FRET measurements at high excitation intensities

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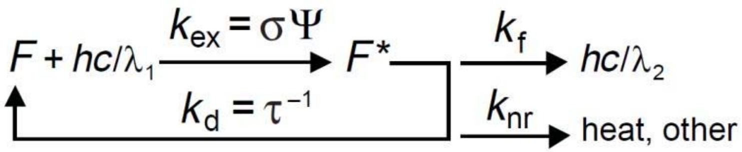
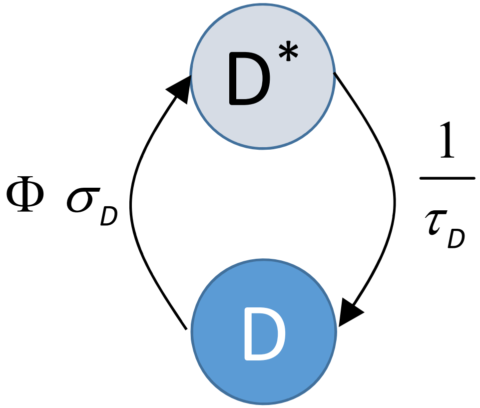
Fluorophore saturation

- High excitation intensity → more emitted photons → better signal/noise ratio
- BUT: risk of **fluorophore saturation**

- Fluorophores can be considered to be "enzymes" converting excitation photons to emitted photons.





In equilibrium:

$$\begin{pmatrix} 0 \\ 0 \\ D_{all} \end{pmatrix} = \begin{pmatrix} -\Phi\ \sigma_D & \frac{1}{\tau_D} \\ \Phi\ \sigma & -\frac{1}{\tau_D} \\ 1 & 1 \end{pmatrix} \begin{pmatrix} D \\ D^* \end{pmatrix}$$

$$\sigma\left[{cm^2}/{molecule}\right] = \frac{1000\ \ln(10)}{6\cdot 10^{23}}\varepsilon\left[M^{-1}cm^{-1}\right]$$

$$D_{sat} = \frac{\sigma_D\ \tau_D\ \Phi}{1+\sigma_D\ \tau_D\ \Phi},\ D_e = D_{all}\ D_{sat}$$

$$D_{sat} = \frac{\sigma_D\ \tau_D\ \Phi}{1+\sigma_D\ \tau_D\ \Phi} = \frac{\Phi}{\frac{1}{\sigma_D\ \tau_D}+\Phi}$$

"K_M" of the fluorophore

The Michaelis-Menten equation for enzymes:

$$v = v_{max}\frac{[S]}{K_M+[S]}$$

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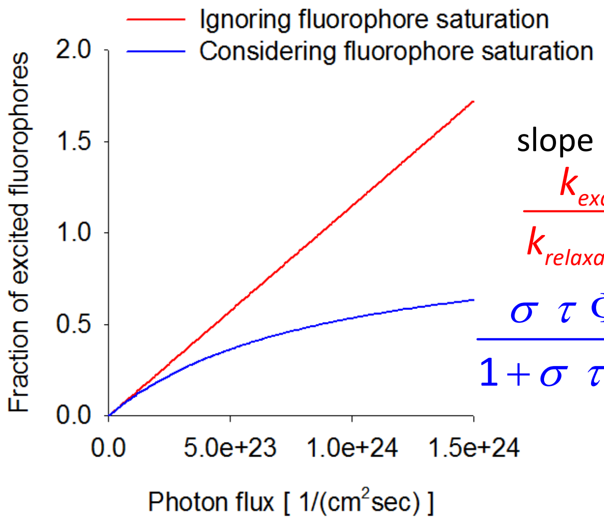
Fluorophore saturation



slope of line (*k* – rate constant):

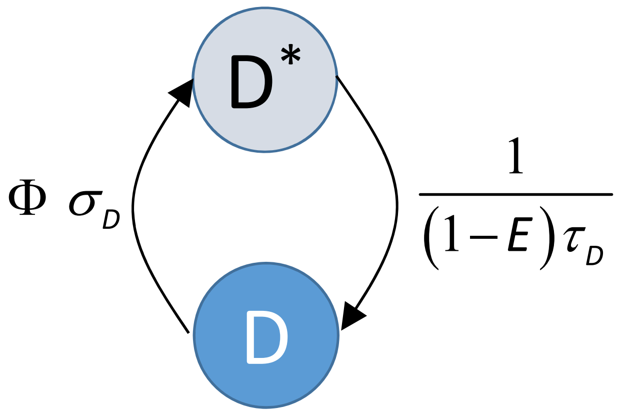
$$\frac{k_{exc}}{k_{relaxation}} = \frac{\sigma \, \Phi}{1/\tau} = \sigma \, \Phi \, \tau$$

$$\frac{\sigma \, \tau \, \Phi}{1 + \sigma \, \tau \, \Phi}$$

- Calculated curves for AlexaFluor 488
- τ=4.1 ns
  ε=71000 M^{-1} cm^{-1}

Consequence: fluorescence intensity is not any more proportional to the excitation intensity.

Effect of fluorophore saturation on FRET: the donor side



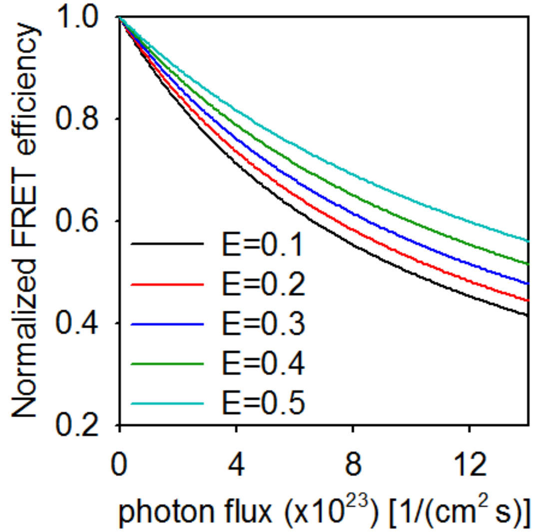
Equilibrium solution for D*: $D^* = \frac{D_{all}(1-E)\sigma_D\ \tau_D\ \Phi}{1+(1-E)\sigma_D\ \tau_D\ \Phi}$

Correction for donor saturation: $E = \frac{E_{apparent}}{1+D_{sat}\left(E_{apparent}-1\right)}$

Predictions of the above for donor quenching:

$$E_{apparent} = 1 - \frac{I_{DA}}{I_D} = 1 - \frac{D^*_A}{D^*_{no\ A}} = 1 - \frac{\frac{(1-E)\sigma_D\ \tau_D\ \Phi}{1+(1-E)\sigma_D\ \tau_D\ \Phi}}{\frac{\sigma_D\ \tau_D\ \Phi}{1+\sigma_D\ \tau_D\ \Phi}} = \frac{E}{1+(1-E)\sigma_D\ \tau_D\ \Phi} = \frac{(1-D_{sat})E}{1-D_{sat}E}$$

Rationalization:
- although the donor is "quenched", it is immediately re-excited → no or smaller decrease in intensity



τ =4.1 ns
ε =71000 $M^{-1}\ cm^{-1}$
σ =2.72 x 10^{-16} cm^2

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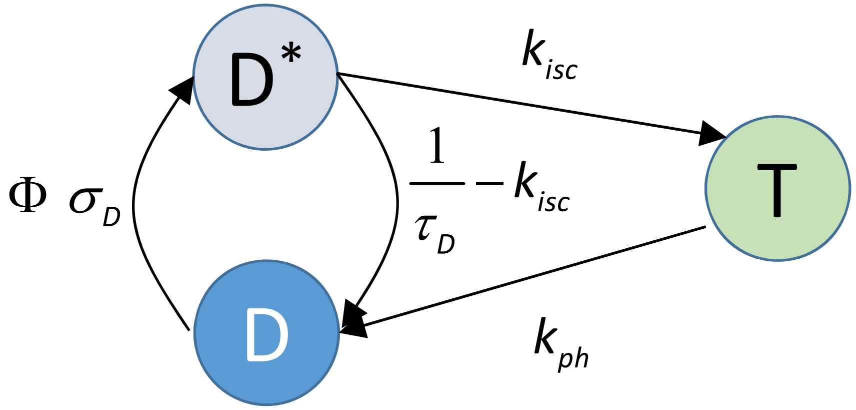
Fluorophore saturation in the presence of the triplet state



In equilibrium:

$$\begin{pmatrix} 0 \\ 0 \\ 0 \\ F_{all} \end{pmatrix} = \begin{pmatrix} -\Phi\ \sigma_D & \frac{1}{\tau_D} - k_{isc} & k_{ph} \\ \Phi\ \sigma_D & -\frac{1}{\tau_D} & 0 \\ 0 & k_{isc} & -k_{ph} \\ 1 & 1 & 1 \end{pmatrix} \begin{pmatrix} S_0 \\ S_1 \\ T_1 \end{pmatrix}$$

$$\Rightarrow$$

$$S_0 = \frac{F_{all}\ k_{ph}}{k_{ph} + \left(k_{isc} + k_{ph}\right)\ \sigma_D\ \tau_D\ \Phi}$$

$$S_1 = \frac{F_{all}\ k_{ph}\ \sigma_D\ \tau_D\ \Phi}{k_{ph} + \left(k_{isc} + k_{ph}\right)\ \sigma_D\ \tau_D\ \Phi}$$

$$T_1 = \frac{F_{all}\ k_{isc}\ \sigma_D\ \tau_D\ \Phi}{k_{ph} + \left(k_{isc} + k_{ph}\right)\ \sigma_D\ \tau_D\ \Phi}$$

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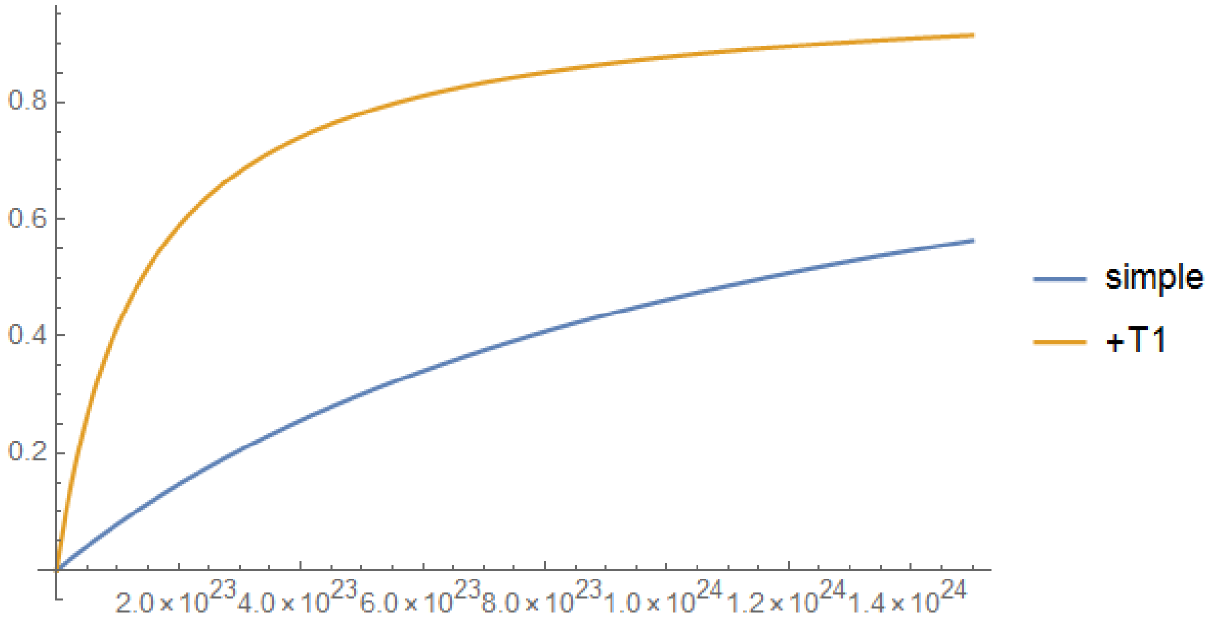
Fluorophore saturation in the presence of triplet state

Fluorescence intensities of a fluorophore normalized to the limit at $\Phi \to \infty$



$$\lim_{\Phi \to \infty} \left(\frac{F_{all}\ k_{ph}\ \sigma_D\ \tau_D\ \Phi}{k_{ph} + \left(k_{isc} + k_{ph}\right)\ \sigma_D\ \tau_D\ \Phi} \right) = \frac{k_{ph}\ F_{all}}{k_{isc} + k_{ph}}$$

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Donor (with T state) + acceptor

$$\begin{pmatrix} 0 \\ 0 \\ 0 \\ F_{all} \end{pmatrix} = \begin{pmatrix} -\Phi\ \sigma_D & \frac{1+(E-1)k_{isc}\tau_D}{(1-E)\tau_D} & k_{ph} \\ \Phi\ \sigma_D & -\frac{1}{(1-E)\tau_D} & 0 \\ 0 & k_{isc} & -k_{ph} \\ 1 & 1 & 1 \end{pmatrix} \begin{pmatrix} S_0 \\ S_1 \\ T_1 \end{pmatrix}$$

$$S_0 = \frac{k_{ph}\ F_{all}}{(E-1)(k_{isc}+k_{ph})\sigma_D\ \tau_D\ \Phi - k_{ph}}$$

$$S_1 = \frac{(E-1)k_{ph}\ F_{all}\ \sigma_D\ \tau_D\ \Phi}{(E-1)(k_{isc}+k_{ph})\sigma_D\ \tau_D\ \Phi - k_{ph}}$$

$$T_1 = \frac{(E-1)k_{isc}\ F_{all}\ \sigma_D\ \tau_D\ \Phi}{(E-1)(k_{isc}+k_{ph})\sigma_D\ \tau_D\ \Phi - k_{ph}}$$

FRET calculated from donor quenching:

$$E_{apparent} = 1 - \frac{I_{DA}}{I_D} = 1 - \frac{S_{1,\,A}}{S_{1,\,no\ A}} = 1 - \frac{\dfrac{(E-1)k_{ph}\ F_{all}\ \sigma_D\ \tau_D\ \Phi}{(E-1)(k_{isc}+k_{ph})\sigma_D\ \tau_D\ \Phi - k_{ph}}}{\dfrac{k_{ph}\ F_{all}\ \sigma_D\ \tau_D\ \Phi}{(k_{isc}+k_{ph})\sigma_D\ \tau_D\ \Phi + k_{ph}}} = \frac{E\ k_{ph}}{(E-1)(k_{isc}+k_{ph})\sigma_D\ \tau_D\ \Phi - k_{ph}}$$

Using $\lim_{\Phi\to\infty}\left(\frac{F_{all}\ k_{ph}\ \sigma_D\ \tau_D\ \Phi}{k_{ph}+(k_{isc}+k_{ph})\ \sigma_D\ \tau_D\ \Phi}\right) = \frac{k_{ph}\ F_{all}}{k_{isc}+k_{ph}}$

$$E_{apparent} = \frac{(1-D_{sat,T})E}{1-D_{sat,T}\ E}$$

Equation not considering the triplet state: $E_{apparent} = \frac{(1-D_{sat})E}{1-D_{sat}E}$

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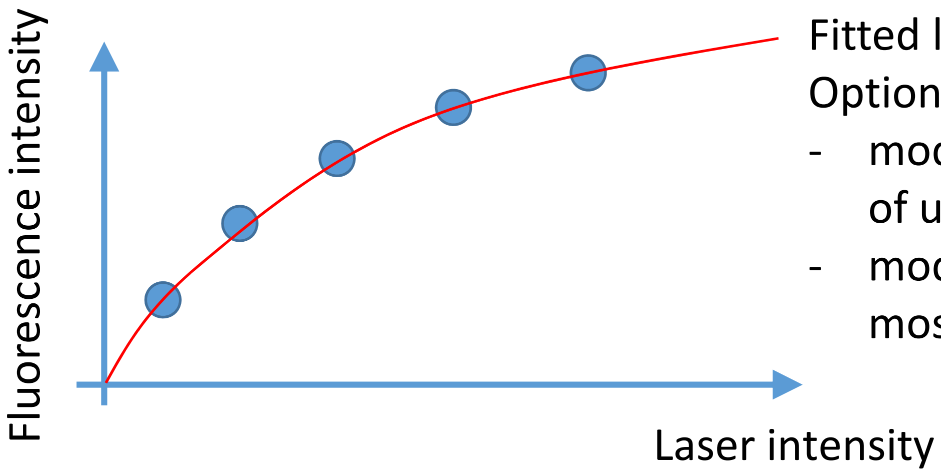
It's only the fractional saturation of the donor that matters.

Intentional misestimation of the photon flux (Φ)



Fitted line

Options

- model involving the triplet state: a lot of unknown parameters
- model disregarding the triplet state: most likely wrong model

Consequence of fitting with the wrong model:

- The degree of saturation of the S_1 state is significantly larger if the triplet state is present.
- If the photon flux is estimated from the degree of S_1 saturation in a system where the triplet state is populated according to a model in which the triplet state is neglected, the photon flux will be **overestimated**

$$\frac{(k_{isc}+k_{ph})\ \sigma_D\ \tau_D\ \Phi}{k_{ph}+(k_{isc}+k_{ph})\ \sigma_D\ \tau_D\ \Phi}=\frac{\sigma_D\ \tau_D\ \Phi_{est}}{1+\sigma_D\ \tau_D\ \Phi_{est}}\Rightarrow\Phi_{est}=\frac{k_{isc}+k_{ph}}{k_{ph}}\Phi$$

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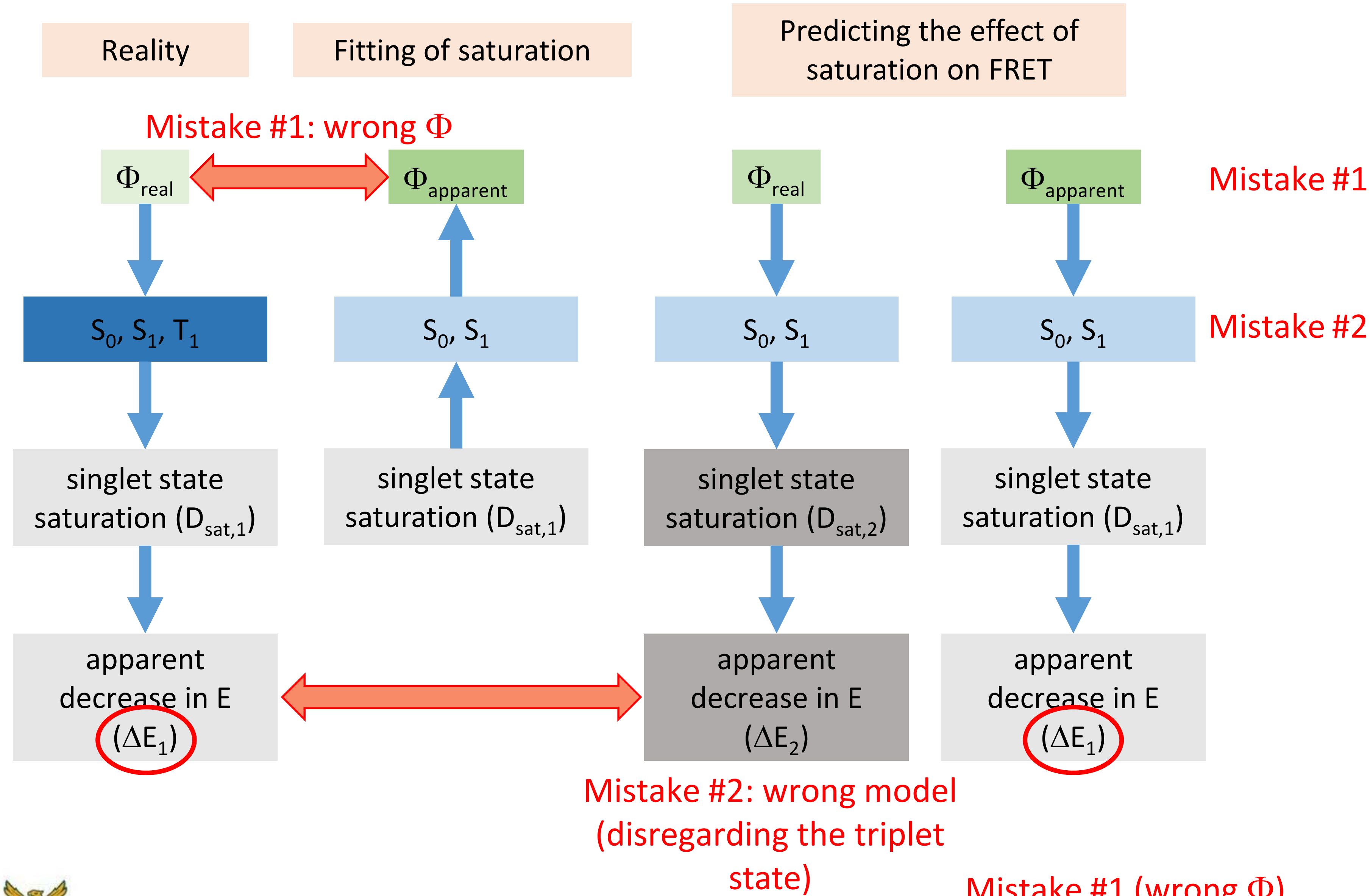
Calculation of the effect of saturation on the FRET efficiency



Mistake #1 (wrong Φ)
\+ Mistake #2 (wrong model)

No mistake in ΔE

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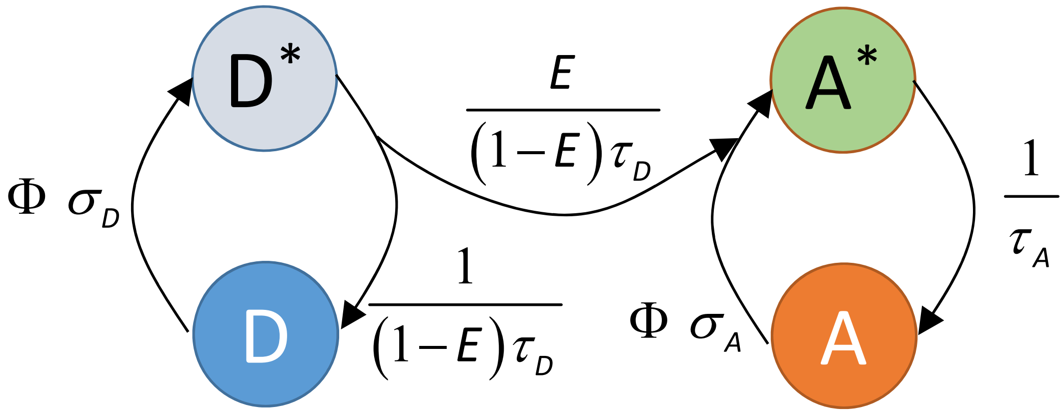
Effect of fluorophore saturation on FRET: both sides



In equilibrium:

$$\begin{pmatrix} 0 \\ 0 \\ 0 \\ 0 \\ D_{all} \\ A_{all} \end{pmatrix} = \begin{pmatrix} -\Phi\ \sigma_D & \frac{1}{(1-E)\tau_D} & 0 & 0 \\ \Phi\ \sigma_D & -\frac{1}{(1-E)\tau_D} & 0 & 0 \\ 0 & -\frac{E}{(1-E)\tau_D} & -\Phi\ \sigma_A & \frac{1}{\tau_A} \\ 0 & \frac{E}{(1-E)\tau_D} & \Phi\ \sigma_A & -\frac{1}{\tau_A} \\ 1 & 1 & 0 & 0 \\ 0 & 0 & 1 & 1 \end{pmatrix} \begin{pmatrix} D \\ D^* \\ A \\ A^* \end{pmatrix}$$

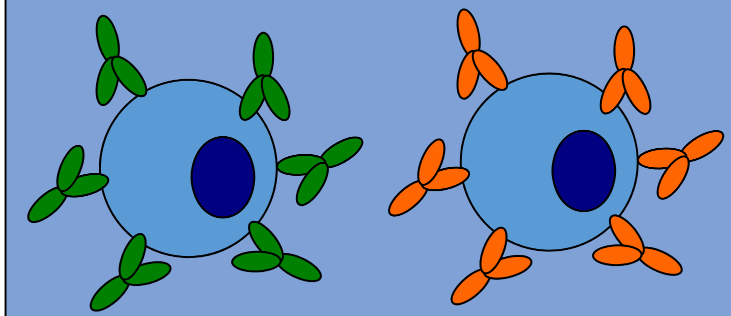
$\Rightarrow$

$$D_e = \frac{D_{all}\ D_{sat}(1-E)}{1-D_{sat}\ E}$$

$$A_e = A_{All}\ A_{sat} + \frac{(1-A_{sat})D_{all}\ D_{sat}\ \mathrm{E}\ \tau_A}{(1-D_{sat}\ E)\tau_D}$$

Parameter α

Antibody-labeled cells



$N_{donor}=N_{acceptor}$

label the first sample with donor-antibody, the second sample with acceptor-antibody against the same epitope.

$$\alpha = \frac{I_2}{I_1}\frac{\varepsilon_D L_D}{\varepsilon_A L_A}$$

α characterizes how efficiently an excited acceptor can be detected in the FRET channel compared to an excited donor in the donor channel.

$$\alpha = \frac{Q_A \ \eta_{A,2}}{Q_D \ \eta_{D,1}}$$

Fluorescence intensities at fluorophore saturation: determination of α

$$I = \frac{D_{all}\ \sigma_D\ \tau_D\ \Phi}{1+\sigma_D\ \tau_D\ \Phi} k_f$$

equilibrium population density

Series expansion about Φ=0 and substituting $k_f = \frac{Q}{\tau}$ yields:

$$I = D_{all} k_f \sigma\ \tau\ \Phi + D_{all} k_f \sigma^2 \tau^2 \Phi^2 + \ldots = D_{all} \frac{Q}{\tau} \sigma\ \tau\ \Phi + D_{all} \frac{Q}{\tau} \sigma^2 \tau^2 \Phi^2 + \ldots =$$

$$= D_{all} Q\ \sigma\ \Phi + D_{all} Q\ \sigma^2 \tau\ \Phi^2 + \ldots$$

Consequence of the above for calculation of α:

$$\left.\begin{aligned} M_D &= B\ L_D\ \sigma_{D(D)}\ \Phi_D\ Q_D\ \eta_{D,1} \\ M_A &= B\ L_A\ \sigma_{A(D)}\ \Phi_D\ Q_A\ \eta_{A,2} \end{aligned}\right\} \alpha = \frac{M_A\ L_D\ \sigma_{D(D)}}{M_D\ L_A\ \sigma_{A(D)}} \text{ (without saturation)}$$

$$\left.\begin{aligned} M_D &= B\ L_D\ D_{sat,D}\ k_{f,D}\ \eta_{D,1} \\ M_A &= B\ L_A\ A_{sat,D}\ k_{f,A}\ \eta_{A,2} \end{aligned}\right\} \alpha_{sat} = \frac{M_A\ L_D\ D_{sat,D}\ \tau_A}{M_D\ L_A\ A_{sat,D}\ \tau_D} \text{ (with saturation)}$$

Limit of α_{sat} in the absence of saturation: $\lim_{\Phi\to 0} \frac{M_A\ L_D\ D_{sat,D}\ \tau_A}{M_D\ L_A\ A_{sat,D}\ \tau_D} = \lim_{\Phi\to 0} \frac{M_A\ L_D \frac{\sigma_{D(D)}\ \tau_D\ \Phi}{1+\sigma_{D(D)}\ \tau_D\ \Phi} \tau_A}{M_D\ L_A \frac{\sigma_{A(D)}\ \tau_A\ \Phi}{1+\sigma_{A(D)}\ \tau_A\ \Phi} \tau_D} = \frac{M_A\ L_D\ \sigma_{D(D)}}{M_D\ L_A\ \sigma_{A(D)}}$

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Solve the intensity-based equation set for *E*

	Donor intensity	FRET intensity	Acceptor intensity
I_1	$I_{D,1}=\frac{F_D\ D_{sat,D}\left(1-E\right)}{1-D_{sat,D}\ E}$	$I_{F,1}=\frac{\left(1-A_{sat,D}\right)F_D\ D_{sat,D}\ \mathrm{E}\ \alpha_{sat}}{\left(1-D_{sat,D}\ E\right)}\frac{S_4}{S_2}$	$I_{A,1}=F_A\ A_{sat,A}\ S_4$
I_2	$I_{D,2}=\frac{F_D\ D_{sat,D}\left(1-E\right)}{1-D_{sat,D}\ E}S_1$	$I_{F,2}=\frac{\left(1-A_{sat,D}\right)F_D\ D_{sat,D}\ \mathrm{E}\ \alpha_{sat}}{\left(1-D_{sat,D}\ E\right)}$	$I_{A,2}=F_A\ A_{sat,A}\ S_2$
I_3	$I_{D,3}=\frac{F_D\ D_{sat,D}\left(1-E\right)}{1-D_{sat,A}\ E}S_3$	$I_{F,3}=\frac{A_{sat,D}\left(1-A_{sat,A}\right)F_D\ D_{sat,A}\ \mathrm{E}\ \alpha_{sat}}{A_{sat,A}\left(1-D_{sat,A}\ E\right)}\frac{1}{S_2}$	$I_{A,3}=F_A\ A_{sat,A}$

$$I_1=\frac{F_D\ D_{sat,D}\left(1-E\right)}{1-D_{sat,D}\ E}+F_A\ A_{sat,A}\ S_4+\frac{\left(1-A_{sat,D}\right)F_D\ D_{sat,D}\ \mathrm{E}\ \alpha_{sat}}{\left(1-D_{sat,D}\ E\right)}\frac{S_4}{S_2}$$

$$I_2=\frac{F_D\ D_{sat,D}\left(1-E\right)}{1-D_{sat,D}\ E}S_1+F_A\ A_{sat,A}\ S_2+\frac{\left(1-A_{sat,D}\right)F_D\ D_{sat,D}\ \mathrm{E}\ \alpha_{sat}}{\left(1-D_{sat,D}\ E\right)}$$

$$I_3=\frac{F_D\ D_{sat,D}\left(1-E\right)}{1-D_{sat,A}\ E}S_3+F_A\ A_{sat,A}+\frac{A_{sat,D}\left(1-A_{sat,A}\right)F_D\ D_{sat,A}\ \mathrm{E}\ \alpha_{sat}}{A_{sat,A}\left(1-D_{sat,A}\ E\right)}\frac{1}{S_2}$$

Solution for *E*:

```
(1/2).*Dsat0d.^(-1).*(Asat0a.*S2.*(Dsat0d.*S3.*(i1.*S2+(-1).*i2.*S4)+Dsat0a.*(i2+(-1).*i1.*S1+(-
1).*i3.*S2+i3.*S1.*S4))+(Asat0a+(-1).*Asat0d).*Dsat0a.*(i1.*S2+(-1).*i2.*S4).*alphaSat).^(-1).*(Asat0d.*(((-
1)+Asat0a).*Dsat0a+(-1).*Asat0a.*Dsat0d).*(i1.*S2+(-1).*i2.*S4).*alphaSat+(-1).*((-
4).*Asat0a.*Dsat0d.^2.*S2.*(i1.*(S1+(-1).*S2.*S3)+i3.*(S2+(-1).*S1.*S4)+i2.*((-
1)+S3.*S4)).*(Asat0a.*S2.*(Dsat0d.*S3.*((-1).*i1.*S2+i2.*S4)+Dsat0a.*((-1).*i2+i1.*S1+i3.*S2+(-
1).*i3.*S1.*S4))+((-1).*Asat0a+Asat0d).*Dsat0a.*(i1.*S2+(-1).*i2.*S4).*alphaSat)+(Asat0a.*Dsat0d.*S2.*(((-
1)+(-1).*Dsat0a).*i2+(1+Dsat0a).*i1.*S1+(-
1).*(1+Dsat0d).*i1.*S2.*S3+(1+Dsat0d).*i2.*S3.*S4+(1+Dsat0a).*i3.*(S2+(-1).*S1.*S4))+((1+(-
1).*Asat0a).*Asat0d.*Dsat0a+Asat0a.*((-1)+Asat0d).*Dsat0d).*(i1.*S2+(-
1).*i2.*S4).*alphaSat).^2).^(1/2)+Asat0a.*Dsat0d.*(i2.*S2.*(1+Dsat0a+(-1).*(1+Dsat0d).*S3.*S4)+(-
1).*i2.*S4.*alphaSat+S2.*((1+Dsat0a).*i3.*((-1).*S2+S1.*S4)+i1.*(((-1)+(-
1).*Dsat0a).*S1+(1+Dsat0d).*S2.*S3+alphaSat))));
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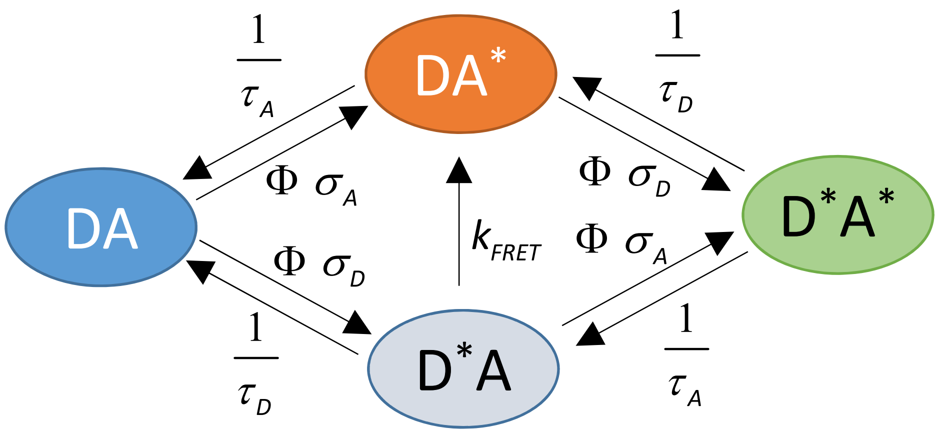
Problems considered:
- donor saturation
- acceptor saturation, but not FRET frustration

Effect of fluorophore saturation and FRET frustration on *E*



$$k_{FRET} = \frac{E}{(1-E)\tau_D}$$

In equilibrium:

$$\begin{pmatrix} 0 \\ 0 \\ 0 \\ 0 \\ DA_{all} \end{pmatrix} = \begin{pmatrix} -\Phi(\sigma_D+\sigma_A) & \frac{1}{\tau_D} & \frac{1}{\tau_A} & 0 \\ \Phi\ \sigma_D & -\frac{1}{(1-E)\tau_D}-\Phi\ \sigma_A & 0 & \frac{1}{\tau_A} \\ \Phi\ \sigma_A & \frac{E}{(1-E)\tau_D} & -\frac{1}{\tau_A}-\Phi\ \sigma_D & \frac{1}{\tau_D} \\ 0 & \Phi\ \sigma_A & \Phi\ \sigma_D & -\left(\frac{1}{\tau_D}+\frac{1}{\tau_A}\right) \\ 1 & 1 & 1 & 1 \end{pmatrix} \begin{pmatrix} DA \\ D^*A \\ DA^* \\ D^*A^* \end{pmatrix}$$

$$DA = \frac{DA_{all}\left(-\tau_D+\tau_A\left(-1+(E-1)(\sigma_A+\sigma_D)\tau_D\ \Phi\right)\right)}{\tau_D\left(-1+(E-1)\sigma_D\ \tau_D\ \Phi\right)+\tau_A^2\ \Phi\left(1+\sigma_D\ \tau_D\ \Phi\right)\left(-\sigma_A-E\ \sigma_D+(E-1)\sigma_A(\sigma_A+\sigma_D)\tau_D\ \Phi\right)+\tau_A\left(-1+\tau_D\ \Phi\left((E-2)(\sigma_A+\sigma_D)+(E-1)\sigma_D(2\sigma_A+\sigma_D)\tau_D\ \Phi\right)\right)}$$

$$D^*A = \frac{DA_{all}(E-1)\sigma_D\ \tau_D\ \Phi\left(\tau_A+\tau_D+(\sigma_A+\sigma_D)\tau_A\ \tau_D\ \Phi\right)}{\tau_D\left(-1+(E-1)\sigma_D\ \tau_D\ \Phi\right)+\tau_A^2\ \Phi\left(1+\sigma_D\ \tau_D\ \Phi\right)\left(-\sigma_A-E\ \sigma_D+(E-1)\sigma_A(\sigma_A+\sigma_D)\tau_D\ \Phi\right)+\tau_A\left(-1+\tau_D\ \Phi\left((E-2)(\sigma_A+\sigma_D)+(E-1)\sigma_D(2\sigma_A+\sigma_D)\tau_D\ \Phi\right)\right)}$$

$$DA^* = \frac{DA_{all}\ \tau_A\ \Phi\left(-(\sigma_A+E\ \sigma_D)(\tau_A+\tau_D)+(E-1)\sigma_A(\sigma_A+\sigma_D)\tau_A\ \tau_D\ \Phi\right)}{\tau_D\left(-1+(E-1)\sigma_D\ \tau_D\ \Phi\right)+\tau_A^2\ \Phi\left(1+\sigma_D\ \tau_D\ \Phi\right)\left(-\sigma_A-E\ \sigma_D+(E-1)\sigma_A(\sigma_A+\sigma_D)\tau_D\ \Phi\right)+\tau_A\left(-1+\tau_D\ \Phi\left((E-2)(\sigma_A+\sigma_D)+(E-1)\sigma_D(2\sigma_A+\sigma_D)\tau_D\ \Phi\right)\right)}$$

$$D^*A^* = \frac{DA_{all}\ \sigma_D\ \tau_A\ \tau_D\ \Phi^2\left(-\sigma_A\ \tau_A-E\ \sigma_D\ \tau_A-\sigma_A\ \tau_D+E\ \sigma_A\ \tau_D+(E-1)\sigma_A(\sigma_A+\sigma_D)\tau_A\ \tau_D\ \Phi\right)}{\tau_D\left(-1+(E-1)\sigma_D\ \tau_D\ \Phi\right)+\tau_A^2\ \Phi\left(1+\sigma_D\ \tau_D\ \Phi\right)\left(-\sigma_A-E\ \sigma_D+(E-1)\sigma_A(\sigma_A+\sigma_D)\tau_D\ \Phi\right)+\tau_A\left(-1+\tau_D\ \Phi\left((E-2)(\sigma_A+\sigma_D)+(E-1)\sigma_D(2\sigma_A+\sigma_D)\tau_D\ \Phi\right)\right)}$$

Effect of fluorophore saturation and FRET frustration on *E*

Applying the same strategy as before:

1. Write the donor and acceptor intensities in $I_1 - I_3$

$$\left.\begin{aligned}
I_{D,X} &= D^* A^* \; k_{f,D} \; \eta_{D,X} + D^* A \; k_{f,D} \; \eta_{D,X} \\
I_{A,X} &= \left(D^* A^* + DA^*\right) k_{f,A} \; \eta_{A,X} + A_f \frac{\sigma_{A(D)} \; \tau_A \; \Phi_D}{1+\sigma_{A(D)} \; \tau_A \; \Phi_D} k_{f,A} \; \eta_{A,X} \\
I_D &= DA_{all} \; k_{f,D} \; \eta_{D,1} \\
I_A &= \left(DA_{all} + A_f\right) k_{f,A} \; \eta_{A,3}
\end{aligned}\right\} I_{D,X}, I_{A,X}$$

2. Solutions for $I_{D,X}$ and $I_{A,X}$ were inserted into the equation set below:

$$I_1 = I_{D,1} + I_{A,1}$$

$$I_2 = I_{D,2} + I_{A,2}$$

$$I_3 = I_{D,3} + I_{A,3}$$

3. A numerical solution of this cubic equation set for *E* was found numerically.

Problems considered:
- donor saturation
- acceptor saturation and FRET frustration

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