorption rates between the two photosystems and might play a role in the metabolic reactions (Cardol et al., 2009). qT happens slower than qE, and doesn’t change the lifetime of the Chl a in PSII, but the lifetime from Chl molecules in photosystem I (PSI) is much shorter than in PSII. The fluorescence intensity will decrease as the light harvesting complex moves away from PS II to PSI. FLIM can differentiate between these two processes because FLIM can determine whether the changes in fluorescence intensity result
from dynamic quenching (such as FRET) or just a change in the number of fluorescent molecules. Another NPQ is photoinhibition, qI. This usually refers to a long term photodamage to one of the proteins in the photosynthesis apparatus under high light illumination. It takes hours or days to repair the damaged PS II molecules; therefore, the fluorescence intensity and lifetime values do not fully recover during the long Chl a fluorescence transient (Papageorgiou et al., 2007). For in vivo measurements, all these pathways of Chl a take place in real time, and the activation and conversion between the pathways depends on the physiological state of the samples. The Chl a fluorescence signal is a non-destructive measurement of the photosynthesis, and FLIM can provide the detailed molecular information of this dynamic molecular complex.

Short preview of results: FLIM measurements of wild-type Chlamydomonas reinhardtii during the long fluorescence transient were carried out. The correlation between the fluorescence lifetime-resolved measurements and the intensity distinguishes the activation of two pathways, qE and qT, during the same time frame. In the 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) treated cells, the movement of Light-Harvesting complex II (LHC II) from PS I to PS II was observed. The other experiment applied the newly developed Spectral-FLIM to study intact avocado leaves. The lifetime pool (we speak of pools because it is not expected that singular, individual lifetimes are present in such a complex system) of the far-red region (~740 nm) is shorter than the one in the red region (~680 nm), but both lifetime pools were quenched during the slow fluorescence transient. The time-course spectrum shows two specific changes with different rates; that is, the peak in the red region decreases faster than the far-red peak. As indicated in the literature (Lambrev et al., 2010, Holzwarth et al., 2009), the spectral feature in the red region is associated with the energy-dependent quenching and PsbS-independent quenching, and the far-red peak depends on the PsbS subunit. Therefore the data suggest that there are two separate binding sites for xanthophylls, because the two peaks have different fluorescence lifetimes and kinetics, and yet they are both quenched during the light induction. We suggest that the binding of xanthophyll molecules is involved in both the PsbS-independent and the PsbS-dependent NPQ mechanisms.
4.2 Materials and Methods

Wild-type *Chlamydomonas reinhardtii* was grown in a liquid media until the exponential growth phase was reached (Holub et al., 2007). *In vivo* measurements were taken to study the fully functional photosynthesis apparatus. The *Chlamydomonas* liquid culture was dark adapted for 5 minutes before chemical treatment, which was followed by 5 minutes of incubation time. For the FLIM measurements, the cells were centrifuged and put in a small vessel which was fabricated on a glass slide, in order to maintain conditions conducive for swimming cells. The cells were illuminated with a 440 nm laser and the fluorescence emission of Chl a was measured between 670~725 nm. The modulation frequency of excitation was 100MHz, and a 40X objective lens was used in the FLIM microscope. After focusing the image, cells were dark adapted for 5 minutes before 60 continuous FLIM measurements were carried out. The recording time for 60 data takes about 600 seconds. Then, 5 minutes of dark adaptation was applied before the 61\textsuperscript{st} measurement. In our experiment protocol, we observed both the induction and the recovery of the Chl a fluorescence transient. For the chemical treatment, 10 µM of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) in ethanol was added to the liquid culture. The final concentration of ethanol in the liquid culture was only 1.2% v/v. The same amount of ethanol was used in the controls.

An avocado plant (*Persea americana* Mill.) from a California nursery was grown in our laboratory. The leaves were cut from the tree immediately before the experiment. During the FLIM measurement, the leaf was placed between the coverslip and a piece of moist towel paper on the glass slide. The sample was excited at 440 or 488 nm; the excitation wavelength is given in the figure legends. A 40X objective was used and the image was focused on the mesophyll layer of the leaves. In the time-course Spectral-FLIM experiment, the leaves were dark adapted for 5 minutes before 15 continuous measurements. The total experiment time was about 300 seconds. The Spectral-FLIM setup and data analysis methods are as discussed in the previous chapters, and are also shown in Fig. 4.1.
4.3 Results

4.3.1 Co-occurrence of Xanthophyll Cycle and State Transition

In Fig. 4.2, the lifetime-resolved fluorescence signal of the *Chlamydomonas* sample was measured during the slow phase of Chl a fluorescence transient. On the polar plot (Fig. 4.2 (a)), the points move from the longer lifetime values (~2 ns) towards shorter lifetime values (~1 ns); this indicates the activation of qE, which quenches dynamically the fluorescence of Chl a. The 61st recovery point on the polar plot was found to return to the lifetime position almost the same as the 1st data point, showing that qI wasn’t triggered, which would have hindered the recovery of the Chl a fluorescence. In the intensity vs. time trace shown in Fig. 4.2 (b), the intensity does not decrease monotonically as the lifetime moves towards the faster lifetime pool. It can also be seen clearly in the 3D polar plot of Fig. 4.2 (c), where the z-axis shows the intensity values. As discussed earlier, qE involves the quenching of Chl a lifetime, and subsequently decreases its fluorescence yield. If qE is functioning by itself, the intensity and lifetime would show a positive correlation (Matsubara et al., 2010, Holub et al., 2007). Between 100-300 seconds of the laser illumination, the fluorescence intensity rises as the lifetime gets shorter. This is an indication that the number of Chl a molecules is increasing, meaning that LHC IIs are moving back from PS I to PS II. In other words, the redox state of the cells activated qT during this period of time.
Figure 4.2. FLIM measurements the slow fluorescence transient of wild-type *Chlamydomonas*, without DCMU treatment (controls). (a) Polar plot representation of the 61 FLIM data. Black dot represents the 1st measurement after 5 minutes of dark adaptation, green dot is the 60th measurement of the whole fluorescence curve, and the red dot is the 61st measurement conducted after another 5 minutes of dark adaptation following the 60 measurements. (b) The time trace curves of the steady-state intensity, modulation, and phase of the 61 FLIM measurements. The value of fluorescence intensity is normalized by the exposure time (arbitrary unit / ms). (c) The 3D polar plot of the 61 data, with the z-axis representing the steady-state intensity.

Figure 4.3 shows the same FLIM measurement but in DCMU treated *Chlamydomonas* cells. DCMU interrupts the electron transfer chain in PS II, and thus blocks the flow from PSII to PS I. As a consequence, it blocks the ΔpH build-up, which is essential for activating qE (Papageorgiou & Govindjee, 2004). Therefore, as expected, the data show no quenching of the lifetime or decease of fluorescence intensity. On the contrary, there is a large increase in fluorescence intensity during the fluorescence transient, accompanied by a relatively small increase in lifetime. This can be explained as the movement of LHC II complexes back to PS II, which means the presence of DCMU can induce qT.
Figure 4.3. FLIM measurements the slow fluorescence transient of wild-type *Chlamydomonas*, with 10 µM DCMU treatment. (a) Polar plot representation of the 61 FLIM data. Black dot represents the 1st measurement after 5 minutes of dark adaptation, green dot is the 60th measurement of the whole fluorescence curve, and the red dot is the 61st measurement conducted after another 5 minutes of dark adaptation following the 60 measurements. (b) The time trace curves of the steady-state intensity, modulation, and phase of the 61 FLIM measurements. The value of fluorescence intensity is normalized by the exposure time (arbitrary unit / ms). (c) The 3D polar plot of the 61 data, with the z-axis representing the steady-state intensity.

4.3.2 *Spectral-FLIM measurements during the slow Chl a fluorescence transient*

The result in Fig. 4.4 (a) is FLIM data of the avocado leaf, acquired after 5 minutes of dark adaptation before the measurement. It shows that there are multiple lifetime pools, and Fig. 4.4 (b) demonstrates a method to discriminate the underlying lifetime pools by using the emission spectrum information. The FLIM data on the polar plot at different wavelengths lie approximately along a straight line. If two lifetime pools contribute to the emission, each with different spectral characteristics, the lifetime data in the spectrum overlapping region would lie along the straight line connecting these two pools. These constitute lifetime pools might each have multiple lifetime components. However, for singular lifetimes for each pool, the extrapolation of a straight line fit to the data will intersect on the semicircle of the polar plot. This will provide estimates of the lifetimes of the two pools. To acquire additional information
available from the emission wavelengths, Spectral-FLIM is used to provide lifetime image with spatial information in one dimension and wavelength spectrum in another axis of the CCD (Fig. 4.1). Fig. 4.4 (b) is the Spectral-FLIM data of the same location of the leaf as in Fig. 4.4 (a), again measured after 5 minutes of dark adaptation. The color code represents the fluorescence intensity, showing a major peak emitted at ~680 nm and a minor peak at longer wavelength. The lifetime values are distributed as a straight line on the polar plot, suggesting two lifetime pools with a longer lifetime pool at shorter wavelength, and shorter lifetime pool at longer wavelength. Compared with the plot in Fig. 4.4 (a), the result of Spectral-FLIM offers direct information on different lifetime pools without the need of curve fitting or prior assumption of a lifetime model.

Figure 4.4. Polar plots of FLIM measurements on intact avocado leaf. The 488 nm excitation light was modulated at 100 MHz. The upper side of leaf was imaged with a 40X objective lens that focused onto mesophyll cells. The fluorescence emission is collected using a dichroic filter with 670-725 nm pass band. (a) FLIM data was acquired after 5 minutes of dark adaptation before the measurement, showing the ensemble lifetime signal collected with a band-pass filter from 670-725 nm; (b) A spectrograph is added to the FLIM emission (see Fig. 4.1) and lifetime data are acquired simultaneously at different wavelengths are shown. The color scale (blue = low intensity, red = high intensity) represents the fluorescence intensity; a peak intensity at ~680 nm can be seen.

Spectral-FLIM measurements of avocado leaves during the slow fluorescence transient are shown in Fig. 4.5 and Fig. 4.6. The spectrum of the young leaf (Fig. 4.6) has a shorter peak at the far-red region than that of the mature leaf (Fig. 4.5); otherwise, the two leaves have similar lifetime and spectroscopic features. In Fig. 4.5 (a) and Fig. 4.6 (a), the lifetime data from 670 nm to 770 nm are presented on the polar plot, with the color code representing wavelengths. All 15 measurements are plotted together, and the data show that lifetimes along the whole spectrum are quenched in a similar way. The data show two lifetime pools at different wavelengths: the longer lifetime is in the red region (670-690 nm) and the shorter lifetime pool is in the far-red region (730-750 nm). The time-course curves of the intensity at those two spectral regions (Fig. 4.5 (b)
and 4.6 (b)) both decrease as the lifetime values move toward faster time. The fluorescence spectra of the 15 measurements are also plotted together in (d). The spectra are normalized to 740 nm, and the arrow in the figures shows the greater decrease of intensity in time at the red peak. The time-course spectra are further analyzed to quantify the differences between the two spectral regions (Lambrev et al., 2010):

$$\frac{S(t, \lambda)}{S(1, 740)} - \frac{S(1, \lambda)}{S(1, 740)} \times 100\%.$$  

Each time course spectrum is first normalized to its 740 nm peak, and then the normalized spectrum of the 1st measured spectrum is subtracted. The ratios of the spectra differences to the 1st measurement are shown in Figure 4.7. The 680 nm peak has a negative fluorescence difference relative to the first Spectral-FLIM data, and the ratio of the fluorescence difference in the red region decreases continuously during the fluorescence transient. In marked contrast, the 730 nm peak has a positive fluorescence difference ratio. It reaches the maximum difference at the 2nd measurement, and stays at the same value during the light induction. Therefore, from the temporal changes of the fluorescence spectra, the red and far-red regions show difference kinetics. Since the intensities and lifetimes of those two regions both decrease during the slow fluorescence curve, they behave as qE mechanisms. The data then suggest that they may reflect two different binding sites for xanthophylls molecules, which quench Chl a fluorescence subsequitually during the light induction. As discussed in the literature (Lambrev et al., 2010), the red peak is a PsbS-independent qE site, and the far-red peak appears only when PsbS is present. We conclude that xanthophylls molecules may be also involved in PsbS-dependent qE quenching.
Figure 4.5. Spectral-FLIM measurements of a mature avocado leaf. The 440 nm excitation light was modulated at 100 MHz. The whole fluorescence emission was collected and dispersed by the spectrograph. (a) FLIM data was acquired after 5 minutes of dark adaptation before 15 continuous measurements. The color bar represents the fluorescence wavelength (from 670~770 nm). (b) The temporal data of the fluorescence intensity, modulation, and phase from the two peaks of the chlorophyll a fluorescence spectrum. One peak is between 670~690 nm, and the other one is between 730~750 nm. The value of intensity is in arbitrary unit (A.U.). (c) The time responses of the steady-state intensity data measured by Spectral-FLIM. It is color-coded by the intensity. (d) The 15 spectra are normalized to the far-red peak (740 nm). The arrows show the relatively faster decrease of the red peak (~680 nm) in time.
Figure 4.6. Spectral-FLIM measurements of a young avocado leaf. Same measurements and analysis are applied as in Fig. 4.5. Even though the far-red peak is shorter than that of the mature leaf, similar lifetime and spectrum changes during fluorescence transient are observed.

Figure 4.7. The fluorescence difference spectrum with respect to the 1st measurement: \[ \frac{S(\lambda)}{S(740)} = \frac{S(740)}{S(740)} \times 100\% \]. Data from avocado leaves in Fig. 4.5 and 4.6. The 680 nm peak decreases continuously during the slow
phase of Chl a fluorescence transient, while the 730 nm peak reaches its maximum difference at the 2\textsuperscript{nd} measurement, and stays at the same value during the light induction. See text for details.

4.4 Discussion

4.4.1 Co-occurrence of Xanthophyll Cycle and State Transition

From the FLIM measurements on Chlamydomonas cells (Fig. 4.2 & 4.3), this chapter has demonstrated that FLIM can distinguish between Chl a signals that are affected by different pathways. It records both lifetime and intensity information; therefore, the activation of qE and qT are easily distinguished. The results clearly show the advantages of applying FLIM to study a complex system.

4.4.2 Spectral-FLIM measurements during the slow Chl a fluorescence transient

In the Spectral-FLIM data on avocado leaves (shown in Fig. 4.5–4.7), the kinetics of Chl a emission at different wavelengths are in good agreement with the findings in the literature (Lambrev et al., 2010), where intact leaves of Arabidopsis (Arabidopsis thaliana) were studied. The measured emission spectra were normalized to 750 nm values, and the intensity differences of the light- and dark-adapted leaves at different wavelength regions were compared (Lambrev et al., 2010). In our Spectral-FLIM time-course experiments, a 5 minute dark adaptation was applied prior to the measurements. The 1\textsuperscript{st} data point is therefore comparable to the dark-adapted leaves in the literature just mentioned. During the 5 minutes when the Spectral-FLIM measurements were made, the laser light was continuously shining on the leaves; thus, the 15\textsuperscript{th} data point can be considered as high-light treated samples. Hence, the spectral features in different wavelength regions measured in our study have similar fluorescence difference ratios as in the literature (Lambrev et al., 2010). As also shown by mutants (Lambrev et al., 2010), the PsbS-deficient mutant npq4 doesn’t have the positive fluorescence ratio in the far-red peak, and the mutant L17 which overexpresses PsbS protein has a higher positive fluorescence ratio in this region. Therefore, the PsbS protein is considered to contribute to the fluorescence feature in the far-red region of the emission spectrum. Based on the similarity of the spectral features found in
this study and in the literature (Lambrev et al., 2010), the fluorescence signal in the far-red region shown in Fig.4.5~4.7 should have the same origin, the PsbS-dependent emission.

Our Spectral-FLIM data on avocado leaves further associate the kinetic spectral features to the corresponding fluorescence lifetime responses to light induction. While the lifetime values in both red and far-red peaks decrease during the fluorescence transient, the two lifetime pools remain well-separated on the polar plot, even though their fluorescence intensity values change with different kinetics. This supports the idea that the far-red peak originates from a different mechanism, and its signal is not merely due to a process that would be visible in the tail of the red peak. Otherwise, the relative distance between the two lifetime pools would be further apart on the polar plot, as the intensity contribution of the red peak decreases at greater pace. On the other hand, the lifetime behavior of the far-red peak on the polar plot is similar to that of the red peak, and the red peak represents the major site modulated by the binding of xanthophyll molecules. Therefore, the lifetime quenching of the far-red peak during light induction suggests it is also affected by qE. Based on the hypothesis that the far-red peak has a different origin, it is therefore suggested that the PsbS-dependent quenching also involves the binding of xanthophylls.

It has been shown that PsbS protein is necessary for qE functioning for plants, but its detailed role is still in debate (Niyogi et al., 2005). Since Spectral-FLIM combines the spectrum and lifetime information, and has the required rapid data acquisition rate, which is able to record the temporal change of those two important properties, new data suggesting that xanthophylls are associated with PsbS-dependent quenching is provided.

The time-resolved spectrum has been previously demonstrated to successfully identify the kinetic processes involving different proteins in LHC II (Gilmore et al., 2000, Kana et al., 2009, Miloslavina et al., 2008b). Different analysis methods have been applied to study kinetics and functions of the core and peripheral antenna: for example, the Gaussian deconvolution of spectral components (Gilmore et al., 2000), and the global target analysis (Miloslavina et al., 2008a, Holzwarth et al., 2009). Spectral-FLIM data provides additional lifetime information that can robustly discriminate components. But a word of caution is important: care must be taken when decomposing FLIM data, as subtracting incorrect contributions of different components to the spectrum will result in mistaken lifetime calculations.
4.5 Acknowledgements

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4.6 References


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5 FLIM of Avocado leaves on Contributions of the Lutein-Epoxide Cycle and the Violaxanthin Cycle

5.1 Overview

This chapter describes the work in collaboration with Shizue Matsubara in Forschungszentrum Jülich, Germany. FLIM measurements of Chlorophyll a (Chl a) were made on leaves of avocado plants (*Persea americana* Mill.) in order to study the energy-dependent quenching (qE) by the two xanthophyll cycles operating in parallel, the lutein-epoxide and violaxanthin cycles (Osmond *et al.*, 2008). FLIM data are presented and analyzed on the polar plot (Chen *et al.*, 2009b, Redford & Clegg, 2005a), which reveal different quenching states of Chl a by xanthophyll molecules, as well as their behavior during the Chl a fluorescence transient. Interconversion between different lifetime pools take place during this transient, and the species fractions of each lifetime quenching state have been correlated well with the pigment analysis data. Based on these observations, it is concluded that both xanthophyll cycles are able to enhance qE during photosynthetic induction.

5.2 Introduction

As mentioned in the previous chapter, light absorbed in excess of the photochemical capacity of the photosynthesis apparatus can be dissipated thermally, which is called the energy-dependent quenching (qE). qE is triggered by the build-up of a transthyakoid H⁺ gradient (ΔpH), which effects conformational changes of molecules in xanthophyll cycles (Demmig-Adams & Adams III, 1996, Muller *et al.*, 2001). The most common xanthophyll cycle in higher plants and algae is the violaxanthin (V) cycle (Fig. 5.1) (Garcia-Plazaola *et al.*, 2007). The light-driven acidification of the thylakoid lumen V activates an enzyme called V de-epoxidase (VDE), which de-epoxidizes V to antheraxanthin (A) and then to zeaxanthin (Z). The reverse reactions are catalyzed by Z epoxidase (ZE) and convert Z and A back to V, when VDE is inactive at high
lumen pH under weak light or during the night. The light-dependent de-epoxidation and epoxidation thus determine the equilibrium position of V, A and Z, or de-epoxidation state of the V cycle (DPS-VAZ) defined as \((A+Z)/(V+A+Z)\). Elimination of VDE activity by an inhibitor (Bilger et al., 1989) or by genetic mutation (Niyogi et al., 1998) diminishes \(\Delta p\text{H}\)-induced thermal energy dissipation, underlining an essential role of the V cycle in light harvesting regulation.

Some plant species possess another xanthophyll cycle, which involves lutein (L) and lutein epoxide (Lx) (Garcia-Plazaola et al., 2007) (Figure 5.1). Lx undergoes light-induced de-epoxidation to L in the same way as V to A and Z, and the resulting retention of DPS in the Lx cycle (DPS-LxL) is calculated as \(L/(Lx+L)\). The difference between the two cycles is that the epoxidation of L to Lx is much slower than the V cycle, and the kinetics of Lx recovery to L are variable in different species (Garcia-Plazaola et al., 2007). Based on the analogous light-dependent responses and the similarity in chemical structures, it has been proposed that the Lx cycle, together with the V cycle, may play a role in fine-tuning the qE pathways.

This chapter shows the temporal response of Chl a fluorescence lifetime during light induction of photosynthesis in leaves of avocado plants, an evergreen woody species in which Lx restoration happens slowly and it does not recover over hours and days after light exposure (Garcia-Plazaola et al., 2007, Osmond et al., 2008). The contrasting dark recovery kinetics of the Lx and V cycles in avocado leaves resulted in different DPS at different recovery time. The \(\Delta p\text{H}\)-dependency of DPS effects was also examined by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) treatment. DCMU inhibits the \(\Delta p\text{H}\) build-up through the linear electron transport, therefore is applied to compare the quenching effects due to \(\Delta p\text{H}\)-dependent qE and \(\Delta p\text{H}\)-independent qI.
5.3 Materials and Methods

5.3.1 Plant material

Two avocado trees (*Persea americana* Mill.) were kept under room light (photosynthetic photon flux density of 1 to 5 μmol photons m\(^{-2}\) s\(^{-1}\)) in the laboratory after they had been transported from a nursery (Clifton’s nursery, Porterville, CA, USA). Following 1 month of acclimation, experiments were conducted by using fully-expanded, dark-green leaves.

5.3.2 Light treatment

Leaves were sampled at 08:30 in the morning, cut into two halves along the midvein and floated on water with the adaxial surface facing the air. For each leaf, one cut-half was placed under a halogen lamp (400 to 500 μmol photons m\(^{-2}\) s\(^{-1}\), ‘light’-samples) for 20 min to induce xanthophyll de-epoxidation, while the other cut-half was kept under room light (1 to 5 μmol photons m\(^{-2}\) s\(^{-1}\), ‘control’-samples). The leaves were floating on water to avoid temperature increase by the 20 minutes of light treatment. At the end of the treatment both *light* and *control*-samples were
wrapped in a moist tissue and put in a plastic bag to avoid dehydration. Subsequently, they were kept in the dark to allow epoxidation of de-epoxidized xanthophylls.

Leaf discs (50 mm$^2$) for FLIM measurements were taken from the light- and control-samples at five different time points (Fig. 5.2). After leaves had been collected at 08:30 in the morning, the first leaf discs of control-samples were taken and dark-adapted on a moist tissue for 10 min. These discs, called 'control-morning', represent the most epoxidized state of the two xanthophyll cycles in avocado leaves in the experiment. After 20-min treatment under room light or the halogen lamp, leaf discs were taken in parallel from both control- and light-samples at four different time points during the subsequent dark recovery treatment in the plastic bag, namely after 10 ('control-10' and 'light-10'), 60 ('control-60' and 'light-60'), 180 ('control-180' and 'light-180') or 360 min ('control-360' and 'light-360'). The light-samples from the dark recovery treatment represent different de-epoxidation states of the two xanthophyll cycles.

![Diagram](image)

Figure 5.2. Sampling and experimental protocol of the FLIM measurements. Leaf disc samples from avocado plants were collected and FLIM measurements were performed at five different time points indicated by gray triangles. The first discs ('control-morning' samples) were taken after harvesting the leaves at 08:30 in the morning under room light (1 to 5 µmol photons m$^{-2}$ s$^{-1}$) and dark-adapting for 10 min. All other samples were taken after 20-min exposure of the leaves to the room light (control-treatment) or a halogen lamp (400 to 500 µmol photons m$^{-2}$ s$^{-1}$, light-treatment) followed by dark adaptation of 10 ('control-10' and 'light-10'), 60 ('control-60' and 'light-60'), 180 ('control-180' and 'light-180') or 360 min ('control-360' and 'light-360'). Twenty consecutive FLIM measurements were made on each leaf disc under continuous laser illumination (~50 µmol photons m$^{-2}$ s$^{-1}$). After the 20$^{th}$ measurement, the laser was turned off for 5 min to dark-adapt the samples, and the 21$^{st}$ measurement was made in the same spot. Then, leaf discs were dark-adapted for another 5 min and frozen in liquid nitrogen for pigment analysis.
5.3.3 Treatment with inhibitor

Inhibitor treatment was used to examine the energy-dependent quenching, or qE. Two avocado leaves were collected at 08:30 in the morning and floated on water without cutting into two halves. One of the leaves was treated as light-sample while the other one was treated as control, according to the treatment protocol described above. Following different treatments, three replicate leaf discs (50 mm²) were removed from both light- and control-leaves and floated on 1.2 mM of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) with the adaxial surface facing the air for 360 min, corresponding to the longest dark recovery treatment of the time-course experiment. DCMU was dissolved in ethanol first, and then diluted by deionized water until the right concentration was reached. In order to promote inhibitor uptake, the samples floating on the DCMU solution were placed under room light (1 to 5 μmol photons m⁻² s⁻¹), instead of darkness, during the 360-min recovery treatment. At the end of the recovery treatment, leaf discs were dark-adapted for 10 min and used for FLIM measurements.

5.3.4 FLIM measurements

Full-field fluorescence lifetime-resolved images were obtained by using frequency-domain homodyne technique. The instrument is described in Chapter 1. The excitation wavelength was 488 nm, and the incident laser intensity on the sample surface was around 50 μmol photons m⁻² s⁻¹. An emission filter with bandpass between 670 and 725 nm was used to collect mostly photosystem II (PS II) fluorescence. Since the contribution of photosystem I (PS I) is very little in this wavelength range at room temperature (Pfündel, 1998), it was assumed to be zero.

Leaf discs were placed between a cover slip and moist tissue which was put on a glass slide. Fluorescence intensity images were obtained with a 40X objective lens from the adaxial surface of the leaf discs, focusing on a mesophyll layer. Figure 5.3 (A) shows a fluorescence intensity image taken from an avocado leaf disc. The gray scale bar represents fluorescence intensity (in arbitrary unit, A.U.). The structure of veins (dark areas) and chloroplasts (bright spots) can be easily distinguished in this image. The lifetime data from Fig. 5.3 (A) are presented on the polar plot in Fig. 5.3 (B). In every FLIM image, an average lifetime data is calculated for further
analysis. The averaged lifetime corrects for the lifetime heterogeneity of cells and the random noise during data acquisition. A measurement protocol was designed for FLIM experiments with avocado leaves in this study (Fig. 5.2). Twenty consecutive FLIM measurements were made under continuous laser during the fluorescence induction transient. Each measurement took about 15 seconds. After the 20th measurement, the laser was turned off for 5 min to dark-adapt the sample, and the 21st measurement was made in the same spot. The purpose of this last measurement was to check if the laser illumination caused photoinhibition (qI).

After the 21st measurement, leaf discs were dark-adapted for another 5 min to minimize a local effect of laser illumination before freezing in liquid nitrogen (77K) for high performance liquid chromatography (HPLC) pigment analysis.

Figure 5.3. FLIM measurement of an intact avocado leaf disc. (A) Fluorescence intensity image. A 40X objective lens is used and focused on a mesophyll cell layer. The gray scale bar represents fluorescence intensity (in arbitrary unit, A.U.). (B) Polar plot representation of the fluorescence lifetime information of every pixel of the image in (A). The gray scale bar indicates the number of pixels.

5.3.5 Polar Plot Analysis of FLIM data

As discussed in previous chapters, the advantage of the polar plot representation is that it provides an immediate visualization and characterization of lifetime data without assuming an a priori model (Redford & Clegg, 2005a, Chen & Clegg, 2009). All points from a fluorescence signal with a single lifetime lie on a universal semicircle of the polar plot; the semicircle is centered at (x, y) = (0.5, 0) and has a radius of 0.5. All points from a fluorescence signal composed of multiple lifetime components lie inside of the semicircle (discussed in Chapter 2). For example, data having two lifetime components lie inside of the semicircle on a straight line
connecting the two single lifetime locations on the semicircle. The position on the straight line depends on the fractional fluorescence intensity contributed by each lifetime component (Redford & Clegg, 2005a).

5.3.6 Two-lifetime model

The measured fluorescence response of a two-lifetime system $F_\delta(t)$ to a very short excitation pulse (a so called $\delta$-function excitation pulse) can be expressed as follows:

$$F_\delta(t) = a_1 \cdot \exp\left(-\frac{t}{\tau_1}\right) + a_2 \cdot \exp\left(-\frac{t}{\tau_2}\right) = (a_1 + a_2) \cdot [A_1 \cdot \exp\left(-\frac{t}{\tau_1}\right) + A_2 \cdot \exp\left(-\frac{t}{\tau_2}\right)].$$

(5.1)

$\tau_1$ and $\tau_2$ are the two lifetimes. $a_1$ and $a_2$ are the corresponding preexponential factors, which are proportional to both the number of fluorescent molecules of each fluorescent component in the sample and the corresponding intrinsic radiative kinetic rate constant of each fluorescent component. In this calculation, the two lifetime components are derived from the same molecular species, but they have different lifetimes because they are in different environments (states or lifetime pools). Therefore, the intrinsic radiative rate constants are the same, and the preexponential factors are proportional to the number of molecules in each state. $A_1$ and $A_2$ are the fractional preexponential factors ($A_1 = \frac{a_1}{a_1+a_2}$ and $A_2 = \frac{a_2}{a_1+a_2}$), which are equal to the fractional concentration of the fluorescent molecules in each environment (state).

In the frequency domain FLIM measurement, the modulated excitation light $E(t)$ with the repetitive modulation frequency $\omega$ and phase $\varphi^E$ can be represented by (in complex exponential notation):

$$E(t) = E_0 + E_\omega e^{i(\omega t + \varphi^E)}.$$  

(5.2)

Here we have assumed that the excitation light is modulated by a single repetitive sinusoidal wave form (if the excitation is not a pure sinusoidal form this can be extended to be any Fourier component of the repetitive wave form). The emitted fluorescence signal following excitation by
this sinusoidal excitation light is the convolution in time of Equations 5.1 and 5.2. After carrying out the convolution, the modulated fluorescence signal, \( F_\delta(t) \ast E(t) \), becomes:

\[
F_\delta(t) \ast E(t) = C \cdot (a_1 + a_2) \cdot \left[ A_1 \tau_1 \cdot \left( E_0 + \frac{E_\omega e^{j(\omega t + \varphi^E)}}{1 + j\omega \tau_1} \right) + A_2 \tau_2 \cdot \left( E_0 + \frac{E_\omega e^{j(\omega t + \varphi^E)}}{1 + j\omega \tau_2} \right) \right]
\]

\[
= C'' \cdot (A_1 \tau_1 + A_2 \tau_2) \cdot \left[ f_1 \cdot (1 + M_1 \cos (\varphi_1 + \varphi^E)) + f_2 \cdot (1 + M_2 \cos (\varphi_2 + \varphi^E)) \right],
\]

where \( C \) accounts for the instrument factors of the FLIM instrument, and \( C'' \) is equal to \( C \cdot E_0 \cdot (a_1 + a_2) \). In Eq. 5.3, the modulations \((M_1, M_2)\) and phases \((\varphi_1, \varphi_2)\) values are the same as defined Chapter 1 and 2. \( f_1 \) and \( f_2 \) represents the fractional fluorescence intensity contributed from the lifetime component \( \tau_1 \) and \( \tau_2 \), respectively. They are related to the fractional preexponential factors by the following equations:

\[
f_1 = \frac{A_1 \tau_1}{A_1 \tau_1 + A_2 \tau_2},
\]

\[
f_2 = \frac{A_2 \tau_2}{A_1 \tau_1 + A_2 \tau_2}.
\]

For the two-lifetime model, least-square fitting of the polar plot data to a straight line is carried out to determine the fractional intensities \( f_1 \) and \( f_2 \) and the two lifetimes \( \tau_1 \) and \( \tau_2 \) (the lifetimes are related to the intercepts of the straight line with the semi-circle). The fractional preexponential factors \( A_1 \) and \( A_2 \) are derived from \( f_1 \) and \( f_2 \). Using these values, the steady state fluorescence intensity, \( C'' \cdot (A_1 \tau_1 + A_2 \tau_2) \), can be simulated and compared to the measured fluorescence intensities. This is a good test for the applicability of the two component model. \( C'' \) is just a constant that is varied to give the best fit to the measured fluorescence intensity.

5.3.7 Pigment analysis

Leaf discs (50 mm²) frozen in liquid nitrogen after the FLIM experiments were lyophilized overnight. Immediately before pigment extraction, leaf discs were ground in a small amount of liquid nitrogen by using a mortar and pestle. Pigments were extracted twice in chilled acetone,
and then, centrifuged at 13000 rpm for 5 min and syringe-filtered prior to the HPLC analysis. Photosynthetic pigments were separated by an Allsphere ODS-1 column (Alltech Associates, Inc., Deerfield, IL) by using solvents and protocols modified from the literature (Gilmore & Yamamoto, 1991). Pigments were identified by retention times and absorption spectra. This part of work was done by Shizue Matsubara and Rosanna Caliandro.

5.4 Results

5.4.1 Pigment composition in avocado leaves

After 20-min of halogen light exposure, 10-min of dark adaptation ('light-10') resulted in de-epoxidation of nearly half of the V-cycle pigments (Fig. 5.4 (A) and (B), Shizue Matsubara and Rosanna Caliandro, personal communication). The V level was gradually restored in the light-samples at the expense of A+Z during the subsequent dark period of 60, 180 and 360 min (light-60, light-180 and light-360, respectively), although the recovery was not yet complete after 6 hour of darkness. Following different durations of dark adaptation, A was the dominant de-epoxidized form of V in light-samples; only a single light-10-sample contained some Z (5.5 mmol per mol Chl).

The light exposure also induced de-epoxidation in the Lx cycle (Fig. 5.4 (C) and (D)). The Lx level was diminished to less than 5 mmol per mol of Chl in light-10-samples, which was accompanied by an increase in DPS-LxL. Compared to the V cycle, the Lx cycle exhibited little recovery during the subsequent dark adaptation of up to 6 hours, confirming extremely slow reversibility of this cycle in avocado leaves (Garcia-Plazaola et al., 2007, Esteban et al., 2008).

No change in pigment composition was detected in control-samples at different time points; both xanthophyll cycles remained maximally epoxidized throughout the experiment (Fig. 5.4).
Figure 5.4. Composition of the xanthophyll-cycle pigments in dark-adapted control- and light-samples of avocado leaves at different times (n = 4, ±SE) (Shizue Matsubara and Rosanna Caliandro, personal communication). (A) Levels of violaxanthin (V). (B) De-epoxidation state of the V cycle (DPS-VAZ) calculated as (A+Z)/(V+A+Z), where A and Z are antheraxanthin and zeaxanthin, respectively. (C) Levels of lutein epoxide (Lx). (D) De-epoxidation state of the Lx cycle (DPS-LxL) calculated as L/(Lx+L), where L is lutein. The different sampling times are: at 08:30 in the morning and after 10, 60, 180 or 360 min of dark adaptation following a 20-min exposure to 1 to 5 (control) or 400 to 500 µmol photons m\(^{-2}\) s\(^{-1}\) (light). None of the control-samples contained A or Z whereas most of the light-samples had A but no Z, except for a single light-10-sample in which a small amount of Z (5.5 mmol mol\(^{-1}\) Chl) was detected.

5.4.2 FLIM measurements in control- and light-samples

FLIM measurements were conducted in vivo to examine effects of de-epoxidation in the two cycles on PSII Chl a fluorescence lifetime. Figure 5.5 shows the average fluorescence intensity measured in three replicate leaves of control- and light-samples during 20 consecutive FLIM measurements under continuous laser illumination. The laser intensity was ~50 µmol photons m\(^{-2}\) s\(^{-1}\), which induced the additional de-epoxidation to the light treatment. The laser illumination of the 20 measurements did not saturate either the two xanthophyll cycles in the sample, as the control-samples always showed higher intensity and longer fluorescence lifetimes than the most de-epoxidated sample, light-10. Each measurement took about 15 s, resulting in 300 s of continuous laser illumination for 20 consecutive measurements. Note that the data of the 1\(^{st}\) measurements, shown at time = 0, were actually recorded during the first 15 s of laser
illumination, and consequently, do not represent the maximal fluorescence intensity of these samples. Following the 20 measurements during the 5-min light induction, the laser was switched off for 5 min, and then a final (21st) measurement was made during the time interval between 600 and 615 s (for the protocol of FLIM measurements, see Fig. 5.2).

The fluorescence intensity of the 1st measurement was higher in control-samples than in light-samples; the highest intensity was found in control-morning and the lowest in light-10 (Fig. 5.5). The initial fluorescence intensity upon illumination varied little among control-samples whereas the values increased in light-samples with increasing length of dark adaptation from 10 to 360 min. Pronounced recovery of the initial fluorescence intensity was observed in light-samples between 10 and 60 min of dark treatment; thereafter the recovery was small.

During the 20 FLIM measurements, the fluorescence intensity first decreased (corresponding to the P to S in the Chl a fluorescence transient) and then increased (corresponding to the S to M in the Chl a fluorescence transient) in all samples under the continuous laser illumination (Papageorgiou et al., 2007). Independent of the light intensity during the pre-treatments, longer dark adaptation resulted in a slower onset of the S-to-M rise, which happened earliest in light-10 (after the 4th measurement) and latest in control-360 (the 19th measurement). In all samples except light-10, the fluorescence intensity of the 21st measurements recovered to 80 or 90% of the original values recorded at the 1st measurements, indicating that the 5-min laser illumination at ~50 μmol photons m⁻² s⁻¹ caused little photoinhibition (qI) in the avocado leaf discs. In the case of light-10 samples, the intensity of the 21st measurements was higher than the 1st measurements.
Figure 5.5. Fluorescence intensity during 20 continuous FLIM measurements of avocado leaves under laser illumination (~ 50 μmol photons m\(^{-2}\) s\(^{-1}\)) and a subsequent 21\(^{st}\) measurement after 5 min of dark adaptation. Fluorescence intensity (in arbitrary unit, A.U.) was normalized to the CCD exposure time (ms). Shown are the averages of three replicates from three different leaves for control- (C) and light-samples (L) at five different sampling times: morning, after 10, 60, 180 or 360 min of dark adaptation following a 20-min exposure to 1 to 5 (control) or 400 to 500 μmol photons m\(^{-2}\) s\(^{-1}\) (light).

The fluorescence lifetime data of control- and light-samples taken from one of the three replicate leaves are presented in a polar plot (Figure 5.6). Comparable results were obtained in all three replicate leaves. The data from control- and light-samples are located inside of the semicircle, indicating that the fluorescence signals of these samples were composed of multiple lifetime components, and fall on a straight line connecting two lifetimes. A linear least-squares regression was used to determine the straight line and the intercepts on the semicircle: one is about 1.5 ns and another 0.5 ns. During the 5-min light induction, the data from control-samples moved along this straight line, starting from the positions near 1.5 ns and moving towards 0.5 ns, then from there gradually moving back to 1.5 ns. This pattern follows the P-S-M change observed in the fluorescence intensity (Fig. 5.5); that is, shorter lifetimes correspond to lower fluorescence intensities. Compared to control-samples, the data from light-samples locate closer to 0.5 ns (Fig. 5.6 (A)), especially in light-10. Yet, the same pattern of data was observed in light-samples as found in control-samples; i.e. the data points first moved towards 0.5 ns and then towards 1.5 ns. This again parallels the intensity changes measured in these samples (Fig. 5.5). Figure 5.6 (B) shows the same data set as in Fig. 5.6 (A), but in a three-dimensional (3D) polar plot, with the fluorescence intensity given on the z-axis. The rise and decay of the intensity along the z-axis of
the 3D polar plot correlates very well with the data progression along the straight line between the two lifetimes.

In order to achieve a more detailed understanding of the underlying mechanism for the observed fluorescence intensity changes, the data were fitted by the two-lifetime model using the lifetime parameters obtained from the polar plot analysis. In this model, it assumes that fluorescing molecules giving rise to the two different lifetime components have the same natural radiative kinetic rate constant; in other words, the two lifetimes represent two states of dynamic quenching of otherwise Chl a molecules with identical fluorescence properties. If fluorescence intensity changes are caused by shifts in the fractional contribution of the two lifetime components due to interconversion between the two quenching states, rather than concentration changes of the fluorescent molecules, the two-lifetime model can predict the fluorescence induction trace. In Fig. 5.6 (C), the fluorescence intensity is plotted against the fractional intensity of the 0.5-ns lifetime pool calculated from the two-lifetime model. The data from both control- and light-samples are distributed along the curve simulated by the two-lifetime model, and the simulated fluorescence intensity matches the intensity measured in these samples. The simulated values were reasonably close to the experimental data in all the individual samples at different time points (Fig. 5.7). These results show that interconversion between the two differently quenched states, represented by the 1.5- and 0.5-ns lifetime pools in the polar plot, can sufficiently explain the fluorescence intensity changes observed in the avocado leaf discs during the P-S-M transient (Fig. 5.5). Concentration changes of Chl a, such as state transition (Iwai et al., 2010), seem to play a minor role in the fluorescence quenching in the avocado leaves under the experimental conditions in this study.
Figure 5.6. Polar plot of FLIM data from *control* - and *light*-samples from avocado leaf discs. (A) The polar plot of all data from *control* - and *light*-samples taken from one of the three replicate leaves at five different time points: morning, after 10, 60, 180 or 360 min of dark adaptation following a 20-min exposure to 1 to 5 (*control*) or 400 to 500 µmol photons m⁻² s⁻¹ (*light*). The straight regression line indicates the approximate positions of two lifetime pools at around 1.5 ns and 0.5 ns. (B) A 3D presentation of the polar plot with the z-axis for fluorescence intensity normalized to CCD exposure time (A.U. ms⁻¹). (C) Measured fluorescence intensity plotted against the fractional intensity of the 0.5-ns lifetime pool calculated from the fitting. The simulation intensity by the two-lifetime model is also shown.
Figure 5.7. Fluorescence intensity measured in control- and light-samples of avocado leaf discs during the slow Chl a fluorescence transient at different times and the simulation line of the data fitting. Samples were collected at 08:30 in the morning and after 20-min exposure to 1-5 (control) or 400-500 µmol photons m$^{-2}$ s$^{-1}$ (light) followed by 10, 60, 180 or 360 min of dark recovery. Similar fluorescence induction traces were found in all replicates and the simulation lines fit reasonably well to the data from all replicates.

Figure 5.8 summarizes the behavior of the species fractions of the two lifetime pools during the light induction in control- and light-samples. The species fractions are calculated from the two-lifetime model. In all control-samples, laser illumination induced a very similar P-to-S decrease in the fraction of Chl a species of the 1.5-ns lifetime pool (Fig. 5.8 (A)); this decline was slightly slower in control-morning. Differences among control-samples emerged in the subsequent S-to-M rise; longer dark treatment delayed the onset of the rise, resulting in a progressive decrease of the lowest 1.5-ns species fraction measured during the light induction. The lowest values occurred in control-samples between 180 and 195 s (control-morning), 120 and 135 s (control-10), 135 and 195 s (control-60), 165 and 180 s (control-180), and 210 and 255 s (control-360). At the 21st measurements, the 1.5-ns species fraction recovered to 60 to 80% of the initial levels, in parallel to the intensity data (Fig. 5.5).
The species fraction of the 1.5-ns lifetime pool was much smaller in light-samples than in control-samples, and this was observed already at the 1st measurements (Fig. 5.8 (B)). The initial species fraction of the 1.5-ns pool recovered in light-samples with increasing duration of dark adaptation (from 10% to 50%). However, unlike in control-samples, the 1.5-ns species fraction decreased to similarly low levels (less than 10%) during the light induction in all light-samples, although this happened fastest in light-10 and slowest in light-360. Thus, the lowest values were recorded in light-samples between 30 and 45 s (light-10), 90 and 135 s (light-60), 120 and 195 s (light-180), and 135 and 225 s (light-360). At the 21st measurements, the 1.5-ns species fraction accounted for about 40% of the entire population of the fluorescent molecules in all light-samples irrespective of the sampling time. Except in light-10, this corresponds to 80% of the initial levels.

The species fraction of the 0.5-ns lifetime pool shows a behavior essentially opposite to that of the 1.5-ns pool (Fig. 5.8 (C) and (D) for control- and light-samples, respectively). This is also evident in the Fig. 5.9, where the species fractions of the two lifetime pools are plotted together for each treatment and time.

Figure 5.8. Species fractions of the 1.5- (A and B) and 0.5-ns lifetime pools (C and D) during the FLIM measurements. Shown are the averages of three replicates from three different avocado leaves for control- (C) and light-samples (L) at five different sampling times: morning, after 10, 60, 180 or 360 min of dark adaptation following a 20-min exposure to 1.5 (control) or 400-500 µmol photons m⁻² s⁻¹ (light).
Figure 5.9. Species fractions of the 1.5- and 0.5-ns lifetime pools in control- and light-samples from avocado leaf discs during the slow Chl a fluorescence transient at different times. The average values of three replicates are shown for control- and light-samples. Samples were collected in the morning and after 20-min exposure to 1-5 (control) or 400-500 µmol photons m\(^{-2}\) s\(^{-1}\) (light) followed by 10, 60, 180 or 360 min of dark recovery.

5.4.3 Xanthophyll de-epoxidation state and the 0.5-ns lifetime pool

If the two xanthophyll cycles in avocado leaves are involved in regulation of light harvesting and thermal energy dissipation, variations in the species fraction of the two lifetime pools (Fig. 5.8) should correlate with changes in their de-epoxidation state (or DPS in short, see Fig. 5.4). In order to directly compare fluorescence lifetime and pigment data, the species fraction of the short (0.5-ns) lifetime pool was plotted against DPS for each sample (Fig. 5.10). As these DPS values represent a dark-adapted condition, only the lifetime data from the first three measurements (within 45 s) were examined; that is, only before substantial de-epoxidation could have been induced by laser illumination. The DPS was analyzed separately for the V cycle (DPS-VAZ, Fig. 5.10 (A)-(C)) and the Lx cycle (DPS-LxL, Fig. 5.10 (D)-(F)), as well as both cycles combined.
(DPS-all, Fig. 5.10 (G)-(I)). In these samples DPS-VAZ was determined by the levels of A and V; none of the samples had Z except one light-10.

Linear regression lines fitted to the data show a significantly positive correlation between the species fraction of the 0.5-ns lifetime pool and DPS of each separately, as well as both, of the two xanthophyll cycles (Fig. 5.10). The 0.5-ns species fraction was best predicted by DPS-all, suggesting the involvement of both A in the V cycle and L in the Lx cycle in interconversion between the 1.5- and 0.5-ns lifetime pools. The highest $R^2$ values were found at the 2nd measurements between 15 and 30 s (Fig. 5.10 (B), (E) and (H)). In the first 15 s both DPS-VAZ and DPS-all explained the variations in the 0.5-ns species fraction similarly well; thereafter, the correlation was better when the two cycles were combined than for the V cycle alone. Between the two cycles, DPS-VAZ indicated a stronger correlation with the 0.5-ns species fraction than DPS-LxL, which may be attributed to a greater impact of the V cycle than the Lx cycle and/or the large pool of L that is not directly involved in the Lx cycle. The data of the light-10-sample with Z did not particularly deviate from the fitting lines; they were found among the data of other light-samples in which A was the only de-epoxidized xanthophyll detected for the V cycle.
5.4.4 *Inhibitor experiment*

The energy-dependent quenching (qE) requires a build-up of ΔpH across the thylakoid membrane (Baker, 2008). To ascertain the effect of ΔpH on fluorescence lifetime, avocado leaf discs were treated with 1.2 mM DCMU during the 6-h recovery period following the 20-min light- or control-treatment. The DCMU inhibits the ΔpH build-up by blocking the linear electron transport at the Qₐ binding site of PSII, hence eliminating ΔpH-dependent qE.

The FLIM measurements were performed in the DCMU treated samples by using the same measurement protocol as in the time-course experiment (Figs. 5.2). After incubation with DCMU, the fluorescence intensity as well as the lifetime parameters remained nearly unchanged.
during laser illumination in five out of six samples (Fig. 5.11). This is a stark contrast to the fluorescence quenching observed during the P-S-M phase of the fluorescence transient in all the samples without DCMU (Fig. 5.5), the latter which was interpreted as interconversion between the 1.5-ns and 0.5-ns lifetime pools based on the polar plot analysis (Fig. 5.6). In one control-sample, the intensity and lifetime values changed slightly with time, probably due to inefficient DCMU binding. Plotting the data from six DCMU-treated samples on a polar plot gave a straight line fit that intercepted the semicircle at about 2 and 0.5 ns (Fig. 5.11 (A)), suggesting a substantial increase in fluorescence lifetime of the long lifetime pool in the presence of DCMU (in the absence of ΔpH), compared to the 1.5-ns pool found in the absence of DCMU (in the presence of ΔpH; see Fig. 5.6 (A)). On the other hand, the lifetimes of the short lifetime pool are comparable in the two experiments with and without DCMU (both about 0.5 ns).

![Polar plot analysis of the FLIM data from leaf discs treated with 1.2 mM DCMU. Sample discs were floated on the DCMU solution for 360 min following a 20-min exposure to 1-5 (control, black symbols) or 400-500 μmol photons m⁻² s⁻¹ (light, gray symbols).](image)

Figure 5.11. Polar plot analysis of the FLIM data from leaf discs treated with 1.2 mM DCMU. Sample discs were floated on the DCMU solution for 360 min following a 20-min exposure to 1-5 (control, black symbols) or 400-500 μmol photons m⁻² s⁻¹ (light, gray symbols).

Similar responses were found in control- and light-samples after the DCMU incubation, suggesting that DPS may not influence fluorescence lifetime in the absence of ΔpH. In marked
contrast to Fig. 5.10, in which the species fraction of the 0.5-ns lifetime pool was strongly correlated with DPS of the two xanthophyll cycles, there was no significant correlation in the DCMU-treated samples between the species fraction of the ΔpH-independent 0.5-ns lifetime pool and any of the three DPS parameters (Fig. 5.12). The DPS values varied greatly among the samples, with DPS-VAZ ranging between 0 and 0.5 and DPS-LxL between 0.9 and 1.0, while the 0.5-ns lifetime pool accounted for about 60% of the whole population of the fluorescing molecules in all the DCMU-treated samples (Fig. 5.12). The results imply that neither DPS-VAZ nor DPS-LxL was involved in this ΔpH-independent photoinhibition (qi) quenching. Thus, it seems that DPS of the V and Lx cycles mainly affects the ΔpH-dependent qE in avocado leaves in the experimental conditions here.

Figure 5.12. Correlation between the de-epoxidation state (DPS) of the two xanthophyll cycles and the species fraction of the 0.5-ns lifetime pool in the control- (black symbols) and light-samples (white symbols) in avocado leaves after a 360-min DCMU treatment (Shizue Matsubara and Rosanna Caliandro, personal communication). (A-C) De-epoxidation state of the V cycle, DPS-VAZ; (D-F) de-epoxidation state of the Lx cycle, DPS-LxL; (G-I) combined de-epoxidation state of the two cycles, DPS-all. The species fractions at the 1st (between 0 and 15 s; A, D and G), 2nd (between 15 and 30 s; B, E and H) and 3rd (between 30 and 45 s; C, F and I) measurements of the 21 FLIM measurements are shown. Each symbol represents an individual leaf disc. Zeaxanthin was not detected in any of these samples.
5.5 Discussion

5.5.1 Slow phase of the chlorophyll a fluorescence transient in avocado leaves

FLIM experiments illustrated the P-S-M phase of the fluorescence transient in avocado leaves (Fig. 5.5). The decreasing fluorescence intensity in the first minutes shows the P-to-S phase, which reflects the build-up of ΔpH and the subsequent activation of qE (Papageorgiou et al., 2007). This is confirmed by disappearance of P-to-S phase after the treatment with DCMU (Fig. 5.11). The P-to-S decline is followed by the S-to-M rise (Fig. 5.5). In higher plants, this rise has been associated with photosynthetic induction (Papageorgiou et al., 2007). In agreement with this, longer dark treatment delayed the onset of the S-to-M rise in both control- and light-samples (Fig. 5.5), suggesting a need of longer photosynthetic induction after prolonged darkness. This pronounced effect of darkness on the photosynthetic induction during the S-to-M phase contrasts with no apparent effect on the reactions in the P-to-S phase (see control-samples in Fig. 5.5).

Changes in the fluorescence intensity recorded during the P-S-M transient can be well-accounted for by the interconversion of Chl a between the two (0.5 and 1.5 ns) fluorescence lifetime pools (Fig. 5.6). Together with the disappearance of the P-to-S decrease in the intensity measurements, changes in the fluorescence lifetimes also vanished in the DCMU-treated samples (Fig. 5.11), suggesting that these changes (without DCMU) in the lifetime pools are related to qE. As the simulated intensity by the two-lifetime model fits the intensity data very well (Fig. 5.6 (C) and Fig. 5.7), it is reasonable to conclude that the number of Chl a molecules in PSII did not change during FLIM measurements, and thus, effect of state transition (qT) can be neglected.

5.5.2 Fluorescence lifetime pools revealed by polar plot analysis

The polar plot representation illustrates the uniform correlation between lifetime and intensity for both control- and light-samples (Fig. 5.6 (A) and (B)). Not only those changes during the P-S-M transient, but also the differences between control- and light-samples can be fitted by a single straight line connecting 1.5 and 0.5 ns on the semicircle. Compared with the FLIM results of Chlamydomonas reinhardtii during the Chl a fluorescence transient shown in Chapter 4, a
distinctive relation between the lifetime and intensity was observed, indicating different mechanisms of non-photochemical fluorescence quenching (NPQ) are functioning in the two different species. *Chlamydomonas* displays pronounced qT, while the avocado leaves show mostly qE effects. Therefore, FLIM data can identify different mechanisms of fluorescence intensity quenching and lifetime transitions in photosynthetic cells and tissues.

For complex and 3D samples like leaves, having high spatial heterogeneity of fluorescence intensity and lifetime (Fig. 5.3 (A); (Baker, 2008)), one should keep in mind that data are mean values of heterogeneous signals over the measurement area. Variations between the samples can also produce small variations in the lifetime values. This will lead to an average lifetime for each lifetime pool, which may consist of a distribution of similar lifetime components. Such pools behave on the polar plot in the same way as expected for pools with singular lifetimes (Chen & Clegg, 2009, Redford & Clegg, 2005a).

5.5.3 Two xanthophyll cycles and quenching of PS II fluorescence lifetime

Earlier studies have shown that ΔpH reduces the lifetime and fractional contribution of the longer (>2 ns) lifetime component of PS II (Gilmore et al., 1995, Moise & Moya, 2004). According to the results in Figs. 5.6 and 5.11, this effect of ΔpH manifested itself as the difference between the long lifetime pools found with DCMU (2 ns) and without DCMU (1.5 ns) treatment. Also shown in the literature (Gilmore et al., 1995, Holub et al., 2000), accumulation of Z and A decreases the longer fluorescence lifetime component giving rise to a short lifetime component at 0.3–0.5 ns, analogous to the 0.5-ns pool in the control- and light-samples (Fig. 5.6 (A)). In avocado leaves having both the V and Lx cycles, the species fraction of the 0.5-ns lifetime pool shortly after switching on the light was tightly correlated with DPS, especially the combined DPS of the two cycles (Fig. 5.10).

The overall DPS of the dark-adapted avocado leaf discs was determined by A and 'photo-converted' L (as opposed to the majority of L not involved in the Lx cycle). The weaker correlation between DPS-LxL and the 0.5-ns pool (Fig. 5.10 (D)-(F)) may suggest a smaller effect of photo-converted L compared with A. Nonetheless, the improved correlation of the 0.5-ns species fraction with DPS-all (Fig. 5.10 (G)-(I)) indicates involvement of A as well as photo-
converted L in the ΔpH-dependent interconversion between the 1.5- and 0.5-ns lifetime pools in avocado leaves.

The ΔpH-dependency of the DPS effect on fluorescence quenching in avocado leaves (Figs. 5.10 and 5.10) suggests that retention of A and photo-converted L per se does not cause qI. This confirms previous reports in plants with the V cycle alone that the formation of the short lifetime component related to DPS-VAZ necessitates ΔpH (Gilmore et al., 1995). Also in Lx-cycle species, different DPS-LxL values found in sun and shade leaves or in shade leaves before and after light exposure do not always affect the maximal PS II efficiency (Matsubara et al., 2008, Matsubara et al., 2005).

The results shown here suggest that mainly A in the V cycle and photo-converted L in the Lx cycle, both of which have a de-epoxidized β-ionone (Figure 5.1), are components of qE associated with the 0.5-ns fluorescence lifetime pool revealed in avocado leaves. While ΔpH acts as the main switch of qE, plants are able to adjust their ΔpH response and level of photoprotective energy dissipation by altering DPS of the xanthophyll-cycle pigments.

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5.7 References


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