A guide to quantitative FRET imaging

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1 Naive ratiometric approach

1.1 Common practices

Many published or reported studies that use FRET ratiometric imaging, usually ignore spectral corrections for their Donor-Acceptor couple, simply defining the FRET ratio as: $F = S^{\mathrm{DA}} / S^{\mathrm{DD}}$. This approach is valid only if:

- Only qualitative / relative comparisons are done.
- Quantitative comparison are done between different conditions measured with the **same biosensor** and imaged in the **same optical configurations**.

1.2 Introduction to the problem

However this assumptions are usually ignored and often broken. To illustrate this problem we can

take a look at figure 1. We can see that even in very common imaging and experimental setup such as PKA FRET biosensor AKAR3ev, which uses ECFP and YPET fluorescent proteins (FPs) as fluorophores, we start finding issues; in this case heavy Donor bleedthrough to the FRET/Acceptor channel.

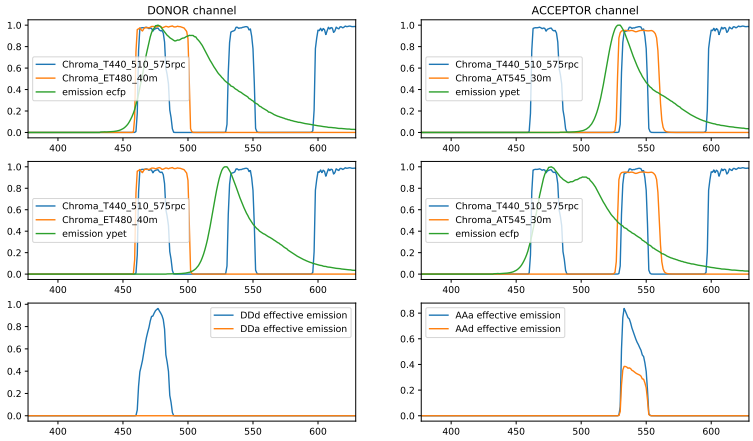

Figure 1. Donor and Acceptor "standard" optical configuration for ECFP-YPET couple (AKAR3ev). On the left column the emission of ECFP (first row) and YPET (second row) is displayed for the Donor optical configuration. On the right column the emission of ECFP (first row) and YPET (second row) is displayed for the Acceptor optical configuration. The third row for both column represents the effective emission of Donor and Acceptor by taking the moltiplication of all the spectra above. It's worth noticing that there is **significant** Donor bleedthrough in the Acceptor channel (bottom right).

Depending on the availability of certain dichroic mirrors, excitation light source and/or excitation and emission filters, sometimes the situation can be ever more dramatic. Consider for example, the case in figure 2. In cases like this one, similar optical configurations and even same Donor fluorophore can lead to dramatically different Donor bleedthough in the Acceptor channel simply because of the nature of the fluorophores spectra. In this case, it'd be impossible to perform any quantitative comparison without any correction.

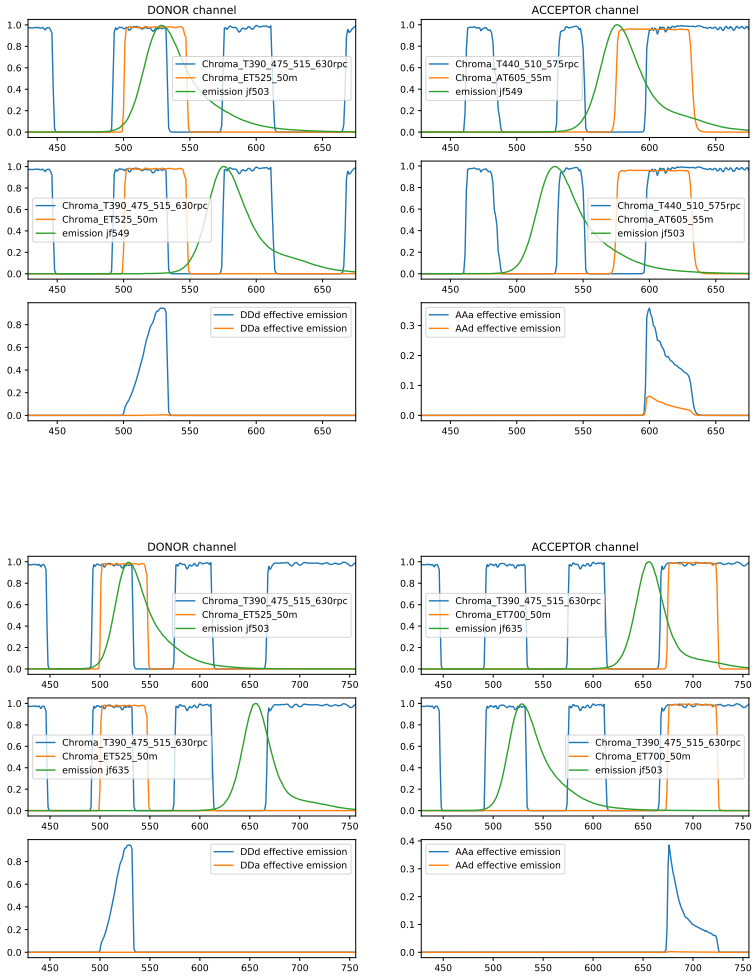

Figure 2. Donor and Acceptor optical configuration for SNAP-AKAR-HALO labeled with JF503Snap and JF549Halo (top graph) and JF503Snap and JF635Halo (bottom graph). Donor bleedthrough can be dramatically different with comparable optical configurations if fluorophores are different.

2 Simple FRET corrections

2.1 Introduction

In most cases, for a **quantitative** and **accurate** evaluation of FRET biosensors we want to use the "real" FRET efficiency value instead of the simple ratio $F = S^{\mathrm{DA}}/S^{\mathrm{DD}}$. In the single molecule FRET field, this is the standard approach and where most of these issues started becoming evident. In order to obtain the FRET efficiency the largest contributing factors are:

- Donor bleedthrough (Donor excitation Donor emission in FRET/Acceptor channel)
- Acceptor cross-excitation (Acceptor excitation Acceptor emission in FRET/Acceptor channel)
- Detection efficiency of Donor and Acceptor in respective channels

Many sources have already addressed these parameters and, especially in smFRET measurements, have tried to provide a uniform framework to properly take these issues into account and compensate for them [1][4][2][3][6].

2.2 Accepted modern framework

In the currently accepted framework for FRET spectral corrections we take into account 3 parameters, each describing a factor described in section 2.1. The follwing explanation is heavily based on [1] excellent main and supplementary material.

We assume that each individual optical configuration (i.e. channel) $S^{\mathrm{DD}}, S^{\mathrm{DA}}, S^{\mathrm{AA}}$ represent the total amount of photons or signal detected on the camera under their respective optical configuration (filters, mirrors, etc..).

Figure 3. Summary of the simple framework for FRET corrections. Each pie slice represents an imaging channel, i.e. Donor, Acceptor and FRET channels. The outer layer shows the main contributions of "detected" photons in each channel. While Donor channel is usually "pure" Donor, Acceptor and FRET channels are usually contaminated with photons coming from other sources.

In summary, following figure 3:

- Signal in the Donor channel comes from the excited non-FRETting Donor emission,
- Signal in the Acceptor channel comes from the directly excited Acceptor emission
- Signal in the FRET channel comes from excited non-FRETting Donor emission that bleedsthrough, excited FRETting Acceptor emission and directly cross-excited Acceptor emission

Simply, we can then formulate the contributions to each channel as:

$$S^{\mathrm{DD}} = [D]\, I_D\, \mathrm{Ex}_D^{\mathrm{DD}}\, (1-E) \phi_D\, \mathrm{Em}_D^{\mathrm{DD}} \tag{1}$$

$$S^{\mathrm{AA}} = [A]\, I_A\, \mathrm{Ex}_A^{\mathrm{AA}}\, \phi_A \mathrm{Em}_A^{\mathrm{AA}} \tag{2}$$

$$S^{\mathrm{DA}} = [D]\, I_D\, \mathrm{Ex}_D^{\mathrm{DA}}\, E\, \phi_A\, \mathrm{Em}_A^{\mathrm{DA}} + [D]\, I_D\, \mathrm{Ex}_D^{\mathrm{DA}}\, (1-E)\, \phi_D\, \mathrm{Em}_D^{\mathrm{DA}} + [A]\, I_D\, \mathrm{Ex}_A^{\mathrm{DA}}\, \phi_A\, \mathrm{Em}_A^{\mathrm{DA}} \tag{3}$$

Where E is the FRET efficiency, $[D]$ and $[A]$ are Donor and Acceptor "real" concentrations in the sample, I_L is the intensity or power for the L light source, ϕ_X is the measured or reported quantum yield for fluorophore X, $\mathrm{Ex}_X^{\mathrm{CH}}$ and $\mathrm{Em}_X^{\mathrm{CH}}$ is the **effective** excitation and emission for fluorophore X in channel CH (DD, AA or DA).

Normally and ideally, the **effective excitation and emission** can be measured if it's possible to image fluorophore X under the *same conditions* of the FRET experiments, alone, *without its respective fluorophore couple*. Alternatively they can also be **inferred** if all spectra for the optics are available, as it was done for the analysis in figure 1 and 2 and in our published work [5].

Typical contributors for the Excitation path are:

- LED or Laser spectrum (**reflection** not transmission)
- LED or Laser excitation filter spectrum

- Dichroic Mirror (DM) spectrum
- Absorption spectrum of fluorophore spectrum
- Molar Exctinction Coefficient ϵ of fluorophore

Typical contributors for the Emission path are:

- DM spectrum (**transmission**, not reflection)
- Emission filter spectrum
- Emission spectrum for fluorophore
- Camera detection efficiency spectrum

Since the contributions for the FRET channel S^{DA} are more complicated and depend on the other two, it's useful to express its equation in the form $S^{\mathrm{DA}} = X_1 S^{\mathrm{DD}} + X_2 S^{\mathrm{AA}}$ where X_n are parameters collecting all other components in the equation.

This can be easily done by expressing S^{DA} in function of S^{DD} and S^{AA} by isolating $[D]$ from equation 1 and $[A]$ from equation 2. We thus, obtain:

$$S^{\mathrm{DA}} = \frac{\phi_A \mathrm{Em}_A^{\mathrm{DA}}}{\phi_D \mathrm{Em}_D^{\mathrm{DD}}} \frac{E}{(1-E)} S^{\mathrm{DD}} + \frac{\mathrm{Em}_D^{\mathrm{DA}}}{\mathrm{Em}_D^{\mathrm{DD}}} S^{\mathrm{DD}} + \frac{I_D \, \mathrm{Ex}_D^{\mathrm{DA}}}{I_A \, \mathrm{Ex}_A^{\mathrm{AA}}} S^{\mathrm{AA}} \tag{4}$$

Thus it's convenient to define the following parameters:

$$\alpha = \frac{\mathrm{Em}_D^{\mathrm{DA}}}{\mathrm{Em}_D^{\mathrm{DD}}} \qquad \delta = \frac{I_D \, \mathrm{Ex}_D^{\mathrm{DA}}}{I_A \, \mathrm{Ex}_A^{\mathrm{AA}}} \qquad \gamma = \frac{\phi_A \mathrm{Em}_A^{\mathrm{DA}}}{\phi_D \mathrm{Em}_D^{\mathrm{DD}}} \tag{5}$$

Now we can achieve a definition of S^{DA} in a simple form in function of S^{DD} and S^{AA}:

$$S^{\mathrm{DA}} = \gamma \frac{E}{(1-E)} S^{\mathrm{DD}} + \alpha \, S^{\mathrm{DD}} + \delta \, S^{\mathrm{AA}} \tag{6}$$

Now we can obtain the "real" FRET efficiency from equation 6, as follows:

$$E = \frac{S^{\mathrm{DA}} - \alpha \, S^{\mathrm{DD}} - \delta \, S^{\mathrm{AA}}}{S^{\mathrm{DA}} - \alpha \, S^{\mathrm{DD}} - \delta \, S^{\mathrm{AA}} + \gamma \, S^{\mathrm{DD}}} \tag{7}$$

A common convention is to further simply equation 7 by defining the **sensitized emission**:

$$F_c = S^{\mathrm{DA}} - \alpha \, S^{\mathrm{DD}} - \delta \, S^{\mathrm{AA}} \tag{8}$$

Leading us to the definition of FRET efficiency:

$$E = \frac{F_c}{F_c + \gamma \, S^{\mathrm{DD}}} \tag{9}$$

Which can also be expressed as:

$$E = \frac{1}{1 + \gamma \, S^{\mathrm{DD}} / F_c} \tag{10}$$

Reminiscent of the physical definition of efficiency in function of distance between fluorophores R and Forster radius R_0.

$$E = \frac{1}{1 + (R/R_o)^6}$$

This approach has been taken and demonstrated useful in a recent work from our group [5].

3 Full FRET corrections

3.1 Introduction

The "simple" corrections proposed in section 2, are based on the intuitive, and often correct, facts:

- Acceptor excitation *does not* excite Donor

- Acceptor emission *does not* emit in the Donor channel
- If there is a component of Acceptor emission into the Donor channel, it can be separated into direct excitation of the Acceptor and FRET-induced Acceptor emission

These are often factual assumptions, as it often seen in noisy or empty images when one acquires the Donor channel upon Acceptor excitation. However, it's important to remember that this ought not to be always the case, especially if the FRET couple is **spectrally very close**.

3.2 Examples in favor of complete FRET corrections

In figure 2, we have seen the case of JF503 and JF549 as a FRET couple. When measuring this sample, for some constraints in the optical availability, the DM had to be changed when imaging S^{AA} (Acceptor channel). In figure 4 (bottom right panel), we can see how the Donor is actually excited more than the Acceptor in the Acceptor optical configurations, and due to a little Donor bleedthrough, the contributions of both Donor and Acceptor into the Acceptor channel are not negligible (thus the need for proper corrections).

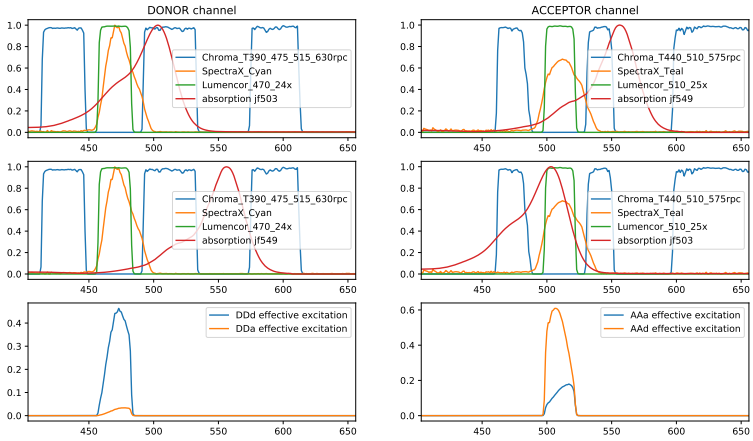

Figure 4. Excitation configuration of SNAP-AKAR-HALO biosensor stained with JF503Snap and JF549Halo corresponding to the emission spectra shown in figure 2. We can observe a massive asimmetry in the excitation of the Donor and the Acceptor in the Acceptor channel. Due to optical constrainsts, in the Acceptor channel, the Donor is excited 3x more than the Acceptor itself, and due to a very small quantity of Donor bleedthrough, signal in the Acceptor channel will have strong contributions from both Donor and Acceptor emissions.

Similarly, in figure 5 we can notice the massive Donor bleedthrough into the Acceptor channel (that would be taken into account by the α factor)(bottom right panel), but more unusually, the very low effective detection of the Donor in the Donor channel (bottom left panel). This is an unusual situation, because tipically one would want the most amount of signal of the Donor in the Donor channel, however, in this case doing so, would imply **considerable amount of Acceptor "reverse" bleedthrough** into the Donor channel (as Donor excitation light also excites Acceptor). In this case, the situation does not demand particular attention to the corrections, because the amount of "reverse" bleedthrough is not significant; however, if the Donor intensity hadn't been enough by taking only a fraction of the emission spectrum into the detection, this correction of the α^r factor would have been fundamental.

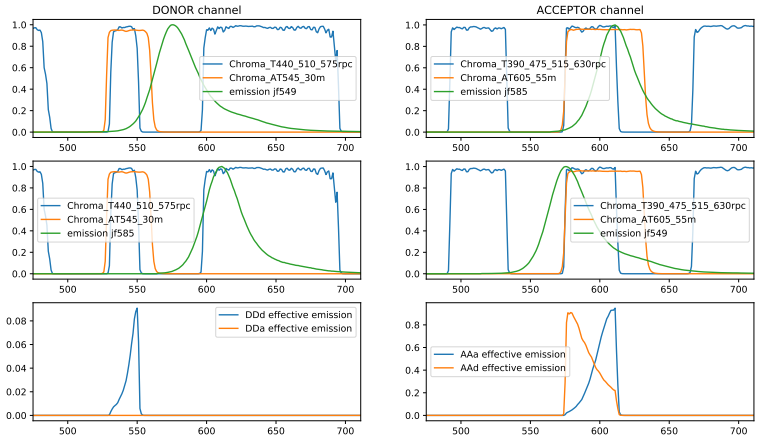

Figure 5. Emission configuration configuration of SNAP-AKAR-HALO biosensor stained with JF549Snap and JF585Halo. It's of notice the significant Donor bleedthrough into the Acceptor channel (50/50) (bottom right) and the small Donor contribution to the Donor channel in order to avoid Acceptor bleedthrough into the Donor channel (reverse bleedthrough)(bottom left).

3.3 Full FRET correction framework

Following what was mentioned in section 3, in some unusual and specific conditions dictated by either **unsuitable optical** components or by particularly **overlapping fluorophore** couples, it might be relevant, more accurate and even recommended to apply full FRET corrections. For this, we propose the following framework.

We start by defining the theoretical components of the signal in each channel similarly to how we did for equations 1-3. This time however, we take into account every possible contributions to each channel, even if, as mentioned they can be unlikely or unusual.

$$S^{\mathrm{DD}} = [D]\, I_D\, \mathrm{Ex}_D^{\mathrm{DD}}\, (1-E) \phi_D\, \mathrm{Em}_D^{\mathrm{DD}} + [D]\, I_D\, E\, \phi_A\, \mathrm{Em}_A^{\mathrm{DD}} + [A]\, I_D\, \mathrm{Ex}_A^{\mathrm{DD}}\, \phi_A\, \mathrm{Em}_A^{\mathrm{DD}} \tag{11}$$

$$S^{\mathrm{AA}} = [A]\, I_A\, \mathrm{Ex}_A^{\mathrm{AA}}\, \phi_A \mathrm{Em}_A^{\mathrm{AA}} + [D]\, I_A\, \mathrm{Ex}_D^{\mathrm{AA}}\, (1-E)\, \phi_D\, \mathrm{Ex}_D^{\mathrm{AA}} + [D]\, I_A\, \mathrm{Ex}_D^{\mathrm{AA}}\, E\, \phi_A\, \mathrm{Em}_A^{\mathrm{AA}} \tag{12}$$

$$S^{\mathrm{DA}} = [D]\, I_D\, \mathrm{Ex}_D^{\mathrm{DA}}\, E\, \phi_A\, \mathrm{Em}_A^{\mathrm{DA}} + [D]\, I_D\, \mathrm{Ex}_D^{\mathrm{DA}}\, (1-E)\, \phi_D\, \mathrm{Em}_D^{\mathrm{DA}} + [A]\, I_D\, \mathrm{Ex}_A^{\mathrm{DA}}\, \phi_A\, \mathrm{Em}_A^{\mathrm{DA}} \tag{13}$$

Notice that equation 13, it's the same as the original equation 3, as all the possible contributions to the FRET channel have already been taken into account.

Note that in equation 11 and 12, we can define 3 different contributors for each channel:

- **Non-FRET** Donor excitation and emission
- **FRET-induced** Acceptor emission
- **Direct** Acceptor excitation and emission

Although it is possible, it is not elementary to re-write this system of equation in function of $[D]$ and $[A]$ to take the same strategy as it was done in equation 4. Instead we take a simpler approach of defining several parameters that summarize the listed 3 contributions for each of the 3 channels.

For example for S^{DD}:

$$S^{\mathrm{DD}} = I_D \mathrm{Ex}_D^{\mathrm{DD}}\, \phi_D\, \mathrm{Em}_D^{\mathrm{DD}}\, [D]\, (1-E) + I_D\, \mathrm{Ex}_D^{\mathrm{DD}} \phi_A\, \mathrm{Em}_A^{\mathrm{DD}}\, [D]\, E + I_D\, \mathrm{Ex}_A^{\mathrm{DD}}\, \phi_A\, \mathrm{Em}_A^{\mathrm{DD}}\, [A]$$

$$S^{\mathrm{DD}} = I_D \mathrm{Ex}_D^{\mathrm{DD}}\, \phi_D\, \mathrm{Em}_D^{\mathrm{DD}}\, [D] - I_D \mathrm{Ex}_D^{\mathrm{DD}}\, \phi_D\, \mathrm{Em}_D^{\mathrm{DD}}\, [D] E + I_D\, \mathrm{Ex}_D^{\mathrm{DD}}\, \phi_A\, \mathrm{Em}_A^{\mathrm{DD}}\, [D]\, E + I_D\, \mathrm{Ex}_A^{\mathrm{DD}}\, \phi_A\, \mathrm{Em}_A^{\mathrm{DD}}\, [A]$$

$$S^{\mathrm{DD}} = I_D \mathrm{Ex}_D^{\mathrm{DD}}\, \phi_D\, \mathrm{Em}_D^{\mathrm{DD}}\, [D] + (I_D\, \mathrm{Ex}_D^{\mathrm{DD}} \phi_A\, \mathrm{Em}_A^{\mathrm{DD}} - I_D \mathrm{Ex}_D^{\mathrm{DD}}\, \phi_D\, \mathrm{Em}_D^{\mathrm{DD}})[D]\, E + I_D\, \mathrm{Ex}_A^{\mathrm{DD}}\, \phi_A\, \mathrm{Em}_A^{\mathrm{DD}}\, [A]$$

$$S^{\mathrm{DD}} = I_D \mathrm{Ex}_D^{\mathrm{DD}}\, \phi_D\, \mathrm{Em}_D^{\mathrm{DD}}\, [D] + \mathrm{Ex}_D^{\mathrm{DD}}\, (I_D\, \phi_A\, \mathrm{Em}_A^{\mathrm{DD}} - I_D\, \phi_D \mathrm{Em}_D^{\mathrm{DD}})[D]\, E + I_D\, \mathrm{Ex}_A^{\mathrm{DD}}\, \phi_A\, \mathrm{Em}_A^{\mathrm{DD}}\, [A]$$

We can simplify the equation above defining X parameters as in table 1.

$$S^{\mathrm{DD}} = X_0\,[D] + X_1\,[D]\,E + X_2\,[A] \tag{14}$$

Similarly we can define the other 2 channels as following:

$$S^{\mathrm{DA}} = X_3\,[D] + X_4\,[D]\,E + X_5\,[A] \tag{15}$$

$$S^{\mathrm{AA}} = X_6[D] + X_7\,[D]\,E + X_8\,[A] \tag{16}$$

X parameter	Definition
X_0	$I_D \mathrm{Ex}_D^{\mathrm{DD}}\,\phi_D\,\mathrm{Em}_D^{\mathrm{DD}}$
X_1	$\mathrm{Ex}_D^{\mathrm{DD}}\,(I_D\,\phi_A\,\mathrm{Em}_A^{\mathrm{DD}} - I_D\,\phi_D \mathrm{Em}_D^{\mathrm{DD}})$
X_2	$I_D\,\mathrm{Ex}_A^{\mathrm{DD}}\,\phi_A\,\mathrm{Em}_A^{\mathrm{DD}}$
X_3	$I_D \mathrm{Ex}_D^{\mathrm{DD}}\,\phi_D\,\mathrm{Em}_D^{\mathrm{DA}}$
X_4	$\mathrm{Ex}_D^{\mathrm{DD}}\,(I_D\,\phi_A\,\mathrm{Em}_A^{\mathrm{DA}} - I_D\,\phi_D \mathrm{Em}_D^{\mathrm{DA}})$
X_5	$I_D\,\mathrm{Ex}_A^{\mathrm{DD}}\,\phi_A\,\mathrm{Em}_A^{\mathrm{DA}}$
X_6	$I_D \mathrm{Ex}_D^{\mathrm{AA}}\,\phi_D\,\mathrm{Em}_D^{\mathrm{AA}}$
X_7	$\mathrm{Ex}_D^{\mathrm{AA}}\,(I_D\,\phi_A\,\mathrm{Em}_A^{\mathrm{AA}} - I_D\,\phi_D \mathrm{Em}_D^{\mathrm{AA}})$
X_8	$I_A\,\mathrm{Ex}_A^{\mathrm{AA}}\,\phi_A\,\mathrm{Em}_A^{\mathrm{AA}}$

Table 1. X parameter definitions according to the full FRET spectral corrections framework.

From equations 14, 15 and 16 and from X parameter definitions in table 1, we can now define:

$$[D] = \frac{(X_2\,X_4 - X_5\,X_1)\,(X_2\,S^{\mathrm{AA}} - X_8\,S^{\mathrm{DD}}) - (X_2\,X_7 - X_8\,X_1)\,(X_2 S^{\mathrm{DA}} - X_5\,S^{\mathrm{DD}})}{(X_2\,X_4 - X_5\,X_1)\,(X_2\,X_6 - X_8\,X_0) - (X_2\,X_7 - X_8\,X_1)(X_2\,X_3 - X_5\,X_0)} \tag{17}$$

$$E = \frac{(X_2\,S^{\mathrm{DA}} - X_5\,S^{\mathrm{DD}}) - (X_2\,X_3 - X_5\,X_0)\,[D]}{(X_2\,X_4 - X_5 X_1)\,[D]} \tag{18}$$

$$[A] = \frac{S^{\mathrm{DD}} - X_0\,[D] - X_1\,[D]\,E}{X_2} \tag{19}$$

Following this full FRET correction framework is possible to obtain **more precise and correct FRET efficiency values** and "real" Donor and Acceptor sample concentrations.

3.4 Limitations

Although the full FRET correction framework shoud lead to more precise and correct corrections, it is often difficult to implement and might lead to overcorrections. Usually all correction factors should be **experimentally measured** to be precise and it's often not worth the effort or it's not easy to achieve (depending on the experiment, imaging setup and sample constructs or fluorophores). Thus one can reserve to estimate the correction factors, like the ones mentioned in table 1, entirely from theoretical or measured spectra. However, it's important to keep in mind that these estimations suffer from large approximations, either due to difference in the real spectra, or to experimental variability and local environment changing the optical properties of the fluorophores (spectra, ϕ and ϵ).

4 Conclusions

It is recommended to take into account the spectral properties and circumstances of every FRET imaging experiment. Unless only qualitative comparisons are done or different experiments are carried with identical fluorophore and optical configurations, it is strongly encouraged to proceed with the **FRET spectral corrections** described in this manuscript.

Ideally, single fluorophore experiments and/or effective excitation/emission can be measured **directly**, but often, estimating the correction parameters from the theoretical spectra leads to reasonable results, although less accurate.

Unless in particular and problematic circumastances discussed in section 3.2, simple but complete FRET correction described in section 2, will be enough to have a more quantitative analysis and comparison between conditions/experiments. A good initiative would be to also try both approaches (simple and full corrections) to evaluate the difference. Theoretically, it is to be expected that FRET couples that are very well discriminated in their optical configurations and that are well spectrally-separated won't show much difference between the full and simple FRET correction approach. On the other hand, difficult couples with severely overlapping dyes or inappropriate optical configurations, not suitable to properly separate Donors and Acceptors, will require a more accurate corrections as described in section 3.3.

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