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 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. The 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 ($k_T$) divided by the sum of all deactivation rates of the excited state of $D$ ($k_T+k_D$), where $k_D$ is the sum of its deactivation rates other than FRET. At the $D$ excitation wavelength, the decay profiles of the excited $D$ [${D}_D(t)$ in Eq. (1)] and the excited $A$ [${A}_D(t)$ in Eq. (2)] are presented, where $k_A$ 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.

\[
{D}_D(t) = D_0 \exp \left[ -\left( k_D + k_T \right) t \right],
\]

\[
{A}_D(t) = A_0 \exp \left[ -\left( k_D + k_T \right) t \right] = \frac{k_A - \left( k_D + k_T \right)}{k_A - \left( k_D + k_T \right)} \exp \left[ -\left(k_D + k_T \right)t \right] + A_0 \exp \left[ -k_A t \right].
\]

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 ($k_{DP}$ and $k_{AP}$) to obtain the emitted intensities of $D$ [$I_{DD}$ in Eq. (3)] and $A$ [$I_{DA}$ in Eq. (4)].

\[
I_{DD} = D_0 \left[k_D/(k_D + k_T)\right] = QY_D D_0 \left[1 - E\right], \quad QY_D = k_D/k_A,
\]

where $QY_D$ is the quantum yield of donor emission.
and A peak emission wavelengths. In our filter-based FRET method known as PFRET,7 at the D excitation wavelength, two images of the donor- and acceptor-labeled specimen are acquired −IBDa in the D emission channel and IBDa in the A emission channel. In contrast to the IBDa image in spectral FRET, the IBDa 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 IBDa image is commonly used to measure the qD signals for the E calculation, as shown in Eq. (6).1–8

\[ E = \frac{\text{coef} \cdot \text{FRET}}{\text{coef} \cdot \text{FRET} + qD}, \quad \text{FRET} = IBDa - \text{ASBT} - \text{DSBT}, \]

\[ qD = IBDa, \]

where coef has the same meaning as described in Eq. (5). FRET is measured by separating both DSBT and ASBT from the IBDa image, and the DSBT and ASBT are determined using the single-label donor and acceptor specimens.7 The DSBT signals appearing in the IBDa image in filter-based FRET are included in the qD (the IBDa image) for the spectral FRET E calculation [Eq. (5)], but is not in the qD (the IBDa image) for the filter-based FRET E calculation [Eq. (6)]. Significantly, DSBT is actually part of qD, so adding DSBT back to the IBDa image can provide a more accurate E measurement when using a filter-based FRET method, as shown in Eq. (7).

\[ E = \frac{\text{coef} \cdot \text{FRET}}{\text{coef} \cdot \text{FRET} + qD}, \quad \text{FRET} = IBDa - \text{ASBT} - \text{DSBT}, \]

\[ qD = IBDa + \text{DSBT}. \]

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 of the C-alone and FRET-standard constructs (Fig. 1) clearly demonstrate a shorter lifetime from C to CTB to C17V to C5V, and C17V to C5V. In both TCSPC and FD FLIM measurements, the $E$ of a FRET-standard construct was estimated from the donor and acceptor alone and FRET-standard constructs measured in the four FRET microscopy methods as compared to column and actual numbers in the inset ($n$=12 for each construct measured in each method; the bar on the right 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 CTB 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 CTB construct results in a poor signal-to-noise ratio (SNR) in the intensity-based methods, and in turn affects the accuracy of their $E$ estimations. 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 510 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](image-url) FRET efficiency ($E$) comparison. The average $E$s of CTB, 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 right 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 CTB 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 CTB construct results in a poor signal-to-noise ratio (SNR) in the intensity-based methods, and in turn affects the accuracy of their $E$ estimations. 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 510 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 DSBT 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


