Using Förster resonance energy transfer to distinguish between proteins that exist as monomers, transient oligomers and stable oligomers in the plasma membrane of living cells

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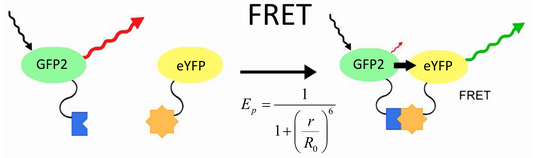
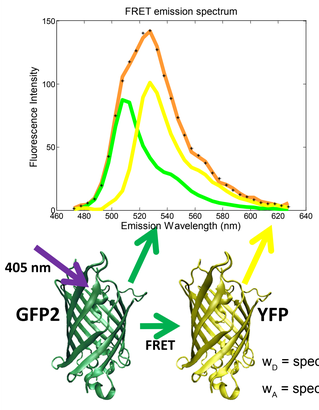
Methods

FRET



Förster resonance energy transfer (FRET) is non-radiative energy transfer that occurs through dipole-dipole interactions between two chromophores that are in close proximity and share an overlap in their emission and excitation spectra. Using a spectrally-resolved microscope, we measured FRET efficiencies between different membrane proteins, tagged with GFP2 or eYFP and expressed in CHO or HEK293 cells.



$$E_{app} = \frac{1}{1+\frac{Q_A k_{DA} w_D}{Q_D k_{AD} w_A}}$$

$$k_{DA} = \frac{\sum(F_M \cdot F_A)\sum(F_D^2) - \sum(F_A \cdot F_D)\sum(F_M \cdot F_D)}{\sum(F_A^2)\sum(F_D^2) - \left[\sum(F_A \cdot F_D)\right]^2}$$

$$k_{AD} = \frac{\sum(F_M \cdot F_A)\sum(F_D^2) - \sum(F_A \cdot F_D)\sum(F_M \cdot F_D)}{\sum(F_A^2)\sum(F_D^2) - \left[\sum(F_A \cdot F_D)\right]^2}$$

E_{app} = apparent FRET efficiency

k_{DA} = fluorescence of donors in the presence of acceptors

k_{AD} = fluorescence of acceptors in the presence of donors

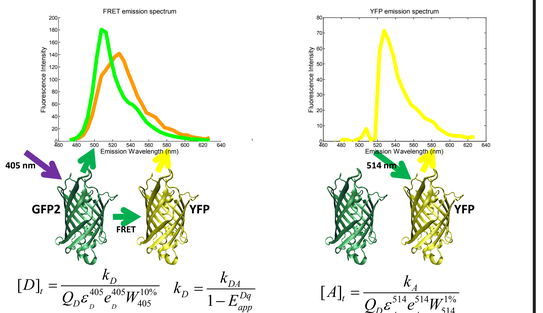
Q_D = quantum yield of donors

Q_A = quantum yield of acceptors

w_D = spectral integral of the donor spectrum

w_A = spectral integral of the acceptor spectrum

The emission spectra from cells expressing both GFP2- and eYFP-tagged proteins (*e.g.* orange line above) were unmixed into the donor (k_{DA}) and acceptor (k_{AD}) components (green and yellow lines above). Using the relative strength of the donor and acceptor signal (equation to the right of the plot), we calculated the apparent FRET efficiency (E_{app}) for each cell that was imaged.



$$[D]_t = \frac{k_D}{Q_D \varepsilon_D^{405} e_D^{405} W_{405}^{10\%}} \qquad k_D = \frac{k_{DA}}{1 - E_{app}^{Dq}}$$

$$[A]_t = \frac{k_A}{Q_D \varepsilon_A^{514} e_A^{514} W_{514}^{1\%}}$$

k_D = total fluorescence of donors

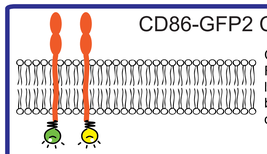
$[D]_t$ = total concentration of donors (in arbitrary units)

k_A = total fluorescence of acceptors

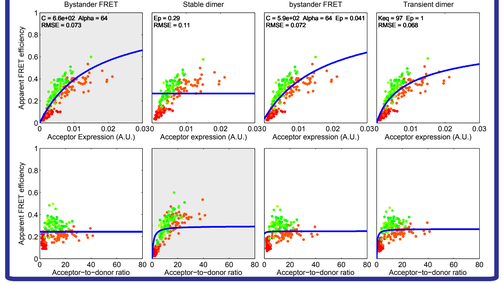
$[A]_t$ = total concentration of acceptors (in arbitrary units)

In addition to the apparent FRET efficiency, we also estimated the amount of donor and acceptor present in each cell. We estimated the amount of donor from the FRET emission spectrum by correcting for the amount of energy lost by the donors through FRET. We estimated the amount of acceptor by taking a second image using the 488 nm laser line, which only excited the acceptors. In each case, fluorescence emission was converted into concentration units using the Beer-Lambert law.

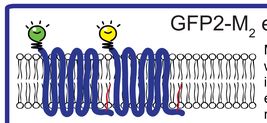
CD86-GFP2 CD86-eYFP



CD86 is the monomeric control. FRET efficiencies approach zero at low levels of expression and cannot be described by a model for a stable dimer.



GFP2-M_2 eYFP-M_2



M_2 musarinic receptor is the receptor whose oligomeric size is being investigated. The pattern of FRET efficiencies cannot be described by a model for a stable dimer.

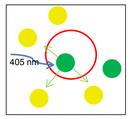
Mathematical models

Bystander FRET

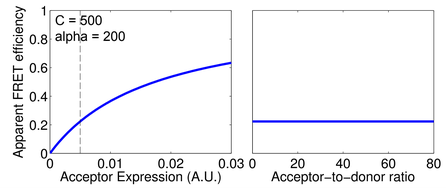
$$E_{bystander} = 1 - \int_0^{\infty} \begin{pmatrix} \exp[-\lambda] \cdot \\ \exp[-\pi \cdot C \cdot [A] \cdot \gamma(2/3, \lambda/\alpha) \cdot \lambda^{1/3}] \cdot \\ \exp[\pi \cdot C \cdot [A] \cdot \alpha^{1/3} \cdot (1 - e^{-\lambda/\alpha})] \end{pmatrix} d\lambda$$

$$\alpha = \left(\frac{R_e}{R_0}\right)^6$$

C = clustering constant



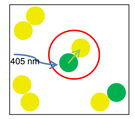
The bystander FRET model predicts the pattern of apparent FRET efficiencies for non-interacting but colocalised donors and acceptors. FRET efficiencies depend on the acceptor expression level but not on the acceptor-to-donor ratio.



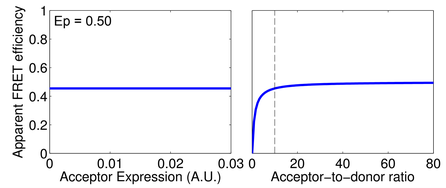
Stable dimer

$$E_{stable} = E_{pairwise} \cdot P_A$$

$E_{pairwise}$ = pairwise FRET efficiency between one donor and one acceptor



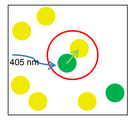
The stable dimer model predicts the pattern of apparent FRET efficiencies for constitutive dimers or higher-order oligomers. FRET efficiencies depend on the acceptor-to-donor ratio but not on the acceptor expression level.



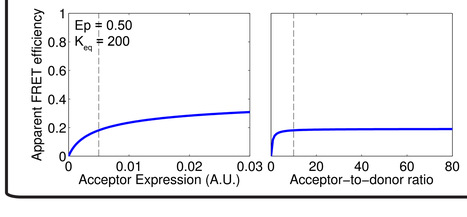
Transient dimer

$$E_{transient} = \frac{E_{pairwise} \cdot P_A}{2} \Big/ \left(1 - \sqrt{1 + 8 \cdot [A] / P_A \cdot K_{eq}}\right)$$

K_{eq} = equilibrium association constant

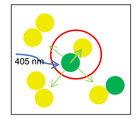


The transient dimer model predicts the pattern of apparent FRET efficiencies for monomers that have a propensity to dimerise but do not form constitutive dimers. FRET efficiencies depend both on the acceptor expression level and the acceptor-to-donor ratio.

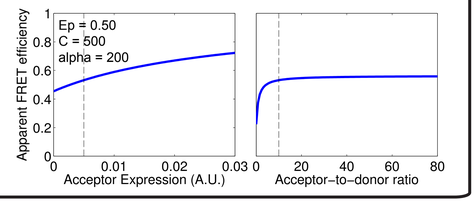


Stable dimer with bystander FRET

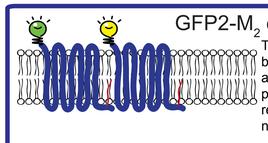
$$E_{stable+bystander} = P_A \cdot \left(\frac{E_{pairwise} - 2 \cdot E_{pairwise} \cdot E_{bystander} + E_{bystander}}{1 - E_{pairwise} \cdot E_{bystander}}\right) + (1 - P_A) E_{bystander}$$



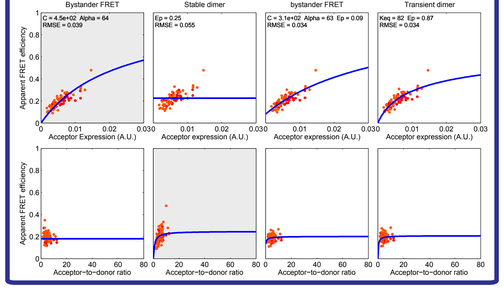
The stable dimer with bystander FRET model predicts the pattern of apparent FRET efficiencies for constitutive dimers colocalised in small compartments. FRET efficiencies depend both on the acceptor expression level and the acceptor-to-donor ratio.



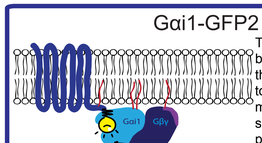
GFP2-M_2 eYFP-β2



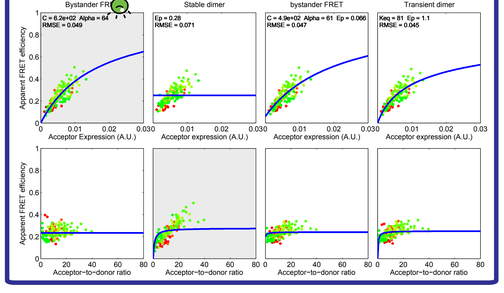
The pattern of efficiencies observed between M_2 muscarinic and β2 adrenergic receptors is similar to the pattern observed between different M_2 receptors, suggesting that the FRET is non-specific.



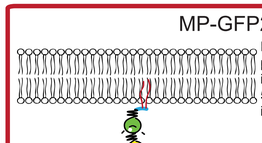
Gαi1-GFP2 M_2-eYFP



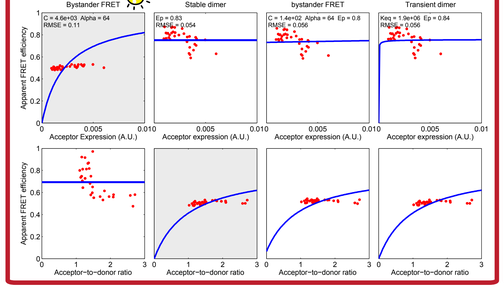
The pattern of efficiencies observed between M_2 muscarinic receptors and their cognate G proteins (Gαi1) is similar to the pattern observed for the monomeric control CD86, which is surprising because M_2 receptors and G proteins are known to interact.



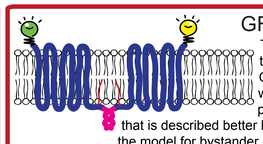
MP-GFP2-eYFP



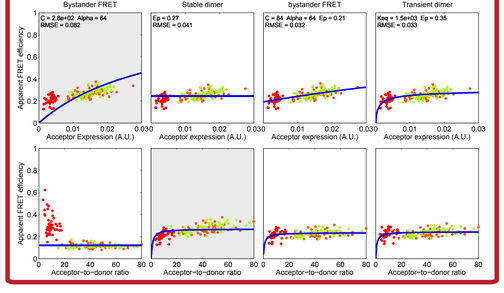
FRET efficiencies for myristoylated palmitoylated GFP2 and eYFP linked in tandem are always greater than 50%, and the acceptor-to-donor ratio is always around one, as expected.



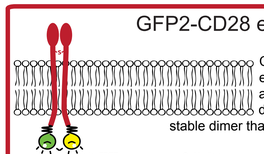
GFP2-M_2R1 eYFP-M_2R2



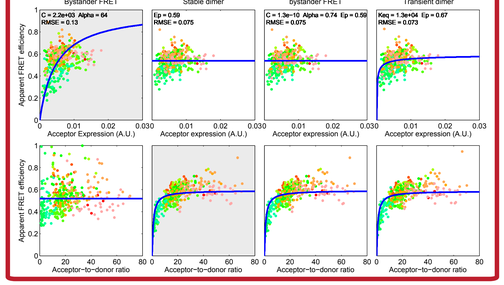
The M_2 muscarinic receptor fused with the coiled-coid domains from the $GABA_B$R1 and $GABA_B$R2 receptors, which are known to induce dimerisation, produced a pattern of FRET efficiencies that is described better by the model for a stable dimer than the model for bystander FRET.



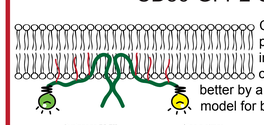
GFP2-CD28 eYFP-CD28



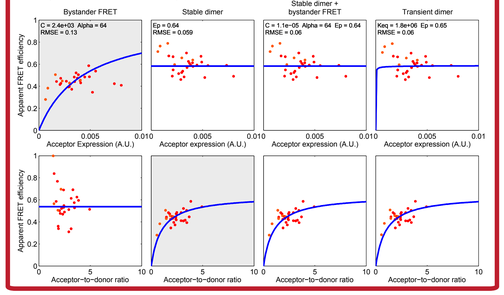
CD86 is the dimeric control. FRET efficiencies are always high at high acceptor-to-donor ratios, and they are described better by the model for a stable dimer than by the model for bystander FRET.



CD86-GFP2 CD86-eYFP



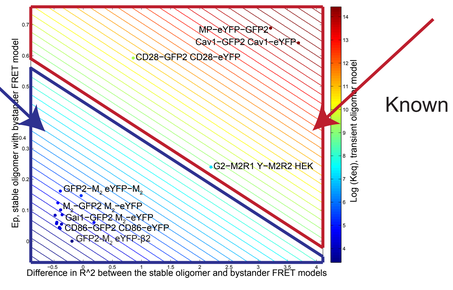
Caveolin-1 is a membrane scaffolding protein and is known to form oligomers in live cells. The pattern of efficiencies observed for caveolin-1 is described better by a model for a stable dimer than the model for bystander FRET.



Data presentation

- In order to show the quality of the fit achieved by each model, when FRET efficiencies were plotted as a function of acceptor expression level, they were transformed to a constant acceptor-to-donor ratio of 10, and when they were plotted as a function of acceptor-to-donor ratio, they were transformed to a constant acceptor expression level of 0.005.
- Uncorrected FRET efficiencies were plotted as a function of acceptor expression level for the bystander FRET model (grey box).
- Uncorrected FRET efficiencies were plotted as a function of acceptor-to-donor ratio for the stable dimer model (grey box).

Monomers, transient oligomers, a combination of monomers and oligomers?



Known oligomers

Conclusions

- M_2 receptors do not appear to exist as constitutive oligomers.
- It is difficult to distinguish between bystander FRET and transient oligomerisation.
- Negative controls can show FRET efficiencies as high as 80%.
- Stable oligomers can have maximum FRET efficiencies of only 30% (M_2 with $GABA_B$R1/2 coiled coils).
- In general, the data are difficult to interpret.
- The plasma membrane of living cells is a very populated and clustered environment.