Hardware implementation algorithm and error analysis of high-speed fluorescence lifetime sensing systems using center-of-mass method

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Abstract. A new, simple, high-speed, and hardware-only integration-based fluorescence-lifetime-sensing algorithm using a center-of-mass method (CMM) is proposed to implement lifetime calculations, and its signal-to-noise-ratio based on statistics theory is also deduced. Compared to the commonly used iterative least-squares method or the maximum-likelihood-estimation-based, general purpose fluorescence lifetime imaging microscopy (FLIM) analysis software, the proposed hardware lifetime calculation algorithm with CMM offers direct calculation of fluorescence lifetime based on the collected photon counts and timing information provided by in-pixel circuitry and therefore delivers faster analysis for real-time applications, such as clinical diagnosis. A real-time hardware implementation of this CMM FLIM algorithm suitable for a single-photon avalanche diode array in CMOS imaging technology is now proposed for implementation on field-programmable gate array. The performance of the proposed methods has been tested on Fluorescein, Coumarin 6, and 1,8- anilinonaphthalenesulfonate in water/methanol mixture.

Keywords: lifetime-based sensing; fluorescence lifetime imaging microscopy; time-resolved imaging; photon counting; single-photon avalanche diode; center-of-mass.

1 Introduction

Time-resolved fluorescence lifetime imaging (FLIM) is widely used in cell-biology research, medical diagnosis, and pharmacological development.1-3 It is based on the measurement of the decay in fluorescence emission across a sample after optical excitation and can be used to quantify physiological parameters, such as pH, Ca2+, pO2, etc., in biological samples. The independence of fluorescence lifetimes from probe concentration makes FLIM more favored than its counterpart—fluorescence intensity imaging. As shown in Fig. 1(a), a laboratory FLIM experiment usually contains a Ti-sapphire laser, a photomultiplier tube (PMT), a time-correlated single-photon counting (TCSPC) photon-counting card, fluorescence lifetime analysis software, and a PC graphical user interface (GUI). Available FLIM systems provide excellent time resolution and light sensitivity, although they are quite expensive and cumbersome. Commercial applications increasingly demand compact and portable system-on-chip (SOC) FLIM solutions. Thanks to the progress of semiconductor technology, high-accuracy time resolution, high sensitivity, low cost, and compactness can be achieved by exploiting CMOS single-photon avalanche diode (SPAD) arrays with low dark count rate to replace PMTs4-7 and by bump-bonding AlInGaN UV micropixelated light-emitting diodes to replace lasers8 in the general direction of lab on chip. The imager can include a CMOS SPAD array with in-pixel digital counters or time-to-digital converters (TDCs)9,10 that allows recording not just the photon counts but also the raw timing data for detailed scientific analysis. The imager also contains Field-programmable gate arrays (FPGAs) allowing data processing. Figure 1(b) shows the SOC solution suited to lab-on-chip applications, which is intended to replace the system of Fig. 1(a). For imaging purposes, a remaining challenge is that the excessive computational demands of available lifetime analysis software such as the iterative least-squares method (LSM) or maximum-likelihood-estimation (MLE) render real-time imaging impossible. A new FLIM algorithm considering the instrument response based on the Laguerre expansion technique9 speeds up lifetime calculations, but the computation time increases with imager size. However, in many applications, such as microfluidic mixing10 and exploratory biological experiments, it is desirable to monitor the instantaneous biochemical interactions to provide quick feedback to corresponding manipulations. The slow speed of LSM- or MLE-based software analysis tools becomes a
bottleneck and has driven the recent development of noniterative, compact, and fast real-time domain FLIM systems,11–17 and real-time frequency-domain FLIM algorithms and systems.18–21 In the past, rapid lifetime determination methods (RLD) were thought to be the simplest algorithms10 and were used in some previously reported video-rate FLIMs.12,13 In Ref. 12, an optomechanical delay control for RLD was proposed; however, its cumbersome optical setup makes it difficult to image a wide range of fluorophore lifetimes, and an electronically controllable delay would be preferable.14 To further achieve compactness for SOC, we can exploit configurable devices such as FPGAs to realize real-time FLIM systems. FPGAs have significantly benefited from the advances of CMOS technology. The latest FPGAs contain over hundreds of millions of transistors and can easily accommodate the output signals from SPAD arrays of growing size. With the ability of configuration, designers can easily reconfigure FPGAs by hardware description language to perform any application-specific logic functions, such as real-time lifetime calculations. We therefore evaluated the possibility of applying RLD either on chip or on FPGA and concluded that RLD can be implemented on FPGA with the possibility of applying RLD either on chip or on FPGA.

Fig. 1 (a) Laboratory FLIM and (b) FLIM system on chip.

Fig. 2 (a) Center of mass of a single-exponential function and (b) concept of single-exponential CMM.

comparison of various fitting algorithms. Moreover, a single-exponential decay model is still useful to contrast different types of fluorophores. For diagnostic applications, obtaining lifetime contrast is probably more important than calculating the absolute values of lifetimes.13 The FPGA implementation and Verilog/Matlab modeling of the center-of-mass (CM) method (CMM) will be introduced. The performance of the proposed algorithm will be tested using Fluorescein, Coumarin 6, and 1,8-anilinonaphthalenesulfonate (ANS) in water/methanol.

2 Theory

2.1 CM of a Single-Exponential Function

For an object with a continuous distribution of mass density $f(r)$ and total mass $M_T$, its CM is defined as

$$\text{CM} = \frac{\int r f(r) dV}{\int f(r) dV} = \frac{\int r f(r) dV}{M_T}.$$  

(1)

For a mass density of a single-exponential function $f(t) = A \exp(-t/\tau)$ in the range $0 \leq t \leq T$, we have

$$\int_0^T tf(t) dt = \int_0^T A t \exp(-t/\tau) dt = A \tau^2 (1 - e^{-T/\tau}) - A \tau T e^{-T/\tau}$$

$$= \tau \int_0^T f(t) dt - A \tau T e^{-T/\tau}.$$
As \( T > \tau \), the center of mass lies at the position with a distance of \( \tau \) from the origin as Fig. 2(a) shows. If \( f(t) \) represents a fluorescence histogram, then the denominator of Eq. (2) will be the total photon count, while the numerator is the sum of temporal information of total photon events. To implement Eq. (2) in hardware, denoted as CMM for simplicity, we need to quantize the temporal information by dividing the measurement window into \( M \) time bins (bin width of \( h \)), as shown in Fig. 2(b), using TDCs in the photon counting module. An interesting analog circuit was proposed to calculate Eq. (2) for single-molecule microscopy,\(^{16}\) however, it did not describe how to remove background noise in the analog domain. In such applications with low fluorescence emission, background-to-signal ratio will be relatively significant. Compared to Ref. 16, CMM works in the digital domain and allows background noise to be removed much more easily.

2.2 Error Analysis of CMM

In Ref. 22, it was shown that Eq. (2) is equivalent to MLE when \( M \to \infty \); therefore, CMM can be viewed as hardware version of MLE. When the ratio of the full width at half maximum (FWHM) of the instrumental response function (IRF) over the lifetime is \( \ll 1 \), we can assume the fluorescence decay histogram \( f(t) = A \exp(-t/\tau) \) with \( \tau \) being the lifetime.\(^{17}\) For the usual measurement setup in a lab, the FWHM of the IRF is on the order of hundreds of picoseconds; thus, it is reasonable to target lifetimes of \( >500 \) ps. With the assumption of single-exponential decay, the lifetime \( \tau \) is related to the decay function as

$$
\tau = \int_0^\infty \frac{\Delta t f(t) dt}{\int_0^\infty f(t) dt} = \frac{\int_{t_0}^{t_1} \Delta t f(t) dt + \cdots + \int_{t_M}^{t_{M+1}} \Delta t f(t) dt}{\int_{t_0}^{t_1} f(t) dt + \cdots + \int_{t_M}^{t_{M+1}} f(t) dt},
$$

where

$$
\Delta t = t_j - t_0 + h/2 = (j + 1 - 1/2)h, \quad N_j = \text{number of recorded counts in the } j\text{'th time bin}, \quad M = \text{the number of time bins}, \quad N_i = \text{the total effective signal count}.
$$

The recorded variables \( N_j \) are independently Poisson distributed with respective mean value \( EN_j = \int_0^{T+h} f(t) dt \) and standard deviation \( \sigma N_j = (EN_j)^{1/2} \), and we thus have

$$
N_j = EN_j + \sigma N_j = N_i \exp(-x)(1-x^{M+1}) + \sigma N_j,
$$

where \( x = \exp(-h/\tau) \). Substituting Eq. (4) into Eq. (3), we have

$$
\tau\text{CMM} = \frac{\sum_{j=0}^{M-1} \Delta t_i EN_j + \sum_{j=0}^{M-1} \Delta t_i \sigma N_j}{\sum_{j=0}^{M-1} (EN_j + \sigma N_j) - \sum_{j=0}^{M-1} \sum_{j=0}^{M-1} \sigma N_j} = \frac{U + \sigma U}{V + \sigma V} = \tau + \frac{\Delta \tau}{\tau} \left( 1 + \frac{\sigma U}{U} - \frac{\sigma V}{V} \right).
$$

From Eqs. (4) and (6), we have

$$
U = \frac{h(1-x) \sum_{j=0}^{M-1} (j + 1 - 1/2)x^j}{1 - x^M} = \frac{h(1-x)}{1-x^M} - \frac{h}{2} = h \left( \frac{1 - (M + 1)x^M + Mx^{M+1}}{(1-x)(1-x^M)} - \frac{1}{2} \right) = hG(x) = \tau + \frac{\Delta \tau}{\tau}.
$$

Therefore, we have the accuracy equation

$$
\frac{\Delta \tau\text{CMM}}{\tau} = \frac{h}{\tau} G(x) - 1,
$$

and

$$
G(x) = \frac{1 + x - (2M + 1)x^M + (2M - 1)x^{M+1}}{2(1-x)(1-x^M)}.
$$

From Eqs. (5)–(7), we have the precision equation

$$
\frac{\sigma \tau\text{CMM}}{\tau} = \frac{\sigma U}{U} - \frac{\sigma V}{V} = \frac{h}{\tau} G(x) \left( \frac{\sigma U}{U} - \frac{\sigma V}{V} \right),
$$

where

$$
\sigma U = \sqrt{\sum_{j=0}^{M-1} \left( \frac{\Delta t_i}{U} \right)^2 \sigma^2 N_j} = \frac{1}{\sqrt{N_i}} G(x) \sqrt{\frac{1 - x}{1 - x^M} \sum_{j=0}^{M-1} \left( \frac{1}{2} G(x)^2 \right)^x \sqrt{P(x)}}
$$

and

$$
\sigma V = \frac{1}{\sqrt{N_i}} G(x) \left( \frac{1}{1 - x} (1-x^M) G(x) \right).
$$
The accuracy of the CMM lifetime estimator is determined by the quantization error in Eq. (3). It is usually predictable and can be calibrated by software, whereas the precision (normalized standard deviation) mainly comes from Poisson noise and can be improved only through increasing photon count. From Eqs. (2) and (3), we can also calibrate the lifetime by

\[ \tau_{\text{CMM, Cal}} = \tau_{\text{CMM}} + \frac{Te^{-Mh/\tau_{\text{CMM}}}}{1 - e^{-Mh/\tau_{\text{CMM}}}} \left( R + \frac{Me^{-Mh/R}}{1 - e^{-Mh/R}} \right) h. \]

This calibration can be easily done by software and can improve the accuracy further to \( T > 4\pi \).

Figure 3 shows the inverse accuracy and precision curves of Eqs. (7) and (9) (for easily transferred to decibels) for \( M = 1024 \) and \( N_c = 2^{17} \). The theoretical results marked as solid lines are compared to Monte Carlo simulations marked with crosses, giving good agreement and proving the correctness of Eqs. (7) and (9). Theoretical precision curves of 1024-bin MLE, and 2-gate RLD (with gate width \( w_g = Mh/2 = 512h \)) are also provided for comparison. From Fig. 3, the optimal window for RLD is from \( Mh/\pi = 1–5 \), whereas that for CMM is from \( Mh/\pi = 7–100 \). Here, we define a new precision value for CMM as

\[ \text{Precision} = \frac{\tau_{\text{CMM}}}{\sqrt{\sigma_{\tau_{\text{CMM}}}^2 + \Delta \tau_{\text{CMM}}}^2}. \]

In applications, there is no need to define this new precision as long as the accuracy can be enhanced by software calibration as described above. For simplicity, we assume there is no software calibration available. The new precision definition facilitates end users to familiarize themselves with the sensing system and easily choose a proper parameter. The new precision curve is also shown in Fig. 3. Its optimal window is the same as that of MLE from \( Mh/\pi = 10–100 \).

In some applications, we need to know the range of lifetimes that a predictor can resolve when the laser repetition rate (LPR) or the measurement window (MW) is fixed. We use the \( F \) value introduced in Ref. 23 to quantify the performance of a lifetime imaging technique. The \( F \) value is defined as \( F = N_c^{12} \sigma \tau / \tau \), where \( \sigma \tau \) is the standard deviation of repeated measurements of the lifetime value \( \tau \). Figure 4 shows \( F \) curves for 1024-bin CMM, 4096-bin CMM, 1024-bin MLE, 15-bin IEM, and 2-gate RLD in terms of \( \tau \) normalized by measurement window (MW=\( Mh \)). The MLE demonstrates the best resolvability range. However, it is not possible to implement it in hardware. For the other three methods, only CMM has a flat optimal \( F \) response. Taking LPR=5 MHz as an example and assuming the TDC full range is equivalent to the measurement window, MW=200 ns. If \( N_c = 2^{17} \), then the lifetime range with a precision of 40 dB (\( F \approx 4 \)) for 2-gate RLD, 15-bin IEM, 1024-bin CMM, and 1024-bin MLE are 16–200, 12–140, 0.6–30, and 0.02–320 ns, respectively. For RLD, the optimal window can be chosen by selecting proper delays, mechanically or electronically. For CMM and IEM, the optimal window can be easily set on FPGA by choosing a proper \( M \). In theory, the lower bound of CMM and MLE can be further reduced by increasing \( M \). However, it is limited by the FWHM of the system IRF, which can be several hundreds of picoseconds, considering jitter contributed by the SPADs, laser, and TDCs. Therefore, increasing \( M \) further for real-time, single-exponential lifetime estimation is not sensible. A comparison summary for the CMM, IEM, RLD, and the MLE algorithms is provided in Table 1. It clearly shows the merits of the CMM in terms of \( F \) value, lifetime resolvability, and on-chip feasibility. From Fig. 4, the advantage of CMM is that its photon collecting efficiency, for a given precision, is 2.5-
Table 1 Comparison summary of the CMM, IEM, RLD, and MLE.

<table>
<thead>
<tr>
<th>Method</th>
<th>$F_{\text{max}}$ at $\tau$</th>
<th>$F_{&lt;4}$ resolvability</th>
<th>On-chip feasibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>1.5</td>
<td>0.08 $&lt; \tau$/MW $&lt; 1$</td>
<td>Yes/LUT</td>
</tr>
<tr>
<td>RLD-2</td>
<td>2.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IEM w/o Calibration</td>
<td>1.6</td>
<td>0.06 $&lt; \tau$/MW $&lt; 0.7$</td>
<td>Yes</td>
</tr>
<tr>
<td>IEM with Calibration</td>
<td>1.2</td>
<td>0.03 $&lt; \tau$/MW $&lt; 0.7$</td>
<td>Yes</td>
</tr>
<tr>
<td>MLE $M=1024$</td>
<td>1.0</td>
<td>$1 \times 10^{-4}$ $&lt; \tau$/MW $&lt; 1.6$</td>
<td>No</td>
</tr>
<tr>
<td>CMM $M=1024$</td>
<td>1.0</td>
<td>$3 \times 10^{-3}$ $&lt; \tau$/MW $&lt; 0.15$</td>
<td>Yes</td>
</tr>
<tr>
<td>CMM $M=4096$</td>
<td>1.0</td>
<td>$7 \times 10^{-4}$ $&lt; \tau$/MW $&lt; 0.15$</td>
<td>Yes</td>
</tr>
</tbody>
</table>

$^a$The optimal $h$ of IEM is independent of $M$. $^b$On a small detector array.

2.3 Error Analysis of CMM with Background Correction

In most practical lifetime analysis tools, background is taken into account by the subtraction of a dc background value $C_0$ ($= N_0/M$, $N_0$ is total background noise within a measurement window of $Mc$) from the measured histogram, and along with lifetime calculations, this is done by software. However, for higher real-time imaging it is desirable that a hardware calibration technique can be integrated into the system by generating the required $C_0$ using the available counts. Figure 5 shows a typical measured histogram (of 1 μM Fluorescein, detailed in Section 4). There always exists a flat response before the peak of the histogram decided by the delay between systems enable signal and laser excitation. A dc value of $C_0$ can be obtained by averaging the counts of several bins on the flat response. Suppose we have a white background noise response, and we can therefore obtain the background count as $N_b = MC_0$, from Eq. (5), and by subtracting $C_0$ from the count in each bin, we have

$$\tau_{\text{CMM,corr}} = \frac{M-1}{h} \sum_{j=0}^{M-1} \frac{\Delta f_j}{h} (N_j - C_0) = \frac{M-1}{N_{\text{total}} - MC_0} \sum_{j=0}^{M-1} \left( j + \frac{1}{2} \right) N_j - \frac{MN_b}{2},$$

where $N_{\text{total}} = N_c + N_b$.

$$N_j = EN_j + \sigma N_j = N_c \frac{x^l(1-x)}{1-x^M} + \frac{N_b}{M} + \sigma N_j,$$

$\frac{\Delta f_j}{h}$ is the count in each bin, we have

$$\tau_{\text{CMM,corr}} = \frac{M-1}{h} \sum_{j=0}^{M-1} \frac{\Delta f_j}{h} (N_j - C_0) = \frac{M-1}{N_{\text{total}} - MC_0} \sum_{j=0}^{M-1} \left( j + \frac{1}{2} \right) N_j - \frac{MN_b}{2},$$

where $N_{\text{total}} = N_c + N_b$.

$N_j = EN_j + \sigma N_j = N_c \frac{x^l(1-x)}{1-x^M} + \frac{N_b}{M} + \sigma N_j,$

**Fig. 5** Measured fluorescence histogram of Fluorescein by a CMOS SPAD.
It is quite easy to implement Eq. (4) because it does not need a clock smaller than an adder for the numerator and a counter for the denominator, we do not even need digital division by only taking the first \( K \) most significant bit (MSB) bits of the register or more than \( K \) MSB bits for decimal accuracy. The second term 1/2 on the right-hand side can be kept in mind or simply merging Eqs. (16) and (17) as

\[
N_c = 2^L, \quad L \text{ is an integer.}
\]

When this condition is reached, a trigger signal is sent to latch the latest \( \tau_{\text{CMM}}/h \) and reset the register to perform the next calculation and keep updating the lifetime. By this arrangement, we do not even need digital division by only taking the first \( K \) most significant bit (MSB) bits of the register or more than \( K \) MSB bits for decimal accuracy. The second term 1/2 on the right-hand side can be kept in mind or simply merging Eqs. (16) and (17) as

\[
\tau_{\text{CMM}}/h = \sum_{i=1}^{N_c} \bar{D}_i + \frac{1}{2^L}.
\]
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Though in practice, there is no need to do so according to Eqs. (16) and (17). Taking a single decay function $f(t) = A \exp[-t/(21h)]$ as an example, if $h$ is of 200 ps (the full range of the TDC is $256h = 51.2$ ns, which is equivalent to a LPR = 20 MHz). The lifetime $\tau = 21h = 4.2$ ns is much larger than a typical jitter of 300 ps such that only tail fitting is applied to extract the lifetime without digital deconvolution.

A measurement window of $M = 200$ (44th to 243rd bin) from the peak of the histogram is chosen for lifetime calculations. Figure 8 shows the decay histogram $N_t$ obtained by the model and the fitted curve $N_f$ by CMM with background correction of Eq. (14) using Verilog. The calculated lifetimes with four extra bits for decimal accuracy obtained by CMM and RLD with/without background correction are listed, respectively. The reduced chi-squared is 1.10 showing a good fit, and Fig. 8 also shows the normalized residual plot of $(N_r - N_f)/N_f^{1/2}$, which is well distributed, implying that the model is Poisson distributed as in real cases.

The second example is $f(t) = A \exp[-t/(2h)]$, with a lifetime $\tau = 2h = 400$ ps at LPR = 20 MHz. We are comparing CMM to other algorithms with $M = 200$. CMM is not sensitive to the timing jitter. For RLD, it is a challenging task to resolve lifetimes, much less than the effective measurement window ($\tau \approx 200h$ in this case). Thus, we use MLE$^3$ instead to calculate the lifetime with software using

$$1 + (x^{-1} - 1)^{-1} - M(x^{-M} - 1)^{-1} = N_r^{-1} \sum_{j=0}^{M-1} (j+1)N_j, \quad (22)$$

where $x = \exp(-h/\tau)$. In this example, all algorithms are performed on the computer for a fair comparison, and therefore, there is no digital quantization error for CMM. The reduced chi-squared is 0.94, and Fig. 9 shows the decay histogram and residual obtained by the model and the fitted curve by CMM with background correction using Matlab. The calculated lifetimes for CMM and MLE are also listed. For CMM and MLE, it is necessary to apply background correction when resolving lifetime much less than the measurement window. It is also interesting to note that the behavior of CMM and MLE are almost identical in terms of precision and sensitivity to background noise in the optimal lifetime range; therefore, CMM can be viewed as a hardware implementation algorithm of...
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4 Experimental Results

4.1 Measurements of Fluorescein and Coumarin 6 Using SPADs

Measurements of the decays of Fluorescein and Coumarin 6 mounted on microcavity slides have been made to test the proposed CMM hardware lifetime calculation algorithm. Table 2 lists the fluorophores under test in terms of solvent, concentration, excitation and emission wavelengths, typical lifetimes provided by the manufacturers, and the calculated lifetimes using CMM, MLE, RLD-2, and Edinburgh Instruments F900 software. Then, 45 μL of each sample was pipetted into a single-cavity (15-mm diam) glass microscope slide (Fisher Scientific, United Kingdom, MNK-140-010A) and sealed with a 0.12-mm-thick borosilicate glass coverslip (Fisher Scientific, MNJ-300-020T). The LPR (PicoQuant pulsed diode laser with wavelength of 470 nm) is 10 MHz, and the average output power is 0.12 mW. Fluorescence decay curves were recorded on a time scale of 100 ns, resolved into 1024 time bins (i.e., $h \sim 0.098$ ns). With LPR of 10 MHz, there is no bleed through observed on measured histograms. The fluorescence emission is captured by a SPAD array fabricated in a 0.35-μm CMOS high-voltage process mounted on a daughter board. Figure 5 shows the measured histogram of Fluorescein, and Fig. 11 shows the logarithmic plot for the measured histogram $N_i$, starting from the bin with the peak intensity and the fitted curve $N_i$ by CMM with background correction, and also the normalized residual count. The reduced chi-squared is 1.40. The last three rows of Table 2 show the calculated lifetimes with background correction for CMM, MLE, RLD, and Edinburgh Instruments F900 software, respectively. Measurement windows of $5−20/30\tau$,

![Figure 10](https://example.com/figure10.png)

**Fig. 10** Inverse precision curves versus total count of 800-bin CMM and 2-gate RLD for different lifetimes.

MLE although their physical definitions are not the same. Figure 10 shows inverse precision curves versus total count for 800-bin CMM and 2-gate RLD ($2w_g=800\tau=1\tau$ or $4\tau$) with Monte Carlo simulations for different lifetimes. CMM displays its uniform performance and higher photon-counting efficiency over a wide range of lifetimes. Therefore, CMM is suitable for low light detection.

<table>
<thead>
<tr>
<th>Table 2 Summary of fluorophores used.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorophore</td>
</tr>
<tr>
<td>Solvent</td>
</tr>
<tr>
<td>Concentration (μM)</td>
</tr>
<tr>
<td>Excitation wavelength (nm)</td>
</tr>
<tr>
<td>Peak emission wavelength (nm)</td>
</tr>
<tr>
<td>Typical lifetime (ns)</td>
</tr>
<tr>
<td><strong>Calculated lifetime (ns)</strong></td>
</tr>
<tr>
<td>using CMM</td>
</tr>
<tr>
<td>$h=0.098;\text{ns}$</td>
</tr>
<tr>
<td>using MLE</td>
</tr>
<tr>
<td>$h=0.098;\text{ns}$</td>
</tr>
<tr>
<td>using RLD-2</td>
</tr>
<tr>
<td>$1\tau &lt; 2w_g &lt; 5\tau$</td>
</tr>
<tr>
<td><strong>Calculated lifetime (ns)</strong></td>
</tr>
<tr>
<td>using Edinburgh Instruments F900</td>
</tr>
<tr>
<td>$h=0.098;\text{ns}$</td>
</tr>
</tbody>
</table>

*Maximum measurement window for Fluorescein $\sim 20\times4.1$ ($\sim 800\tau$).

*Maximum measurement window for Coumarin 6 $\sim 30\times2.5$ ($\sim 800\tau$).
0.5–20/30τ, 1–5τ, and 1–20/30τ are chosen for CMM, MLE, RLD-2, and F900, respectively. The mean lifetimes of Fluorescein and Coumarin 6 calculated by CMM, are 4.15 and 2.42 ns, respectively, in good agreement with the data provided by the manufacturers and are also comparable to other algorithms.

4.2 Measurements of ANS in Water/Methanol Using PMTs

Fluorescent dye ANS is widely used in biological experiments due to its extreme sensitivity to the composition of water/methanol mixtures, showing a drastic variation in lifetime from 250 ps in pure water to 6 ns in pure methanol. The excitation light comes from a Ti-sapphire laser (LPR = 4.75 MHz using a pulse picker) with a laser power of 0.1 mW. The concentration of ANS is 1 mM. The ANS in the water/methanol mixture with a concentration of water of 0, 10, 20, 30, and 100% \( \nu/\nu \), respectively, is measured by a PMT. Figure 12 shows the measured and fitted fluorescence histograms obtained by the PMT and CMM, respectively. The fluorescence histograms of ANS display a single-exponential decay as stated in the previously reported literature, making it an ideal probe of solvent composition. The calculated lifetimes in terms of water concentration obtained by CMM, the prediction function in Ref. 10, and Edinburgh Instruments F900 software are listed in Table 3. They are in a good agreement with one another. The reduced chi-squared is also listed in Table 3.

Table 3 Comparison of calculated lifetimes of ANS between CMM, prediction function, and Edinburgh Instruments F900 software.

<table>
<thead>
<tr>
<th>Percentage ( \nu/\nu ) (%)</th>
<th>CMM (ns)</th>
<th>Ref. 10 (ns)</th>
<th>F900 (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>reduced chi-squared</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>6.08/1.7</td>
<td>5.97</td>
<td>6.13</td>
</tr>
<tr>
<td>10</td>
<td>4.87/1.01</td>
<td>4.76</td>
<td>4.86</td>
</tr>
<tr>
<td>20</td>
<td>3.49/1.10</td>
<td>3.38</td>
<td>3.47</td>
</tr>
<tr>
<td>30</td>
<td>2.49/1.02</td>
<td>2.4</td>
<td>2.48</td>
</tr>
<tr>
<td>100</td>
<td>0.28/1.22</td>
<td>0.25</td>
<td>0.27</td>
</tr>
</tbody>
</table>

5 Conclusion

We have proposed a very simple FLIM algorithm called CMM [Eq. (3)] for real-time applications and derived the theoretical error equations [Eqs. (7) and (9)] for easily obtaining the best recording parameters, such as measurement window, width of a time bin, and bit resolution of the TDC. The method has the potential of using the available photons efficiently, provided that the recording parameters are correctly optimized. For single-exponential lifetime imaging, the algorithm provides the same precision level as the MLE in the optimal window with \( F \) of 1.0. An interesting result of our study is that the optimum performance of \( F \sim 1.0 \) can be obtained at...
10h < τ < 0.1Mh

(or 10h < τ < 0.14Mh, with software calibration)

(23)

or for \( F \sim 1.5 \) comparable to RLD-2 (\( \nu_g \sim 2.5\tau \))

\[ 5h < \tau < 0.35Mh. \]

(24)

The advantage of CMM over the other hardware algorithms is that CMM has higher photon-collecting efficiency (\( \gg 2.5 \)) than RLD/IEIM. For CMM of Eq. (3), without any differential term similar to that of IEM,\(^\text{17}\) the design specifications of in-pixel TDCs can be more relaxed. The FPGA implementation of this FLIM algorithm is proposed for the first time as Eqs. (17) and (18). Hardware implementation of CMM with background correction can also be easily implemented on FPGA with Eq. (14). The performance of CMM is successfully tested not only on Verilog/Modelsim synthetic data but also on real data collected by CMOS SPAD pixels and PMTs. CMM on the latest developed CMOS SPAD arrays has single-photon sensitivity and provides an efficient way of video-rate FLIM implementations; it is promising for imaging applications.

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**References**