FRAP-FLIP-FCS-FRET-FLIM

Ian Dobbie

The FXXX words

- Advanced fluorescence techniques can be used to measure properties such as dynamics (FRAP, FLIP, FLAP, FSC) or molecular interactions such as (FRET and FCCS).

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.

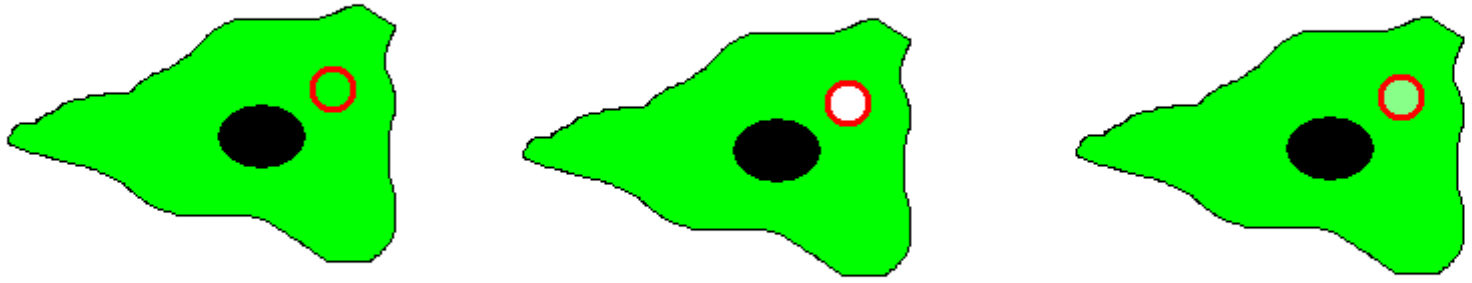
However, reduced sensitivity and slow speed make a widefield microscope better if it has the ability to photobleach.

Fluorescence Recovery After Photobleaching (FRAP)

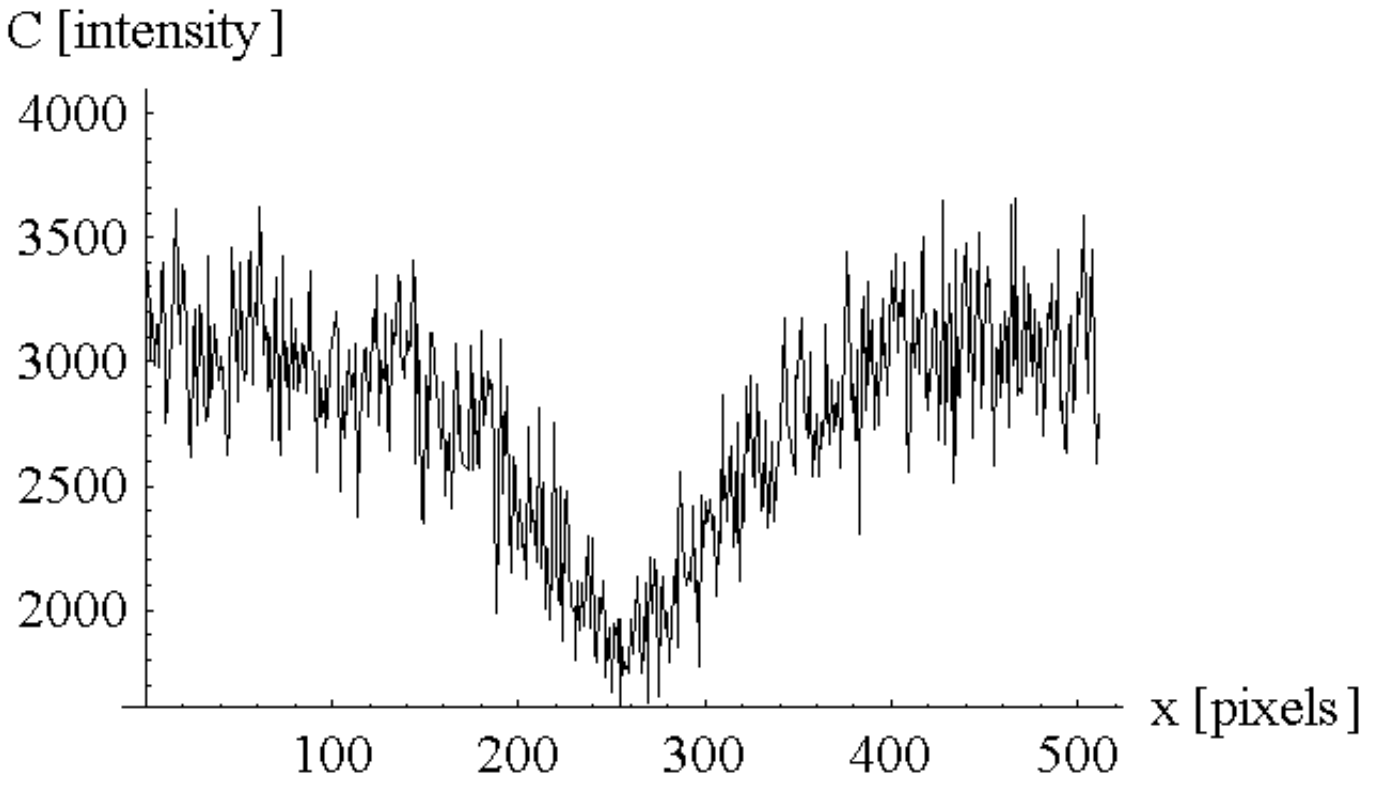
A region is rapidly bleached and the rate at which fluorescence fills the bleach region is determined by the diffusion of unbleached molecules.

Small objects – fast diffusion

Large objects – slow diffusion

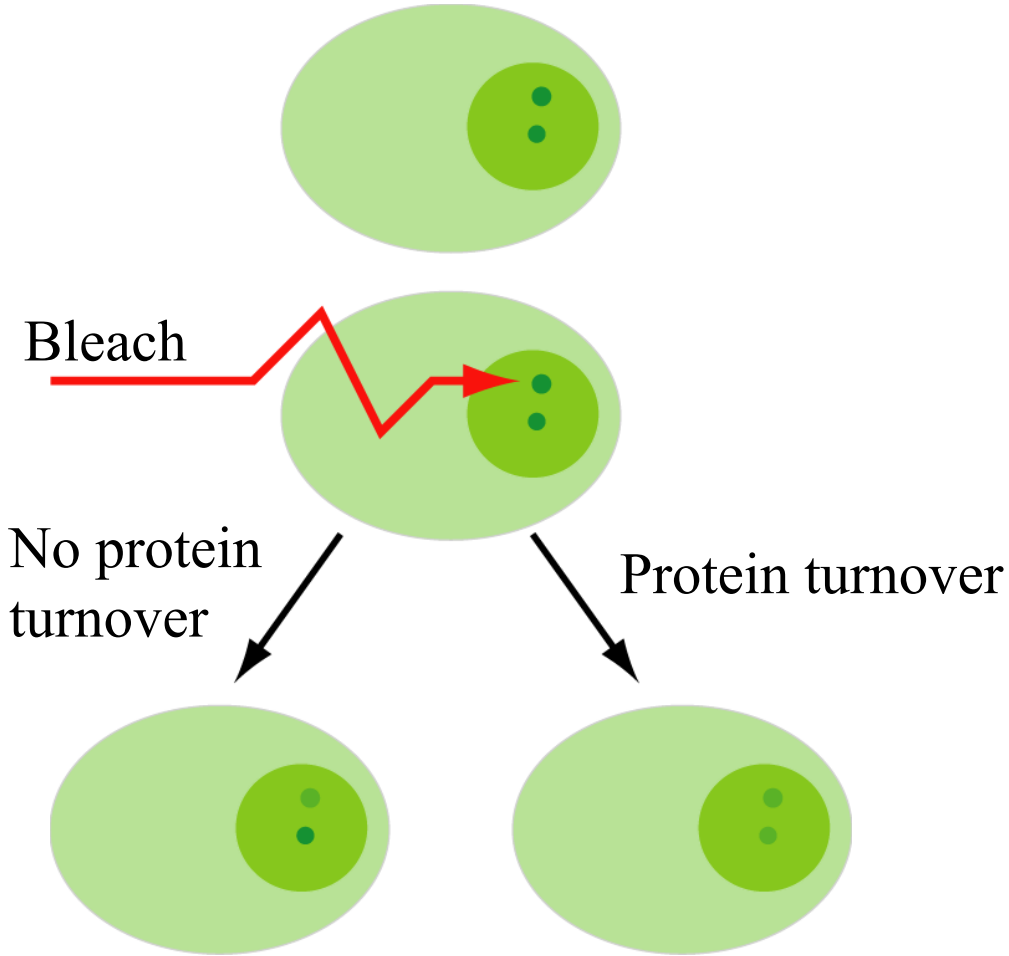


Raw data acquired from a profile crossing a bleach region (FITC-IgG solution)

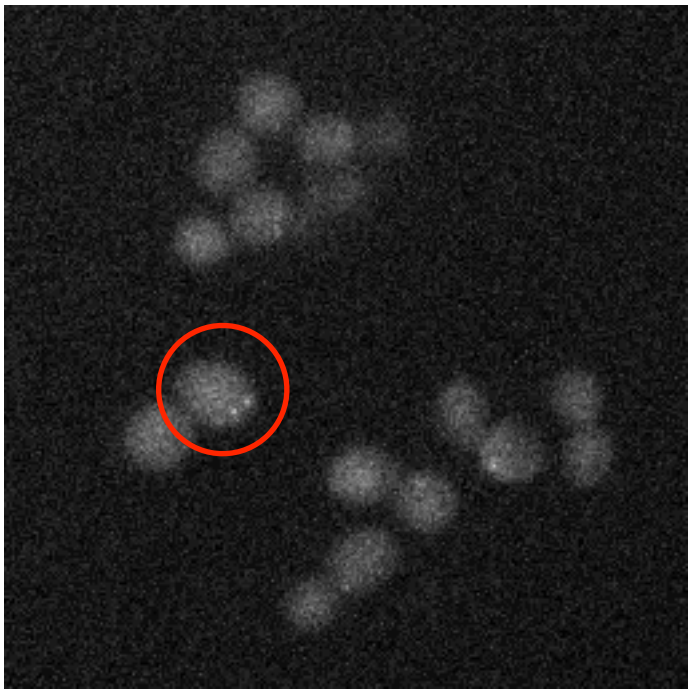


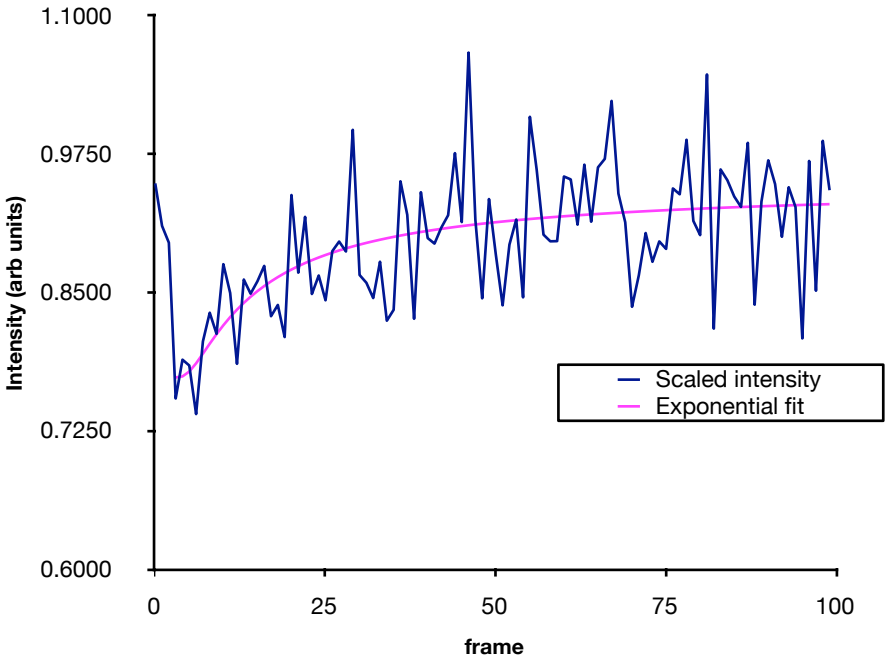
Alastair Nicol, Colin Gray, Daniel Zicha

FRAP for foci



FRAP on DNA repair foci in Yeast



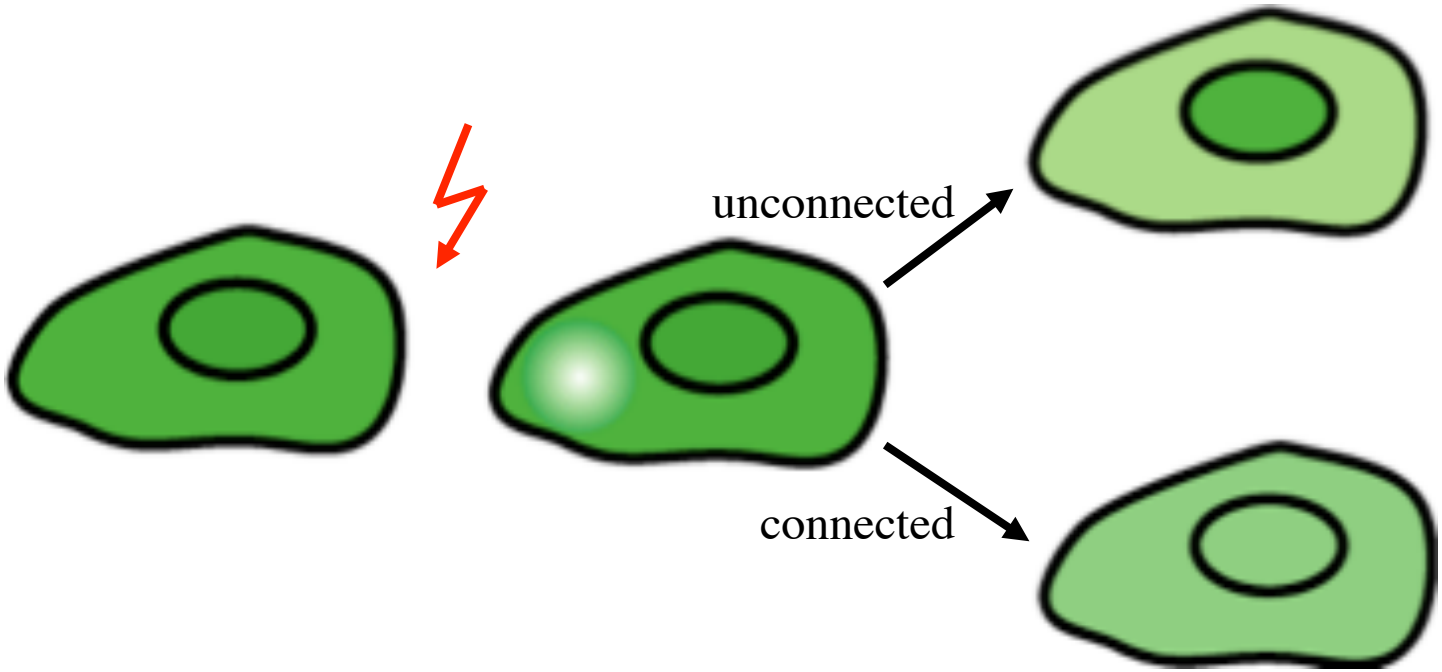


Ddc2-GFP 1.5 hours after 200 Gy IR,
G2 arrested cells.

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.

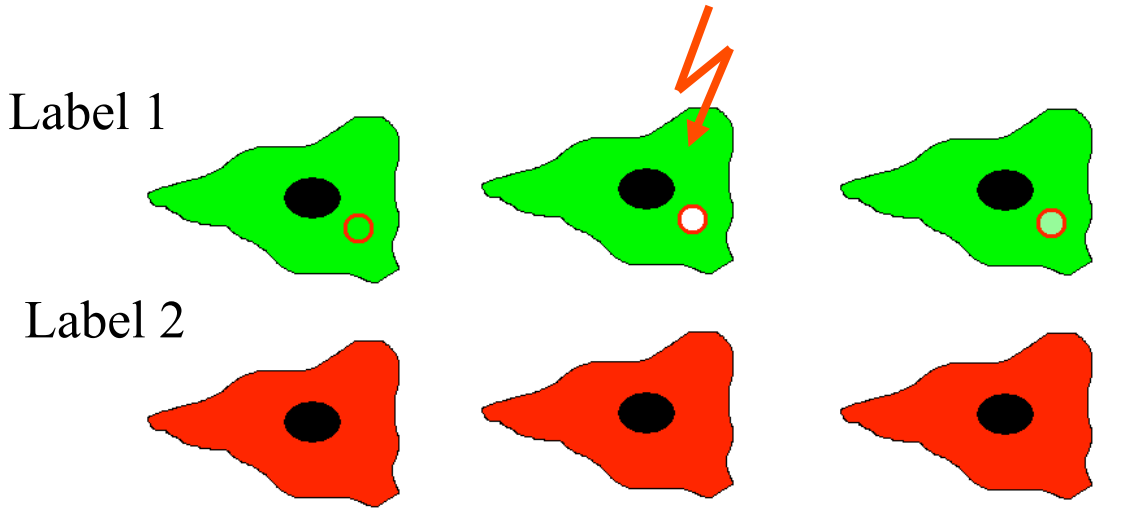
But

Bleached molecules are lost to detection.

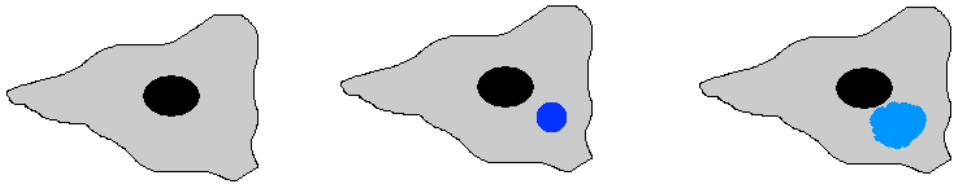
FRAP & FLIP are not good for following localisation of a subpopulation.

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.



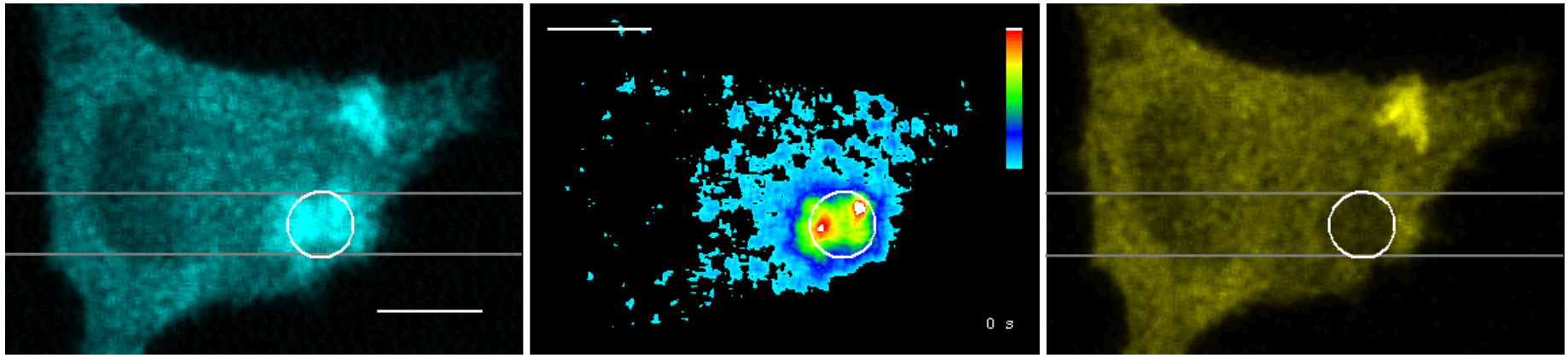
FLAP difference image = red - green



FLAP procedure

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

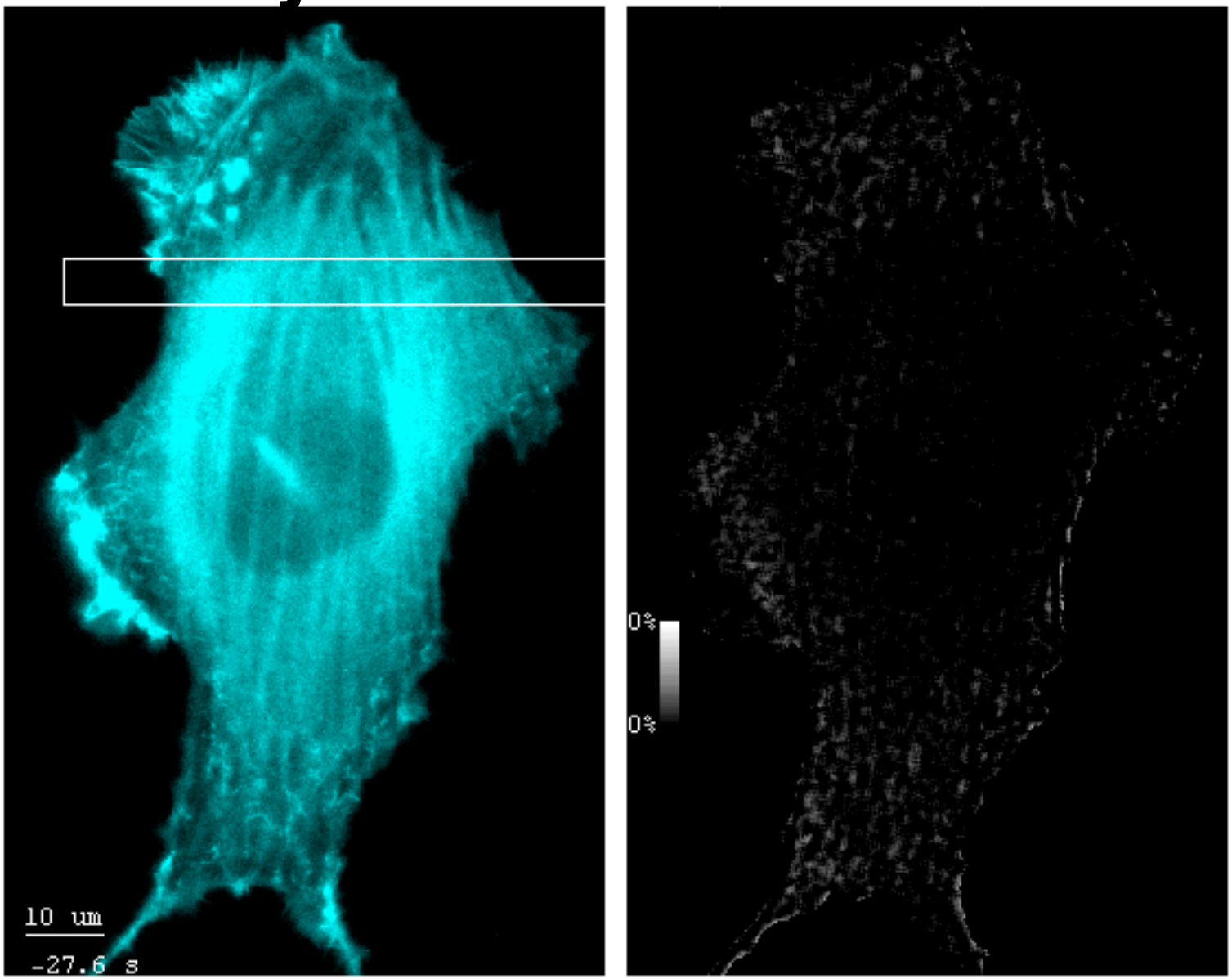
CFP - actin YFP - actin



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

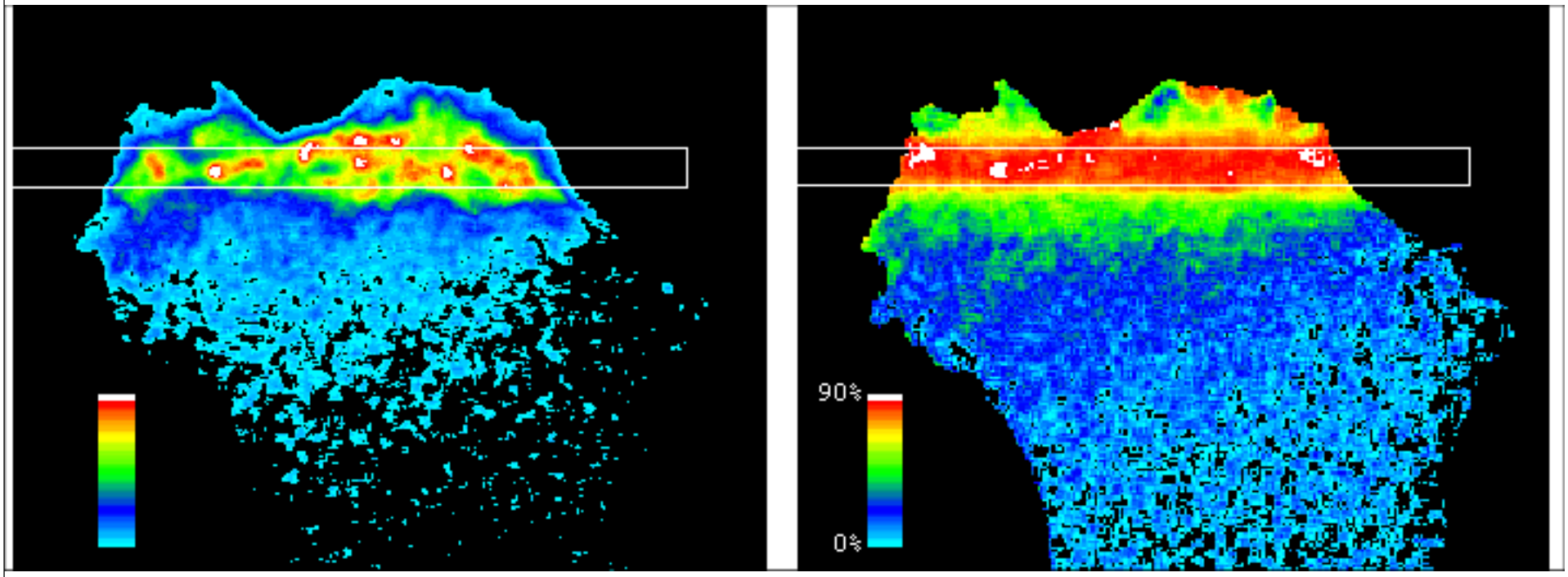
The FLAP signal is simply the image of this difference signal shown in pseudo-colour.

Dynamics of actin bundles

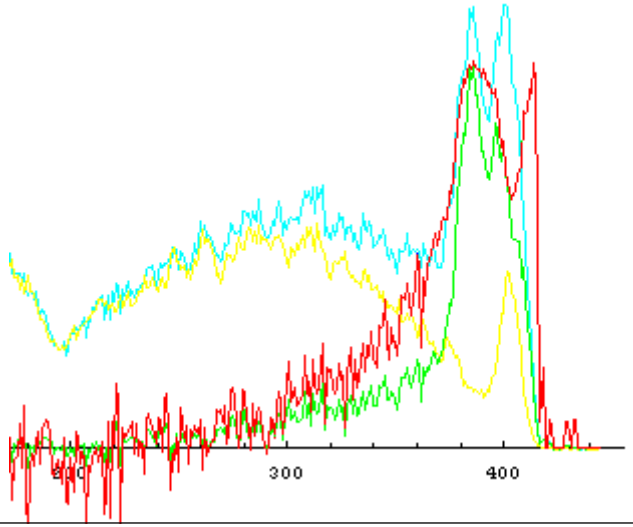


CFP-actin *r-FLAP*

Dynamics at the leading edge



a-FLAP



r-FLAP

Fluorescence Techniques to measure molecular binding

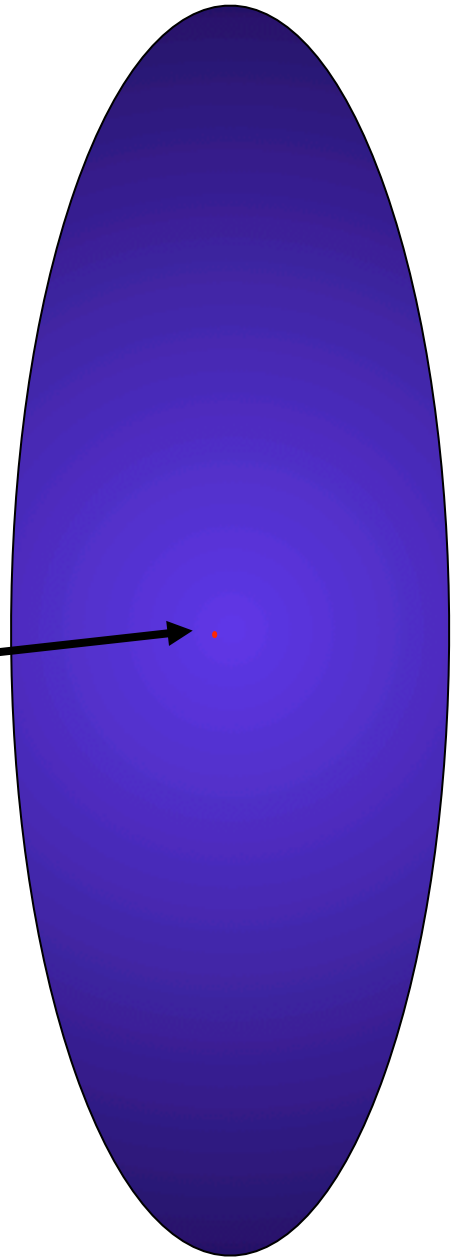
Fluorescence Correlation Spectroscopy – FCS

This allows the measurement of diffusion rates and hence molecular/ complex size. Multiple channels allow measurements of interactions.

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 Ca^{2+}.

Why colocalisation isn't enough

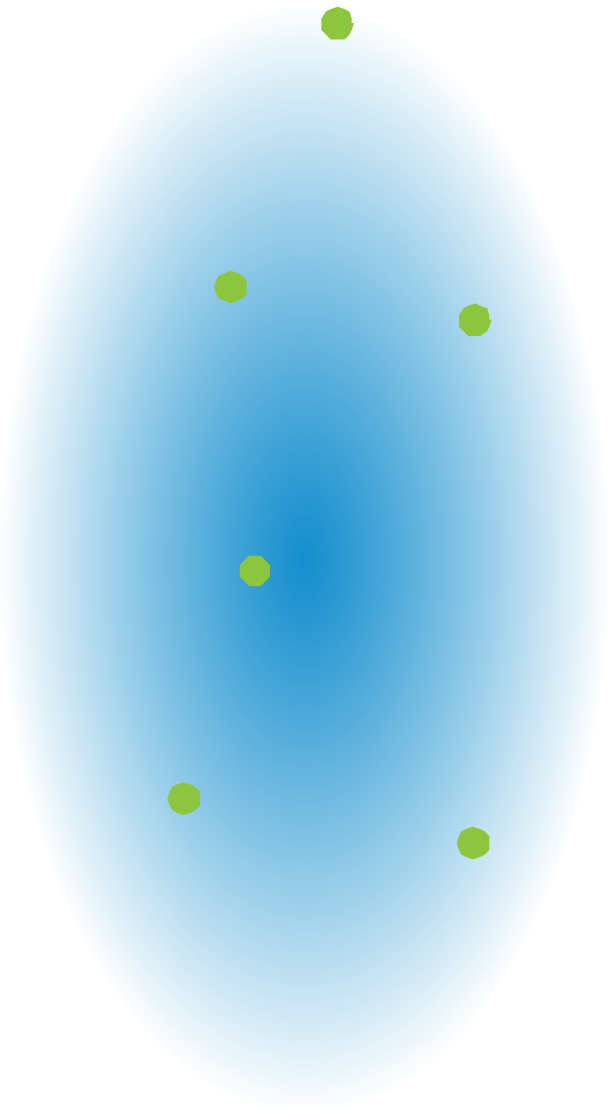


Confocal volume ~
250x250x750 nm

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?

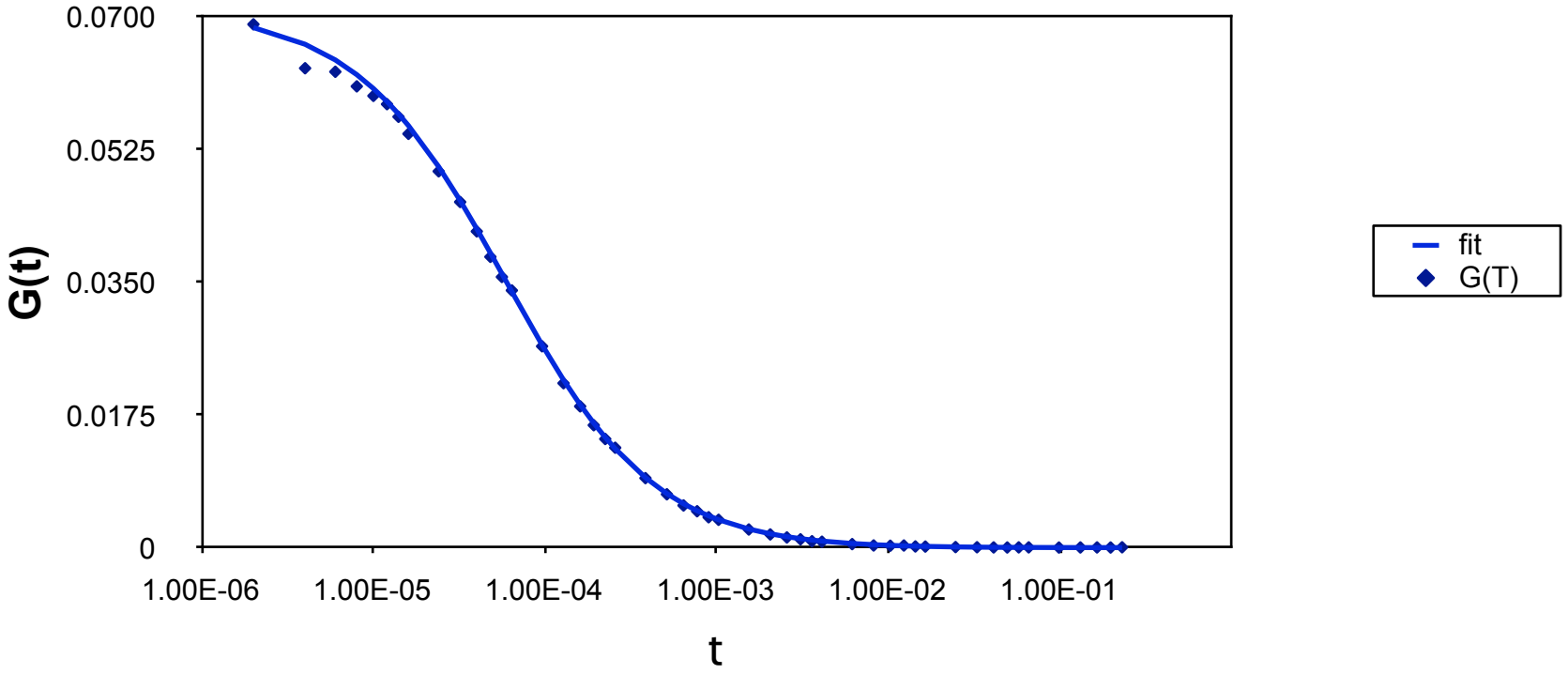
1) Poison noise.

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

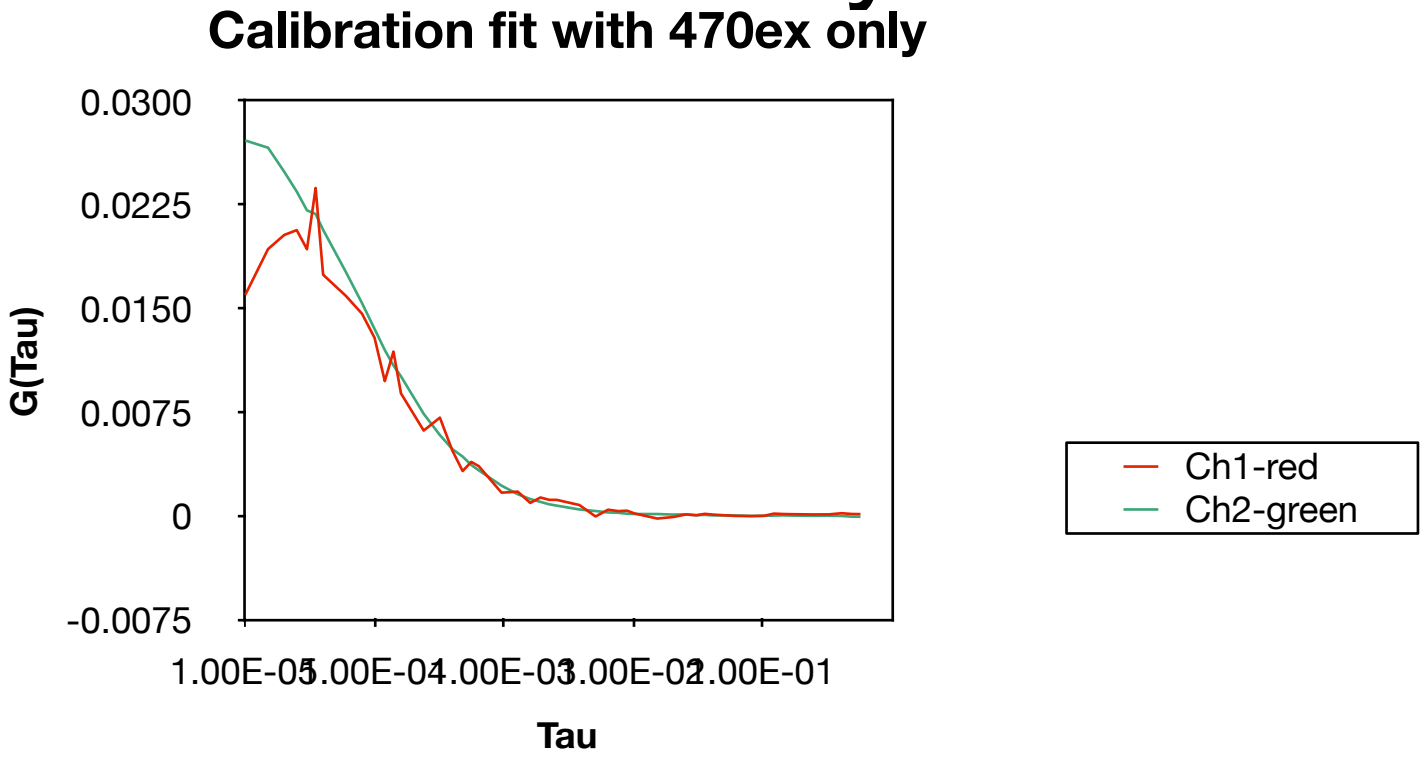
Typical FCS autocorrelation curve



What Does FCS tell us?

- Diffusion rate - hence complex size - however only good for order of magnitude, $D \propto m^{1/3}$.
- Tells us the number of fluorescent objects in volume and hence concentration.
- Can be used to measure flow.

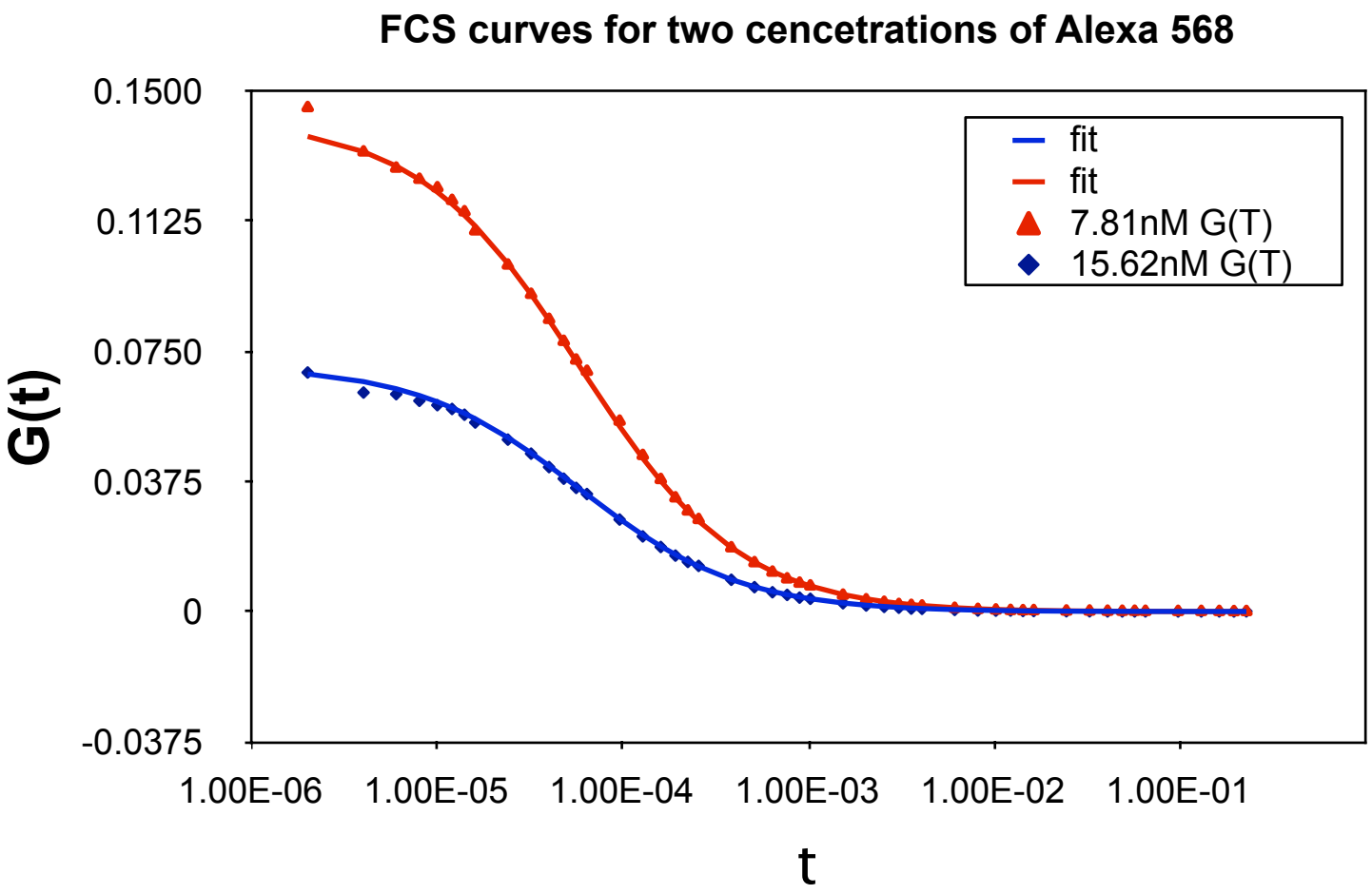
FCS curve is independent of intensity



Green channel count rate ~ 148k/s

Red channel count rate ~ 13k/s

FCS curve amplitude but not shape depends on concentration



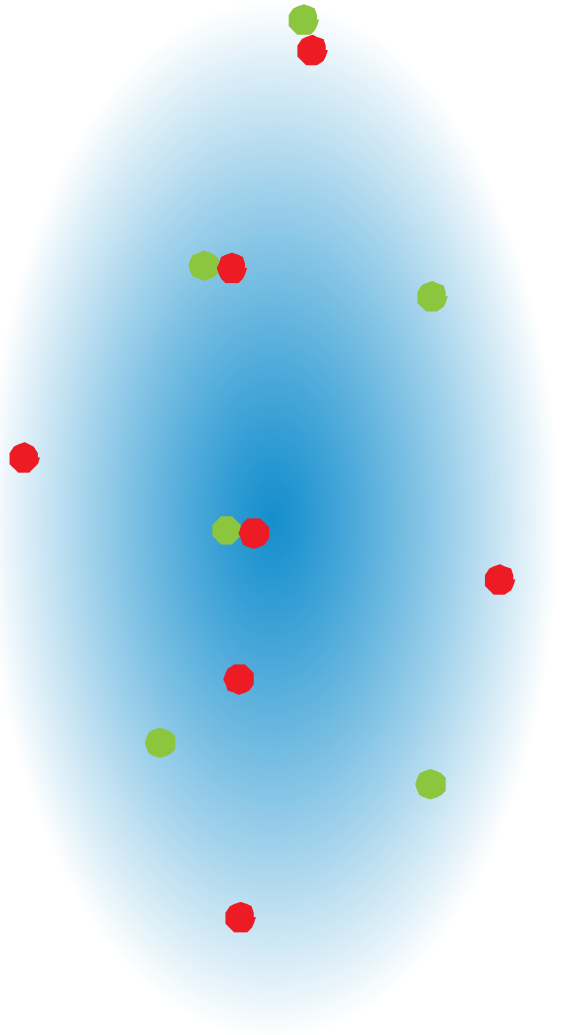
Limitations of FCS

1. Low sensitivity to changes in mass of diffusing particles, $D \propto m^{1/3}$.

2. Small range of useful concentrations, need ~1-100 molecules in your volume, typically nM concentrations.

3. Only works on diffusing particles.

Fluorescence Cross-Correlation Spectroscopy



Take a confocal volume with a few molecules in it.

How do fluctuations in the two channels vary?

1) Poison noise.

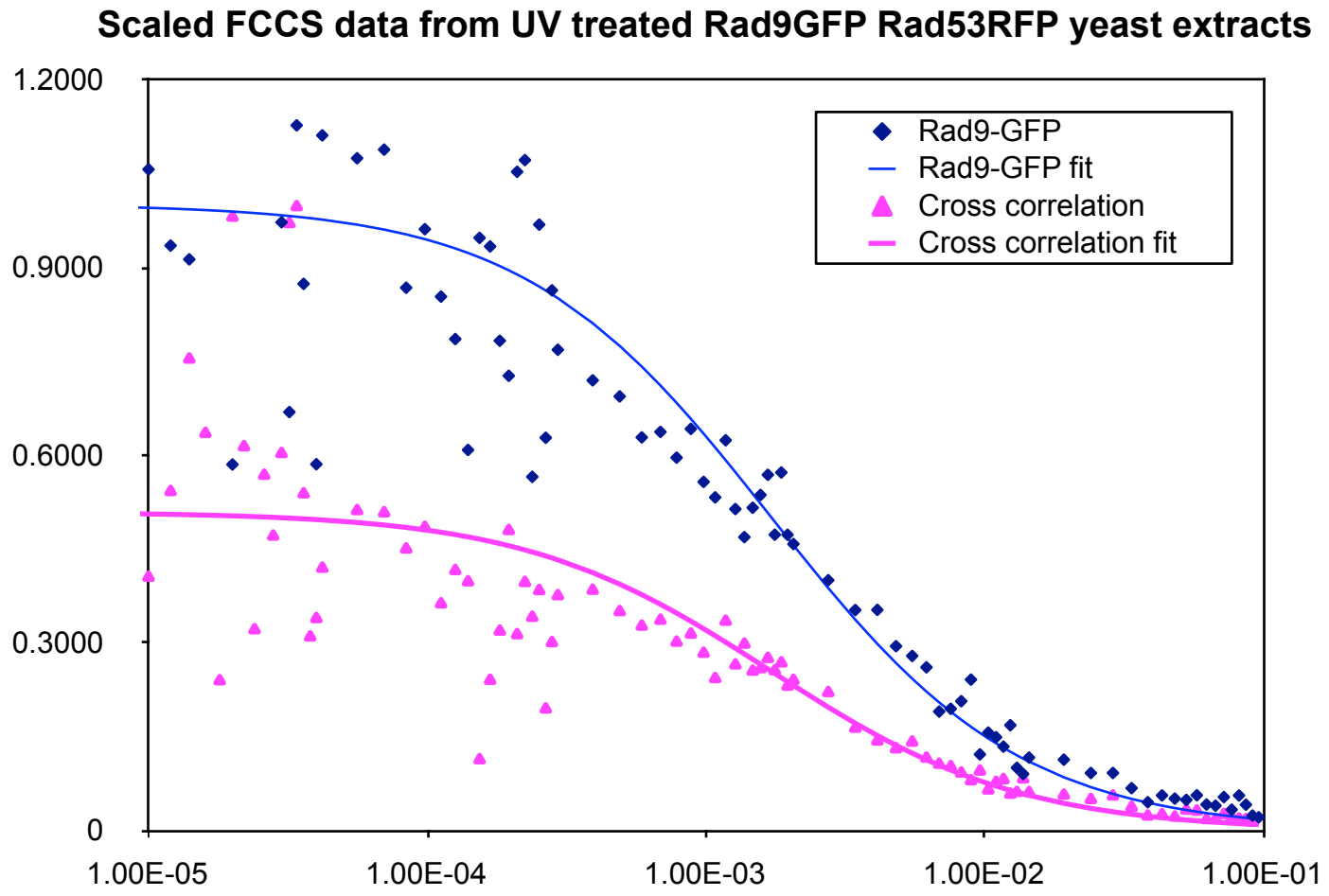
2) Single colour molecules entering or leaving the volume.

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
- Alignment critical - max interaction < 1.

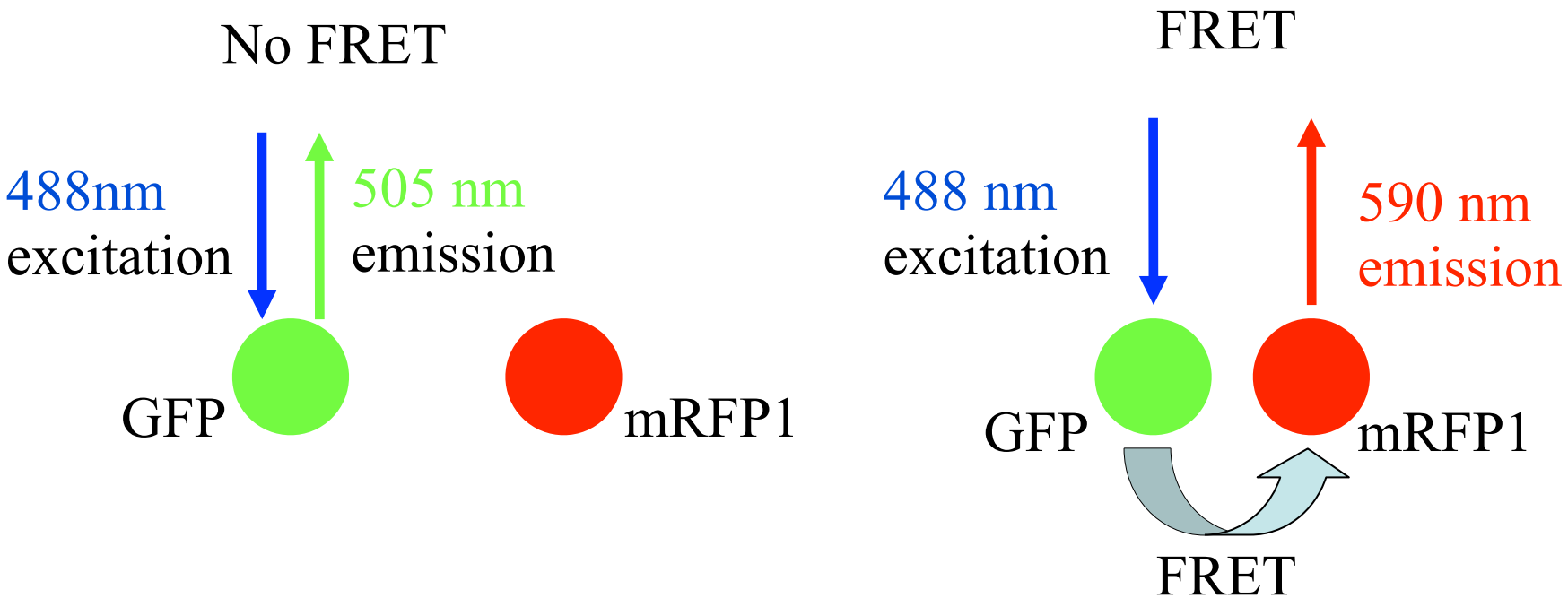
Rad9-Rad53 FCCS



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

FRET - Fluorescence Resonance Energy Transfer

Two fluorophores are used to label two proteins. If the two proteins are less than ~10 nm apart some fraction of the energy used to excite the shorter wavelength fluorophore is transferred to the longer wavelength fluorophore and emitted as longer wavelength light and reduces the lifetime of the shorter wavelength species.



How to detect FRET?

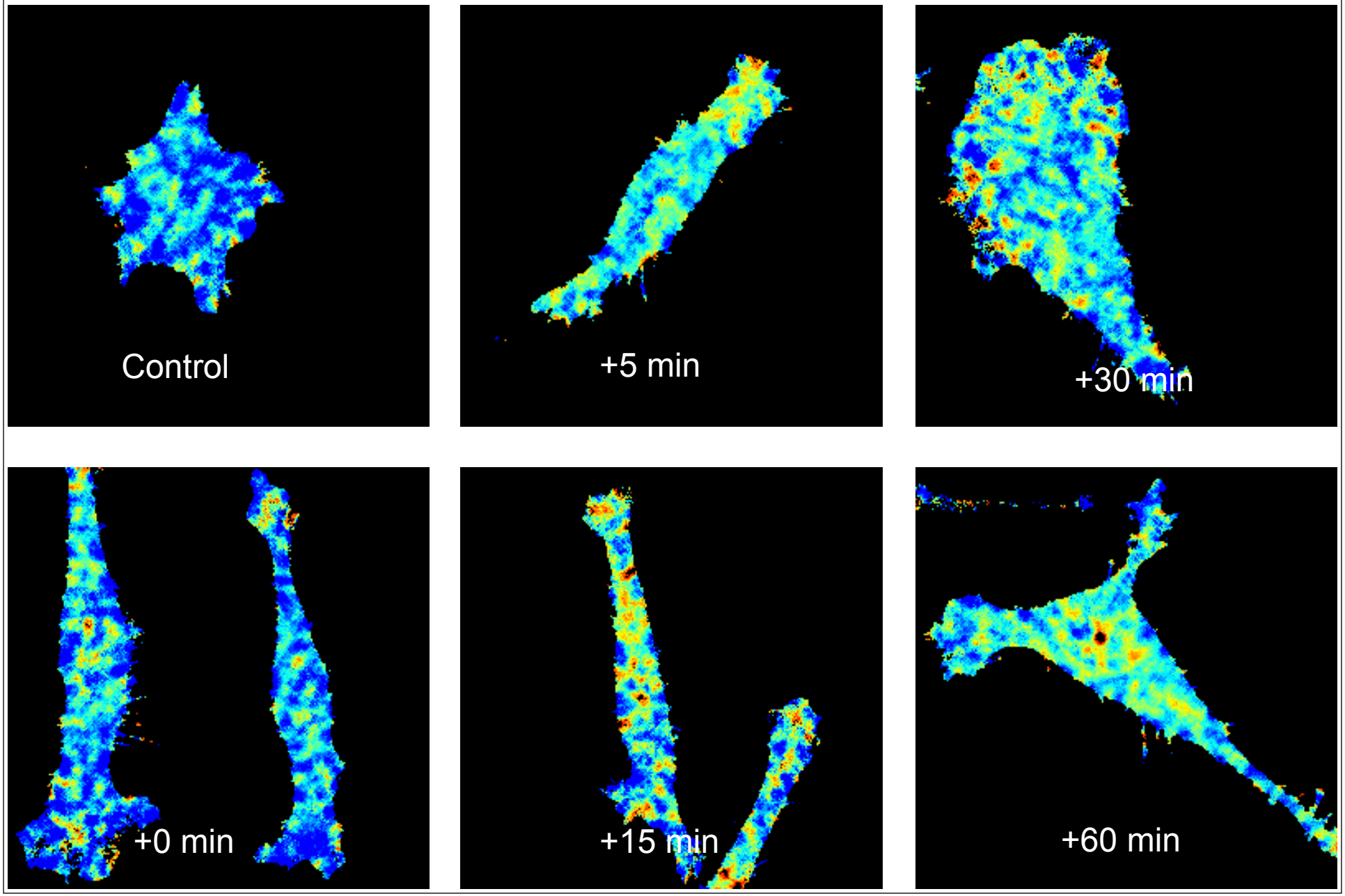
When FRET occurs

1. The donor emission intensity is reduced
2. The acceptor emission intensity is increased
3. The donor fluorescence lifetime is reduced
4. All 3 of these changes can be measured

Fluorescence Lifetime IMaging

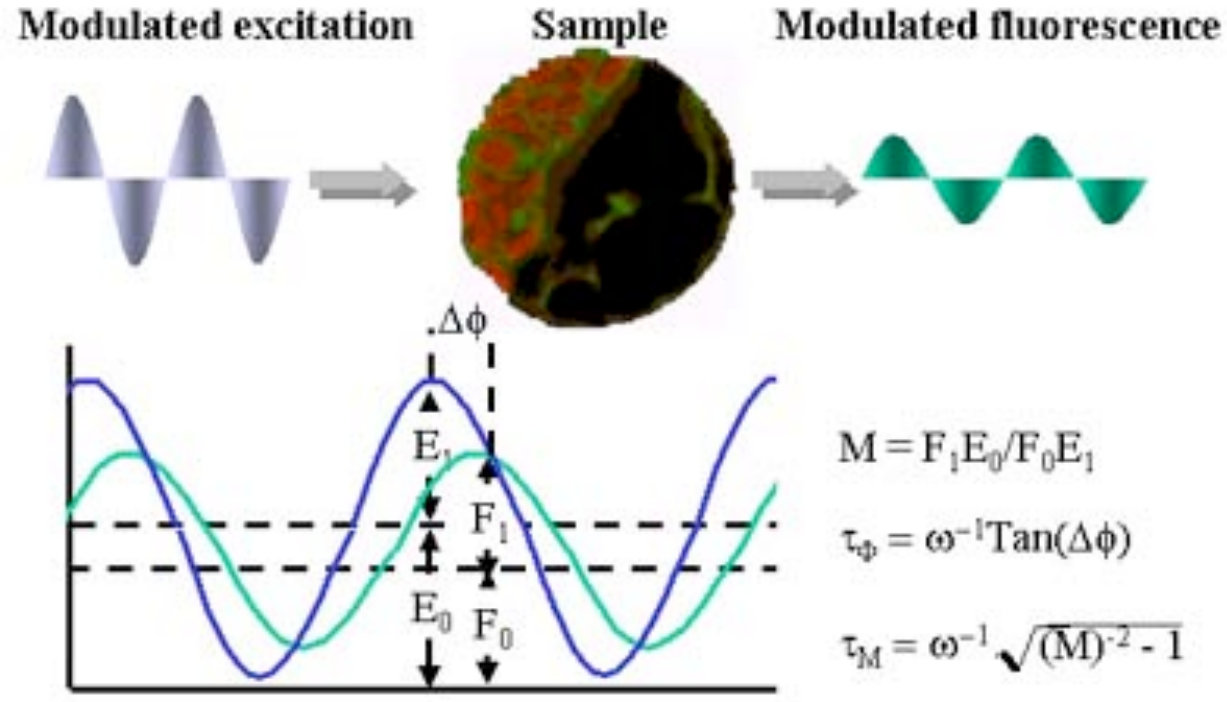
- FLIM is the best way to measure FRET
- It is concentration and hence intensity independent.
- Pulsed illumination allows direct measurement of fluorescence lifetime.
- Sinusoidal illumination intensity allows life measurement by detecting phase lag between excitation and emission.

Some TCSPC FLIM images

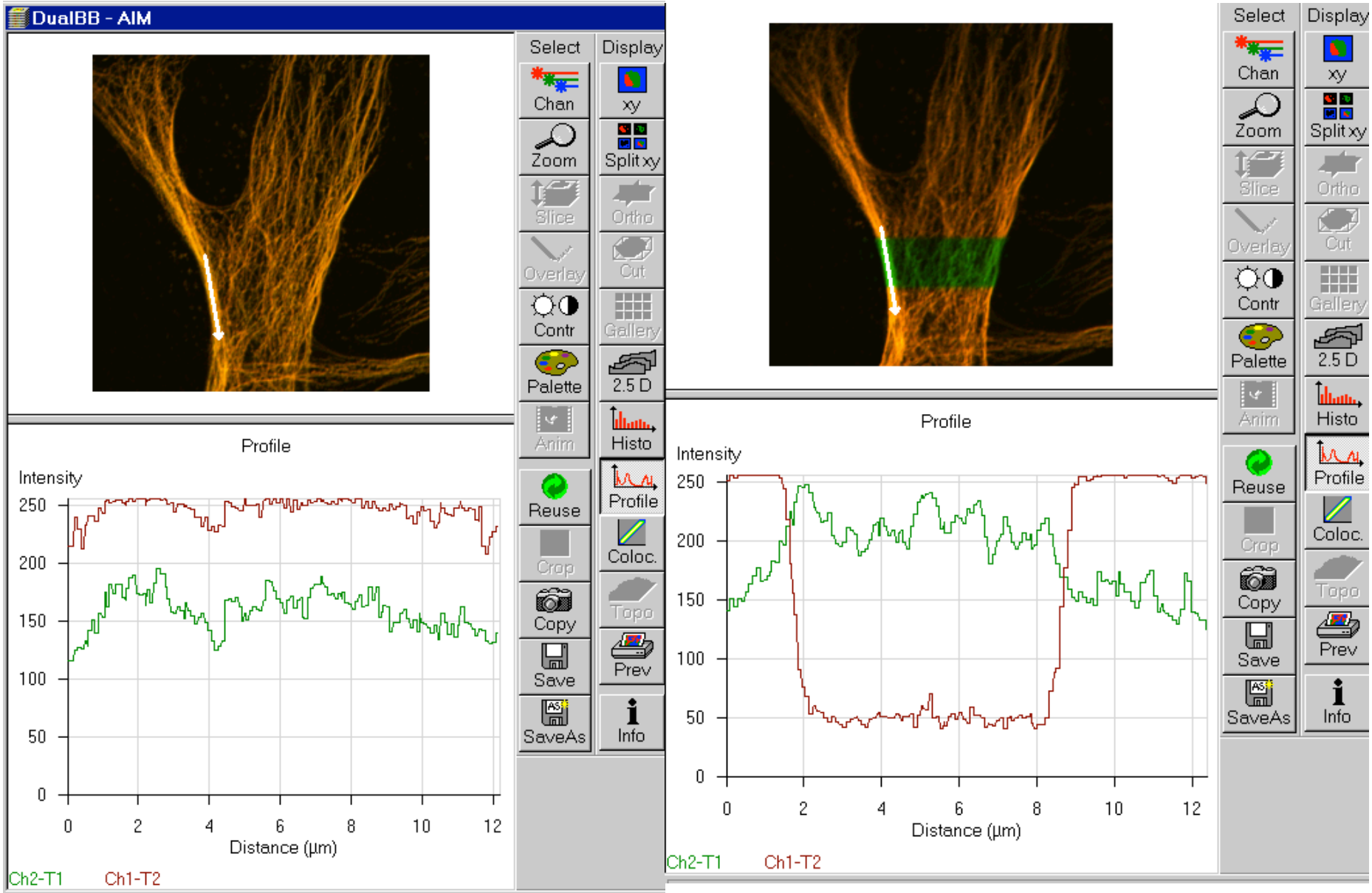


Frequency Domain FRET Measurements

A slight variation on the methods for time domain measurment of FRET is to use frequency domain techniques. Instead of a pulsed light source the illumination is varied sinusodally. The emission intensity is then measured over the same sinusodal cycle. The intensity modulation and the phase shift between the illumination and emmision can then be used to work out the amount of FRET occuring.

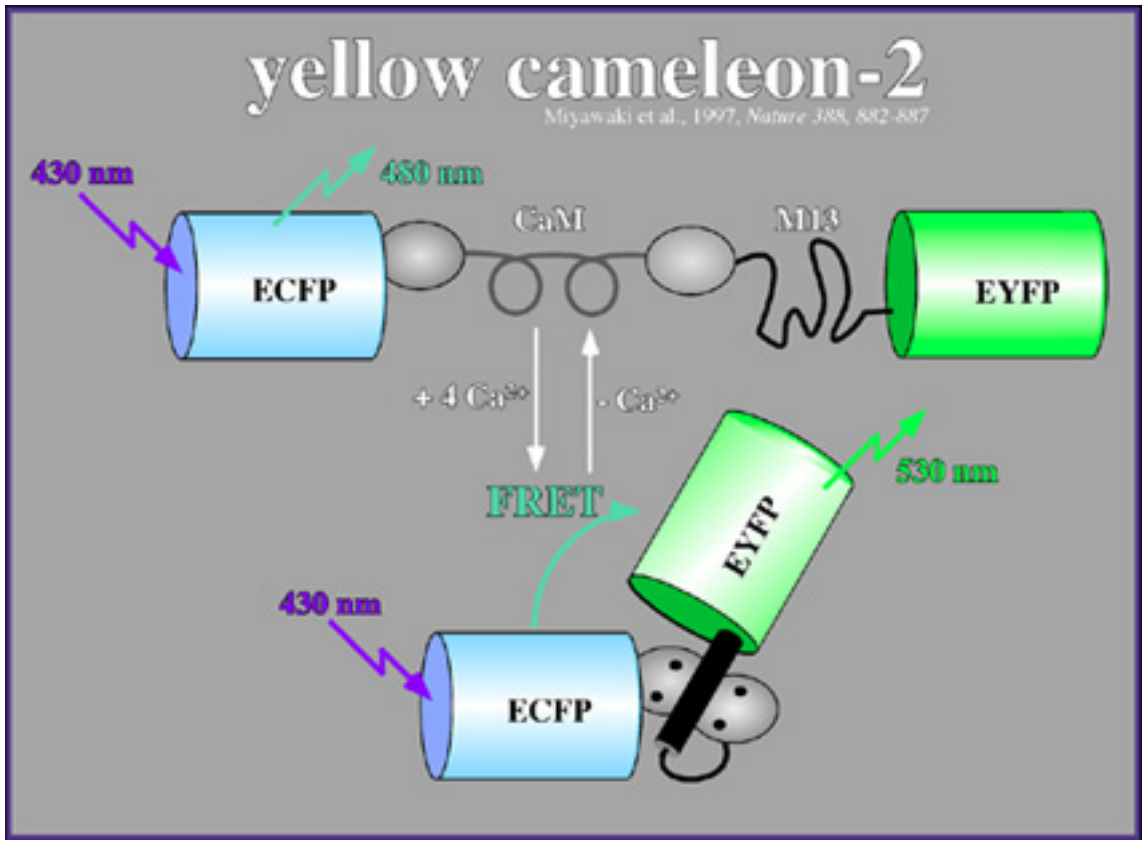


FRET by photobleaching



FRET based biosensors

The Chameleon construct uses the Ca2+ binding domain of calmodulin to join YFP and CFP. When calcium binds the domain folds, causing FRET.

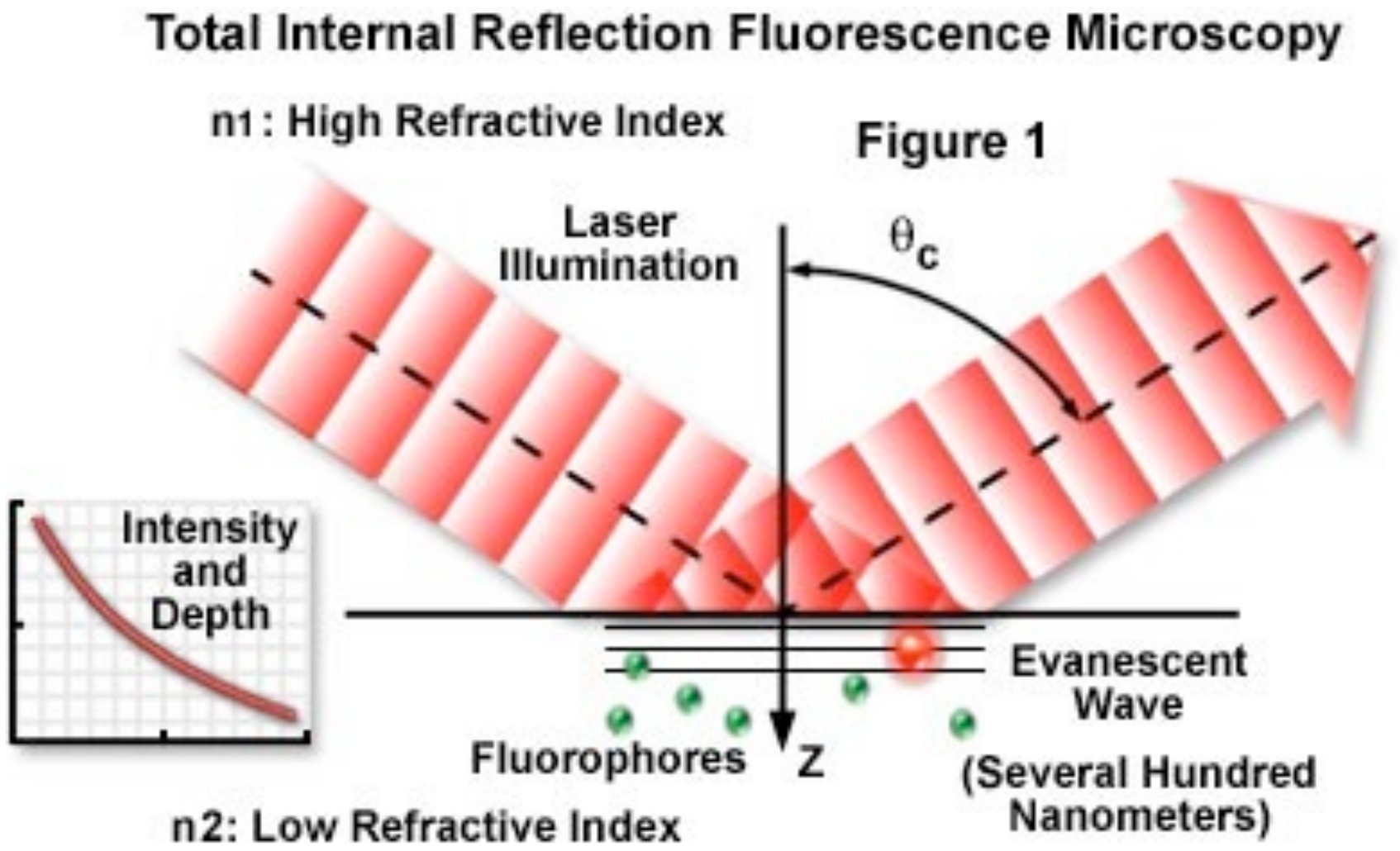


Quick intro to Total Internal Reflection Microscopy(TIRF)

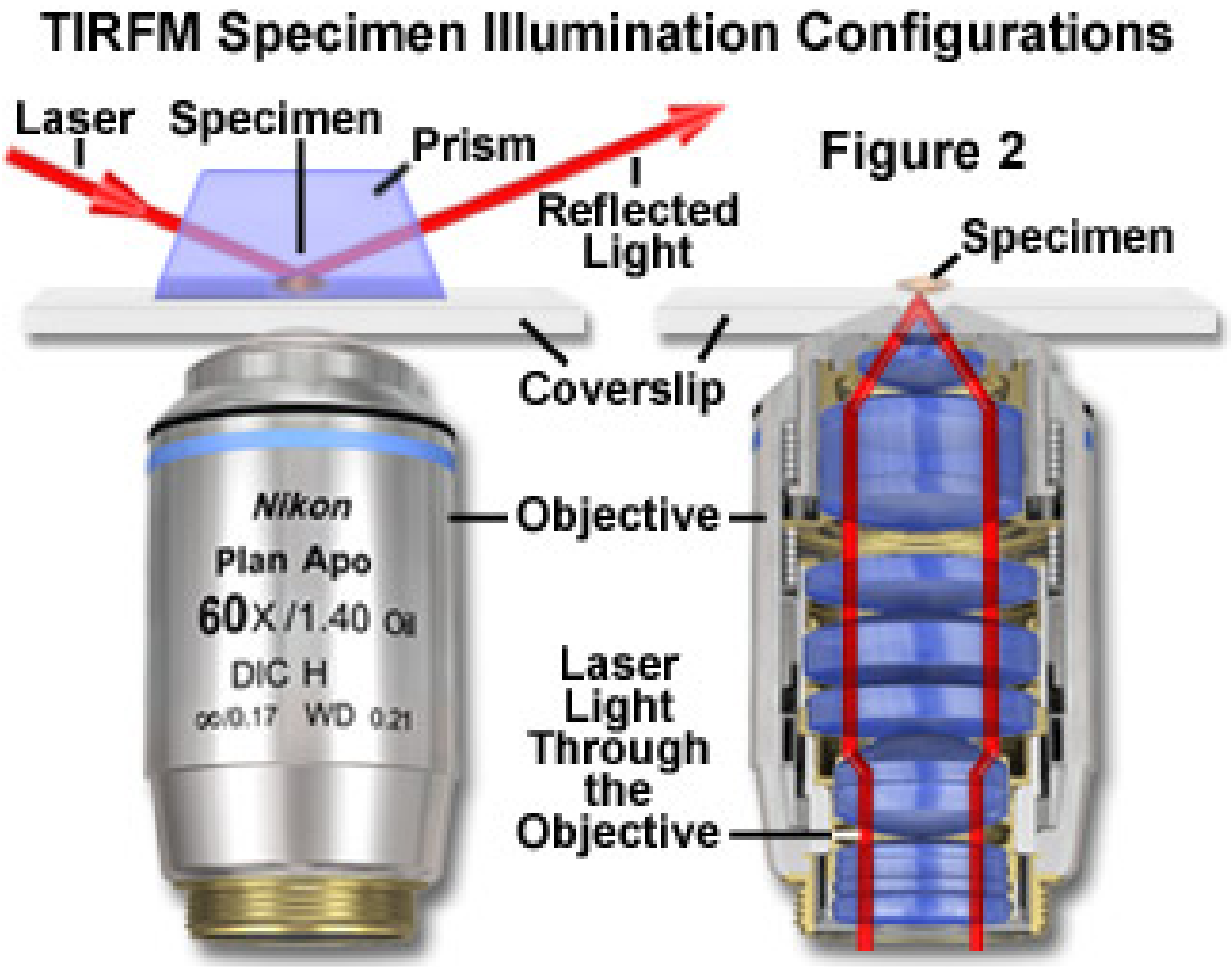
What is TIRF

- Light striking an interface between a high refractive index material and a lower one is totally reflected above a critical angle.
- None of the illumination light enters the experimental sample.
- The field penetrates the interface, called an evanescent wave, allowing fluorescence excitation up to a depth of ~100nm.

Total Internal Reflection Fluorescence (TIRF)



Two common setups



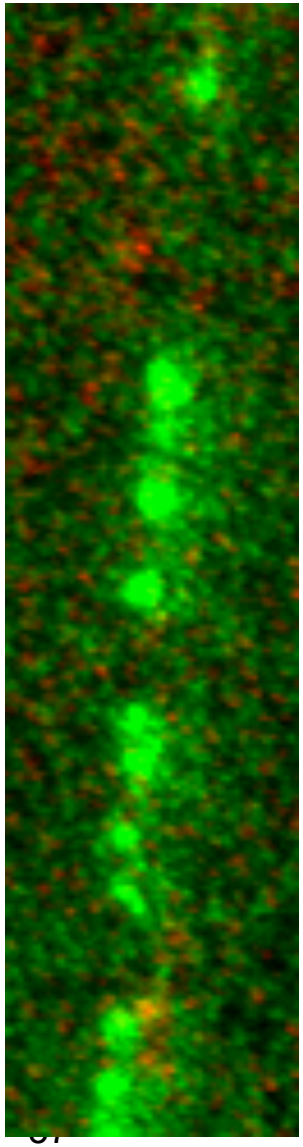
Prism based TIRF Objective based TIRF[36]

Uses of TIRF

Two Key advantages

1. Extremely low background
2. High Z resolution (100 nm) due to localisation to the glass surface

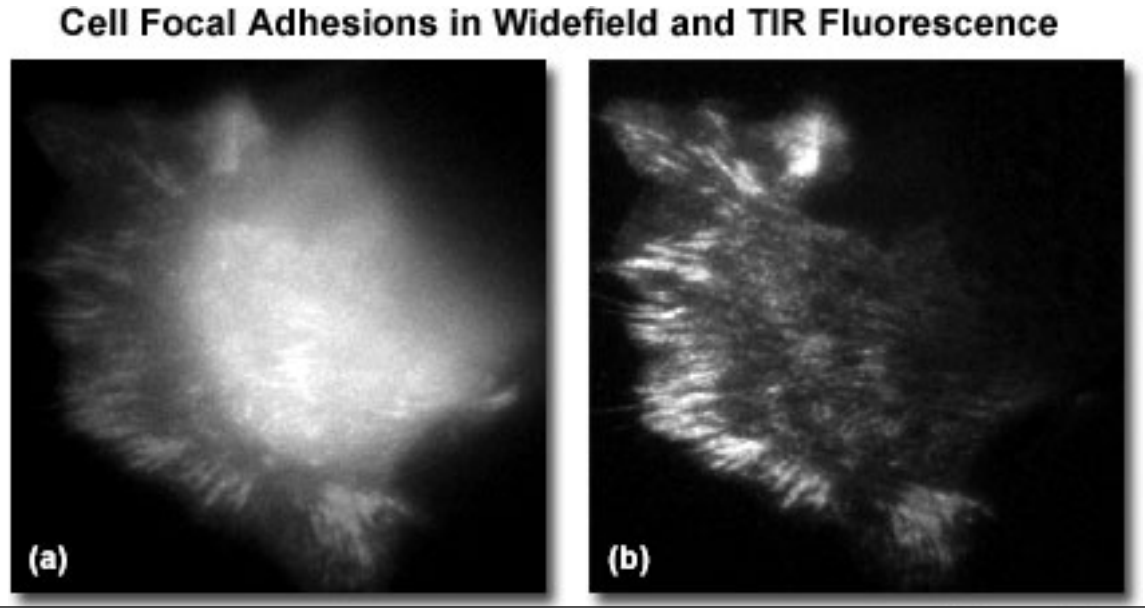
TIRF has often been used to visualise single fluorescent molecules or localisation specific structures such as cell surface receptor binding or a single motor molecule.



Dynein and Kinesin on a microtubule in TIRF

TIRF for Cell adhesions

- TIRF is ideal for visualising focal adhesions
- Only the region very near the coverslip is excited and so cellular background is greatly reduced.



Key points

- There are a range of fluorescence techniques to probe dynamics and molecular interactions.
- They have different strengths and weaknesses.
- All of them require careful experiment design and data analysis.