FLUORESCENCE LIFETIME IMAGES OF DYE MOLECULES

by

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ABSTRACT

FLUORESCENCE LIFETIME IMAGES OF DYE MOLECULES

In this thesis, Fluorescence Lifetime Images of perylene and bodipy dye molecules mixture are studied using a time-correlated single photon-counting technique. Lifetimes of dye molecules are calculated pixel-by-pixel from these time-resolved images, and the spatial variations of the lifetimes are then displayed in a pseudocolor format. Three images with resolutions 77 x 39 μm^2, 9.7 x 9.7 μm^2 and 7.7 x 7.7 μm^2 are obtained, lifetimes are changing from 4.78 ns to 5.38 ns, from 4.78 ns to 4.98 ns and from 4.78 ns to 4.98 ns, respectively.

ÖZET

BOYA MOLEKÜLLERİNİN FLORESANS YARI ÖMÜR GÖRÜNTÜLERİ

Bu tezde, zaman korelasyonlu tek foton sayma tekniği kullanılarak perylene ve bodipy boya molekülleri karışımının Floresans Yarıömür görüntüleri çalışılmıştır. Boya moleküllerinin yarıömürleri bu zaman korelasyonlu gorüntülerden her piksel için hesaplandı; ve yarıömurlerin konumlarındaki değişimler daha sonra psödo renk formatında gösterildi. Çözünürlükleri 77 x 39 μm^2, 9.7 x 9.7 μm^2 ve 7.7 x 7.7 μm^2 olan sırsıyla yarıömürleri 4.78 ns'den 5.38 ns'ye, 4.78 ns'den 4.98 ns'ye ve 4.78 ns'den 4.98 ns'e değişen üç görüntü elde edildi.

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LIST OF SYMBOLS/ABBREVIATIONS

I	Fluorescence intensity
I_0	Initial intensity
k_{nr}	Nonradiative decay rate
$n(t)$	Number of excited molecules at time t
n_0	Initial number of fluorophores
Γ	Emissive rate
τ	Lifetime
ϕ	Phase shift
$FLIM$	Fluorescence lifetime imaging microscopy
$FRET$	Fluorescence resonance energy transfer
$IUPAC$	International Union of Pure and Applied Chemistry
MRI	Magnetic resonance imaging
PDL	Picosecond diode laser
PMT	Photomultiplier tube
TAC	Time-to-amplitude converter
$TCSPC$	Time-correlated single photon counting
$TTTR$	Time-tagged time resolved mode

1. INTRODUCTION

Fluorescence lifetime imaging microscopy (FLIM) allows the lifetimes of one or more fluorophores to be spatially resolved and can be used to provide information about the state of the fluorescent species and their immediate molecular environment. The fluorescence lifetime is sensitive to environmental conditions such as pH, and excited state reactions such as fluorescence resonance energy transfer (FRET) or collisional quenching, properties that have been exploited for resolving physiological parameters in the cell [1].

The information from a steady-state scan (a plot of fluorescence intensity versus wavelength) represents the averaged behavior of what occurs during the entire scan. Fluorescence itself, however, occurs on the nanosecond timescale. Therefore, if one could take snapshots at that speed, he/she would learn much more about the mechanisms that lead to chemical or biochemical processes hence the appeal of lifetime spectrofluorometers. For example, among the experiments possible with lifetime instruments include: determination of the environment that the sample molecules inhabit, e.g., viscosity, pH, temperature, polarity, salvation, etc [2]; uncovering the size and shape of the sample molecules, and the distances between different parts of the molecules; learning about the contributions of each component in a mixture of sample molecules, through time-resolved spectra of overlapping emissions.

Fluorescence is also a powerful tool for investigating the structure and dynamics of matter or living systems at a molecular or supramolecular level. Polymers, solutions of surfactants, solid surfaces, biological membranes, proteins, nucleic acids and living cells are well-known examples of systems in which estimates of local parameters such as polarity, fluidity, order, molecular mobility and electrical potential is possible by means of fluorescent molecules playing the role of probes [3].

The chapters in the thesis are ordered as follows: In Chapter 2, the theoretical background of fluorescence process and fluorescence lifetime is given. Also, time-

correlated single photon-counting (TCSPC) technique, time-tagged time resolved mode (TTTR), confocal microscopy are presented. Lastly, the imaging is explained. In Chapter 3, sample preparation and optical setup for FLIM and FLIM results of dye molecules are given. Finally, these results are discussed.

2. FLUORESCENCE LIFETIME IMAGING MICROSCOPY

2.1. Luminescence

Luminescence is the emission of ultraviolet, visible or infrared light from the electronically excited states. The root of the word "luminescence" is the Latin word "lumen" (light). It was used firstly by the physicist Eilhardt Wiedemann in 1888 as *luminescenz* meaning that 'all those phenomena of light which are not solely conditioned by the rise in temperature'. Different excitation modes correspond to different types of luminescence (Table 2.1) [3]. If the excitation is occurred with the absorption of light, the process is called photoluminescence. Once a molecule is excited to an electronic state by absorbtion of a photon, it can return to the ground state with emission of light, which is one of the possible physical effects resulting from interaction of light with matter, as shown in Figure 2.1.

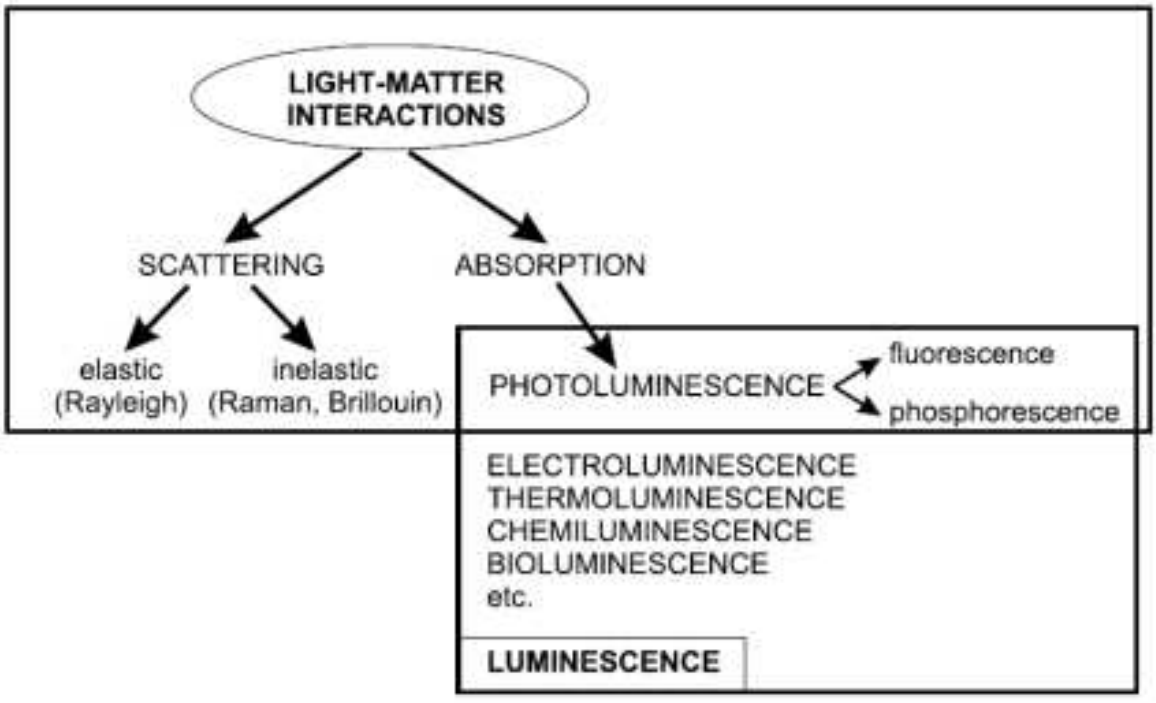

Figure 2.1. Position of fluorescence and phosphorescence in the frame of light-matter interactions.

Fluorescence and phosphorescence are the two types of photoluminescence, depending on the nature of the excited state (Table 2.1) [3]. Speaking for the fluorescence case which takes place in excited singlet states, the spin of the electron in the excited state is the opposite of the spin of the electron in the ground-state. Therefore, the electron turns back to the ground state emitting a photon in approximately 10 ns time, which is the lifetime for a fluorophore representing the average time between its excitation and return to the ground state. Observing the lifetime of fluorescence can only be made with advanced optic and electronic devices due to the short lifetime. Secondly, in the phosphorescence case, which takes place in excited triplet states, the spin of the electron in the excited state is identical with the spin of the electron in the ground-state leading to the delay of transition to the ground state. Thus, the lifetime of phosphorescence ranges between milliseconds and seconds.

Table 2.1. Various types of luminescence

Phenomenon	**Mode of excitation**
Photoluminescence (fluorescence, phosphorescence)	Absorption of light(photons)
Radioluminescence	Ionizing radiation X-rays,(α, β, γ)
Cathodoluminescence	Cthode rays (electron beams)
Electroluminescence	Electric field
Thermoluminescence	Heating after prior storage of energy (e.g. radioactive irradiation)
Chemiluminescence	Chemical process (e.g. oxidation)
Bioluminescence	Biochemical process
Triboluminescence	Frictional and electrostatic forces
Sonoluminescence	Ultrasounds

2.2. Jablonski Diagram

Jablonski Diagrams, which are named after Professor Alexander Jablonski, demonstrate the processes that occur between the absorption and emission of light. A typical

Jablonski diagram shown in Figure 2.2 [4] represents the electronic states where S_0, S_1, and S_2 are the singlet ground, first, and second electronic states, respectively. These electronic states include some vibrational levels numbered like 0, 1, 2, etc. Vertical lines show the transitions between states. An upward arrow means that the atom absorbed light and a downward arrow means that light is emitted from the atom.

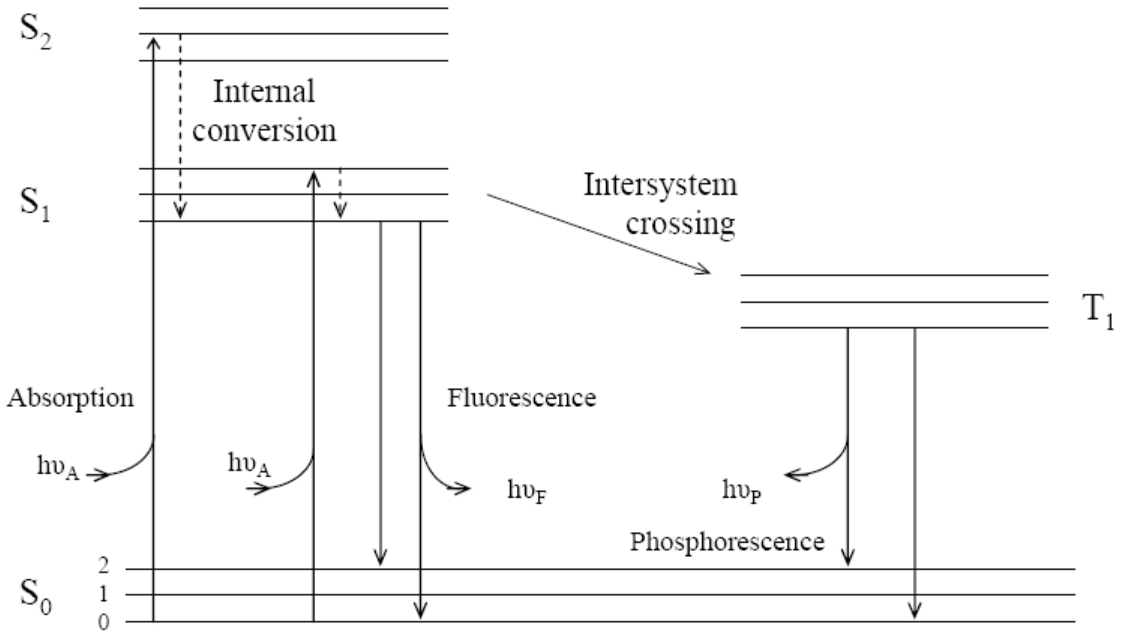

Figure 2.2. One form of a Jablonski diagram.

After the absorption of light, there occurs different processes. The electrons are excited to S_1 or S_2 states however in most cases the electron in S_2 state quickly passes to vibrational S_1 state. This process is called internal conversion and generally occurs within 10^{-12} s which is less than typical fluorescence lifetime, 10^{-8} s. After turning back to S_1 state, the electrons can follow two steps. Firstly, the electron can directly pass to a higher excited vibrational ground state at the level of S_0 state and emits a photon. This is the fluorescence process. Secondly, instead of immediately turning back to S_0 state, it goes to the first triplet state T_1 providing a spin conversion and turns back to the S_0 state. This transition from S_1 to T_1 is called intersystem crossing. Emission from T_1 is called phosphorescence, and phosphorescence produces longer wavelengths compared to the fluorescence.

2.3. Steady-State and Time-Resolved Fluorescence

Fluorescence can be split into two types of measurements, steady-state and time-resolved. The most common type was the steady-state measurement. In steady-state measurements, the sample is exposed to a beam of light continuously, without any break and the data of intensity and the spectrum of emission is recorded continuously. On the other hand, the second type of measurement which is time-resolved measurement observes the intensity decays. In that method, a pulse of light is subjected to the sample and the intensity decay is recorded. A high-speed detection system is required for the detection of the decay which happens in ns time scale. Note that a steadystate observation is simply an average of the time-resolved phenomena over the intensity decay of the sample. The advantage of using time-resolved measurement instead of steady-state is avoiding the overlaps which occurrs during the absorbtion or emission in steady-state measurement [4].

2.3.1. Time-Domain and Frequency-Domain Measurements

Two time-resolved techniques, time-domain fluorometry and frequency-domain fluorometry, are commonly used to record the data of lifetimes and to observe the δ-pulse response of a fluorescent sample. Time-domain fluorometry uses a short exciting pulse of light and measures the δ-pulse response of the sample. Frequency-domain fluorometry uses modulated light at variable frequency and gives the harmonic response of the sample, which is the Fourier transform of the δ-pulse response. The methods of time-domain and frequency-domain fluorometries are the same however the instruments they use are different. In time-domain fluorometry, the sample is excited with a pulse of light (Figure 2.3(a)). The intensity width must be much shorter compared to the decay time, τ. The time dependent intensity is measured just after the excitation pulse, and the slope of a plot of $I(t)$ versus t gives us the decay time. In addition, the decay time can also be found from the time at which the intensity decreases to $1/e$ of the intensity at $t = 0$.

Another method used in time-resolved fluorometry is the frequency-domain method.

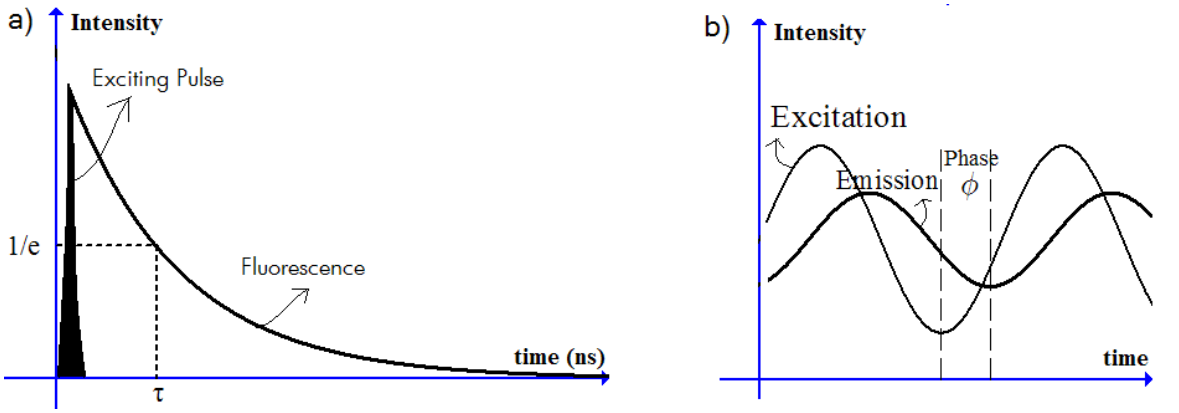

Figure 2.3. a)Pulse or time-domain lifetime measurements. b)Phase-modulation or frequency domain lifetime measurements.

In this case, the sample is excited with intensity-modulated light, typically sine-wave modulation provided that the frequency of emission is the same with the frequency of excitation (Figure 2.3(a)). There occurs a phase shift because of the lifetime of the fluorophore, (ϕ). Consequently, the lifetime is calculated using the phase shift [4].

2.3.2. Meaning of the Lifetime or Decay Time

In the previous sections, the meaning of lifetime was briefly described as the time passing between absorbtion and emission. At this point, it is more useful to give a detailed information about lifetime, τ. When a fluorophore is excited, the initial number of fluorophores, (n_0) is increased and the rate of change of excited fluorophores [4] is given with the formula

$$\frac{dn(t)}{dt} = -(\Gamma + k_{nr})n(t) \tag{2.1}$$

where $n(t)$ is the number of excited molecules at time t, Γ is the emissive rate, and k_{nr} is the nonradiative decay rate. Solving the equation (2.1) gives the number of excited molecules in the exponential form

$$n(t) = n_0 e^{-t/\tau} \tag{2.2}$$

We note that, instead of measuring the number of excited molecules, $n(t)$, we measure the fluorescence intensity, which is proportional to $n(t)$. Therefore, if we rewrite equation (2.2) we obtain

$$I(t) = I_0 e^{-t/\tau} \tag{2.3}$$

where I_0 is the initial intensity. The lifetime τ is the inverse of the total decay rate, $\tau = (\Gamma + k_{nr})^{-1}$.

In order to show that the lifetime is the average amount of time between the absorbtion and emission of the fluorophore we must calculate the average time, $< t >$, in the excited state and it is found by averaging t over the intensity decay of the fluorophore:

$$< t >= \frac{\int_0^\infty tI(t)dt}{\int_0^\infty I(t)dt} = \frac{\int_0^\infty texp(-t/\tau)dt}{\int_0^\infty exp(-t/\tau)dt} \tag{2.4}$$

The denominator is equal to τ and the numerator is equal to τ^2. Hence the lifetime equals the average time for a single exponential decay:

$$< t >= \tau. \tag{2.5}$$

2.4. Time-Correlated Single Photon Counting

Time-correlated single photon counting (TCSPC) is the best way for fluorescence lifetime measurements in the time domain since this method can record electron dynamics on extremely short time scales (nanoseconds). The basic concept is the analyzing the transition from an excited state to a lower energy state. After the simultaneous excitation by a pulse of laser, molecules do not emit the photons simultaneously. It takes a certain rate not a specific time. A graph (intensity vs. time) is drawn by recording the relaxation duration of the photons with these data points. This graph displays the exponential decay curve typical to these processes. Because of the difficulty

of observing simultaneously multiple molecules, individual excitation-relaxation events are recorded and then averaged to generate the curve. The pulsed laser beam splits into two ways. One travels to a photomultiplier tube (PMT) and the other one travels through the sample. The pulse which detected by a photomultiplier tube, activates a time-to-amplitude converter (TAC) circuit. This circuit begins to build a charge on a capacitor which will only be discharged once the PMT sends another electrical pulse to the circuit. This electrical pulse comes after the second laser pulse excites the molecule to a higher energy state, and a photon is eventually emitted from a single molecule upon returning to its original state. Thus, the longer a molecule takes to emit a photon, the higher the voltage of the resulting pulse. The central concept of this method is that only a single photon is needed to discharge the capacitor. After that, a calibrated computer converts the voltage sent out by the TAC into a time and records the event in a histogram of time since excitation. Lastly, a decay curve can be analyzed to find out the decay rate of the event [5].

2.5. Time-Tagged Time-Resolved Mode

The desired capturing of the complete fluorescence dynamics can be achieved by recording the arrival times of all photons relative to the beginning of the experiment (time tag), in addition to the picosecond TCSPC timing relative to the excitation pulses. This is called Time-Tagged Time-Resolved (TTTR) mode [6]. Figure 2.4 shows the relationship of the time figures involved. As in conventional TCSPC, a picosecond

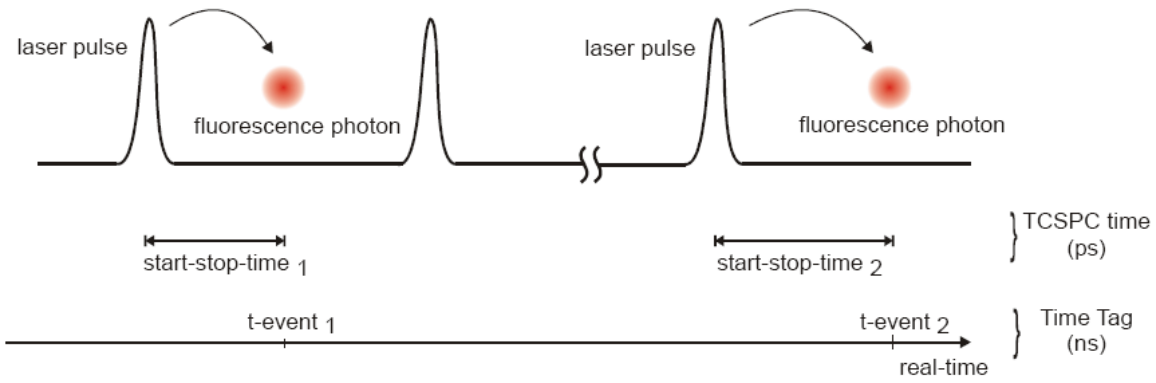

Figure 2.4. Timing figures in TTTR data acquisition.

timing between laser pulse and fluorescence photon is obtained. In addition to that, a coarser timing is performed on each photon with respect to the start of the experiment. This is done with a digital counter running at typically 50 or 100 ns resolution. Even though this is much higher than what most applications mentioned above would require, modern hardware provides this resolution at no extra cost. Since the TCSPC timing typically covers the time scale just below 100 ns, it is indeed sensible to chose a time tag resolution just above that range, thereby covering the whole time range for ultimate flexibility in further data analysis. This makes possible to reconstruct the 2D image from the stream of TTTR records, since the relevant XY position of the Piezo-scanner can be determined during the data analysis. The image size is virtually unlimited. Restrictions arise only form hard disk and/or computer memory size, which is not a practical limitation with modern computers [7].

2.6. Confocal Microscopy

Confocal microscopy is an optical imaging technique used to increase micrograph contrast and/or to reconstruct three-dimensional images by using a spatial pinhole to eliminate out-of-focus light or flare in specimens that are thicker than the focal plane [8]. This technique has gained popularity in the scientific and industrial communities. Typical applications include life sciences and semiconductor inspection. The principle of confocal imaging was patented by Marvin Minsky in 1957 [9]. In a conventional (i.e., wide-field) fluorescence microscope, the entire specimen is flooded in light from a light source. Due to the conservation of light intensity transportation, all parts of the specimen throughout the optical path will be excited and the fluorescence detected by a photodetector or a camera. In contrast, a confocal microscope uses point illumination and a pinhole in an optically conjugate plane in front of the detector to eliminate out-of-focus information. Only the light within the focal plane can be detected, so the image quality is much better than that of wide-field images. As only one point is illuminated at a time in confocal microscopy, 2D or 3D imaging requires scanning over a regular raster (i.e. a rectangular pattern of parallel scanning lines) in the specimen. The thickness of the focal plane is defined mostly by the inverse of the square of the numerical aperture of the objective lens, and also by the optical properties of the

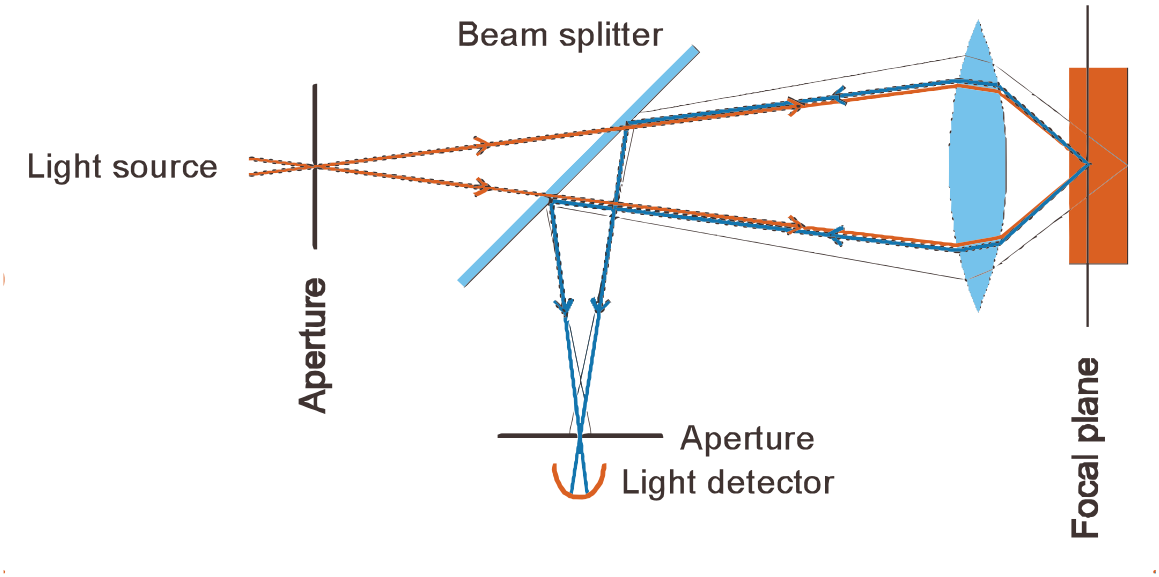

Figure 2.5. Principle of confocal microscopy.

specimen and the ambient index of refraction. These microscopes also are able to see into the image by taking images at different depths.

2.7. Fluorescence Lifetime Imaging Microscopy

Fluorescence lifetime imaging microscopy (FLIM) is a technique to obtain an image which shows the lifetimes of each pixel as a third dimension. Flourescence lifetime is the contrast parameter that is represented on the false color scale.

The fluorescence lifetime is dependent upon excited state reactions but independent upon intensity variations therefore it is also independent of the local concentration of fluorophores, the optical path of the microscope, the local excitation light intensity, or on the local fluorescence detection efficiency.

Fluorescent lifetimes are sensitive to polarity, pH, oxygen tension and other environmental factors. The fluorescence lifetime (τ) depends on both the radiative decay rate (Γ) and nonradiative decay rate (k). Via radiative decay rate, the fluorescence lifetime is useful to contrast different fluorophore species and via k, it is useful to contrast different local fluorophore environments. FLIM thus contains information about the

environment of the fluorophore molecules as well as their location. FLIM also provides to study the dynamics of the environment such as fluidity. In addition, FLIM is useful to examine protein interaction inside cells, dynamics of certain proteins.

The usual method to get a FLIM is scanning method when the lifetime is measured at discreate location. The spatial resolution of a florescence lifetime image is usually 256 x 256 and it is measured using a microscope.

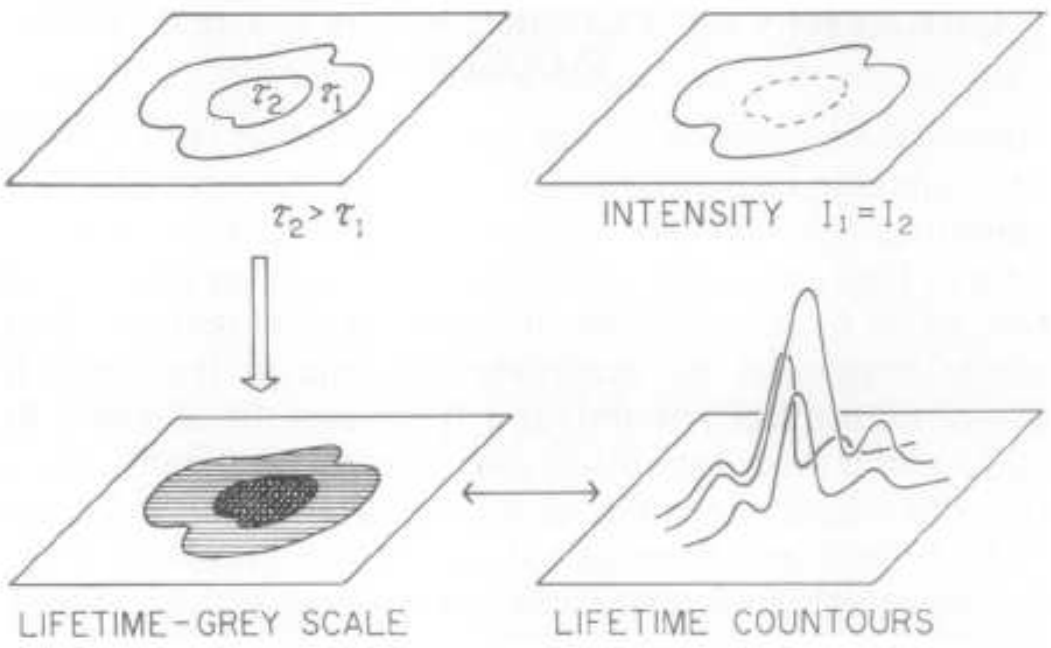

Figure 2.6. Principle of FLIM.

The concept of fluorescence lifetime imaging (FLIM) is illustrated in Figure 2.4 [10]. Suppose the sample is composed of two regions, each with an equal fluorescence intensity. Assume further that the lifetime of the probe is several-fold higher in the central region of the object (τ_2) as compared with the larger outer region (τ_1). The longer lifetime in the central region could be due to the presence of a chemical species that increases the probe lifetime because of binding of the probe to a macromolecule or other environmental factors. The intensities of the central and outer regions could be equal because of dye exclusion or other mechanisms. Observation of the intensity image (Figure 2.4 Upper Right) will not reveal the different environments in regions 1

and 2. However, if the lifetimes were measured, then the distinct environments would be detected. The FLIM method allows direct visualization of the spatially dependent decay times, which can be presented on a grey or color scale (Figure 2.4 Lower Left) or as a three-dimensional (3D) projection in which the height represents the local decay time (Figure 2.4 Lower Right). The concept of FLIM is an optical analogue of magnetic resonance imaging (MRI), in which the proton relaxation times at each location are used to create contrast in the calculated image [10].

3. EXPERIMENTAL WORK AND RESULTS

3.1. Sample Preparation

3.1.1. Perylene as a fluorescent probe

In fluorescence experiments, aromatic molecules are generally used as fluorescent probes [11]. Polynuclear aromatic hydrocarbons, such as anthracane and perylene, are also fluorescent, and the emission from such species is used for environmental monitoring of oil pollution [4]. We have used perylene as a fluorescent molecule in our experiments. Perylene, $C_{20}H_{12}$ is the member of a type of polynuclear aromatic hydro-

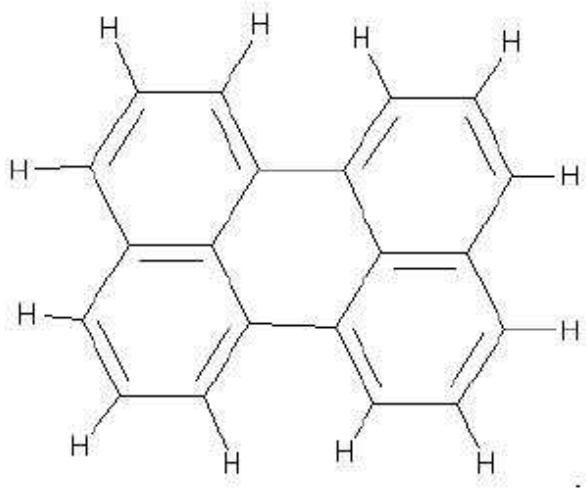

Figure 3.1. Chemical Structure of Perylene.

carbon which is different from naphthalene, anthhrocene, pyrene, dibenzanthracene, benzperylene, coronene, and ovalene [12]. The chemical structure of perylene is shown in Figure 3.1 [3]. In the experiment we put perylene in gel which is a solid. Gels are defined as a substantially dilute crosslinked system, which exhibits no flow when in the steady-state [13]. By weight, gels are mostly liquid, yet they behave like solids due to a three-dimensional crosslinked network within the liquid. It is the crosslinks within the fluid that give a gel its structure (hardness) and contribute to stickiness (tack). A solid three-dimensional network spans the volume of a liquid medium. This internal network

structure may result from physical or chemical bonds, as well as crystallites or other junctions that remain intact within the extending fluid. Virtually any fluid can be used as an extender including water (hydrogels), oil, and air (aerogel). Both by weight and volume, gels are mostly liquid in composition and thus exhibit densities similar to those of their constituent liquids. Jell-O is a common example of a hydrogel and has approximately the density of water. Hydrogel (also called Aquagel) is a network of polymer chains that are water-insoluble, sometimes found as a colloidal gel in which water is the dispersion medium. Hydrogels are highly absorbent (they can contain over 99 % water) natural or synthetic polymers. Hydrogels also possess a degree of flexibility very similar to natural tissue, due to their significant water content.

3.1.2. Bodipy as a fluorescent probe

BODIPY, short for boron-dipyrromethene, is a class of fluorescent dyes. It is comprised of dipyrromethene complexed with a disubstituted boron atom, typically a BF2 unit. The IUPAC (International Union of Pure and Applied Chemistry) name for the BODIPY core is 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene. This compound, representing the unsubstituted BODIPY, has been prepared only recently.

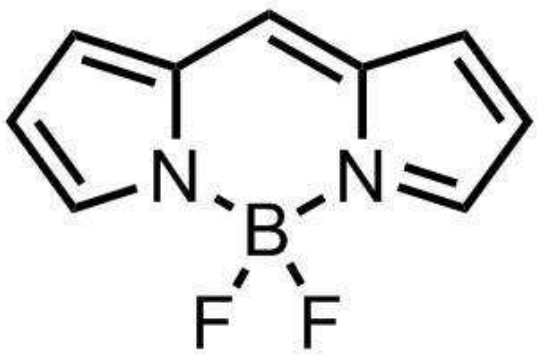

Figure 3.2. Chemical Structure of Bodipy.

BODIPY dyes are notable for their uniquely small Stokes shift, high, environment-independent quantum yields, often approaching 100 % even in water, and sharp ex-

citation and emission peaks contributing to overall brightness. The combination of these qualities makes BODIPY fluorophore an important tool in a variety of imaging applications. The position of the absorption and emission bands remain almost unchanged in solvents of different polarity as the dipole moment and transition dipole are orthogonal to each other. In the experiment Bodipy dyes are on the hydrogel perylene mixture which is shown in Figure 3.3 [14]-[16].

Figure 3.3. Picture of the sample

3.2. Optical Setup

3.2.1. Optical Setup for Fluorescence Lifetime Measurements

The experimental setup for FLIM measurements is shown in Figure 3.4. In this setup, the picosecond diode laser (PDL 800-B), which is triggered by its internal oscillator (settable at 2.5, 5, 10, 20 and 40 MHz), is used as the excitation source. Single mode optical fiber is used to obtain a Gaussian beam profile. Another instrument which is used to focus the excitation beam onto the sample is a microscope objective (Nikon 50X). After excitation, the fluorescent sample emits light at a longer wavelength than that of the excitation light. A photon dedector PMT is used with TimeHarp 200 which is a compact user friendly TCSPC system on a single PCI board. After the

excitation of sample with same picosecond diode laser(PDL 800-B), emission from the sample is collected by means of the microscope objective. The other essential equipment in this setup is the dichroic mirror, which is mounted at a 45^o angle with respect to the excitation light to filter out the fluorescence emission from the scattered excitation light. The fluorescence light is directed to the photon dedector(PMT) via some appropriate collection optics (microscope objective or a lens) [11]. Before the lens, a pinhole (Confocal Aperture) is mounted to eliminate out-of-focus information. Also, to scan the sample an xyz positioner , TRITOR101, and its amplifier NV 40/3 CLE is connected to the board, TimeHarp 200, via Two-channel piezo control voltage driver, SCX200. Finally, the SymPhoTime software (from PicoQuant) allows to directly image the average fluorescence lifetime already during the scanning process.

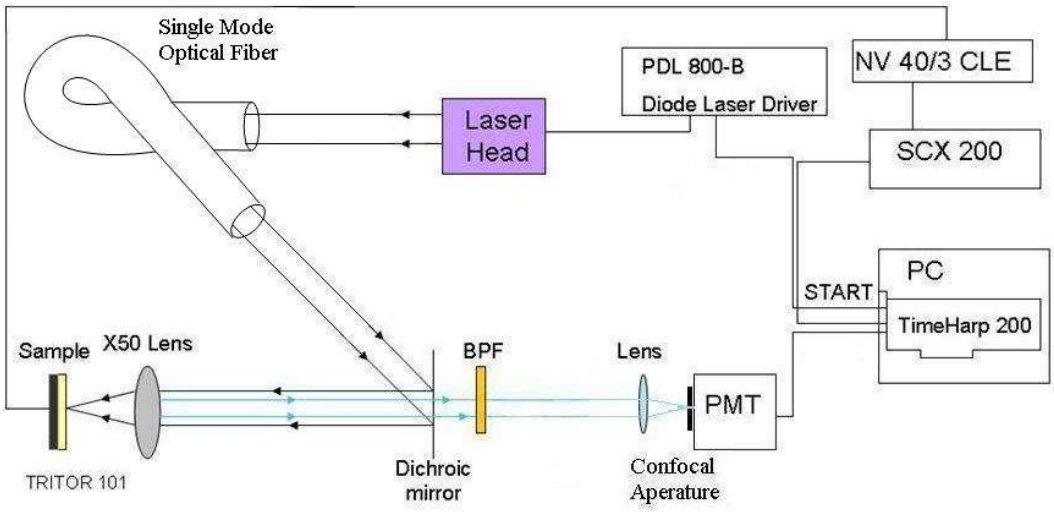

Figure 3.4. Optical setup.

3.3. Results

The fluorescence lifetime images of perylene and bodipy dye molecules are obtained using the optical setup shown in Figure 3.4. The fluorescence lifetime is determined using TimeHarp 200, which is a compact easy-to-use TCSPC system on a single

PCI board. The time-tagged time-resolved (TTTR) mode should be activated to get the image. In TTTR, every detected photon is tagged with two times, the macroscopic arrival time and the picosecond TCSPC time. Both times are directly stored on the PCs hard disc. As in the timetagged mode, TTTR mode preserves the complete macroscopic temporal resolution of the data acquisition board, and generates data flow only when a photon is detected [17]. Also the fluorescence lifetime imaging controller SCX 200 should control the devices. SCX200 is an accessory for the TimeHarp 200 time-correlated single photon counting board. The product allows us to connect a high resolution 2-D piezo scanner and collect fluorescence lifetime data synchronous with high speed scanning. Scan and data acquisition in Time-Tagged Time Resolved (TTTR) mode are driven from the same 100 nanosecond clock, while at the same time picosecond timing is performed on laser induced fluorescence photons. This allows to acquire fluorescence lifetime images with picosecond lifetime resolution and nanometer spatial resolution. Knowing the exact correlation between detected photons and image position, the TCSPC times of the photons can be used for calculating lifetime images [18]. In this study, the lifetime of an image pixel was simply calculated as the average lifetime of all the photons falling into that pixel, taking into account only photons arriving within a time window after the exciting laser pulse.

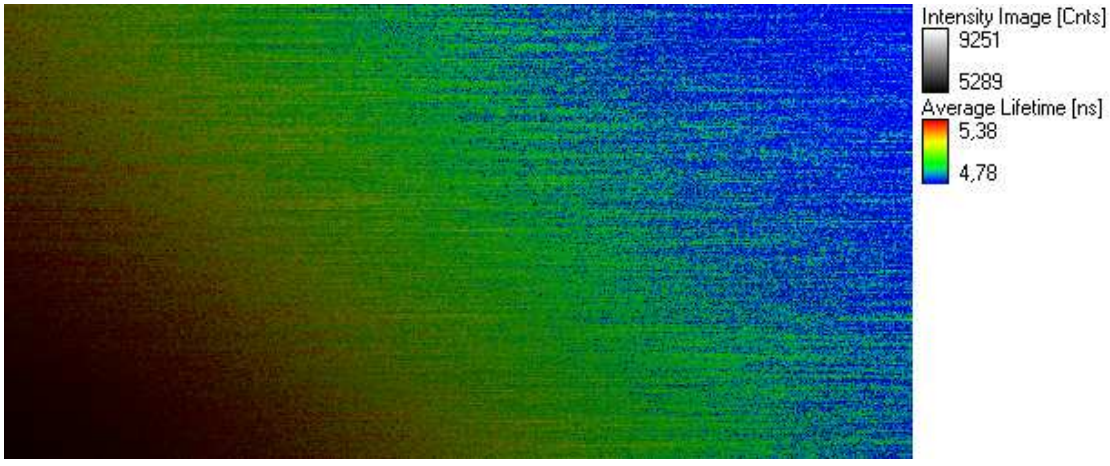

Figure 3.5. FLIM of the sample with resolution 4000 x 2000 pixels.

The first image shown in Figure 3.5 was recorded with 4000 x 2000 pixels (77 μm x 39 μm). Data collection of each pixel is 200 μs. Lifetimes are changing from

4.78 ns (represented blue) to 5.38 ns (represented red), which show perylene molecules and bodipy molecules, respectively. The green region is the mixture of the two type molecules. Also intensity is high in perylene side, which is expected.

The second image shown in Figure 3.6 was recorded 500 x 500 pixels (9.7 μm x 9.7 μm) but it starts at the point 15.4 μm in x-axis and 0 μm in y-axis. Data collection of each pixel is 1000 μs. Lifetimes are changing from 4.78 ns (represented blue) to 4.98 ns (represented red). The image shows us that this region is dominated with perylene molecules but some bodipy molecules increased the lifetime of the area.

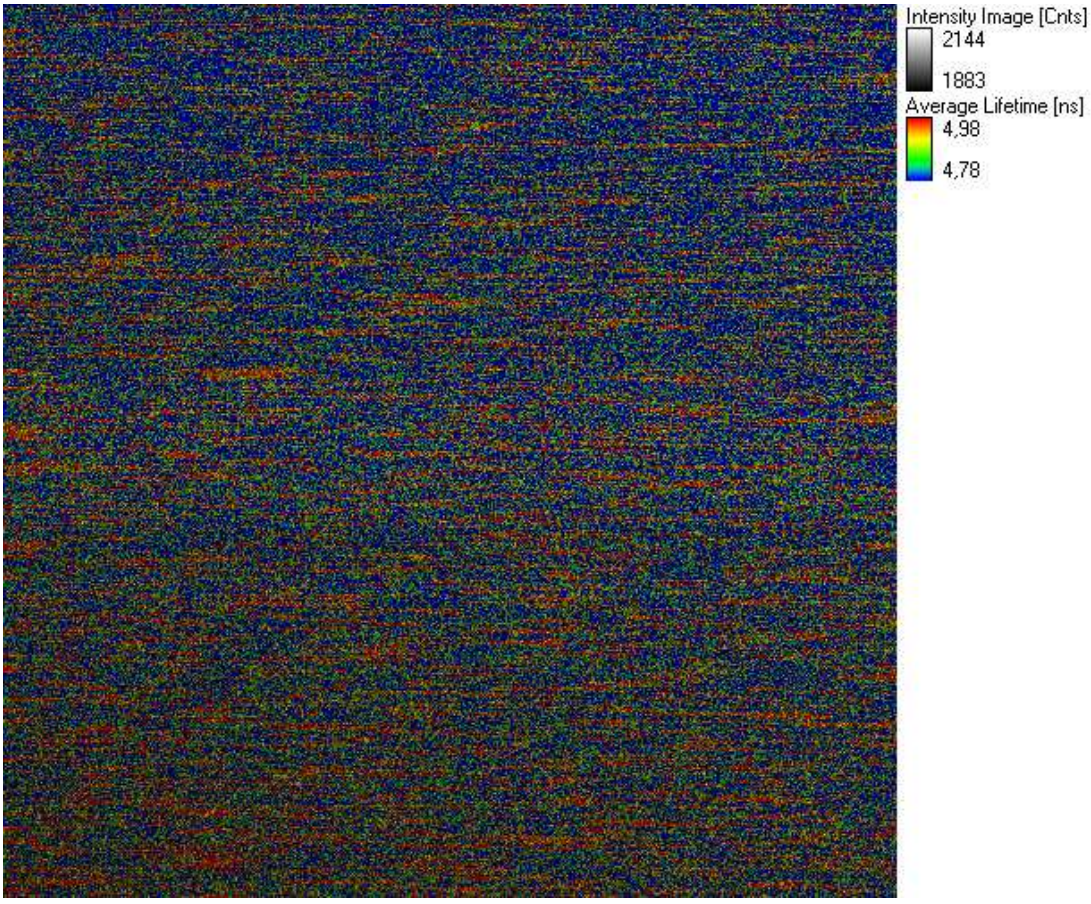

Figure 3.6. FLIM of the sample with resolution 500 x 500 pixels.

The third image shown in Figure3.7 was recorded 400 x 400 pixels (7.7 μm x 7.7 μm) but it starts at the point 19.25 μm in x-axis and 0 μm in y-axis. Data collection of each pixel is 3200 μs. Lifetimes are changing from 4.78 ns (represented blue) to 4.98 ns (represented red). The image shows us more clearly the second image. The lifetimes

are the same in both images but the intensity is greater than the second image since data collection at each pixel is threefold higher than the second image.

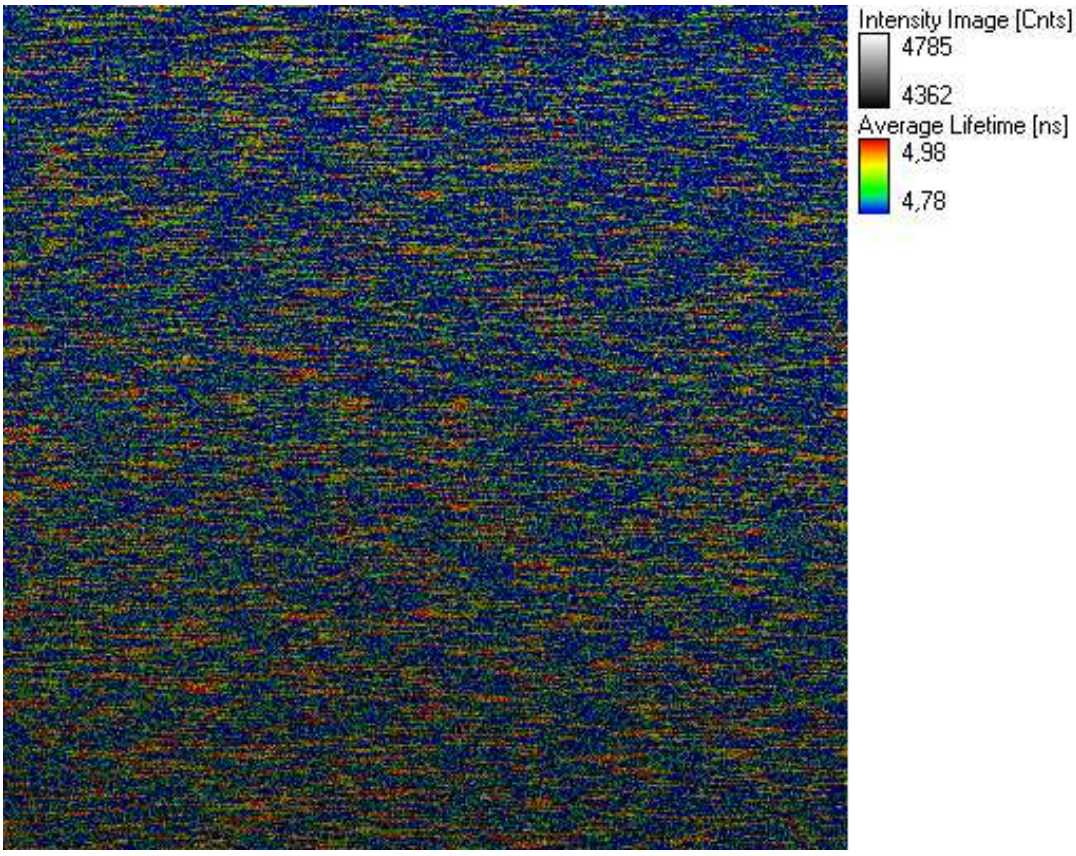

Figure 3.7. FLIM of the sample with resolution 400 x 400 pixels.

4. CONCLUSIONS

In this study, images of a dye molecule mixture is studied. The images of perylene and bodipy dye molecules mixture are obtained using fluorescence lifetime imaging microscopy technique. These images have different resolutions. As a result, we could examine lifetime images of molecules and see their location in micro scale.

As a future work, these dye molecules, especially perylene, since it is more flourescent than bodipy molecules, could inject into living cells so that information about the molecular organization of cells and tissues can be investigated. The mapping of cell parameters such as pH, ion concentrations or oxygen saturation by fluorescence quenching, or fluorescence resonance energy transfer (FRET) between different chromophores in the cell could be studied since the non-radiative (k) decay rate can vary according to how the fluorophore interacts with its local environment. For some fluorophores, k is sensitive to physical factors such as the local viscosity, or to chemical factors such as the local pH or to calcium ion concentration [19].

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