Additional correction for energy transfer efficiency calculation in filter-based Förster resonance energy transfer microscopy for more accurate results

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Abstract. Förster resonance energy transfer (FRET) microscopy is commonly used to monitor protein interactions with filter-based imaging systems, which require spectral bleedthrough (or cross talk) correction to accurately measure energy transfer efficiency (E). The double-label (donor+acceptor) specimen is excited with the donor wavelength, the acceptor emission provided the uncorrected FRET signal and the donor emission (the donor channel) represents the quenched donor (qD), the basis for the E calculation. Our results indicate this is not the most accurate determination of the quenched donor signal as it fails to consider the donor spectral bleedthrough (DSBT) signals in the qD for the E calculation, which our new model addresses, leading to a more accurate E result. This refinement improves E comparisons made with lifetime and spectral FRET imaging microscopy as shown here using several genetic (FRET standard) constructs, where cerulean and venus fluorescent proteins are tethered by different amino acid linkers. © 2010 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.3407655]

Keywords: Förster resonance energy transfer microscopy; filter-based Förster resonance energy transfer microscopy; bandpass filters; quenched donor; spectral bleedthrough; Förster resonance energy transfer standards.

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1 Introduction
In Förster resonance energy transfer (FRET) microscopy, detection of the sensitized emission from the acceptor—the FRET signal—is obtained by exciting the specimen containing both the donor and acceptor with the donor excitation wavelength. Accurate quantification of FRET signals requires the removal of spectral bleedthrough (SBT) contaminations, which include the donor SBT (DSBT) resulting from the donor emission that is detected in the FRET channel, and the acceptor SBT (ASBT) caused by the direct excitation of the acceptor at the donor excitation wavelength. Algorithms have been developed for various microscopy techniques to identify and remove the SBT contaminations, allowing accurate measurements of the energy transfer efficiency $E$. In filter-based FRET microscopy, signals measured in the donor and acceptor emission channels are separated using bandpass filters. In contrast, spectral FRET microscopy uses a spectral detector to measure signals over a continuous emission spectrum. Comparing FRET measurements from cells expressing FRET-standard proteins obtained by filter-based or spectral methods suggested a source of error in the filter-based measurements. Our model, which includes the DSBT signals in the quenched donor $qD$ for E calculation provides a more accurate measurement of $E$ in filter-based FRET microscopy not previously considered in commonly used algorithms.

This source of error is not an issue in spectral FRET microscopy, since DSBT is included in the qD used for calculating E. The new model was tested with measurements from GHFT1 cells, expressing several different genetic (FRET standard) constructs, including C5V, C17V, and C32V, where cerulean (C) and venus (V) are directly tethered by either a 5, 17, or 32 amino acid linker. Additionally, a CTV construct was used, where C and V are separated by a 229 amino acid linker encoding the tumor necrosis factor receptor-associated factor (TRAF) domain. The results are confirmed with both time-correlated single photon counting (TCSPC) and frequency-domain (FD) lifetime measurements.

2 Methods
In our theoretical model, for a donor (D)-acceptor (A) FRET system, the energy transfer efficiency $E$ is defined as the energy transfer rate $kT$ divided by the sum of all deactivation rates of the excited state of D $(kT + kD)$, where $kD$ is the sum of its deactivation rates other than FRET. At the D excitation wavelength, the decay profiles of the excited D $[Dd(t)$ in Eq. (1)] and the excited A $[Ad(t)$ in Eq. (2)] are presented, where $kA$ is the sum of the rates for deactivation of the excited A, and $D_0$ and $A_0$ are the absorbed intensities of the D and A at $t=0$, respectively.

\[
D_d(t) = D_0 \exp[-(kD + kT)t],
\]
\[
A_d(t) = D_0 \left[\frac{\exp[-(kD + kT)t] - \exp(-kA)t}{kA - (kD + kT)}\right] + A_0 \exp(-kAt).
\]

Integrating $D_d(t)$ and $A_d(t)$ over time (0 to $\infty$) yields the absorbed intensities of D and A, which are then multiplied by their radiative rates $(kDR$ and $kAR$) to obtain the emitted intensities of D $[IB_{DD}$ in Eq. (3)] and A $[IB_{DA}$ in Eq. (4)].

\[
IB_{DD} = D_0 [kD/(kD + kT)] = QYD D_0 [1 - E], \quad QYD = kD/kD,
\]

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\[ \text{IBDA} = D_0 \left( \frac{k_A}{k_D + k_T} \right) + A_0 \left( \frac{k_A}{k_A} \right) = QY_A D_0 E + QY_A A_0, \]

\[ QY_A = \frac{k_A}{k_A}. \]  

Based on \( E = k_T (k_D + k_T) \), \( k_T + k_T \) is substituted by \((1 - E)/k_D \) in Eq. (3), and \( k_T (k_D + k_T) \) is substituted by \( E \) in Eq. (4). \( QY_D \) and \( QY_A \) are the respective natural quantum yields of D and A. IBDA refers to the signals emitted from the donor, which are called quenched donors (qD). IBDA represents the signals emitted from the acceptor and is composed of the FRET \( (QY_A D_0 E) \) and ASBT \( (QY_A A_0) \) signals (see Sec. 1).

In spectral microscopy imaging, the IBDD in Eq. (3) and IBDA in Eq. (4) can be directly measured using the combination of linear unmixing and ASBT correction to estimate \( E \). In our spectral FRET method known as sFRET, using the D \( (IDD_D) \) and A \( (IAA_A) \) reference spectra obtained from the respective single-label to unmix a double-label \( \lambda \) stack obtained under the donor excitation \( (IBDS) \) produces IBDD and IBDA images. Thus, \( E \) can be calculated by Eq. (5), which is derived from Eqs. (3) and (4).

\[ E = \frac{\text{coef} \cdot \text{FRET}}{\text{coef} \cdot \text{FRET} + qD}, \]

\[ qD = IBDD, \quad \text{coef} = \frac{SSD}{SSA} \left( \frac{QY_D}{QY_A} \right), \]  

where a coefficient (coef) is introduced with \( SSD \) and \( SSA \), which are the detector quantum efficiencies at the D and A peak emission wavelengths, respectively. The \( qD \) signals are quantified by the IBDD image. The FRET signals are measured by separating the ASBT signals from the IBDA image, and the ASBT signals are determined using the single-label acceptor specimens.

In filter-based microscopy imaging, separate bandpass filters are usually used to measure the signals emitted close to D and A peak emission wavelengths. In our filter-based FRET method known as PFRET, at the D excitation wavelength, two images of the donor- and acceptor-labeled specimen are acquired \( \text{IBDa} \) in the D emission channel and IBDD in the A emission channel. In contrast to the IBDA image in spectral FRET, the IBDD image acquired using the filter-based method also contains the DSBT signals emitted from the donor, in addition to the FRET and ASBT signals emitted from the acceptor. The IBDD image is commonly used to measure the \( qD \) signals for the \( E \) calculation, as shown in Eq. (6).

\[ E = \frac{\text{coef} \cdot \text{FRET}}{\text{coef} \cdot \text{FRET} + qD}, \]

\[ qD = IBDD, \]  

where coef has the same meaning as described in Eq. (5). FRET is measured by separating both DSBT and ASBT from the IBDD image, and the DSBT and ASBT are determined using the single-label donor and acceptor specimens. The DSBT signals appearing in the IBDD image in filter-based FRET are included in the \( qD \) (the IBDD image) for the spectral FRET E calculation [Eq. (5)], but it is not in the \( qD \) (the IBDD image) for the filter-based FRET E calculation [Eq. (6)].

Significantly, DSBT is actually part of \( qD \), so adding DSBT back to the IBDD image can provide a more accurate \( E \) measurement when using a filter-based FRET method, as shown in Eq. (7).

\[ E = \frac{\text{coef} \cdot \text{FRET}}{\text{coef} \cdot \text{FRET} + qD}, \]

\[ qD = IBDD + DSBT. \]  

3 Results and Discussions

The use of FRET-standard fusion proteins allowed direct comparison of the energy transfer efficiencies (\( E \)) measured...
in intensity- and lifetime-based FRET microscopy. The representative decays and phasor plots \( \text{Em. 458~561 nm} \) and \( \text{C5V} \) are compared as columns and actual numbers in the inset \( n > 12 \) for each construct measured in each method; the bar on the top of each column indicates the standard deviation. \( E_s \) in filter-based FRET were calculated using Eqs. (6) and (7) (see Sec. 2). For the C5V, C17V, or C32V construct, ANOVA analyses indicate conventional Eq. (6) results in a statistically different \( E \) of the same construct estimated by other methods (spectral, TCSPC, and FD) \( p < 0.05 \). In contrast, Eq. (7) statistically matches the other methods \( p > 0.05 \). For the CTV construct, the intensity-based \( E_s \) are found to be different than the FLIM-FRET \( E_s \) based on ANOVA analysis, because the very low FRET signal level of the CTV construct results in a poor signal-to-noise ratio (SNR) in the intensity-based methods, and in turn affects the accuracy of their \( E \) estimates. However, it is still clearly shown that the average \( E \) obtained with Eq. (7) is closer to those obtained by other methods than the average \( E \) obtained with Eq. (6) within their small variations. [For filter based and spectral FRET: Zeiss S10 Meta; 63X/1.4NA Oil, Ex. 458 nm (donor), Ex. 415 nm (acceptor); filter-based: Em. 470–500 nm (donor) and Em. 535–590 nm (acceptor); spectral: Em. 458–561 nm.]

Fig. 2 FRET efficiency \( (E) \) comparison. The average \( E \)s of CTV, C32V, C17V, and C5V constructs measured in the four FRET microscopy methods are compared as columns and actual numbers in the inset \( n > 12 \) for each construct measured in each method; the bar on the top of each column indicates the standard deviation. \( E_s \) in filter-based FRET were calculated using Eqs. (6) and (7) (see Sec. 2). For the C5V, C17V, or C32V construct, ANOVA analyses indicate conventional Eq. (6) results in a statistically different \( E \) of the same construct estimated by other methods (spectral, TCSPC, and FD) \( p < 0.05 \). In contrast, Eq. (7) statistically matches the other methods \( p > 0.05 \). For the CTV construct, the intensity-based \( E_s \) are found to be different than the FLIM-FRET \( E_s \) based on ANOVA analysis, because the very low FRET signal level of the CTV construct results in a poor signal-to-noise ratio (SNR) in the intensity-based methods, and in turn affects the accuracy of their \( E \) estimates. However, it is still clearly shown that the average \( E \) obtained with Eq. (7) is closer to those obtained by other methods than the average \( E \) obtained with Eq. (6) within their small variations. [For filter based and spectral FRET: Zeiss S10 Meta; 63X/1.4NA Oil, Ex. 458 nm (donor), Ex. 415 nm (acceptor); filter-based: Em. 470–500 nm (donor) and Em. 535–590 nm (acceptor); spectral: Em. 458–561 nm.]

Fig. 2 clearly demonstrate that the theoretically derived Eq. (7) produces an \( E \) for filter-based confocal microscopy that more closely matches the lifetime or spectral FRET measurements. Based on our PFRET results, the accuracy of \( E \) increases by including the DSRT in \( qD \) [Eq. (7)]. The refinement of the \( E \) calculation presented here is of particular interest to those researchers who use multiple FRET methods, sometimes simultaneously, to achieve their research goals.

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References


