FRET for biomolecular imaging I

Ion channels
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Color mutants of GFP make FRET potentially easy in cells

- FRET: Fluorescence Resonance Energy Transfer

No FRET

FRET
Imaging molecular interactions and biological signals in live cells

- Review of FRET principles
- Unscrambling FRET crosstalk via $3^3$-FRET
- Unscrambling FRET crosstalk via E-FRET
- Application 1—Bimolecular binding curves quantified in live cells
- Application 2—Unimolecular FRET sensor monitors calcium dynamics in cardiac myocytes
Fluorescence in the absence of FRET

Fluorophore (CFP)

Energy

hv

$E = \frac{hc}{\lambda}$

$k_D = k_{D/\text{photon}} + k_{D/\text{heat}}$

$QY = \frac{k_{D/\text{photon}}}{k_{D/\text{photon}} + k_{D/\text{heat}}}$

extinction coefficient = $\varepsilon(\lambda)$ (optical capture profile)

shape invariant
Fluorescence in the presence of FRET

\[ E = \frac{k_T}{k_T + k_D} = \frac{R_0^6}{R_0^6 + R^6} \]

\[ R_0 \sim 49 \text{ Å} \]
Intensity-based quantification of FRET is potentially complicated by CFP/YFP crosstalk.

Aggregate emission with 440 nm excitation

YFP component via FRET and direct excitation

CFP component via direct excitation

excitation at 440 nm
emission (readout) at 535 nm
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Kinetic model of FRET

\[ D + A + h_\text{ex} \rightarrow D^\ast + A \]
\[ A + D + h_\text{D} \]

\[ k_D = k_{D/\text{photon}} + k_{D/\text{heat}} \]

\[ QY = \frac{k_{D/\text{photon}}}{k_{D/\text{photon}} + k_{D/\text{heat}}} \]
Kinetic model of FRET

- Efficiency measures the fraction of excited donor molecules that have their energy “stolen” by an acceptor.
- Ranges from 0 to 1

More refined definition of FRET efficiency:

\[ E = \frac{k_T}{k_T + k_{D/\text{photon}} + k_{D/heat}} \]

\[ D + A \quad D + A \]
\[ \uparrow k_{D/heat} \quad \uparrow k_{A/heat} \]

\[ D + A + h\nu_{ex} \rightarrow D^* + A \xrightarrow{k_T} D + A^* \]
\[ \downarrow k_{D/\text{photon}} \quad \downarrow k_{A/\text{photon}} \]

\[ A + D + h\nu_D \quad A + D + h\nu_A \]
Kinetic model of FRET
‘complete’ system

\[ D + A + h\nu_{\text{ex}} \rightarrow D^* + A \]
\[ A + D + h\nu_D \]

\[ k_D = k_{D/\text{photon}} + k_{D/\text{heat}} \]

\[ QY = \frac{k_{D/\text{photon}}}{k_{D/\text{photon}} + k_{D/\text{heat}}} \]

No FRET

FRET

excitation (440 nm)  
emission (475 nm ~ max)

excitation (440 nm)  
emission (527 nm ~ max)
Kinetic model of FRET
‘complete’ system

Supplemental Data for:

Supplemental Appendices on the Three-Cube FRET Method (3³-FRET) and Extensions of the Method to Characterize Properties of Binding Between Donor and Acceptor Molecules, 2\textsuperscript{nd} Edition
Kinetic model of FRET
‘complete’ system

 excitation subsystem

 fluorophore-rate-constant subsystem

 emission-detection subsystem

(includes QY)

(includes QY)

(includes QY)
7.1 Kinetic model of FRET

(‘complete’ system - low excitation limit)

\[ P_{D^*} = (1-D_b) \cdot I_o \cdot G_x(D,\lambda_{ex,x}) / k_D + D_b \cdot I_o \cdot G_x(D,\lambda_{ex,x}) / (k_T+k_D) \quad \text{[A1]} \]

\[ P_{A^*} = I_o \cdot G_x(A,\lambda_{ex,x}) / k_A + A_b \cdot [ I_o \cdot G_x(D,\lambda_{ex,x}) / (k_T+k_D) ] \cdot k_T / k_A \quad \text{[A2]} \]

\[
\text{CFP}_x(\lambda_{ex,x},\lambda_{em,x},\text{direct}) = \\
N_D \cdot k_D \cdot [(1-D_b) / k_D + (D_b / (k_T+k_D))] \cdot I_o \cdot G_x(D,\lambda_{ex,x}) \cdot F_x(D,\lambda_{em,x}) \quad \text{[A3]} \\
\text{YFP}_x(\lambda_{ex,x},\lambda_{em,x},\text{direct}) = N_A \cdot k_A \cdot [ I_o G_x(A,\lambda_{ex,x}) / k_A ] \cdot F_x(A,\lambda_{em,x}) \quad \text{[A4]} \\
\text{YFP}_x(\lambda_{ex,x},\lambda_{em,x},\text{FRET}) = N_A \cdot A_b \cdot [ I_o G_x(D,\lambda_{ex,x}) / (k_T+k_D) ] \cdot k_T \cdot F_x(A,\lambda_{em,x}) \quad \text{[A5]} \\
\]

Fluorophore photon outputs of various classes. *Master equations.*
3³-FRET unscrambles the crosstalk

\[
FR = \frac{\text{YFP(direct)} + \text{YFP(FRET)}}{\text{YFP(direct)}}
\]

\[
E \cdot A_b = (FR - 1) \cdot \frac{\varepsilon_{\text{YFP}}(440 \text{ nm})}{\varepsilon_{\text{CFP}}(440 \text{ nm})}
\]
3³-FRET unscrambles the crosstalk

$$E \cdot A_b = (FR - 1) \cdot \frac{\varepsilon_{YFP} (440 \text{ nm})}{\varepsilon_{CFP} (440 \text{ nm})}$$

$$FR = \frac{YFP(\text{direct}) + YFP(\text{FRET})}{YFP(\text{direct})}$$

$$FR - 1 = \frac{YFP(\text{FRET})}{YFP(\text{direct})}$$

$$FR - 1 = \frac{N_A \cdot A_b \cdot I_O \cdot G_D \cdot E \cdot F_D}{N_A \cdot I_O \cdot G_A \cdot F_D} = E \cdot A_b \cdot \frac{G_D}{G_A} \quad ; \text{from Eqs. A4 and A5}$$

$$E \cdot A_b = (FR - 1) \cdot \frac{G_A}{G_D} \approx (FR - 1) \cdot \frac{\varepsilon_{YFP} (440 \text{ nm})}{\varepsilon_{CFP} (440 \text{ nm})}$$
Obtaining CFP(direct) at 535 nm (points 3 & 4)

but, $S_{\text{CFP}}$ (output of CFP cube [excite 440 nm, emit 480 nm]) = point 2 = CFP(direct at 480 nm)

Point 1, the FRET readout, will be complicated
Obtaining CFP(direct) at 535 nm (points 3 & 4)

- CFP(direct) at 480 nm
- CFP(direct) + YFP(direct) + YFP(FRET)
- YFP(direct) + YFP(FRET)
- YFP(direct)

So, in cells expressing only CFP, we can determine this invariant ratio using 440 nm excitation:

\[ S_{CFP} = \text{CFP(direct at 480 nm)} \]

Then, in cells with both CFP and YFP present, CFP(direct @ 535 nm) = \( S_{CFP} \cdot R_{D1} \) = point 3.

And point 4 = \( S_{FRET} \) (point 1) - point 3.
Obtaining YFP(direct) at 535 nm (point 5)

440-nm excitation induces YFP(direct @ 535 nm) = point 5, and CFP(direct @ 535 nm) and YFP(FRET @ 535 nm)

But, exciting at 515 nm only produces YFP(direct @ 535 nm)
Obtaining YFP(direct) at 535 nm (point 5)

Exciting at 440 nm makes YFP(direct @ 535 nm) = point 5, and CFP(direct @ 535 nm) and YFP(FRET @ 535 nm)

But, exciting at 515 nm only produces YFP(direct @ 535 nm)

So, if in cells expressing only YFP, we determine this invariant ratio using 440 and 515 nm excitation

\[ R_A = \frac{\text{YFP emission at 535 nm with 440 excitation}}{\text{YFP emission at 535 nm with 515 excitation}} \approx 0.016 \]

Then, in cells with both CFP and YFP are present,

\[ \text{YFP(direct @ 535 nm with 440 excitation)} = S_{\text{YFP}} \cdot R_A = \text{point 5}, \]

where \( S_{\text{YFP}} \) = signal from YFP cube with both CFP and YFP present.
3³-FRET unscrambles the crosstalk

\[ FR = \frac{YFP(\text{direct}) + YFP(\text{FRET})}{YFP(\text{direct})} \]

\[ FR = \frac{S_{\text{FRET}} - R_{D1} \cdot S_{\text{CFP}}}{R_A \cdot S_{\text{YFP}}} \]

\[ E \cdot A_b = (FR - 1) \cdot \frac{\varepsilon_{\text{YFP}}(440 \text{ nm})}{\varepsilon_{\text{CFP}}(440 \text{ nm})} \]

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Unscrambling FRET crosstalk via E-FRET

Measure $S_{CFP}$ (440 ex, 480 em, actual signal) before and after selective photobleaching of YFP, yielding $S_{CFP/after}$ and $S_{CFP/before}$.

$$E \cdot D_b = 1 - \frac{S_{CFP/before}}{S_{CFP/after}}$$; from Eq. A3, yielding the 'donor dequenching method'

But can we measure this entity nondestructively. Enter, E-FRET.
Unscrambling FRET crosstalk via E-FRET

\[ E \cdot D_b = 1 - \frac{S_{\text{CFP/before}}}{S_{\text{CFP/after}}} = \frac{S_{\text{CFP/after}} - S_{\text{CFP/before}}}{S_{\text{CFP/after}}} = \frac{(\text{CFP}_{\text{FRET/after}} - \text{CFP}_{\text{FRET/before}})}{\text{CFP}_{\text{FRET/after}}/R_{D1}} = \frac{(\text{CFP}_{\text{FRET/after}} - \text{CFP}_{\text{FRET/before}})}{\text{CFP}_{\text{FRET/after}}} \]

From Eq. A3 we have

\[ \text{CFP}_{\text{FRET}} \text{(direct)} = N_D \cdot I_O \cdot G_{\text{FRET}}(D) \cdot F_{\text{FRET}}(D) \cdot (1 - E \cdot D_b) \]

\[ = N_D \cdot I_O \cdot C \cdot M_D \cdot (1 - E \cdot D_b) \]

\[ = N_D \cdot I_O \cdot C \cdot M_D - N_D \cdot D_b \cdot E \cdot I_O \cdot C \cdot M_D \]

\[ = N_D \cdot I_O \cdot C \cdot M_D - N_A \cdot A_b \cdot E \cdot I_O \cdot C \cdot M_D \]

Substituting from Eq. A32 for \( N_A \)

\[ = N_D \cdot I_O \cdot C \cdot M_D - \frac{YFP_{\text{FRET}} \text{(direct)}}{I_O \cdot C \cdot M_A} \cdot A_b \cdot E \cdot I_O \cdot C \cdot M_D \]

\[ \text{CFP}_{\text{FRET}} \text{(direct)} = \frac{N_D \cdot I_O \cdot C \cdot M_D - YFP_{\text{FRET}} \text{(direct)} \cdot A_b \cdot E \cdot M_D / M_A}{\text{CFP}_{\text{FRET}} \text{(direct, after)}} \quad \text{[A33M]} \]
Unscrambling FRET crosstalk via E-FRET

\[ CFP_{FRET}(direct, before) = \frac{N_D \cdot I_O \cdot C \cdot M_D - YFP_{FRET}(direct) \cdot A_b \cdot E \cdot M_D}{M_A} \]  [A33M]

\[ E \cdot D_b = 1 - \frac{S_{CFP/before}}{S_{CFP/after}} \]

\[ = \frac{S_{CFP/after} - S_{CFP/before}}{S_{CFP/after}} = \left( \frac{CFP_{FRET/after} - CFP_{FRET/before}}{R_{D1}} \right) = \frac{(CFP_{FRET/after} - CFP_{FRET/before})}{CFP_{FRET/after}} \]

\[ = \frac{(CFP_{FRET/after} - CFP_{FRET/before} + YFP_{FRET}(direct) \cdot A_b \cdot E \cdot M_D \cdot M_A)}{YFP_{FRET}(direct) \cdot A_b \cdot E \cdot M_D \cdot M_A + CFP_{FRET/before}} \]
Unscrambling FRET crosstalk via E-FRET

recalling that

\[ E \cdot A_b = (FR - 1) \cdot \frac{\varepsilon_{YFP}(440\,\text{nm})}{\varepsilon_{CFP}(440\,\text{nm})} = \frac{YFP_{FRET}(FRET)}{YFP_{FRET}(direct)} \cdot \frac{YFP_{FRET}(direct)}{\varepsilon_{YFP}(440\,\text{nm})} \cdot \frac{\varepsilon_{CFP}(440\,\text{nm})}{\varepsilon_{YFP}(440\,\text{nm})} \]

\[ E \cdot D_b = \frac{YFP_{FRET}(direct) \cdot \frac{YFP_{FRET}(FRET)}{YFP_{FRET}(direct)} \cdot \frac{\varepsilon_{YFP}(440\,\text{nm})}{\varepsilon_{YFP}(440\,\text{nm})} \cdot M_D / M_A}{M_D / M_A + CFP_{FRET/before}} \]

\[ E \cdot D_b = \frac{YFP_{FRET}(FRET) \cdot \frac{\varepsilon_{YFP}(440\,\text{nm})}{\varepsilon_{CFP}(440\,\text{nm})} \cdot M_D / M_A}{YFP_{FRET}(FRET) \cdot \frac{\varepsilon_{YFP}(440\,\text{nm})}{\varepsilon_{CFP}(440\,\text{nm})} \cdot M_D / M_A + CFP_{FRET/before}} \]

\[ E \cdot D_b = \frac{YFP_{FRET}(FRET)}{YFP_{FRET}(FRET) + CFP_{CFP/before} \cdot R_D \cdot (\frac{\varepsilon_{CFP}(440\,\text{nm})}{\varepsilon_{YFP}(440\,\text{nm})}) \cdot M_A / M_D} \]

\[ E \cdot D_b = \frac{YFP_{FRET}(FRET)}{YFP_{FRET}(FRET) + CFP_{CFP/before} \cdot G} = \frac{(S_{FRET} - R_D \cdot S_{CFP} - R_A \cdot S_{YFP})}{(S_{FRET} - R_D \cdot S_{CFP} - R_A \cdot S_{YFP}) + S_{CFP} \cdot G} \]

This is the E–FRET equation!
Unscrambling FRET crosstalk via E-FRET

so we have

\[ E \cdot A_b = (FR - 1) \cdot \frac{\varepsilon_{YFP}(440 \text{ nm})}{\varepsilon_{CFP}(440 \text{ nm})} = \left( \frac{S_{FRET} - R_{D1} \cdot S_{CFP}}{R_A \cdot S_{YFP}} - 1 \right) \cdot \frac{\varepsilon_{YFP}(440 \text{ nm})}{\varepsilon_{CFP}(440 \text{ nm})} \]

and

\[ E \cdot D_b = \frac{YFP_{FRET}(\text{FRET})}{YFP_{FRET}(\text{FRET}) + CFP_{CFP/before} \cdot G} = \frac{(S_{FRET} - R_{D1} \cdot S_{CFP} - R_A \cdot S_{YFP})}{(S_{FRET} - R_{D1} \cdot S_{CFP} - R_A \cdot S_{YFP}) + S_{CFP} \cdot G} \]
A brilliant way to deduce constants experimentally

\[
E = \frac{F_c}{F_c + \text{Supp} \cdot \frac{(E_G) \cdot M_A}{(E_C) \cdot M_D}}
\]

\[
\Rightarrow \quad \frac{F_c}{\text{Supp}} + \frac{\text{Supp} \cdot \frac{E_D}{\text{Supp}}}{\text{Supp}} = \left(\frac{F_c}{\text{Supp}}\right) \left[\frac{(E_G)}{(E_C) \cdot R_A}\right]
\]

\[
\text{Solve for } x\text{ and } y\text{ as: } F_c = \frac{E_G}{R_A}
\]

\[
R_A \left[\frac{(E_C)}{(E_G)}\right] = \frac{F_c}{S_Y} + \frac{S_Y \cdot E_D}{S_Y} \cdot \frac{E_D}{E_G}
\]

\[
\left(\frac{F_c}{S_Y}\right) = -E_D \cdot G_i \cdot \left(\frac{S_Y}{S_Y} + R_A \left[\frac{(E_C)}{(E_G)}\right]\right)
\]

This is key geometric intuition

1. Y-intercept: \( P_A \cdot \frac{(E_C)}{(E_G)} \)
2. Then calculate \( \lambda \text{ and } \mu \)

Ikeda's \( G \) calc is just

\[
\frac{\Delta Y}{\Delta X} = -E_D \cdot G_i
\]
A brilliant way to deduce constants experimentally

CV constructs

Sc/Sy

Fc/Sy

CTV 6-11-09
C40V 6-11-09
C32V 6-11-09
C50V 6-11-09
C50V 6-16-09
C32V 6-16-09
C40V 6-16-09
CTV 6-16-09
CTV 6-18-09
C5V 7-05-09
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Application #1—Binding curves measured in live cells

Case 1: Low Affinity + Optimal Geometry

Case 2: High Affinity + Poor Geometry

\[ FR = (FR_{\text{max}} - 1) \cdot A_b + 1 = (FR_{\text{max}} - 1) \cdot \frac{D_{\text{FREE}}}{D_{\text{FREE}} + K_{d,\text{EFF}}} + 1 \]

where \( A_b \) is the fraction of YFP-tagged molecules bound, \( D_{\text{FREE}} \) is the relative number of unbound CFP-tagged molecules, and \( K_{d,\text{EFF}} \) is the effective dissociation constant.
optional for channel geometry

\[ A_b = \frac{1}{1 + 2 \cdot K_d / [D_{\text{free}}]} \]

\[ A_b = \frac{1}{\left[ 1 + 2 \cdot K_d \cdot V \cdot N_{\text{avogadro}} / (N_D - A_b \cdot N_A) \right]} \quad [A25] \]

\[ A_b = \frac{N_D + N_A + (2 \cdot N_{\text{avogadro}} \cdot K_d \cdot V) - \sqrt{(N_D + N_A + (2 \cdot N_{\text{avogadro}} \cdot K_d \cdot V))^2 - 4 \cdot N_D \cdot N_A}}{2 \cdot N_A} \quad [A26] \]

\[ R_{A1} \cdot S_{YFP}(DA,530,535) = \]

\[ YFP_{FRET}(440,535,\text{direct}) = N_A \cdot I_0 \cdot G_{FRET}(A,440) \cdot F_{FRET}(A,535) \quad [A28] \]

\[ R_{D1} \cdot S_{CFP}(DA,440,480) = \]

\[ CFP_{FRET}(440,535,\text{direct}) = N_D \cdot k_D \cdot \left[ ((1-D_b) / k_D) + (D_b / (k_T+k_D)) \right] \cdot I_0 \cdot G_{FRET}(D,440) \cdot F_{FRET}(D,535) \quad [A27] \]

\[ R_{D1} \cdot S_{CFP}(DA,440,480) = \]

\[ CFP_{FRET}(440,535,\text{direct}) = [ N_D - E_{\text{EFF}} \cdot N_A ] \cdot I_0 \cdot G_{FRET}(D,440) \cdot F_{FRET}(D,535) \]

where \( E_{\text{EFF}} = E \cdot A_b \).

\[ [A27M] \]
$M_A$ and $M_D$ (gfa, gfd in spreadsheet) can be calculated from optical parameters

\[
G_{\text{FRET}}(A,440) \cdot F_{\text{FRET}}(A,535) \approx C \cdot \[\varepsilon_A(\lambda)\]_{\lambda=430-450\,\text{nm}} \cdot \[f_A(\lambda)\]_{\lambda=505-575\,\text{nm}}
\]

\[
G_{\text{FRET}}(D,440) \cdot F_{\text{FRET}}(D,535) \approx C \cdot \[\varepsilon_D(\lambda)\]_{\lambda=430-450\,\text{nm}} \cdot \[f_D(\lambda)\]_{\lambda=505-575\,\text{nm}}
\]

\[
R_{A_1} \cdot S_{YFP}(DA,530,535) = YFP_{\text{FRET}(440,535,\text{direct})} \approx N_A \cdot I_0 \cdot C \cdot M_A \quad [A32]
\]

\[
R_{D_1} \cdot S_{CFP}(DA,440,480) =
\]

\[
\begin{align*}
\text{CFP}_{\text{FRET}(440,535,\text{direct})} & \approx N_D \cdot I_0 \cdot C \cdot M_D - E_{\text{EFF}} \cdot YFP_{\text{FRET}(440,535,\text{direct})} \cdot M_D / M_A \\
& \quad [A33M]
\end{align*}
\]

\[
N_A \cdot I_0 \cdot C = YFP_{\text{EST}} = \frac{YFP_{\text{FRET}(440,535,\text{direct})}}{M_A} \quad [A36]
\]

\[
N_D \cdot I_0 \cdot C = CFP_{\text{EST}} = \frac{\text{CFP}_{\text{FRET}(440,535,\text{direct})} + E_{\text{EFF}} \cdot YFP_{\text{FRET}(440,535,\text{direct})} \cdot M_D / M_A}{M_D} \quad [A35M]
\]
If we want to do binding by $3^3$-FRET

\[
A_b = \frac{N_D + N_A + (2 \cdot N_{\text{avogadro}} \cdot K_d \cdot V)}{2 \cdot N_A} - \sqrt{(N_D + N_A + (2 \cdot N_{\text{avogadro}} \cdot K_d \cdot V))^2 - 4 \cdot N_D \cdot N_A}
\]

\[
A_b = \frac{\text{CFP}_{\text{EST}} + \text{YFP}_{\text{EST}} + K_{\text{d,eff}}}{2 \cdot \text{YFP}_{\text{EST}}} - \sqrt{\left(\frac{\text{CFP}_{\text{EST}} + \text{YFP}_{\text{EST}} + K_{\text{d,eff}}}{2 \cdot \text{YFP}_{\text{EST}}}\right)^2 - 4 \cdot \text{CFP}_{\text{EST}} \cdot \text{YFP}_{\text{EST}}}
\]

\[
K_{\text{d,eff}} = 2 \cdot K_d \cdot V \cdot N_{\text{avogadro}} \cdot I_o \cdot C
\]

\[
FR_{\text{pred}} = 1 + (FR_{\text{max}} - 1)A_b = 1 + E(\varepsilon_D/\varepsilon_A)A_b
\]

\[
\text{Square Error} = (FR - FR_{\text{pred}})^2
\]

determined by measurements

guess parameter

prediction based on guess and measurements

vary guess parameters to minimize this, thereby yielding best guess parameters
If we want to do binding by $3^3$-FRET (optional worries about only $Ma/Md$ ratio determination)

$$A_b = \frac{N_D + N_A + (2 \cdot N_{\text{avogadro}} \cdot K_d \cdot V) - \sqrt{(N_D + N_A + (2 \cdot N_{\text{avogadro}} \cdot K_d \cdot V))^2 - 4 \cdot N_D \cdot N_A}}{2 \cdot N_A}$$

If we calibrated to actual $N_A$ and $N_D$ could use above equation directly. One correspondence to in vitro would set average $V$.

Multiplying numerator and denominator of our standard $A_b$ equation by $M_a$ yields:

$$A_b = \frac{R \cdot C + (E_{\text{EFF/A}} + 1) \cdot Y + Y + \bar{K}_{d\text{EFF}} - \sqrt{(R \cdot C + (E_{\text{EFF/A}} + 1) \cdot Y + Y + \bar{K}_{d\text{EFF}})^2 - 4 \cdot (R \cdot C + E_{\text{EFF/A}} \cdot Y) \cdot Y}}{2 \cdot Y}$$

Where

$R = M_a / M_d$

$C = CFP_{\text{FRET (direct)}} = R_D \cdot S_{\text{CFP (DA)}}$

$Y = YFP_{\text{FRET (direct)}} = R_A \cdot S_{\text{YFP (DA)}}$

$\bar{K}_{d\text{EFF}} = 2 \cdot K_d \cdot V \cdot N_{\text{avogadro}} \cdot I_O \cdot C \cdot M_a$

So an error in the absolute value of $M_a$ would simply scale the hat form of $K_{d\text{EFF}}$ much as our lack of knowledge of $I_O \cdot C$ currently impacts our estimate of $K_{d\text{EFF}}$. We could adjust Ma slightly from our current value to match current $K_{d\text{EFF}}$ values for robust construct.
If we want to do binding by E-FRET

\[
D_b = \frac{N_D + N_A + (2 \cdot N_{\text{avogadro}} \cdot K_{d} \cdot V) - \sqrt{(N_D + N_A + (2 \cdot N_{\text{avogadro}} \cdot K_{d} \cdot V))^2 - 4 \cdot N_D \cdot N_A}}{2 \cdot N_D}
\]

\[
D_b = \frac{\text{CFP}_{\text{EST}} + \text{YFP}_{\text{EST}} + K_{d,\text{EFF}}}{2 \cdot \text{CFP}_{\text{EST}}} - \sqrt{\left(\text{CFP}_{\text{EST}} + \text{YFP}_{\text{EST}} + K_{d,\text{EFF}}\right)^2 - 4 \cdot \text{CFP}_{\text{EST}} \cdot \text{YFP}_{\text{EST}}}
\]

\[K_{d,\text{EFF}} = 2 \cdot K_d \cdot V \cdot N_{\text{avogadro}} \cdot I_0 \cdot C\]  \[[A37]\]

\[E_{\text{pred}} = E_{\text{max}} \cdot D_b\]

\[\text{Square Error} = (E_{\text{measure}} - E_{\text{pred}})^2\]

determined by measurements

guess parameter

prediction based on guess and measurements

vary guess parameters to minimize this, thereby yielding best guess parameters
If we want to do binding by E-FRET (optional worries about only Ma/Md ratio determination)

\[
D_b = \frac{N_D + N_A + (2 \cdot N_{\text{avogadro}} \cdot K_d \cdot V) - \sqrt{(N_D + N_A + (2 \cdot N_{\text{avogadro}} \cdot K_d \cdot V))^2 - 4 \cdot N_D \cdot N_A}}{2 \cdot N_D}
\]

If we calibrated to actual \(N_A\) and \(N_D\) could use above equation directly. One correspondence to in vitro would set average \(V\).

Multiplying numerator and denominator of our standard \(A_b\) equation by \(M_a\) yields:

\[
D_b = \frac{R \cdot C + (E_{\text{EFF/A}} + 1) \cdot Y + Y + \tilde{K}_{d\text{EFF}} - \sqrt{(R \cdot C + (E_{\text{EFF/A}} + 1) \cdot Y + Y + \tilde{K}_{d\text{EFF}})^2 - 4 \cdot (R \cdot C + E_{\text{EFF/A}} \cdot Y) \cdot Y}}{2 \cdot (R \cdot C + E_{\text{EFF/A}} \cdot Y)}
\]

where

\[
R = M_a / M_d
\]

\[
C = CFP_{\text{FRET}} (\text{direct}) = R_{D_1} \cdot S_{CFP} (DA)
\]

\[
Y = YFP_{\text{FRET}} (\text{direct}) = R_A \cdot S_{YFP} (DA)
\]

\[
\tilde{K}_{d\text{EFF}} = 2 \cdot K_d \cdot V \cdot N_{\text{avogadro}} \cdot I_O \cdot C \cdot M_a
\]

So an error in the absolute value of \(M_a\) would simply scale the hat form of \(K_{d\text{EFF}}\) much as our lack of knowledge of \(I_O\) currently impacts our estimate of \(K_{d\text{EFF}}\). We could adjust \(M_a\) slightly from our current value to match current \(K_{d\text{EFF}}\) values for robust construct.
Application #1—Binding curve measured in live cells

\[ FR = (FR_{\text{max}} - 1) \cdot A_b + 1 = (FR_{\text{max}} - 1) \cdot \frac{D_{\text{FREE}}}{D_{\text{FREE}} + K_{d,\text{EFF}}} + 1 \]

where \( A_b \) is the fraction of YFP-tagged molecules bound, \( D_{\text{FREE}} \) is the relative number of unbound CFP-tagged molecules, and \( K_{d,\text{EFF}} \) is the effective dissociation constant.

\[ FR_{\text{max}} \leftrightarrow \text{distance/orientation} \]

\[ K_{d,\text{EFF}} \leftrightarrow \text{relative binding affinity} \]

Application #1—Practical pointers on bimolecular FRET: What genuine binding looks like
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Imaging molecular interactions and biological signals in live cells

- Review of FRET principles
- Unscrambling FRET crosstalk via $3^3$-FRET
- Unscrambling FRET crosstalk via E-FRET
- Application 1—Bimolecular binding curves quantified in live cells
- Application 2—Unimolecular FRET sensor monitors calcium dynamics in cardiac myocytes
TNXL calcium sensor in adult ventricular myocytes
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Doing quantitative biochemistry in live cells

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Michael Erickson – $3^3$-FRET, bimolecular binding curves
Michael Tadross – $3^3$-FRET for confocal imaging
Lingjie Sang – Spurious FRET and genuine IQ/ICDI binding
Lai Hock Tay – TNXL calcium imaging of cardiomyocytes

AHA, NHLBI, NIMH, NINDS, Kleberg Foundation