Extended output phasor representation of multi-spectral fluorescence lifetime imaging microscopy

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Abstract: In this paper, we investigate novel low-dimensional and model-free representations for multi-spectral fluorescence lifetime imaging microscopy (m-FLIM) data. We depart from the classical definition of the phasor in the complex plane to propose the extended output phasor (EOP) and extended phasor (EP) for multi-spectral information. The frequency domain properties of the EOP and EP are analytically studied based on a multiexponential model for the impulse response of the imaged tissue. For practical implementations, the EOP is more appealing since there is no need to perform deconvolution of the instrument response from the measured m-FLIM data, as in the case of EP. Our synthetic and experimental evaluations with m-FLIM datasets of human coronary atherosclerotic plaques show that low frequency indexes have to be employed for a distinctive representation of the EOP and EP, and to reduce noise distortion. The tissue classification of the m-FLIM datasets by EOP and EP also improves with low frequency indexes, and does not present significant differences by using either phasor.

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References and links
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1. Introduction

The ultimate goal in fluorescence imaging and sensing is to detect and identify the sources of the measured fluorescence signal [1, 2]. Spectral approaches attain this by identifying flu-
orophores based on differences in their emission/excitation spectra. If the fluorophores have excitation/emission spectra that are highly overlapped (which is usually the case for endogenous sensing, when the fluorophores cannot be selected), fluorophore identification becomes more challenging [1]. Conventional fluorescence lifetime imaging microscopy (FLIM) methods measure the fluorescence lifetime at a specific emission band, but do not take into consideration the spectral properties of the fluorophores. Being able to measure the fluorescence lifetime at different emission bands, would allow more accurate identification of the fluorophores and the possibility of multiplexing a larger number of fluorescence molecules [3]. In the context of clinical diagnosis, the goal is to identify the normal and pathological state of the tissue, based on the tissue endogenous fluorescence characteristics. Being able to measure the fluorescence lifetime at different emission bands would facilitate the identification of the state of the tissue.

In this context, multi-spectral FLIM (m-FLIM) is a promising optical diagnosis tool that has been successfully applied to characterize skin and oral cancer [4–6], colonic dysplasia [7] and atherosclerotic plaques [8, 9], among others pathologies. In time-domain m-FLIM, the sample is excited with a pulse of light and the fluorescence emission intensity decay is recorded over a time-window at different wavelength bands according to the spectral properties of the studied components [9]. The band-limited responses can be concatenated to obtain a multi-spectral vector for each spatial point in the sample. This m-FLIM information can then be processed to study the quantitative biochemical structure of the tissue: the number of molecules in the sample, the multi-spectral profiles or end-members of the components, and their spatial concentrations or abundances [10–13]. This information can be extracted by considering a linear mixing model at each spatial point and assuming that the multi-spectral end-members are invariant throughout the sample [10, 14]. An alternative biochemical characterization of the sample can be achieved by quantifying the average lifetime and normalized intensity per spectral band and pixel in the estimated fluorescence impulse response [6, 8, 9].

Both m-FLIM processing approaches [6, 8–10, 14] can provide quantitative characterization of the sample fluorescence emission, but the required computational power can be prohibited for real-time applications. Moreover, time-resolved m-FLIM measurements are represented by high-dimensional vectors, whose time-domain properties are sometimes difficult to visualize for classification purposes. As a result, a low-dimensional and model-free representation of the m-FLIM measurements with a fast computation time will be appealing towards an intuitive real-time visualization. With this perspective, phasors in [15] were suggested for frequency domain fluorometry by mapping into a plane the modulation ratio and phase angle obtained for each studied frequency. Later this tool was proposed in [16] for time-domain FLIM by computing the Fourier transform of the tissue impulse response at the studied wavelengths and angular frequency of the light excitation. Other phasor representation relies on the process of fluorescence emission spectra [17] to achieve this low-dimensional mapping. Nonetheless, all phasors are bidimensional vectors that are quantified per wavelength band and at each spatial location in the sample. As result, the visualization of the phasors can be achieved in a plane, which produces a very intuitive graphical tool for data analysis. In this sense, there are important applications of the phasor tool in FLIM studies [18–23]. Furthermore, the phasor representation has been recently suggested to compute spectral unmixing [17], estimate average lifetimes in time-gated methods [24], achieve blind estimations [25], and perform simultaneous analysis of decay kinetics of donor and acceptor molecules in multispectral lifetime imaging [26]. Meanwhile, a quantitative comparison between the phasor approach and the standard fitting method based on the multi-exponential model was carried in [27], where the phasor technique produced more accurate estimations of the fluorescence lifetime values in synthetic and experimental datasets.

In this context, we propose a new alternative to the low-dimensional and model-free graphical visualization of the traditional phasor representation (TPR) by defining the extended output
phasor (EOP) and the extended phasor (EP). Our approach considers time-domain m-FLIM measurements sampled at a constant frequency and with enough samples to characterize accurately the fluorescent decays per channel [16]. Our proposed phasors have three important properties: (i) The EOP and EP fuse the multi-spectral fluorescence information at all wavelengths of the m-FLIM measurements; (ii) The EOP and EP have low complexity since they can be computed by a simple vector multiplication; and (iii) The EOP and EP provide distinctive features in a low-dimensional domain that can be used for classification purposes. In addition, the EOP can be directly evaluated without deconvolution of the instrument response from the measured m-FLIM data, so its implementation does not require extra processing of the dataset nor a previous knowledge of the instrument response. Furthermore, for low frequency indexes, the unique characterization through the TPR is preserved by EOP and EP, as well as the linear mixing feature of the corresponding end-members in the low-dimensional representation. However, our analysis shows that this low-dimensional representation has a limitation to characterize linear mixing models with high number of characteristic components.

The notation used in this document is described next. Scalars are denoted by italic letters, and vectors and matrices by lower and upper-case bold letters, respectively. \( \mathbb{R} \) and \( \mathbb{C} \) represent the real and complex numbers, respectively, and \( \mathbb{R}^N \) stands for \( N \)-dimensional real vectors. The imaginary unit is represented by \( j = \sqrt{-1} \), and for a complex scalar \( z \), its absolute value is represented by \( |z| \), its complex conjugate by \( z^* \), its phase by \( \angle z \), and its real and imaginary parts by \( \Re \{ z \} \) and \( \Im \{ z \} \), respectively. For a real vector \( \mathbf{x} \), the transpose operation is denoted by \( \mathbf{x}^\top \), its Euclidean norm by \( \| \mathbf{x} \| = \sqrt{x^\top x} \), and \( x \geq 0 \) represents that each component in the vector is non-negative. An \( N \)-dimensional vector filled with ones is represented by \( \mathbf{1}_N \), and for a set \( S \), \( \text{card}(S) \) denotes its cardinality. For an one-sided discrete time signal \( x[n] \) \( (x[n] = 0 \text{ for } n < 0) \), its discrete-time Fourier transform is defined by

\[
X(\omega) = \sum_{n=-\infty}^{\infty} x[n] e^{-j\omega n}
\]

where \( \omega \) denotes the discrete-time angular frequency. Finally, \( x[n]*y[n] \) stands for the convolution between discrete-time signals \( x[n] \) and \( y[n] \), and \( 1[n] \) denotes the step function.

2. Phasor representation

2.1. Traditional phasor

From the original descriptions in [16] and [24] for continuous time signals, we adopt a discrete-time formulation for the phasor computation of time-domain FLIM measurements. In our framework, we assume that the FLIM measurements consider \( \Lambda \) distinctive wavelengths and \( K \) spatial points. The fluorescence impulse response \( h_{k,\lambda}[n] \) at \( k \)-th spatial position and \( \lambda \)-th wavelength is assumed to be causal, i.e. \( h_{k,\lambda}[n] = 0 \) for all time indexes \( n < 0 \); and since it represents FLIM intensity values, all its observations are non-negative, i.e. \( h_{k,\lambda}[n] \geq 0 \) for all \( n \geq 0 \). Furthermore, we assume that there are \( I \) fluorophores in the sample. Hence for the \( i \)-th fluorophore, its multi-spectral fluorescence impulse response is characterized by its average lifetimes \( (\tau_{i,1}, \ldots, \tau_{i,\Lambda}) \) and normalized intensities \( (\delta_{i,1}, \ldots, \delta_{i,\Lambda}) \) at all wavelengths. As a result, based on a multieponential model [1, 2], the impulse response at \( k \)-th spatial point and \( \lambda \)-th wavelength \( h_{k,\lambda}[n] \) can be constructed by the convex combination [28] of \( I \) particular exponential modes \( \{ \delta_{i,\lambda} e^{-n/\tau_{i,\lambda}} \}_{i=1}^{I} \) [1, 2, 15]:

\[
h_{k,\lambda}[n] = \sum_{i=1}^{I} \alpha_{k,i} \{ \delta_{i,\lambda} e^{-n/\tau_{i,\lambda}} \} \cdot 1[n]
\]

where the coefficients \( \alpha_{k,i} \) are called the abundances or concentrations of \( i \)-th fluorescent decay end-member at \( k \)-th spatial position \( (\alpha_{k,i} \geq 0 \text{ and } \sum_{i} \alpha_{k,i} = 1) \). In this linear mixture model, the abundance \( \alpha_{k,i} \) does not depend on the measured wavelength of the m-FLIM response, and it describes the spatial concentration of all wavelengths of \( i \)-th component at \( k \)-th location.
Furthermore, the exponential scalings $\delta_{i,\lambda}$ are needed to identify the contribution of $i$-th single exponential component in each wavelength that later will be used to define the fluorescence end-member signals. Hence the TPR is defined as the normalized Fourier transform of Eq. (1):

$$ D_{k,\lambda}(\omega) \triangleq \frac{H_{k,\lambda}(\omega)}{H_{k,\lambda}(0)} = \frac{1}{H_{k,\lambda}(0)} \sum_{i=1}^{l} \alpha_{k,i} \frac{\delta_{i,\lambda} e^{j\omega \tau_{i,\lambda}}}{e^{j\omega \tau_{i,\lambda}} - e^{-1/\tau_{i,\lambda}}} \in \mathbb{C} \quad \omega \in [0, 2\pi]. $$

In [16], the frequency $\omega$ is chosen as the discrete-time angular frequency of light modulation. By the exponential modes in Eq. (1), the frequency response $H_{k,\lambda}(\omega)$ will exhibit a monotonic low-pass profile [29], and as a result, $H_{k,\lambda}(0) > H_{k,\lambda}(\pi)$ since $1/(1 - e^{-1/\tau_{i,\lambda}}) > 1/(1 + e^{-1/\tau_{i,\lambda}})$. Another important property is that for any specific frequency $\omega \in (0, 2\pi]$, the complex numbers expressed by $\{e^{j\omega} / (e^{j\omega} - e^{-1/\tau_{i,\lambda}})\}_{i=1}^{l}$ are all different. Consequently, the resulting TPR $D_{k,\lambda}(\omega)$ in Eq. (2) will be the sum of $l$ different and scaled ($0 \leq \alpha_{k,i} \leq 1$) complex numbers, so it will be a distinctive point in the complex plane which also satisfies

$$ \lim_{\omega \to 0} D_{k,\lambda}(\omega) = 1, \quad \& \quad \lim_{\omega \to \pi} D_{k,\lambda}(\omega) < 1. $$

2.2. Output phasor

In the previous analysis, the phasors are constructed departing from the fluorescence impulse responses $h_{k,\lambda}[n]$ of the m-FLIM measurements. However, in this section, we will show that in the low frequency range, the measured fluorescence decays can be employed to construct the phasor without a major distortion. For this purpose, the discrete-time fluorescence decay at $k$-th spatial point and $\lambda$-th wavelength is defined by a convolution operation as $y_{k,\lambda}[n] = h_{k,\lambda}[n] * u[n]$, where $u[n]$ stands for the instrument response in the m-FLIM system (causal signal), which is common for all spatial points in the dataset and wavelengths [1, 2]. Therefore, the output phasor (OP) is defined by

$$ P_{k,\lambda}(\omega) \triangleq \frac{Y_{k,\lambda}(\omega)}{Y_{k,\lambda}(0)} \frac{H_{k,\lambda}(\omega)U(\omega)}{H_{k,\lambda}(0)U(0)} \in \mathbb{C} \quad \omega \in [0, 2\pi] $$

Without loss of generality, and to allow an analytic derivation, we assume the following description for the instrument response $u[n]$: 

$$ u[n] = u_{n} n e^{-n/\tau_{u}} 1[n] \quad n \geq 0, $$

where $u_{n} > 0$ is a scaling parameter, and $\tau_{u} > 0$ is the equivalent time constant. Nonetheless, a similar result is obtained by assuming a delayed Gaussian pulse as instrument response. Note that as $\tau_{u} \to 0$ in Eq. (5) and with a proper scaling in $u_{n}$, the resulting pulse $u[n]$ has a narrower time support. Moreover, the time constant of the instrument response $\tau_{u}$ is assumed much smaller than the characteristic times of the fluorescence responses in Eq. (1), i.e. $\tau_{u} \ll \tau_{i,\lambda}$ for all $i, \lambda$. Hence the frequency response of the instrument response $U(\omega)$ is given by

$$ U(\omega) = u_{n} \frac{e^{-1/\tau_{u}} e^{j\omega}}{[e^{j\omega} - e^{-1/\tau_{u}}]^{2}}, $$

so the magnitude of $U(\omega)$ exhibits a monotonic low-pass response, but with a larger bandwidth than the components in $H_{k,\lambda}(\omega)$ for all $\lambda$. As a result, in the low-frequency range $\omega \approx 0$, $U(\omega)/U(0) \approx 1$ and we obtain

$$ P_{k,\lambda}(\omega) \approx \frac{H_{k,\lambda}(\omega)}{H_{k,\lambda}(0)} = D_{k,\lambda}(\omega), $$
i.e. the low frequency response of the TPR is roughly maintained by the OP, so there is no need for an initial processing to deconvolve the m-FLIM measurements just for \( \omega \approx 0 \) [30]; although the resulting values of TPR and OP will be in fact different. Meanwhile, if there is additive Gaussian noise influencing the measured fluorescence decays \( y_{\lambda}[n] \), the noise effect will be more severe at high frequencies in the output phasor \( P_{\lambda}(\omega) \), since the magnitude of \( P_{\lambda}(\omega) \) will decrease with frequency by the low-pass properties of \( H_{\lambda}(\omega) \) and \( U(\omega) \). In general, the frequency characteristics of the instrument response \( U(\omega)/U(0) \) will have a common effect on the resulting OPs, since the phase and magnitude shifts will be shared by all components in the linear mixture model and at all spatial points. Previously in [16, 26], the authors suggested to cancel the effect of the instrument response in the OP by subtracting the rotation angle and compensating for the magnitude scaling induced at the evaluated frequency. However, this approach still requires a previous measurement of the instrument response in the experimental setup.

2.3. Extended phasor

By the \( I \) exponential modes in the fluorescence impulse response in Eq. (1) at \( \lambda \)-th wavelength, there exists a time index \( M_{\lambda} > 0 \) such that the fluorescence decays \( y_{\lambda}[n] \) will reach steady-state for all components after a time-limited instrument response, i.e. \( M_{\lambda} \gg \tau_{\lambda} \) for all \( i \). Based on these maximum settling-times \( M_{\lambda} \), an extended fluorescence impulse response \( \tilde{h}_{\lambda}[n] \) is constructed by the delayed signals at all wavelengths \( \lambda \) such that overlapping is avoided in time-domain:

\[
h_{\lambda}[n] = \sum_{i=1}^{I} \alpha_{\lambda,i} \tilde{h}_{\lambda i}[n],
\]

where the characteristic extended impulse response of \( i \)-th component is given by

\[
\tilde{h}_{\lambda i}[n] = \sum_{k=1}^{\Lambda} \delta_{\lambda,k} e^{-n(M_{\lambda} + \tau_{\lambda,i})} \left[ 1 - M_{\lambda} \right]
\]

where the accumulated delays are described as

\[
\tilde{M}_{\lambda} = \left\{ \begin{array}{ll}
0 & \lambda = 1 \\
\sum_{i=1}^{\lambda-1} M_{i} & \lambda > 1 \\
\end{array} \right. \quad \lambda \in [1, \Lambda]
\]

An illustration of the resulting concatenated responses is shown in Fig. 1. The discrete-time Fourier transform of this extended response \( \tilde{h}_{\lambda i}[n] \) for \( i \)-th component is

\[
\tilde{H}_{\lambda i}(\omega) = e^{-j\omega \tilde{M}_{\lambda}} \sum_{i=1}^{\lambda} \delta_{\lambda,i} e^{j\omega} \left[ e^{j\omega} - e^{-1/\tau_{\lambda,i}} \right] + \sum_{i=2}^{\lambda} \delta_{\lambda,i} e^{j\omega} \left[ e^{j\omega} - e^{-1/\tau_{\lambda,i}} \right] + \ldots + e^{-j\omega \tilde{M}_{\lambda}} \delta_{\lambda,\Lambda} e^{j\omega} \left[ e^{j\omega} - e^{-1/\tau_{\lambda,\Lambda}} \right].
\]

Therefore, the delay components \( \{e^{-j\omega \tilde{M}_{\lambda}}\}_{i=1}^{\lambda} \) will provide large negative phase contributions in Eq. (11), specially for high frequencies. However, if the phase terms in the exponential modes \( e^{j\omega}/(e^{j\omega} - e^{-1/\tau_{\lambda,i}}) \) are small compared to the phase jumps by the delay terms \( e^{-j\omega \tilde{M}_{\lambda}} \), then the uniqueness of the low-dimensional mapping could be preserved. Hence, in the low frequency range \( \omega \approx 0 \), there is no phase cancelling among the complex numbers \( F_{i,1, \ldots, F_{i,\Lambda}} \), and the extended impulse response of \( i \)-th component \( \tilde{H}_{\lambda i}(\omega) \) will be a distinctive point in the complex plane. Therefore, the EP at \( k \)-th spatial point is defined by

\[
D_{k}(\omega) \triangleq \frac{H_{k}(\omega)}{H_{k}(0)} = \frac{1}{H_{k}(0)} \sum_{i=1}^{I} \alpha_{k,i} \tilde{H}_{i}(\omega) \in \mathbb{C} \quad \omega \in [0, 2\pi].
\]
whose properties are

\[
\lim_{\omega \to 0} D_k(\omega) = 1, \\
\lim_{\omega \to \pi} D_k(\omega) = \sum_{i=1}^{N} \alpha_{k,i} \tilde{H}_k(\pi) \tilde{H}_k(0) < 1. \tag{13}
\]

with an inherited low-pass profile in the magnitude, due to the frequency response of \(\tilde{H}_i(\omega)\) for all \(i \in [1, I]\). On the other hand, an important observation is that if all the delay times \(\tilde{M}_\lambda\) are equal to a specific value \(M > 0\), the concatenation of the fluorescence impulse responses in Eq. (9) will naturally induce an artificial frequency component at \(\omega = 2\pi/M\) and its harmonics, and as a consequence, the EP at these frequencies will have a larger magnitude. Therefore, this artificial frequency component and its harmonics should be avoided for the phasor evaluation and subsequent classification purposes.

2.4. Extended output phasor

In this section, the EOP is proposed to characterize graphically in a low-dimensional domain m-FLIM measurements. This mapping employs the concatenated measured fluorescence decays of the multi-spectral channels \(y_{k,\lambda}[n]\) in the m-FLIM dataset. Therefore, the discrete-time concatenated fluorescence decay signal is defined as

\[
y_k[n] = \sum_{\lambda=1}^{\Lambda} y_{k,\lambda}[n - \tilde{M}_\lambda] 1[n - \tilde{M}_\lambda] = h_k[n] \ast u[n]
\]

and from the notation in previous section,

\[
y_k[n] = \sum_{i=1}^{I} \alpha_{k,i} \left[ \sum_{\lambda=1}^{\Lambda} \delta_{i,\lambda} e^{-\left(n - \tilde{M}_\lambda\right)/\tau_{i,\lambda}} \cdot 1[n - \tilde{M}_\lambda] \right] \ast u[n] \tag{15}
\]
where $q_i[n]$ denotes the fluorescence end-member signal for $i$-th component. With this notation at hand, the EOP at $k$-th spatial point is defined as

$$P_k(\omega) \triangleq \frac{Y_k(\omega)}{Y_k(0)} = \frac{U(\omega)}{U(0)} \sum_{l=1}^{L} \alpha_{k,l} \tilde{H}_k(\omega) \in \mathbb{C} \quad \omega \in [0, 2\pi].$$ \hspace{1cm} (16)

In this way, the previous properties of the TPR, OP and EP in Eqs. (2), (7) and (12) are maintained, i.e. $\lim_{\omega \to 0} P_k(\omega) = 1$ and $\lim_{\omega \to \infty} P_k(\omega) < 1$; and in the low frequency range $\omega \approx 0$, we have $P_k \approx D_k$, i.e. the effect of the instrument response can be minimized.

3. Numerical implementations of extended output phasor and extended phasor

In a real-time implementation, the m-FLIM dataset collects the discrete-time information of the fluorescence decays in the sample over a certain time window. Moreover, we assume that for the $\lambda$-th wavelength ($\lambda \in [1, \Lambda]$) and for any spatial location $k$ ($k \in [1, K]$), there are $M_\lambda$ samples $\{y_{k,\lambda}[n]\}_{n=0}^{M_\lambda-1}$ in the dataset. In this way, the vector of measured fluorescence decays $y_{k,\lambda}$ at $\lambda$-th wavelength and $k$-th spatial location is given by

$$y_{k,\lambda} = [y_{k,\lambda}[0] \ldots y_{k,\lambda}[M_\lambda - 1]]^\top \in \mathbb{R}^{M_\lambda}$$ \hspace{1cm} (17)

and the multi-spectral measurement vector $y_k$ is built by stacking the readings for each wavelength:

$$y_k = [y_{k,1}^\top \ldots y_{k,\Lambda}^\top]^\top \in \mathbb{R}^L$$ \hspace{1cm} (18)

where $L = \sum_{\lambda=1}^{\Lambda} M_\lambda$ is the total number of measurements at each spatial location. To avoid signal variability, in practice the vector $y_k$ is normalized to sum one, i.e. $1^\top y_k = 1$ and since the readings represent intensity decays, its components are always non-negative $y_k \succeq 0$. Therefore, the EOP is computed based on the discrete Frequency transform [29] for $l$-th frequency index as

$$\phi_{k,l} = W_l^\top y_k \in \mathbb{C} \quad k \in [1, K], \ l \in [1, L - 1],$$ \hspace{1cm} (19)

where

$$W_l^\top = [1 \ e^{-j2\pi l/L} \ e^{-j2\pi 2/L} \ldots \ e^{-j2\pi (L-1)/L}].$$ \hspace{1cm} (20)

On the other hand, from the measured fluorescence decay vector $y_{k,\lambda}$ and the instrument response samples $\{u[n]\}_{n=0}^{M_\lambda-1}$, the fluorescence impulse response vector $h_{k,\lambda} = [h_{k,\lambda}[0] \ldots h_{k,\lambda}[L - 1]]^\top$ at $\lambda$-th wavelength can be estimated by a Laguerre basis or a multi-exponential model [6, 8, 9]. Similar to the fluorescence decay vector, the multi-spectral fluorescence impulse response vector $h_k$ is constructed as

$$h_k = [h_{k,1}^\top \ldots h_{k,\Lambda}^\top]^\top \in \mathbb{R}^L.$$ \hspace{1cm} (21)

This vector is also normalized for a sum to one property ($1^\top h_k = 1$) to compute the EP:

$$\pi_{k,l} \triangleq W_l^\top h_k \in \mathbb{C} \quad k \in [1, K], \ l \in [1, L - 1].$$ \hspace{1cm} (22)

As carefully elaborated in Section 2 by a frequency domain analysis, there are three important reasons to consider low frequency indices $l$ in the computation of EOP and EP in Eqs. (19) and (22), respectively:

- The phase rotations induced by the time concatenation of the multi-spectral measurements are reduced.
The magnitude and phase adjustments produced by the instrument response frequency properties are minimized.

The measurement noise distortion in the resulting phasor is decreased.

Under these considerations, the EOP and EP will have similar locations in the complex plane for low frequency indices \( l \). In practice, the EOP low dimensional representation is more appealing since there is no need to measure the instrument response in the experimental setup and perform a time consuming deconvolution process of the m-FLIM dataset, as for EP. Therefore, in the rest of this section, we focus on the geometrical properties of EOP and uniqueness of this representation.

Recalling the results in Section 2, in order to have a distinctive value \( \phi_{k,l} \) in the complex plane for any m-FLIM measurement, the frequency index \( l \) has to be small to avoid large phase jumps by the delay terms obtained by the time concatenation in Eq. (14). In Section 2.3, we discuss that if the time indexes, or in this case, the time window sizes \( M_k = M \) are all equal for any \( \lambda \), the induced periodicity in the concatenation of the measured fluorescence decay vectors \( \{ y_{k,l} \}_{k=1}^{M} \) in Eq. (18) will enhance the frequency \( 2\pi/M \) and its harmonics. In this way, these frequency indexes will provide a larger magnitude during the computation of the EOPs. On the other hand, there are two important properties of the EOP with respect to its graphical interpretations:

- **Geometric interpretation:** Let the elements of the multi-spectral measurement vector \( y_k \) be denoted by \( \{ y_{k,l} \}_{l=0}^{L-1} \), then the EOP in Eq. (19) can be written as

\[
\phi_{k,l} = \sum_{l=0}^{L-1} y_{k,l} e^{-j2\pi l/L}
\]

where \( y_{k,l} \geq 0 \) and \( \sum_{l=0}^{L-1} y_{k,l} = 1 \), and consequently, \( \phi_{k,l} \) lies in the convex hull of the finite set \( S = \{ 1, e^{-j2\pi l/L}, \ldots, e^{-j2\pi l(L-1)/L} \} \). Hence, the EOP can be interpreted as a convex combination of the elements in \( S \) by using the scaling values \( \{ y_{k,l} \}_{l=0}^{L-1} \), as is shown in Fig. 2. Hence by selecting \( l \geq 1 \), the order in the linear combination of complex exponentials in \( S \) will be adjusted, and the EOP will exhibit a different position in the complex plane. This previous observation is consistent with the frequency domain analysis in Section 2. Meanwhile, for any index \( l \in [1, L-1] \) define the set

\[
\Omega_l = \left\{ \sum_{\ell=0}^{L-1} a_{\ell} e^{-j2\pi \ell l/L} : a_{\ell} \geq 0 & \sum_{\ell=0}^{L-1} a_{\ell} = 1 \right\}
\]

then any element in this set is a convex combination of the vertices \( \{ 1, e^{-j2\pi l/L}, \ldots, e^{-j2\pi l(L-1)/L} \} \) that lie on the unit circle, and consequently \( \Omega_l \subset \mathcal{C} \), where \( \mathcal{C} = \{ z \in \mathbb{C} : |z| \leq 1 \} \). In this way, the EOP is always inside the unit circle, i.e. \( \phi_{k,l} \in \mathcal{C} \) (see Fig. 2).

- **Linear mixture and dimensionality:** By considering a linear mixture model for any m-FLIM measurement \([10–13]\):

\[
y_k = \sum_{i=1}^{d} a_{k,i} q_i
\]

where \( q_i \in \mathbb{R}^d \) represents the \( i \)-th fluorescent component end-member \( (q_i \geq 0 \text{ and } 1^\top q_i = 1) \). In this way, from Eq. (19), the EOP satisfies

\[
\phi_{k,l} = \sum_{i=1}^{d} a_{k,i} W_l^\top q_i
\]
where the complex value $v_{i,l}$ denotes the phasor fluorescent end-member for $i$-th component at $l$-th frequency index. Therefore, as long as $l$ is a small value, all the end-members will be distinctive points inside the unit circle, i.e. $v_{i,l} \in \mathbb{C}$; and as a result, the EOP $\phi_{k,l}$ lies inside the convex hull of the phasor fluorescent end-members $(v_{1,l}, \ldots, v_{I,l})$. However, this two-dimensional representation of the m-FLIM measurements can only characterize a linear mixture of at most 3 components by Caratheodory’s theorem [28]. As a result, if the dataset presumably contains 4 basic fluorescent components, to have a unique representation by the linear mixture model in Eq. (25), the EOP must be computed at two low frequency indexes $l$ and $m$ to extract a three-dimensional visualization by combining the real and imaginary parts of the resulting EOPs. For example, one possible representation is

$$\phi_{k,l,m} = \sum_{i=1}^{I} \alpha_{k,l,i} v_{i,l,m} \in \mathbb{R}^3 \quad (27)$$

where

$$\phi_{k,l,m} = [\Re\{\phi_{k,l}\} \Im\{\phi_{k,l}\} \Re\{\phi_{k,m}\}]^T$$

$$v_{i,l,m} = [\Re\{v_{i,l}\} \Im\{v_{i,l}\} \Re\{v_{i,m}\}]^T. \quad (28)$$

Moreover, the location of the EOPs in the complex plane will depend on the number of characteristic components in Eq. (25) and positions of the phasor fluorescent end-members $\{v_{i,l}\}_{i=1}^{I}$ [16, 23]: (i) $I = 1$, the EOPs will be gathered around a fixed point $v_{1,l}$, (ii) $I = 2$, the EOPs will be inside the line segment joining the phasor fluorescent end-members $(v_{1,l}, v_{2,l})$, (iii) $I = 3$, the EOPs will be inside a triangle whose vertices are the phasor fluorescent end-members $(v_{1,l}, v_{2,l}, v_{3,l})$. However, for $I = 4$, in a three-dimensional domain (considering two low frequency indexes $l$ and $m$), the EOPs will lie inside a tetrahedron whose vertices are $(v_{1,l}, v_{2,l}, v_{3,l}, v_{4,l})$ [28]. In addition, additive noise in the m-FLIM measurements will incorporate some random dispersion to the EOP locations, but if this noise energy is not large, the EOPs will not suffer a major disruption.

In the next section, we employ the EP and EOP to represent synthetic and experimental m-FLIM datasets, and illustrate the equivalence of both characterization for low frequency indexes. We consider also different noise levels in the synthetic data and different number of components in
a linear mixture model. Moreover, our next validation results highlight the importance of the previous geometric and dimensionality properties of the EOP.

4. Validation results

4.1. Synthetic data

The synthetic datasets used for validation were constructed as $60 \times 60 \times 600$ arrays, i.e. there are $K = 60 \times 60 = 3,600$ spatial locations and the time window has $L = 600$ samples. The datasets emulate the experimental setup described in [9] for FLIM datasets of atherosclerotic plaques. Until four fluorophores or synthetic end-members are considered in our evaluation ($I \leq 4$) to represent: high collagen (HC), high lipids (HL), low collagen and lipids (LCL) and high elastin (HE), evaluated at three characteristic wavelengths ($\Lambda = 3$): $390 \pm 2$ nm, $452 \pm 22.5$ nm, $550 \pm 20$ nm. The sampling time is set to $T_s = 250$ ps. Each fluorescence impulse response is described by a multi-exponential model whose parameters are presented in Table 1 by considering the lifetimes and normalized intensities reported in [9]. The instrument response is assumed to have the time profile described in Eq. (5) with parameters $\tau_u = 1 \text{ ns}/T_s$, $u_o = T_s$, where its magnitude is next normalized to sum one. All the signal processing of the synthetic m-FLIM datasets was computed in Matlab. We consider in this section two synthetic cases

- **Case 1**: the datasets contain three synthetic end-members (HC, HL and LCL) ($I = 3$) with the abundances described in the subplots (A), (B) and (C) in Fig. 3. The resulting output end-member vectors ($q_1, q_2, q_3$) are illustrated in Fig. 3(D). In this case, we evaluate the effect of measurement noise and the selection of the frequency index $l$ in Eq. (19). The noise intensity is quantified by the peak signal-to-noise ratio (PSNR) [10]:

$$
\text{PSNR} = 10 \log_{10} \frac{\max_{l} y_{k}^l}{\sigma^2}
$$

(29)

where $\sigma$ represents the equivalent variance, and the noisy fluorescent decay vector $\tilde{y}_{k}$ is constructed by a multiplicative noise model to emulate a random term with a signal-to-noise ratio proportional to the intensity of the observation $y_{k}^l$:

$$
\tilde{y}_{k}^l = y_{k}^l + \sigma \varepsilon_{k}^l \sqrt{y_{k}^l}, \quad l \in [0, L - 1]
$$

(30)

where $\varepsilon_{k}^l$ is an i.d.d. Gaussian random variable with zero mean and unitary variance. The noise free $y_{k}$ and noisy measurements $\tilde{y}_{k}$ at spatial point 100 ($k = 100$) are visualized in Fig. 3(E) for a PSNR=20 dB.

- **Case 2**: the dataset considers now four synthetic fluorescent end-members (HC, HL, LCL and HE) ($I = 4$), whose abundances are described in the subplots (A), (B), (C) and (D) in Fig. 4. Therefore, according to the discussion in Section 2, an EOP in a three dimensional space has to be considered now. The resulting output end-member vectors ($q_1, q_2, q_3, q_4$) are illustrated in Fig. 4(E) for HC, HL, LCL and HE, respectively. The PSNR is fix to 20 dB, and the noise free $y_{k}$ and noisy measurement $\tilde{y}_{k}$ at spatial point 100 ($k = 100$) are visualized in Fig. 4(F).

The results for Case 1 are presented in Fig. 5, where the first and second columns illustrate the resulting EPs (subplots (A) and (C)) and EOPs (subplots (B) and (D)), respectively. Meanwhile, the first row (subplots (A) and (B)) in Fig. 5 describes the phasors at PSNR 30 dB, and the second row (subplots (C) and (D)) at 20 dB. In each subplot, the phasors for the whole dataset of 3,600 spatial points are plotted for three frequency indexes $l = 1, 16$ and 31, as well as
Table 1. Multi-Spectral Properties of Fluorophores Employed in the Synthetic Datasets [9].

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Characteristic Times</th>
<th>Scaling</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\tau_1 \cdot T_s$</td>
<td>$\delta_1$</td>
</tr>
<tr>
<td>High Collagen ($i = 1$)</td>
<td>5 ns</td>
<td>0.57</td>
</tr>
<tr>
<td>High Lipids ($i = 2$)</td>
<td>5.3 ns</td>
<td>0.42</td>
</tr>
<tr>
<td>Low Collagen and Lipids ($i = 3$)</td>
<td>4.0 ns</td>
<td>0.48</td>
</tr>
<tr>
<td>High Elastin ($i = 4$)</td>
<td>2.5 ns</td>
<td>0.45</td>
</tr>
</tbody>
</table>

Fig. 3. Case 1 of Synthetic Evaluation: (A) Noise-free Abundance of Component 1 (HC), (B) Noise-free Abundance of Component 2 (HL), (C) Noise-free Abundance of Component 3 (LPL), (D) Output End-Member Vectors, and (E) Sample 100 of Noisy and Noise Free Measurement for PSNR=20 dB.

the corresponding phasor fluorescence end-members. This ascendant pattern in the frequency indexes was selected to sequentially illustrate the similarity between EOP and EP at low frequency, and subsequently the induced noise distortion in both phasors at high frequency. Since from the abundance maps in subplots (A) to (C) in Fig. 3, none of the components has a pure concentration in the spatial locations, i.e. none of the abundances attains an exact value of 1.0 (100%), then in the complex plane, the EPs and EOPs do not reach the phasor fluorescence end-members ($v_{1,l}, v_{2,l}, v_{3,l}$) that represent the triangle vertices in the convex combinations in Eq. (26). In the overall, these results confirm that for $l = 1$, the EPs and EOPs have a similar position in the complex plane, and due to the monotonic low-pass property of the phasors, as $l$ increases, the phasors tend to the origin. As a result, as $l$ increases, the effect of measurement noise is also increased by distorting the phasor locations. Finally, by the low-pass frequency pattern of the instrument response, the EOPs approach the origin faster compared to the EPs as $l$ increases. Consequently, as previously highlighted in Section 2, the frequency index $l$ in the EOP has to be a small value to have a distinct phasor in the complex plane, to avoid the effect of the instrument response and to reduce the distortion of measurement noise.
The results for the three-dimensional phasor representation in Case 2 are presented in Fig. 6. By the results in the previous evaluation, just the EOPs are computed for a PSNR = 20 dB, and since two frequencies has to be selected, the frequency indexes are set to the lowest values, i.e. \( l = 1 \) and \( m = 2 \). Four possible EOPs could be defined in a three-dimensional space by combining real and imaginary parts at both frequency indexes \( l \) and \( m \):

\[
\begin{align*}
\phi_{1,1,2}^1 &= \left[ \Re\{\phi_{k,1}\} \Im\{\phi_{k,1}\} \Re\{\phi_{k,2}\} \right]^\top, \\
\phi_{1,1,2}^2 &= \left[ \Re\{\phi_{k,1}\} \Im\{\phi_{k,1}\} \Im\{\phi_{k,2}\} \right]^\top, \\
\phi_{1,1,2}^3 &= \left[ \Re\{\phi_{k,1}\} \Re\{\phi_{k,2}\} \Im\{\phi_{k,2}\} \right]^\top, \\
\phi_{1,1,2}^4 &= \left[ \Im\{\phi_{k,1}\} \Re\{\phi_{k,2}\} \Im\{\phi_{k,2}\} \right]^\top.
\end{align*}
\] (31)

The resulting EOPs in Eqs. (31) are shown in Fig. 6, and they highlight distinctive points in the three-dimensional space. As in Case 1, the abundance maps in subplots (A) to (D) in Fig. 4 do not present pure components in the dataset, since none of the abundances attains a 100% value, then once more the EOPs do not reach the phasor fluorescence end-members \((v_1,1,2, v_2,1,2, v_3,1,2, v_4,1,2)\) that represent the vertices in the tetrahedron, which is obtained by the convex combinations in Eq. (26). In this way, any of the four representations for the EOP could be applied to visualize graphically the m-FLIM measurements for the four characteristic fluorescence components.

4.2. Experimental data

The experimental m-FLIM datasets of human coronary atherosclerotic plaques reported in [9] were employed in this evaluation. The multi-spectral measurements were recorded at three
Fig. 5. Case 1 of Synthetic Evaluation: EOPs of the Dataset for Frequency Components $l = 1, 16$ and $31$ (A) EP for PSNR=30 dB, (B) EOP for PSNR=30 dB, (C) EP for PSNR=20 dB, (D) EOP for PSNR=20 dB.

Fig. 6. Case 2 of Synthetic Evaluation: EOPs of the Dataset at PSNR=20 dB and frequency components $l = 1$ and $m = 2$, (A) $\phi_{1,1,2}^1$, (B) $\phi_{1,1,2}^2$, (C) $\phi_{1,1,2}^3$, and (D) $\phi_{1,1,2}^4$. 

(C) 2015 OSA 1 Jun 2015 | Vol. 6, No. 6 | DOI:10.1364/BOE.6.002088 | BIOMEDICAL OPTICS EXPRESS 2101
wavelengths (\(\lambda = 3\)), which were described in the previous section. Each m-FLIM measurement had a field of view of \(2 \times 2 \text{mm}^2\) at \(60 \times 60\) pixels with a sampling rate of 250 ps. Twenty-six datasets are studied with an uniform and similar histology, and based on this histopathological evaluation, from the 26 datasets, five were labelled as “High-Collagen” (HC), five as “High-Lipids” (HL) and sixteen as “Low Collagen and Lipids” (LCL). The time length of the recordings is 598 samples, where the lengths of the measurement per wavelength are \(M_1 = 184\), \(M_2 = 167\) and \(M_3 = 247\), i.e. \(L = M_1 + M_2 + M_3 = 598\). In order to reduce the signal variability and to increase the signal-to-noise ratio in the datasets, a \(15 \times 15\) pixel block (\(500 \times 500 \mu\text{m}^2\)) in each m-FLIM image was counted as a single data point by spatial averaging for the computation of EP and EOP. In this way, there are \(4 \times 4 \times 5 = 80\) classification points labelled as HC, \(4 \times 4 \times 5 = 80\) as HL, and \(4 \times 4 \times 16 = 256\) as LCL in the whole dataset. These groups were used for training and validation purposes in a 10-fold cross-validation strategy [31]. In order to deconvolve the m-FLIM measurements to compute the EP for comparison purposes, the methodology based on the Laguerre basis expansion (8-th order with shape parameter \(\alpha = 0.85\)) with a constrained third-derivative proposed in [32] was applied to all datasets. The signal processing and classification methodology of the m-FLIM information was carried out in Matlab.

First, the computed extended impulse responses and fluorescence decay m-FLIM measurements for the three studied classes (HC, HL, LCL) are illustrated in Fig. 7. The EOPs and EPs in Eqs. (19) and (22), respectively, were computed for the 26 datasets at three frequency indexes \(l = 1, 2\) and 3, and the resulting locations are illustrated in Fig. 8. In these plots, the mean fluorescence decay measurement for the whole dataset is pointed out as a large circle for HL, a diamond for HC, and a square for LCL. Consequently from the graphical display, as expected, the EPs and EOPs have similar locations in the complex plane, and the subgroups (HC, HL, LCL) present just a small overlap with good spatial separability. Similarly, the EPs and EOPs for each dataset change with the frequency index \(l\), and visually, the largest separability is observed for \(l = 3\). As will be shown next, this visual appreciation is consistent with the result of the cross-validation strategy.

To evaluate the classification performance, for the 26 datasets, linear discriminants were trained and validated by using the Statistics Toolbox [33] from Matlab, and the resulting true test error [31] is illustrated as a function of the frequency index \(l \in [1, 75]\) in Fig. 9. As a result, the true test error has a similar performance for EP and EOP in the interval \(l \in [1, 19]\), and after \(l = 20\) the difference between the error by EP and EOP increases, which is in line with our previous analysis that for low frequency components the classification by EP and EOP is comparable. The resulting confusion matrices are presented for the linear classifier and frequency indexes \(l \in [1, 3]\) in Table 2. This table shows that the classification (sensitivity and specificity) by EOP improves for \(l = 3\), and for this frequency index, the EOP produces a similar classification performance with respect to [9], but with less complexity in the features extraction and processing; since the deconvolution of the m-FLIM information can be avoided by the EOP, and the responses at the three wavelengths can be analyzed jointly.

Finally, we present the validation stage of the EOP linear discriminant trained with the previous samples by considering three new m-FLIM datasets, and the three studied classes (LCL, HC, HL). In this testing data, the three samples consider a mixed histopathology of high and low collagen and lipids, as shows Fig. 10. Each m-FLIM image has \(60 \times 60\) pixels, where each pixel in the sample was classified by the linear discriminant as LCL, HC or HL for the frequency index \(l = 1\). We observed that the classification generalization was slightly better by this frequency index compared to \(l = 2\) and \(l = 3\). Here the top panel of Fig. 10 illustrates a plaque with areas of significant content of elastin, collagen and/or lipids, as shown in the histopathology image. The classification map indicates the presence of the three studied classes all over the sample. The second panel in Fig. 10 corresponds to a plaque with a thick region rich in lipids.
Fig. 7. Extended Impulse Responses (1st Column) and m-FLIM Measurements (2nd Column) for the Experimental Datasets: (A)-(B) Low Collagen and Lipids, (C)-(D) High Collagen, and (E)-(F) High Lipids.

Table 2. Confusion Matrix by Linear Classifier (10-Fold Cross-Validation) for Experimental Datasets with Frequency Indexes \( l = 1, 2 \) and \( 3 \) in EOP.

<table>
<thead>
<tr>
<th>Frequency Index ( l )</th>
<th>LCL</th>
<th>HC</th>
<th>HL</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>( l = 1 )</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LCL</td>
<td>244</td>
<td>12</td>
<td>0</td>
<td>95%</td>
<td>94%</td>
</tr>
<tr>
<td>HC</td>
<td>4</td>
<td>75</td>
<td>1</td>
<td>94%</td>
<td>92%</td>
</tr>
<tr>
<td>HL</td>
<td>6</td>
<td>15</td>
<td>59</td>
<td>74%</td>
<td>100%</td>
</tr>
<tr>
<td>( l = 2 )</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LCL</td>
<td>247</td>
<td>7</td>
<td>2</td>
<td>96%</td>
<td>97%</td>
</tr>
<tr>
<td>HC</td>
<td>4</td>
<td>76</td>
<td>0</td>
<td>95%</td>
<td>94%</td>
</tr>
<tr>
<td>HL</td>
<td>1</td>
<td>14</td>
<td>65</td>
<td>81%</td>
<td>99%</td>
</tr>
<tr>
<td>( l = 3 )</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LCL</td>
<td>243</td>
<td>13</td>
<td>0</td>
<td>95%</td>
<td>99%</td>
</tr>
<tr>
<td>HC</td>
<td>1</td>
<td>79</td>
<td>0</td>
<td>99%</td>
<td>93%</td>
</tr>
<tr>
<td>HL</td>
<td>0</td>
<td>10</td>
<td>70</td>
<td>88%</td>
<td>100%</td>
</tr>
</tbody>
</table>

(left side on histopathology), and a thin region rich in elastin (right side on histopathology). In this case, the classification map highlights two distinctive regions in the sample associated to high lipids, and the rest of the image shows low collagen and lipids. The last panel in Fig. 10 shows a plaque with a thin region rich in elastin (left side on histopathology) and a thick region rich in collagen (right side on histopathology). So, the linear discriminant recognizes roughly half of the image as a region with high collagen, and the rest as low collagen and lipids. In this way, for the validation with the 3 testing datasets, we observe a precise characterization of the distinctive classes by the EOP.
5. Conclusions

In this paper, we introduced the concepts of EOP and EP to provide graphical tools to visualize and classify m-FLIM measurements in a low-dimensional domain. The frequency domain properties of the EOP and EP were analytically studied to consider the effects of the concatenation of the multi-spectral measurements and the instrument response, and we conclude that the EOP and EP have to be evaluated at low frequency indexes to reduce the effect of the instrument response and noise, and to have a distinctive point in the complex plane for each m-FLIM measurement. For practical implementations, the EOP is more appealing since there is no need to measure the instrument response and deconvolve it from the FLIM dataset. The real-time implementations of the EOP and EP consider a simple vector multiplication for a multi-spectral vector of FLIM information. Furthermore, our analysis show that by considering a linear mixture for the m-FLIM response, a 2-dimensional representation in the complex plane for the EOP can only characterize until 3 distinctive components, and a 3-dimensional visualization could distinguish uniquely until 4 components. Nonetheless, higher order representations of EOP could be employed for dimensional reduction purposes when dealing with more than 4 distinctive components. Our synthetic and experimental evaluation confirmed our previous remarks that low frequency indexes, preferable the first components, have to be employed for a distinctive representation of the EOP and EP, and to reduce noise distortion. Meanwhile, the tissue classification of m-FLIM datasets by EOP and EP also improves by low frequency indexes, and does not present significant differences by using either phasor. Consequently, for low-dimensional visualization and classification, the EOP can be employed in m-FLIM datasets. As a future work, we will study the problem of blind linear unmixing for abundance and end-
Fig. 9. Experimental Classification Performance for EOP and EP with Linear Classifier (10-Fold Cross-Validation) and Frequency Indexes $l \in [1, 75]$.

Fig. 10. Experimental Classification Performance for EOP with First Frequency Index ($l = 1$) and Linear Discriminant in Testing Samples (Mixed Histopathology): Elastin-Collagen-Lipids, Lipids-Elastin and Collagen-Elastin (Histopathology Slide Corresponds to the Middle Cross-Section in the Classification Map).

members extraction by the EOP.

Acknowledgments
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