mb-FLIM: Model-based fluorescence lifetime imaging

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ABSTRACT

We have developed a model-based, parallel procedure to estimate fluorescence lifetimes. Multiple frequencies are present in the excitation signal. Modeling the entire fluorescence and measurement process produces an analytical ratio of polynomials in the lifetime variable $\tau$. A non-linear model-fitting procedure is then used to estimate $\tau$. We have analyzed this model-based approach by simulating a 10 $\mu$M fluorescein solution ($\tau = 4$ ns) and all relevant noise sources. We have used real LED data to drive the simulation. Using 240 $\mu$s of data, we estimate $\tau = 3.99$ ns. Preliminary experiments on real fluorescent images taken from fluorescein solutions (measured $\tau = 4.1$ ns), green plastic test slides (measured $\tau = 3.0$ ns), and GFP in U2OS (osteosarcoma) cells (measured $\tau = 2.1$ ns) demonstrate that this model-based measurement technique works.

Keywords: fluorescence lifetime imaging microscopy, lifetime estimation, quantitative microscopy, non-linear estimation

1. INTRODUCTION

FLIM (fluorescence lifetime imaging microscopy) is a standard tool for studying the biochemical and biophysical environment of molecules within a cell. FLIM has been successfully used to measure local phenomena such as pH, oxygen concentration, calcium concentration and NADH concentration\textsuperscript{1-4}. FLIM is also a standard tool when FRET (fluorescence resonance energy transfer) is used to study the physical distance between molecules. There are several standard approaches to measuring the fluorescence lifetime of one or more species of molecule.

1.1 Time-domain FLIM

In time-domain FLIM one uses a short, intense pulse of light to excite the molecule\textsuperscript{5-8}. One can then measure the time it takes for the first photon to be emitted from a test volume, repeat this procedure for a number of pulses, and then take the average time for the emission of the first photon over the data recorded for a collection of pulses. Alternatively one can measure the photon response to a single impulse, a decaying exponential, by sampling the decay at a suitably high rate. For lifetimes in the common range of 1 to 10 ns, this implies using a very short pulse of light with a time duration measured in ten of picoseconds and with a sufficient number of excitation photons in each pulse to ensure that the molecules in the test volume produce emission photons. Whether one is looking at the arrival time of the first emission photon for each pulse or the arrival times of “all” the emission photons produced by a single pulse, a sensor with a very fast response time and little dead-time is required as is a very high-speed sampling system. Bandwidths involved certainly exceed 1 GHz for both the sensor and the sampling system. None of these system components is inexpensive.

1.2 Frequency-domain FLIM

Frequency-domain FLIM uses a different approach\textsuperscript{9-12}. The excitation light is produced by a source such as an LED that has the proper wavelength spectrum to act as an excitation source. The current to the LED can be varied to produce a periodically varying light signal that in turn produces a fluorescence emission that has a similar, periodic modulation.

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Figure 1 shows a system model for what is happening at the excitation level of this process as well as the light measured at the output of an LED ($\lambda_{peak} = 455$ nm, Lumiled, LXML-PR01-0225). The fundamental frequency of the signal driving the LED is $25$ MHz for a period of $40$ ns and the sampling rate of the recording process is $2.5$ GHz or $400$ ps per sample implying 100 samples per period.

![System model diagram]

Figure 1. (a) A square-wave, periodic signal, period $T$, of duty cycle 50% is the input to a current-driven LED; (b) Output light from a real LED with a resulting duty cycle less than 50%; (c) Simulated fluorescence emission for fluorescein ($\tau = 4$ ns) in black given the real LED excitation signal in red.

The periodic variation of the excitation light intensity (modulation) means that the emission light will undergo a similar periodic variation. The harmonically-related frequencies of the excitation light will be reproduced in the emission light but with a change in the amplitude and phase of each harmonic. This is illustrated in Figure 1c. The basis for this is discussed in the next section. The analysis of these amplitude and phase changes forms the basis for the frequency-domain procedure for estimating the fluorescence lifetime components within each test volume.

### 1.3 Frequency-domain model

If the excitation light could be produced as a unit impulse, $L(t) = \delta(t)$, then the fluorescence emission, an impulse response, would be a decaying exponential with lifetime $\tau$ and described by:

$$f(t) = \left(\frac{1}{\tau}\right)e^{-t/\tau}u(t) = \begin{cases} \frac{1}{\tau}e^{-t/\tau} & t > 0 \\ 0 & t < 0 \end{cases}$$

(1)

The signal $f(t)$ has been intentionally normalized to a unit area corresponding to the fact that this function describes the probability that a single molecule in the test volume will emit a photon in a time interval $\Delta t$ around time $t = t_n$. This is given by $f(t_n)\Delta t$. Because our test volumes contain a number of fluorescent molecules, this ensemble generates an exponentially-decaying light pulse in response to the input light impulse $\delta(t)$. This result is the basis for the time-domain measurement techniques.

Further, if an impulse of area $A$ is used instead of a unit impulse, that is $L(t) = A\delta(t)$ and $A > 1$ means more excitation photons, then barring photo-destruction of the target molecules, the resulting emission light will be $A f(t)$. The conclusion we are tempted to draw is that the system composed of many fluorescent molecules is a linear system. We must be a bit careful about this because true linearity requires that this scaling property (and another the additivity property)\(^\text{13}\) hold for any value of $A$ including $A < 0$. But we can never produce a negative amount of light, neither excitation nor emission. This implies that we must be careful that, in using this linearity assumption, we restrict ourselves to scaling fluorescent signals in a manner consistent with this positivity constraint.

Let us now consider what happens if an impulse of excitation light is delayed in its arrival at the test volume by $t_1$ seconds, that is $\delta(t) \rightarrow \delta(t - t_1)$. Experience has shown us that, if $t_1$ is not too long, the resulting emission pulse will also be delayed by the same amount, $f(t) \rightarrow f(t - t_1)$. Barring chemical changes in the fluorophore that alter its fluorescent character, the system consisting of fluorescent molecules is time-invariant.
Together these two—not unreasonable—assumptions allow us to treat an ensemble of fluorescent molecules in a test volume as a linear, time-invariant (LTI) system and to use linear system theory techniques to measure fluorescent lifetimes. We make a point of mentioning these assumptions, linearity and time-invariance because, should either of them not be true, the applicability of the measurement techniques we have described will be doubtful.

The effect of the impulse response $f(t)$ on a sinusoidal input is well-understood. The Fourier transform of the impulse response yields the transfer function, $F(\omega)$ that describes how the amplitude and phase of the sinusoidal excitation light are changed. This transfer function is given by:

$$F(\omega) = \mathcal{F}\{f(t)\} = \frac{1}{1+j\omega \tau} = |F(\omega)| e^{j\angle F(\omega)} = \left(\frac{1}{\sqrt{1+(\omega \tau)^2}}\right) e^{-\frac{1}{2} \angle \omega \tau} (2)$$

We now consider an excitation signal $L(t) = 1 + \cos(\omega_o t - \theta)$, where $\theta$ is a phase term (delay) that we can control at the electrical input to the LED. Taking into account the fact that $F(\omega = 0) = 1$, the fluorescent emission will be

$$E(t) = 1 + |F(\omega)| \cos(\omega_o t + \angle F(\omega) + \theta)$$

The effect of the fluorescence emission on the sinusoidal excitation is clear; both the amplitude and phase of the sinusoidal input have changed. In conventional frequency-domain FLIM, the next step is to process $E(t)$ in such a way as to recover the phase. This is generally accomplished by demodulation: multiplying $E(t)$ with $\cos(\omega_o t)$ yielding $R(t) = E(t) \cos(\omega_o t)$ and then averaging the resulting over $N$ periods of $T_o = \frac{2\pi}{\omega_o}$. It is not difficult to show that the multiplication yields:

$$R(t) = E(t) \cos(\omega_o t) = \left[1 + \frac{1}{\sqrt{1+(\omega_o \tau)^2}} \cos(\omega_o t - \theta - \arctan(\omega_o \tau))\right] \cos(\omega_o t)$$

$$= \cos(\omega_o t) + \frac{1}{\sqrt{1+(\omega_o \tau)^2}} \cos(\omega_o t - \theta - \arctan(\omega_o \tau)) \cos(\omega_o t)$$

$$= \cos(\omega_o t) + \frac{1}{2} \left[ \frac{1}{\sqrt{1+(\omega_o \tau)^2}} \right] \left( \cos(2 \omega_o t - \arctan(\omega_o \tau) - \theta) + \cos(\arctan(\omega_o \tau) + \theta) \right)$$

(4)
and averaging the result gives:

$$< R > = \frac{1}{NT_\alpha} \int_{t=0}^{NT_\alpha} R(t) dt = \frac{1}{2} \left( \frac{1}{\sqrt{1 + (\omega_0 \tau)^2}} \right) \cos(\arctan(\omega_0 \tau) + \theta)$$

(5)

By stepping through various values of $\theta$ we can determine a value $\theta_o$ for which the cosine function achieves its maximum value of one. This then leads to:

$$\cos(\arctan(\omega_0 \tau) + \theta) = 1 \Rightarrow -\arctan(\omega_0 \tau) = \theta \Rightarrow \tau_o = -\tan(\theta_o) / \omega_0$$

(6)

It is instructive to look at the consequences of this result in terms of choosing a proper frequency $\omega_0$. If $|\omega_0 \tau| < < 1$ then the $\arctan(\omega_0 \tau) \approx 0$ and it will be difficult to estimate the correct value of $\theta$ in the presence of noise. If $|\omega_0 \tau| >> 1$ then $|F(\omega_0)| \approx 0$ as can be seen in Eq. (2). This means the fluorescent signal amplitude will be small and once again phase estimation in the presence of noise will be next to impossible. From this we conclude that the appropriate range for $\omega_0$ is $\omega_0 \tau \approx 1$. This can be manipulated into the following rule-of-thumb:

$$\omega_0 \tau \approx 1 \Rightarrow f_0 = \frac{1}{2\pi \tau} \Rightarrow f_0 \approx \frac{160 \text{ [MHz•ns]}}{\tau \text{ [ns]}}$$

(7)

If, for example, one is working with fluorescein with $\tau \approx 4$ ns$^{14}$, then the appropriate frequency is about $f_0 = 40$ MHz. For GFP with $\tau \approx 2$ ns$^{15}$, this would be about $f_0 = 80$ MHz.

The demodulation step in frequency-domain FLIM is generally accomplished through use of an image intensifier where the $\cos(\omega_0 t)$ term (or a similar periodic signal) is applied as a voltage. The frequency $f_o = \omega_0 / 2\pi$ can then be driven to values in excess of 100 MHz. As described in a companion paper, however, we are developing a new system for FLIM based upon a totally solid-state approach. That is, we do not use an image intensifier but instead recover the necessary measurements on a specially-designed CCD camera chip.

Our use of a CCD chip means that we must consider what the effects of clock frequencies such as 80 MHz are on the clock driver circuitry as well as the CCD chip itself. A thermal image of the clock driver in our first implementation running at 20 MHz indicated that the temperature at the driver was approximately 92 °C. This is shown in Figure 2. While the driver had no problem running at this frequency (and temperature), should the frequency be raised to 80 MHz the heat production would increase by at least a factor of four with the associated consequences.

![Figure 2. (a) CCD chip and associated electronics; (b) Thermal image of the board showing a hot spot in red (92 °C) at the location of the clock driver. At 20 MHz the CCD chip remains at ≈35 °C. Ambient temperature was 22 °C.](image-url)

What is apparent, however, is that a periodic clock (modulation) signal running at a fundamental frequency $f_0$ has a series of higher, harmonic frequencies that can provide the frequency components necessary to satisfy Eq. (7). Looking at the data from an LED being driven by a 50% duty cycle square wave running at 25 MHz, we see that the output signal
shown in Figure 1b has a number of significant harmonic components above the fundamental frequency. These are shown in Figure 3 and we see that the first six harmonics have amplitudes that are non-negligible.

We can now use these first six harmonics to produce an analytical model for the LED signal, \( L(t) \). With the complex coefficients of the Fourier spectrum being characterized by a set of magnitudes \( \{a_k\} \) and phases \( \{\phi_k\} \), this is given by:

\[
L(t) = a_0 + 2 \sum_{k=1}^{6} a_k \cos(k\omega_0 t + \phi_k + k\theta)
\]

Note that the delay, \( \theta \), used in Eq. (3) is to be found in this expression as well but now as a harmonic term. This follows from the Fourier property that a delay in time becomes a multiplication in the Fourier domain with a phase factor that increases linearly with frequency. Formally, if \( x(t) \) and \( X(\omega) \) are a Fourier transform pair, then \( x(t - \psi) \) has the Fourier transform \( e^{-j\omega \psi}X(\omega) \).

The use of only six harmonic terms to describe the LED pulse appears sufficient as shown in Figure 3c.

The continuous curve in Figure 3c follows from Eq. (8) and the sampled data, shown in red, is one period taken from Figure 3a. The match is excellent.

The fluorescence emitted by a sample is then:

\[
E(t) = L(t) \otimes f(t) = a_0 + 2 \sum_{k=1}^{6} a_k \left( \frac{\cos(k\omega_0 t + \phi_k + k\theta)}{1+(k\omega_0 \tau)^2} + \frac{(k\omega_0 \tau)\sin(k\omega_0 t + \phi_k + k\theta)}{1+(k\omega_0 \tau)^2} \right)
\]

We have made explicit use of the form of Eq. (3). This result indicates that the emitted light is a function of \( \tau \) (as expected), the phase \( \theta \) (which we control), \( \omega_0 \) (the clock frequency), and the average Fourier coefficients of the excitation light as described by \( \{a_k, \phi_k\} \). We assume that we are dealing with a single lifetime. In the case of multiple lifetimes, it is straightforward to expand Eq. (9) to include each of the lifetime components \( \{\tau_i\} \) and the associated mixing components \( \{p_i\} \) that indicate the (unknown) proportion of each lifetime species. At this time, however, we will confine our remarks to the mono-exponential case.

It is the use of this model to describe the emitted fluorescent light—a model that has at this point only one unknown term \( \tau \)—that forms the basis for our model-based procedure for fluorescent lifetime estimation. We are not the first to explore this possibility \(^1\), \(^4\), \(^7\), \(^15-18\) but it represents an important aspect in the consideration of our MEM-FLIM camera system.

2. MEM-FLIM MODEL

Our CCD chip is designed so that each pixel (photogate) is sandwiched between two storage gates, one which we term PLUS and one which we term MINUS. The periodic clock signal, as illustrated in Figure 1a has two states, one “positive” and one “negative”. Photoelectrons produced during the “positive” state are collected (integrated) and transferred to the PLUS gate and photoelectrons produced during the “negative” state are collected and transferred to the MINUS gate. This is illustrated in Figure 4.
The output of the **PLUS** and **MINUS** storage gates over one clock period are given by:

\[
\text{PLUS}(\tau) = \int_{t=0}^{T_o/2} E(t)dt \\
\text{MINUS}(\tau) = \int_{t=T_o/2}^{T_o} E(t)dt
\]  

(10)

For the 25 MHz clock (\(T_o = 40\) ns) with a 50% duty cycle, this means the **PLUS** gate collects photoelectrons from the first 20 ns of each period and the **MINUS** gate collects the photoelectrons from the second 20 ns of each period. The signal associated with one period then becomes:

\[
\text{PeriodMeasure}(\tau) = \text{strength} \cdot (\text{PLUS}(\tau) - \text{MINUS}(\tau)) + \text{offset}
\]  

(11)

We have added two parameters, **strength** and **offset**, to account for (unknown) electronic gain and offset in the system. Further, we look at the difference between the two storage gates as they represent 180° phase shifts of the clock signal. Equations (9) and (10) can be substituted into Eq. (11) to arrive at the formidable-looking but nevertheless understandable result:

\[
\text{PeriodMeasure} = \text{offset} - \frac{0.655519 \cdot \text{strength} \cdot \cos(0.179621 - 5\theta - 5\psi)}{16 + 9.8696 \cdot \tau^2} - \\
\frac{14.9426 \cdot \text{strength} \cdot \cos(0.130426 - 3\theta - 3\psi)}{400 + 88.8264 \cdot \tau^2} + \\
\frac{2670.85 \cdot \text{strength} \cdot \cos(0.29512 - \theta - \psi)}{400 + 9.8696 \cdot \tau^2} + \\
\frac{0.514844 \cdot \text{strength} \cdot \tau \cdot \cos(1.39118 + 5\theta + 5\psi)}{16 + 9.8696 \cdot \tau^2} + \\
\frac{7.04152 \cdot \text{strength} \cdot \tau \cdot \sin(0.130426 - 3\theta - 3\psi)}{400 + 88.8264 \cdot \tau^2} + \\
\frac{419.536 \cdot \text{strength} \cdot \tau \cdot \sin(0.29512 - \theta - \psi)}{400 + 9.8696 \cdot \tau^2}
\]  

(12)

\(\text{PeriodMeasure}\) depends upon five parameters \(\{\tau, \theta, \text{strength}, \text{offset}, \psi\}\). The first four have been described. The term involving \(\psi\) represents the unknown delay between the clock driving the LED and the same clock driving the CCD electronics. The cable length difference introduces a delay of more than 1 ns per 30 cm and thus must be included in the model.

The specific numbers in Eq. (12) come from the measured Fourier coefficients of the periodic LED signal and the \((k\omega_0)^2\) terms in Eq. (9). In this result we are using the first six harmonics.

### 3. MODEL-BASED ESTIMATION

Starting from Eq. (11) or the more specific example of Eq. (12), we have, for each pixel in an image, a number of measurements made for various values of \(\theta\) over a number of integration periods. At \(T_o = 40\) ns, a 10 ms total integration time would yield 250,000 integration periods. The model is then fit to the data by using the non-linear fitting procedure **FINDFIT** (or the equivalent **NONLINEARMODELFIT**) in Mathematica 8.0 (Wolfram Research Inc.). A non-linear procedure is required because **PeriodMeasure** is a non-linear function of \(\{\tau, \theta, \psi\}\).
Before going into some details of the fitting results for both simulations and real fluorescent samples, it is useful to look at the agreement that is found between the model as described in Eq. (12) and experimental data. This is shown in Figure 5 for experiments with a 10 μM fluorescein solution (τ = 4 ns) and total exposure times of 100 ms for Figure 5a and 180 ms for Figure 5b.

Figure 5. Model-based PeriodMeasure versus θ after finding optimal values for the five parameters {τ, θ, strength, offset, ψ}. The grey curve is the theory; the red dots are the experimental data. (a) For 6 values of the controllable phase θ, (b) For 24 values of the controllable phase θ.

In both cases in Figure 5, the fit is quite good. Both curves are almost perfectly sinusoidal and this should be a bit of a surprise as Eq. (12) allows the possibility for a much more complicated form. Examining the coefficients of each term in Eq. (12), however, shows that with the exception of one term all of the coefficients are quite small. The term that has the dominant coefficient is the term that is functionally dependent on cos(θ) and not sin(θ), cos(3θ), etc. Thus it is this term that we see in Figure 5. This is important because it means that the 50% duty-cycle clock input is not providing a significant amount of spectral power in the higher harmonics of the LED output. A shorter duty cycle would remedy this problem without changing the fundamental clock frequency.

We have evaluated this model-based approach to determine τ, the fluorescence lifetime, using simulations of the entire system and experiments with fluorescein14 (τ = 4 ns), GFP15 (τ = 2.2 ns), and a green plastic test slide (τ = 2.8 ns).

3.1 Simulations

In the simulations we model the Poisson distribution of the photoelectrons. We do not model the Poisson-distributed dark current because we have shown in the companion paper19 that, at the maximum total integration times of 200 ms used in the experiments and the simulations, the dark current has an average value of (200 ms)(0.33 e−/ms)(0.38 ADU/e−) ≈ 25 ADU, a negligible effect when compared to simulated and experimental amplitudes of 2500 ADU. Similarly, the Gaussian readout noise has an average, negligible value of 5.9 ADU, a value that is independent of the integration time. The results of various simulations are given in Table 1.

The data in Table 1 show that precise and accurate estimation of the lifetime is possible within the context of the model. The Total Clock Time refers to the time necessary to acquire data for one pixel including all phase measurements. As an example, if the clock period \( T_o = 40 \text{ ns} \), \( N = 1000 \) periods, and 6 phases are used, then the total clock time is 40 ns × 1000 × 6 = 240 μs as shown in the table. The \( \text{SE}(\sigma) \) value refers to the standard-error-of-the-estimate for the average τ found in the fitting process. The computation time was determined by 1) running the fitting algorithm on each data set to find values for the parameters, 2) measuring the time required to find the fit, 3) repeating the procedure 100 times, and finally 4) determining the average time. All computations were performed on an Apple iMac 2.4 GHz Intel Core 2 Duo with 4 GB of 667 MHz RAM. The calculations, as mentioned earlier, were performed in Mathematica 8.0 and no parallelization or compilation was explicitly requested.

As acquisition and processing time are two separate issues, it should be noted that 1) pixel data in the MEM-FLIM camera, like most CCD cameras, are acquired in parallel so that the Total Clock Time will remain constant except for data transfer out of the camera chip and 2) unless a form of parallelism is invoked the Total Processing Time will be the number of pixels in the image times the value given in the last column. The processing power represented here, however,
is based on 2007 technology so that represents no major bottleneck. A significant reduction in processing speed can be realized, for example, with the use of a graphics processing unit (GPU).

Table 1. Simulation results for model-based estimation of fluorescence lifetimes assuming a single lifetime.

<table>
<thead>
<tr>
<th>Simulated Fluorophore</th>
<th>True $\tau$ [ns]</th>
<th>Phases</th>
<th>Total Clock Time [µs]</th>
<th>Estimated $\tau$ [ns]</th>
<th>SE($\sigma_\tau$)</th>
<th>Computation Time/ pixel [ms]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescein</td>
<td>4.0</td>
<td>6</td>
<td>240</td>
<td>3.99613</td>
<td>$1.9 \times 10^{-3}$</td>
<td>2.8</td>
</tr>
<tr>
<td>Fluorescein</td>
<td>4.0</td>
<td>24</td>
<td>960</td>
<td>3.99775</td>
<td>$1.5 \times 10^{-3}$</td>
<td>3.0</td>
</tr>
<tr>
<td>Green plastic</td>
<td>2.8</td>
<td>6</td>
<td>240</td>
<td>2.80130</td>
<td>$2.7 \times 10^{-3}$</td>
<td>3.1</td>
</tr>
<tr>
<td>Green plastic</td>
<td>2.8</td>
<td>24</td>
<td>960</td>
<td>2.80126</td>
<td>$7.1 \times 10^{-4}$</td>
<td>3.0</td>
</tr>
<tr>
<td>GFP</td>
<td>2.2</td>
<td>6</td>
<td>240</td>
<td>2.19514</td>
<td>$5.1 \times 10^{-3}$</td>
<td>2.5</td>
</tr>
</tbody>
</table>

3.2 Experiments

We have used the MEM-FLIM system together with the previously described LED to look at the fluorescent samples that have been simulated in Table 1. The “green plastic slide” is from Chroma Technology (Rockingham, VT, #92001 Autofluorescent Plastic Slides). The fluorescein solution was prepared at 10 µM and deposited on a cover slip. Cell samples were fixed U2OS (osteosarcoma) cells that expressed green fluorescent protein (GFP) supplied from Leiden University Medical Center. Details concerning the microscope imaging system can be found in our companion paper. Table 2 shows the results of the experimental measurements.

Table 2. Experimental results for model-based estimation of fluorescence lifetimes assuming a single lifetime.

<table>
<thead>
<tr>
<th>Experimental Fluorophore</th>
<th>True $\tau$ [ns]</th>
<th>Phases</th>
<th>Total Clock Time [ms]</th>
<th>Estimated $\tau$ [ns]</th>
<th>SE($\sigma_\tau$)</th>
<th>Computation Time/ pixel [ms]</th>
</tr>
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<tbody>
<tr>
<td>Fluorescein</td>
<td>4.0</td>
<td>6</td>
<td>100</td>
<td>4.11291</td>
<td>$2.9 \times 10^{-3}$</td>
<td>9.4</td>
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<tr>
<td>Fluorescein</td>
<td>4.0</td>
<td>24</td>
<td>180</td>
<td>4.00209</td>
<td>$1.9 \times 10^{-3}$</td>
<td>13.2</td>
</tr>
<tr>
<td>Green plastic</td>
<td>2.8</td>
<td>6</td>
<td>180</td>
<td>2.99719</td>
<td>$2.6 \times 10^{-3}$</td>
<td>8.7</td>
</tr>
<tr>
<td>Green plastic</td>
<td>2.8</td>
<td>24</td>
<td>180</td>
<td>2.99054</td>
<td>$2.1 \times 10^{-3}$</td>
<td>12.5</td>
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<tr>
<td>GFP</td>
<td>2.2</td>
<td>6</td>
<td>100</td>
<td>2.05773</td>
<td>$2.5 \times 10^{-2}$</td>
<td>11.5</td>
</tr>
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</table>

In each of these experiments, four distinct parameters {$\tau$, strength, offset, $\psi$} were estimated through the non-linear fitting procedure.

4. DISCUSSION AND CONCLUSIONS

The results are not as clear-cut as in the simulations but there are a number of possible explanations for this. First, the images show a general shading in intensity and in the lifetime estimates as a function of position. These represent a deterministic distortion of the data that we will eliminate. Second, the MEM-FLIM camera chip has a mask protecting parts of the surface from exposure to photons. In the current version there is a slight displacement of the mask from its
intended position. This means that photoelectrons that we measure are to a small extent caused by contributions from the wrong source. This will be corrected in the next chip. Third, the clock signals that switch between the PLUS gate and the MINUS gate have finite rise and fall times. This means the switching from one gate to the other does not occur as quickly as indicated in Eq. (10). Fourth, as indicated earlier, the model as described in Eqs. (9) – (11) depends explicitly on the Fourier coefficients associated with the periodic LED signal. The coefficients that we are now using were measured before the LED was actually installed in the microscope’s lamp-house. We are working on a real-time, feed-forward mechanism to track the LED signal and thus its Fourier coefficients. Finally, as we described earlier, the duty cycle (and rise and fall times) of the LED are probably too long and need to be reduced in order to provide sufficient power in the higher order harmonics.

The results of the entire process are shown in Figure 6.

![Figure 6](image)

**Figure 6.** Model-based estimation of lifetime per pixel for the green plastic slide with 24 values of the controllable phase $\theta$: (a) 100 $\times$ 100 pixel intensity image; (b) LED signal in red and fluorescence signal in black. The arrows (↓) indicate the start and end of integration times; (c) 100 $\times$ 100 pixel lifetime image; (d) histogram of intensity image; (e) measured pixel lifetime starting at the bottom-left pixel and going to the top-right pixel; (f) histogram of lifetime image.

Figure 6a shows the distribution of intensity in a 100 $\times$ 100 pixel region in the fluorescence image measured from the green plastic slide described above. Non-uniform shading can be seen from top-left to bottom right. Figure 6b shows an enlargement of one period of Figure 1c but with the lifetime $\tau = 2.8$ ns, the value associated with the sample. The amplitude and phase change associated with the model are obvious. The three arrows (↓) point to the integration time intervals for PLUS (left interval) and MINUS (right interval). The LED excitation signal (measured) and the fluorescence signal (modeled) are shown in red and black, respectively. Figure 6c shows the lifetime measured at each pixel using 24 values of the phase $\theta$. The average value over the entire image, 10^4 pixels, is given in Table 2 at $\tau = 2.99$ ns.

Figure 6d shows the intensity histogram for the image in Figure 6a. Figure 6e shows the lifetimes starting at the bottom-left pixel in Figure 6c and moving raster-like to the top-right pixel. There is a systematic decrease in the lifetime from bottom-left to top-right. Fitting a linear curve to this shows a decrease of about 6.3%. We do not, as yet, have an explanation for this effect. Figure 6f shows the lifetime histogram for the image in Figure 6c.

We are currently designing a new MEM-FLIM chip that will include a number of improvements, designing a simple sensor to provide calibration information about the LED waveform shape so as to improve our estimation of the Fourier
coefficients, investigating the cause of the “shading” shown in Figure 6e, and shortening the duty cycle of the LED pulse to provide more power in the higher harmonics. We view model-based FLIM, mb-FLIM, as a strategy that will allow us straightforward access to single and multiple fluorescent lifetime information.

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