Fundamentals in Biophotonics

Optical tweezers

Optogentics –manipulating objects, mind, movement and more….+smFRET (catch up)

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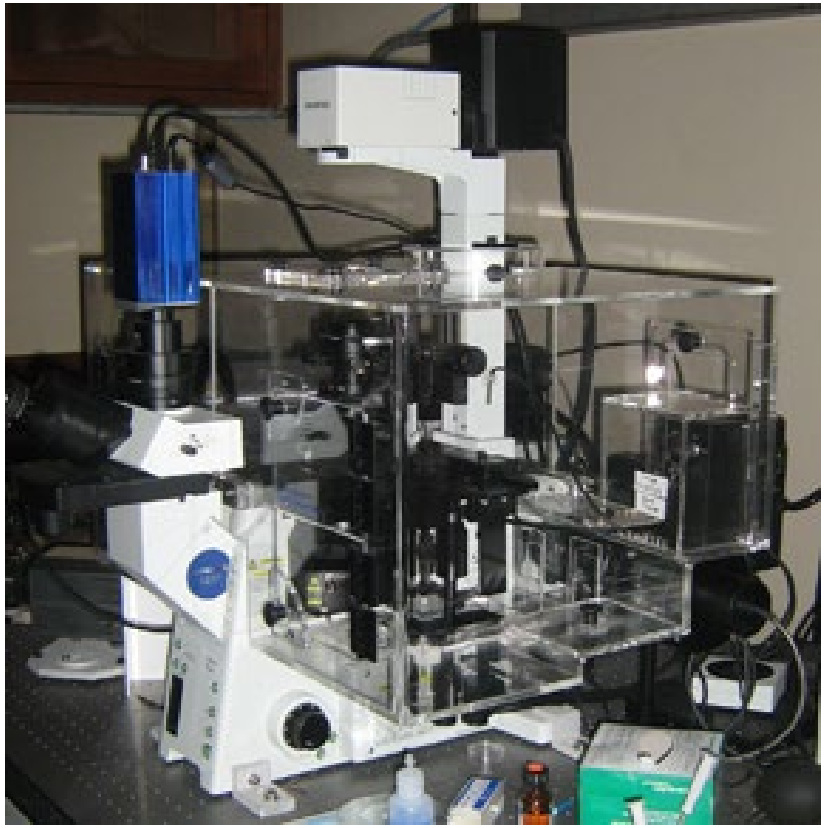
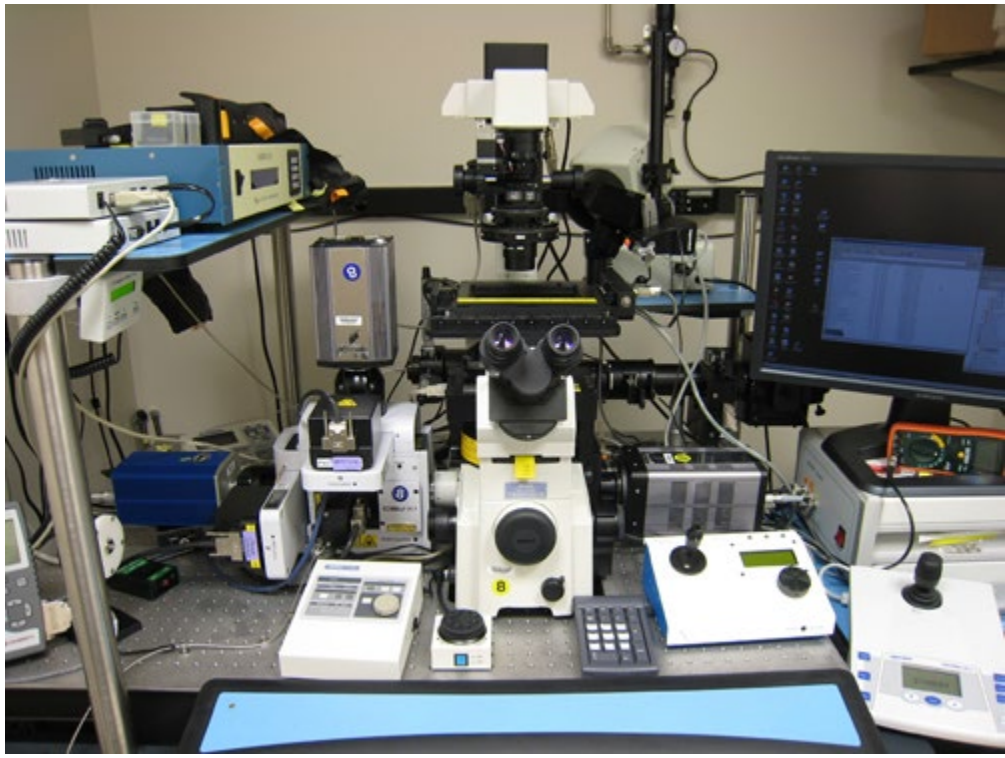
TIRF setup



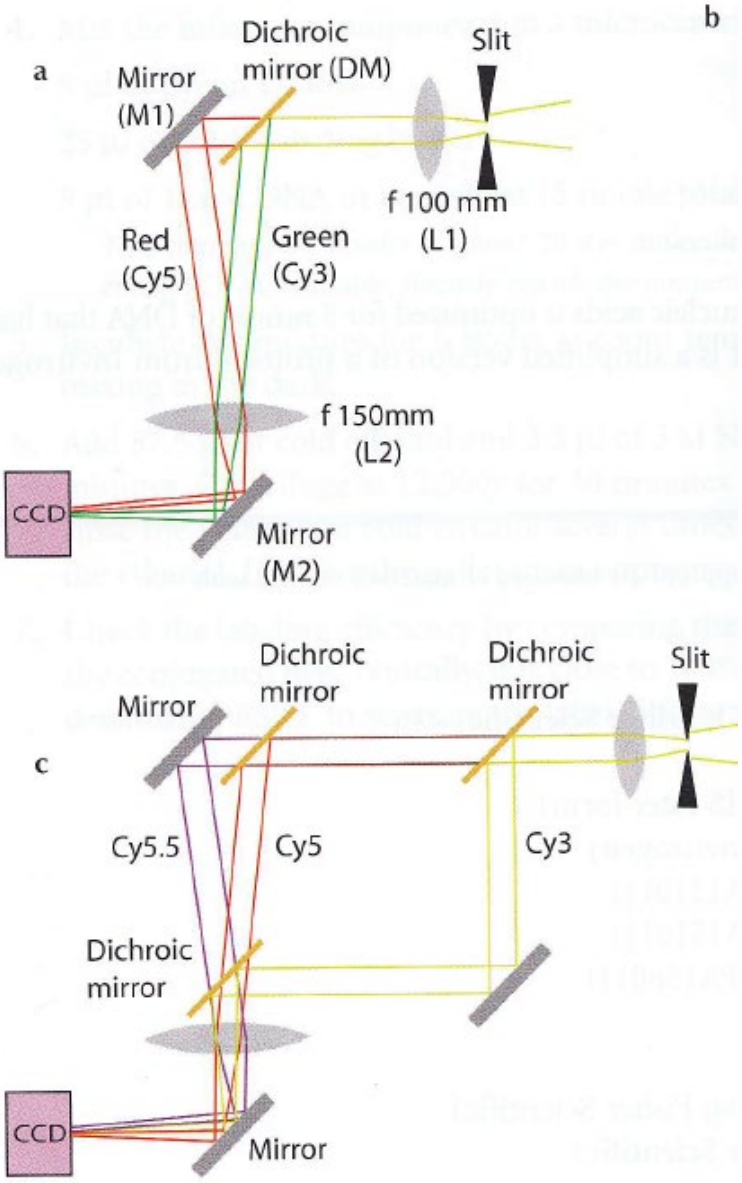


smFRET TIRF setup multicolor detection

- Build around Olympus IX 71 microscope
- Two color emission optics
  Collimated beam goes through a dichroic mirror and the donor and acceptor images are projected on one-half of the CCD camera each

- Three color setup

  donor color is separated first , same trick is employed to split acceptors signals



Typical DATA from smFRET

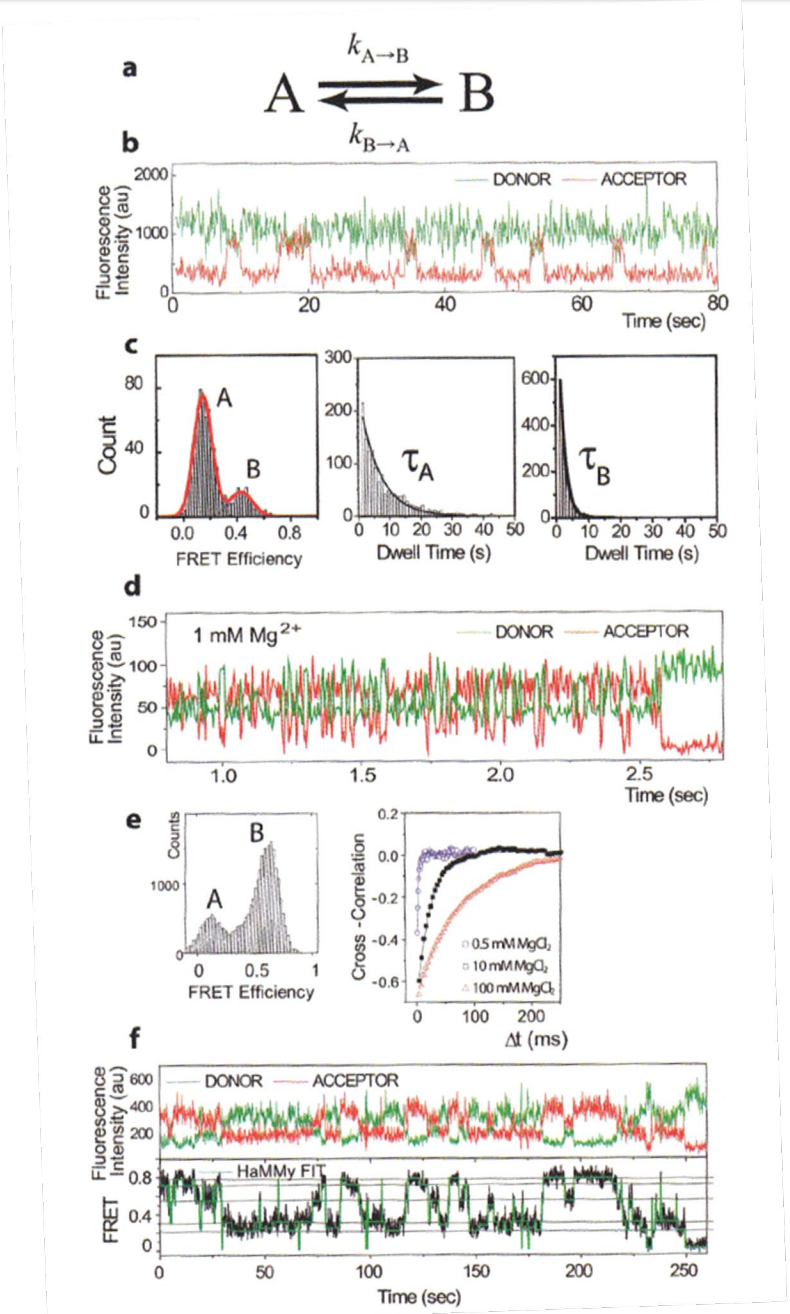
- FIRST find **FRET efficiency**

  apparent FRET efficiency through

$$E_{app} = \frac{I_A}{\left(I_A + I_D\right)}$$

What we actually measure are the raw intensities of the donor and acceptor channels I_{A0} and I_{D0} we need than to correct for leakage of donor signal to the acceptor channel

$$E_{app} = \frac{I_A^0 - \alpha \cdot I_D^0}{\left(I_A^0 + I_D^0 - \alpha \cdot I_D^0\right)}$$

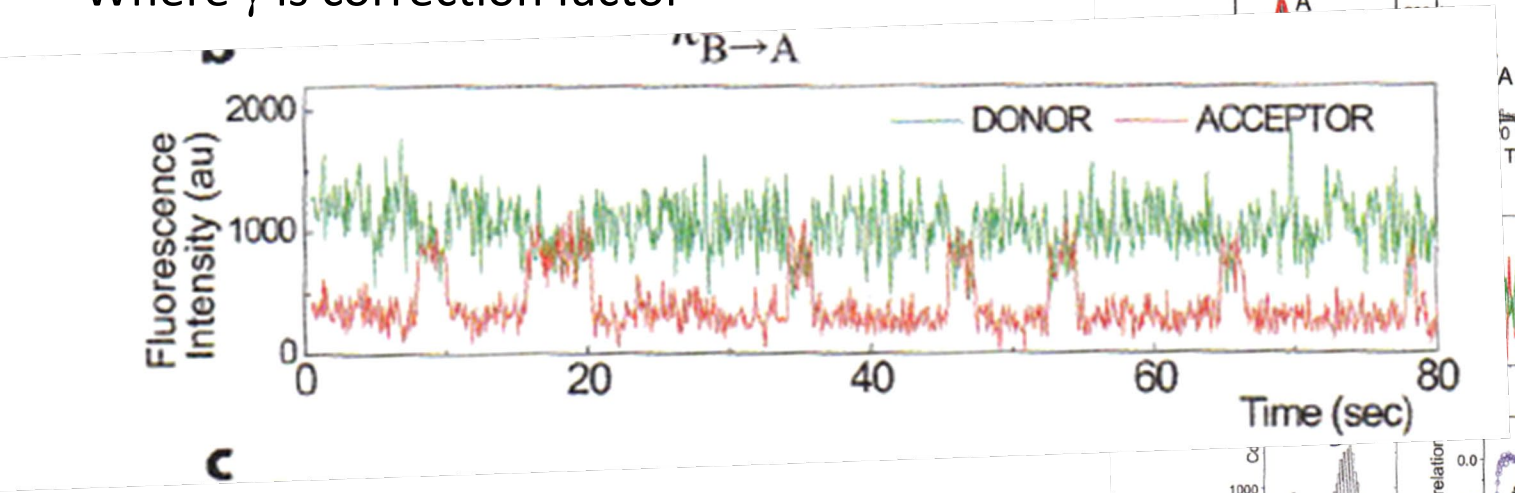


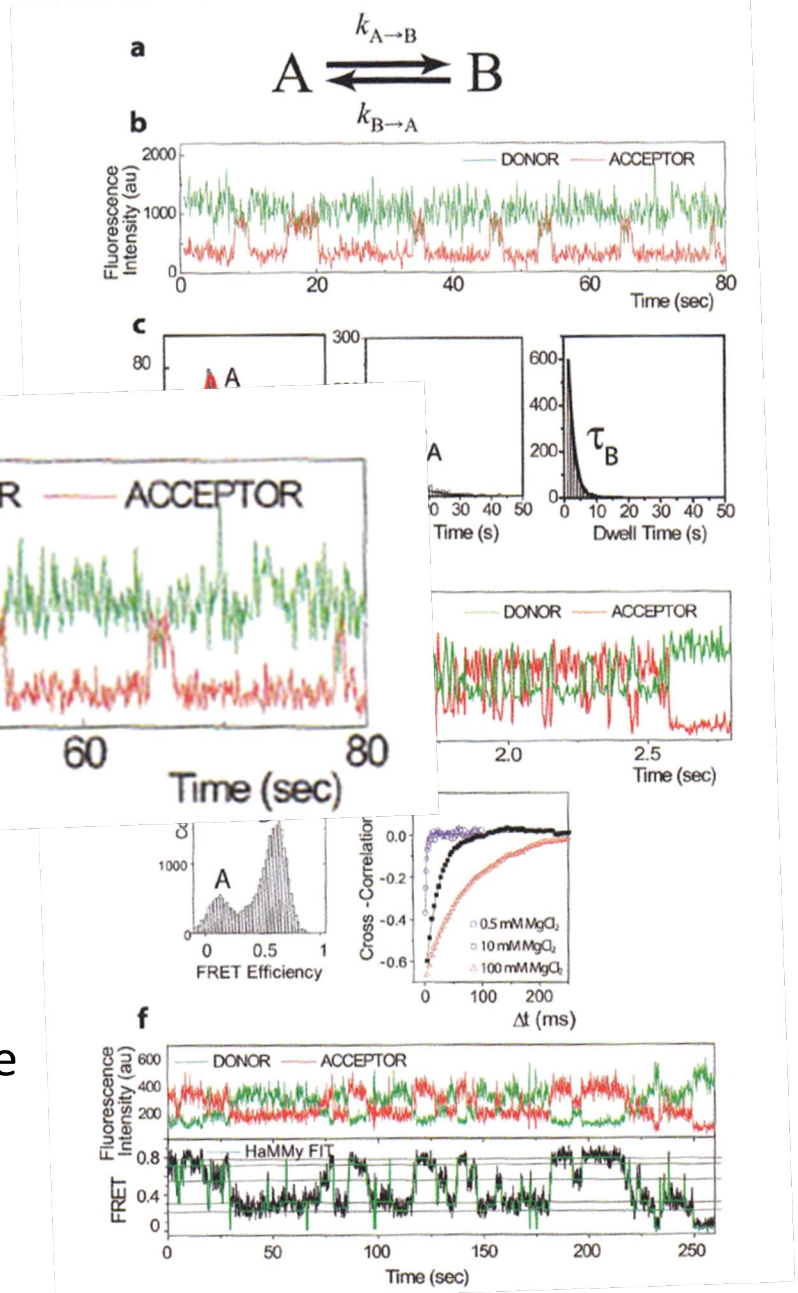
Typical DATA from smFRET

FRET efficiency

$$E = \frac{I_A}{\left(I_D + \gamma \cdot I_D\right)}$$

Where γ is correction factor





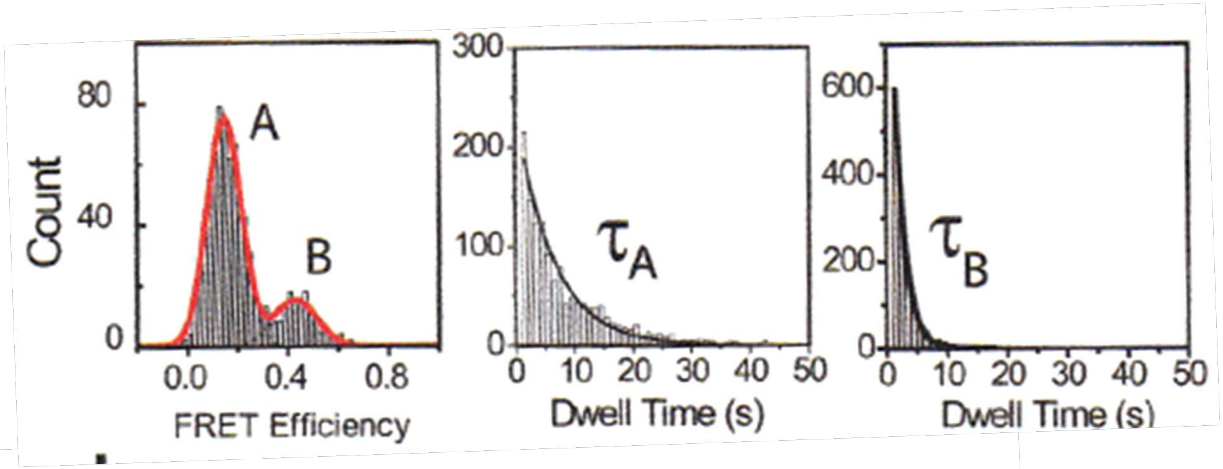
Easy to notice two states

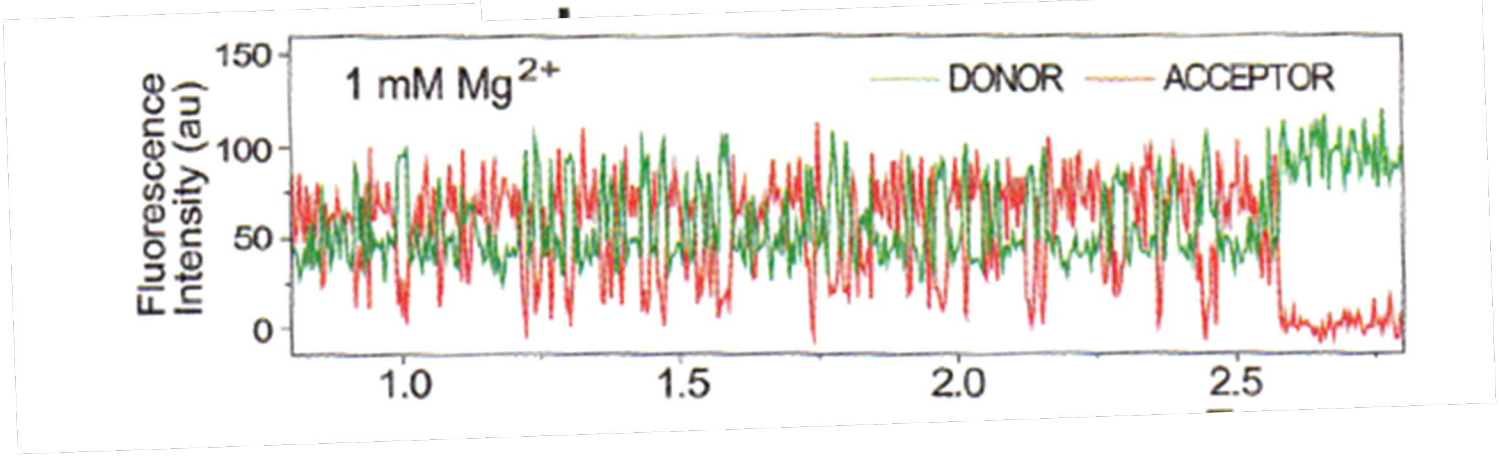
Apply automated threshold analysis

We fit the dwell time histogram to of each state to obtain lifetimes τ_A and τ_B

Typical DATA from smFRET

- Dwell time



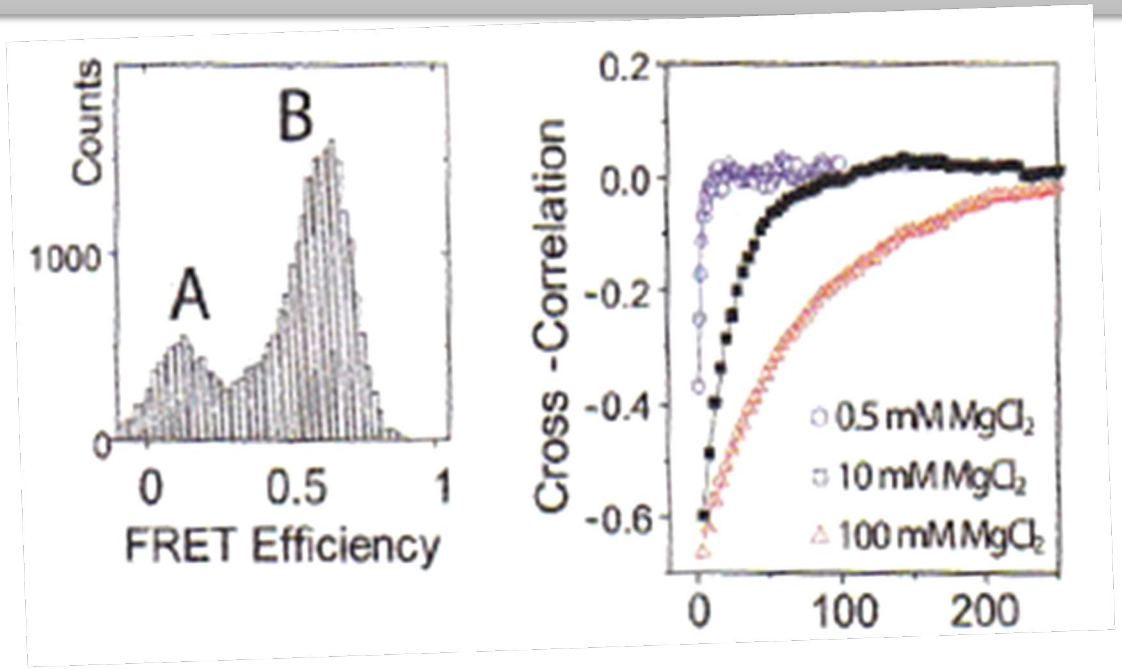


If the timescale of the fluctuations is **very fast** use **cross correlations** this type of analysis can tell us if donor and acceptor are fluctuating in anticorrelated manner

$$CC(\tau) = \int I_D(t) I_A(t-\tau) dt$$

$$-Ae^{-(k_{A\to B}+k_{B\to A})\tau}$$

Typical DATA from smFRET



$$-Ae^{-(k_{A\to B}+k_{B\to A})\tau}$$

From the cross correlation curve fit we can obtain the sum of two rates and if FRET efficiency histogram is clean enough in combination we can determine both

$$k_{A\to B} \quad \text{and} \quad k_{B\to A}$$

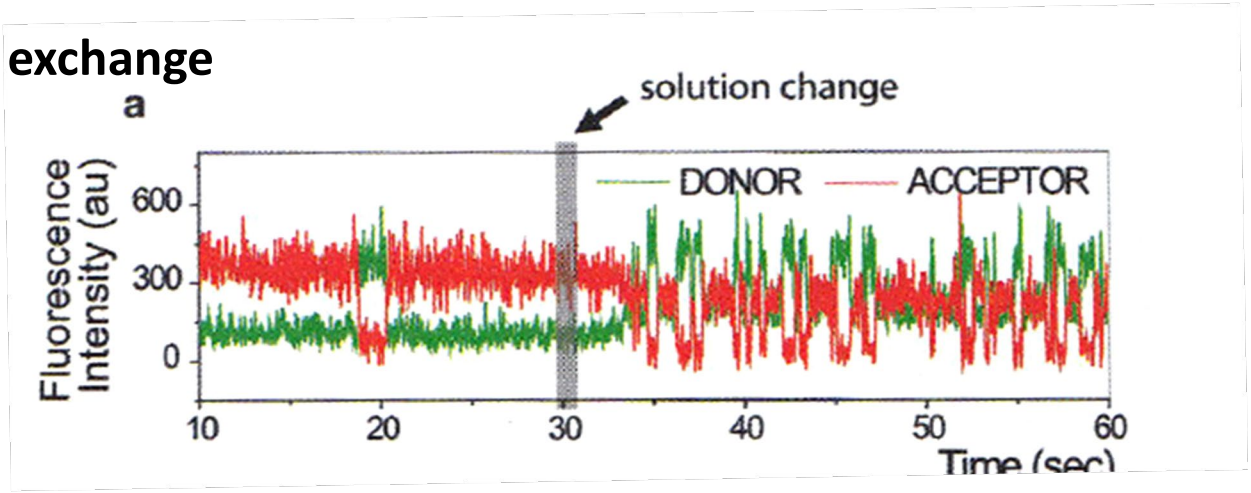
For more than one state more evolved algorithms are need than just simple treshholding And cross correlation analysis – **USE HIDDEN MARKOV MODELING**

Hidden Markov Modeling tools

- Rule inspect your traces if states are well separated no need for Hidden Markov modeling
- For multiple states or unclean data use it ! HaMMy is tool developed in Ha lab
- http://bio.physics.illinois.edu/HaMMy.html

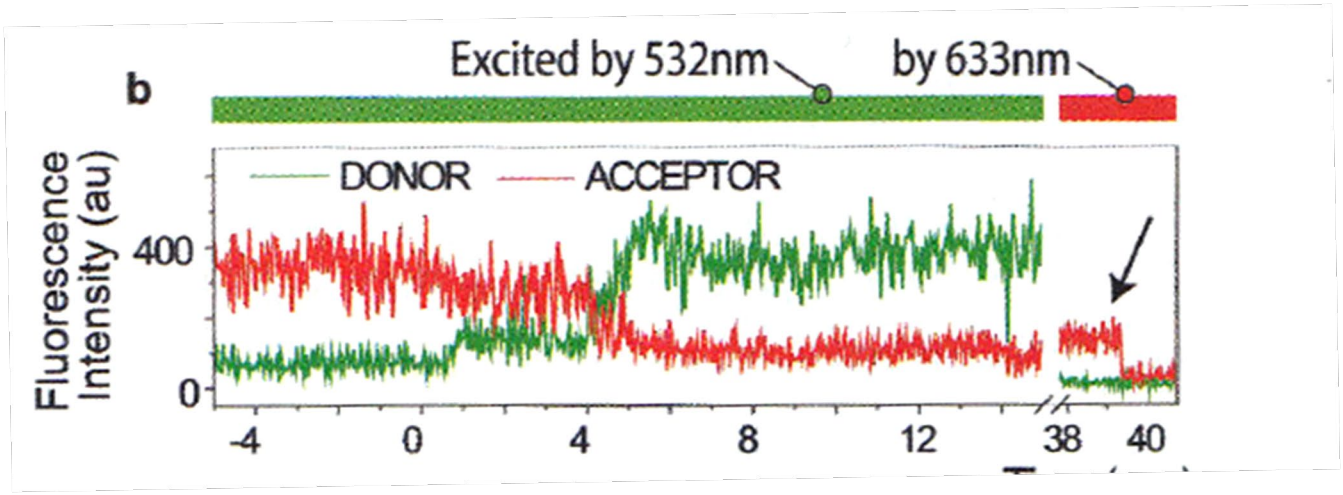
Other types of traces

- **Solution exchange**



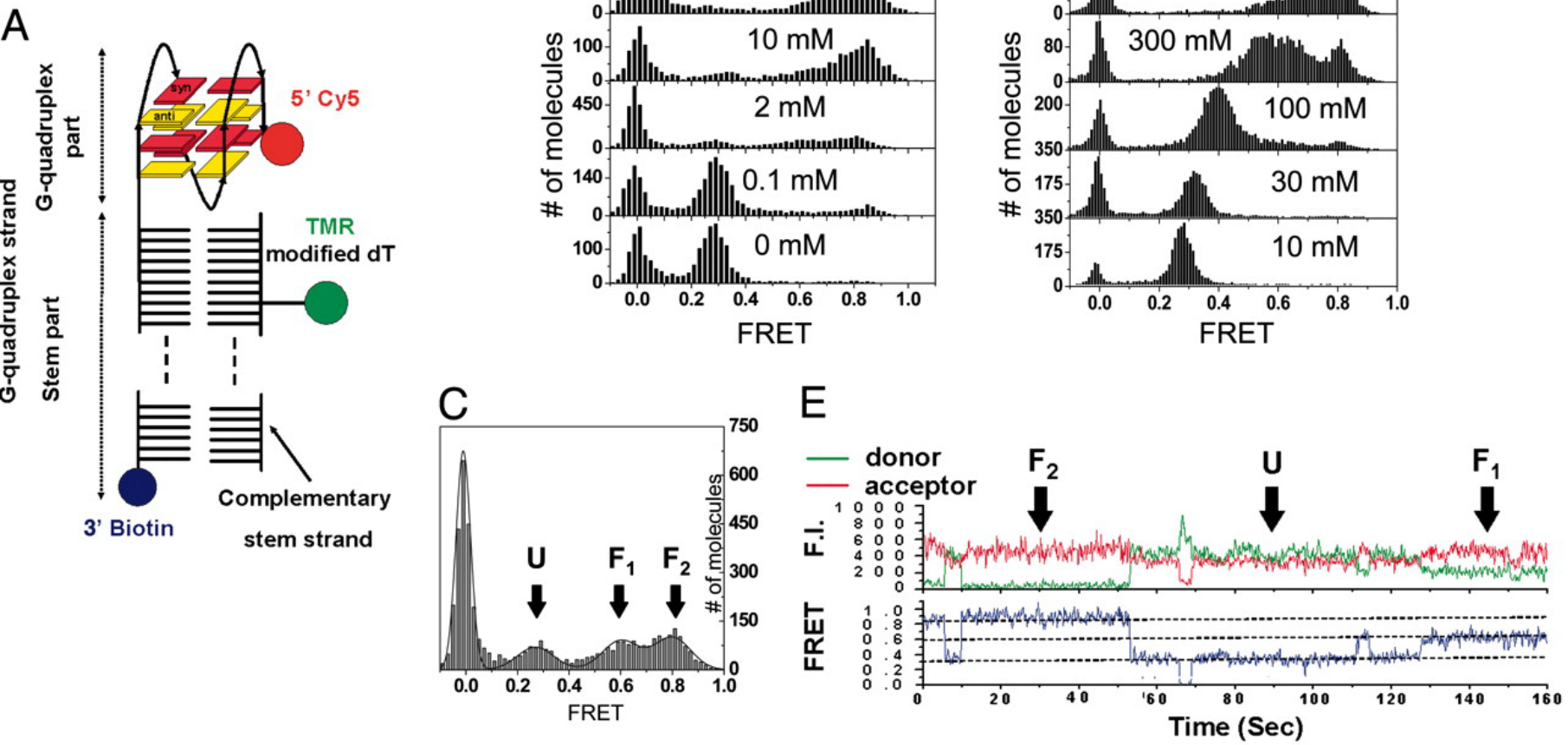
- **Disappearance of signal**

When FRET signal drops to zero to be sure that this comes form the relative dye positioning and not simply form photo bleaching of the donor direct acceptor excitation uses to confirm the data



Other types of traces

- **FRET histograms**

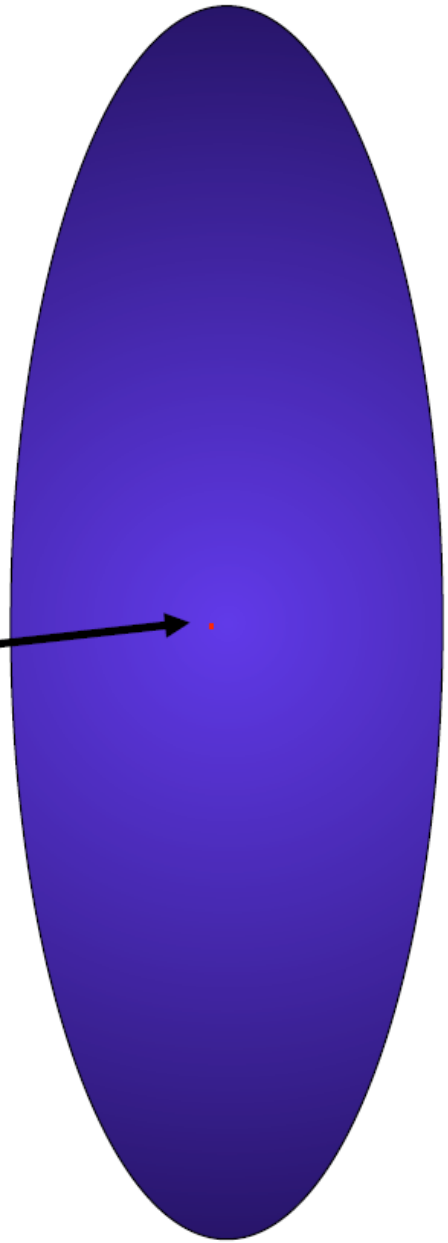


Fluorescence Techniques to measure molecular binding

- Fluorescence Resonance Energy Transfer – FRET
- Good for showing interactions between tagged molecules. It can be used to measure rearrangements within or between proteins or concentrations of ions such as H+ or Ca2+.

- Fluorescence Correlation Spectroscopy – FCS
- This allows the measurement of diffusion rates and hence molecular/ complex size. Multiple channels allow measurements of interactions.

Why colocalisation isn't enough

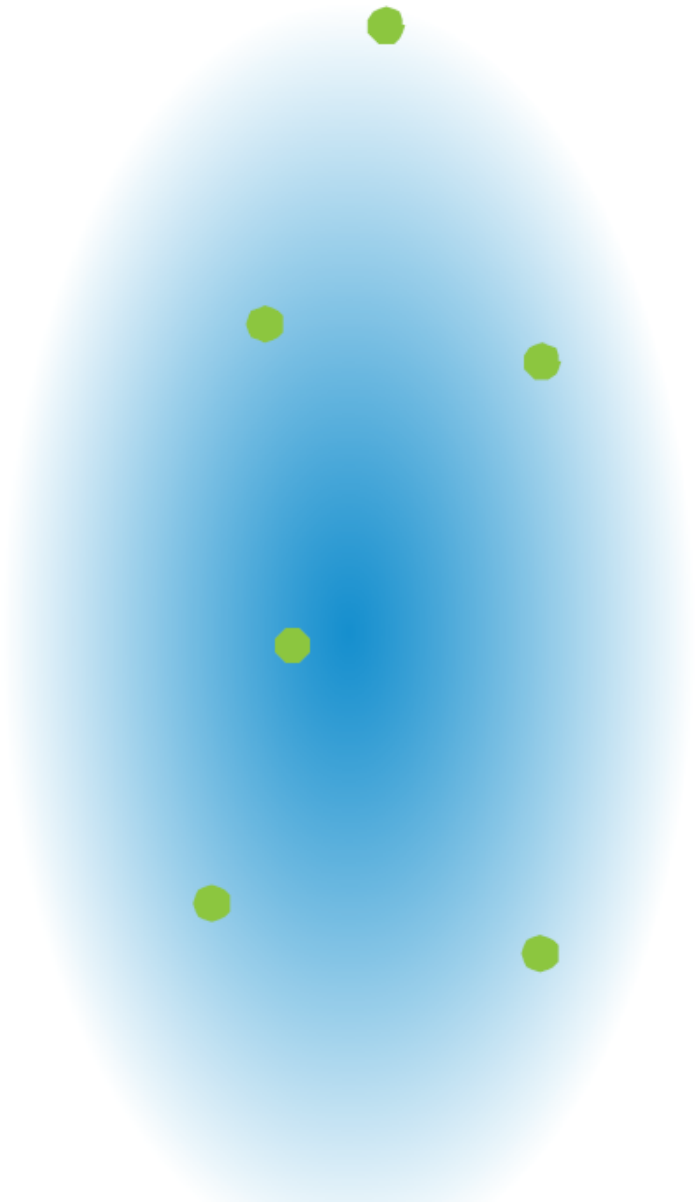


Confocal volume ~
250x250x750 nm

GFP ~ 3x3x4 nm

Fractional volume ~ 10^6

What is Fluorescence Correlation Spectroscopy?



Take a confocal volume with a few molecules in it.

What causes fluctuations in intensity?

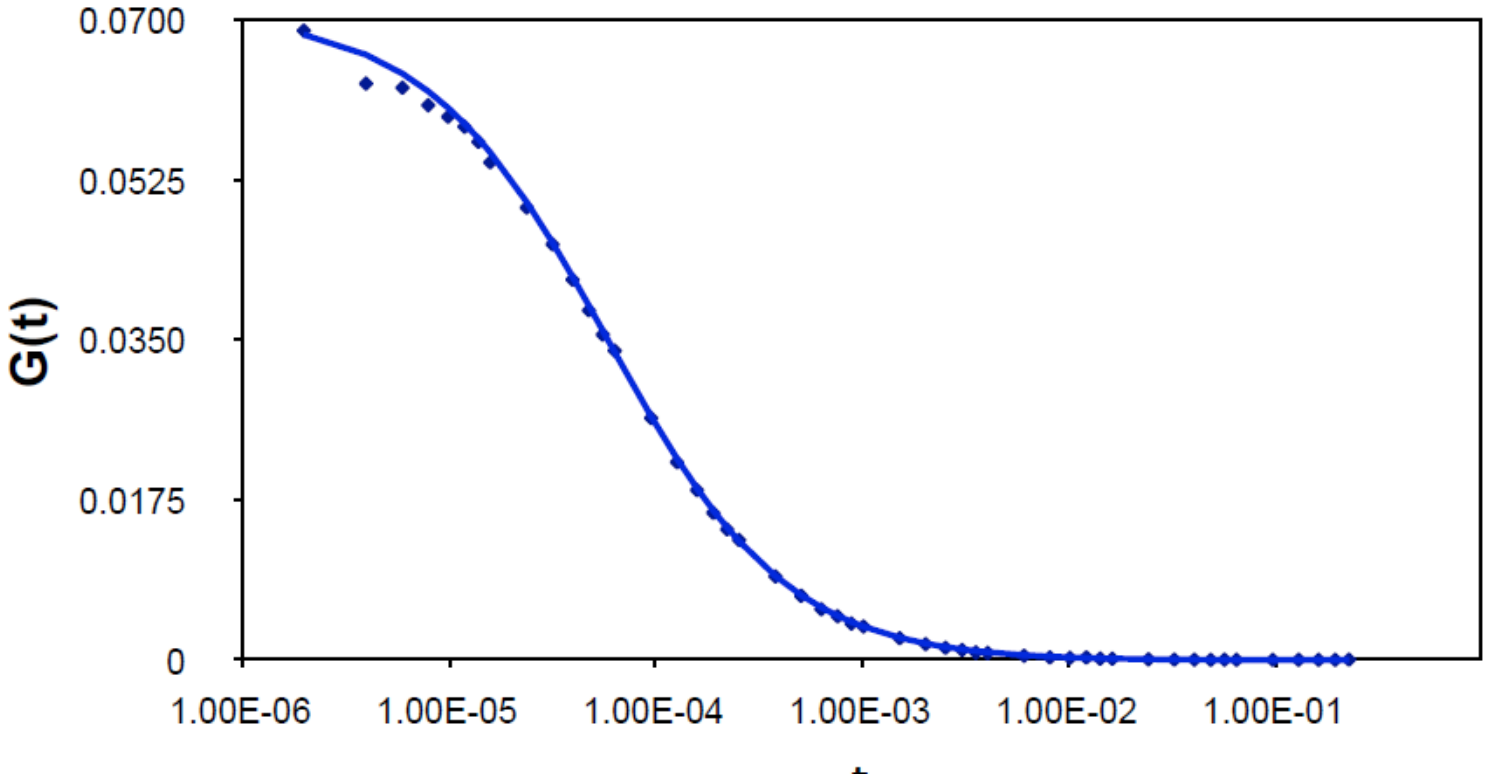
1) Poison noise.

2) Molecules entering or leaving the volume.

How do you do FCS?

- Look at the intensity of fluorescence from a small volume < 1 femto-litre (10^{-15}l)
- Calculate the auto-correlation function :- how similar is the intensity now to some time in the future?
- Fit a theoretical model to measure various parameters - concentration, diffusion rate and flow

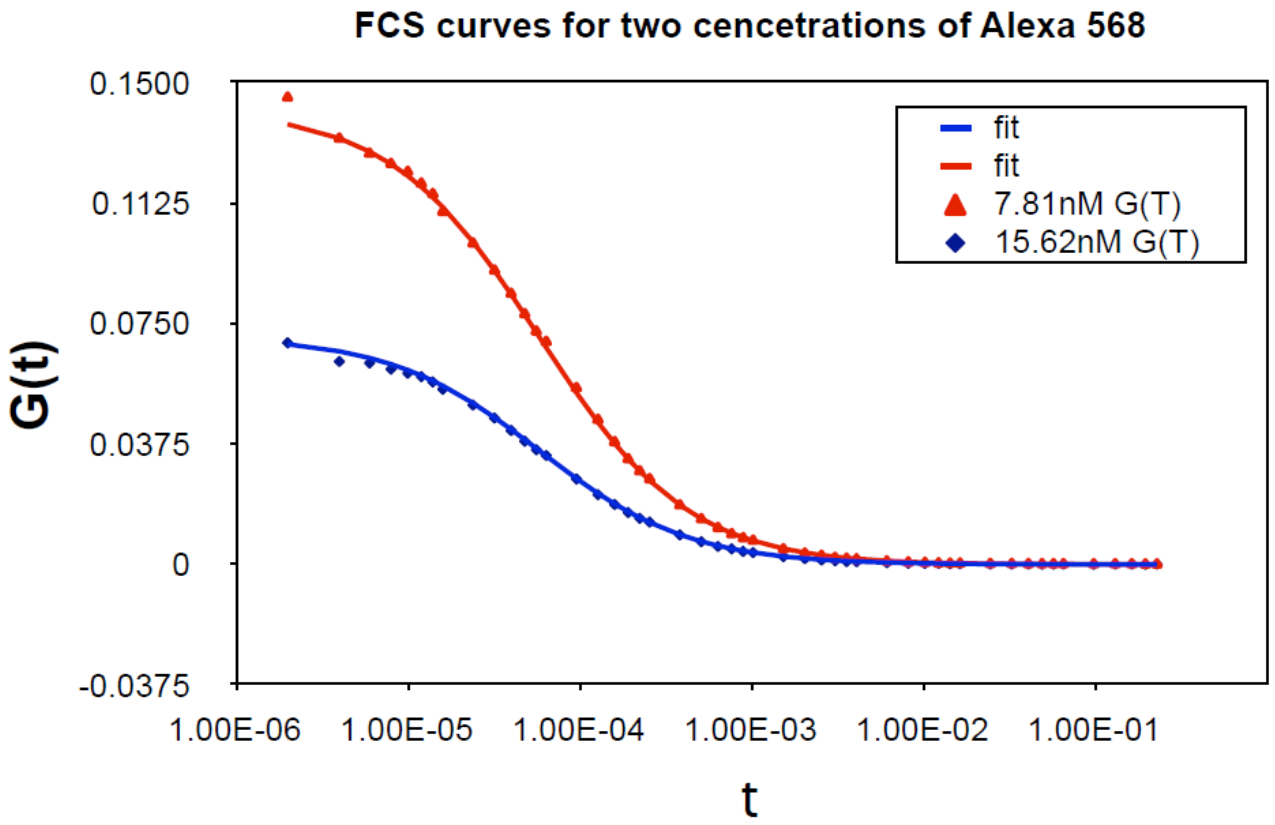
Typical FCS autocorrelation curve



What Does FCS tell us?

- Diffusion rate - hence complex size – however only good for order of magnitude, D ∝ m1/3.
- Tells us the number of fluorescent objects in volume and hence concentration.
- Can be used to measure flow.

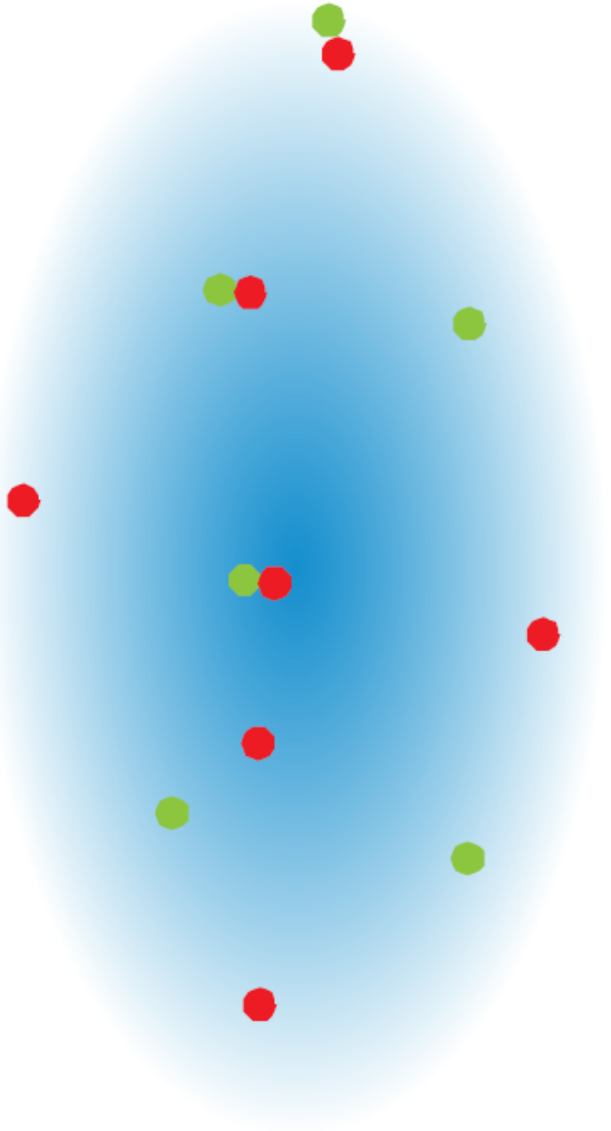
FCS curve amplitude but not shape depends on concentration



Limitations of FCS

- Low sensitivity to changes in mass of diffusing particles, $D \propto m^{1/3}$.
- Small range of useful concentrations, need ~1-100 molecules in your volume, typically nM concentrations.
- Only works on diffusing particles.

Fluorescence Cross-Correlation Spectroscopy FCCS



Take a confocal volume with a few molecules in it.

How do fluctuations in the two channels vary?

1) Poison noise.

2) Single colour molecules entering or leaving the volume.

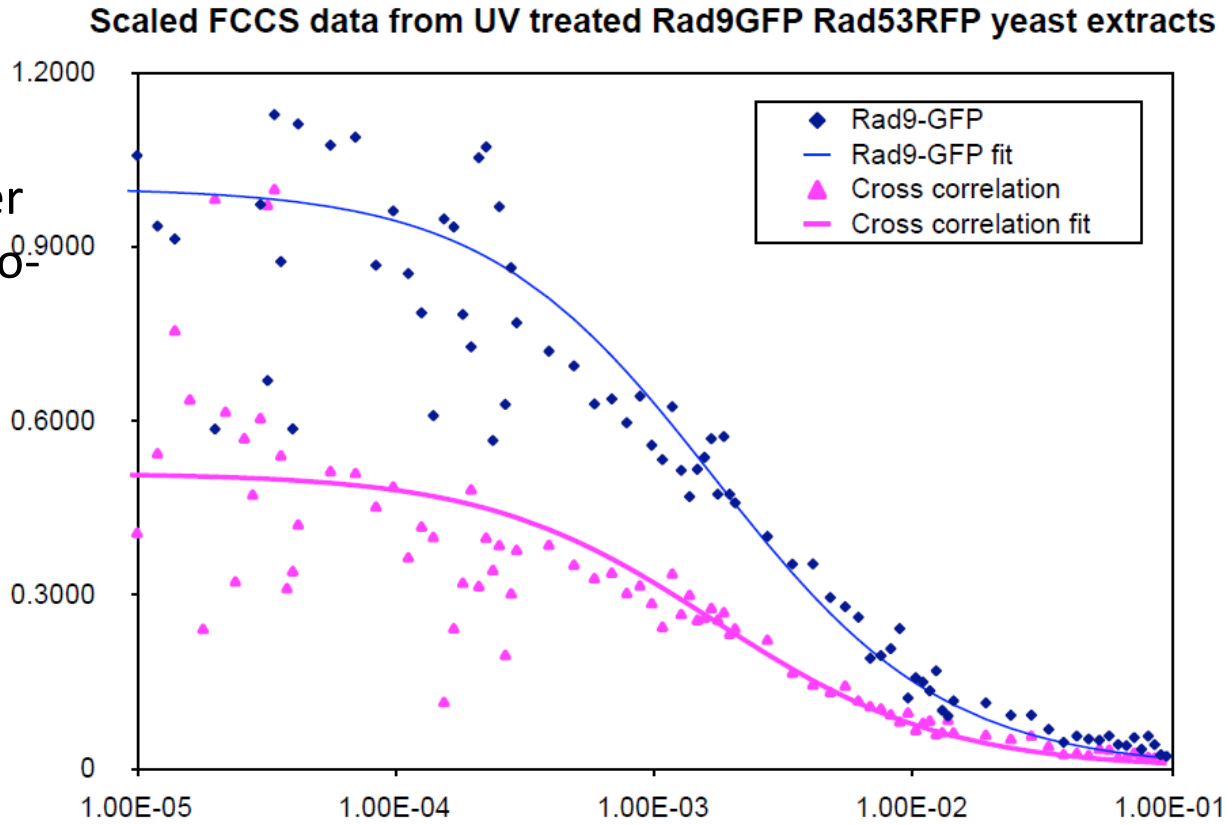
3) Dual colour molecules entering or leaving the volume.

FCCS

- Like FCS, but with two different fluorescent labels at once. By looking at the cross-correlation, does the red signal go up when the green signal goes up etc…, we can measure interacting fractions.

The interacting fraction of each species with the other is the ratio of the auto- to cross-correlation curves

Alignement critical - max interaction < 1.



Ratio of Rad9 auto-correlation to cross-correlation ~0.5

Photobleaching Techniques

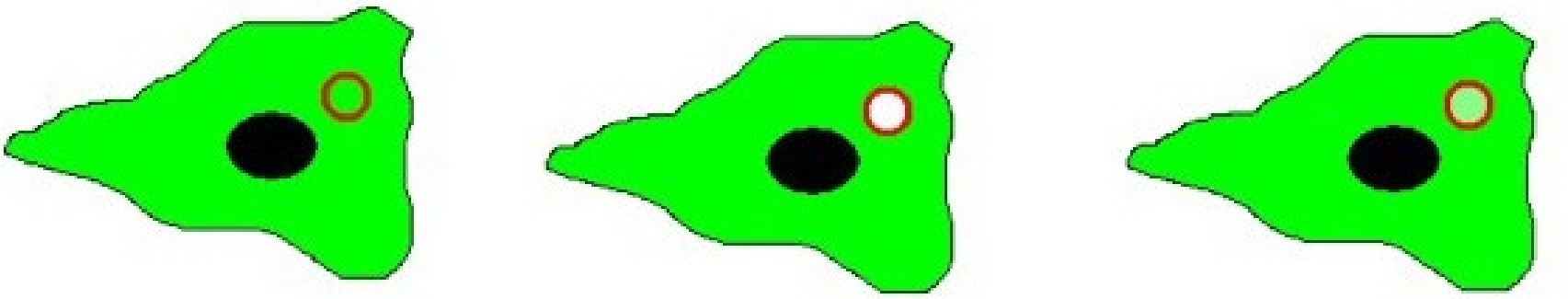
- Illumination with bright light will lead to photobleaching of a fluorophore. Once photobleached the fluorophore is no longer fluorescent.

- Two related techniques of Fluorescence Recovery After Photobleaching (**FRAP**) and Fluorescence Loss in Photobleaching **FLIP**) use photobleaching to measure molecular dynamics.

- Laser Scanning Confocal microscopes are good for these photobleaching techniques due to their high intensity laser light sources and their flexible illumination control. They allow selective bleaching of arbitrary regions within the filed of view and can then rapidly switch between bleaching and imaging. However, reduced sensitivity and slow speed make a widefield microscope better if it has the ability to photobleach Illumination with bright light will lead to photobleaching of a fluorophore. Once photobleached the fluorophore is no longer fluorescent.

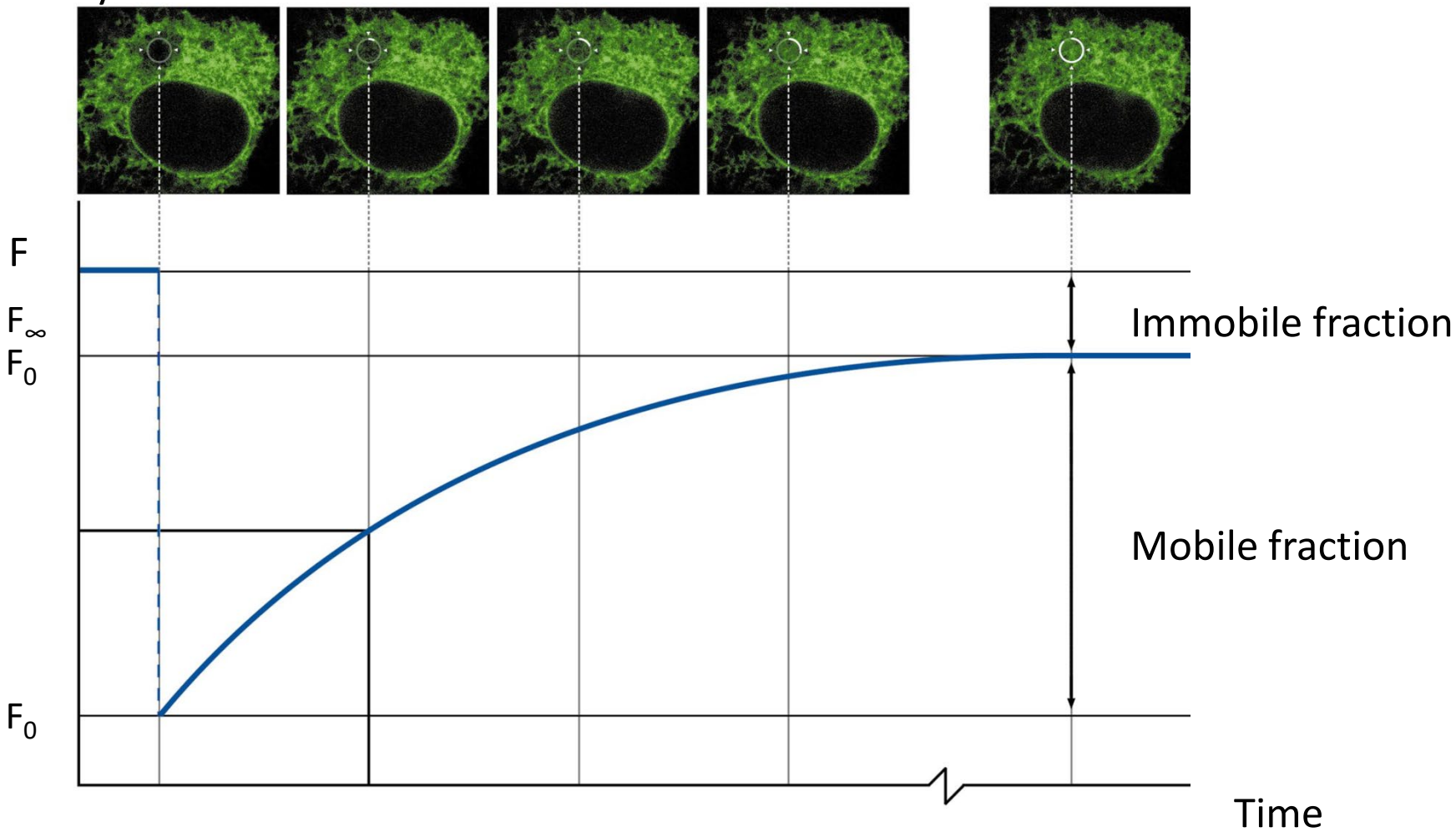
Fluorescence Recovery After Photobleaching -FRAP

- A region is rapidly bleached and the rate at which fluorescence fills
- the bleach region is determined by the diffusion of unbleached molecules.
- Small objects – fast diffusion
- Large objects – slow diffusion

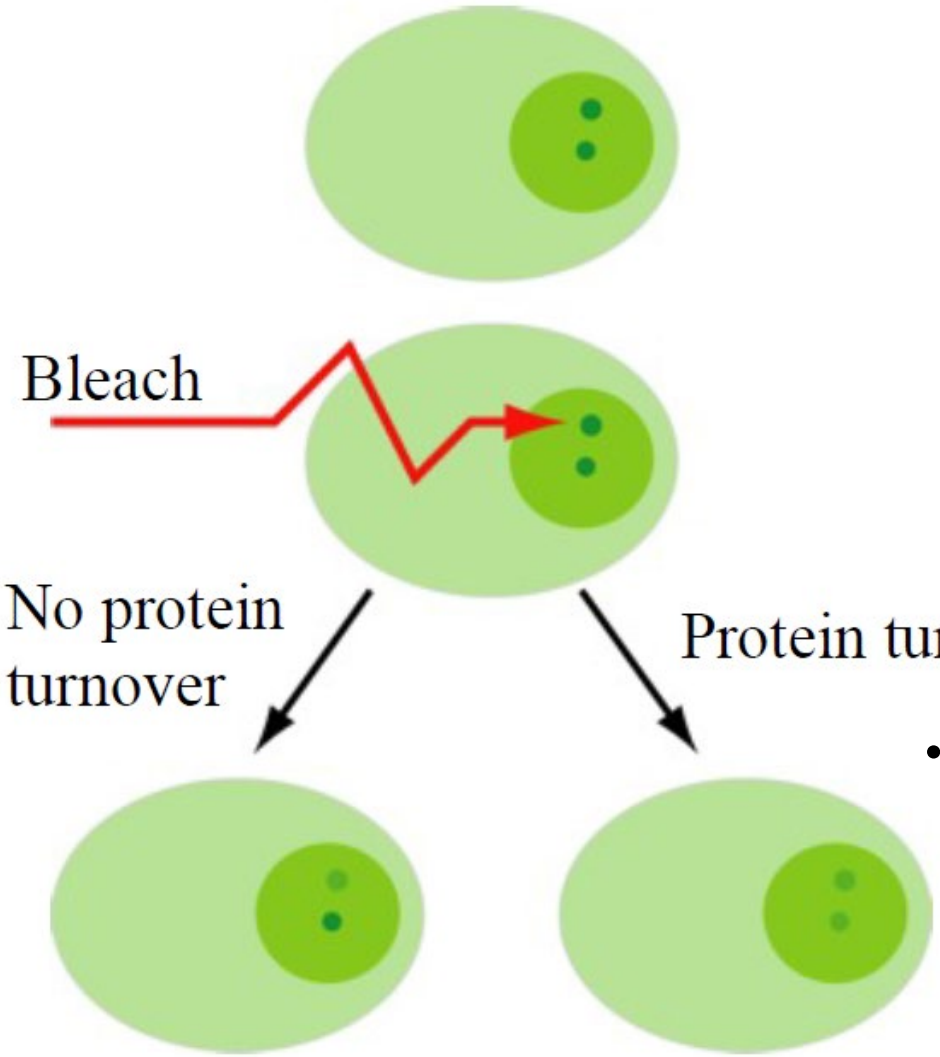


FRAP

- FRAP can be used to address a number of questions about protein localization, dynamics and interactions with other components in living cells. The mobility of molecules within specific cell compartments has been visualized, as has membrane continuity.



FRAP



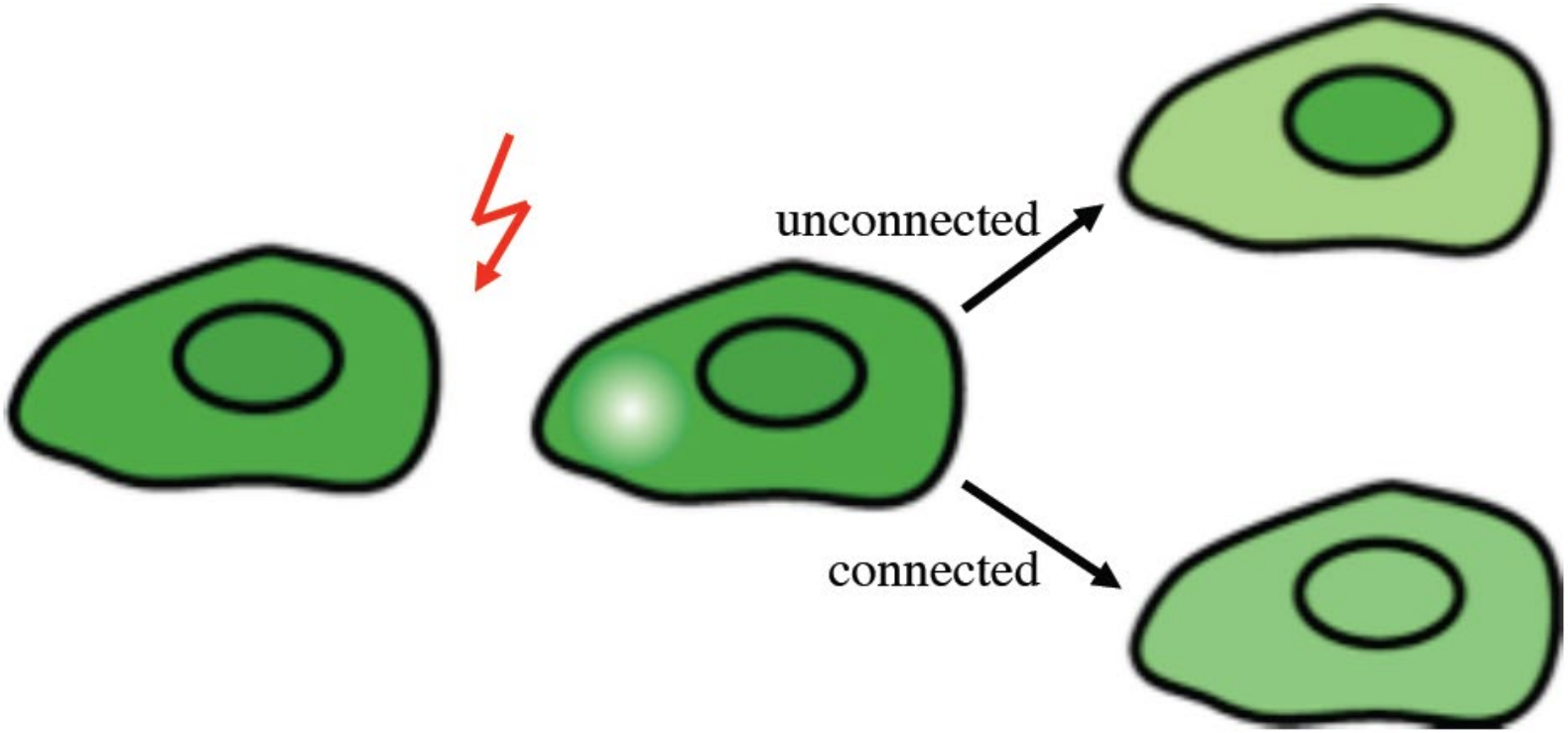
- When a region in the fluorescent area (here the endoplasmic reticulum) is bleached at time t0 the fluorescence decreases from the initial fluorescence Fi to F_0. The fluorescence recovers over time by diffusion until it has fully recovered (F_∞). The characteristic diffusion time tD indicates the time at which half of the fluorescence has recovered.

- The mobile fraction can be calculated by comparing the fluorescence in the bleached region after full recovery (F_∞) with that before bleaching (F_i) and just after bleaching (F_0).

Fluorescence Loss in Photobleaching (FLIP)

- Related to FRAP, a small region is repeatedly bleached and the loss of fluorescence in another region is measured.

- Useful to show connectivity of compartments or for measuring turnover between compartments.

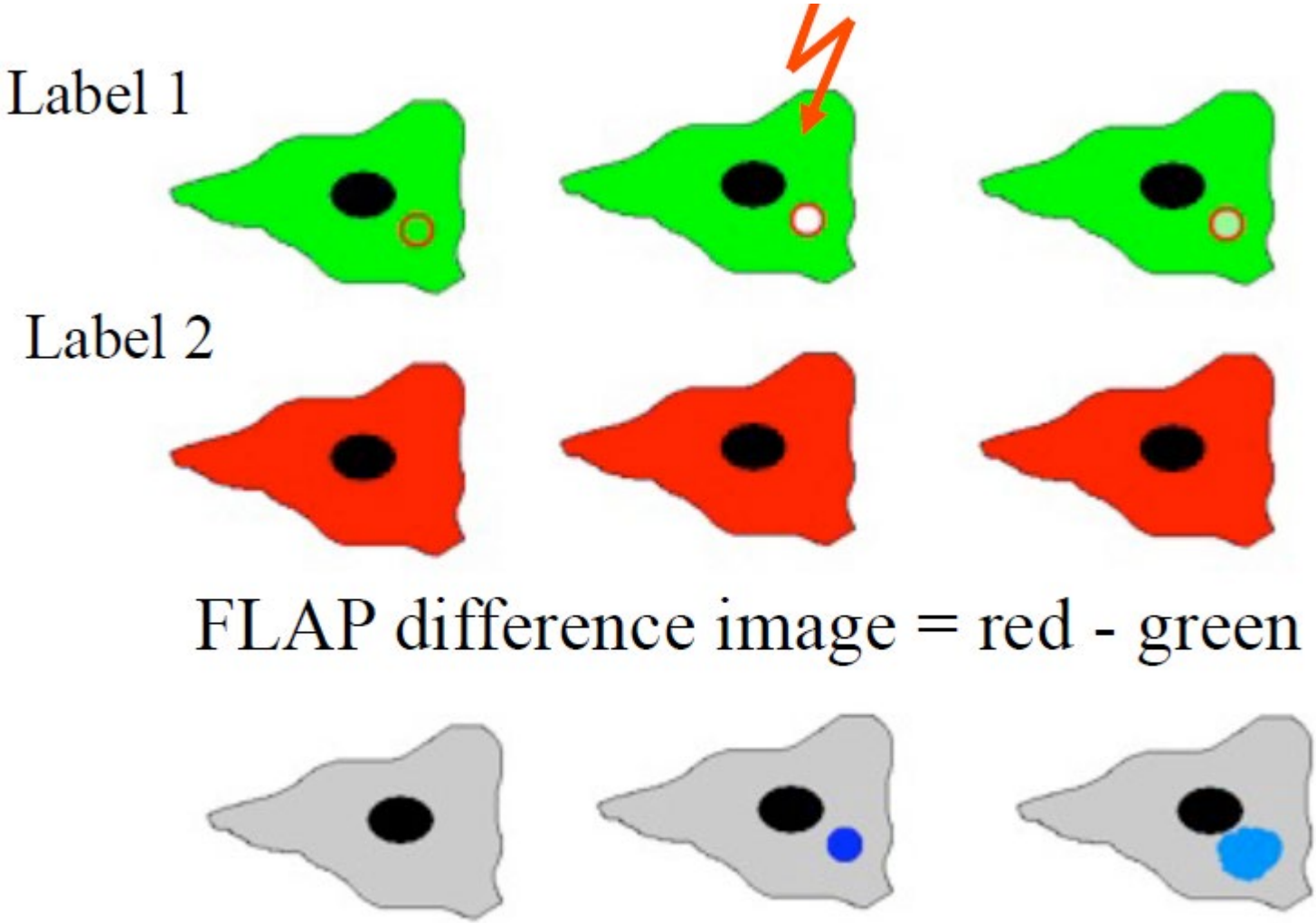


Summary of FRAP and FLIP

- FRAP good for diffusion rates (complex formation).
- FLIP good for connectivity studies.
- **But**
- Bleached molecules are lost to detection.
- FRAP & FLIP are not good for following localisation of a
- subpopulation.
- Friday, 16

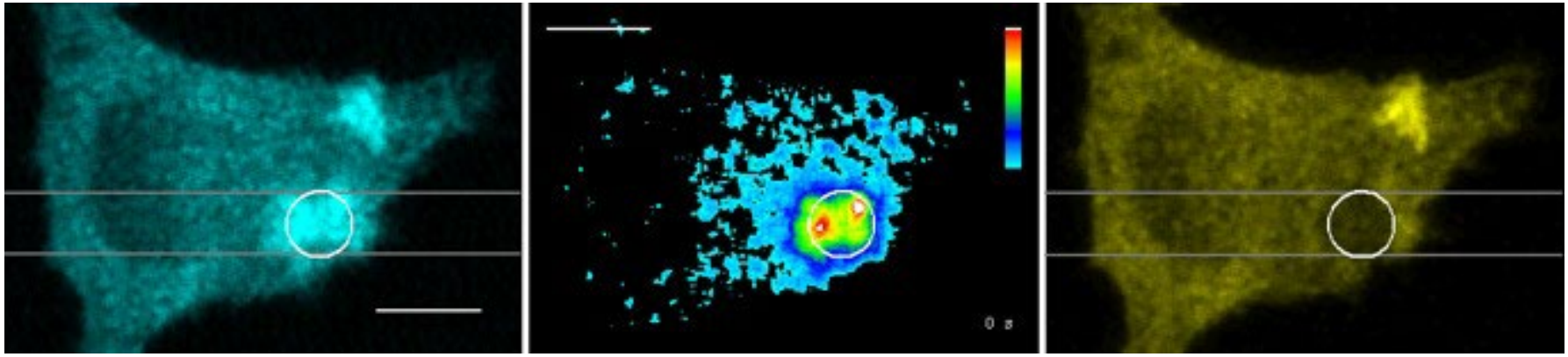
Fluorescence Localisation After Photobleaching (FLAP)

- Related to FRAP and FLIP, by having a single species labelled with two fluorophores FLAP allows localisation of both the unbleached and the bleached molecules.



FLAP procedure

- If the images of the two fluorophores are accurately matched before bleaching, the difference signal (red) is everywhere zero.
- CFP – actin Yfp actin



but after bleaching it reveals the location of the bleached molecules.

The FLAP signal is simply the image of this difference signal

shown in pseudo-colour.

FLAP-Dynamics at the leading edge

- *α-FLAP*