Investigations on Average Fluorescence Lifetimes for Visualizing Multi-Exponential Decays

Yahui Li1,2,3, Sapermsap Natakorn4, Yu Chen4, Mohammed Safar1, Margaret Cunningham1, Jinshou Tian2,3 and David Day-Uei Li*1

1 Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, United Kingdom, 2 Key Laboratory of Ultra-fast Photoelectric Diagnostics Technology, Xi’an Institute of Optics and Precision Mechanics, Xi’an, China, 3 University of Chinese Academy of Sciences, Beijing, China, 4 Department of Physics, Scottish Universities Physics Alliance, University of Strathclyde, Glasgow, United Kingdom

INTRODUCTION

Fluorescence lifetime imaging (FLIM) is a crucial technique for assessing microenvironments of fluorescent molecules [1, 2], such as pH [3], Ca2+ [4, 5], O2 [6], viscosity [7], or temperature [8]. Combining with Förster Resonance Energy Transfer (FRET) techniques, FLIM can be a powerful “quantum ruler” to measure protein conformations and interactions [9–12]. Compared with fluorescence intensity imaging, FLIM is independent of the signal intensity and fluorophore concentrations, making FLIM a powerful quantitative imaging technique for applications in life sciences [13], medical diagnosis [14–16], drug developments [17–19], and flow diagnosis [20–22]. FLIM techniques can build on time-correlated single-photon counting (TCSPC) [23–25], time-gating [26–28], or streak cameras [29]; they record time-resolved fluorescence intensity profiles to extract lifetimes with a lifetime determination algorithm (LDA) [1]. There is a rapid growth of real-time applications that fast analysis is sought after [12, 30]. Traditional LDAs usually use the least square method (LSM) or maximum likelihood estimation (MLE) [31] to analyze decay models chosen by users, and model-fitting analysis follows a reduced chi-squared criterion [1]. In reality, however, it is difficult to know the exact decay model as fluorescent molecules in biological systems can demonstrate...
complex multi-exponential decay profiles. For instance, a mixture of fluorophores, a multi-tryptophan protein, single fluorophores in varied environments, and single-tryptophan proteins in multiple conformational states [1] can show multi-exponential decays as

\[ f(t) = A \sum_{i=1}^{p} q_i \exp(-t/\tau_i), \text{where} \sum_{i=1}^{p} q_i = 1, \quad (1) \]

where \( A \) represents the amplitude, \( q_i \) and \( \tau_i \) \((i = 1, \ldots, p)\) denote the amplitude fractions and lifetimes, respectively, and \( p \) is the number of lifetime components. There are time-domain or frequency-domain [32–35] FLIM systems to measure a fluorescence decay. In this work, we focus on time-domain approaches.

Suppose the instrument response function (IRF) of the measurement system is \( \text{irf}(t) \), the task performed by FLIM analysis tools is to extract \( f(t) \) from the measured decay \( h(t) \), as

\[ h(t) = \text{irf}(t) * f(t). \quad (2) \]

The problems with traditional LSM or MLE are two-fold. (1) It is challenging to categorize a fluorescence emission into a specific exponential model described by Equation (1) in complex biological processes. An arbitrary choice of \( p \) in Equation (1) simply based on reduced chi-squared tests [36] would lead to totally different interpretations. As the fitting routine is not mathematically unique; a measured decay could be fitted to totally different interpretations. As the fitting routine is

\[ \text{measurement system is} \quad \text{irf}(t), \quad \text{the task performed by FLIM} \]

(2) To ensure the accuracy, it usually needs a high photon count (long acquisition time) when \( p \geq 2 \) [37]. Instead of completely extracting \( q_i \) and \( \tau_i \) \((i = 1, \ldots, p)\), which is doubtful as mentioned above and time-consuming, in many applications, it is often useful to determine only the average lifetime which can be expressed in two forms [1]: the intensity-weighted average lifetime

\[ \tau_i = \frac{\sum_{i=1}^{p} q_i \tau_i^2}{\sum_{i=1}^{p} q_i \tau_i}, \quad (3) \]

and the amplitude-weighted average lifetime

\[ \tau_A = \frac{\sum_{i=1}^{p} q_i \tau_i}{\sum_{i=1}^{p} q_i} = \frac{\sum_{i=1}^{p} q_i \tau_i}. \quad (4) \]

The question about which average lifetime we should use according to the applications has been investigated in [38]. For instance, they suggested:

(a) \( \tau_A \) can estimate the energy transfer efficiency in FRET [39],

\[ E = 1 - \frac{I_{DA}}{I_D} = 1 - \frac{\tau_{DA,A}}{\tau_{DA}}, \quad (5) \]

where \( E \) is the energy transfer efficiency, \( I_{DA} \) and \( I_D \) are the fluorescence intensities of the donor in the presence and absence of energy transfer, respectively, and \( \tau_{DA,A} \) and \( \tau_{DA} \) are \( \tau_A \) of the donor in the presence and absence of energy transfer, respectively. \( E \) can further estimate the donor-acceptor distance.

(b) \( \tau_A \) can also assess dynamic quenching behaviors, described by the Stern-Volmer equation [40],

\[ \frac{I_0}{I_1} = 1 + K_D [Q] = \frac{\tau_{0A}}{\tau_{1A}}, \quad (6) \]

where \( I_0 \) and \( I_1 \) are fluorescence intensities, \( \tau_{0A} \) and \( \tau_{1A} \) are \( \tau_A \) of the fluorophore in the absence and presence of the quencher, respectively, \( K_D \) is the Stern-Volmer quenching constant, and \( [Q] \) is the concentration of the quencher. Additionally, the average radiative rate constant can be expressed as, \( k_r = QE/\tau_{1A} \), where \( QE \) is the quantum yield.

(c) \( \tau_A \) can be used to estimate the average collisional constant \( k_q \) from the Stern-Volmer constant \( K_D \).

Average lifetimes can either be calculated by extracting the lifetime components using model-based LDAs and then using Equations (3) and (4). Or they can be directly obtained with model-free LDAs, such as hardware-friendly center-of-mass methods (CMM) [41–44], the phasor method (Phasor) [45–47], the rapid lifetime determination method (RLD) [30, 48–51], or the integral extraction method (IEM) [52, 53], without assuming any decay model.

In this work, we theoretically investigated two types of average lifetimes evaluated by model-free LDAs, examined the performances of \( \tau_A \) and \( \tau_A \) estimations using different LDAs, and suggested the choices of LDAs in terms of accuracy, precision, and estimation speeds according to the applications. We also described a multi-exponential decay visualization tool using the ratio \( \tau_{1A}/\tau_{1} \). Experimental results demonstrate the performance of \( \tau_{1A}/\tau_{1} \) in comparison with Phasor.

2. THEORY

In this section, we derived the average lifetimes determined by the model-free methods, CMM, Phasor, and IEM and described the general work flow of average lifetime estimations with the model-free and model-based LDAs.

As Equation (2), the measured signal \( h(t) \) is the convolution of \( f(t) \) with \( \text{irf}(t) \). Here we focus on the signal \( h_m \) and \( \text{irf}_m \) obtained from a TCSPC system, as shown in Figure 1,

\[ h_m = \sum_{k=0}^{m} \text{irf}_{k-m} f_m, m = 0, 1, 2, \ldots, M - 1, \quad (7) \]

\[ \text{irf}_m = \int_{m\Delta t}^{(m+1)\Delta t} \text{irf}(t)dt, \]

\[ f_m = \int_{m\Delta t}^{(m+1)\Delta t} f(t)dt = A \sum_{i=1}^{p} q_i e^{-\frac{\Delta t}{\tau_i}} \left[ e^{-\frac{\Delta t}{\tau_i}} - 1 \right], \]

where \( h_m \) is the photon count collected in Bin \( m \) at \( t_m = (m + 1/2) \Delta t \), \( M \) is the number of bins, and \( \Delta t \) is the time resolution.

(a) CMM
The average lifetime evaluated with CMM is

$$\tau_{\text{CMM}} = \frac{\int_0^\infty t \cdot h(t) dt}{\int_0^\infty h(t) dt} = \frac{\sum_{i=1}^p q_i \tau_i^2}{\sum_{i=1}^p q_i \tau_i},$$

which is equal to $\tau_f$. The derivation of Equation (8) is shown in the Appendix.

(b) Phasor

The average lifetime evaluated with Phasor is

$$\tau_p = \frac{s}{g\omega} = \frac{\sum_{i=1}^p q_i \tau_i^2 / (1 + \omega^2 \tau_i^2)}{\sum_{i=1}^p q_i \tau_i / (1 + \omega^2 \tau_i^2)},$$

where $\omega = 2\pi/T$, $T = M\Delta t$ is the measurement window, and $g$ and $s$ are the phasor components expressed as

$$g = \frac{\int_0^\infty f(t) \cdot \cos(\omega t) dt}{\int_0^\infty f(t) dt} = \sum_{i=1}^p q_i \tau_i (1 + \omega^2 \tau_i^2),$$

$$R_h + s \cdot I_{\text{irf}} = \frac{g}{R_{\text{irf}}},$$

$$s = \frac{\int_0^\infty f(t) \cdot \sin(\omega t) dt}{\int_0^\infty f(t) dt} = \sum_{i=1}^p q_i \tau_i / (1 + \omega^2 \tau_i^2),$$

$$I_h \cdot R_{\text{irf}} - R_h \cdot I_{\text{irf}} = \frac{s}{R_{\text{irf}}^2 + I_{\text{irf}}^2},$$

where

$$R_h = \frac{\int_0^\infty h(t) \cdot \cos(\omega t) dt}{\int_0^\infty h(t) dt} \approx \frac{\sum_{m=0}^{M-1} h_m \cdot \cos(\omega t_m)}{\sum_{m=0}^{M-1} h_m},$$

$$I_h = \frac{\int_0^\infty h(t) \cdot \sin(\omega t) dt}{\int_0^\infty h(t) dt} \approx \frac{\sum_{m=0}^{M-1} h_m \cdot \sin(\omega t_m)}{\sum_{m=0}^{M-1} h_m},$$

$$R_{\text{irf}} = \frac{\int_0^\infty \text{irf}(t) \cdot \cos(\omega t) dt}{\int_0^\infty \text{irf}(t) dt} \approx \frac{\sum_{m=0}^{M-1} \text{irf}_m \cdot \cos(\omega t_m)}{\sum_{m=0}^{M-1} \text{irf}_m},$$

$$I_{\text{irf}} = \frac{\int_0^\infty \text{irf}(t) \cdot \sin(\omega t) dt}{\int_0^\infty \text{irf}(t) dt} \approx \frac{\sum_{m=0}^{M-1} \text{irf}_m \cdot \sin(\omega t_m)}{\sum_{m=0}^{M-1} \text{irf}_m}.$$
3.1. Case A: Model Mismatch

Ideally, a bi-exponential signal should be analyzed by a bi-exponential model. For instance, BCMM, VPM, and LSM-2 are used for bi-exponential decay models, and LSM-j for j-exponential models, j > 2. However, in realistic biological processes, it is difficult to know precisely how many lifetime components a decay profile contains. In traditional FLIM analysis tools, users usually need to select an exponential model to fit measured decays and use the reduced chi-squared to evaluate the goodness-of-fit. If the reduced chi-squared is not satisfactory, then a different exponential model is chosen. This process continues until the reduced chi-squared is acceptable. Often different exponential models can produce similar reduced chi-squared values, and the question is which fitting we should use? It is quite common that a j-exponential model might analyze a signal containing p lifetime components and j ≠ p. We would like to know if p is unknown to the user, whether using a different analysis model (j ≠ p) would lead to a different biological story.

We generated exponential decay signals \( h_m \) (\( m = 0, \ldots, M-1 \)) to test the LDAs for \( \tau_j \) and \( \tau_A \) estimations. \( h_m \) can be artificially generated with \( f(t) = A \sum_{q=1}^{p} q \exp(-t/\tau_q) \), where \( p = [1, 2, 3, 4] \), \( q_i = 1/p \), and the IRF is approximated with a Poisson distribution \( \exp(\lambda) \lambda^m/m! \) with \( \lambda = 500 \) ps, FWHM ≈ 300 ps, and \( M = 256 \). The measurement window \( T = 10 \) ns, and the total photon count \( N_{tot} = 10^3 \). \( \tau_t \), \( \tau_f \), and \( \tau_A \) for each \( p \) are summarized in Table 1.

The performances of \( \tau_t \) and \( \tau_A \) estimations with the simulated exponential decays are shown in Figures 3A–D for \( B_1, F_1, B_A \), and \( F_A \), respectively. For model-free LDAs, \( B_1 \) and \( B_A \) are below 10% and are independent of \( p \). For LSM-1, when \( p = 1, B_1 \), and \( B_A \) are zero, whereas when \( p > 1, B_1 \) and \( B_A \) increase especially for \( p = 2 \). For model-based LDAs, \( j > 1 \), BCMM, VPM, and LSM-j have similar performances even for \( p \neq j \), seemingly suggesting that a bi-exponential model can well approximate a signal following an arbitrary p-exponential model. We generated 500 signals with \( \tau_t \) and \( \tau_q \) chosen randomly in the ranges of 0.1 ∼ 2.5 ns and 0.1 ∼ 0.9 ns, respectively, for each \( p \). BCMM, VPM, and LSM-2 were used to fit the signals with bi-exponential decays. The goodness-of-fit is judged by the reduced chi-squared \( \chi^2 = \frac{1}{M} \sum_{i=1}^{M} (f_m - f_{cm})^2 / f_m \), where \( f_m \) and \( f_{cm} \) are actual and fitted signals of Bin \( m \). The box plots of \( \chi^2 \) for BCMM, VPM, and LSM-2 are shown in Figures 3E–G, respectively. The \( \chi^2 \) values are insensitive to \( p \) for the three LDAs so that we conclude that a bi-exponential decay is suitable to approximate an arbitrary \( p \)-exponential decay (\( p \leq 4 \)).

Therefore, if the decay model of the signal is inaccessible, model-free and model-based LDAs, BCMM, VPM, and LSM-2 are enough for \( \tau_t \) and \( \tau_A \) estimations.

In practice, users can choose an optimization algorithm and set initial conditions to analyze FLIM images when LSM-2 is used. We would like to know how they can affect \( \tau_t \) and \( \tau_A \) estimations. Four bi-exponential decays, \( \tau_1 \sim 4 \), with different parameters \( (q_1, \tau_1, \tau_2) \) were analyzed using LSM-2 with different initial conditions \( (q_{10}, \tau_{10}, \tau_{20}) \), denoted as Init. 1 ∼ 4 listed in Table 2 with \( N_{tot} = 10^3 \). When \( \tau_1 \) and \( \tau_2 \) are the estimates larger than \( T \) (10 ns), we say that the estimation fails. The probabilities of producing a failed trial, \( P(\tau_1 \sim 10) \) and producing biased \( \tau_1 \) and \( \tau_A \) with \( B_1 \) and \( B_A \) > 0.3, i.e., \( P(B_n > 0.3) \), \( n = I \) or \( A \), are shown in Figure 4. Figures 4A–F are the LSM-2 results with the unconstrained and constrained trust-region-reflective (TRR) algorithms, respectively. The constraints \( 0 < q_1 < 1 \) and \( 0 < \tau_1, \tau_2 < 10 \) ns. Figures 4G–I are the LSM-2 results using the Levenberg-Marquardt (LM) algorithm. For the unconstrained TRR, the performances are relatively sensitive to initial conditions. \( P(\tau_1 \sim 10) \) for Init. 4 is quite significant which results in large \( P(B_n > 0.3) \), \( n = I \) or \( A \).
or $A$, for all four decays. Although Init. 3 leads to a low $P$ for Decays $2 \sim 3$, $P(B_n > 0.3)$ for Decay 1 rises to 0.7. Thus, if the initial conditions are not chosen properly, the quality of $\tau_1$ and $\tau_A$ images cannot be guaranteed. The constrained TRR and LM are insensitive to initial conditions. Although the LM has failed trials, they barely affect $P(B_n > 0.3)$, $n = I$ or $A$. Therefore, to ensure accurate $\tau_1$ and $\tau_A$ estimations, the constrained TRR and LM are recommended for LSM-2.

### 3.1.2. Case B: Performances of Average Lifetime Estimations

As mentioned above, it might be challenging to use a proper exponential model to describe realistic biological processes; a bi-exponential model might well approximate them. Here we will use a bi-exponential model to explain why model-free LDAs have the benefits of higher photon efficiency and faster analysis than model-based LDAs for $\tau_1$ and $\tau_A$ estimations.

$h_m$ can be artificially generated with the same IRF used in Case A and $f(t) = A[q_1\exp(-t/\tau_1) + (1 - q_1)\exp(-t/\tau_2)]$, where $\tau_1 < \tau_2$ and $q_1$ is the amplitude fraction of $\tau_1$. Figure 5A shows the signal and IRF. In FRET and dynamic quenching applications, the fluorescence lifetime of the donor fluorophore is in general decreasing, and we assume $\tau_2 = 2.5$ ns and $\tau_1$ varying from 0.1 to 2.5 ns to emulate FRET or quenching. The theoretical $\tau_1$, $\tau_A$, and $\tau_P$ with $q_1 = 0.5$ are shown in Figure 5B. $\tau_P$ has a negative bias from $\tau_1$. With $T/\tau_2$ increasing, $\tau_P$ approaches $\tau_1$. Figure 5B that two different ($\tau_1$, $\tau_2$) sets can deliver the same $\tau_1$, for instance, (0.32, 2.5) ns and (2.1, 2.5) ns have the same $\tau_1$ of 2.3 ns.

Therefore, only estimating $\tau_1$ can be misleading. Figure 5B also shows that the dynamic range of $\tau_1$ is only 2.5–2.23 = 0.27 ns and within which the above problem persists. Whereas $\tau_A$ does not have this problem for this case. We conducted Monte Carlo simulations to estimate $\tau_1$ and $\tau_A$ with the simulated signals, including Poisson noise under different conditions $q_1 = 0.2, 0.5$, and 0.8.

The performances of $\tau_1$ and $\tau_A$ estimations with bi-exponential decay signals are shown in Figures 6A–D for $B_1$, $F_1$, $B_A$, and $F_A$, respectively. For $\hat{\tau}_1$, $B_{1,CMM}$, and $B_{1,BCMM}$ are roughly 10 and 8%, respectively determined by $T/\tau_2$. The larger $T/\tau_2$ is, the smaller $B_I$ becomes (with $F_{1,CMM}$ and $F_{1,BCMM}$ being closer to 1). Phasor has a lower accuracy when $q_1$ becomes larger and $\tau_1$ smaller, and it is less precise than CMM. VPM and LSM-2 both have a smaller $B_I = 3\%$ but higher $F_I (1.5 \sim 5)$ than CMM and BCMM. For $\hat{\tau}_A$, $B_A$ is 7% except for $\tau_1 = 0.1$ ns, and $F_A$ is around 5 for the four LDAs. Figures 6C,D show that if only $\tau_A$ is needed, there is no need to resort to slower model-based LDAs.

For $\tau_1$ estimations, LSM-2 and VPM are preferred when high accuracy is required. Still, they are slower and have lower photon efficiency than CMM and BCMM which means the photon count should be higher to have similar precision, for instance, a relative standard deviation of 5% can be reached with $N_{tot} = 3,600$ for LSM-2 and $N_{tot} = 500$ for CMM and BCMM. When the

### TABLE 2 | Bi-exponential decays and initial conditions for $\tau_1$ and $\tau_A$ estimations with LSM-2.

| Decay | Parameters | Init. | Initial conditions |
|-------|------------|------|--------------------|
|       | $q_1$ | $\tau_1$ | $\tau_2$ | $q_1^0$ | $\tau_1^0$ | $\tau_2^0$ |
| 1     | 1/2  | 0.1  | 2.5  | 1  | 1/2  | 0.1  | 0.5  |
| 2     | 4/5  | 0.1  | 2.5  | 2  | 1/2  | 0.1  | 2.5  |
| 3     | 1/2  | 0.5  | 1    | 3  | 1/2  | 2    | 4    |
| 4     | 1/2  | 1    | 2.5  | 4  | 1/2  | 4    | 6    |

FIGURE 3 | Performances of $\tau_1$ and $\tau_A$ estimations with exponential decay signals using different algorithms. (A) $B_1$, (B) $F_1$, (C) $B_A$, and (D) $F_A$. (E–G) Box plots of $\chi^2$ for BCMM, VPM, and LSM-2.
accuracy of CMM or BCMM (10% @ $T/\tau_2 = 4$) is acceptable, CMM or BCMM should be employed for their high photon efficiency and estimation speeds. CMM is faster than BCMM as it can work without deconvolution. For $\tau_A$ estimations, since the performances of IEM, BCMM, VPM, LSM-2 are similar, IEM can be the right candidate for fast analysis. Notice that the $\tau_A$ method is less photon efficient than the $\tau_I$ method as $F_A$ is higher than $F_I$.

### 3.2. Experimental Results

tSA201 cells, which are a transformed human kidney cell line, were co-transfected with hP2Y$_{12}$-eCFP and hP2Y$_1$-eYFP receptors. After 48 h of transfection, the cells on the coverslips were washed once gently with PBS followed by fixation with ice-cold methanol for 10 min at room temperature. After being washed three times with PBS, they were mounted on to glass microscope slides with Mowiol. The microscope slides were then stored in the dark at room temperature overnight to allow the coverslips to dry, then stored at 4°C for later use.

Cells were imaged on LSM510 (Carl Zeiss) equipped with a TCSPC module (SPC-830, Becker & Hickl GmbH), to determine the fluorescence lifetime and consequently the amount of FRET. The donor is CFP with the excitation wavelength range of 350 ~ 500 nm and the emission wavelength range of 450 ~ 600 nm. The acceptor is YFP. The sample is scanned pixel by pixel by a femtosecond Ti:Sapphire laser (Chameleon,
Coherent) with an average output laser power of 3.8 W at 800 nm, as a two-photon excitation source to reduce cellular damage. The laser power is controlled with two polarizers. The repetition rate is 80 MHz with illuminating duration <200 fs. The emitted fluorescence signal from the donor is collected through a 63× water-immersion objective lens (N.A. = 1.0), a 480 ~ 520 nm bandpass filter, and transferred into a photomultiplier tube (PMT) detector. The FLIM scanning was performed in a dark room containing the microscope. A set of experimental data (256 × 256 pixels, M = 256, T = 10 ns) was collected over an exposure period of up to 15 min. The IRF is obtained from the measurement of dried urea [(NH$_2$)$_2$CO]$_2$ with histogram classification methods (we will report the details soon), as shown in Figure 7E. Although Fast-IEM causes a small bias in some pixels, the mean square error is acceptable with 0.005 ns$^2$. The color bar represents lifetimes and the pixel brightness represents photon counts. The Figures 7F,G are histograms of $\tau_I$ and $\tau_A$, respectively. Although the histogram of $\tau_I$ with CMM deviates slightly from the one with LSM-2, CMM is 1,800-fold faster than LSM-2. If $T/\tau_I > 4$, the bias of $\tau_I$ with CMM would become smaller. The $\tau_A$ images are almost the same with IEM and LSM-2, whereas IEM and Fast-IEM are much faster than LSM-2.

Since the FRET efficiency E has a linear relationship with the average lifetimes as shown in Equation (5), Figures 7A–E can also be used to represent E images with the color bar in the range of 0 ~ 100%. As we mentioned in Introduction, it is straightforward to obtain E images from $\tau_A$ images, so that Figures 7C–E are proper E images. If $\tau_I$ images are misused for E images, the results would be different, as shown in Figures 7A,B, leading to a different biological story.

### 3.2.2. Visualization of Multi-Exponential Decays With $\tau_A/\tau_I$

$\tau_I$ and $\tau_A$ can not only access the essential parameters in FRET and dynamic quenching processes but also indicate the positions where multi-exponential decays occur. As mentioned previously, a fluorescence signal can be approximated by a bi-exponential decay, so that the ratio of $\tau_I$ and $\tau_A$ can be expressed as

$$\frac{\tau_A}{\tau_I} = \frac{[1 + q_1(R - 1)]}{1 + q_1(R^2 - 1)},$$

where $R = \tau_1/\tau_2$. The distribution of $\tau_A/\tau_I$ (Figure 8) shows that when $R \simeq 1$ or $q_1 \simeq 0$ or $1$, $\tau_A/\tau_I \simeq 1$. With a decrease of $R$ or an increase of $q_1$, $\tau_A/\tau_I$ decreases. Therefore, the ranges of $q_1$ and $R$ of a pixel can be determined by $\tau_A/\tau_I$.

To present the multi-exponential decay visualization performance of $\tau_A/\tau_I$, the $\tau_I$ and $\tau_A$ images evaluated by LSM-2,
FIGURE 7 | (A,C) \(\tau_I\) and \(\tau_A\) images evaluated with LSM-2; (B,D) \(\tau_I\) and \(\tau_A\) images evaluated with CMM and IEM, respectively; (E) \(\tau_A\) image with Fast-IEM. (F) Histograms of \(\tau_I\) with LSM-2 (blue) and CMM (black); (G) Histograms of \(\tau_A\) with LSM-2 (blue), IEM (magenta), and Fast-IEM (yellow). The color bar represents lifetimes and the pixel brightness represents photon counts. (A–E) Can also represent FRET efficiency (\(E\)) images evaluated with the corresponding lifetime images with the color bar representing the range of \(E\), 0 \(\sim\) 100%. (F, G) Can also be used to show the histograms of \(E\) with the upper x label.

Figures 9A,B, were used to generate the \(\tau_A/\tau_I\) image as shown in Figure 9C. The histograms of \(\tau_I\) and \(\tau_A\) and the phasor plot are shown in Figures 9D,E. Figure 9F shows the possible range of \(q_1\) and \(R\) of the selected pixels in Figure 9C. Figures 9C,F share the same color bar. Figure 9D shows that \(\tau_A\) has a broader lifetime dynamic range than \(\tau_I\), which is consistent with the theoretical lines shown in Figure 5B. The \(\tau_A\) histogram shows two clusters with different peaks, whereas the \(\tau_I\) histogram only indicates a single merged group, meaning that there is no way to differentiate these two clusters. It is why using \(\tau_I\) to analyze samples with a strong FRET can be misleading.

The results of the selected pixels within different \(\tau_A/\tau_I\) ranges are shown in Figure 10. \(\tau_A/\tau_I = 0.2 \sim 0.5\), and Figure 11, \(\tau_A/\tau_I = 0.5 \sim 1\). For the pixels with \(\tau_A/\tau_I = 0.2 \sim 0.5\), the histograms clearly show that \(\tau_A\) is much smaller than \(\tau_I\), which means the difference between \(\tau_1\) and \(\tau_2\) is significant. Figure 10F shows that
the ranges of $q_1$ and $R$ are approximately $0.5 \sim 1$ and $0 \sim 0.2$, respectively. For the pixels with $\tau_A/\tau_I = 0.5 \sim 1$, $\tau_A$ is closer to $\tau_I$, meaning the pixels have decays close to mono-exponential. Separating the average lifetime images with $\tau_A/\tau_I$ is easier than phasor plots because $\tau_A/\tau_I$ is one dimensional and phasors are two dimensional. Furthermore, $\tau_A/\tau_I$ can show the $q_1$ and $R$ ranges more intuitively than phasor plots. $\tau_A/\tau_I$ can be a useful tool to visualize the properties of the fluorescence decays within a lifetime image.

4. DISCUSSION

In realistic samples, fluorescence signals always follow multi-exponential decay models. However, extracting lifetime components with a traditional fitting method is a time-consuming process. For some applications that require calculating FRET efficiency and accessing dynamic quenching behaviors, average lifetimes are satisfactory. Model-free lifetime determination algorithms can be used to evaluate average lifetimes directly, for instance, CMM and Phasor for intensity-weighted average lifetimes $\tau_I$ and IEM for amplitude-weighted average lifetimes $\tau_A$. Discussions of the influence of the model mismatch between the real signal and the model-based LDAs on $\tau_I$ and $\tau_A$ estimations suggest that a bi-exponential model can well-approximate a signal following a multiple-exponential model. The results of the Monte-Carlo simulations suggest that VPM and LSM based on a bi-exponential model can be used for applications requiring high accuracy. The constrained TRR and LM algorithms with proper initial conditions are supported for LSM to guarantee accuracy. In contrast, CMM and IEM are recommended for applications requiring high estimation speeds.
FIGURE 10 | (A) $\tau_I$-intensity image, (B) $\tau_A$-intensity image evaluated by LSM-2, (C) $\tau_A/\tau_I$ ratio image, (D) histograms of $\tau_I$ (yellow) and $\tau_A$ (blue), (E) phasor plot, and (F) distribution of $\tau_A/\tau_I$ of the selected pixels in (C) with $\tau_A/\tau_I = 0.2 \sim 0.5$.

FIGURE 11 | (A) $\tau_I$-intensity image, (B) $\tau_A$-intensity image evaluated by LSM-2, (C) $\tau_A/\tau_I$ ratio image, (D) histograms of $\tau_I$ (yellow) and $\tau_A$ (blue), (E) phasor plot, and (F) distribution of $\tau_A/\tau_I$ of the selected pixels in (C) with $\tau_A/\tau_I = 0.5 \sim 1$. 
We also explained why τJ models can be misleading, and τA and τΔ models should be considered. Experimental data were used to compare the performances of LSM-2, CMM, and IEM for evaluating τJ and τΔ images. Similar τJ and τΔ images were generated, whereas CMM and IEM are much faster than LSM-2. The data were further analyzed with τΔ/τJ, which is capable of indicating the possible ranges of the amplitude proportion of the short lifetime and the ratio of the short and long lifetimes. We believe τΔ/τJ is a useful and intuitive tool for visualizing multi-exponential decays in a lifetime image.

**DATA AVAILABILITY STATEMENT**

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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APPENDIX

Derivation of $\tau_{\text{CMM}}$. Take the integration of $t \cdot h(t)$ and $h(t)$,

$$
\int_0^\infty t \cdot h(t) dt = \int_0^\infty t \int_0^\infty \text{irf}(t-t') \cdot f(t') dt' \, dt \\
= \int_0^\infty \int_0^\infty (t-t') \cdot \text{irf}(t-t') \cdot f(t') dt' \, dt \\
+ \int_0^\infty \int_0^\infty \text{irf}(t-t') \cdot t' \cdot f(t') dt' \, dt \\
= \int_0^\infty [t \cdot \text{irf}(t)] * f(t) dt + \int_0^\infty \text{irf}(t) * [t \cdot f(t)] dt \\
= \int_0^\infty t \cdot \text{irf}(t) dt \int_0^\infty f(t) dt \\
+ \int_0^\infty \text{irf}(t) dt \int_0^\infty t \cdot f(t) dt, \quad (A1)
$$

$$
\int_0^\infty h(t) dt = \int_0^\infty \text{irf}(t) dt \int_0^\infty f(t) dt. \quad (A2)
$$

Dividing Equation (A1) by Equation (A2) gives

$$
\frac{\int_0^\infty t \cdot h(t) dt}{\int_0^\infty h(t) dt} = \frac{\int_0^\infty t \cdot \text{irf}(t) dt}{\int_0^\infty \text{irf}(t) dt} + \frac{\int_0^\infty t \cdot f(t) dt}{\int_0^\infty f(t) dt}. \quad (A3)
$$

Then,

$$
\tau_{\text{CMM}} = \frac{\int_0^\infty t \cdot f(t) dt}{\int_0^\infty f(t) dt} = \frac{\sum_{i=1}^p q_i \tau_i^2}{\sum_{i=1}^p q_i \tau_i} \\
= \frac{\int_0^\infty t \cdot h(t) dt}{\int_0^\infty h(t) dt} - \frac{\int_0^\infty t \cdot \text{irf}(t) dt}{\int_0^\infty \text{irf}(t) dt} \\
\approx \frac{\sum_{m=0}^{M-1} t_m \cdot h_m}{\sum_{m=0}^{M-1} h_m} - \frac{\sum_{m=0}^{M-1} t_m \cdot \text{irf}_m}{\sum_{m=0}^{M-1} \text{irf}_m}. \quad (A4)
$$