Impact of Folivory on Red Fluorescence Emission using Fluorescence Lifetime Imaging Microscopy (FLIM)

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Abstract

Many previous studies have used fluorescence intensity measurements to reveal different aspects of photosynthesis and the effect of folivory (action of herbivores eating the leaves of a plant) on leaves. In this experiment, we employ fluorescence lifetime imaging microscopy (FLIM) to study the fluorescence lifetime of healthy leaves and leaves that have been damaged by caterpillars. Previous studies have shown an increase in fluorescence intensity of the area adjacent to the biomass removal. The fluorescence lifetime is a new parameter that can assist the biologist and entomologist to understand the fundamental changes of the plant's physiology and morphology after being damaged by caterpillars.

1 Introduction

1.1 Fluorescence Overview

For the past 50 years, fluorescence has been employed as a primary research tool in biophysics and biochemistry. Fluorescence is a type of luminescence which is the emission of light from a substance in an electronically excited state. When light strikes an absorbing molecule, the molecule can be excited to a higher energy electronic state (*excited state*). The electrons remain in this excited state for a short duration of time before returning to the lower energy state (*ground state*). If the excited molecule can emit a photon; that is if the molecule is a fluorophore, then one of the pathways from the excited to the ground state is by light emission (fluorescence). Some of the original energy absorbed by the molecule is dissipated as heat as the molecule relaxes to the lower vibrational states, and hence the light emitted by the relaxing electron has a lower energy than the absorbed light and a longer wavelength, as shown in Figure 1.1.1.

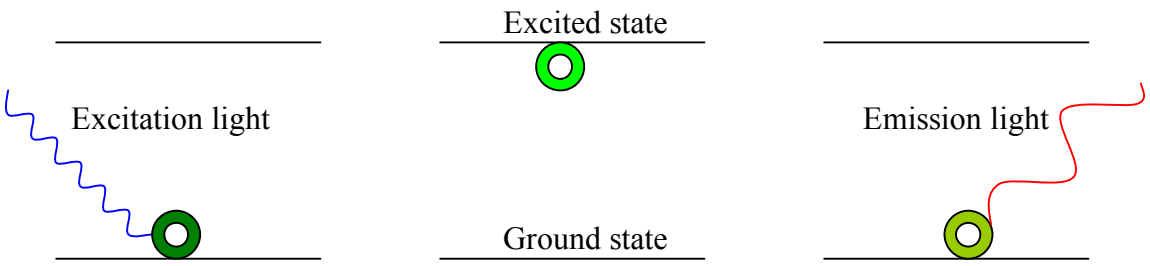

Figure 1.1.1. The fluorescence process (from left to right), the excitation light comes in and excites the fluorophore to a higher energy level. It then releases some of this energy in the form of the emission wave. Note that the excitation light has a shorter wavelength that the emission light.

1.2 Fluorescence Lifetime and Fluorescence Intensity

It is important differentiate between fluorescence intensity and fluorescence lifetime. In order to do this we need to define the *fluorescence quantum yield* - the ratio of the number of photons emitted to the number absorbed. A typical Jabłoński diagram is shown in Figure 1.2.1. The transitions between states are depicted as vertical lines to illustrate the instantaneous nature of light absorption.

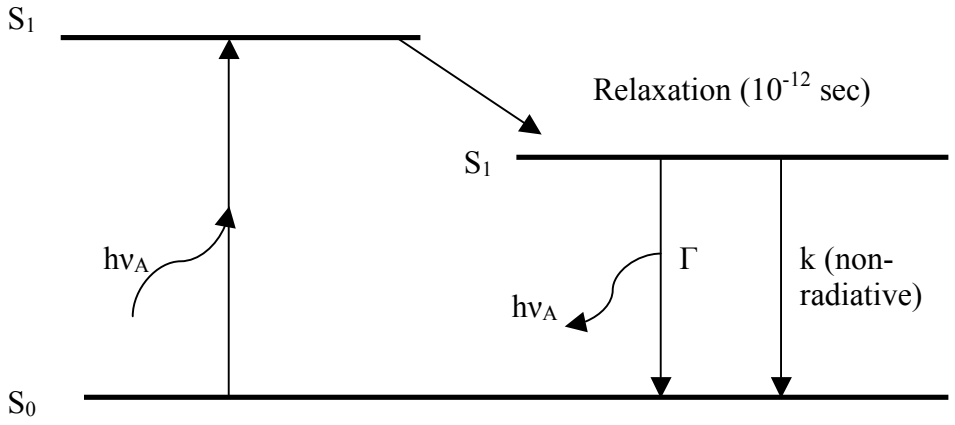

Figure 1.2.1. Modified Jabłoński diagram to illustrate some of the different processes which can occur between the absorption and emission of light. The ground and the first electronic states are depicted as S_0 and S_1 respectively.

If the emissive rate of the fluorophore is Γ, and the radiationless decay rate (the sum of all rates of dissipation other than emission) is *k,* then the quantum yield is given by [1]

$$Q = \frac{\Gamma}{\Gamma + k} \quad . \tag{1.2.1}$$

The fluorescence *intensity* is proportional to the number of fluorescence photons emitted per unit time and per excited volume [2]. Therefore the intensity of fluorescence is directly proportional to the fluorescence quantum yield.

The fluorescence *lifetime* (τ) of a fluorophore is the average period of time the fluorophore remains in the excited state. For the fluorophore illustrated in Figure 1.2.1 the lifetime is:

$$\tau = \frac{1}{\Gamma + k} \ . \qquad (1.2.2)$$

The fluorescence lifetime and quantum yield are important characteristics of a fluorophore [1]. When a fluorophore absorbs light, it can undergo fluorescence emission, only after a certain delay known as the *mean decay time*. It is referred to as a 'mean decay time' and not an 'exact decay time' because fluorescence emission is a random process; only the probability of emission per unit time is constant. Very few molecules emit their photons precisely at $t = \tau$. [3].

Fluorescence lifetime values are independent of concentration of fluorophores (unlike fluorescence intensity, which is dependent on fluorophore concentration); making lifetime a more versatile value as fluorophore population density measurements are not required.

1.3 Photosynthesis

There are a variety of fluorophores in plants. The major fluorophores in plants are chlorophylls and flavin nucleotides. Photosynthesis uses some of these fluorophores to capture light energy, shuttle this energy through a series of electron transfer mechanisms and store it in the organism; the stored energy is used to drive cellular processes [4].

Photosynthesis is localized in sub-cellular structures known as chloroplasts. The chloroplast contains all the chlorophyll pigments and, in most organisms, carries out all the main phases of photosynthesis [4]. The photosynthetic process in all plants and algae, as well as in certain types of photosynthetic bacteria, involves the reduction of CO_2 to carbohydrate, and the ultimate removal of electrons from H_2O, resulting in the release of O_2. In this process, known as oxygenic photosynthesis, water is oxidized by the photosystem-II reaction center, a multi-subunit protein located in the photosynthetic membrane. The two different reaction centers, photosystem I and photosystem II, work concurrently with one another. Photosynthesis has two distinct reaction stages, the “light reactions” and “dark reactions”. Photosystem II provides electrons to photosystem I in the light stage.

When a photon is absorbed by an antenna pigment molecule in the plant antenna system several outcomes are possible: it could be emitted as fluorescence, it could be converted to heat, or the excitation could undergo energy migration until it is trapped by a reaction center. This kinetic competition (each of the above processes are competing with each other) is then exploited by photophysicists to understand the inner workings of

photosynthesis. If fluorescence increases, it indicates that the other processes are receiving less of the absorbed energy; this shows that the leaves are not metabolizing the energy as a normal healthy leaf (a healthy leaf would show less fluorescence because more of the absorbed energy is used in photosynthesis). Figure 1.3.1 should help you get a better sense of where these photosynthetic structures are:

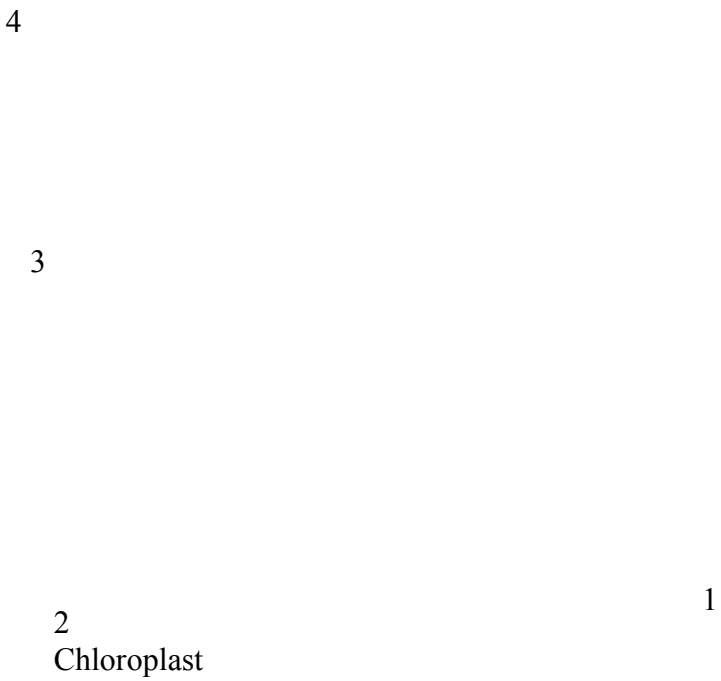

Figure 1.3.1. Diagram showing the photosynthetic apparatus of a plant. The first bubble (1) shows a cross-section of a leaf, and its different types of cells; the dark spots are chloroplasts. The second bubble (2) is a chloroplast; the thylakoid membranes are the

dark lines. The third bubble (3) shows a stack of thylakoids, and the fourth bubble (4) shows the molecular structure of the thylakoid membrane [4].

Two types of chlorophyll molecules are found in plants and green algae. They are Chlorophyll a and Chlorophyll b. Chlorophyll (Chl)* *a* fluorescence is red and bright and provides information about the electron transfer processes in photosystem II. The absolute *quantum yield of fluorescence* ($\mathbf{\Phi}_f$) is related to the ratio of k_f to the sum of all rate constants (k's) of de-excitation; (*f* for fluorescence, *h* for heat dissipation, *t* for excitation energy transfer, *q* for quenching by quenchers and *p* for photochemistry) [5].

$$\mathbf{\Phi}_f = \frac{k_f}{k_f + k_h + k_t + k_q + k_p} \tag{1.3.1}$$

Effects of leaf damage on photosynthesis have been studied using conventional gas exchange methods and compared to fluorescence intensity measurements. The number of Chlorophyll *a* molecules in a plant is dependant on a variety of external factors such as sunlight concentration. Therefore it is hard to know the number of centers for proper normalization. We are using fluorescence lifetime instead of fluorescence intensity to make our measurements, because the information on photosynthesis activity can be obtained without knowing the concentration of the fluorophores.

Figure 1.3.2 illustrates how chlorophyll *a* fluorescence allows a method to probe the photochemical reactions of photosystem II.

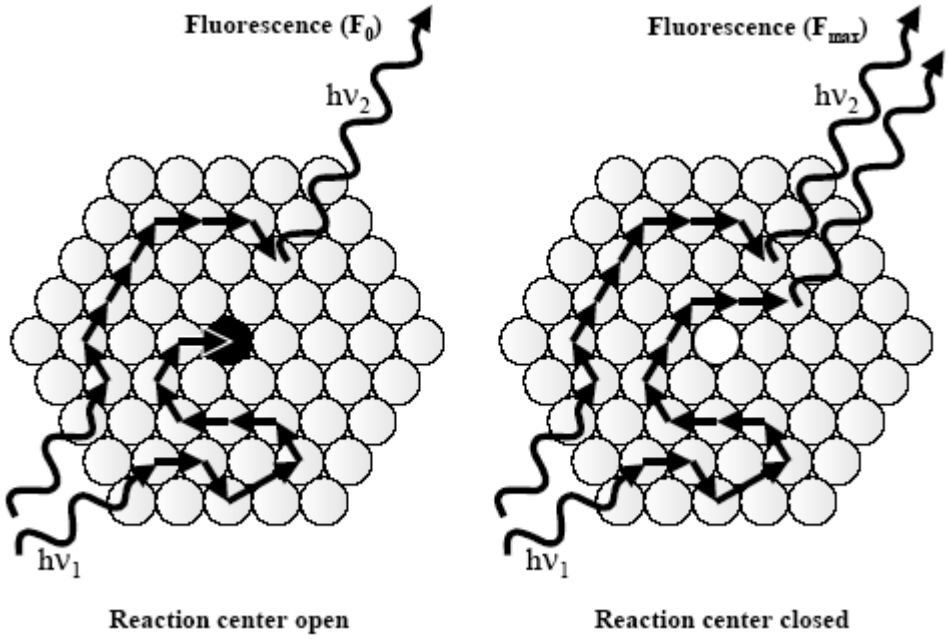

Figure 1.3.2. Antenna Chlorophyll (grey circles) excitation and then FRET (Forster fluorescence resonance energy transfer; the physical process by which energy is transferred non-radiatively from one excited molecule to another [6]) allows migration to an open reaction center (black circle). The fate of two photons are shown, a closed reaction center (white circle) leads to an increase in fluorescence intensity and lifetime [3].

2 Theory and literature overview

2.1 Fluorescence Intensity Experiment

An experiment to understand the effect of folivory (action of herbivores eating the leaves of a plant) was carried out by A. Zangerl *et al*.[7]. There, he and his collaborators used an instrument for imaging chlorophyll fluorescence to map the effects of caterpillar feeding on whole-leaf photosynthesis in wild parsnip (*Pastinaca sativa*). In this experiment cabbage looper caterpillars, Trichoplusia ni (Lepidoptera: Noctuidae) were used. They

found that the adverse effects of caterpillar feeding on photosynthesis extended well beyond the areas of the leaflet in which the caterpillars removed tissue. They found that the impact of folivory on photosynthesis is greater than the sum of holes made by the caterpillars (in fact the damage was six times larger than the area directly damaged by the caterpillars.) The results from their experiment are shown in Figure 2.1.1.

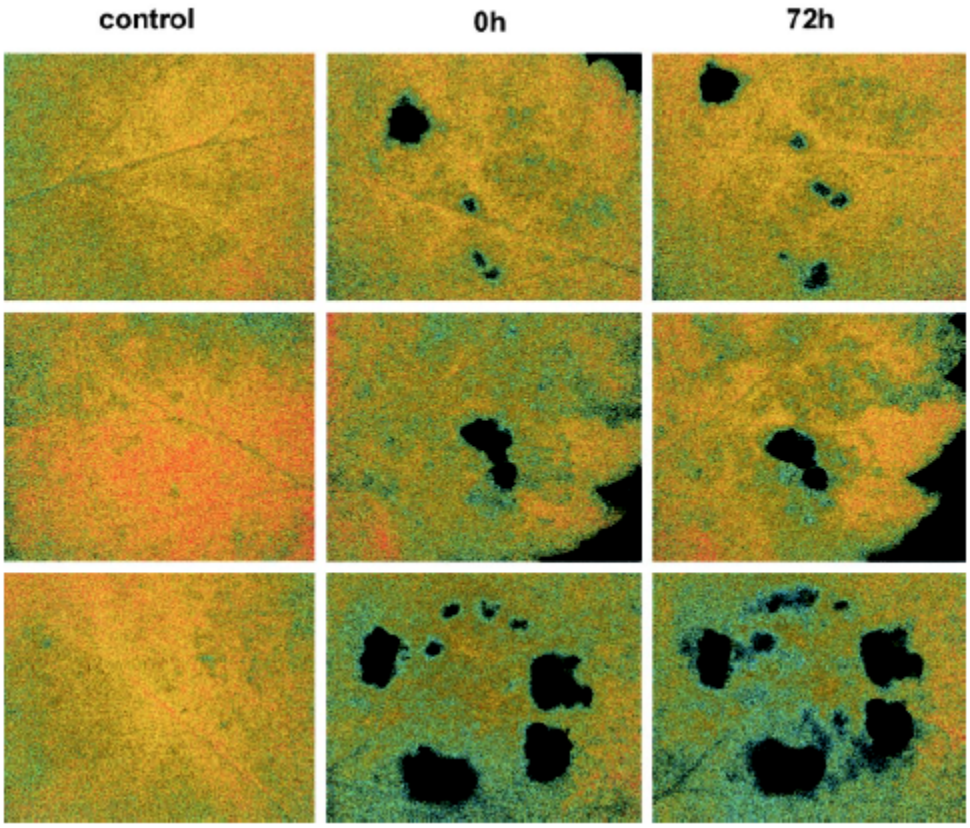

Figure 2.1.1. Patterns of photosynthesis in control leaflets and paired leaflets that were damaged by caterpillars. The three pairs of control (undamaged) and damaged leaflets are arranged from top to bottom. The measurements at times 0 and 72 hours postfeeding by the caterpillars are shown only for the damaged leaflet. The false coloring in this image corresponds to electron flux through photosystem II as follows: red = 0.55, yellow = 0.35, blue = 0.15, black = 0 [7].

The electron flux through photosystem II (chlorophyll *a* fluorescence) depends on the molecular environment of the chlorophyll molecule. We decided to use the lifetimes of the natural leaf fluorophores to record changes in the kinetics of electron transfer during photochemistry [3]. This additional information would be valuable in determining the pathway of electron flux in photosystem II, and give us information regarding the damage to the plant. Investigating whether the fluorophores exist with a variety of different lifetimes or if they maintain a constant lifetime could also provide information concerning the mechanism of changes in the physiology of the leaves. Also investigating lifetime change with folivory would shed light on the micro-environment of the reaction centers. We repeated similar experiments as conducted by A. Zangerl *et al.*; however, instead of using only the fluorescence intensity we measured also the fluorescence lifetime. We also wanted to investigate and compare punched holes (created by a syringe) and caterpillar holes.

2.2 Obtaining Fluorescence Lifetimes

When we send an intensity modulated (at frequency f) light beam into a system containing fluorophores, the fluorescence emission, which has a longer wavelength than the excitation wavelength, is modulated at the same frequency, f. However, the phase of the modulated fluorescence emission is delayed relative to the phase of the excitation light, and the amplitude of the dynamic signal change of the fluorescence relative to the long-time average (this ratio is called the modulation ratio) is less than the corresponding amplitude ratio of the excitation light. These variables are shown in Figure 2.2.1.

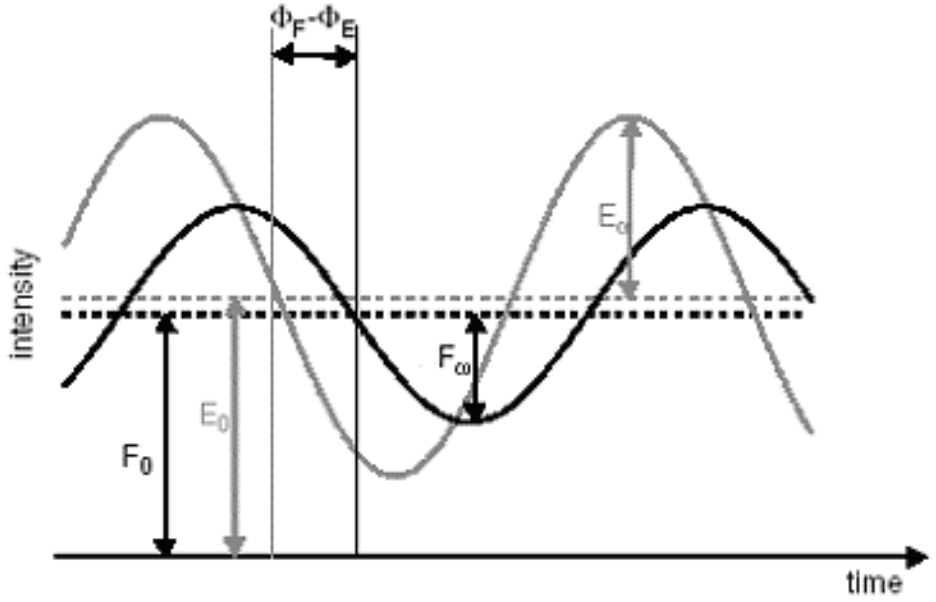

Figure 2.2.1. Time profiles of excitation light (—) and fluorescence emission (—). E_0 and F_0 indicate the amplitude of the DC components of the excitation and the fluorescence, respectively, while E_ω and F_ω represent half the amplitude of the modulated components. The phase shift between the two curves is also shown ($\varphi_F - \varphi_E$).

From this frequency phase and modulation data we extract several different pieces of information. One is the phase lifetime (τ_φ) and another is the modulation lifetime (τ_M). The modulation depth M is the amplitude of the sinusoidal component relative to the DC component (called the modulation ration above). For the excitation light $M_0 = E_\omega/E_0$ and for the fluorescence emission $M = F_\omega/F_0$. By using the change in modulation depth and the phase delay from the excitation to the emission, we can extract the time constants of the system.

$$\tau_\varphi = \frac{1}{\omega}\tan(\varphi - \varphi_0) \qquad (2.2.1)$$

and

$$\tau_M = \frac{1}{\omega}\sqrt{\left(\frac{M_0}{M}\right)^2 - 1}\,. \qquad (2.2.2)$$

If a system has a single lifetime, the phase and modulation lifetime are the same ($\tau_\varphi = \tau_M$). and they correspond to true values However, if, we have a several lifetimes in our system, then $\tau_\varphi < \tau_M$. In this case, in order to determine the true values of the lifetimes we would have to determine the phase and modulation at different frequencies to resolve the components [1].

2.2.1 Frequency domain theory: Homodyne and Heterodyne detection

There are two methods of obtaining fluorescence lifetime information. They are the time domain method and the frequency domain method (which is further divided into heterodyne and homodyne methods). The homodyne method measures the phase by comparing the intensities of two sinusoidal signals. By contrast, the heterodyne method measures phase by timing the arrival of zero crossings on a heterodyned sinusoidal signal. In homodyne detection, the modulation frequencies of the excitation light and the modulation of the detector (usually a photomultiplier or intensifier) are the same $\Delta\omega = 0$ and therefore the detected signal is constant in time. The quantities of $\Delta\varphi$ and M are obtained by changing the phase of the modulation of the detection device relative to that of the excitation light [8]. We use the homodyne method because it allowed us to use photon counting hardware for higher sensitivity. The heterodyne method modulates the detector at a different frequency that the excitation light (that is, the difference frequency is not zero) and the phase and modulation of the difference signal (the heterodyne signal) is analyzed. We use the homodyne method in our lifetime-resolved imaging instrument.

3 Experimental Setup and Method

3.1 Fluorescence Lifetime Imaging Microscopy (FLIM)

Fluorescence lifetime imaging microscopy (FLIM) is a valuable tool to study systems where the lifetime of the fluorophores provide us with additional information. Our discussion of the instrument is divided into two parts: the first consists of all the hardware components and the second is the software. The data acquisition programs used: Flish, Phaseview and Lifer [9] were written by Glen Redford. The hardware is assembled (by Glen Redford) as shown in the Figure 3.1.1:

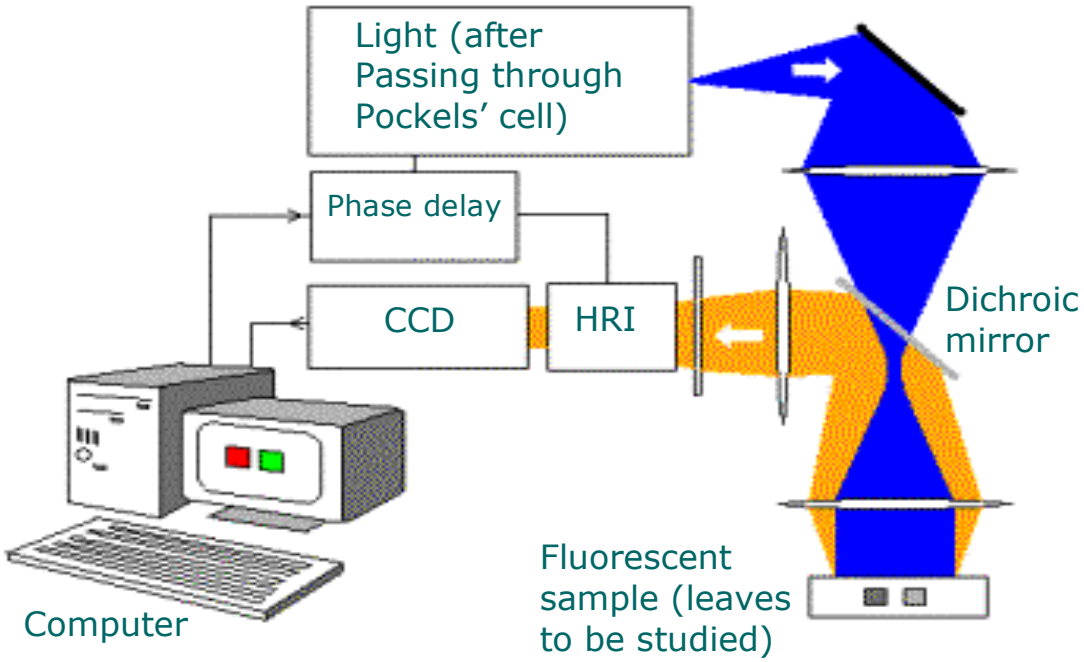

Figure 3.1.1. Schematic of the experimental setup. The CCD (charged coupled device) is used to capture the final image; the intensifier (HRI) used as the high frequency modulated image detection device (not to enhance the detection ability of the signal, which is the usual application of an image intensifier). The Dichroic mirror lets a narrow

band of light pass through and reflects all others. The Pockels' cell acts as a shutter to modulate the amplitude of the excitation light at a very high frequency, f (which is in the 100 MHz range).

The experiment works in this order:

Laser➔ Pockels' Cell ~~>Target ~~>Modulated Intensifier➔ Camera ➔ Computer
|_____Phase Shifter_____|

The laser light is modulated by a Pockels' cell at 100 MHz. The light illuminates the target. The target emits fluorescence with a different wavelength, but same modulated frequency as the excitation light. The emission light is then filtered out using a Dichroic and band-pass filter in the microscope. The fluorescence light is detected using an intensifier modulating its gain at the same frequency. The relative phase of the modulation between the Pockels' cell and the intensifier is changed for each image taken. These images form a series that gives a measured sine wave for each pixel. The phase and modulation of these sine waves are then calculated. A photograph of the experimental setup is shown in Figure 3.1.2.

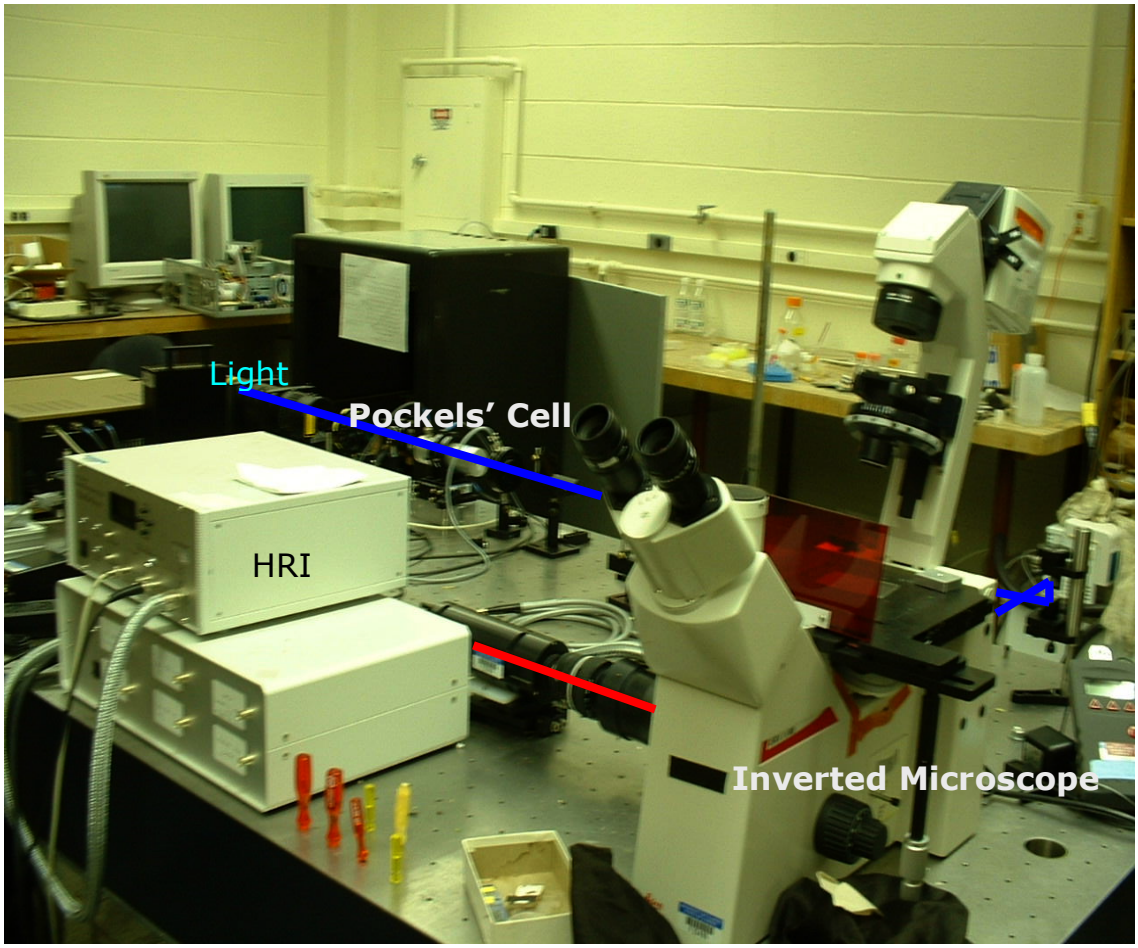

Figure 3.1.2. Experimental setup. As illustrated with the overlay, the excitation light has a shorter wavelength than that of the emission light (here the emission light is red because we looked at Chlorophyll *a* fluorescence).

3.2 Method

We excited the wild parsnip plant (Pastinaca sativa) with 488nm light (which is blue-green) and filtered out all colors other than red (Chl *a* emission). In the vegetative state, wild parsnip are rosettes [7] (a circular cluster of leaves that radiate from a center at or close to the ground) with several compound leaves. Previous studies have shown that sister leaflets are physiologically and chemically equivalent. We used one of the leaves

for testing and the other was used as a control. The plants were grown in a greenhouse under long days (16 hr light / 8 hr dark) at 27ºC from seeds collected from a wild population in Champaign County, IL.

The caterpillars used in this experiment were black swallowtails (Papilio polyxenes) and cabbage loopers (Trichoplusia ni). We used four caterpillars, each at third instar (a stage of an insect between molts). (See Figure 3.2.1)

Cabbage Loopers (Trichoplusia ni)

Black swallowtails (Papilio polyxenes)

Figure 3.2.1. Pictures showing the third instar of the caterpillars used in this experiment

The caterpillars were reared in the laboratory on wild parsnip plants and then placed inside a clip cage on the upper side of each leaflet. The cage had an internal diameter of 3 cm with nylon screening on the top and bottom. This is shown in Figure 3.2.2.

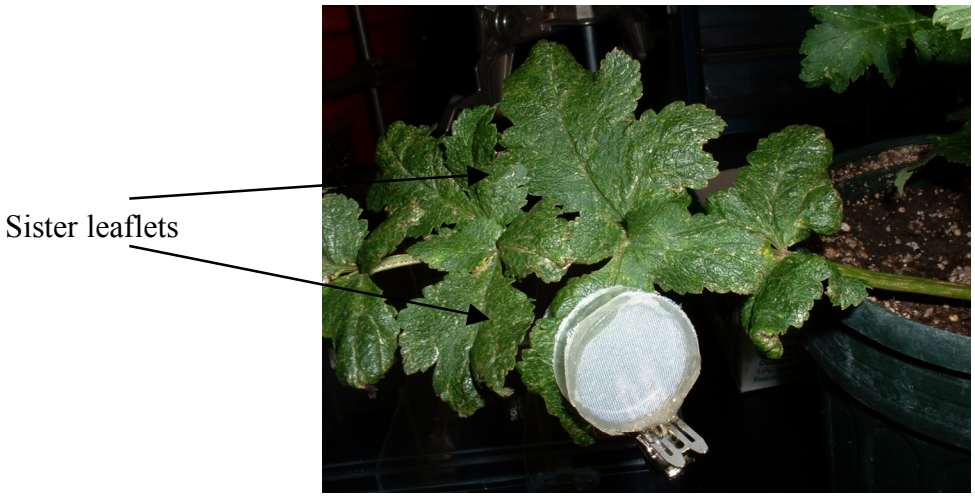

Figure 3.2.2. Wild parsnip (Pastinaca sativa) with a clip cage attached to a leaf.

In this part of the experiment we included two plants, studying both damaged and control leaflets. The caterpillars were permitted to feed for 24 hours before the first measurement, at which time the cage and the caterpillar were removed.

4 Results and Conclusions

4.1 Comparing Images

The following pictures (see Figure 4.1.1 and Figure 4.1.2) show phase lifetime superimposed with fluorescence intensity. The hue corresponds to lifetime and brightness corresponds to intensity. The intensity has been auto scaled (this is an intrinsic feature of the software that could be changed later if the need arises). The lifetimes, however, can be compared visually to one another.

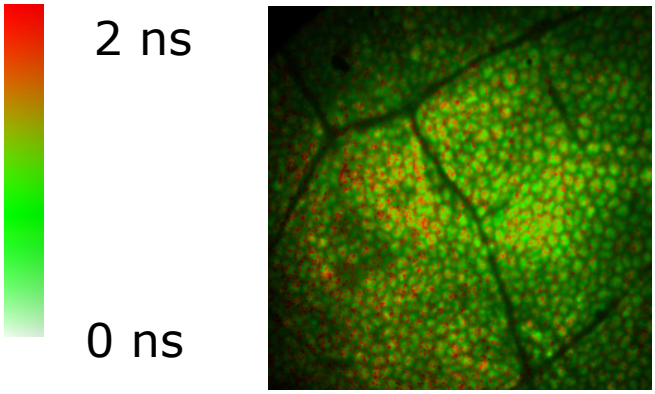

Figure 4.1.1 FLIM image of a healthy leaf. The scale on the right depicts the phase lifetime. Looking at the picture itself we see that the leaf is made up of a variety of lifetimes.

From Figure 4.1.1 we can see that in a healthy leaf, fluorescence intensity is almost evenly distributed (the brightness of one area of the picture is almost the same as other areas). The center of the picture appears slightly brighter than the corners and edges. This is probably because the laser did not illuminate the leaf uniformly.

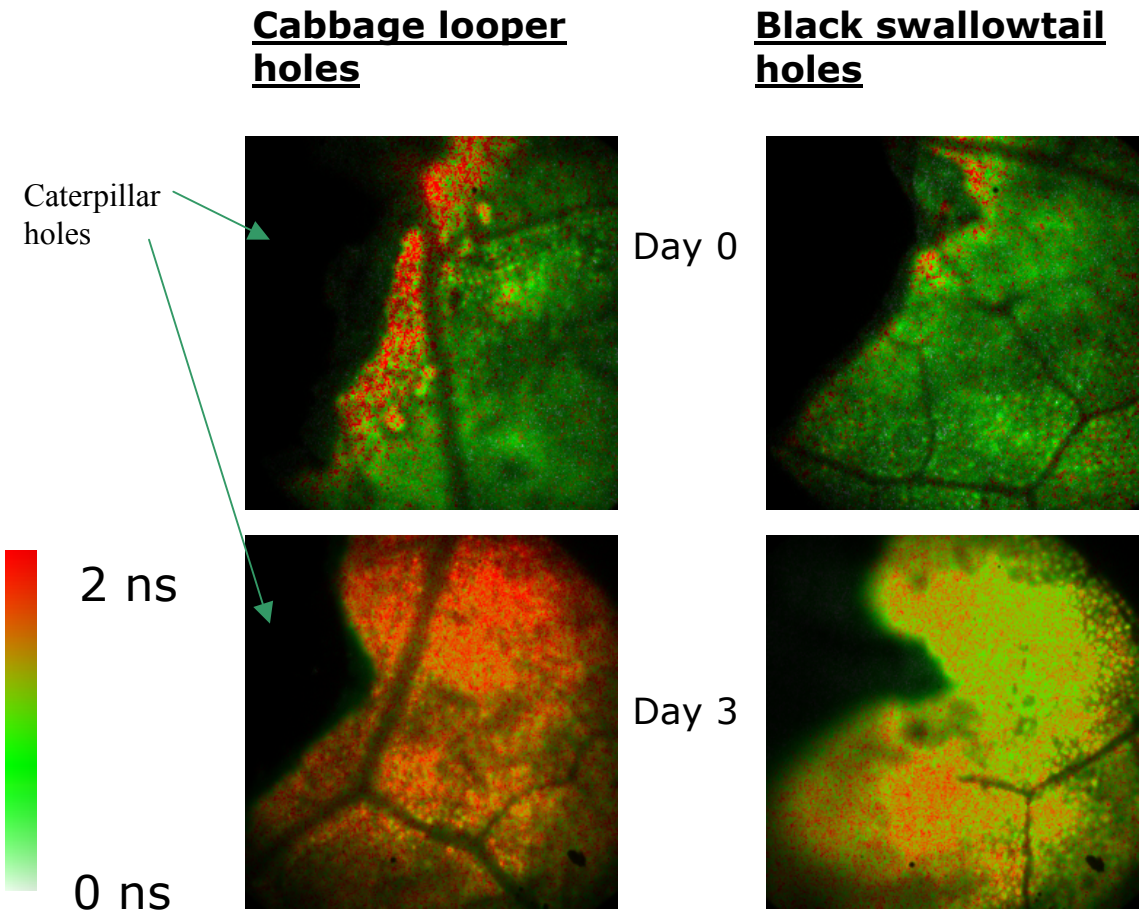

Figure 4.1.2. FLIM images from the two different caterpillars on the day we removed the caterpillars from the plants and three days after that.

Looking at Figure 4.1.2 we note that the area around biomass removal (around the caterpillar holes) has a bright ring of fluorescence intensity. Looking at the hue, which

corresponds to fluorescence lifetime, we find that the area around biomass removal has (on day 0) a ring of longer lifetime, about 2 ns in comparison the healthy leaf (shown in Figure 4.1.1) which has a lifetime of about 1.3 ns (this is true for both species of caterpillars). On day 3, the damage seems to spread further out (a greater area of the leaf has a longer lifetime).

In comparing the effect of the different species of caterpillars, we find that the cabbage loopers cause more damage to the reaction centers (there is a greater area around the hole that has longer lifetimes) in comparison to the black swallowtail caterpillars (this is shown by repeated tests). Wild parsnip is a plant that contains a number of furanocoumarins. Furanocoumarins are toxic compounds found primarily in species of the Apiaceae and Rutacea. They come in a variety of flavors and have adverse affects on a wide variety of organisms, ranging from bacteria to mammals. Some of the furanocoumarins are photoactive (as in the ones found in wild parsnip); their toxicity is enhanced in the presence of ultraviolet radiation [10]. Black swallowtail caterpillars are known to have an antioxidant enzyme that allows them to feed on plants that contain furanocoumarins. In fact, black swallowtail caterpillars almost exclusively feed on furanocoumarin-containing plants. Furanocoumarins are toxic to cabbage looper caterpillars unlike black swallowtail caterpillars. This fact might be correlated to the observation that cabbage looper caterpillars cause more damage to the leaves as opposed to the Black swallow tail caterpillars. It has been proposed that the Black swallow caterpillars have actually coevolved with wild parsnip, thereby causing less damage to the photo-reactive centers when they feed. In the experiment the black

swallowtail caterpillars caused more total damage (this could be attributed to the fact that the third instar of black swallowtail caterpillars are much bigger than the third instar of cabbage looper caterpillars), but less damage to surrounding tissue.

Another explanation for the differences seen between the holes caused by the two caterpillars could be that the cabbage loopers have toxic saliva that inflicts even more damage on the plant than the damage caused by biomass removal alone.

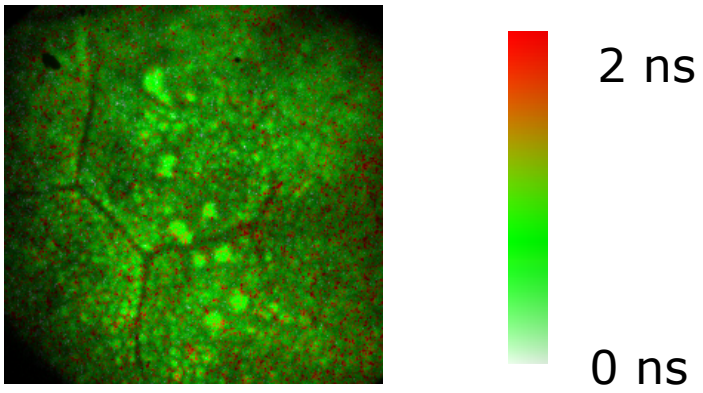

Figure 4.1.3 Cabbage looper crawling over the leaf shows that it may have left behind footsteps on the leaf

Figure 4.1.3 is a clear example as to why fluorescence lifetime data more accurately depicts the health of the leaf. In this part of the experiment, we allowed the Cabbage looper to crawl on the leaf and then we took the images. The spots where the Cabbage looper's feet covered the leaf have higher fluorescence intensity. This is could possibly be due to the fact that the caterpillar's feet actually covered that area on the leaf causing dark adaptation. Dark adaptation is what happens when the leaf is placed in darkness, because it is dark the leaves photo-reactive centers close down. When re-exposed to

light, the photo-reactive centers, due to inertia, undergo less photosynthesis and more fluorescence. That is why the footprints show up in the intensity. The footprints do not show up in fluorescence lifetime (there is no change of coloration / hue in those areas) because there has not been any physical damage to the plant and therefore the fluorophores have the same lifetime as that of a healthy leaf.

4.2 Dark Adaptation Time Test

In order to reconfirm the hypothesis stated above, we carried out another experiment to test the dark adaptation time of the leaf to see the effect of varying the amount of time the leaves remain in the dark on the lifetime of the leaves.

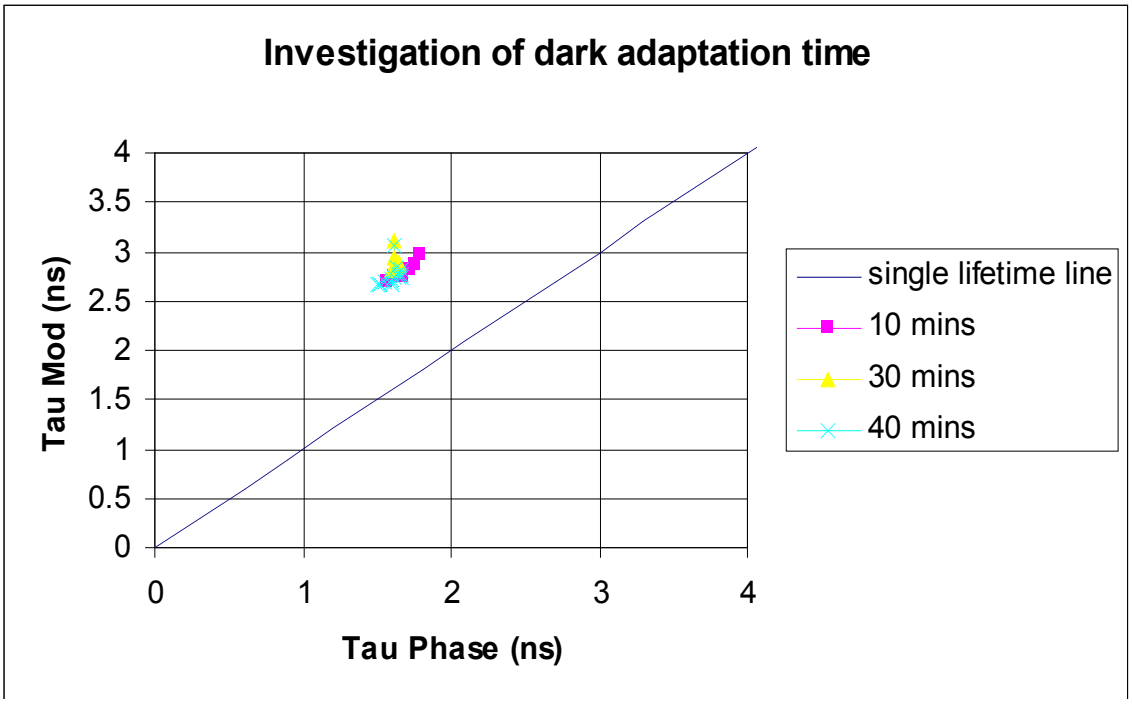

Figure 4.2.1. Data from dark adaptation time test. The lifetime results from 10 minutes, 30 minutes and 40 minutes dark adaptation time do not show much difference.

As shown in Figure 4.2.1, individual data points from the program are shown. The single lifetime line (following the equation y = x) shows the results of having only one

lifetime in our system ($\tau_\varphi = \tau_M$). The results show that the lifetimes at varying dark adaptation time remain very close. This observation validates the claim that the lifetime depicts the health of the leaf in comparison to the intensity, which relies on external factors.

4.3 Fluorescence transient curves

In the dark-adapted oxygen evolving system, the intensity of Chl *a* fluorescence emission shows a characteristic variation in time, known as the fluorescence transient or induction curve [11]. The kinetics of the fluorescence arise from the minimum yield F_0 to the maximum yield F_m [12]. (See Figure 4.3.1).

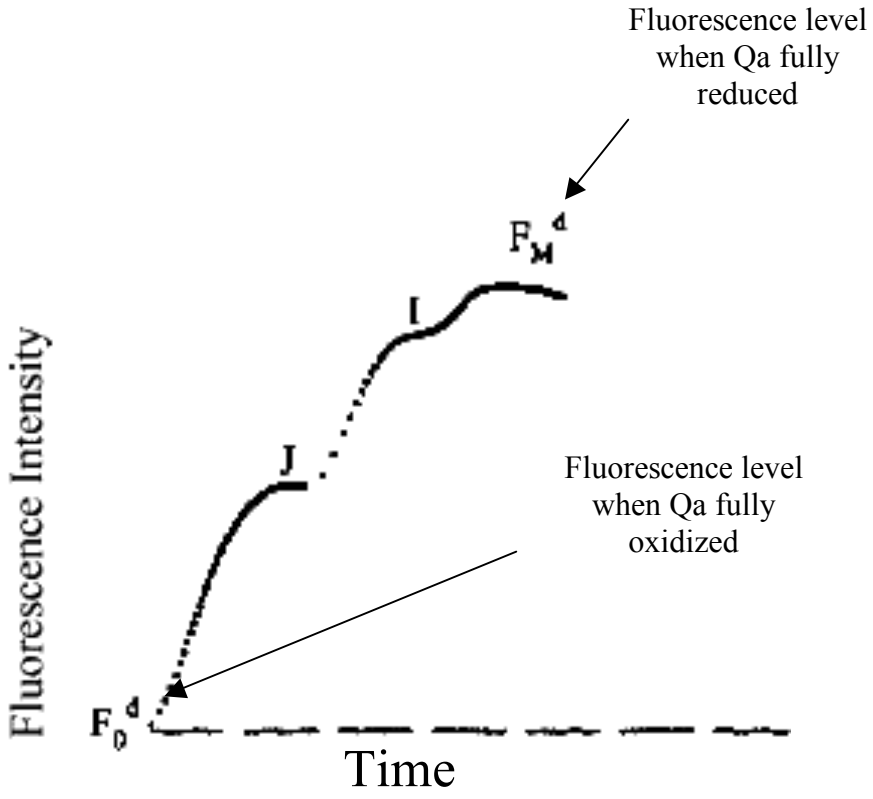

Figure 4.3.1. Fluorescence transient for chlorophyll *a* fluorescence over time.

The next experiment was aimed at comparing the lifetimes of the fluorophores in the leaf at different parts of the fluorescence transient. This was accomplished by modifying the data acquisition program to allow varying phase measurements with time.

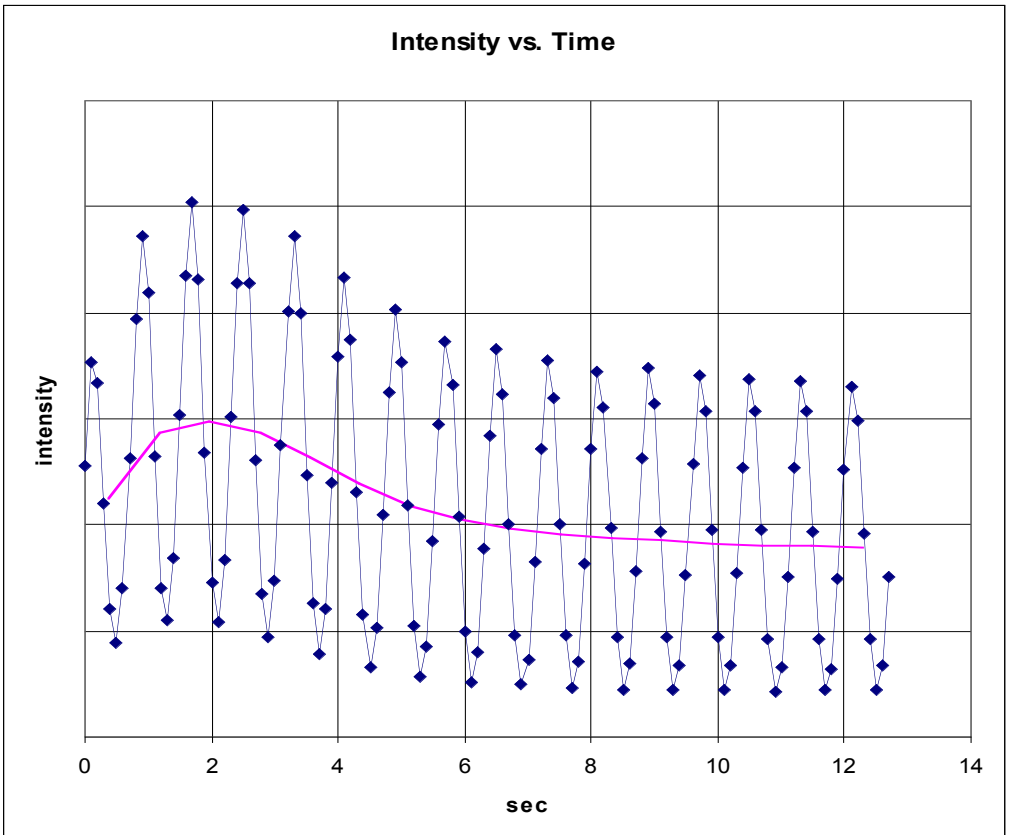

Figure 4.3.2 Experimental results from FLIM with a dark-adaptation time of 10 minutes. The blue curve is the actual data that contains the lifetime information. The pink curve is the average fluorescence intensity.

As one can notice in Figure 4.3.2, we can see the fluorescence transient curve in between 0 seconds and 6 seconds. After this range saturation of the intensity occurs. The lifetimes of the fluorophores are in the sinusoidal waves. We now need to extract the data to understand the evolution of the lifetime with respect to the fluorescent transient curve.

More experiments need to be carried out to compare the results found in this paper with mechanically punched holes. This is done to validate the claim that the damage spread is due to the caterpillars eating in a particular way rather than the damage to the plant by biomass removal.

6 Acknowledgments

I have used many resources to understand the biophysics, plant biology and entomology that has been used in these experiments. This work would not have been possible without the guidance of Professor Robert Clegg, Professor Govindjee, Professor May Berenbaum, Dr. Art Zangerl and Glen Redford. Glen Redford, a graduate student in Professor. Clegg's group was a valuable resource and great teacher. The data acquisition program: Flish, analysis programs: Phaseview and Lifer, and image rendering program: Dyeit were all written by G. Redford. I would also like to thank Bridget O'Neill for supplying me with the caterpillars and plants. Lastly, I would like to thank the National Science Foundation for funding this work and the REU program. This work has been supported by the National science foundation under Grant No. PHY-0243675. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author and do not necessarily reflect the views of the National Science Foundation.

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