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Refractive index sensing using Fluorescence Lifetime Imaging (FLIM)

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Abstract. The fluorescence lifetime is a function of the refractive index of the fluorophore’s environment, for example in the case of the biologically important green fluorescent protein (GFP). In order to address the question whether this effect can be exploited to image the local environment of specific proteins in cell biology, we need to determine the distance over which the fluorophore’s lifetime is sensitive to the refractive index. To this end, we employ Fluorescence Lifetime Imaging (FLIM) of fluorescein in NaOH buffer at an interface. This approach allows us to map the fluorescence lifetime as a function of distance from a buffer/air and buffer/oil interface. Preliminary data show that the fluorescence lifetime of fluorescein increases near a buffer/air interface and decreases near a buffer/oil interface. The range over which this fluorescence lifetime change occurs is found to be of the order several μm which is consistent with a theoretical model based on the full width at half maximum of the emission spectrum proposed by Toptygin.

1. Introduction
Optical microscopy, and in particular fluorescence microscopy, is a powerful and effective tool in the biological and biomedical sciences today, because it is minimally invasive and can be applied to living cells and tissues. In fluorescence microscopy, the exciting light can be eliminated from the image by filters so that only fluorescence on a dark background is detected, leading to a high contrast. In addition to fluorescence intensity and location, additional spectroscopic parameters such as fluorescence spectrum, lifetime, or polarization can be imaged. [1]

1.1. Fluorescence Lifetime Imaging (FLIM)
Fluorescence Lifetime Imaging (FLIM) has emerged as a key technique to image not only the location, but also the environment and interaction of specific proteins in living cells. [2] It provides image contrast according to the fluorescence lifetime $\tau$ which is the average time a fluorophore remains in the excited state after excitation.

$$\tau = \frac{1}{k_r + k_{nr}}$$

(1)

where $k_r$ and $k_{nr}$ are the radiative and non-radiative rate constants respectively. $\tau$ depends on the environment, the local viscosity, pH, or refractive index, as well as interactions with other molecules (e.g. by collision or energy transfer). Imaging $\tau$ thus allows this molecular information to be mapped. $k_r$ is a function of the refractive index due to the polarizability of the medium surrounding the fluorophore.
\[ k_r = \frac{1}{\tau_0} = 2.88 \times 10^{-9} n^2 \int \frac{I(v)dv}{[I(v)v^{-3}dv]} \frac{\epsilon(v)}{\nu} dv \]  

Equation 2 is known as the Strickler Berg formula, where \( \tau_0 \) is the natural lifetime, \( I \) is the fluorescence emission, \( \epsilon \) is the extinction coefficient, \( \nu \) is the wavenumber, and \( n \) is the refractive index. [3] It is derived from the Einstein A and B coefficients for spontaneous emission and absorption.

The natural radiative lifetime \( \tau_0 \) and the fluorescence lifetime \( \tau \) are related through the quantum yield \( \phi \) by

\[ \phi = \frac{\tau}{\tau_0} = \frac{k_r}{k_r + k_{nr}} \]  

The fluorescence quantum yield can be thought of as the ratio of the number of fluorescence photons emitted to the number of photons absorbed.

1.2. Green Fluorescent Protein (GFP)

The Green Fluorescent Protein (GFP) of the jellyfish Aequorea victoria is extensively used in biological imaging as it can be genetically encoded in many other organisms and still maintain its ability to fluoresce. [4] GFP has had a huge impact on biological research and has influenced the advancement of many fluorescence techniques, for example imaging. The fluorescence decay of GFP in aqueous solution is a function of the refractive index of its environment, [5,6] and the fluorescence decay of the spectral variants cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) have also recently been shown to be affected by the refractive index. [7]

FLIM of GFP has been used to identify Förster Resonance Energy Transfer (FRET), a bimolecular quenching process to detect protein interaction, but could the GFP fluorescence decay also be used to directly probe the local refractive index environment of GFP? For example, it is known that different regions of the cell have different refractive indices. On average, the cell membrane has a higher refractive index than the cytoplasm, \( n_{membrane} = 1.46 - 1.60 \), and \( n_{cytoplasm} = 1.35 \) [8,9]. Different domains within a cell membrane may also differ in refractive index. To assess whether it is feasible to use the GFP fluorescence decay to probe the local environment, we need to investigate over which distance the GFP fluorescence decay senses the refractive index. If it is a near-field effect acting over nanometer distances, shorter than the optical resolution of the microscope, then we would be probing the local environment beyond the spatial resolution limit of light microscopy, not dissimilar to FRET. In such a case, GFP-lifetime-based refractive index sensing may open up the possibility of fast real-time FLIM to detect raft-like environments for individually tagged receptors in living cells.

There is a dearth of imaging data to quantify the distance over which the fluorescence lifetime senses the refractive index in general. Therefore, as a first step, we employ Fluorescence Lifetime Imaging (FLIM) of fluorescein in NaOH buffer (refractive index \( n = 1.33 \)) at an interface to map the fluorescence lifetime as a function of distance from a buffer/air \((n = 1.00)\) and buffer/oil \((n = 1.51)\) interface. The quantum yield \( \phi \) of fluorescein is 93%, and thus \( k_{nr} \) is over an order of magnitude smaller than \( k_r \) [5]. As a negative control, we also image fluorescein in ethylene glycol \((n = 1.45)\) at an oil interface, where the refractive index difference is significantly smaller.

A theoretical model based on the full width at half maximum of the emission spectrum predicts a maximum refractive index sensing distance beyond which there is no sensing, [13] and we show that our preliminary data are in broad agreement with this.
2. Theory

The Strickler Berg formula (eq 2) predicts that the fluorescence lifetime is inversely proportional to the square of the refractive index, and there have been recent experimental data to confirm this, eg [5,6,7,11,13]. The distance over which the refractive index affects radiative transitions can be gauged by investigating the fluorescence decay at an interface with a medium of a different refractive index.

Studies by Drexhage as far back as the 1970s have shown that there is a fluorescence lifetime difference at an interface of media with two different refractive indices. [12] He predicts that the fluorescence decay time of a molecule with its electric dipole oscillator perpendicular or parallel to a interface with a plane mirror is not constant, but a function of the distance from the mirror. Toptygin [13] extends the Drexhage model to predict that the cut-off distance beyond which the refractive index has no effect on the fluorescence decay is given by:

$$R_c = 1/(2n\Delta \nu)$$  \hspace{1cm} (4)

Where $R_c$ is the cut-off distance at which the medium no longer has an effect, $n$ is the refractive index of the fluorophore containing medium and $\Delta \nu$ is the full width at half maximum of the emission spectrum in wavenumbers. This is derived by assuming that the fluorophore is at the centre of a spherical mirror cut in half. The resulting radiative decay rate is then:

$$A = 2\sin^2\left(\frac{2\pi R}{\lambda}\right)A_{wm}$$  \hspace{1cm} (5)

Here $A$ is the spontaneous emission rate at the centre of the sphere, $A_{wm}$ is the spontaneous emission rate if the mirror were not there, $R$ is the radius of the sphere, $n$ is the refractive index and $\lambda$ is the emission wavelength.

It can then be determined that $A = 0$ when $2nR=\lambda$ and is maximum ($A= 2A_{wm}$) when $2nR=(m+\frac{1}{2})\lambda$. As the radius of the sphere increases the value for $A$ keeps oscillating between these two values and there is no cut-off. However if there are two emission wavelengths the emission of one wavelength reaches a maximum when the other is a minimum if:

$$2nR/\lambda_1 - 2nR/\lambda_2 = \frac{1}{2}$$  \hspace{1cm} (6)

is satisfied. Therefore converting wavelength to frequency equation 4 results.

Calculations have been carried out for GFP using the FWHM of its emission spectrum and a value of $R_c=4\mu m$ was predicted [6]. A similar result is predicted for fluorescein as its emission spectrum is similar to that of GFP [5].

3. Experimental Method

An inverted confocal microscope (Leica TCS SP2) was adapted for Time-Correlated Single-Photon Counting (TCSPC [14]) FLIM with a Becker & Hickl SPC 830 card. A 467nm pulsed diode laser (optical pulse width 90ps, Hamamatsu) was employed to excite the sample at a repetition rate of 20 MHz. The fluorescence was detected through a 500nm cut-off filter. To ensure that there were maximum counts in the y direction, the image size was reduced to 512 x 64 pixels, with a line scan speed of 200Hz. We used a 63× water immersion objective (NA 1.2) at maximum zoom (32×) for an image size of 7.44μm in the x direction by 0.93μm in the y direction. On the FLIM system the pixels were reduced even further to 256 x 32. A few microliters of fluorescein in NaOH buffer (n=1.33) were placed on a microscope slide to form an interface with air (n=1), an interface with oil (n=1.51) or an interface with ethylene glycol (n=1.45) (a few microliters of which were placed on the microscope
slide before). Problems with the liquid interfaces moving were solved by creating a ring of nail varnish sealant between the cover slip and the microscope slide. This therefore lifted the cover slip slightly and so reduced pressure on the sample, minimising any interface movement. A fluorescence intensity image of the interface was taken before and after the FLIM acquisition, ensuring that no interface movement had taken place. The FLIM images were acquired for 1000s with an average fluorescence count rate below 10000cts/s with the pinhole fully open (4.5 Airy units). SPCImage software (Becker & Hickl) based on a Levenberg-Marquardt fitting algorithm was used to fit an mono-exponential decay to the fluorescence decay curve in each pixel of the image. A colour was assigned to each lifetime value (red for a long lifetime and blue for a short lifetime), yielding FLIM maps.

4. Results and Discussion

Intensity images and FLIM images for the buffer/oil and buffer/air interfaces are shown in Fig 1. The fluorescence intensity images (Fig 1A) and 1B) reveal the position of the interface, as the fluorescein is only dissolved in the buffer. A cross section through the intensity image along the rows is shown in Fig 1C) and 1D). The interface is not sharp but blurred, probably due to the lateral optical resolution of the confocal microscope (the pinhole is 4.5 Airy units), distortion due to imaging a refractive index discontinuity, and possibly also due to the properties of the interface itself (meniscus). In any case, the boundary (0µm) was defined as the point where the bulk fluorescence intensity has more or less vanished which is at about 1/20 of its maximum value. The rows were then all shifted for the position of the boundary to be the same in each row, so that they could all be compared on one graph.

Limited information can be taken from the intensity image, however even before numerical analysis the FLIM image reveals a fluorescence lifetime change at the boundary, as shown in Fig 1E) and 1F). To compare the two different interfaces quantitatively, the fluorescence lifetime values were averaged over the 32 pixels in the y direction and plotted versus x (Fig 1G)). It shows the average fluorescence lifetime of fluorescein as a function of distance from the boundary at 0 µm for the buffer/air and buffer/oil interfaces.

Now studying Fig 1G showing the lifetime as a function of distance across the image, it is possible to achieve a more quantitative idea of what is shown in Fig 1E and 1F. For the fluorescein in buffer at an air interface an increase in the fluorescence lifetime is seen in the region 0µm to 3µm. Conversely for the oil interface a decrease in fluorescence lifetime is seen from approximately 0µm to 2µm.

To calculate the expected cut-off distance from Toptygin’s model, [13] an emission spectrum of the fluorescein in NaOH buffer was measured and is shown in Fig 2. Using equation 4 it is possible to show that \( R \approx 3\mu m \), which is approximately the same as for GFP. [6] Therefore our results are in agreement with the micrometer cut-off distance predicted by Toptygin’s method, despite his model being based on a spherical mirror rather than a plane mirror. (For a more rigorous treatment this point would perhaps warrant further investigation.)

As an experimental control, an interface of fluorescein in ethylene glycol with oil was imaged, two immiscible liquids. The refractive index difference \( \Delta n \) between ethylene glycol and oil is 0.06, significantly smaller than the \( \Delta n \) of the interfaces imaged in Fig 1. Therefore if our predictions are correct we should expect to see very little or no change at the boundary. This is exactly what we observe and our results can be seen in Fig 3 below.

To allow all three curves to be compared on the same graph without having to consider the different absolute fluorescence lifetime values in the ethylene glycol and NaOH buffer, we calculate the fluorescence lifetime ratio \( \tau_{\text{interface}} / \tau_{\text{bulk}} \), where \( \tau_{\text{interface}} \) is the fluorescence lifetime near the interface, and \( \tau_{\text{bulk}} \) is the fluorescence lifetime in bulk solution beyond the cut-off distance. A plot of the relative change of the fluorescence lifetime ratio for all three interfaces as a function of distance from the interface is shown in Fig 4. The value for ethylene glycol is between the values in oil and air.
**Fig 1A.** (Left) Fluorescence intensity image of the fluorescein in NaOH buffer / air interface. **Fig 1B** (Right). Fluorescence intensity image of the fluorescein in NaOH buffer / oil. The dimensions of the images are 7.44μm x 0.93μm.

![Intensity for Air interface](image1)

![Intensity for Oil interface](image2)

**Fig 1C** (Left). Fluorescence intensity cross section across the air interface. **Fig 1D** (Right). Fluorescence intensity cross section across the oil interface. The boundary is defined where counts fall to ~ 1/20 of original value.

![3800ps](image3)

**Fig 1E** (Left). FLIM of the fluorescein in NaOH buffer / air interface, Δn=0.33. This clearly shows that the lifetime is longer at the interface indicated by the red region. The blue at the edge is probably due to poor fits of noisy fluorescence decay data as there are few counts at that point. **Fig 1F** (Right). FLIM of the fluorescein in NaOH buffer / oil (Δn=0.18) a clear decrease in the fluorescence lifetime is shown by the blue region. The colour scale bar is the same for both images.

![Lifetime vs Distance](image4)

**Fig 1G**: The change in average fluorescence lifetime over the boundary of fluorescein in water with air and oil. A clear increase is seen at the air interface and a decrease at the oil interface.
**Fig 2.** Emission spectrum of fluorescein in NaOH buffer. Its a full width at half maximum is 35nm.

**Fig 3A** (Top). Cross Section of fluorescein in ethylene glycol at an oil interface, Δn=0.06. **Fig 3B** (Bottom). FLIM image of the interface of ethylene glycol with oil, demonstrating that there is no discernible change in the fluorescence lifetime. (Note that the fluorescence lifetime is shorter than for fluorescein in NaOH buffer as ethylene glycol has a higher refractive index than NaOH buffer.)
Fig 4. Plot of normalised fluorescence lifetime versus distance from the interface. The fluorescein in ethylene glycol at an oil interface ($\Delta n=0.06$) does not change the fluorescence lifetime whereas the fluorescein in water at an interface with air ($\Delta n=0.33$) or oil ($\Delta n=0.18$) does.

as expected. We note here that the a re-definition of the interface would change the values on the x-axis, but it would not change the fact that the curves for oil and air diverge. Optical distortions may cause distortions in the x-axis, and further experiments are needed to address these points.

5. Conclusion
Fluorescence Lifetime Imaging (FLIM) of fluorescein in NaOH buffer at air, oil and ethylene glycol interfaces allows the mapping the fluorescence lifetime as a function of distance from the interface. Preliminary images show that the range over which this fluorescence lifetime change occurs is found to be of the order $3 \mu m$. This is consistent with a theoretical model based on the full width at half maximum of the emission spectrum of a fluorophore in front of a spherical mirror, as proposed by Toptygin.

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