Kinetics model for the wavelength-dependence of excited-state dynamics of hetero-FRET sensors
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ABSTRACT

Förster (or fluorescence) resonance energy transfer (FRET) is a powerful tool for investigating protein-protein interactions, in both living cells and in controlled environments. A typical hetero-FRET pair consists of a donor and acceptor tethered together with a linker. The corresponding energy transfer efficiency of a hetero-FRET pair probe depends upon the donor-acceptor distance, relative dipole orientation, and spectral overlap. Because of the sensitivity of the energy transfer efficiency on the donor-acceptor distance, FRET is often referred to as a “molecular ruler”. Time-resolved fluorescence approach for measuring the excited-state lifetime of the donor and acceptor emissions is one of the most reliable approaches for quantitative assessment of the energy transfer efficiency in hetero-FRET pairs. In this contribution, we provide an analytical kinetics model that describes the excited-state depopulation of a FRET probe as a means to predicts the time-resolved fluorescence profile as a function of excitation and detection wavelengths. In addition, we used this developed kinetics model to simulate the time-dependence of the excited-state population of both the donor and acceptor. These results should serve as a guide for our ongoing studies of newly developed hetero-FRET sensors (mCerulean3–linker–mCitrine) that are designed specifically for in vivo studies of macromolecular crowding. The same model is applicable to other FRET pairs with the careful consideration of their steady-state spectroscopy and the experimental design for wavelength-dependence of the fluorescence lifetime measurements.

Keywords: FRET, fluorescence lifetime, excited-state dynamics, kinetics model
1. INTRODUCTION

Fluorescence resonance energy transfer, or FRET, is a powerful tool\textsuperscript{1-5} for investigating conformational changes in biomolecules\textsuperscript{6}, intermolecular and protein-protein interactions\textsuperscript{7-9}. The energy transfer efficiency between a donor and acceptor (i.e., FRET pair) depends on (i) the spectral overlap between the donor’s emission and the acceptor’s absorption, (ii) the intermolecular donor-acceptor distance, and (iii) the relative orientation of the dipole moments of the FRET pair\textsuperscript{10-11}. FRET methods have been used successfully for intermolecular interactions in both solution and living cells\textsuperscript{12}. The energy transfer efficiency in FRET studies can be determined using steady-state spectroscopy of a solution in a cuvette\textsuperscript{12}, multichannel confocal microscopy\textsuperscript{13} or total-internal refraction fluorescence (TIRF) microscopy\textsuperscript{14} for intracellular investigations. However, these steady-state spectroscopy based methods suffer from complications due to the spectral overlap and inability to resolve an increase in the energy transfer efficiency versus an increase in the FRET population.\textsuperscript{15-16} These challenges can be overcome using time-resolved fluorescence measurements to investigate energy transfer efficiency of FRET pairs in both well-controlled environments\textsuperscript{12} and in living cells using fluorescence lifetime imaging microscopy (FLIM) modality\textsuperscript{5, 17}.

In this report, we provide an analytical kinetics model that describes the excited-state depopulation of a FRET probe as a means to predict the time-resolved fluorescence profile as a function of excitation and detection wavelengths. In addition, we applied this kinetics model to simulate the time-dependence of the excited-state population of both the donor and acceptor. These results should serve as a guide for our ongoing studies of newly developed hetero-FRET sensors (mCerulean3–linker–mCitrine) that are designed specifically for \textit{in vivo} studies of macromolecular crowding (see Figure 1). The same model can be applied to other FRET pairs with the careful consideration of their steady-state spectroscopy and the experimental design for wavelength-dependence of the fluorescence lifetime measurements.

![Figure 1](image-url). A sketch describing a novel hetero-FRET probe designed for macromolecular crowding studies\textsuperscript{5, 18}. The donor (mCerulean3) and acceptor (mCitrine) absorb maximally at 425 nm and 480 nm, respectively\textsuperscript{19}. The corresponding emission of mCerulean and mCitrine peaks around 475 and 530 nm, respectively. This FRET sensor and a number of its derivatives with variable linkers are currently being investigated in biomimetic crowding using time-resolved fluorescence methods. Note that cleaving the linker region using proteinase K can disrupt energy transfer in this construct. The resulting cleaved FRET probe is a useful control for measuring the fluorescence lifetime of donor alone because mCerulean3 and mCitrine are no longer in close proximity to each other.
2. A KINETICS MODEL FOR THE DYNAMICS OF HETERO-FRET PAIRS

Consider a FRET probe such as mCerulean3–linker–mCitrine, where mCerulean3 is the donor (D) and mCitrine is the acceptor (A). Based on steady-state spectroscopy of this FRET probe, the donor absorbs maximally around 433 nm and emits fluorescence at 475 nm in the presence (intact probe) and absence (cleaved probe) of the acceptor. Using a kinetic model as described in Figure 2, one can predict the nature of the fluorescence decay of the FRET probe under different conditions of excitation and detection wavelengths. Such a model would allow for flexibility in experimental design towards the determination of energy transfer efficiency under different crowding environments. Below, we discuss our model under different scenarios of excitation and detection wavelengths combinations.

Figure 2. A schematic representation of the wavelength-dependence of the excitation and detection of a FRET pair. The excitation wavelengths shown here are specific for the donor (mCerulean3) and acceptor (mCitrine) in the FRET pair shown in Figure 1. Once the donor in a FRET probe is excited at a rate $k_x$, the donor’s emission (detected around 475 nm) will decay at a rate that depends upon both its excited state lifetime ($k_f^D = 1/\tau_f^D$) and the energy transfer rate ($k_{ET}$) to the acceptor. Under 425-nm excitation of the donor, the emission of the excited acceptor ($A^*$) will build up at energy transfer rate ($k_{ET}$) and decays at a rate $k_f^A$ that depends in its intrinsic excited-state lifetime ($\tau_f^A$).

2.1 Exciting and detecting the donor in a FRET pair:

Under 425-nm excitation of the FRET probe, the donor alone will be excited from the ground electronic state (D) to the excited electronic state (D*). The time-dependent D* population ($N_{D*}^D = N_{D*}^D + N_{D*}^S$) in an intact FRET probe, where the donor is excited at 425 nm and its emission is detected at 475/50 nm, can be described as:

$$\frac{dN_{D*}^D}{dt} = -\left( k_f^D + k_{ET} \right) N_{D*}^D$$  \hspace{1cm} (1)

Let us assume the initial conditions where at time $t = 0$, $N_{D*}^D = N_{D*}^D$. Following the excitation of the donor and during the excited-state lifetime, we assumed that $N_{D*}^D = N_{D*}^D + N_{D*}^S$, due to mass conservation. Upon integrating equation (1) we find:

$$\int_{N_{D*}^D}^{N_{D*}^D} \frac{dN_{D*}^D}{N_{D*}^D} = -\int_0^t \left( k_f^D + k_{ET} \right) dt$$  \hspace{1cm} (2)
Assuming that all the excited population of a FRET probe will undergo energy transfer, equation (3) indicates that the donor emission in the FRET probe will decay as a single exponential at a rate of \((k_f^D + k_{ET})\). Using polarized laser pulses for excitation, however, one may expect a snapshot of subpopulation of the FRET probe that undergoes energy transfer while another subpopulation does not due to either unfavorable dipole orientations of the acceptor with respect to the excited donor and/or different sampling of the donor-acceptor distances during thermal fluctuation. In this case, one would expect to observe a biexponential fluorescence decay of the FRET probe that can be described as the sum of equation (3) and (4); see below.

If we disrupt FRET by cleaving the FRET probe in the linker region so that mCerulean3 and mCitrine are no longer in close proximity to one another, we would expect that the donor emission will now decay as a single exponential at a rate of \((k_f^D)\) such that:

\[
N_D^*(t) = N_{D0}^* e^{-(k_f^D + k_{ET}) t}
\]

As a result, the energy transfer rate constant \(k_{ET}\) can be determined using equations (3) and (4) for the intact (energy transfer occurs) and cleaved (energy transfer is disrupted) FRET probe, measured under the same experimental conditions, such that:

\[
E = \frac{k_{ET}}{k_f^D + k_{ET}}
\]

Keep in mind that the fluorescence lifetime of the donor alone \(\tau_f^D = 1/k_f^D\) can be measured directly using the cleaved version of the FRET probe and the time-correlated single photon counting (TCSPC) technique.

### 2.2 Exciting the donor and detecting the acceptor’s emission in a FRET pair:

Under 425-nm excitation of the FRET probe, the donor alone will be excited from the ground electronic state \((D)\) to the excited electronic state \((D^*)\). The time-dependent \(D^*\) population \(N_D^* = N_D^{S1}\) in an intact FRET probe, where the donor is excited at 425 nm and the acceptor’s emission is detected at 530/40 nm, can be described as:

\[
\frac{dN_D^*}{dt} = k_{ET} N_D^* - k_f^A N_A^*
\]

But, as we described above, the excited-state population of the donor in the FRET probe decays as a single exponential:

\[
N_D^*(t) = N_{D0}^* e^{-(k_f^D + k_{ET}) t}
\]

As a result, equation (6) can be rewritten as:
\[
\frac{dN_A^*}{dt} = k_{ET}N_{D0}e^{-(k_f^D + k_{ET})t} - k_f^A N_A^*
\]  
(7)

By rearranging, we obtain:

\[
\frac{dN_A^*}{dt} + k_f^A N_A^* = k_{ET}N_{D0}e^{-(k_f^D + k_{ET})t}
\]  
(8)

Equation (8) is a standard differential equation with the solution:

\[
N_A^*(t) = \frac{\left(k_{ET} + k_f^D\right)N_{D0}^*}{\left[k_{ET} + k_f^D\right] - k_f^A} \left(e^{-k_f^A t} - e^{-(k_f^D + k_{ET})t}\right)
\]  
(9)

Equation (9) indicates that the temporal behavior of the acceptor’s emission in the FRET probe, exciting the donor alone at 425 nm, will have a double exponential feature, with a build up component (i.e., rise with a negative amplitude) plus a decay component (i.e., positive amplitude) depending on the relative values of the rate constants \((k_f^A)\) as compared with \((k_f^D + k_{ET})\) as described below:

(2.a) \((k_{ET} + k_f^D) > k_f^A\): The acceptor emission will build up at a rate constant of \((k_f^D + k_{ET})\), which is the sum of the energy transfer rate of the intact FRET probe plus the fluorescence decay rate of the donor alone. In addition, the acceptor emission will decay at a rate constant of \(k_f^A\), which is the inverse of the fluorescence lifetime of the acceptor alone.

(2.b) \((k_{ET} + k_f^D) < k_f^A\): The acceptor emission will build up at a rate constant of \(k_f^A\), which is the inverse of the fluorescence lifetime of the acceptor alone. In addition, the acceptor emission will decay at a rate constant of \((k_f^D + k_{ET})\), which is the sum of the energy transfer rate in the FRET probe plus the fluorescence decay rate of the donor alone.

For the cleaved FRET probe under these excitation and detection conditions, however, the fluorescence emission will decay as a single exponential with a decay rate of \((k_f^D)\) due to the spectral overlap between the donor emission and the detection window (530/40 nm), similar to equation (3) above. However, this is correct only in the case of zero-probability of direct excitation of the acceptor under 425-nm illumination. Assuming that there is a small probability of direct excitation of the acceptor at 425-nm illumination, then the detected fluorescence decay of the cleaved FRET probe will decay as a biexponential such that:

\[
N_{DA}^*(t) = \alpha_1e^{-k_f^D t} + \alpha_2e^{-k_f^A t}
\]  
(10)
where \( \alpha_1 \) and \( \alpha_2 \) depend on the initial excited-state population (\( N_{D0}^* \) and \( N_{A0}^* \)) as well as the relative emission intensity of the donor and acceptor, respectively, detected using 530/40 nm filter.

2.3 Exciting and detecting both the donor and acceptor together in a FRET pair due to spectral overlap:

Under 465-nm excitation of the FRET probe, both the donor and acceptor will be excited relatively at rate that depends on the corresponding 465-nm absorption cross-section. In addition, the corresponding donor and acceptor emissions would also be detected at efficiencies that depend on their spectral overlap of their emission within the detection window (530/40 nm). Accordingly, the time-dependent (D*+A*) population (\( N_D^* + N_A^* = N_D^S + N_A^S \)) in an intact FRET probe, where the FRET pair is excited at 465 nm and their relative emissions are detected at 530/40 nm, can be described as:

\[
N_{total}(t) = \beta_1 \cdot N_{D0}^* + \beta_2 \cdot N_{A0}^* \tag{11}
\]

where \( \beta_1 \) and \( \beta_2 \) depend on the excitation cross-section and the relative emission intensity of the donor and acceptor, respectively, under 465-nm illumination and 530/40-nm detection. The excited-state population of the donor (equation 3) and the acceptor (equation 9) can be used to predict the decay profile of the donor-acceptor total emission, which will depend on the relative rate constants, \( (k_{ET}^D + k_f^D) \) and \( k_f^A \) as described for the following conditions.

(3.a) \( (k_{ET}^D + k_f^D) > k_f^A \):

\[
N_{total}^*(t) = \left[ \beta_1 \cdot N_{D0}^* - \frac{\beta_2 \cdot (k_{ET}^D + k_f^D) \cdot N_{D0}^*}{(k_{ET}^D + k_f^D) - k_f^A} \right] \cdot e^{-(k_f^A + k_{ET}^D)t} + \left[ \frac{\beta_2 \cdot (k_{ET}^D + k_f^D) \cdot N_{D0}^*}{(k_{ET}^D + k_f^D) - k_f^A} \right] \cdot e^{-k_f^A t} \tag{12}
\]

(3.b) \( (k_{ET}^D + k_f^D) < k_f^A \):

\[
N_{total}^*(t) = \left[ \beta_1 \cdot N_{D0}^* + \frac{\beta_2 \cdot (k_{ET}^D + k_f^D) \cdot N_{D0}^*}{k_f^A - (k_{ET}^D + k_f^D)} \right] \cdot e^{-(k_f^A + k_{ET}^D)t} - \left[ \frac{\beta_2 \cdot (k_{ET}^D + k_f^D) \cdot N_{D0}^*}{k_f^A - (k_{ET}^D + k_f^D)} \right] \cdot e^{-k_f^A t} \tag{13}
\]

2.4 Exciting and detecting the acceptor alone in a FRET pair:

Under 490-nm excitation of the FRET probe, the acceptor alone will be excited from the ground electronic state (A) to the excited electronic state (A*). The time-dependent A* population (\( N_A^* = N_A^S \)) in an intact FRET probe, where the acceptor is excited at 490 nm and its emission is detected at 530/40 nm, can be described as:
\[ \frac{dN_A^*}{dt} \bigg|_{490\text{ nm}} = -k_f^A N_A^* \]  

(14)

Let us assume the initial conditions where at time \( t = 0 \), \( N_A^* = N_{A0}^* \). Following the excitation of the donor, and during the excited-state lifetime, \( N_{A0}^* = N_A^* + N_A = N_A^S + N_A^0 \), due to mass conservation. By integrating equation (14) we get:

\[ \int_{N_{A0}^*}^{N_A^*} \frac{dN_A^*}{N_A^*} = -\int_0^t k_f^A \, dt \]

(15)

\[ N_A^*(t) = N_{A0}^* e^{-k_f^A t} = N_{A0}^* e^{-t/\tau_f^A} \]

(16)

Equation (13) indicates that the fluorescence emission of the acceptor in the intact FRET probe, excited at 490 nm and detected at 530/40 nm, will decay as a single exponential at a rate of \( k_f^A \), which is the inverse of the acceptor fluorescence lifetime \( (\tau_f^A) \). As a control, both the cleaved and intact FRET probes would decay as a single exponential at a rate of \( k_f^A \) under the same excitation and detection conditions.

3. SIMULATION OF THE WAVELENGTH-DEPENDENCE OF THE FLUORESCENCE EMISSION OF A FRET PAIR

The model mentioned above allows us to predict the time-resolved fluorescence profile of a FRET probe as a function of the energy transfer rate \( (k_{ET}) \) and excitation/detection wavelength for flexible experimental design. Using OriginPro, we simulated the corresponding fluorescence decays for different scenarios of excitation and detection wavelengths that depend on the absorption and emission spectra of a given FRET probe.

![Figure 3](image-url)

Figure 3. Simulation of the fluorescence decay, or the excited-state population, of the donor alone in an ensemble of FRET probes undergoing energy transfer at variable rates. In this simulation, we used equation 3 of a single exponential decay with a fluorescence lifetime of 4 ns (i.e., \( k_f^D = 0.25 \text{ ns}^{-1} \)) for the donor and variable energy transfer rate \( (k_{ET} = 10, 4.2, 1.05, 0.33, 0.25, 0.17, 0.125, \text{ and } 0.11 \text{ ns}^{-1}) \). The arrow shows the direction of increased energy transfer rate. The values used here were selected relative to the fluorescence lifetime of the donor, during which energy transfer to the acceptor may take place.
Using equation 3, we simulated the time-resolved fluorescence of the donor alone in the hetero-FRET pair, when excited at 425 nm and 475 nm, as shown in Figure 3. In the absence of energy transfer ($k_{ET} = 0$), the fluorescence of the donor alone decays as a single exponential with a time constant that is the excited-state lifetime of the donor (assumed here to be 4.0 ns). Such a scenario is a good control and can be carried out on the donor alone, with wavelength-dependent selectivity, and/or the cleaved FRET probe under identical experimental conditions. In the presence of energy transfer that occurs in the intact FRET probe, the donor fluorescence will decay also as a single exponential but will decay at a rate that is equal to the sum of both the fluorescence decay rate plus the energy transfer rate from the donor to the acceptor. As the energy transfer rate increases, the fluorescence decay of the donor in an intact FRET probe becomes faster as shown in Figure 3.

In Figure 3, it is assumed that the excited population of the donor in a FRET probe undergoes energy transfer. However, it is likely that a fraction of that population might not undergo energy transfer due to unfavorable relative dipole orientations or intermolecular distances due to thermal fluctuations. As a result, we simulated the fluorescence decay of a mixture of two populations excited at 425 nm, one that undergoes FRET (fraction = $f_1$) and another that does not (fraction = $f_2$). Figure 4 shows that the time-resolved fluorescence of such a donor is a biexponential according to the sum of equations (3) and (4) such that:

$$N_D^*(t) = f_1 e^{-(k_F^0 + k_{ET}) t} + f_2 e^{-(k_F^0) t}$$

(17)

Importantly, the faster decays component depends on the energy transfer rate ($k_{ET}$). As shown in Figure 4, as the $k_{ET}$–value increases, the decay time constant of the fraction undergoing energy transfer also increases. At any given time during pulsed excitation, the population fraction of the FRET probe undergoing energy transfer may vary and therefore different time-resolved fluorescence profiles can be expected.

![Figure 4](image-url)
Figure 4 also shows that the slow decay component depends only on the excited-state lifetime of the donor alone. The corresponding fluorescence lifetime of the donor can be measured directly and under the same experimental conditions using the cleaved FRET probe or the donor alone. Here, we assume that the direct excitation of the acceptor is negligible under 425-nm pulsed illumination. Such an assumption can be experimentally met through the careful selection of the excitation wavelength, where the absorption of the acceptor is negligible, and the intensity of the laser used for excitation. Otherwise, the corresponding fluorescence decay is likely to be more complicated, which reflects the excited-state dynamics of the acceptor of an intact FRET probe. Such a complex scenario is also possible for FRET pairs with spectral overlap and an experimental design where both the donor and acceptor can be excited and detected simultaneously.

The energy transfer rate can also be observed directly by exciting the donor alone in a FRET probe while detecting the acceptor’s emission. Based on equation 9, the acceptor’s emission will build up (or rise) at a rate equal to the sum of \((k_{\text{ET}} + k_{D}^0)\) and decay at a rate of \(k_{A}^t\), which is the inverse of the excited-state lifetime of the acceptor alone (see Figure 5). Figure 5 also shows that the acceptor’s fluorescence rise increases as the energy transfer rate increases as compared with the fluorescence decays rate of the corresponding donor. As the energy transfer decreases, the build up of the acceptor fluorescence becomes slower. It is worth mentioning that high temporal resolution would be required here to be able to observe such behavior experimentally for a given FRET probe. Unlike cuvette-based measurements where the fluorescence is detected at the right angle with respect to the laser propagation, these potential high temporal resolution measurements of the acceptor’s fluorescence can also be complicated when using epifluorescence in a microscope setting due to internal reflections, which complicate the fitting near time-zero.

![Figure 5. Simulation of the time-resolved fluorescence of the acceptor in a FRET probe, exciting the donor alone and detecting the acceptor’s emission alone. The fluorescence lifetime of the acceptor here is assumed to be 3.6 ns as compared with 4.0 ns for the donor in the same FRET probe. The acceptor’s fluorescence build up (or rise) depends on the energy transfer rate \((k_{\text{ET}}=10, 4, 2, 1, 0.5, 0.33, 0.25, 0.17, 0.125, \text{ and } 0.11 \text{ ns}^{-1})\). The arrow shows the direction of increased energy transfer rate. In this simulation, we also assumed that \((k_{\text{ET}} + k_{D}^0) > k_{A}^t\) as described in case (2.a).](image-url)
Upon selective excitation and detecting of the acceptor alone in a FRET probe, the corresponding fluorescence of the acceptor will decay as a single exponential with a decay time constant that equal the excited-state lifetime of the free acceptor. Experimentally, however, these measurements can be limited by the availability of the excitation laser pulses at the proper wavelength.

The kinetics model and simulation presented here reveal the potential wavelength-dependent complexity of experimental designs and the data interpretation of hetero-FRET system with a likely spectral overlap. As a result, care must be taken during experimental design and data interpretation using time-resolved fluorescence methods for investigating FRET probes. These challenges can be better understood with careful control experiments such as using the cleaved counterpart of the FRET probe that is incapable of undergoing FRET or the free donor and acceptor for independent studies under the same conditions. For example, the purity and quality of sample preparation must be tested prior to experimentation as shown in Figure 6, which shows a SDS-PAGE gel that reveals the molecular weight of both the cleaved and intact FRET probe shown in Figure 1.

Figure 6. SDS-PAGE image that shows the molecular weight of the intact and cleaved FRET probe shown in Figure 1. This is one of the many control experiments required in quantitative hetero-FRET studies. The purity and quality of the FRET probe samples are routinely examined prior to the experiment.
4. CONCLUSIONS
We have developed a kinetics model that describes the excited-state population (or fluorescence decay) of a hetero-FRET probe as a function of excitation/detection wavelength. We have also used the equations in this model to simulate the corresponding fluorescence decays when exciting and detecting the donor and/or acceptor in a FRET probe. Taken together, this model and the associated simulations can be tested experimentally using our wavelength-dependent measurements of the time-resolved fluorescence of the FRET probes as a function of both the linker length and/or flexibility and the surrounding environment. In addition, we predict that exciting and detecting the emission of the donor selectively in a hetero-FRET pair will allow us to calculate the energy transfer rate ($k_{ET}$) and therefore the energy transfer efficiency ($E$) for each FRET pair. Importantly, the developed model here is likely to be used in a wide range of FRET probes beyond the molecular system described here, with the critical caveat that the experimental design and the donor-acceptor spectral overlap must be optimized for those other FRET probes.

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