Phasor based analysis of FRET images recorded using spectrally resolved lifetime imaging

Farzad Fereidouni, Gerhard A Blab and Hans C Gerritsen

Utrecht University, Department of Molecular Biophysics, Utrecht, The Netherlands

E-mail: F.Fereidouni@spechron.com

Received 14 December 2013, revised 7 February 2014
Accepted for publication 10 February 2014
Published 14 May 2014

Abstract
The combined analysis of spectral and lifetime images has the potential to provide more accurate and more detailed information about Förster resonance energy transfer (FRET). We have developed a novel FRET analysis method to analyze images recorded by multispectral lifetime imaging. The new method is based on a phasor approach and facilitates the simultaneous analysis of decay kinetics of donor and acceptor molecules. The method is applicable to both molecules that exhibit a mono-exponential decay and a bi-exponential decay. As an example we show the possibility of extracting the energy transfer efficiency and the fraction of interacting molecules even in the presence of non-interacting molecules. The reliability of the method is investigated by comparing it with conventional FRET-FLIM analyses. We show that, with the same number of detected photons, the spectrally resolved phasor approach provides higher accuracy than other analysis methods; the confidence interval is improved and the FRET efficiency is closer to the real value.

Keywords: fluorescence microscopy, lifetime imaging, multispectral imaging, resonance energy transfer

1. Introduction
Förster resonance energy transfer (FRET) refers to the transfer of excited state energy between two fluorophores that exhibit spectral overlap and are in close proximity to each other [1]. Upon excitation, the donor molecule transfers its energy via a non-radiative dipole–dipole interaction to an acceptor molecule. The energy transfer results in shortening of the donor lifetime and reduction of its fluorescence intensity. In contrast, both the intensity and the average lifetime of the acceptor increase. For a given spectral overlap and transition dipole moment orientation, the changes in intensity and lifetime are a measure of the distance between the two chromophores. The sensitivity of FRET to distance variations is high and it has been successfully employed as a ‘molecular ruler’ to measure distance in the 1–10 nm range [1]. FRET has been successfully used to overcome the spatial resolution limitations of optical microscopes and monitor the interactions of molecules. FRET imaging is at present the technique of choice to visualize molecular interactions in cells [2].

Different approaches have been employed to estimate the FRET efficiency, including donor lifetime measurements [3], donor or acceptor photo bleaching [4, 5] and spectral ratio methods [6]. An excellent overview of the different approaches can be found in the review by Berney et al [7]. Among these methods, fluorescence lifetime measurement of the donor molecule has been extensively used to estimate the efficiency of energy transfer to the acceptor molecule and is preferred over fluorescence lifetime measurements of the acceptor due to comparatively simpler behavior of the donor. Studying the acceptor behavior is more complicated but has also been investigated in several studies [8, 9]. Including the whole spectral range in the FRET analysis, and thus information of both donor and acceptor, offers the prospect of obtaining more
accurate and detailed information about the system. Spectrally resolved fluorescence lifetime imaging methods have been employed for the analysis of FRET. It has been shown that simultaneous monitoring of both donor and acceptor through measuring the whole spectral range, indeed enhances the sensitivity of FRET measurements [10]. This is expected since more photons are collected compared to approaches where only either the donor or acceptor is monitored.

A spectrally resolved, frequency domain, fluorescence lifetime imaging system has been used to study FRET [11] in a multi-fluorophore system. Here, it turned out to be possible to discriminate between situations where all or only some of the fluorophores are interacting. In another application spectrally resolved lifetime imaging has been used to estimate the FRET efficiency in a system with a mixture of interacting (FRET) and non-interacting donor molecules. It was proven that the accuracy of the FRET estimation improves with the number of spectral channels used [12]. Inclusion of the emission from both donor and acceptor in the analysis can both increase the sensitivity of the measurement and provide more information on the state of the system. However, the analysis of the emission in the acceptor channel is complicated. It includes both the signal from direct excitation of donor and acceptor, and the sensitized emission of the acceptor. The latter corresponds to a signal with a negative lifetime (in growth of the acceptor emission) and analysis using conventional techniques is far from trivial.

In this paper we introduce a method to extract the FRET efficiency by simultaneous analysis of the time and spectrally resolved signals from the whole spectral emission including contributions from both donor and acceptor molecules. A model is presented to also include situations when the molecules exhibit a bi-exponential decay.

The method was tested on well-defined specimens of fluorescently labeled DNA. The tests demonstrate the possibility of extracting the FRET efficiency in the case of a mixture of non-interacting and interacting donor molecules.

2. Materials and methods

2.1. Theory

Upon excitation of a binary system of donor and acceptor molecules, both can relax to the ground state via radiative and non-radiative decay pathways. The donor, however, can exhibit an additional decay process when it is in close proximity of an acceptor: it can transfer its excited state energy via a long-range non-radiative decay pathways. The donor, however, can exhibit a bi-exponential decay.

The acceptor can be directly excited by the excitation source. Now, the acceptor kinetics is determined by the convolution of the exponential acceptor decay with the sum of the excitation source profile and the donor decay profile:

\[
A(t) = \sigma_A I(t) e^{-\frac{t}{\tau_A}} + \left( \frac{k}{\tau_D \tau_A} \right) e^{-\frac{t}{\tau_D \tau_A}}
\]

Both the fluorescence of the donor and the acceptor are wavelength dependent. This can be incorporated by multiplying equations (3) and (4) by the spectrally resolved transition rates:

\[
f_D(t, \lambda) = D(t) \gamma_D \sigma_D(\lambda) = \sigma_D(\lambda) \gamma_D(\lambda) I(t) e^{-\frac{t}{\tau_D}}
\]

where \( f_D \) is the spectral and temporal resolved fluorescence emission of the donor, \( \gamma_D \) the transition rate for the donor and \( \sigma_D(\lambda) \) normalized emission profile of donor. Similarly we can write for the acceptor:

\[
f_A(t, \lambda) = A(t) \gamma_A \sigma_A(\lambda) = \sigma_A(\lambda) \gamma_A(\lambda) I(t) e^{-\frac{t}{\tau_A}} + \left( \frac{k \sigma_D \tau_A}{\tau_A \tau_D + k \tau_A} \right) \gamma_A(\lambda) I(t)
\]

where again \( f_A \) is the spectral and temporal resolved fluorescence emission of the acceptor, \( \gamma_A \) the transition rate for the acceptor and \( \sigma_A(\lambda) \) the emission profile of the acceptor. Adding the equations (5) and (6) and replacing \( \gamma \) with \( Q/\tau \), where \( Q \) is the quantum efficiency, yields the full

\[
\frac{d}{dt} D(t) = \sigma_D I(t) - \frac{D(t)}{\tau_D} - \frac{k}{\tau_D} D(t)
\]

where \( D(t) \) is the number density of donor molecules at time \( t \), \( \sigma_D \) is the absorption cross section of the donor, \( I(t) \) is the temporal excitation profile of the excitation source, \( \tau_D \) is the donor lifetime without acceptor, and \( k \) is the energy transfer efficiency. The latter depends on the spectral overlap between donor emission and acceptor excitation spectra, donor and acceptor transition dipole orientations and, importantly, the distance \( R \) between the donor and the acceptor. The energy transfer efficiency is defined by \( k = (R_0/R)^6 \) where \( R_0 \) is the distance where the energy transfer efficiency is 50%. The acceptor can be excited directly by the laser pulse and by energy transfer from the donor:

\[
\frac{d}{dt} A(t) = \sigma_A I(t) - \frac{A(t)}{\tau_A} + \frac{k}{\tau_D} D(t).
\]
description of the time and wavelength dependent fluorescence emission:

\[
    f(t, \lambda) = \left( \sigma_D \frac{Q_D}{\tau_D} a_D(\lambda) - \frac{k_D}{\tau_A} \right) \frac{\tau_A}{\tau_D + k_D} Q_A a_A(\lambda) \\
    \times I(t) e^{-\frac{t}{\tau_D}} + \left( \sigma_A \frac{Q_A}{\tau_A} a_A(\lambda) + \frac{k_D}{\tau_A} \right) Q_A a_A(\lambda) \\
    \times I(t) e^{-\frac{t}{\tau_A}}. \quad (7)
\]

This equation contains a linear superposition of donor and acceptor fluorescence. The second term between the brackets of the bottom part of the equation is responsible for the ingrowth of the acceptor signal; it is only observed in the acceptor signal because it is multiplied by the acceptor emission spectrum.

The lifetime reduction of the donor and the acceptor ingrowth contain valuable information about the FRET process and the donor–acceptor stoichiometry. This includes the ratio of donor to acceptor molecules exhibiting FRET and the number of donors and acceptors that are not involved in the energy transfer process.

We use a phasor approach to analyze the spectrally resolved lifetime images. This graphical approach provides an easy and fast way of analyzing lifetime and spectral images. A detailed description on temporal phasor can be found in [13, 14]. Briefly, the real and imaginary parts of the first harmonic of the Fourier transform of the decay curve are used as coordinates in a scatter plot. The effect of the instrument response function on the fluorescence decay can be simply taken into account by either a rotation of the phasors over a fixed angle obtained from a reference measurement on a dye with a known lifetime or by direct measurement of the instrument response. The phasor approach can also be employed to analyze spectral images. This approach to spectral analysis was recently introduced by our group [15].

In Fourier space the convolution of two functions is simply the product of the transforms of the individual functions, therefore the effect of the instrument response function can be removed by dividing the phasors by the instrument response function phasor.

The linear superposition of the donor and acceptor appears as a line in the spectrally resolved phasor diagram; the temporal phasors from the spectral channels fall on a line connecting the acceptor temporal phasor and donor temporal phasor. The normalized phasors of the spectral lifetime channels \( F(\lambda) \) are given by:

\[
    F(\lambda) = \left\{ \left( \sigma_D \frac{Q_D}{\tau_D} a_D(\lambda) - \frac{k_D}{\tau_A} \right) \frac{\tau_A}{1 - j \omega \tau_D} \right\} \frac{\tau_A}{1 - j \omega \tau_A} \\
    + \left( \sigma_A \frac{Q_A}{\tau_A} a_A(\lambda) + \frac{k_D}{\tau_A} \right) Q_A a_A(\lambda) \\
    \times \left\{ \left( \sigma_D \frac{Q_D}{\tau_D} a_D(\lambda) - \frac{k_D}{\tau_A} \right) \tau_A \\
    + \left( \sigma_A \frac{Q_A}{\tau_A} a_A(\lambda) + \frac{k_D}{\tau_A} \right) \tau_A \right\}^{-1} \quad (8)
\]

where we have used the phasor transformation \( e^{\omega \tau_A} \) and introduced \( A = \frac{k}{\tau_A - \tau_D + \tau} \) which denotes the ingrowth factor. Equation (8) can be rewritten as:

\[
    F(\lambda) = \left\{ \left( \frac{1}{\tau_D} a_D(\lambda) - \frac{1}{\tau_A} a_A(\lambda) \right) \frac{\tau_A}{1 - j \omega \tau_D} \\
    + \left( \sigma A \frac{Q}{\tau_A} a_A(\lambda) + \frac{\sigma Q a_A(\lambda)}{\tau_D} \right) \tau_A \right\} \\
    \times \left\{ \left( \frac{1}{\tau_D} a_D(\lambda) - \frac{1}{\tau_A} a_A(\lambda) \right) \tau_D \\
    + \left( \sigma A \frac{Q}{\tau_A} a_A(\lambda) + \frac{\sigma Q a_A(\lambda)}{\tau_D} \right) \tau_A \right\}^{-1} \quad (9)
\]

where: \( Q' = \frac{Q_A}{Q_D} \), \( \sigma Q = \frac{\sigma a_A}{\tau_D Q_D} \) and \( r(t) = \frac{1}{\tau_A} \). The latter defines the phasor semicircle as a function of lifetime and frequency. It is generated by mapping the imaginary versus real part of \( r(t) \). The frequency is calculated at multiple integers of \( 2\pi / T \) where \( T \) is the width of the total measurement window.

Equation (9) can be used to calculate the temporal phasors for different spectral channels of a system exhibiting FRET. This representation reduces the number of fit parameter for analyzing the experimental data. Moreover \( \sigma Q \) is a parameter that can be easily measured independently and used to verify the goodness of the fit of the FRET data. This parameter denotes the ratio of the integrated intensities of the acceptor and the donor measured at the same concentrations and under the same experimental conditions. Figure 1(a) shows the behavior of the phasors in a simulated spectral lifetime FRET experiments for different energy transfer efficiencies using equation (9). Figure 1(b) shows the spectra of donor and acceptor and the positions of the spectral channels. In these graphs the normalized lifetime of the donor and acceptor are equal to \( \tau_D / T = 0.25 \) and \( \tau_A / T = 0.1 \) respectively. Here we used \( \sigma Q = 0.045 \) and \( Q' = 2 \). The spectra are simulated for a spectograph with 7 channels as used in the experiments. Here, the donor and acceptor emission maxima are chosen at the 3rd and 5th channel respectively. For both spectra we used a FWHM of 2 spectral channels. When the FRET efficiency is equal to zero, the temporal phasors from different spectral channels fall on a line extending from the pure donor phasor to the pure acceptor phasor. This is due to the linear superposition of donor and acceptor phasors and the varying ratio of the contributions of donor and acceptor with wavelength. Here we assume an invariant lifetime for both the donor and acceptor molecules over their emission spectra. Figure 1(a) also shows the phasor behavior for non-zero FRET efficiencies in the range from 10% to 50%. Again the phasors fall onto a straight line and the intersection of this line with the reference circle is a direct measure of the FRET efficiency. We note that this intersection correspond to the lifetime of the donor in the presence of FRET as measured in a conventional FRET-FLIM experiment. In the case of increasing FRET efficiency, the line containing the phasor points rotates along the phasor of the pure acceptor, i.e. the phasor of the acceptor in the absence of FRET.

Due to the negative sign in front of A in equation (9), the non-zero FRET efficiency phasors from the acceptor channels
Figure 1. (a) The spectrally resolved phasor for different FRET efficiencies ranging from 0 to 50% simulated for a spectrograph with 7 spectral channels. The colors in the figure correspond with the channel numbers. Low numbers correspond to short wavelengths (mainly donor emission) and high numbers longer wavelengths (mainly acceptor emission). (b) Spectra of donor and acceptor used in simulations.

move outside the reference semicircle. The slope of the line is determined by the FRET efficiency; the position of phasor points on the line are correlated with the ratio of the absorption cross sections and quantum efficiencies of donor and acceptor molecules.

2.2. Bi-exponential behavior

In many cases the measured fluorescence decay curve is modeled as a mono-exponential function. Often, however, the fluorophores exhibit bi-exponential decays; therefore we also consider a FRET system that exhibits bi-exponential decays for both the donor and the acceptor [16]. It is assumed that each component of the donor interacts independently with each acceptor component. In addition we assume that the donor components have the same spectral properties and we make the same assumption for the acceptor. This means that the spectral overlap and the energy transfer efficiency are constant for all possible combinations of donor and acceptor components. Then the equation (9) can be written as:

\[ F(\lambda) = \left\{ \sum_q \left( Q' a_D^q a_D(\lambda) - A_q Q' a_A(\lambda) \right) \frac{\tau_q^D}{\tau_{DA}} \right\} \times \left\{ \sum_p \left( Q' a_A^p a_A(\lambda) + A_q Q' a_A(\lambda) \right) \frac{\tau_p^A}{\tau_{DA}} \right\}^{-1} \]

where

\[ A_q = \sum_p \frac{\alpha_D^q \alpha_A^p}{\tau_p^A - \tau_D^A + k \tau_p^A} \]

\[ q \text{ and } p \text{ run over the number of components; from 1 to 2 for a bi-exponential decay} \]

\[ \alpha_D^q \text{ and } \alpha_A^p \text{ are the fractional contributions of donor and acceptor lifetimes respectively.} \]

For a donor molecule with a single-exponential decay the FRET trajectory falls on the reference semicircle but in the case of a bi-exponential decay, the FRET trajectory for the donor molecule is more complicated and expressed by:

\[ r_f(E) = \alpha_1^D r(\tau_D^1(1 - E)) + \alpha_2^D r(\tau_D^2(1 - E)) \]

where \( E \) is the FRET efficiency and \( E = k/1 + k \). Here we assume that both decay components of the donor show equal energy transfer efficiencies. A schematic view for a case when donor and acceptor show bi-exponential behavior is shown in figure 2.

In this figure the phasors for both the pure donor and the pure acceptor fall inside the semicircle; this is due to the bi-exponential decay behavior of these molecules. The
The equation includes three terms: one for the interacting donor which includes the term ‘\(A_q\)’ inside the bracket, one for the interacting acceptor which also includes the term ‘\(A_q\)’ and the last term which is responsible for the fraction of pure donor. This equation is the result of adding the equations (5) and (6) and taking the Fourier transform. This equation can be employed to extract the pure donor fraction and the true FRET efficiency in a solution which includes a mix of pure donor and donor–acceptor construct.

2.3. Instrumental details

The FRET experiments are carried out using an inverted microscope equipped with a super continuum white light laser source operating at 80 MHz. The laser is tuned to 532 nm for exciting the Alexa 532 efficiently. The laser light is scanned in the XY direction using a galvanometer mirror scanner (040EF, LSK, Stallikon, Switzerland) and focused on the sample by a microscope objective. The fluorescence emission is collected by the same objective lens. The results reported here are acquired using an infinity-corrected water-immersion objective (CFI Fluar 60×, NA = 1.2, Nikon, Japan). The emission passes through a dichroic mirror (532 nm long pass) and is filtered by an emission filter (532 nm long pass). Next, the fluorescence is coupled into a 900 µm multimode fiber which is connected to a prism based spectrograph equipped with a linear multichannel PMT with 32 anodes of which only 7 anodes are used. The output of the PMT is amplified with a 8 channel amplifier (Philips scientific 774-s-50). The amplified signals are connected to the multispectral lifetime detection system (Lambda–Tau) described in [17]. The spectral width of each channel is about 20 nm and the time bins of the Lambda–Tau detector are 200 ps wide. The 80 MHz repetition rate of the laser yields a 12.5 ns total time measurement window. All experiments were carried out at room temperature and the size of the recorded images is 160 × 160 pixels.

2.4. Sample details

Short, 5’ labeled, complementary DNA oligos were purchased from Invitrogen. Stocks were diluted to 1 µM in PBS from both donor-labeled (Alexa 532, AAGCATGACCGCACAGAT) and acceptor-labeled (Alexa 647, ATCTGTGCGGTCATGCTT) DNA. The sequences were generated by a genetic algorithm and the size of the recorded images is 160 × 160 pixels.

3. Results

3.1. FRET efficiency estimation

Spectral lifetime images of fluorescently labeled DNA were recorded and analyzed. The fluorescence lifetimes and fractions of acceptor and donor molecules were calculated using the Weber method [19]. This requires the recording of pure donor and acceptor decays in separate measurements. With this method we found average lifetimes of \(\tau_D = 3.15\) ns.
and $\tau_A = 1.37$ ns for donor and acceptor respectively. Figure 3 shows the temporal phasors of the pure donor and acceptor molecules. As expected the phasors don’t fall on the semicircle due to multi-exponential behavior of the dyes. The experimental phasors of different spectral channels of a DNA sample exhibiting FRET are shown in figure 3(a). We note that the phasor from the 1st channel is not shown here because of very low counts collected in this channel. The theoretical FRET trajectory for donor molecules is also indicated in the figure; it starts at the pure donor phasor for zero FRET efficiency and eventually ends up at the zero lifetime phasor for 100% FRET efficiency. The phasors of channels 2–4 mainly contain the signal from the donor molecules and fall on this FRET trajectory. Channels 5–6 are dominated by the acceptor signal and fall outside the reference semicircle due to the ingrowth term in the acceptor lifetime. The experimental phasor points were fitted to equation (10) using $Q$, $\sigma Q$ and $k$ as fit parameter; the fitted points are shown in figure 3(a). The fit yields a FRET efficiency $E$ of $24.34 \pm 0.11\%$, a ratio of the quantum efficiencies of $Q_A/Q_D = 1.02 \pm 0.06$ and $\sigma Q = 0.027 \pm 0.0035$. All the fit parameters including the FRET efficiency are considered as invariant over the spectral channels.

Figure 3. The spectrally resolved phasors from experiment and theory from (a) DNA sample and (b) mixture of pure donor and DNA sample.

Figure 4. The spectrally resolved FRET efficiency for DNA sample and mixture of pure donor and DNA sample.

Figure 3(b) shows the phasors of a mixture containing 30% donor only stock solution and 70% donor–acceptor DNA construct stock solution. Both solutions had the same concentration. Fitting the experimental data to the modified equation (10) and employing the values of $Q$ and $\sigma$ from the previous fit yield the energy transfer efficiency $k$ and the fraction of pure donor $\alpha$. This fit yields a global FRET efficiency $E$ of $25.2 \pm 0.39\%$ and the molar fraction of pure donor is estimated to be $48.0 \pm 1.7\%$. The difference with the 30% donor fraction is explained by the effect of the wavelength dependency of the detection efficiency response which is not corrected in this work.

Figure 4 shows the spectrally resolved FRET efficiency for the two samples. The FRET efficiencies are found by intersecting the line connecting the pure acceptor phasor with the FRET phasor trajectory of each spectral channel (calculated using equation (11)). While the FRET efficiency stays constant over the whole spectral range for the sample with only DNA, the DNA—pure donor mixture shows a lower FRET efficiency at the first channels and then it goes up to about 23%. This behavior can be explained by the presence of the pure donor in the first channels; this reduces the average FRET efficiency by the presence of the pure donor lifetimes. As we move to the red part of spectrum, the signal from the donor molecule decreases (see figure 5) and the FRET efficiency becomes less sensitive to the presence of the pure donor molecules and it approaches the true FRET efficiency of the DNA construct.

Experiment on labeled DNA (1:1 donor to acceptor stoichiometry) was carried out for different numbers of detected photons. FRET efficiencies were calculated using both the phasor approach which uses the whole signal over the complete spectral range and using conventional, single channel, time domain lifetime detection of the donor emission. The latter data was analyzed using nonlinear least square analysis [20]. The variation in the number of detected photons was obtained by recording ten separate images and adding images to create the required number of counts. According
to figure 5 the contribution of signal from the acceptor can be ignored in channels 1–4 and it can be assumed that the signal is, to a good approximation, originating from donor molecule only. The results of the count rate dependent FRET efficiency measurements are shown in figure 6. While the FRET estimation from the phasor approach shows an almost constant FRET efficiency, the result from the single channel method shows an increasing trend for decreasing number of counts. By decreasing the total number of counts the detected photons at later times after excitation goes down and the estimated lifetime biased toward shorter lifetimes which is translated to higher FRET efficiency. This behavior is not observed in the global phasor approach. Here, the whole signal is employed and the proposed model describes the FRET process in a more accurate way by including all parameters which are included in this process like quantum efficiencies and absorption cross sections.

4. Conclusion and discussion

Resonance energy transfer has been extensively used as a ruler to estimate distances on a molecular scale and to study molecular interactions. So far the technique of choice to measure FRET has been fluorescence lifetime imaging. Here, the donor fluorescence lifetime is measured and used to obtain the FRET efficiency. This is a reliable technique but it requires comparatively high signals to realize accurate results. Current lifetime methods only use the donor channel to quantify FRET. Using the whole spectral range increases the amount of signal available for the analyses and can increase the accuracy of the FRET estimation.

We introduce a novel method for estimation of FRET efficiency using spectrally resolved fluorescence lifetime images in combination with phasor analysis. This method has the potential to considerably improve FRET measurements. It is shown that the phasors from all spectral channels fall on a line with the phasor of the pure acceptor as one of the end points. The intersection of this line with the reference phasor circle yields the FRET efficiency. The behavior of the acceptor fluorescence is complicated by the ingrowth of the fluorescence due to FRET. This is translated into a simple shift of the phasors on the line produced by the phasors from all spectral channels.

The phasor analysis can take into account bi-exponential behavior of both donor and acceptor molecules. This yields a modified, but well predictable, FRET trajectory. Phasor analysis of the experimental results for bi-exponentially decaying donors and acceptors are in good agreement with the predicted behavior. Here spectrally invariant lifetime are assumed and that both donor decay components experience the same rate of energy transfer to the acceptor molecule. This assumption requires similar spectral overlap between the different decay components of donor and acceptor.

The use of spectrally resolved lifetime imaging systems can provide improved estimates of FRET efficiencies but also additional information about the specimen. The behavior of the spectrally resolved FRET efficiency provides information about the donor fraction involved in FRET. This was demonstrated using a sample consisting of a mixture of pure donor and DNA coupled donor–acceptor. The presence of low FRET efficiency in the donor channel compared to the acceptor channel is indicative of the presence of donors not involved in FRET. Using our phasor analysis the fraction of donors involved in FRET and the correct FRET efficiency are obtained. More importantly our new proposed method is shown that provides higher accuracy with same number of photon counts; while the FRET estimation by conventional methods shows a bias toward larger FRET efficiencies, the global phasor approach estimates the transfer efficiency with higher accuracy.

Currently we are considering the possibility of studying systems consisting of donor molecules interacting with multiple acceptor molecules. This will show a higher FRET efficiency compared to a situation with only one donor and one acceptor and this is reflected in the relative position of the phasor point on the line. There are prospects to generalize the formalism to analyze arbitrary combinations of interacting and non-interacting donors and acceptors. However, resolving more parameters will in general reduce the accuracy of the
fitted parameters. Currently we use only seven spectral channels and more channels may be required for full analysis of arbitrary systems of donors and acceptors.

Although our analyses method yields higher accuracies than existing methods and provides additional information, it does need separate reference measurements of both donor and acceptor. In conventional lifetime imaging techniques the only requirement is the separate measurement of the donor lifetime. The FRET efficiency can be simply calculated by comparing the lifetime of the donor in the FRET sample with the reference lifetime. In our phasor based method the analysis is slightly more complicated; it requires fitting of the equation (10) to the experimental phasor points.

Follow up experiments are planned to further validate this novel FRET analyses method. Analysis of FRET imaging experiments on (living) cells to visualize molecular interactions is a logical next step. In addition further characterization of the analyses in terms of figures of merit as used in [17] would be valuable to better understand the statistics, in particular the number of photons required for obtaining a certain accuracy in FRET efficiency.

References

[1] Wallrabe H and Periasamy A 2005 Imaging protein molecules using FRET and FLIM microscopy Curr. Opin. Biotechnol. 16 19–27
[2] Wouters F S 2011 Imaging molecular physiology in cells using FRET-based fluorescent nanosensors Optical Fluorescence Microscopy (Berlin, Heidelberg: Springer) pp 131–52
[3] Gerritsen H, Agronskaia A, Bader A and Esposito A 2009 Time domain FLIM: theory, instrumentation, and data analysis Lab. Tech. Biochem. Mol. Biol. 33 95–132
[4] Kinoshita A, Whelan C M, Smith C J, Mikhailenko I, Rebeck G W, Strickland D K and Hyman B T 2001 Demonstration by fluorescence resonance energy transfer of two sites of interaction between the low-density lipoprotein receptor-related protein and the amyloid precursor protein: role of the intracellular adapter protein Fe65 J. Neurosci. 21 8354–61
[5] Wouters F S, Bastiaens P I H, Wirtz K W A and Jovin T M 1998 FRET microscopy demonstrates molecular association of non-specific lipid transfer protein (nSL-TP) with fatty acid oxidation enzymes in peroxisomes EMBO J. 17 7179–89
[6] Chen H, Puhl H L, Koushik S V, Vogel S S and Ikeda S R 2006 Measurement of FRET efficiency and ratio of donor to acceptor concentration in living cells Biophys. J. 91 L39–41
[7] Berney C and Danuser G 2003 FRET or no FRET: a quantitative comparison Biophys. J. 84 3992–4010
[8] Laptenok S P, Borst J W, Mullen K M, van Stokkum I H M, Visser A J W G and van Amerongen H 2010 Global analysis of Förster resonance energy transfer in live cells measured by fluorescence lifetime imaging microscopy exploiting the rise time of acceptor fluorescence Phys. Chem. Chem. Phys. 12 7593–602
[9] Biskup C, Zimmer T, Kelbassa L, Hoffmann B, Klöcker N, Becker W, Bergmann A and Berndorf K 2007 Multidimensional fluorescence lifetime and FRET measurements Microsc. Res. Tech. 70 442–51
[10] CHEN Y C and Clegg R 2011 Spectral resolution in conjunction with polar plots improves the accuracy and reliability of FLIM measurements and estimates of FRET efficiency J. Microsc. 244 21–37
[11] Forde T S and Hanley Q S 2006 Spectrally resolved frequency domain analysis of multi-fluorophore systems undergoing energy transfer Appl. Spectrosc. 60 1442–52
[12] Strat D, Dolp F, von Eenem B, Steinmetz C, von Arnim C A F and Rueck A 2011 Spectrally resolved fluorescence lifetime imaging microscopy: Förster resonant energy transfer global analysis with a one-and two-exponential donor model J. Biomed. Opt. 16 026002
[13] Digman M A, Caiolfa V R, Zamai M and Gratton E 2008 The phasor approach to fluorescence lifetime imaging analysis Biophys. J. 94 L14–6
[14] Fereidouni F, Esposito A, Blag B and Gerritsen H 2011 A modified phasor approach for analyzing timedegated fluorescence lifetime images J. Microsc. 244 248–58
[15] Fereidouni F, Bader A N and Gerritsen H C 2012 Spectral phasor analysis allows rapid and reliable unmixing of fluorescence microscopy spectral images Opt. Express 20 12729–41
[16] Lakowicz J R 2006 Principles of Fluorescence Spectroscopy (Berlin: Springer)
[17] Fereidouni F, Reitsma K and Gerritsen H 2013 High speed multispectral fluorescence lifetime imaging Opt. Express 21 11782
[18] Kovacic S, Samii L, Woolfson D N, Curmi P M G, Linke H, Forde N R and Blag B A 2012 Design and construction of a one-dimensional DNA track for an artificial molecular motor J. Nanomater. 2012 109238
[19] Schlachter S, Elder A, Esposito A, Kaminski G, Frank J, Van Geest L and Kaminski C 2009 mhFLIM: resolution of heterogeneous fluorescence decays in widefield lifetime microscopy Opt. Express 17 1557–70
[20] O’connor D, Ware W and Andre J 1979 Deconvolution of fluorescence decay curves. A critical comparison of techniques J. Phys. Chem. 83 1333–43