FRET and Biosensors

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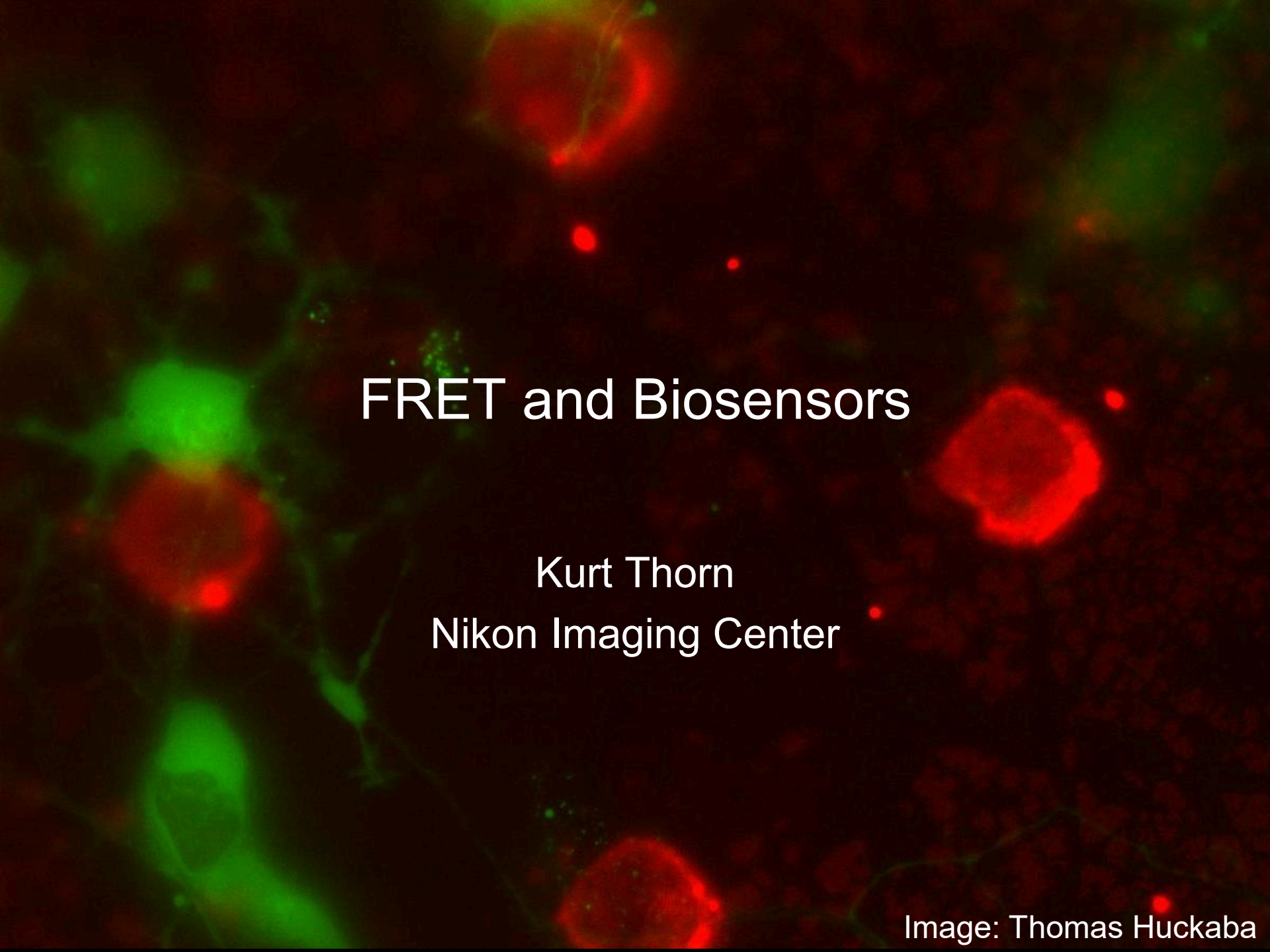
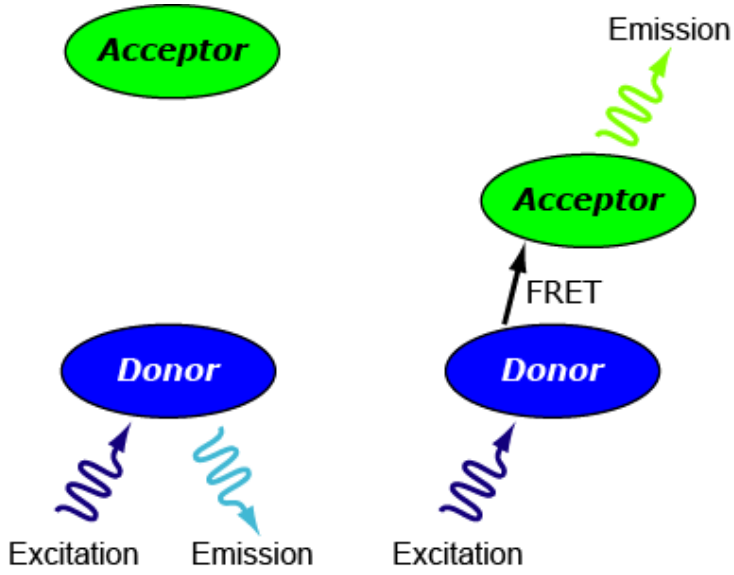
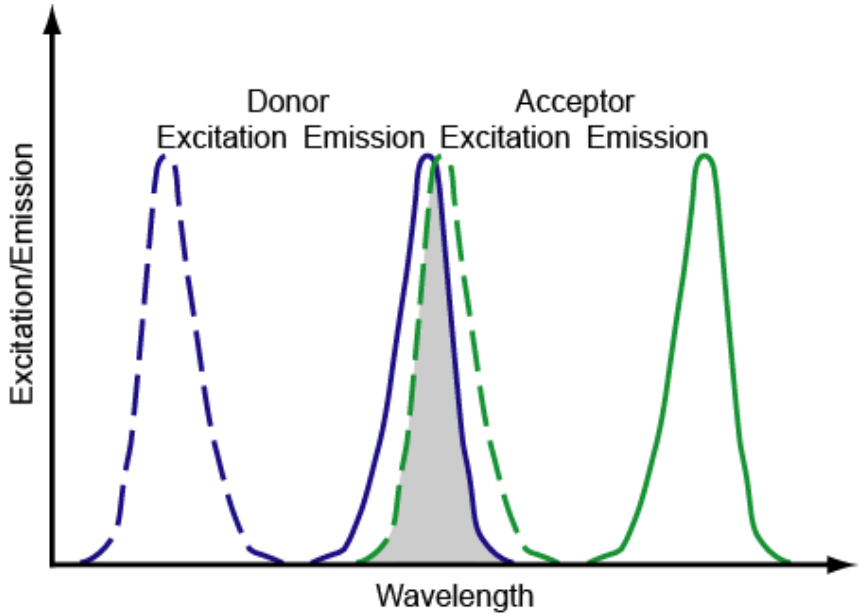


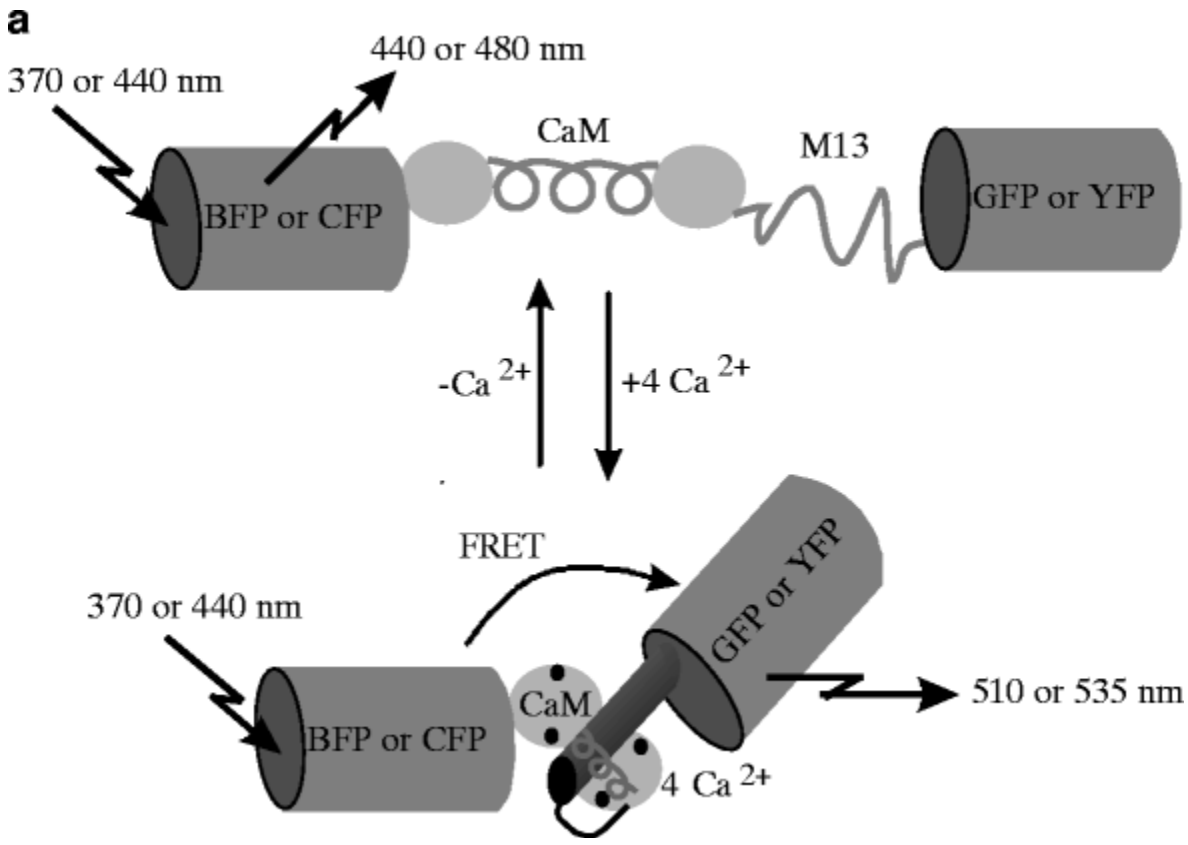
Image: Thomas Huckaba

Fluorescence Resonance Energy Transfer



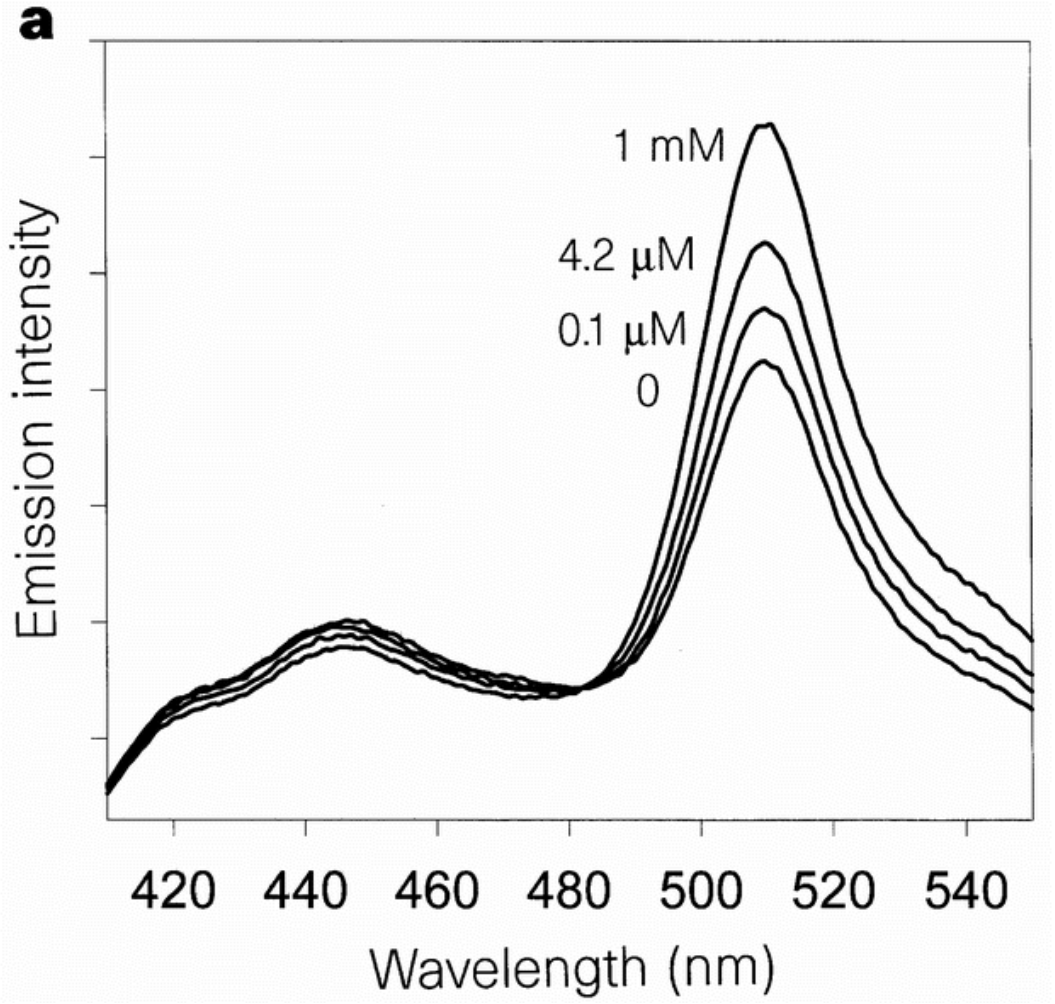


Cameleons: FRET-based Ca^{2+} sensors



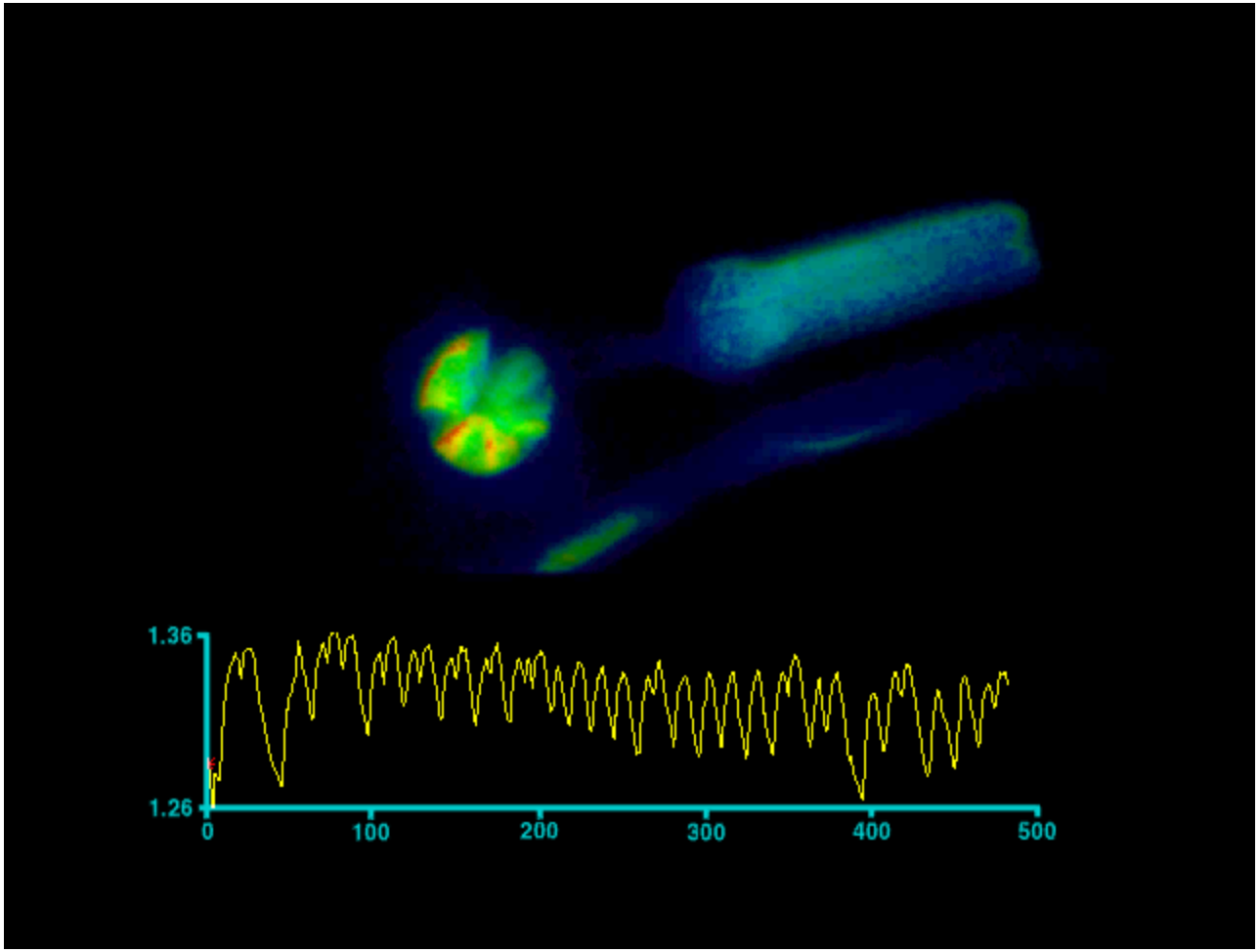
Miyawaki et al. 1997. *Nature* 388, p882-887

Cameleons: FRET-based Ca^{2+} sensors



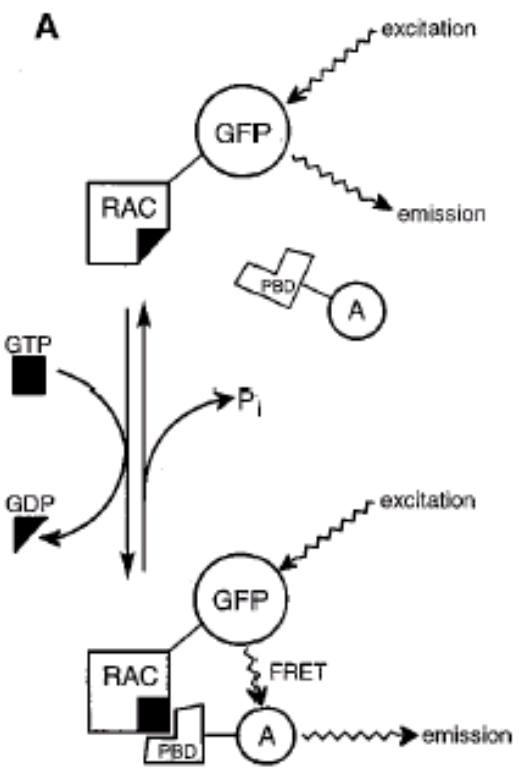
Miyawaki et al. 1997. *Nature* 388, p882-887

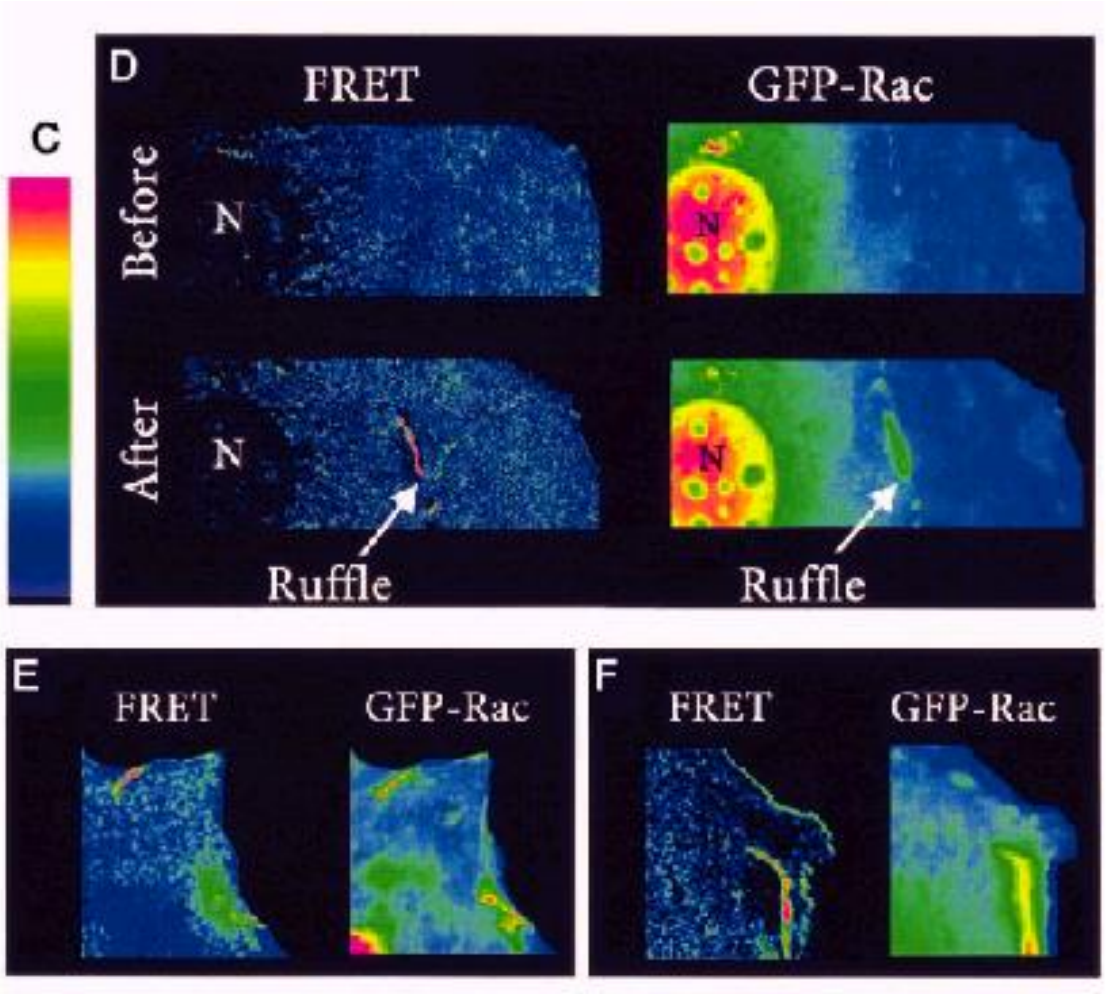
Calcium transients in *C. elegans* pharynx



Kerr et al. 2000. *Neuron* 26, p. 583-594

Using FRET to monitor Rac activation





Kraynov et al. 2000, Science 290: 333

Good FRET pairs

- CFP/YFP – use A206R mutants if dimerization is problematic
- GFP/mCherry, YFP/mCherry, mTFP/mKO, many other FP pairs – not so well validated
- Fluorescein/Rhodamine
- Cy3/Cy5 or Rhodamine/Cy5
- Many other small molecule pairs

Distance dependence of FRET

$$E = \frac{1}{1 + (r^6/R_0^6)}$$

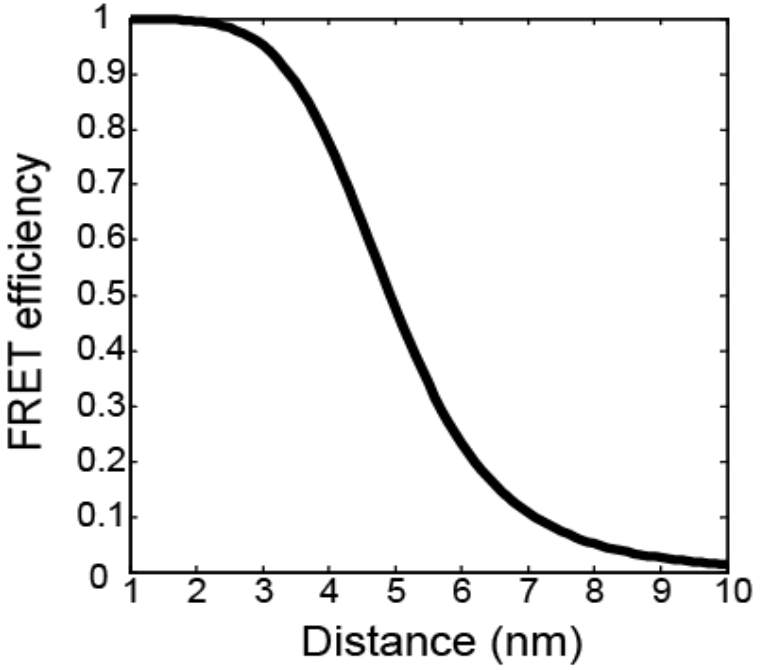
$$R_0^6 \propto \kappa^2 \, n^{-4} \, Q_D \, J(\lambda)$$

Overlap between donor emission and acceptor excitation

Donor quantum yield

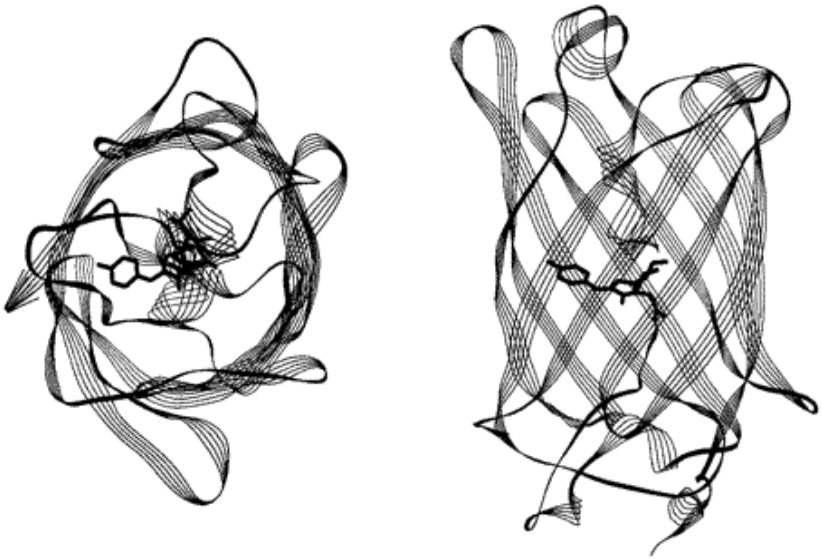
Refractive index

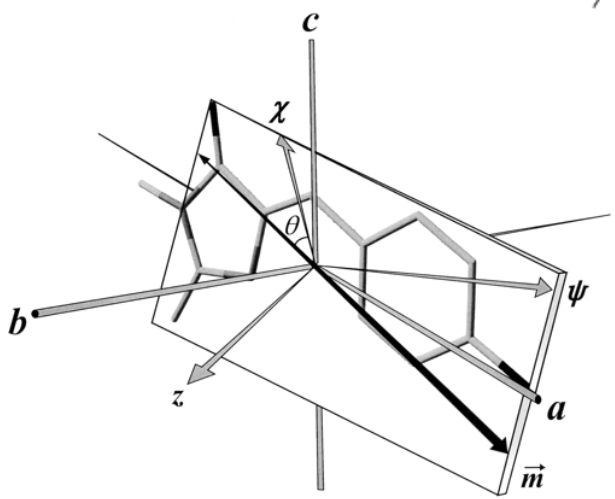
Orientation between fluorophores

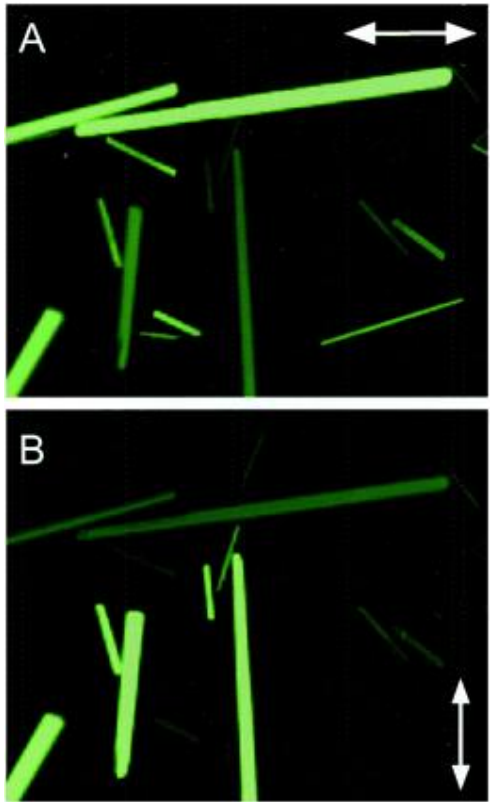


For CFP-YFP,
50% transfer at R_0 = 4.9 nm

Transition dipole of GFP







Rosell and Boxer 2002; Inoue et al. 2002

Angular dependence of FRET

$$R_*^6 \propto n^{-4} Q_D J(\lambda)$$

$$E = \frac{1}{1 + (r^6 / R_*^6 \kappa^2)}$$

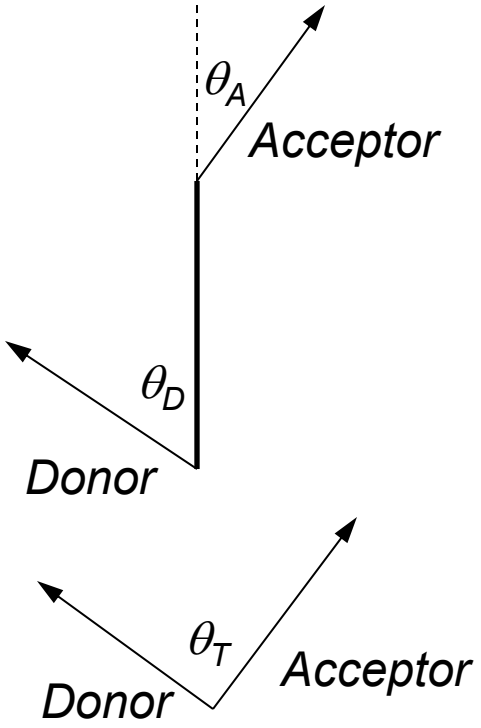
κ^2 depends on the relative orientations of the donor and acceptor excitation dipoles.

κ^2 ranges between 0 and 4 and is 0 for whenever the donor and acceptor dipoles are perpendicular to one another.

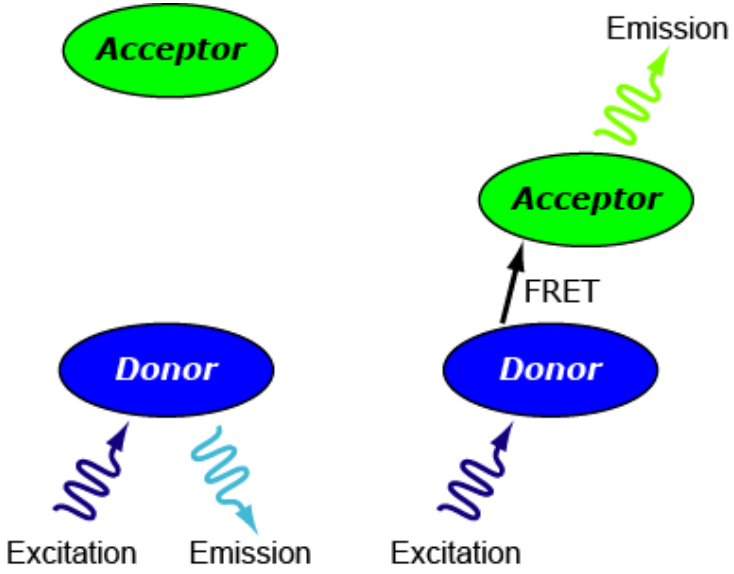
For rapidly-rotating dyes $\kappa^2 = 2/3$

FRET Theory

- $\kappa^2 = (\cos \theta_T - 3 \cos \theta_D \cos \theta_A)^2$
- For rapidly tumbling molecules, can average over all possible orientations to give $\kappa^2 = 2/3$
- But rotational correlation time for GFP is ~16 ns; fluorescence lifetime is ~3ns

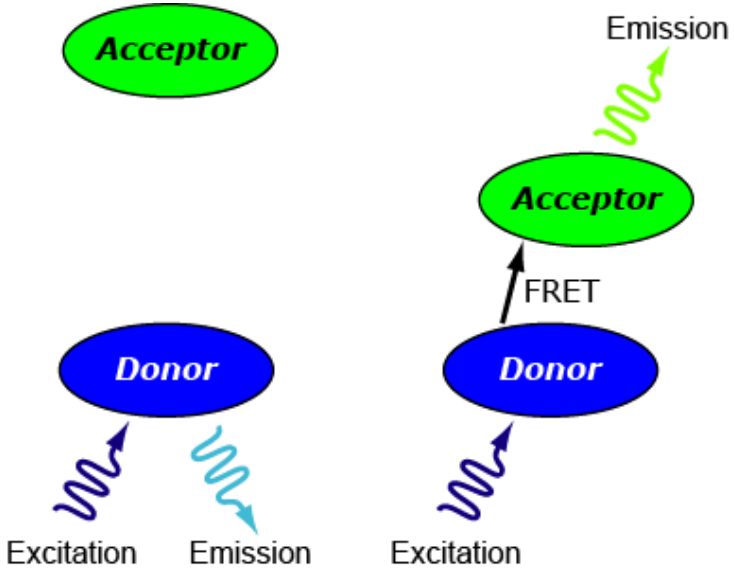


Effects of FRET



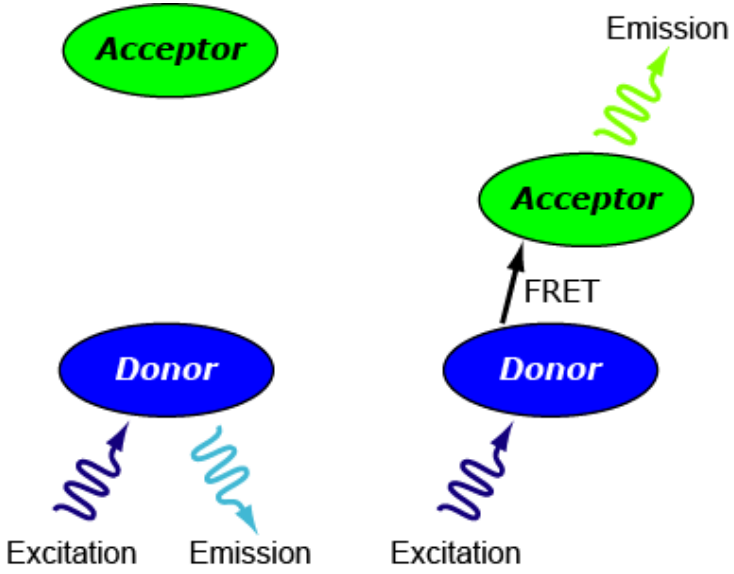
- Donor lifetime shortened
- Acceptor emission depolarized
- Donor fluorescence quenched
- Acceptor fluorescence enhanced on donor excitation

Measuring FRET



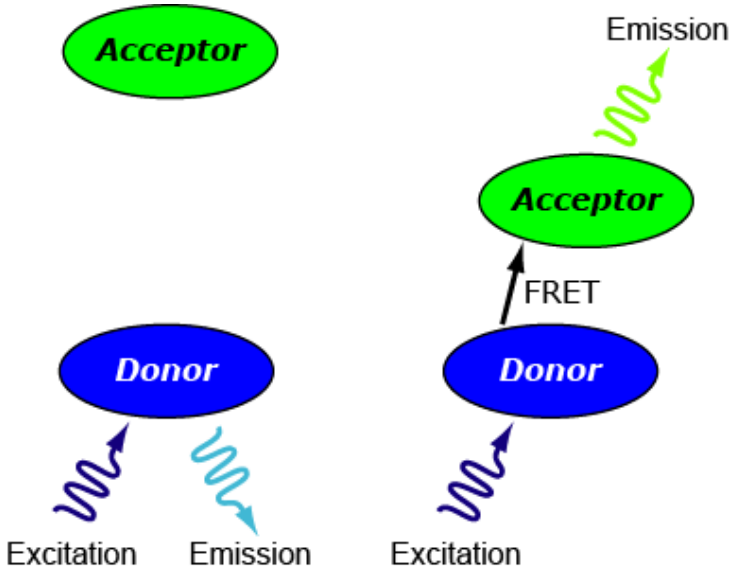
- Donor lifetime shortened
- Can measure by fluorescence lifetime imaging, but requires specialized instrumentation

Measuring FRET



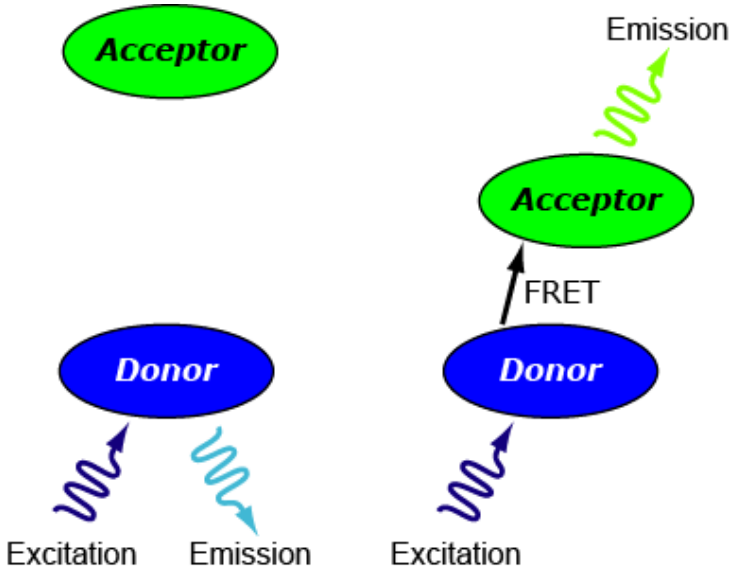
- Acceptor emission depolarized
- Can measure by fluorescence polarization microscopy

Measuring FRET



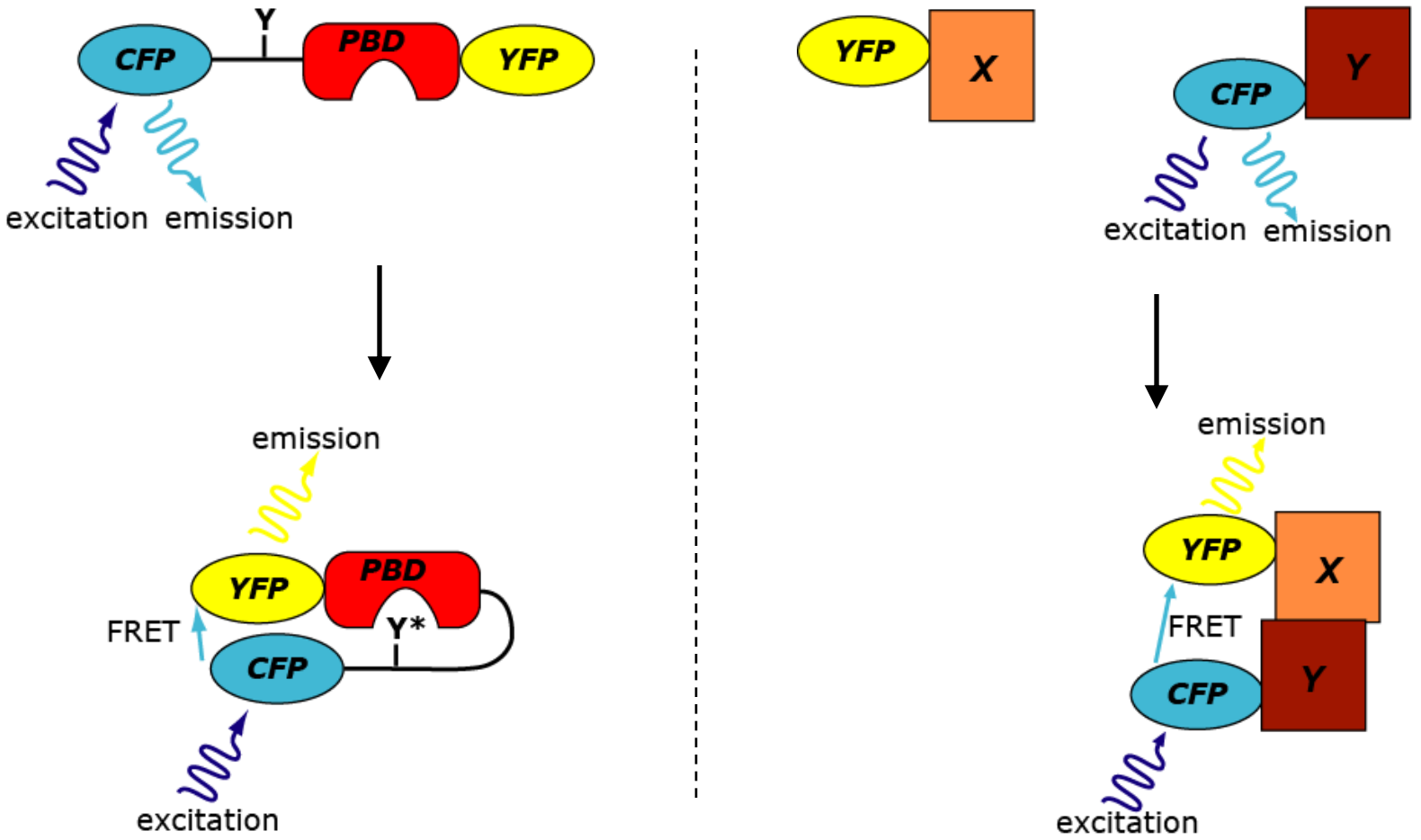
- Donor fluorescence quenched
- Acceptor fluorescence enhanced on donor excitation
- Can measure by donor recovery after acceptor photobleaching
  - Easy, but very sensitive to degree of photobleaching

Measuring FRET



- Donor fluorescence quenched
- Acceptor fluorescence enhanced on donor excitation
- Can measure by quantitative measurement of acceptor enhancement on donor excitation

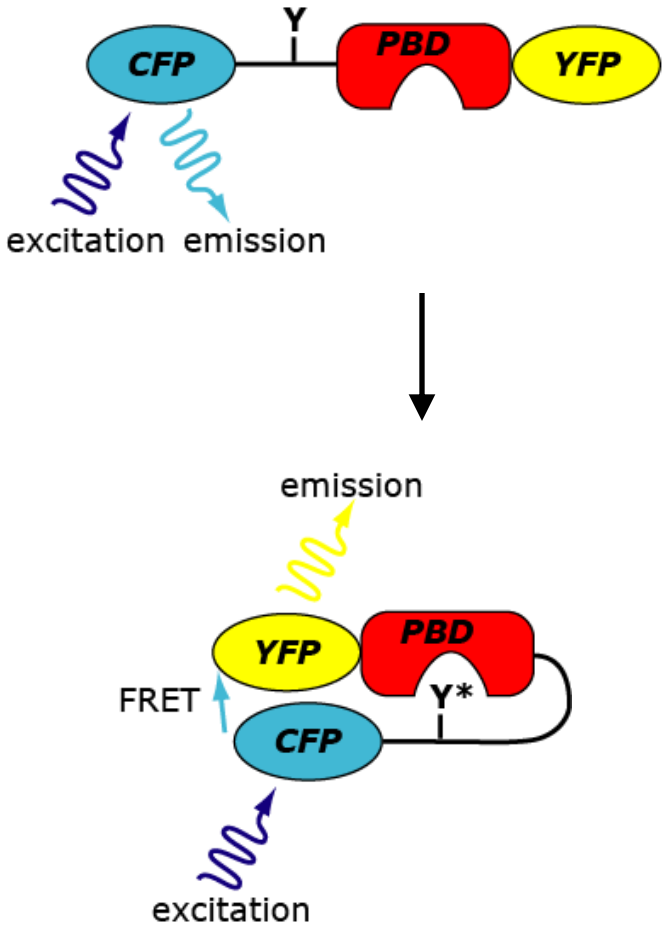
Types of FRET experiments



Intramolecular

Intermolecular

Types of FRET experiments

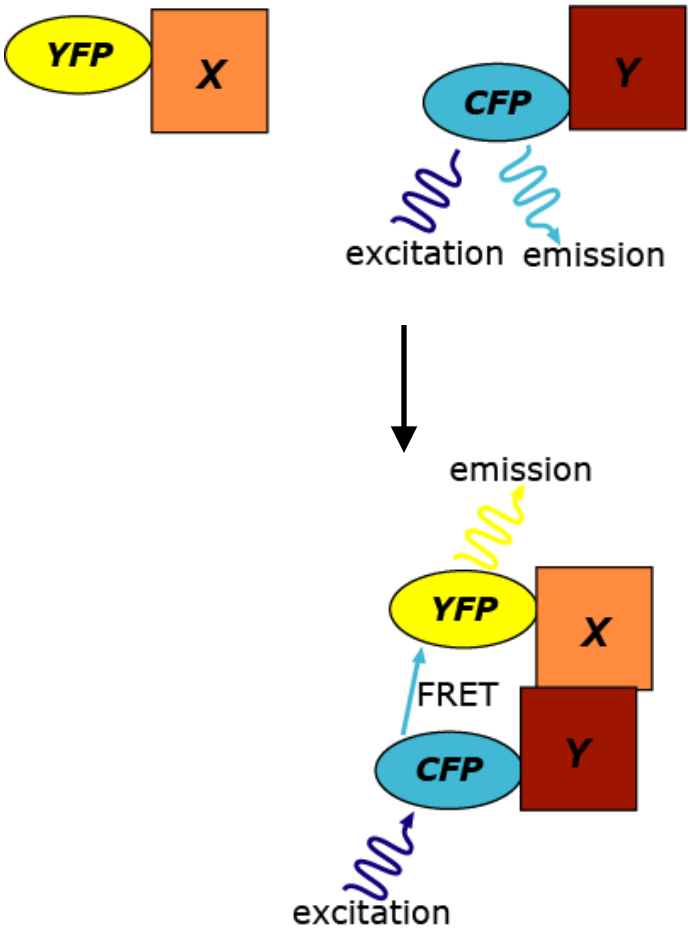


Intramolecular

For intramolecular FRET, CFP and YFP are always present in a 1:1 ratio

Ratiometric imaging can be used as a rough measure of the amount of energy transfer

Types of FRET experiments



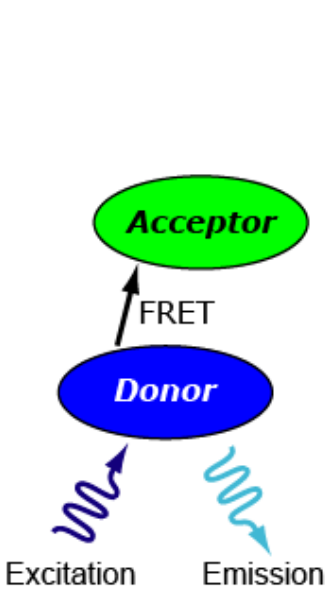
Intermolecular

For intermolecular FRET, the relative abundance of CFP and YFP is not controlled and can change over time.

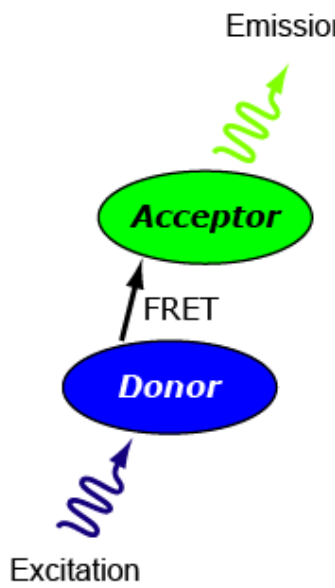
Ratiometric imaging is no longer possible, and additional corrections are necessary.

Data Acquisition

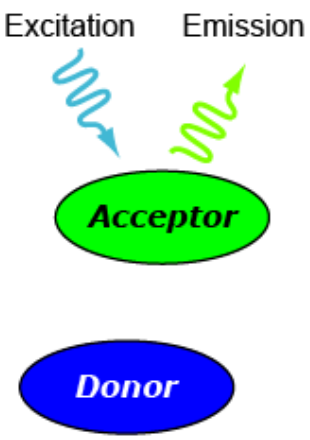
Three things to measure:



Donor Intensity



FRET Intensity



Acceptor Intensity

Data Acquisition

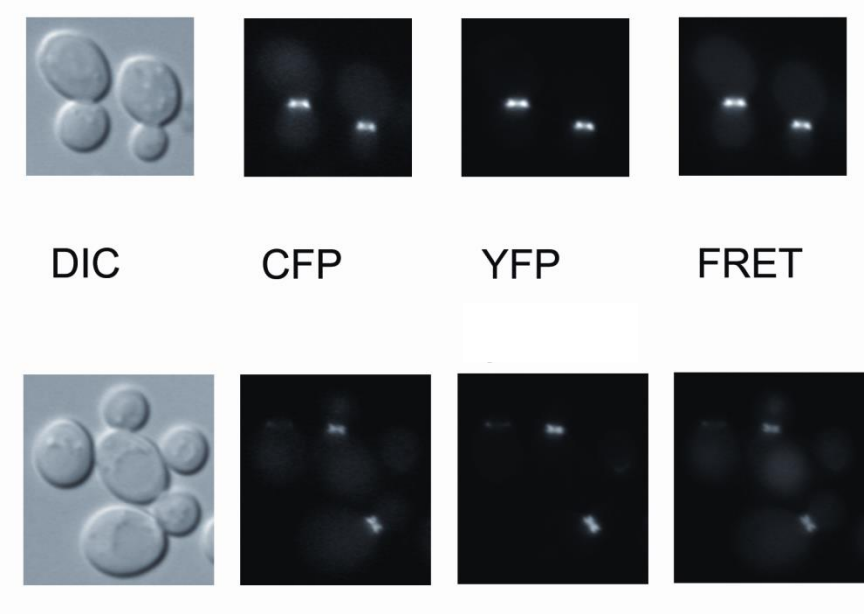
- Maximize signal-to-noise: use high NA objective, sensitive, low-noise camera, high-transmission filters

- Minimize shifts between wavelengths
  - Fluor or apochromatic objective
  - Multipass dichroic with external excitation and emission filters

Image preprocessing

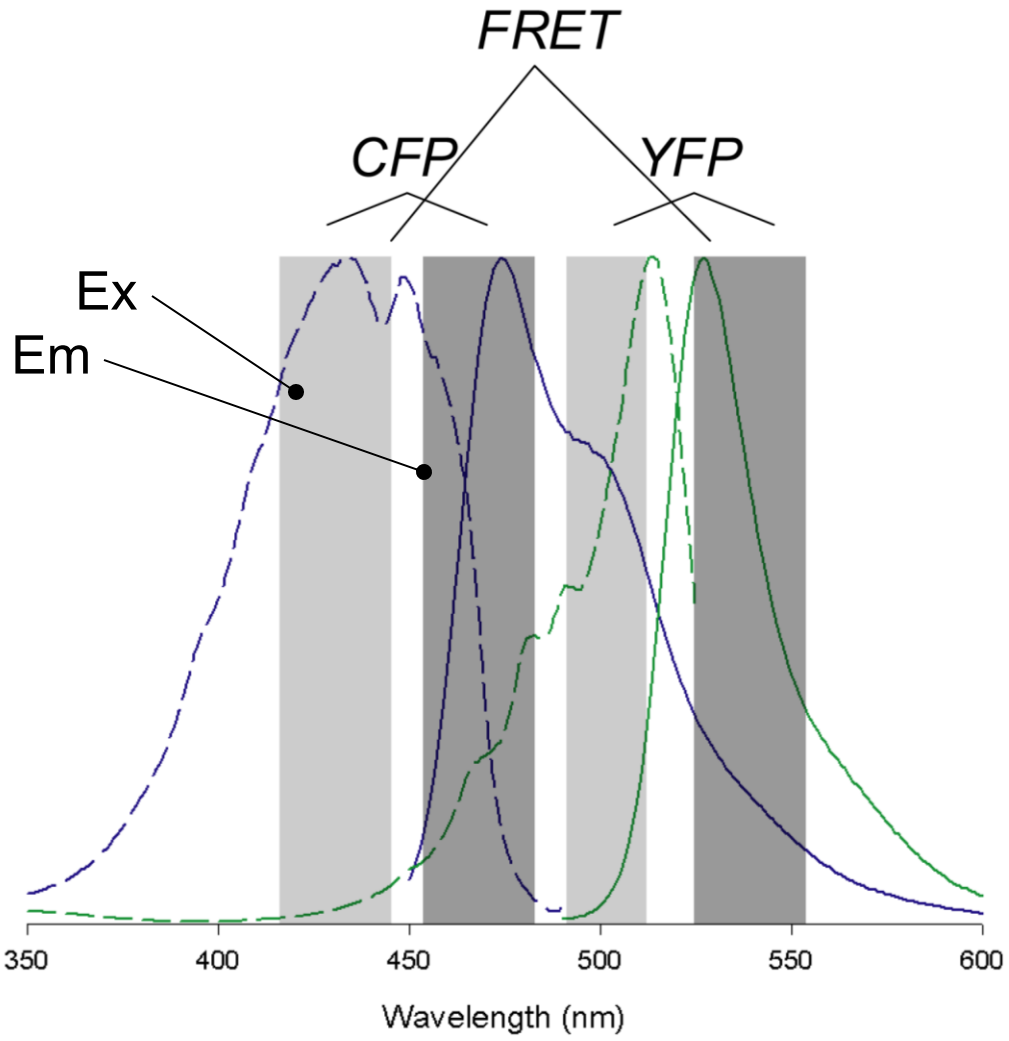
- Background subtraction
- Register images by maximizing correlation with FRET image

Data Acquisition



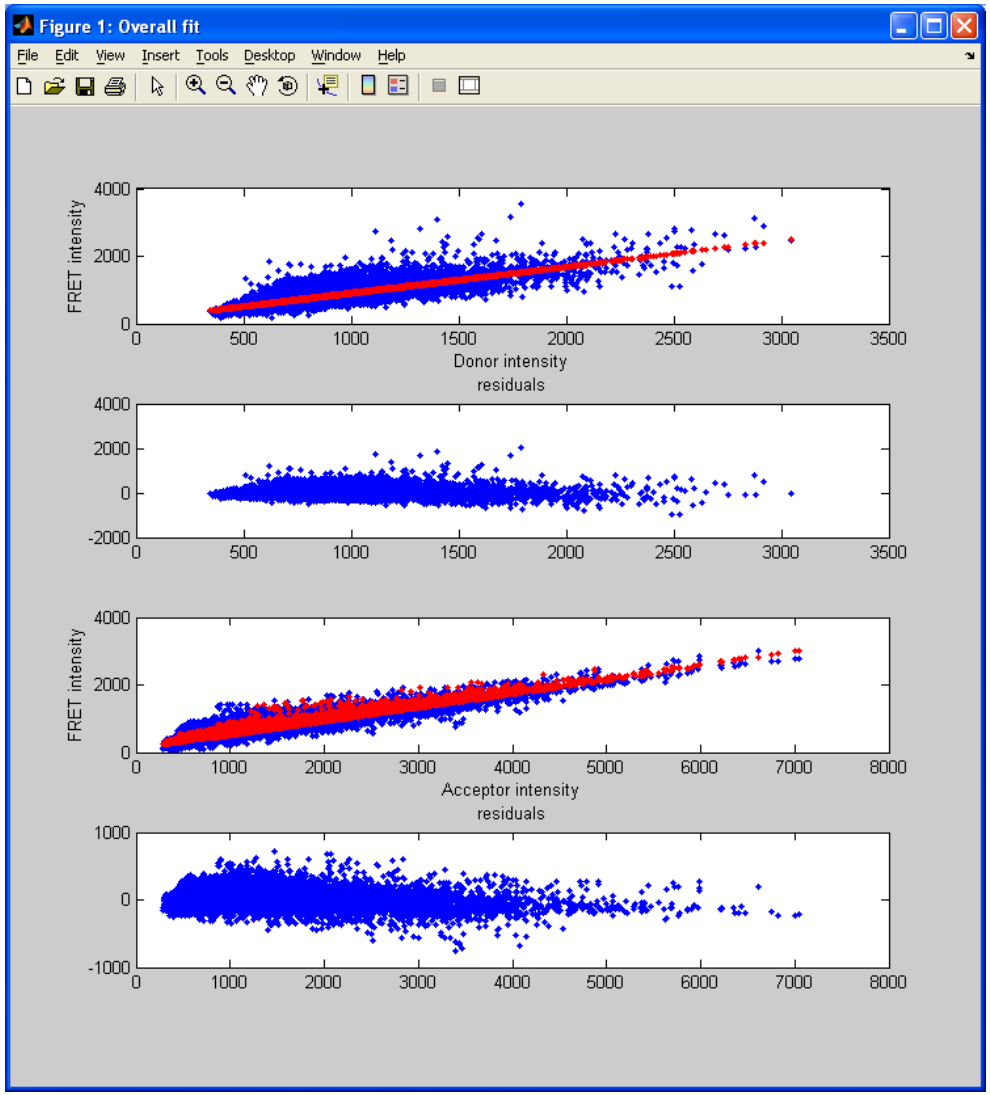
Acquire sequential images of FRET, YFP, CFP, and DIC

A problem: crosstalk into FRET channel



Correct using measurements from CFP- and YFP- only cells

A problem: crosstalk into FRET channel



For strains with only CFP and YFP,
$FRET_C = 0$

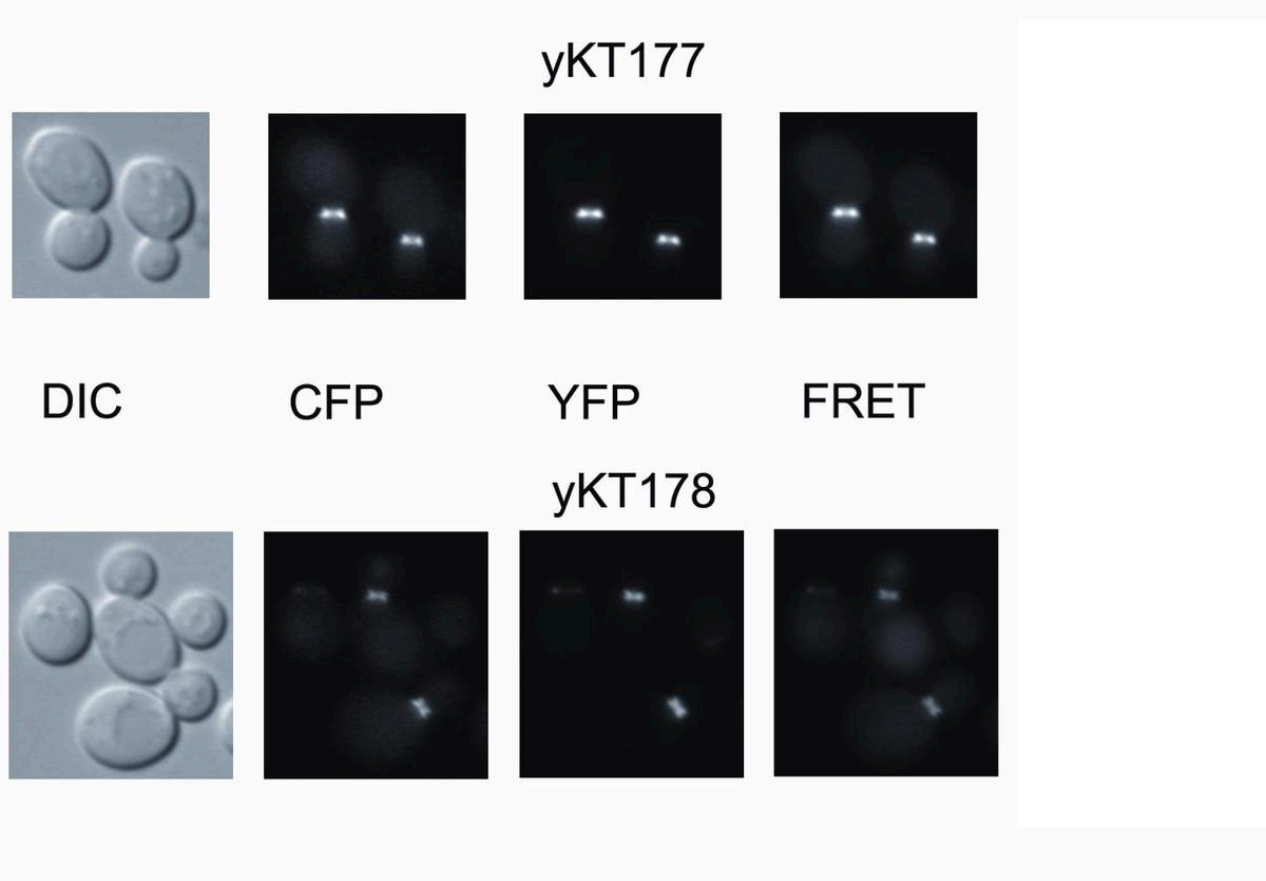
Fit
$FRET_C = FRET_m - \alpha CFP - \beta YFP - \gamma$

Typical values:

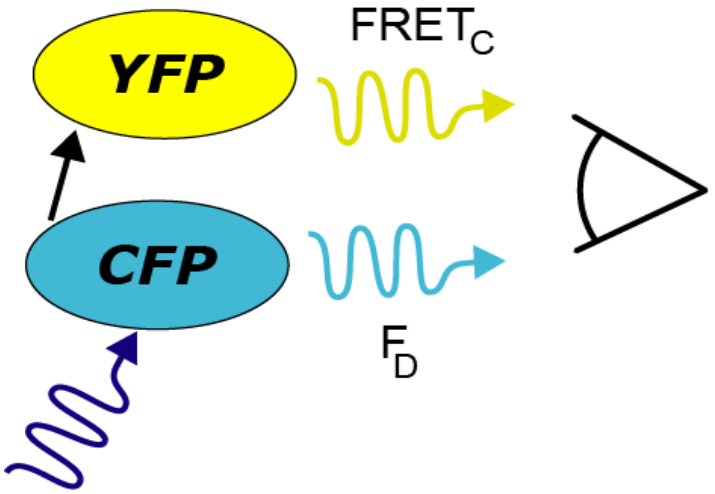
$\alpha \sim 0.9$

$\beta \sim 0.4$

Crosstalk correction



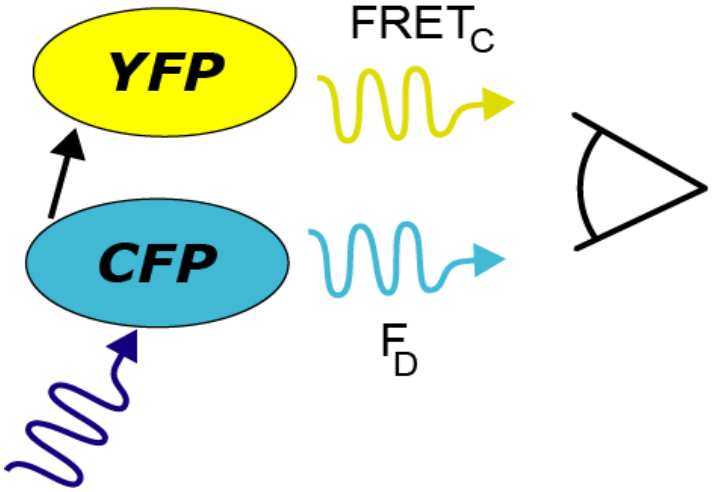
Calculating FRET efficiency



Traditionally:

$$E = 1 - \frac{F_D \text{ (Donor+Acceptor)}}{F_D \text{ (Donor alone)}}$$

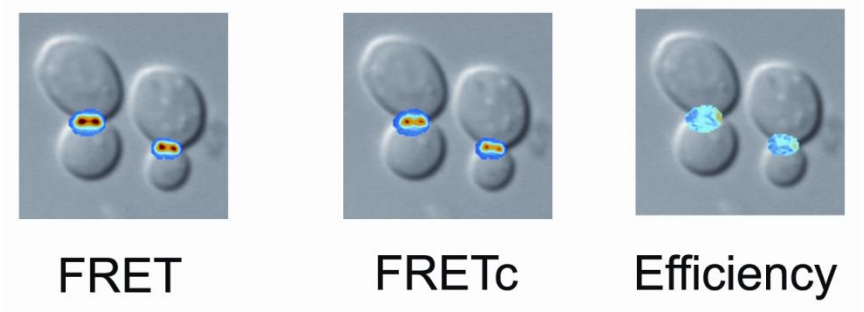
Calculating FRET efficiency



$$E = 1 - \frac{FRET_C \cdot G + F_D}{F_D}$$

G corrects for detection efficiencies of CFP and YFP

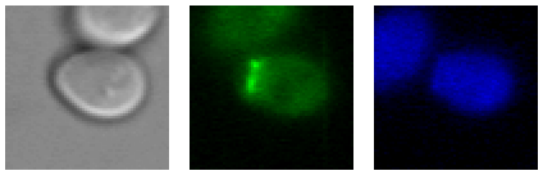
$$G = Q_D\Phi_D / Q_A\Phi_A$$



One final issue: Autofluorescence

We correct for autofluorescence in the FRET channel by inclusion of γ

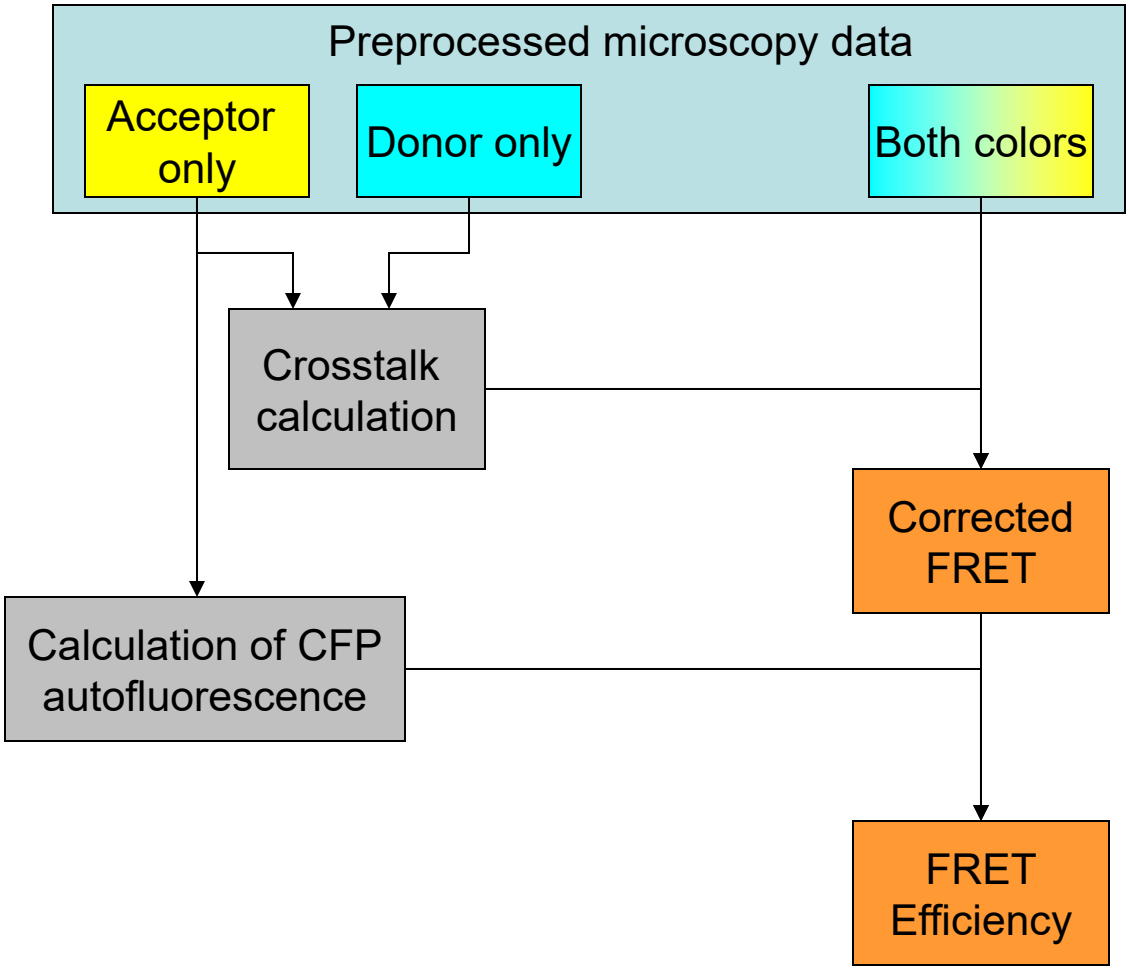
But we also need to correct for autofluorescence in the donor channel



$$E = \frac{FRET_C \cdot G}{FRET_C \cdot G + F_D}$$

Correct donor autofluorescence by subtracting median donor fluorescence of untagged cells

Data analysis procedure



Preprocessing:

Background subtraction

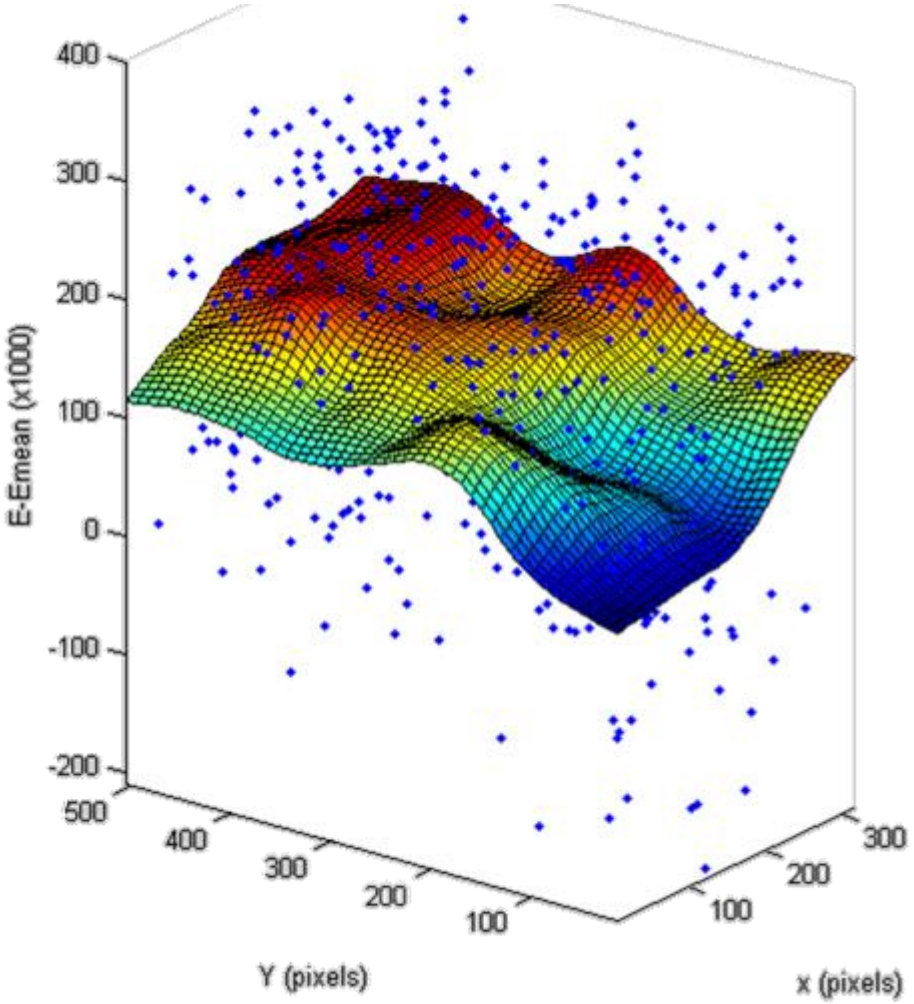
Image alignment by maximizing the correlation of donor and acceptor with the FRET image.

Typical shifts are <2 pixels

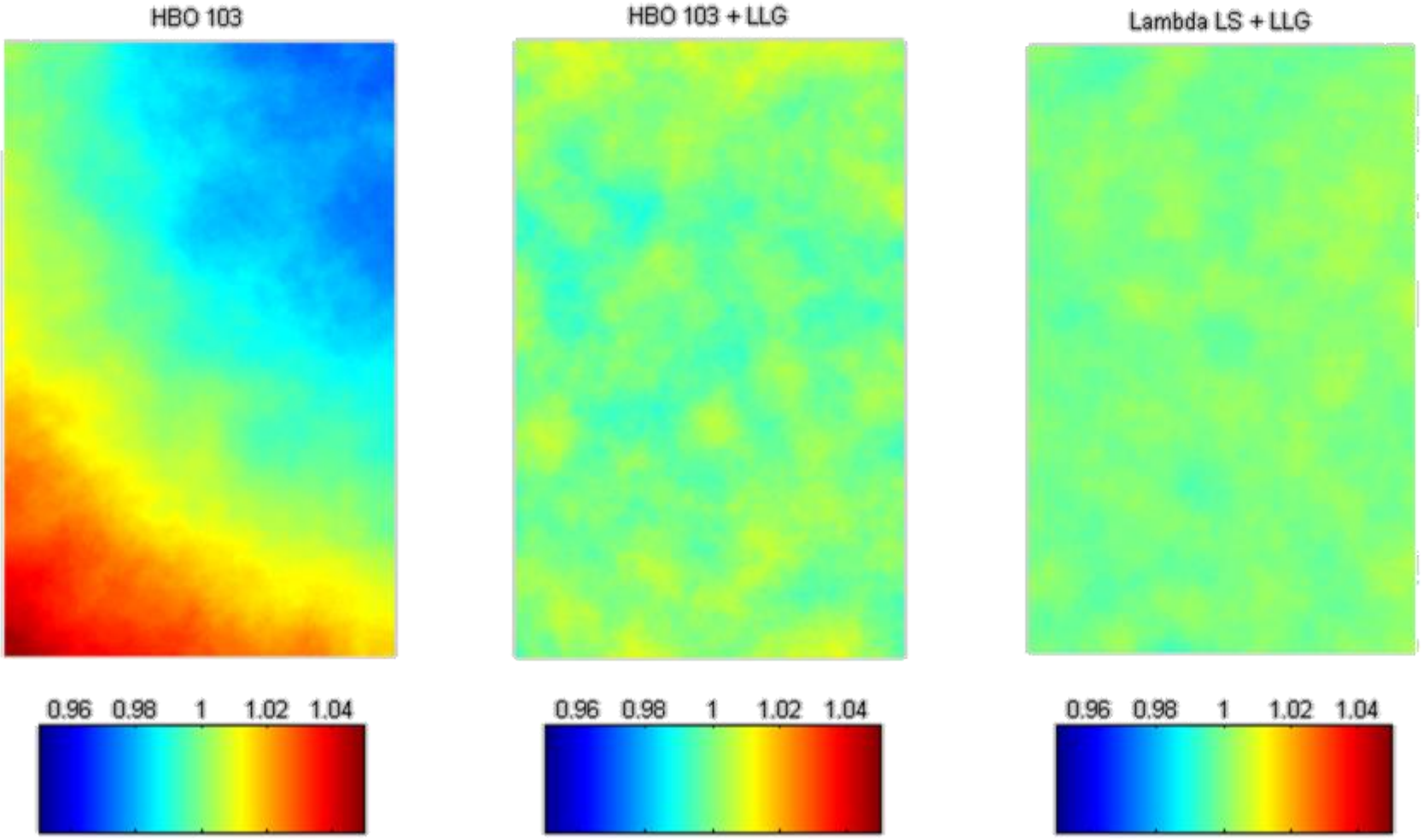
Photobleaching

- Some dyes photobleach quite easily (prime offenders: fluorescein, YFP)
- Correction procedures are available but are non-trivial
- Photobleaching can lead to peculiar artifacts

Spatial variation of efficiency



Illumination Uniformity

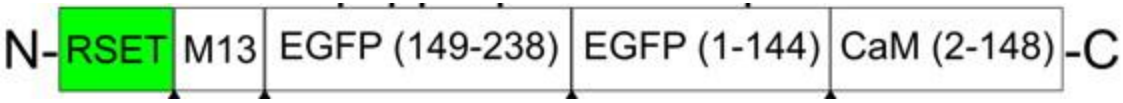


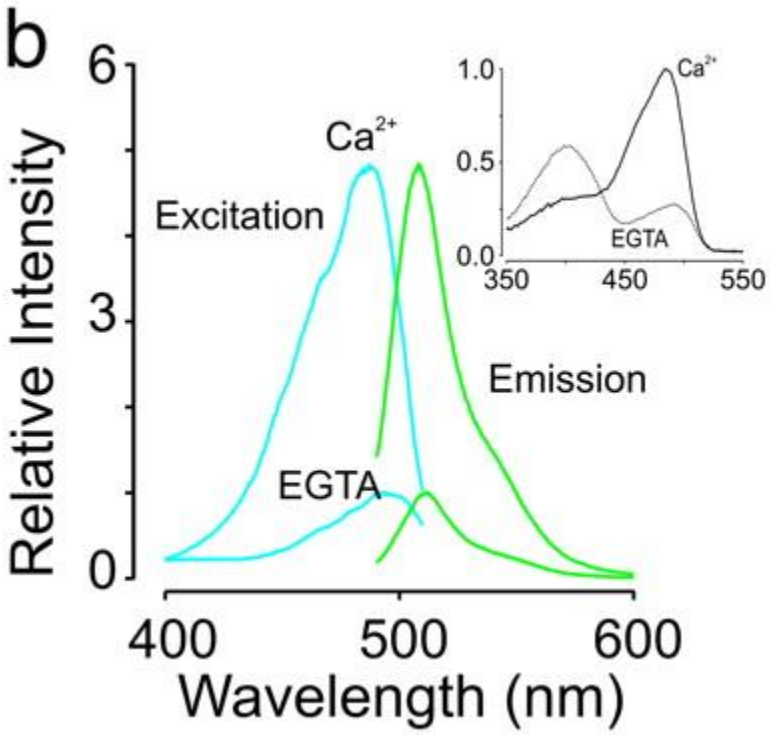
FRET Conclusions

- Using FRET as a qualitative reporter is relatively straightforward.
- Quantitative FRET is challenging and requires correction of a large number of potential artifacts.
- Trying to use FRET to infer distances *in vivo* is probably best avoided.
- Choice of fluorescent proteins for FRET is likely to be idiosyncratic and system dependent.

Single domain sensors – GCaMP2

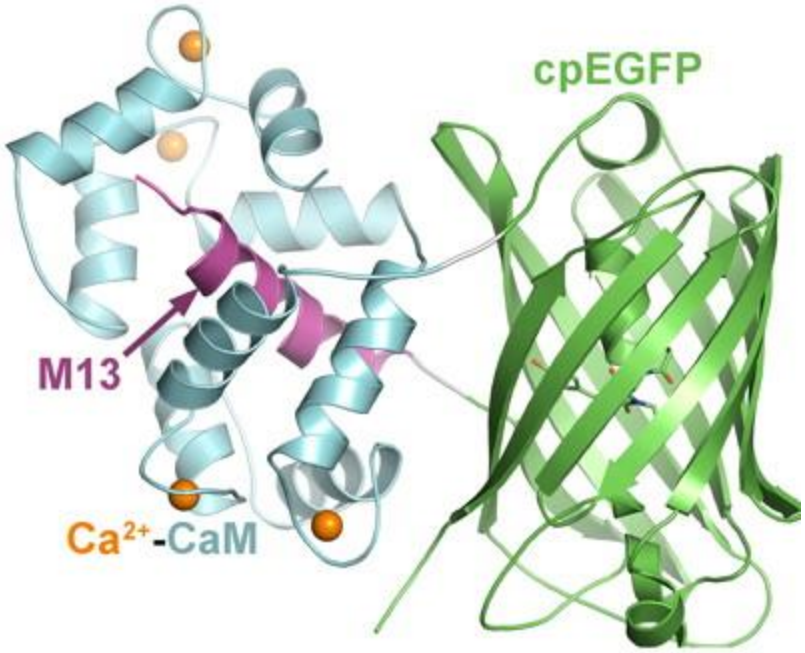
Intensity-based sensor

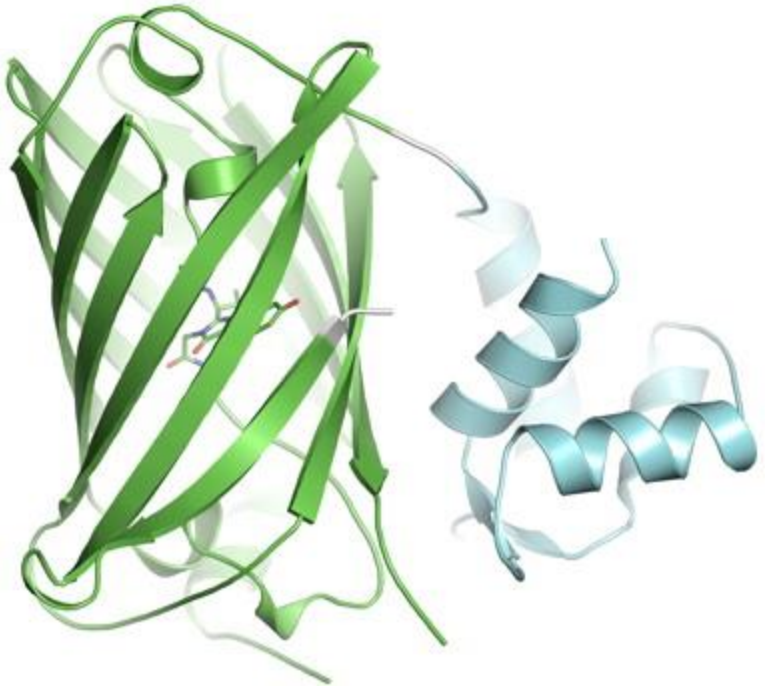




Tallini et al. PNAS 103: 4753 (2006); Akerboom et al. JBC 284: 6455 (2009)

Single domain sensors – GCaMP2





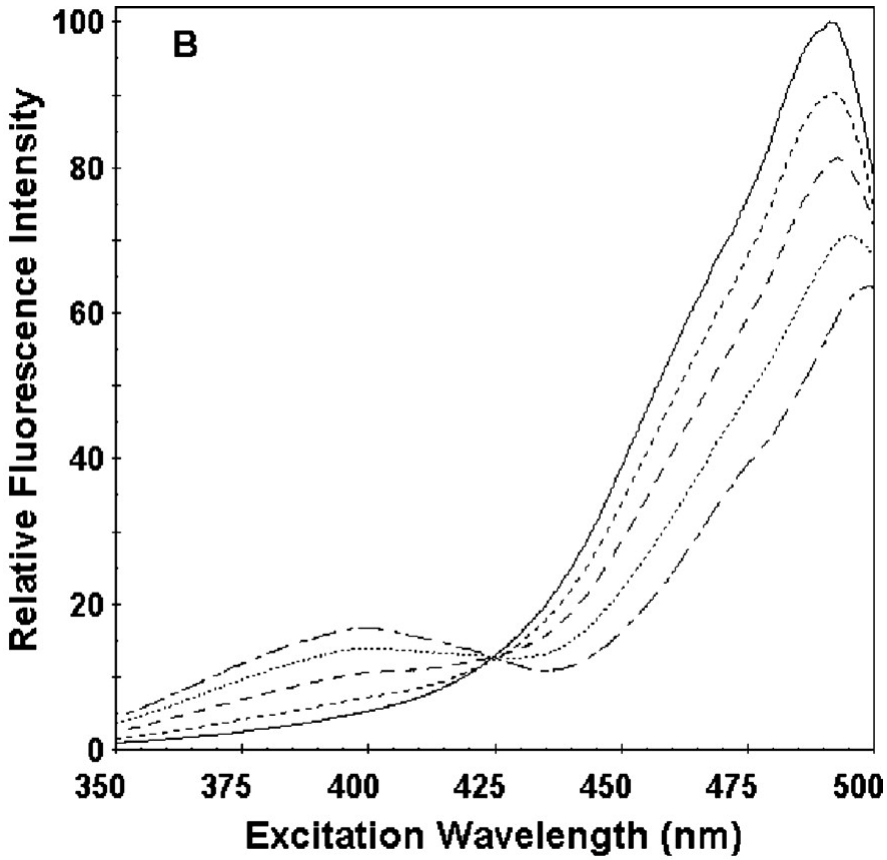
Ca^{2+} bound

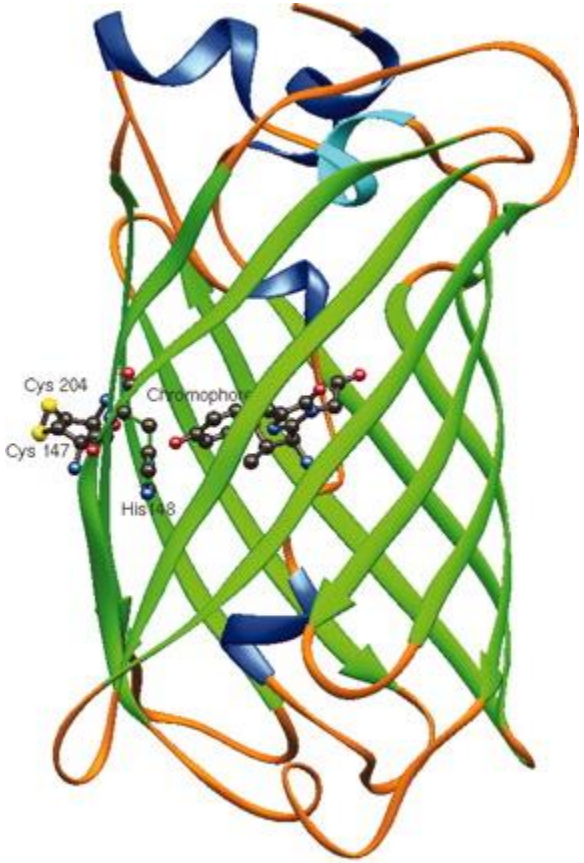
Ca^{2+} free

Tallini et al. PNAS 103: 4753 (2006); Akerboom et al. JBC 284: 6455 (2009)

Single domain sensors - roGFP

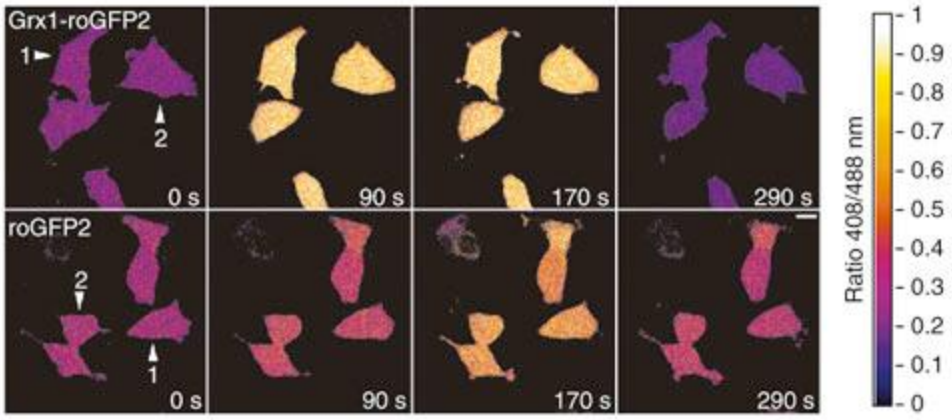
Ratiometric sensor

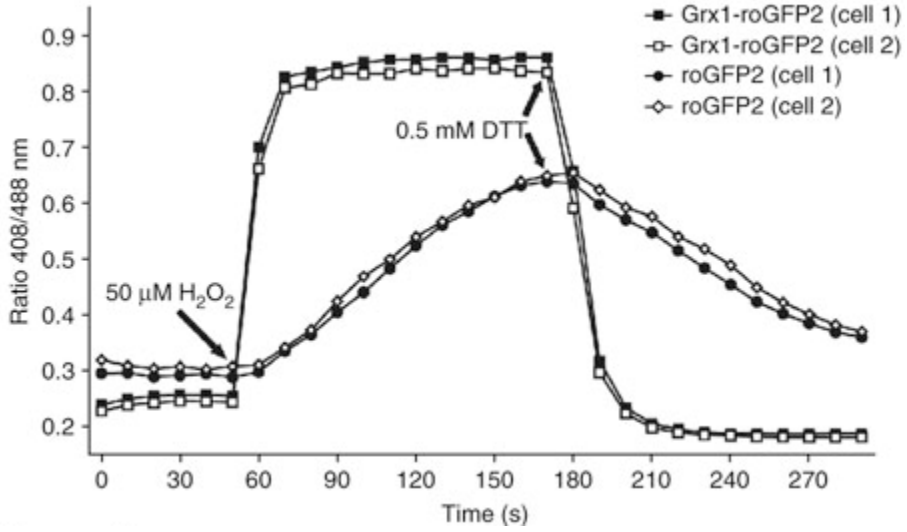




Hanson et al. J. Biol. Chem. 279: 13044 (2004)

Imaging glutathione redox potential *in vivo*



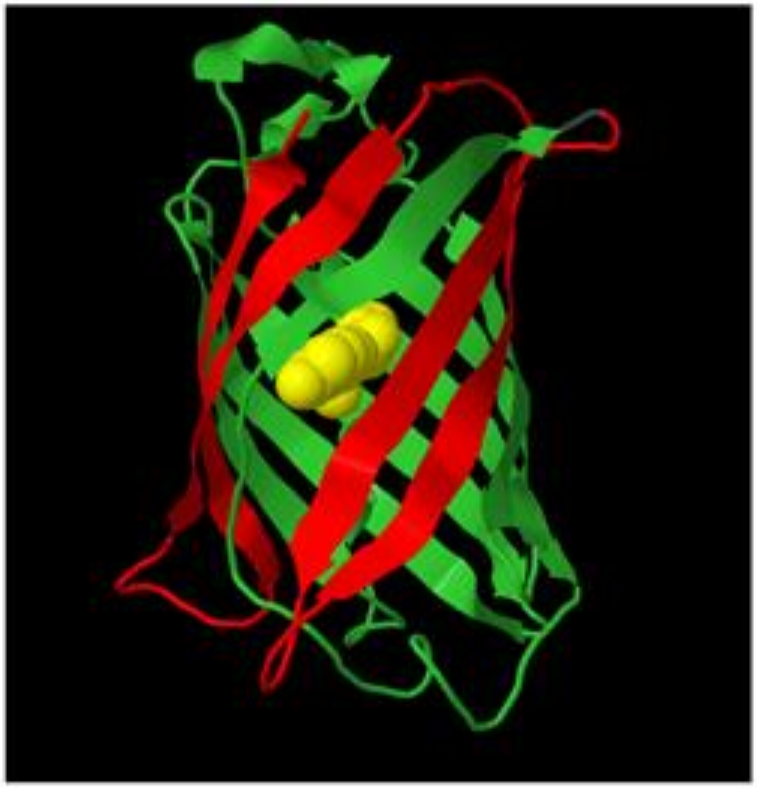


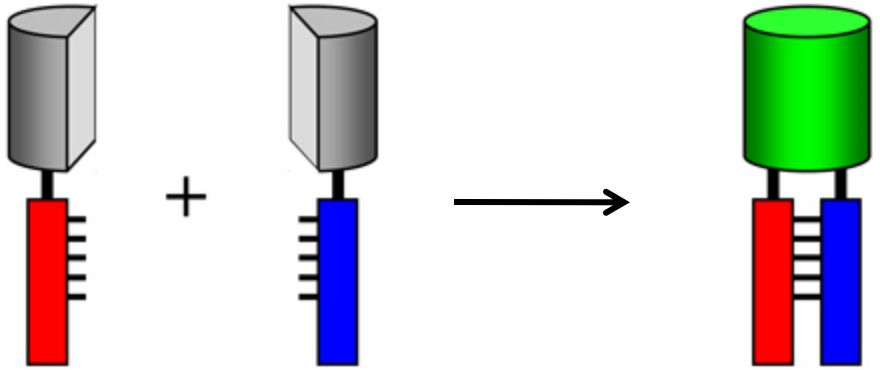
Coupling glutaredoxin-1 to roGFP makes it specifically sensitive to glutathione redox potential and accelerates its response

Gutscher et al. Nat Meth. 5: 553 (2008)

Bimolecular fluorescence complementation

BiFC (aka split GFP)





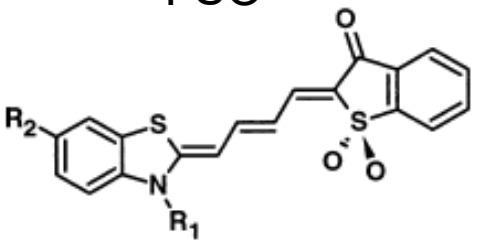
Has speed and reversibility issues, so most useful as a screening tool.

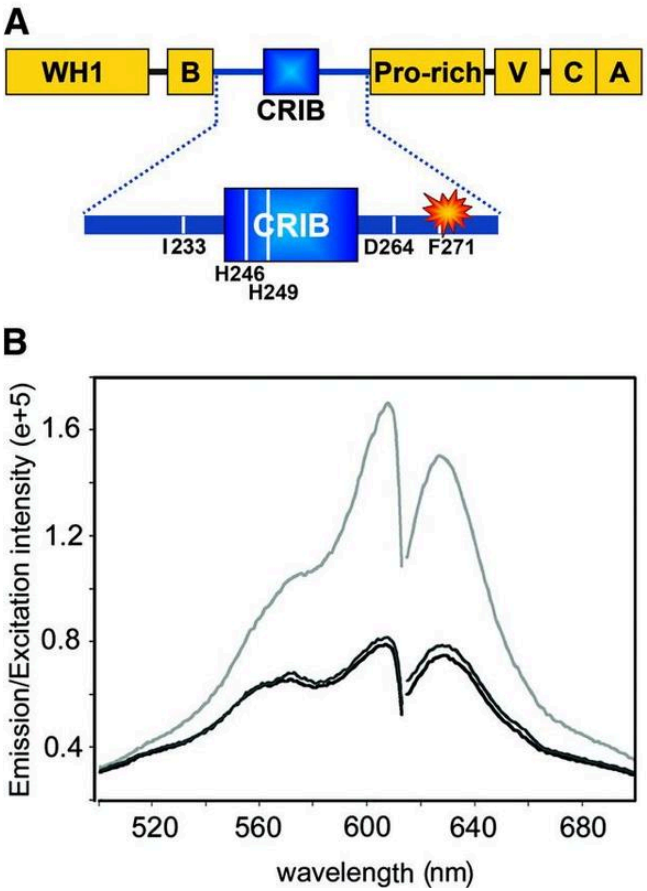
Kerppola, Annu. Rev. Biophys. 2008, 37: p.465

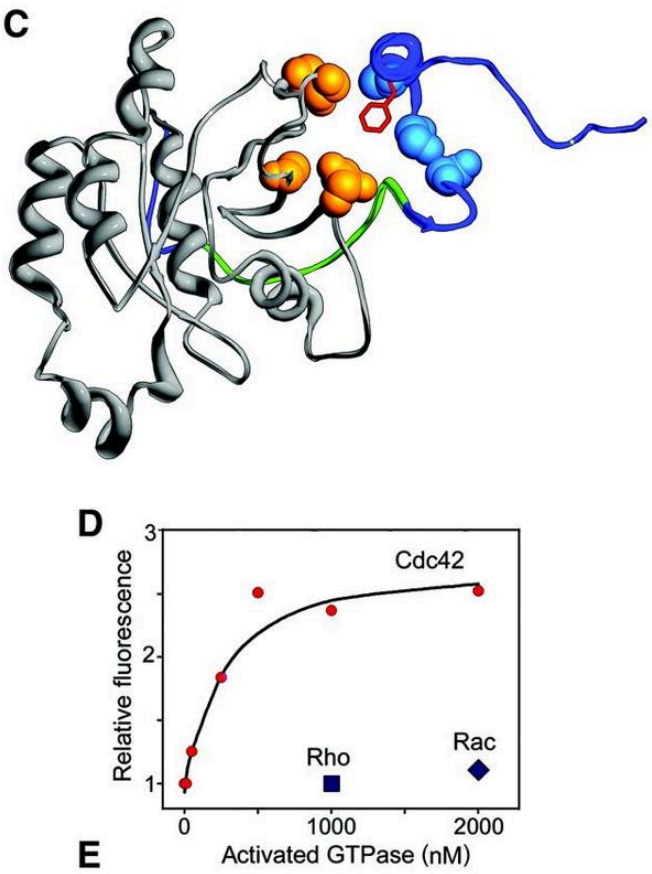
Environment-sensitive fluorophores

Cdc42-binding domain of WASP as sensor for active Cdc42

I-SO

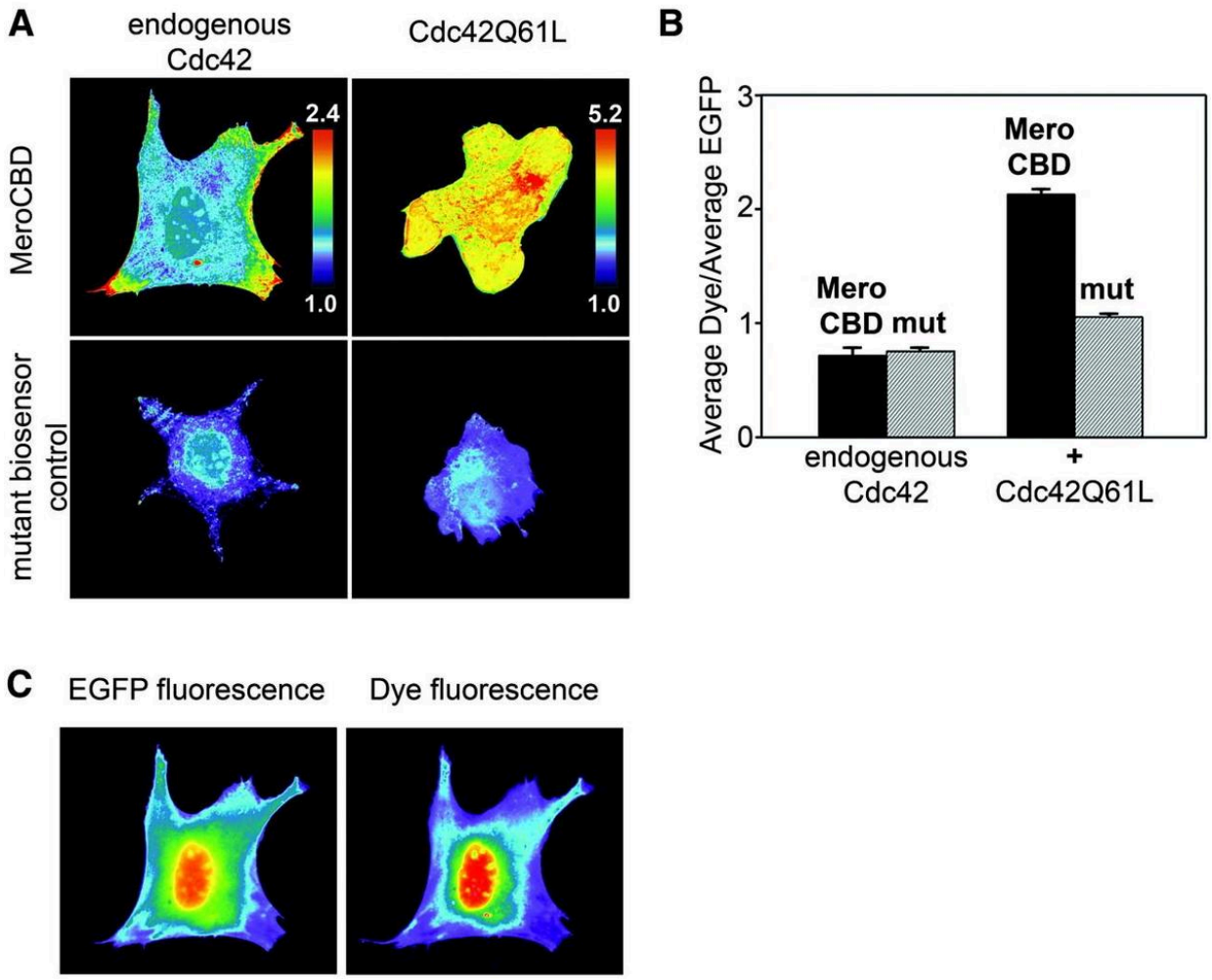






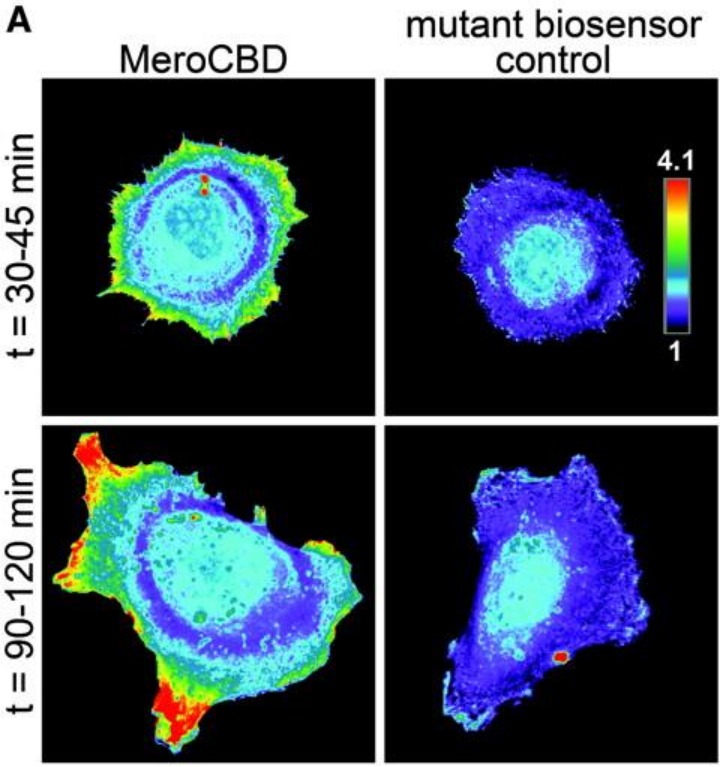
Nalbant et al. 2004, Science 305: 1615-1619

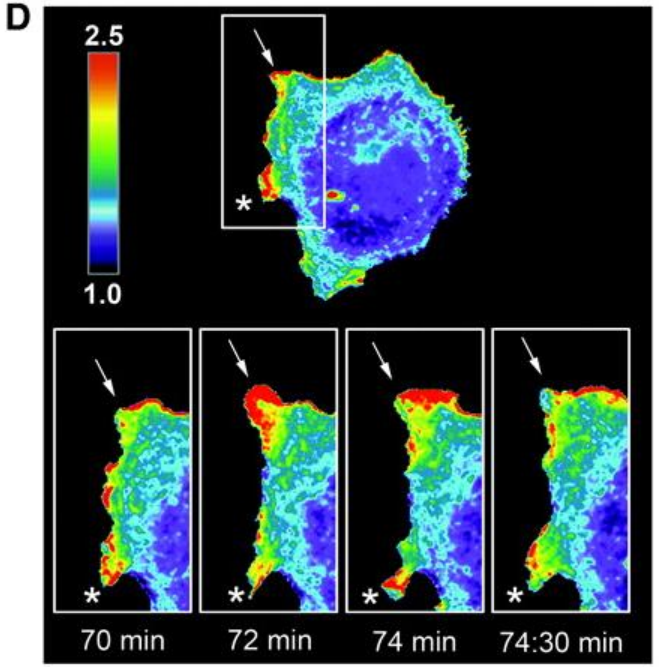
Imaging Cdc42 activation



Fuse sensor to GFP; I-SO/GFP ratio is proportional to degree bound

Monitoring Cdc42 activation





Additional reading

- Lakowicz, “Principles of Fluorescence Spectroscopy”, Chapters 13-15
- Gordon et al. 1998, Biophys. J. **74** p. 2702-2713
- Berney and Danuser 2003, Biophys. J. **84** p.3992-4010
- Zal and Gascoigne 2004, Biophys. J. **86** p 3923-3939
- FRET code is at: https://github.com/kthorn/fretproc