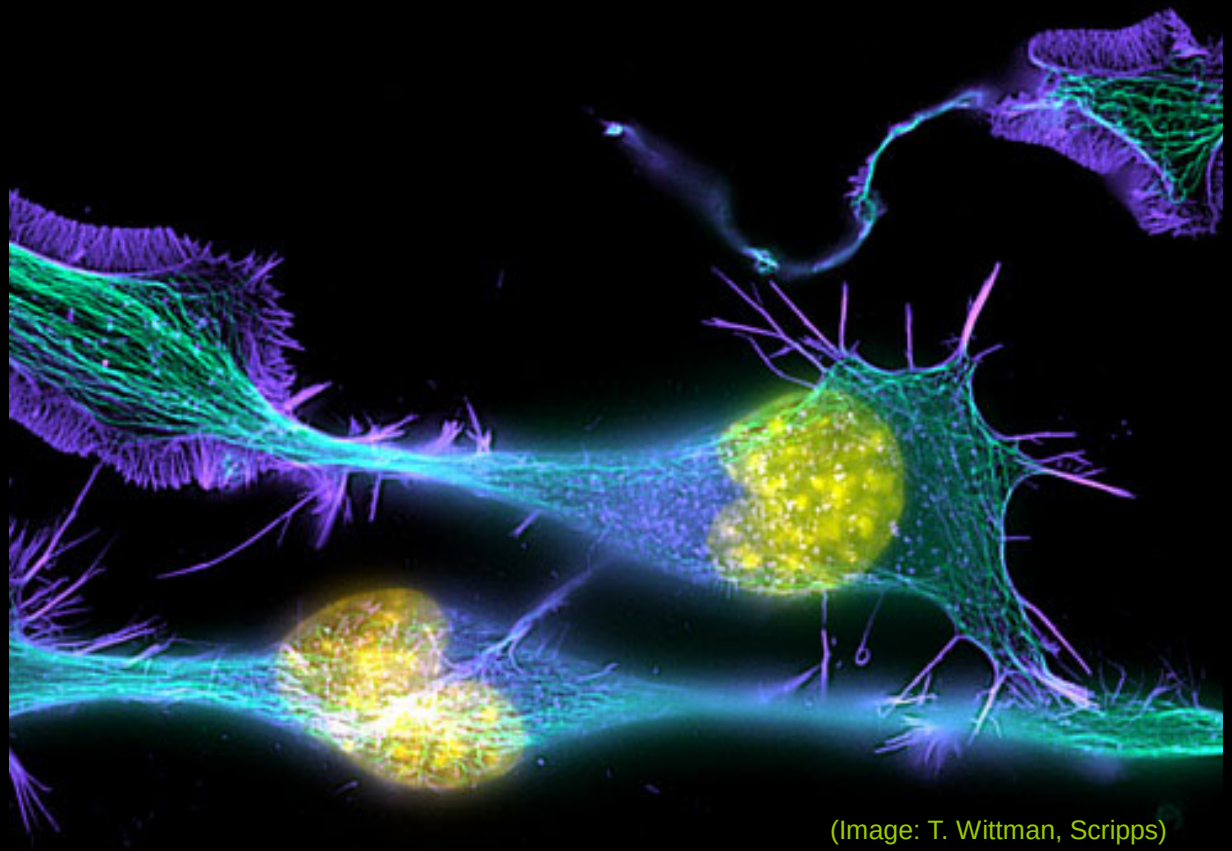


(Image: T. Wittman, Scripps)

Monitoring molecular movement by microscopy

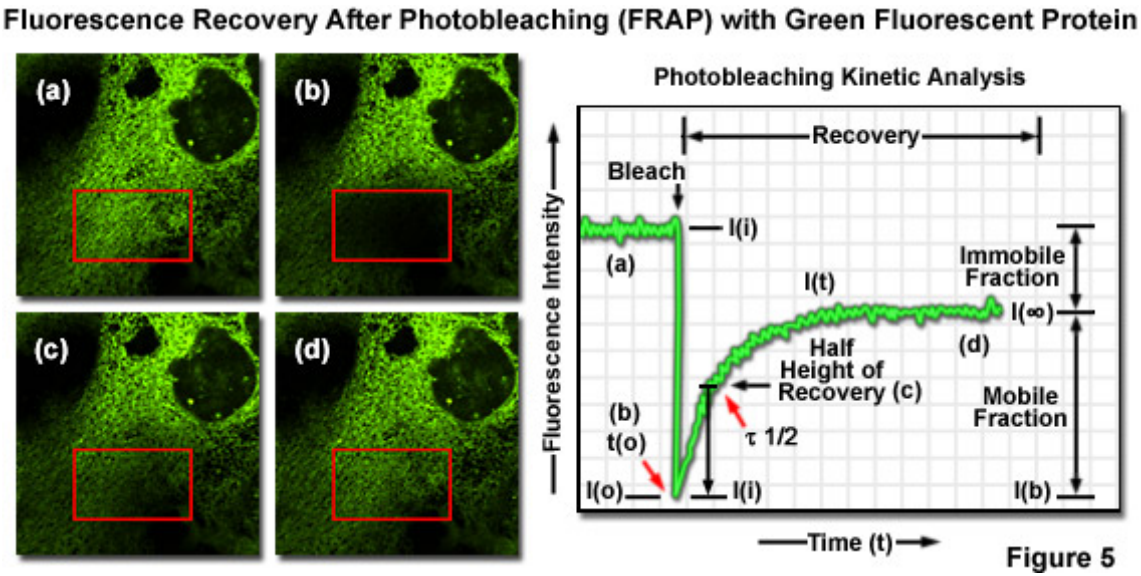
- General idea: optically mark one region of the cell, follow where it goes at later times.

- Implementation
  - Uncaging (old)
  - FRAP, FLIP: photobleaching
  - Photoactivation

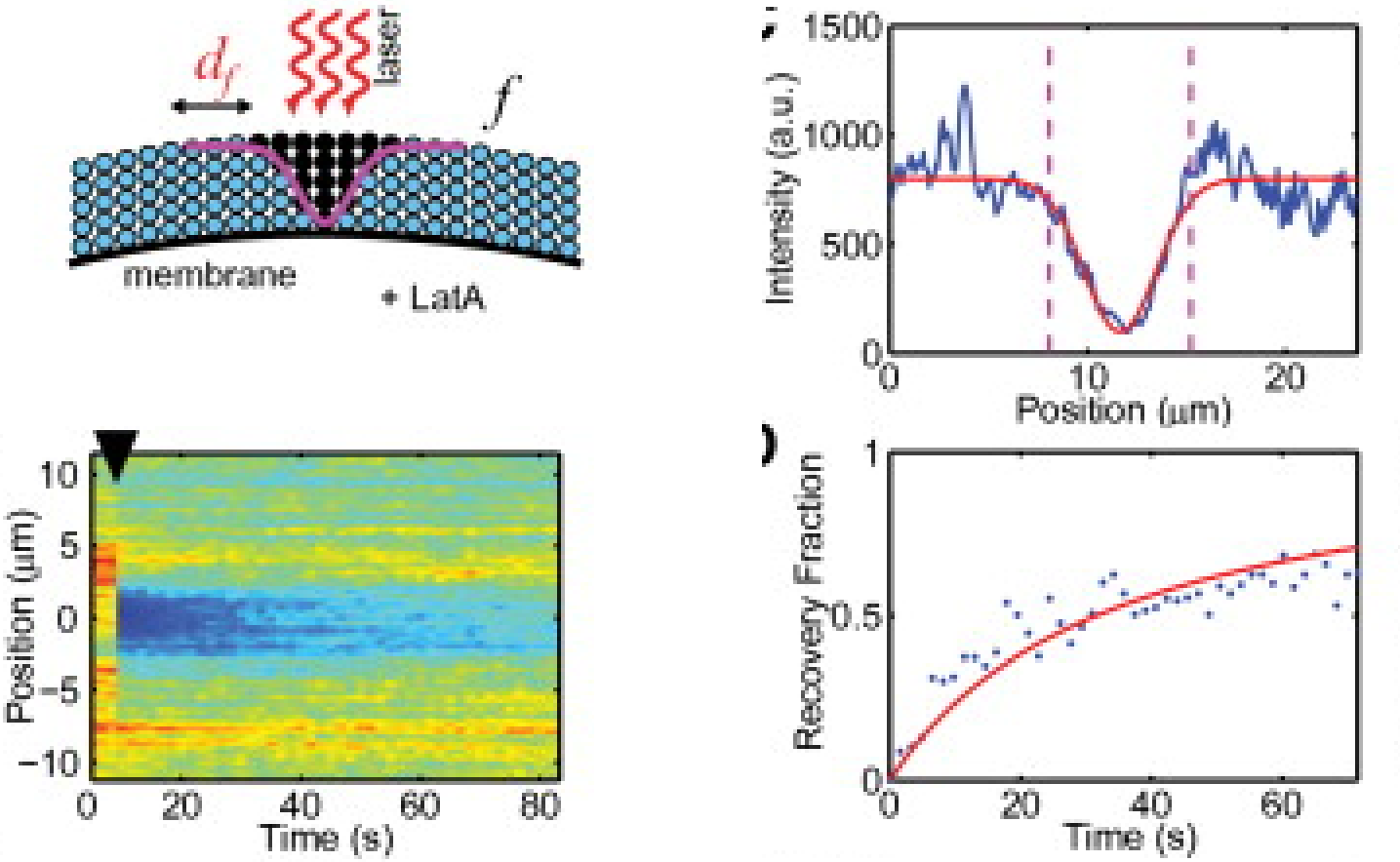
FRAP

Fluorescence Recovery after Photo-bleaching

Need: to probe transport
Idea: bleach in one area,
watch recovery by transport from other areas



Measuring Cdc42 diffusion constant in yeast



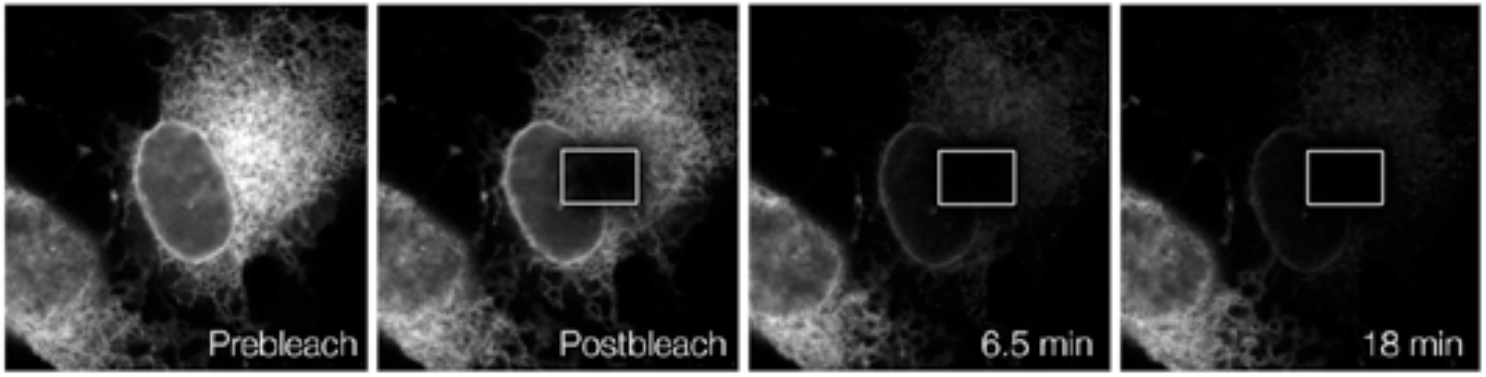
Result: df = (0.036 ± 0.017) μm2/s

Marco et al. 2007 Cell 129:411-422

FLIP

Fluorescence Loss in Photo-bleaching

Need: probe connectivity
Idea: bleach in one compartment,
watch loss in connected compartments by exchange



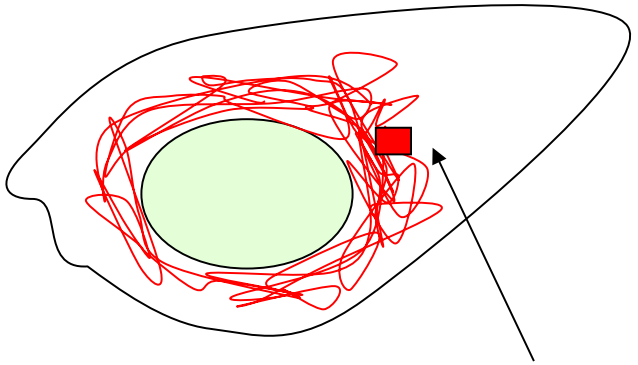
Bleach one area repeatedly. Entire ER dims.
$\Rightarrow$ ER is contiguous

Photo-Activation
(Better?) FRAP/FLIP alternative

Some fluorophores can be activated by light

- Photo-uncagable dyes
- GFP-family proteins

Look for weak light
against dark background
Instead of slight dimming
of bright background



Activate a small area
Watch fluorescence *spread*

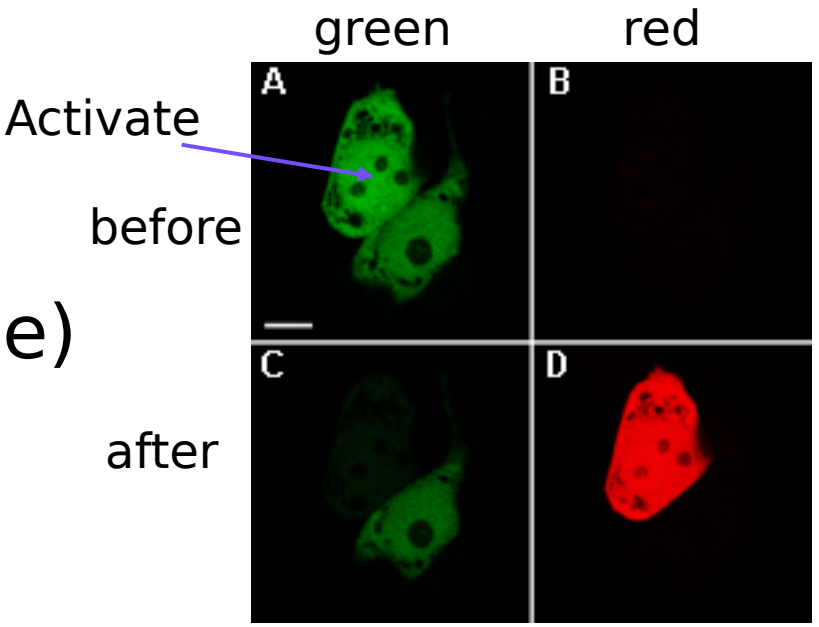
Photo-Activativatable Proteins

Off-On
- PA-GFP, PS-CFP

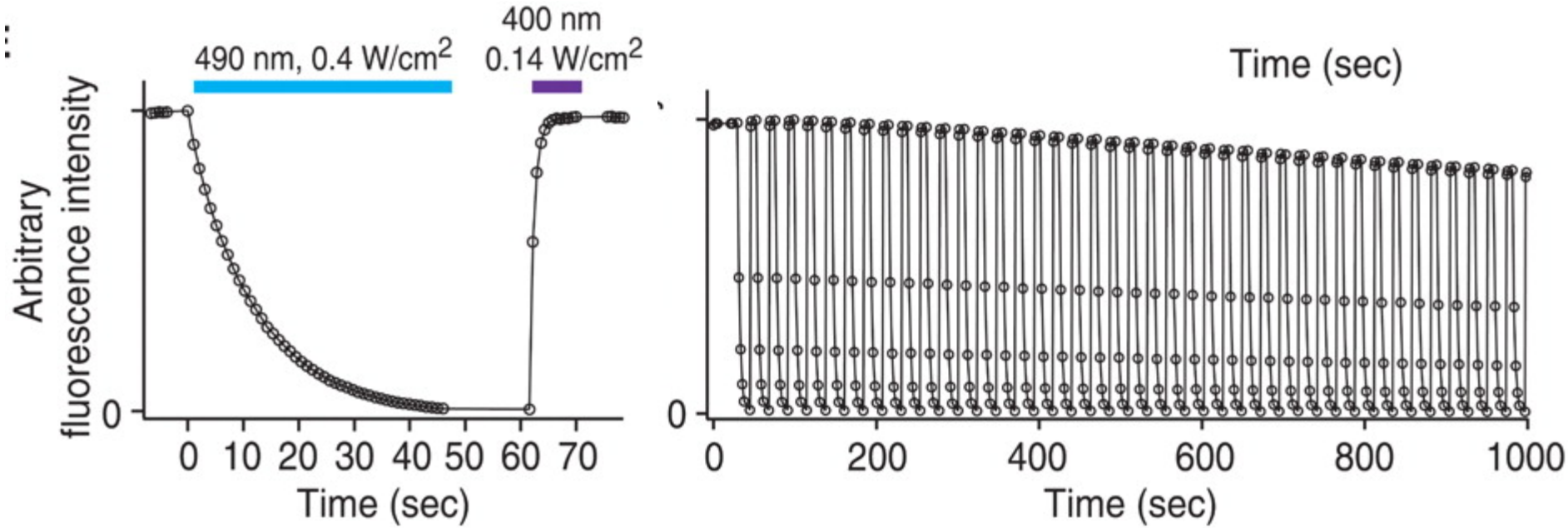
Color change
- Kaede, KikGR, Eos,
- Dendra (activatable by blue)

Reversibly Switchable
- asCP, KFP (tetrameric)
- Dronpa

Dendra2 demo

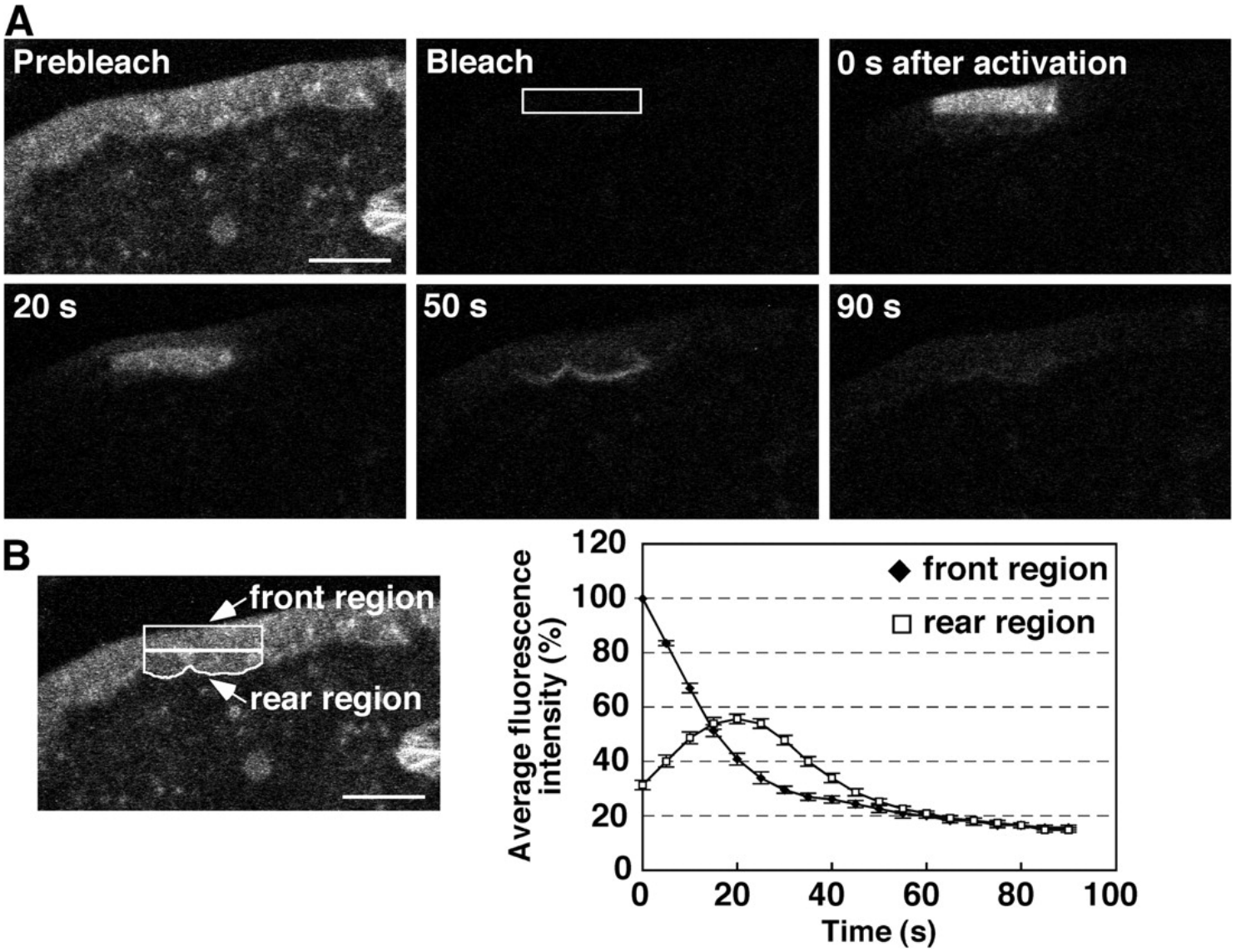


Dronpa – photoswitchable on and off



Ando et al. 2004, Science 306: 1370-1373

Tracking actin flow with Dronpa

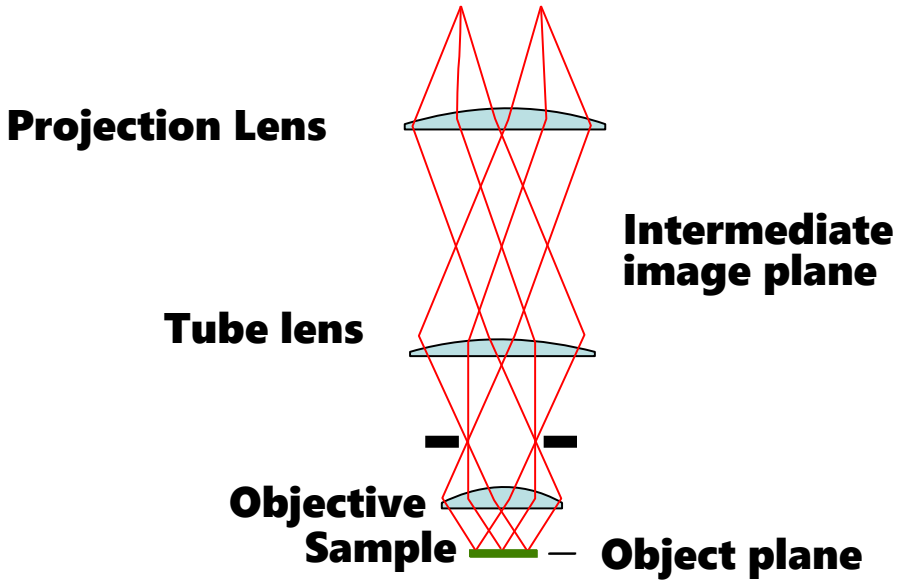


Kiuchi, T. et al. J. Cell Biol. 2007;177:465-476

Hardware for photoactivation / photobleaching

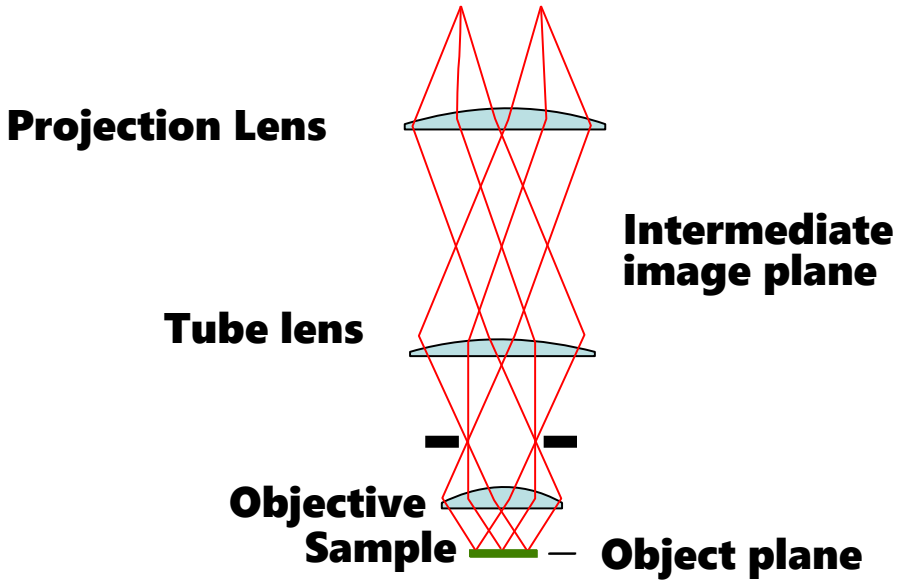
- Need: controlled illumination of a region in the sample

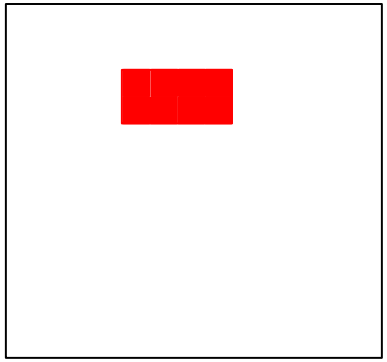
Laser-scanning confocal microscope



Use mirrors to adjust angle at back focal plane – changes location of laser spot at sample

Laser-scanning confocal microscope

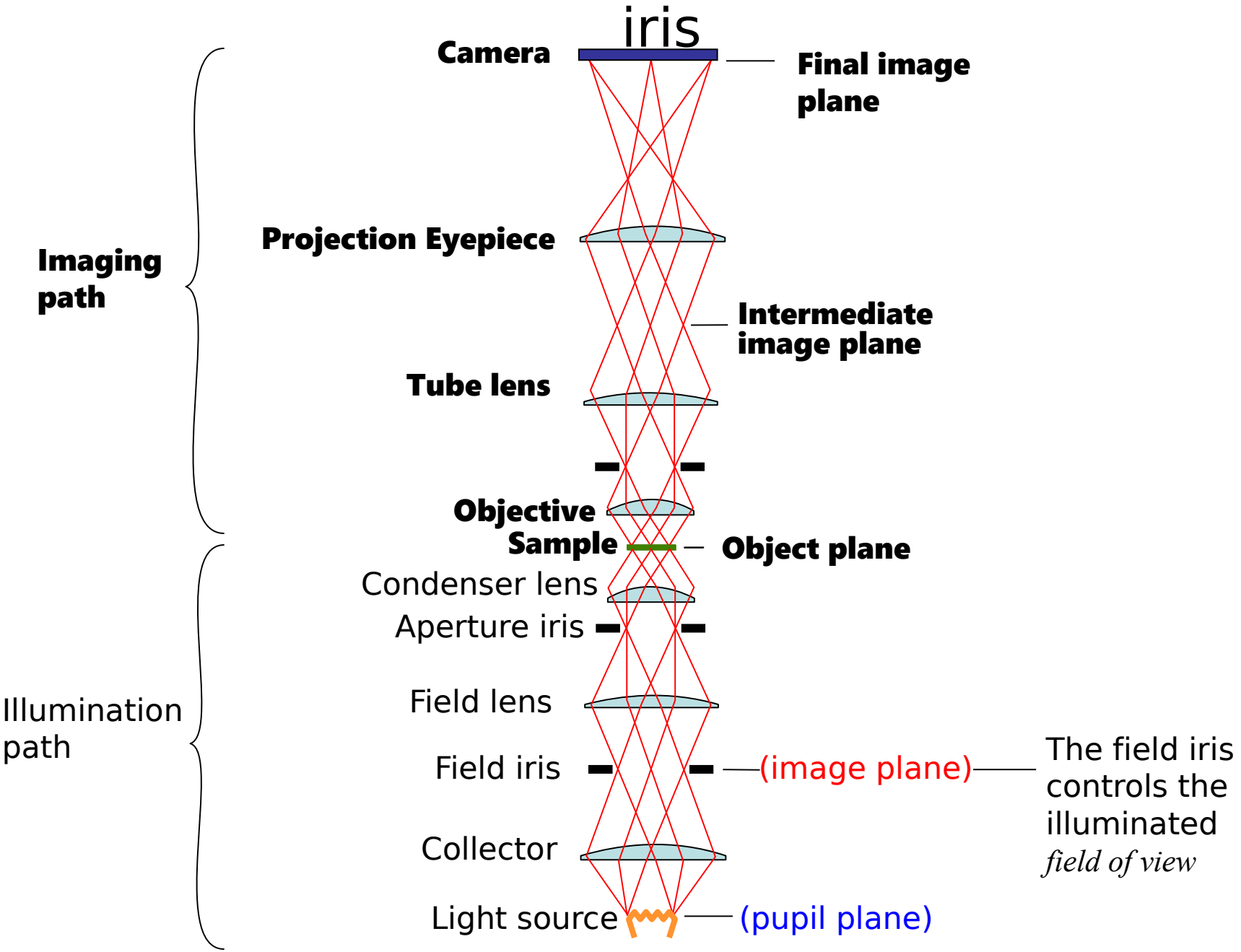




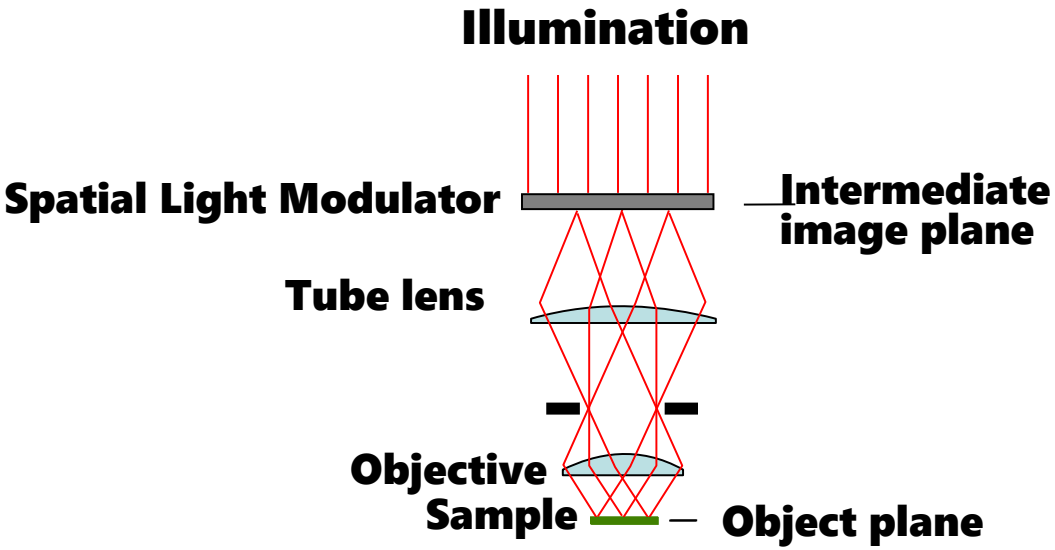
Laser-scanning confocal microscope

- Pros
  - can bleach / activate any shape ROI by scanning point by point
- Cons
  - Slow
  - Need a laser scanning confocal

Can control field of view with field iris

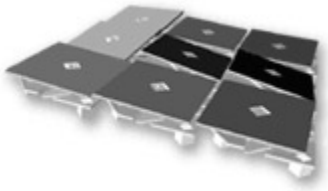


Better idea: Spatial Light Modulator

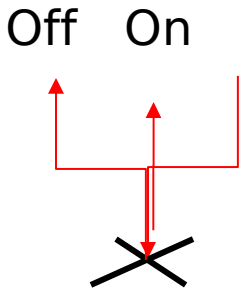


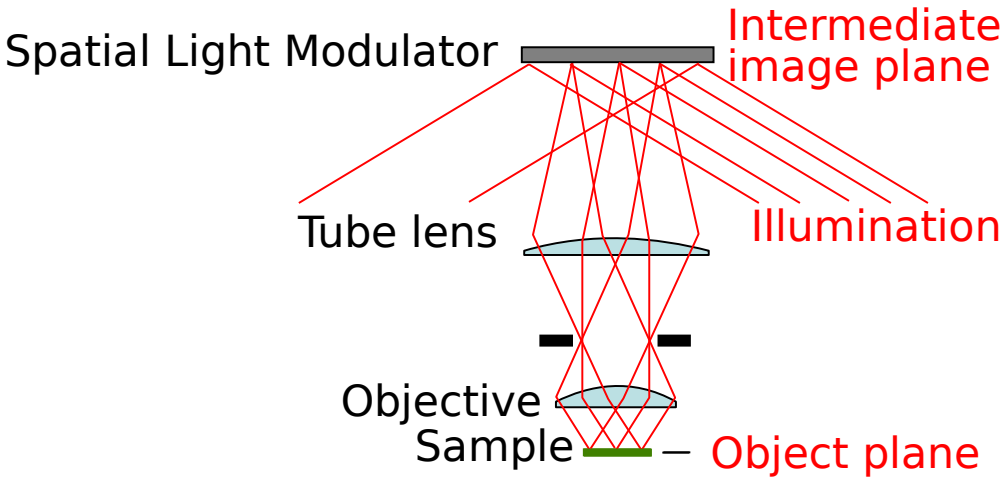
Can project any image onto sample

Digital Micromirror Device



Array of rotatable mirrors





Pros: Fast, can project any arbitrary image

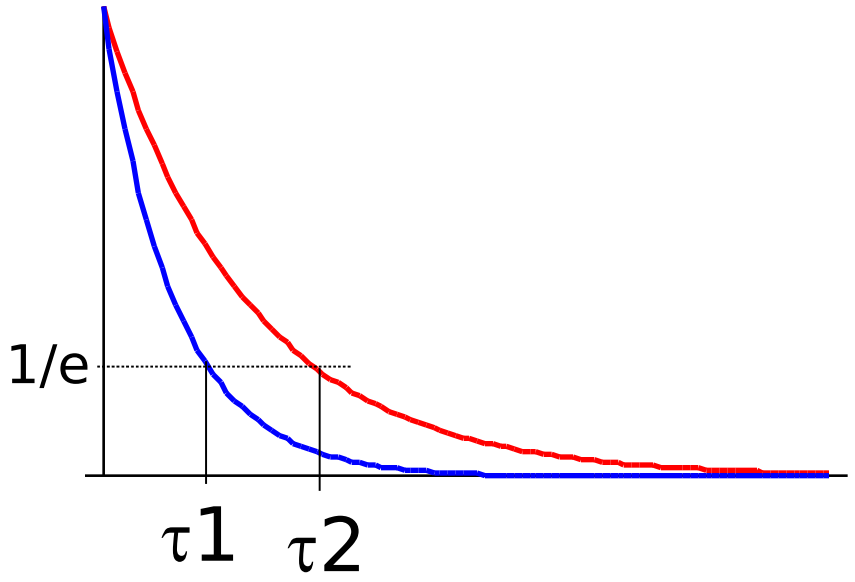
Cons: Intensity is relatively low

FLIM

Fluorescence Lifetime Imaging

Measure the lifetime of the excited state

- Separate fluorophores with similar spectra
- Detect environmental parameters that affect lifetime (FRET, pH, ...)



FLIM

Measurement approaches

- Frequency domain
  - Modulated excitation
  - Lock-in detect emission phase

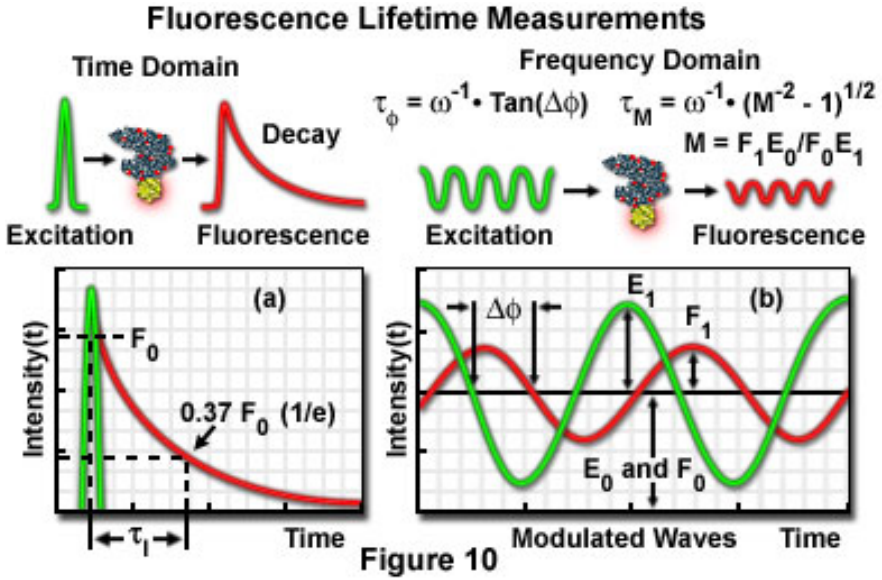
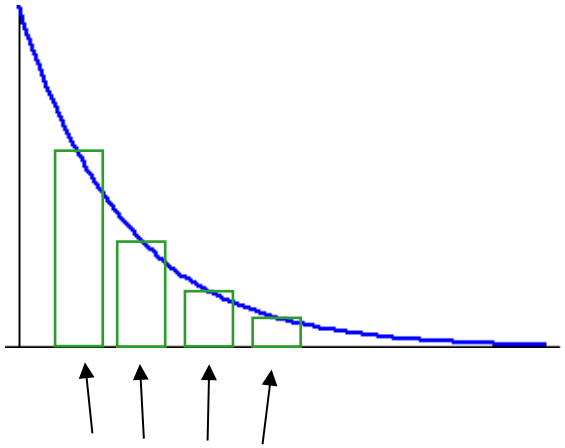


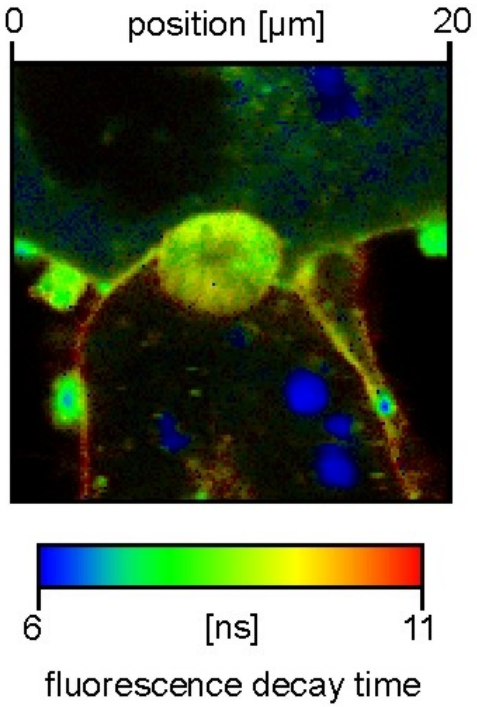
Figure 10

Time domain (pulsed exc.)

- Gated intensifier
  Photon inefficient
- Time-correlated
  single photon counting
  Very efficient
  ≤ one photon per pulse ⇒ slow



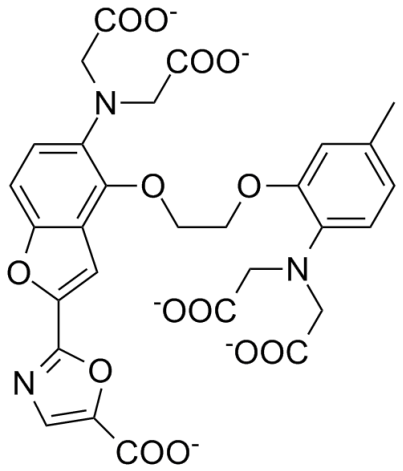
FLIM
Examples

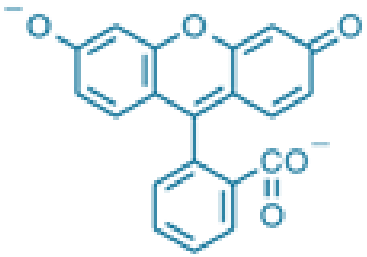


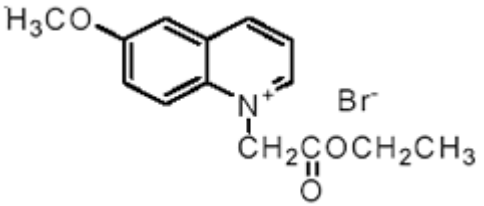
Hepatocyte membrane-stained with NBD, which has a hydrophobicity-dependent lifetime (TCSPC, 3 minutes for 300x300 pixels)

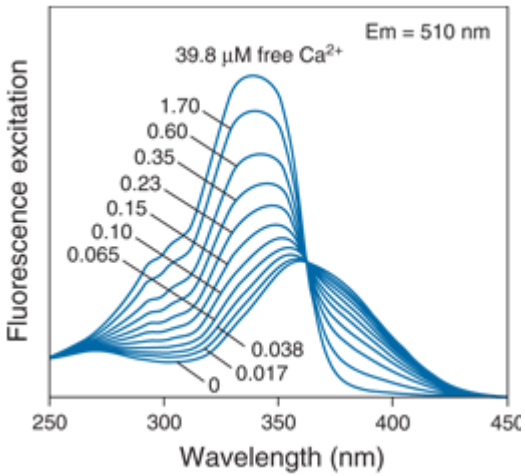
Environment-sensitive fluorophores

Many: pH, ions (Ca2+, Mg2+, Na+, K+, etc.), voltage, hydrophobicity,…

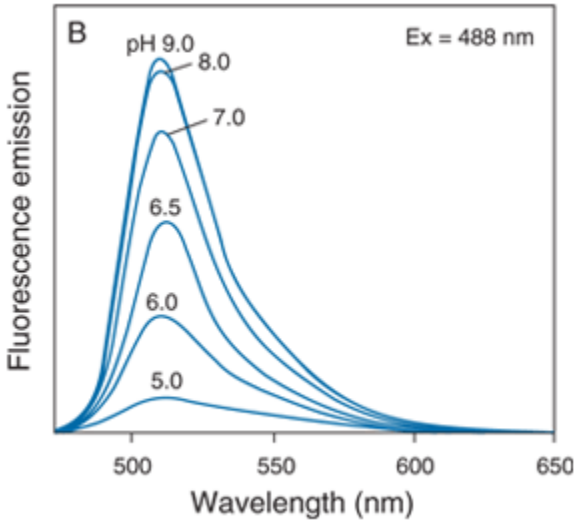




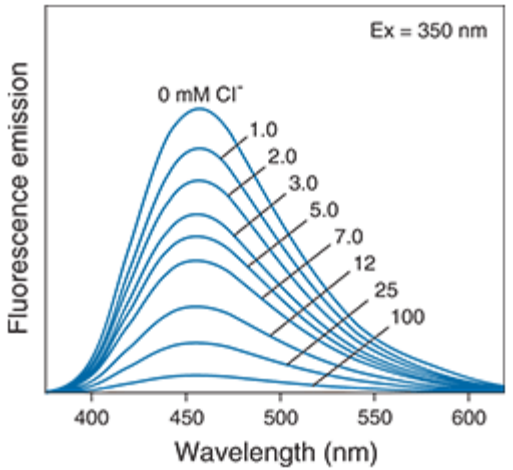




Fura-2



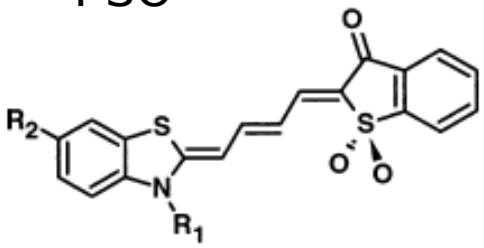
Fluorescein

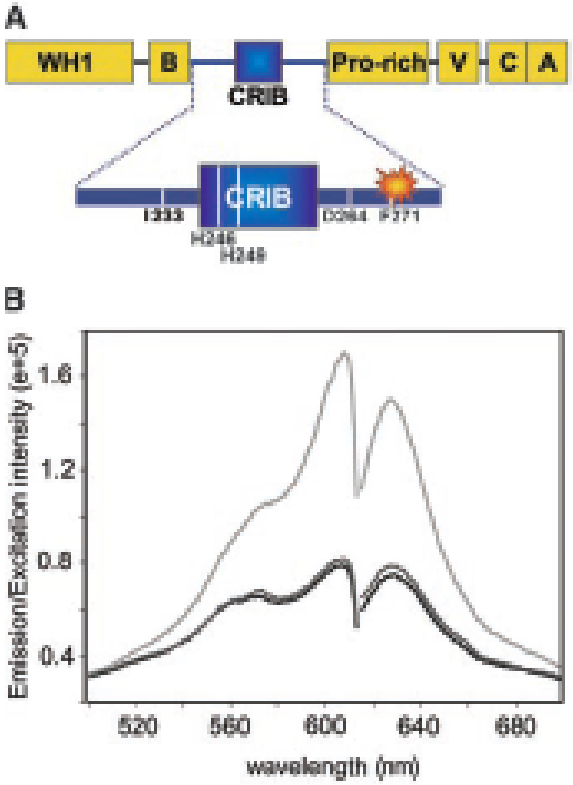


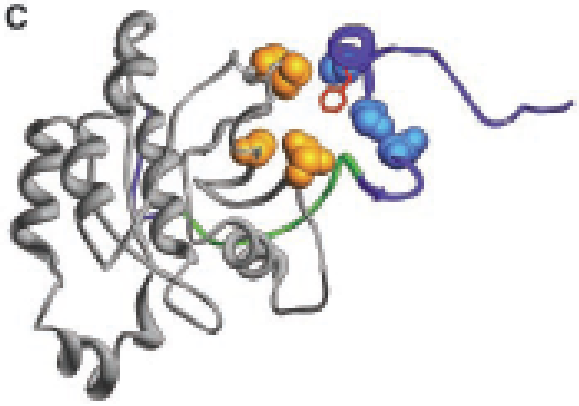
MQAE

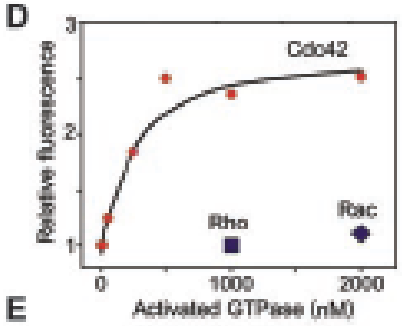
Environment-sensitive fluorophores

I-SO



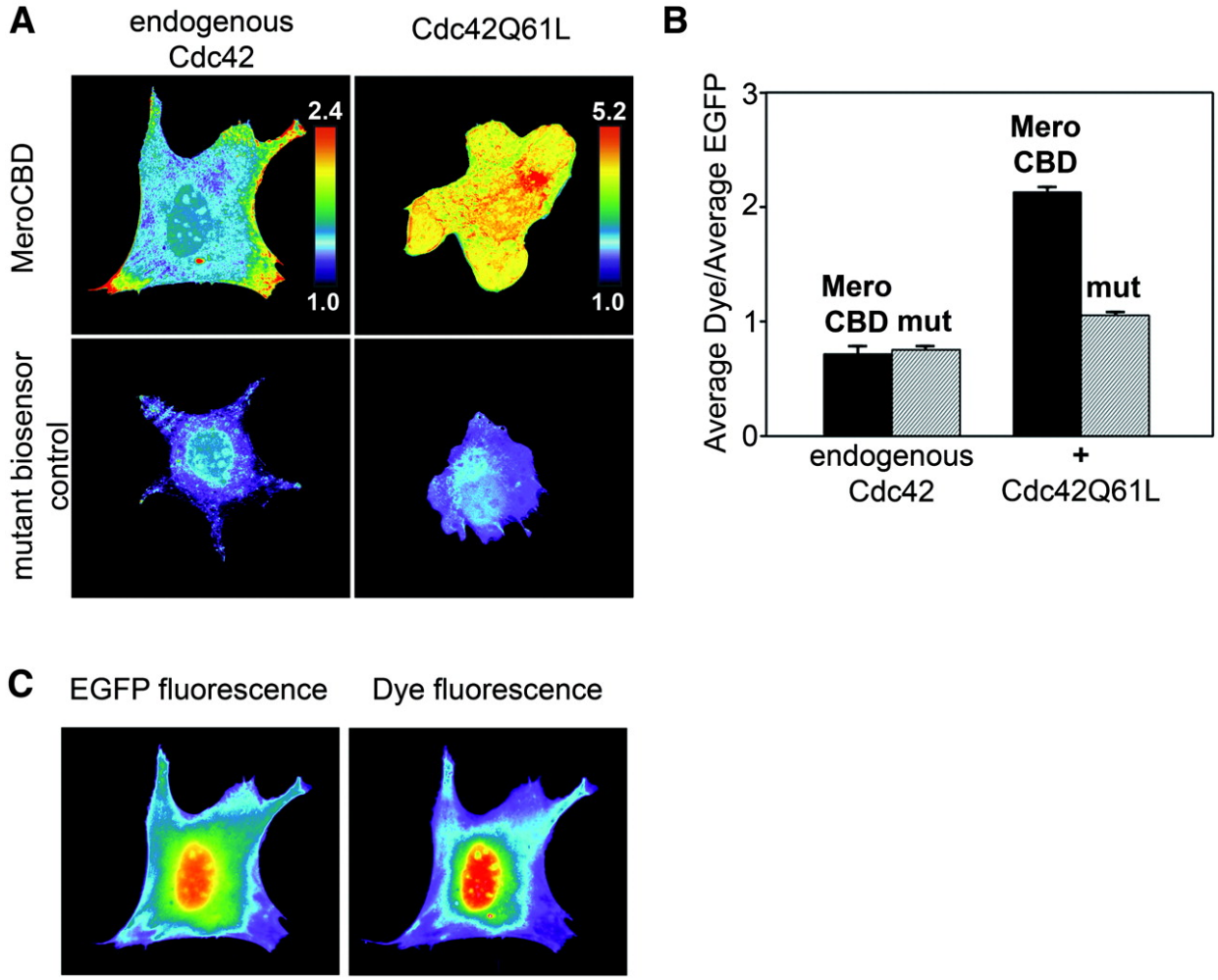






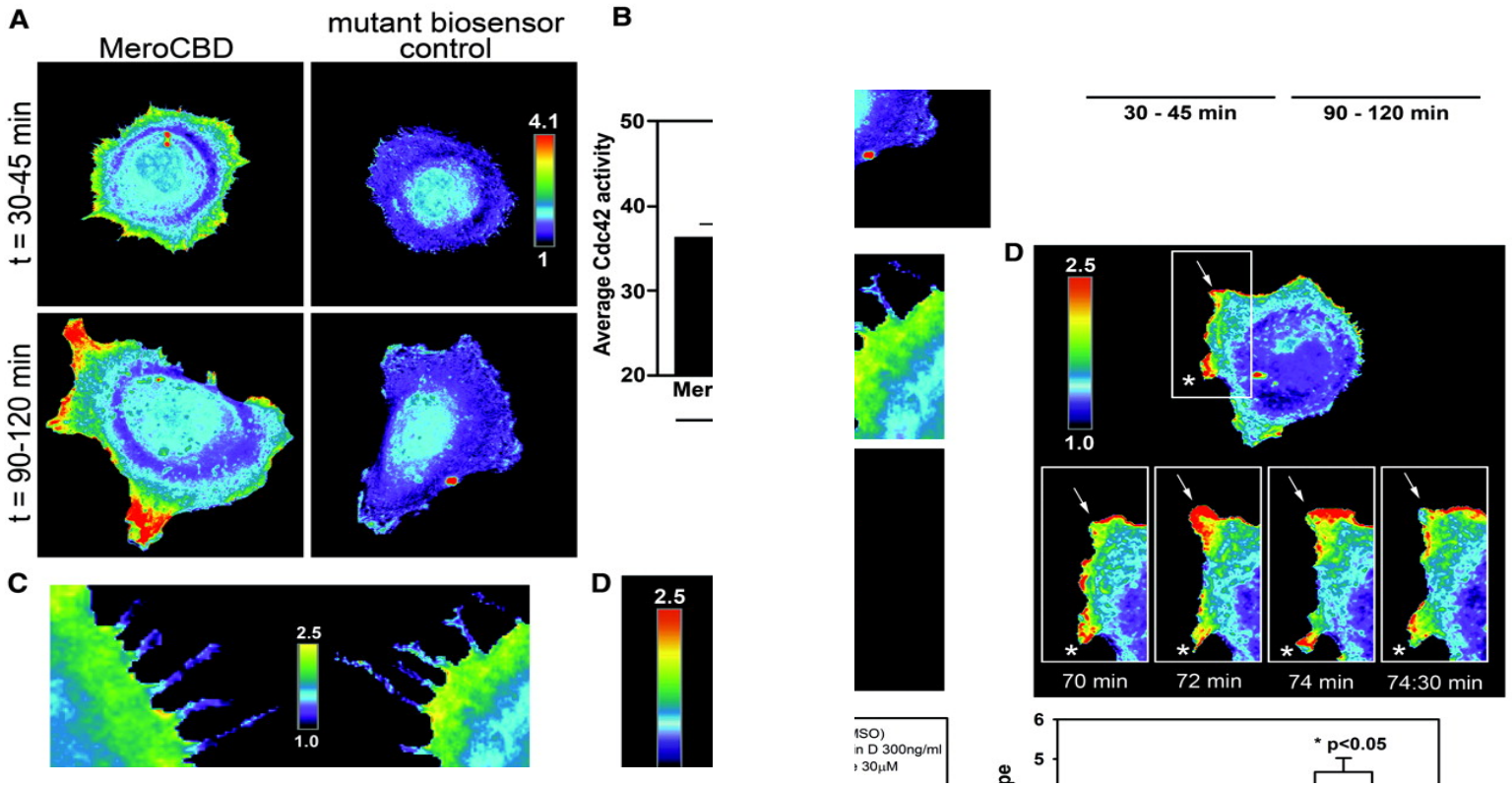
Nalbant et al. 2004, Science 305: 1615-1619

Imaging Cdc42 activation

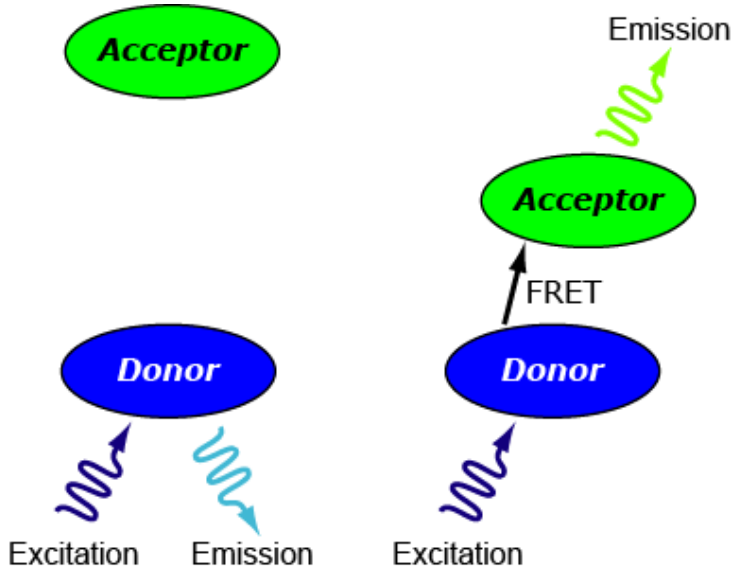


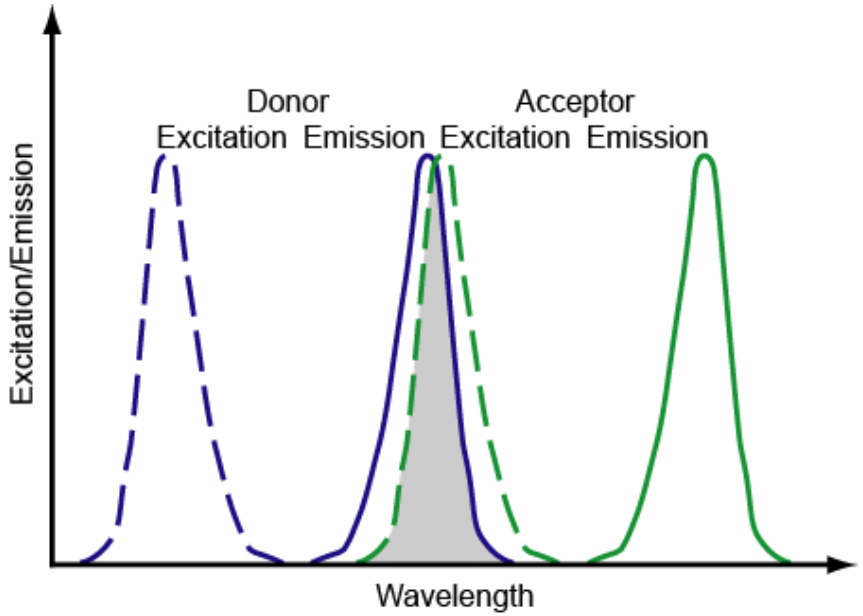
Fuse sensor to GFP; I-SO/GFP ratio is proportional to degree bound

Monitoring Cdc42 activation



Fluorescence Resonance Energy Transfer





Sensitive to distances on the order of 10 nm, 20-fold below resolution limit

Distance dependence of FRET

$$E = \frac{1}{1+(r6/R06)}$$

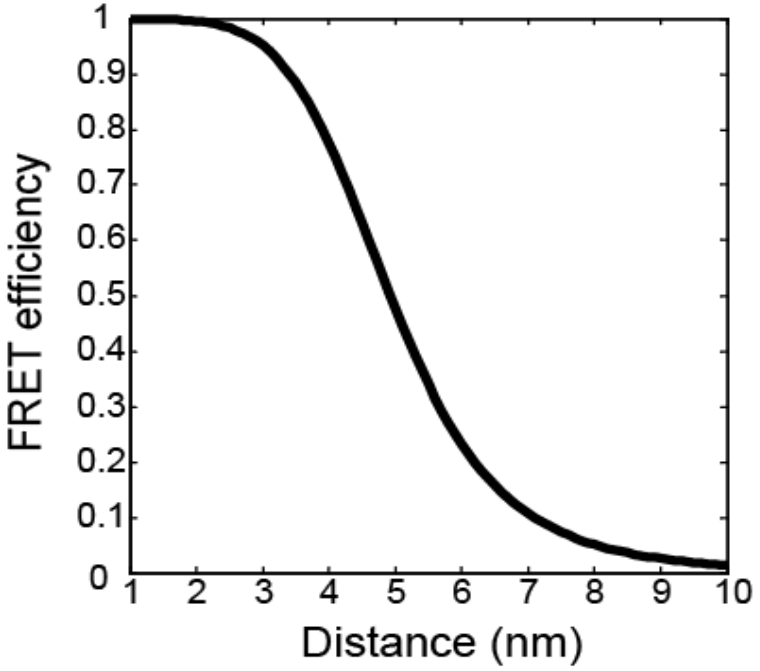
$$R06 \propto k2\ n\text{-}4\ QD\ J(l)$$

Orientation factor

Refractive index

Donor quantum yield

Overlap between donor emission and acceptor excitation



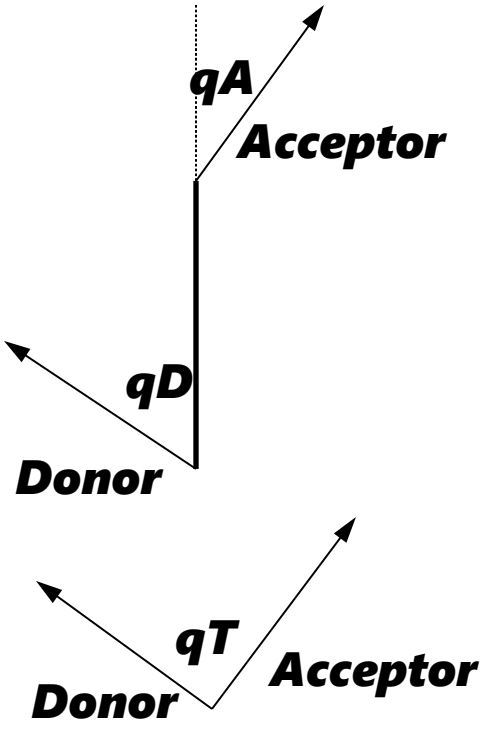
For CFP-YFP,
50% transfer at R0 = 4.9 nm

Good FRET pairs

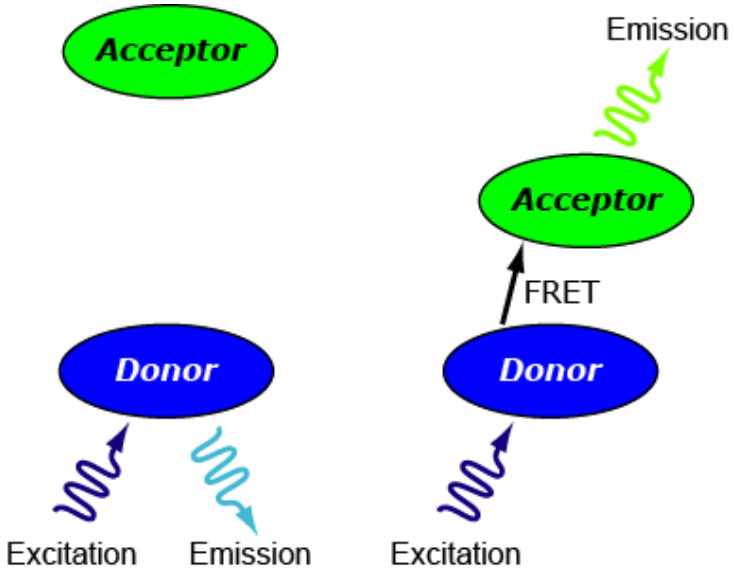
- CFP/YFP – use A206R mutants if dimerization is problematic
- GFP/mCherry or other FP pairs – not so well validated
- Fluorescein/Rhodamine
- Cy3/Cy5 or Rhodamine/Cy5
- Many other small molecule pairs

FRET Theory

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

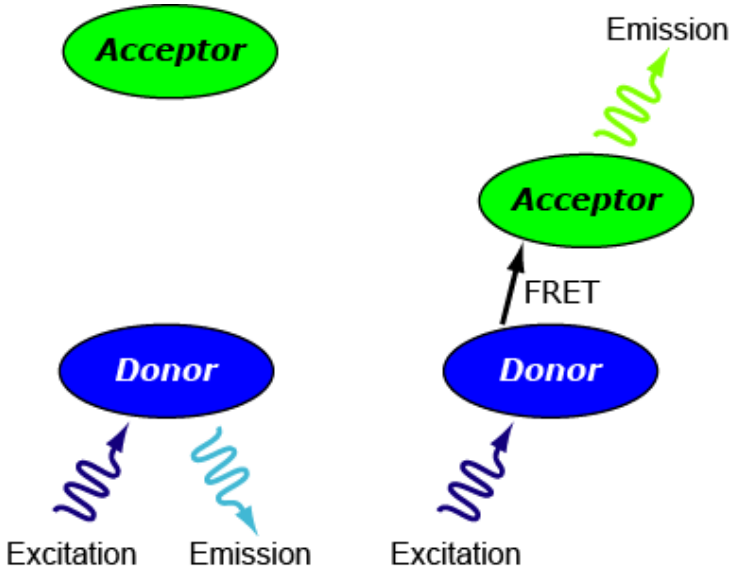


Effects of FRET



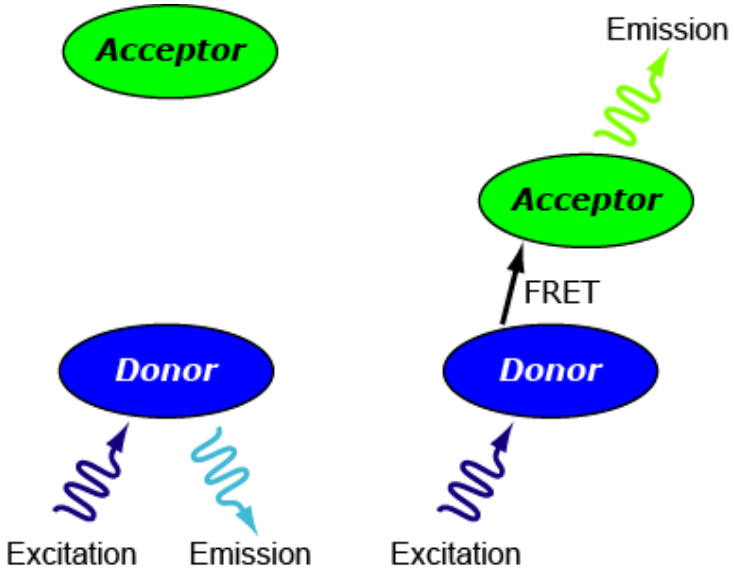
- Donor lifetime shortened
- Acceptor emission depolarized
- Donor fluorescence quenched
- Acceptor fluorescence enhanced on donor excitation

Measuring FRET



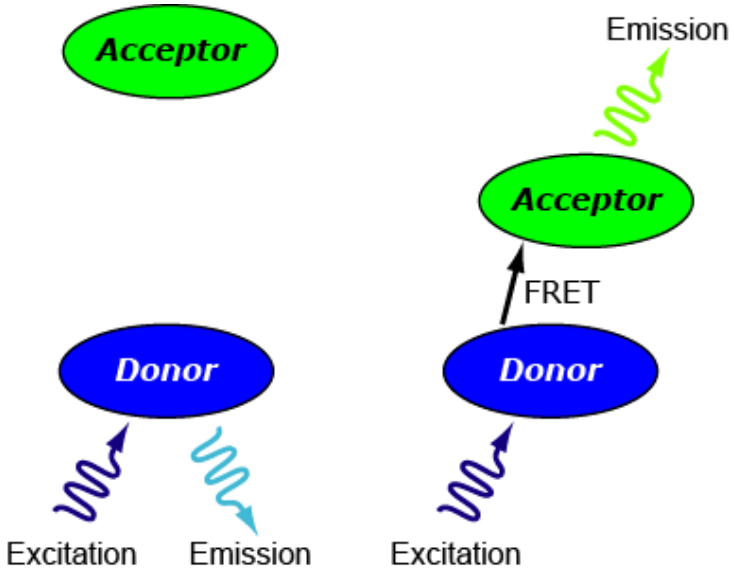
- Donor lifetime shortened
- Can measure by fluorescence lifetime imaging, but requires specialized instrumentation

Measuring FRET



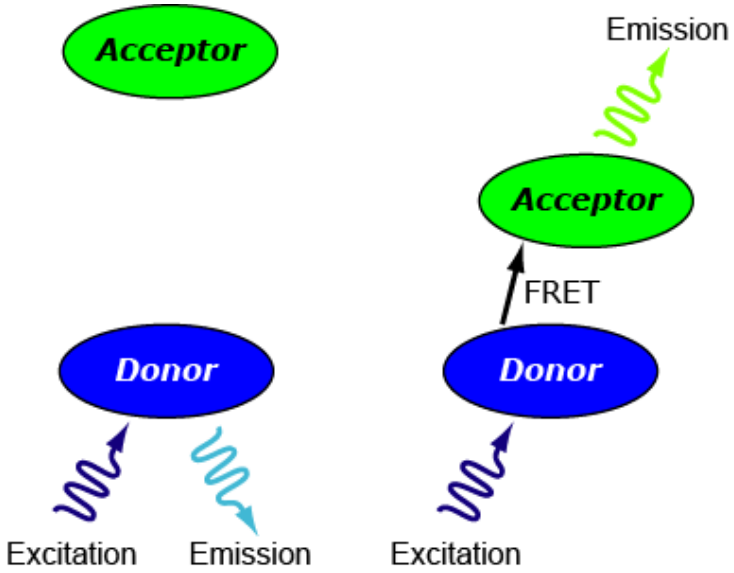
- Acceptor emission depolarized
- Can measure by fluorescence polarization microscopy

Measuring FRET



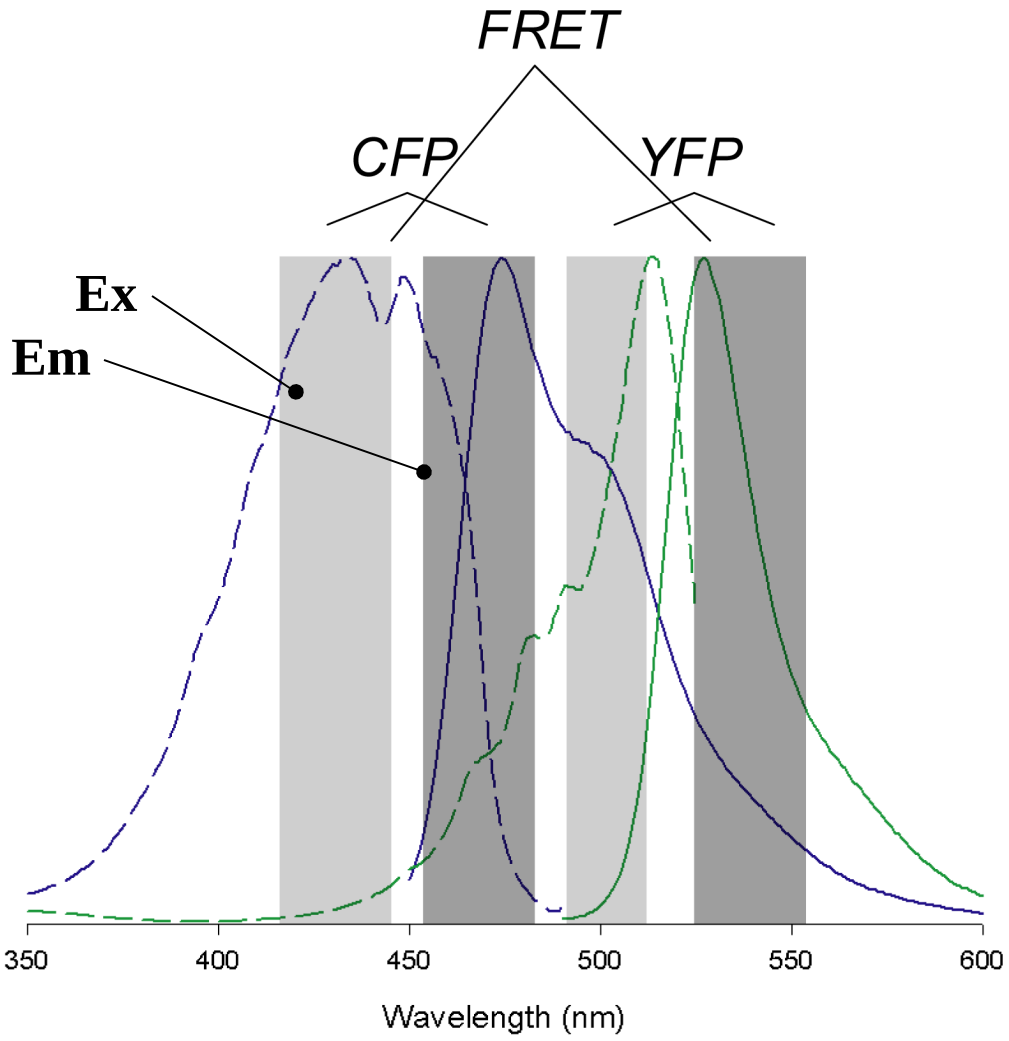
- Donor fluorescence quenched
- Acceptor fluorescence enhanced on donor excitation
- Can measure by donor recovery after acceptor photobleaching
  - Easy, but very sensitive to degree of photobleaching

Measuring FRET



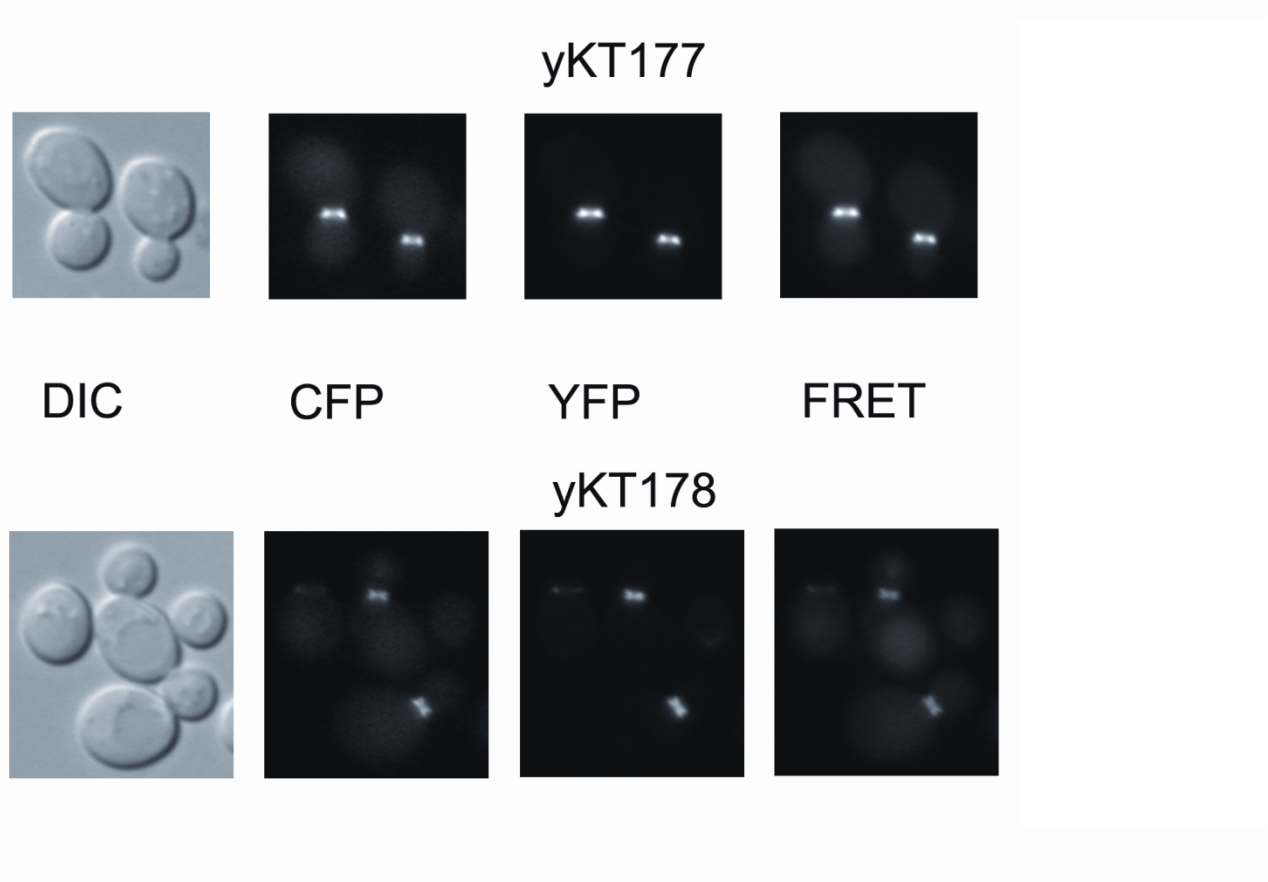
- Donor fluorescence quenched
- Acceptor fluorescence enhanced on donor excitation
- Can measure by quantitative measurement of acceptor enhancement on donor excitation

A problem: crosstalk into FRET channel

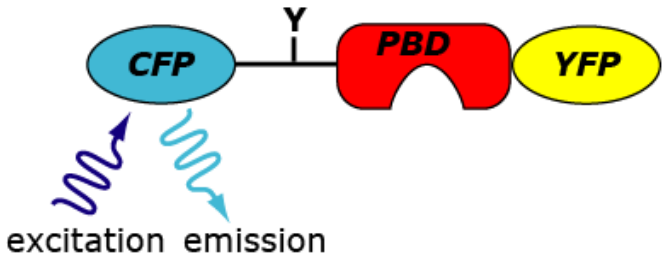


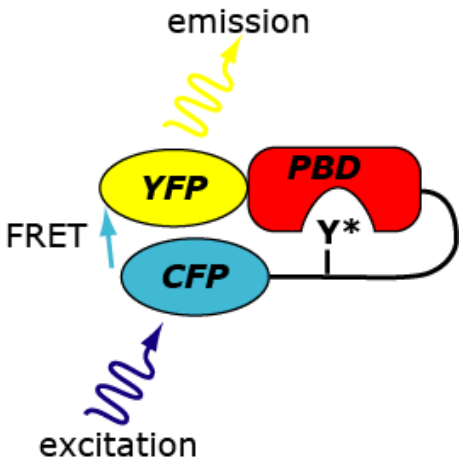
Correct using measurements from CFP- and YFP- only cells

Crosstalk correction

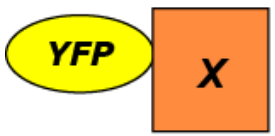


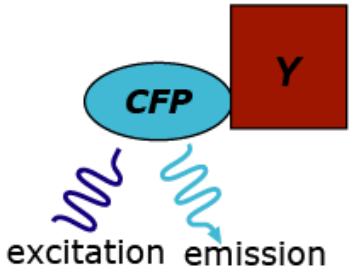
Types of FRET experiments

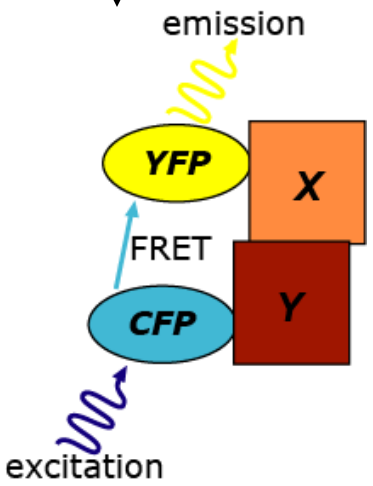




Intramolecular

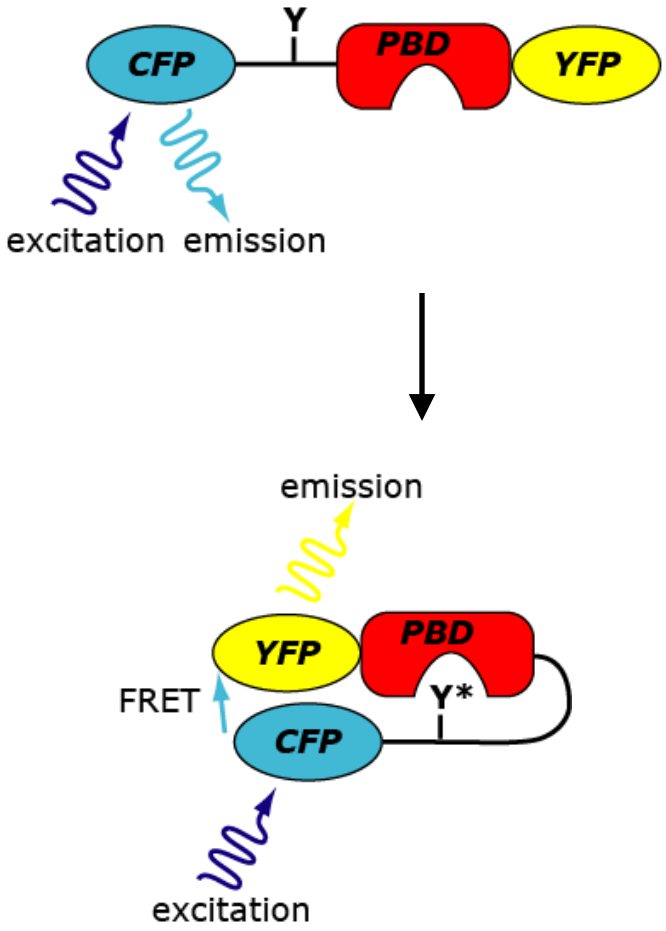






Intermolecular

Types of FRET experiments

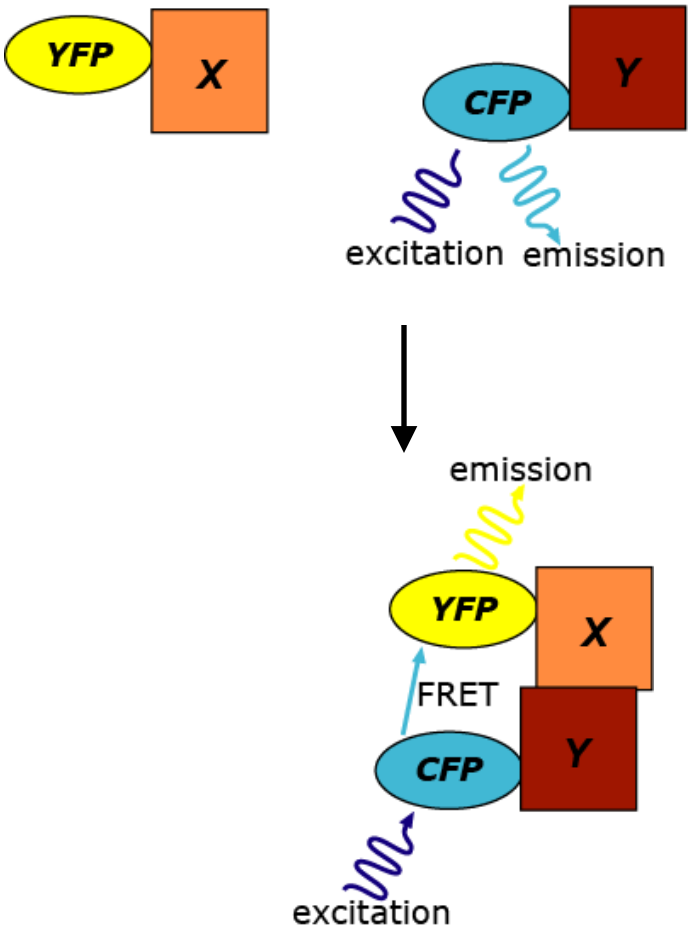


Intramolecular

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

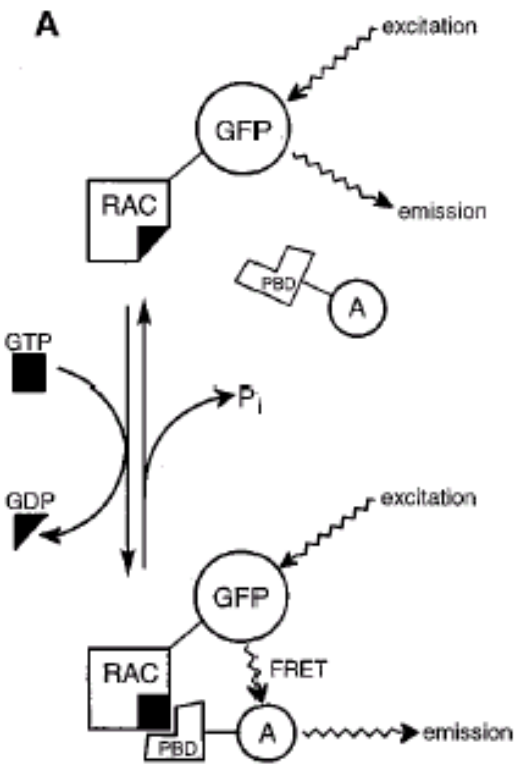


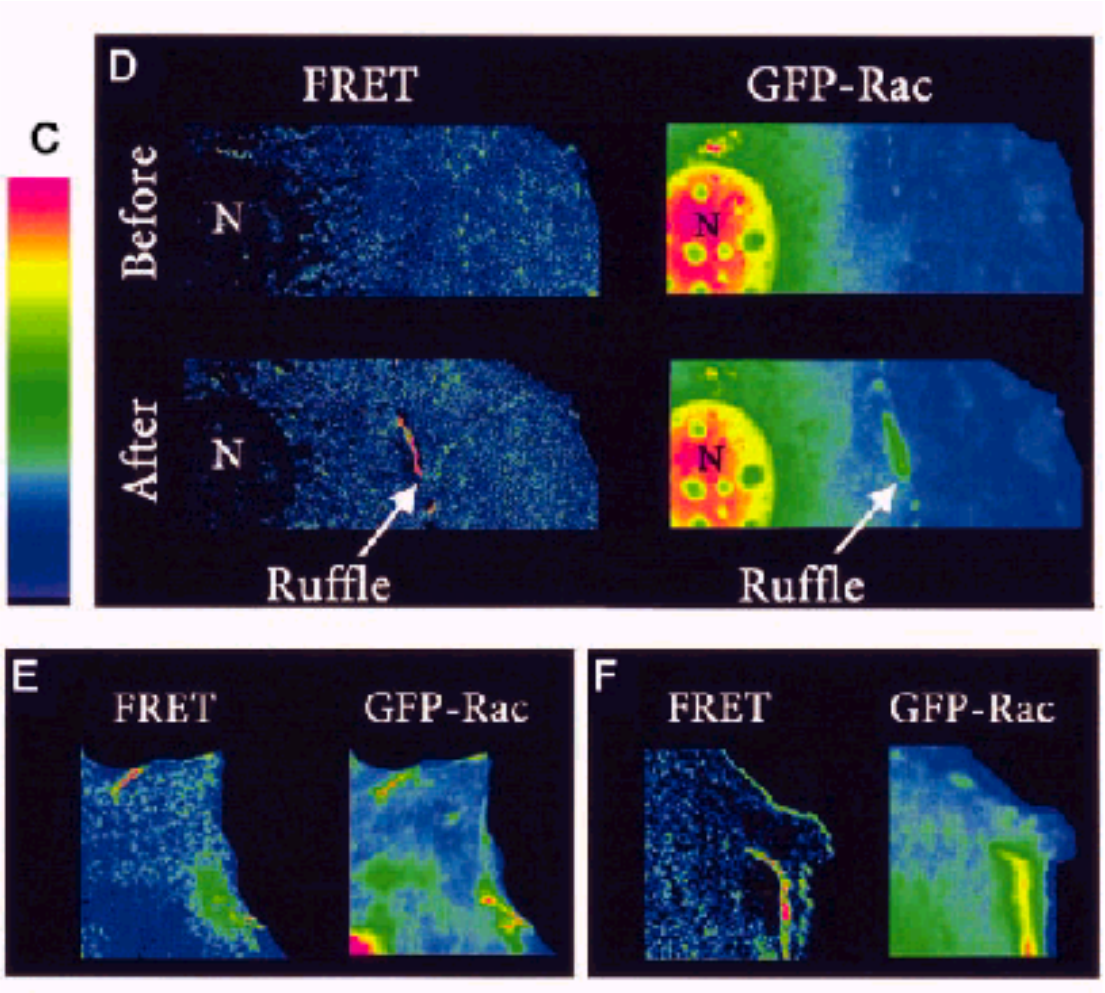
Intermolecular

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.

Using FRET to monitor Rac activation





Kraynov et al. 2000, Science 290: 333

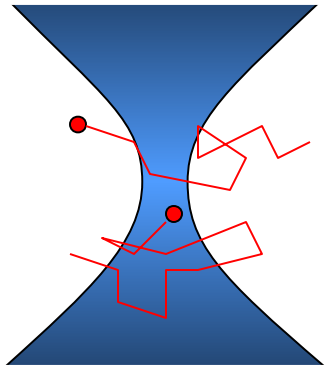
FCS
Fluorescence Correlation Spectroscopy

Small volume → only a few molecules → random fluctuations

- Study the *noise*
- Conclude about *random processes* at different *time scales*

Small excitation volume
Random molecular processes:

- Diffuse in and out
- Adopt different states
- Bind or react
- Photobleach
- …



Study the auto-
(or cross-) correlation

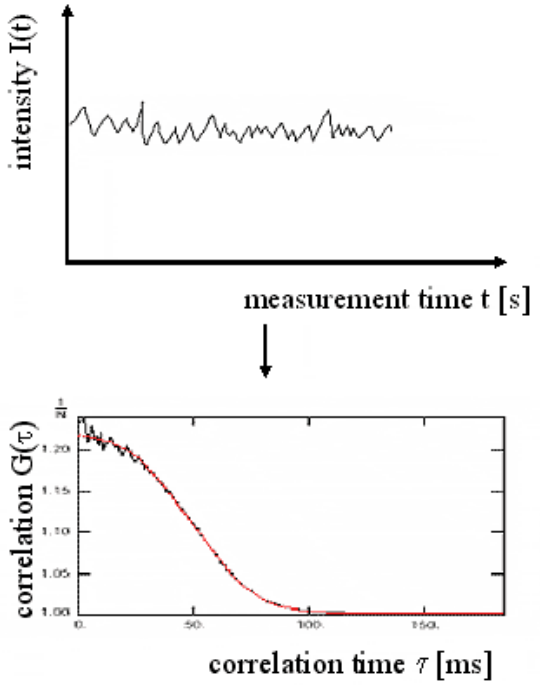


Image Correlation Spectroscopy
Image FCS

Sense the random fluctuations of fluorescence within an *image* or *image sequence*

Much slower than point FCS, but get whole area
Can see where you are & deal with motion
Can do spatio-temporal analysis

Image series
Measure time and space variations in intensity

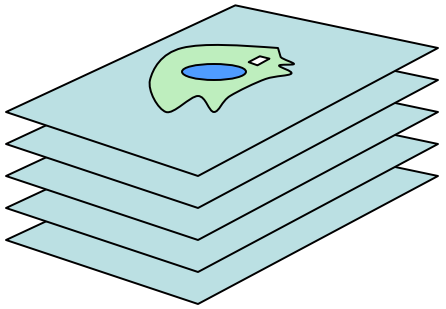
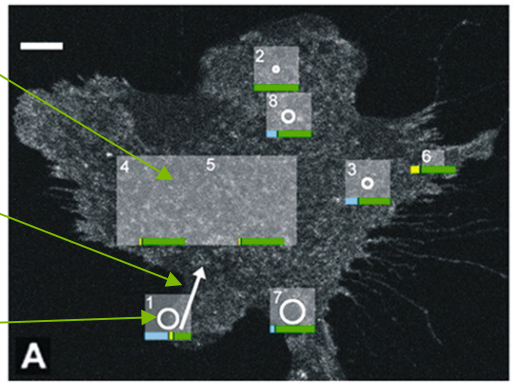


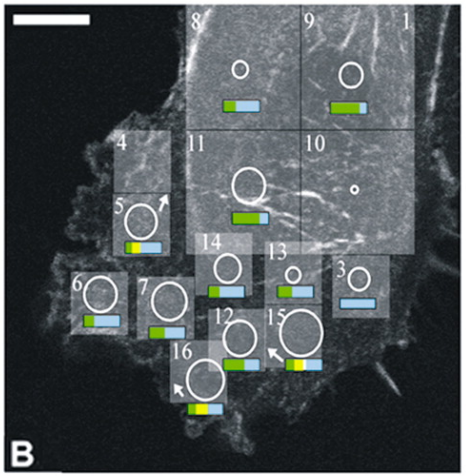
Image Correlation Spectroscopy

Areas analyzed

Flow vector

=10min diffusion length

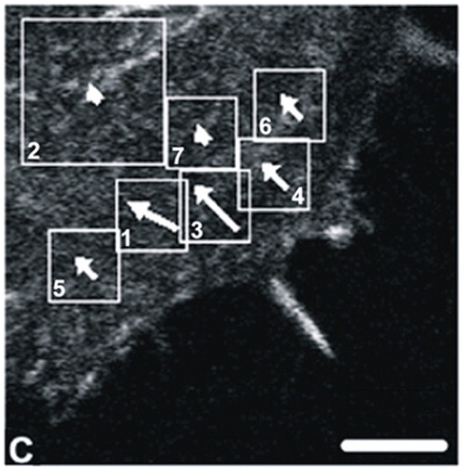


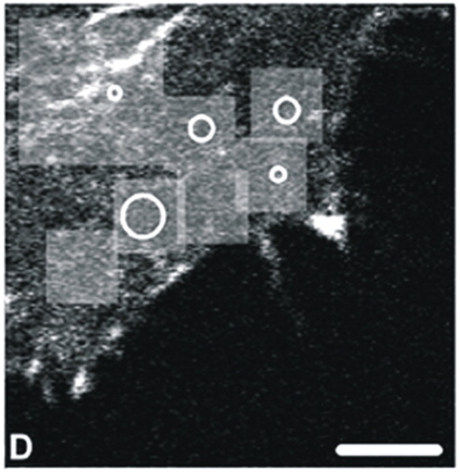


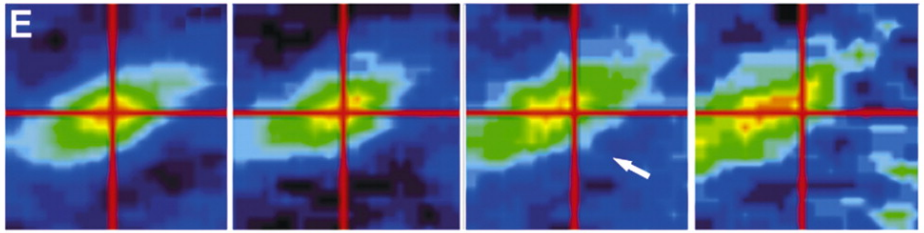
Stationary fraction

Flowing fraction

Diffusing fraction



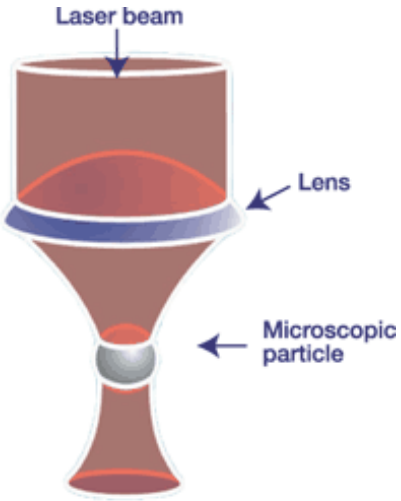




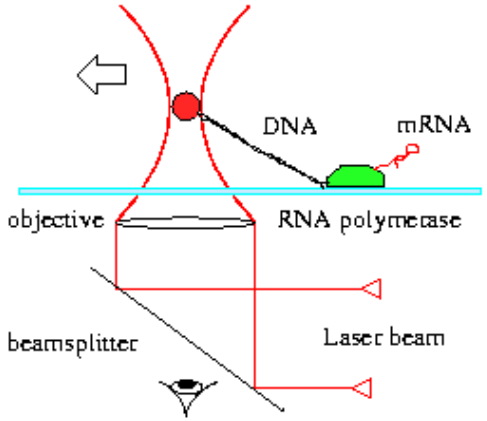
Spatio-temporal autocorrelation

Optical Tweezers

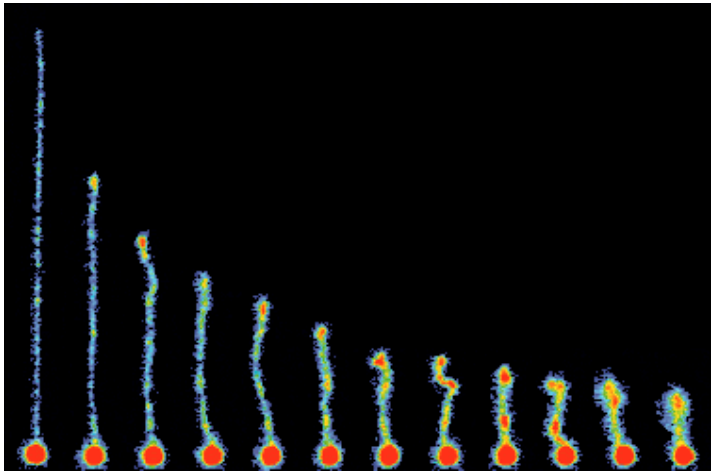
Mechanically manipulate the specimen with light



Why?



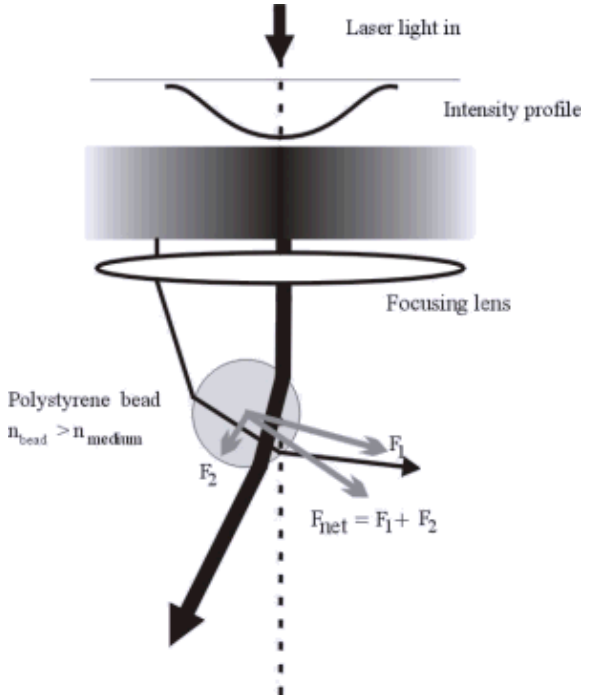
Measuring force and displacement of a single polymerase molecule



DNA recoil after stretching

Optical Tweezers
How does it work?

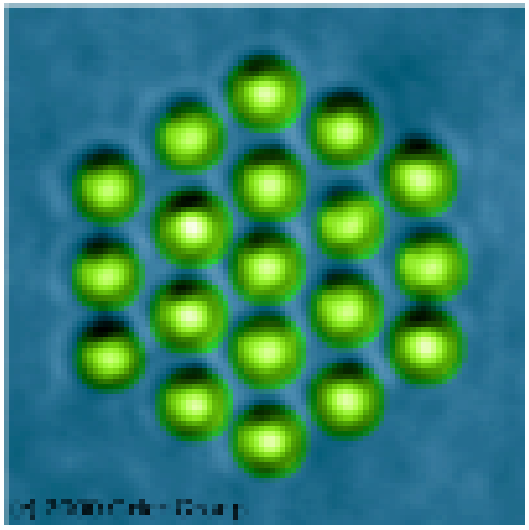
Photons carry momentum
Changing photon direction requires a force
More light refracted away from high intensity region
→ Force toward intensity maximum

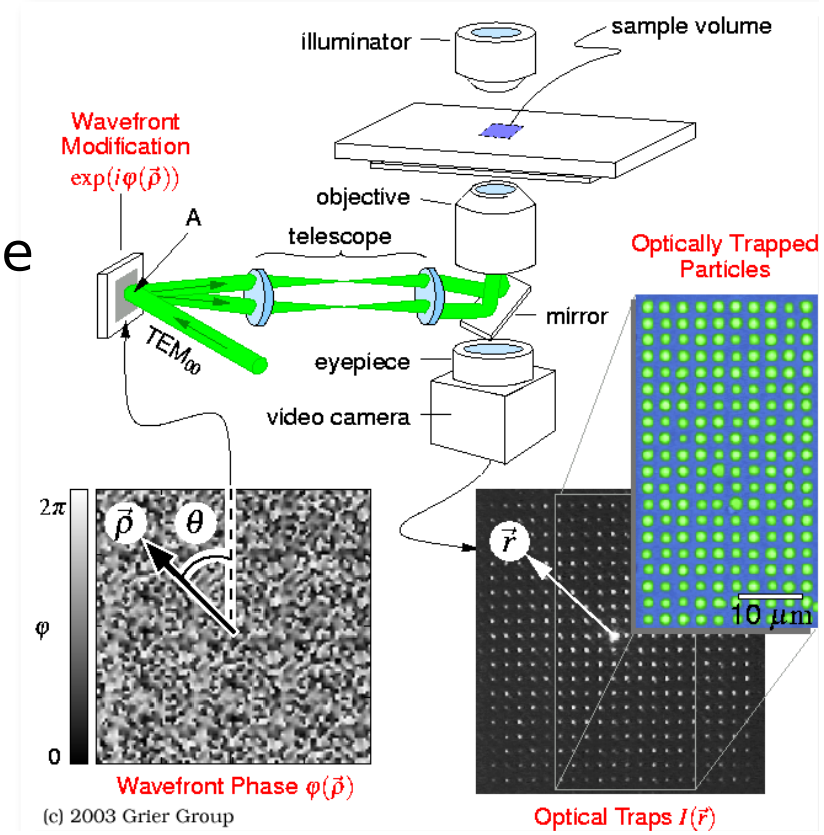


Alternative way to look at it:
Field energy is less in higher index
System energy depends on particle position
dE/dx = Force

Holographic Optical Tweezers

Many traps at once
Independently movable
Made using a computer-controlled
spatial light modulator in a pupil plane





Further reading

www.microscopyu.com

micro.magnet.fsu.edu

Molecular Probes Handbook (probes.com)

James Pawley, Ed. “Handbook of Biological Confocal Microscopy, 3rd ed.”

Acknowledgements

Mats Gustafsson