Applications of confocal fluorescence microscopy in biological sciences

B R Boruah

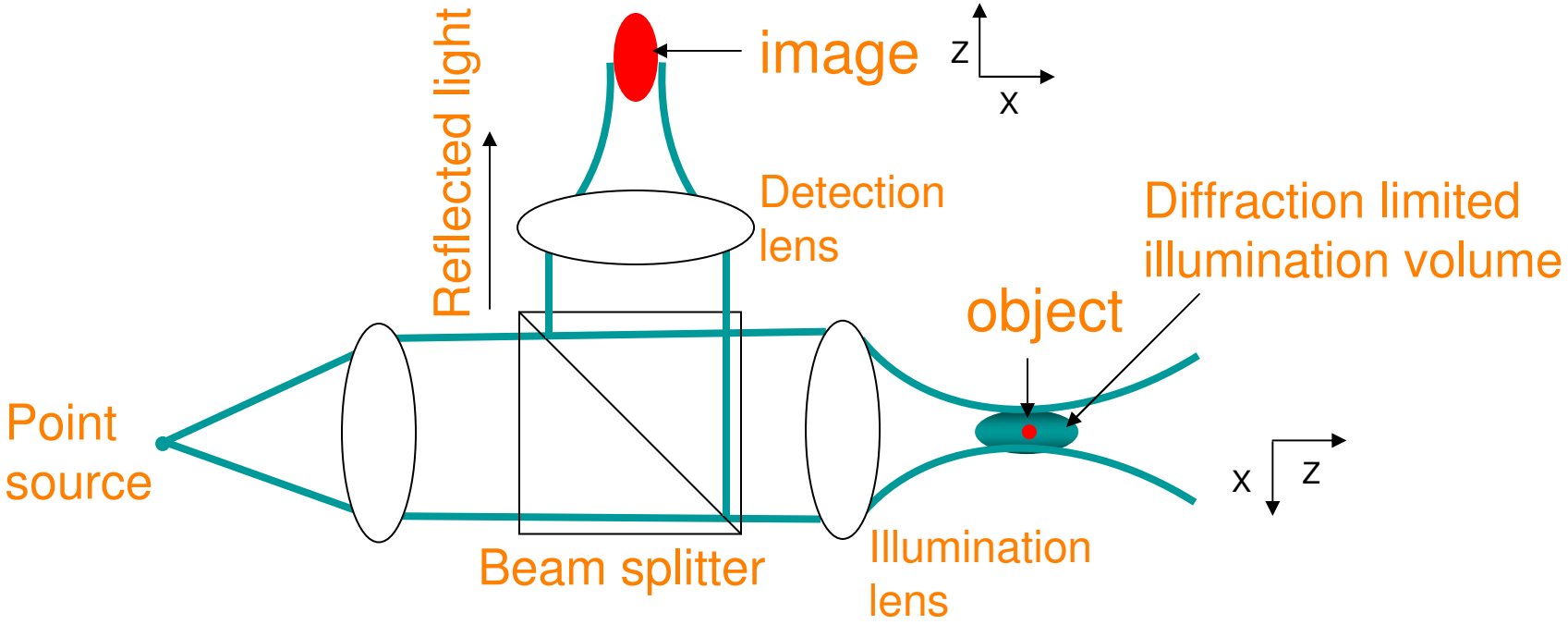
Department of Physics

IIT Guwahati

Email: brboruah@iitg.ac.in

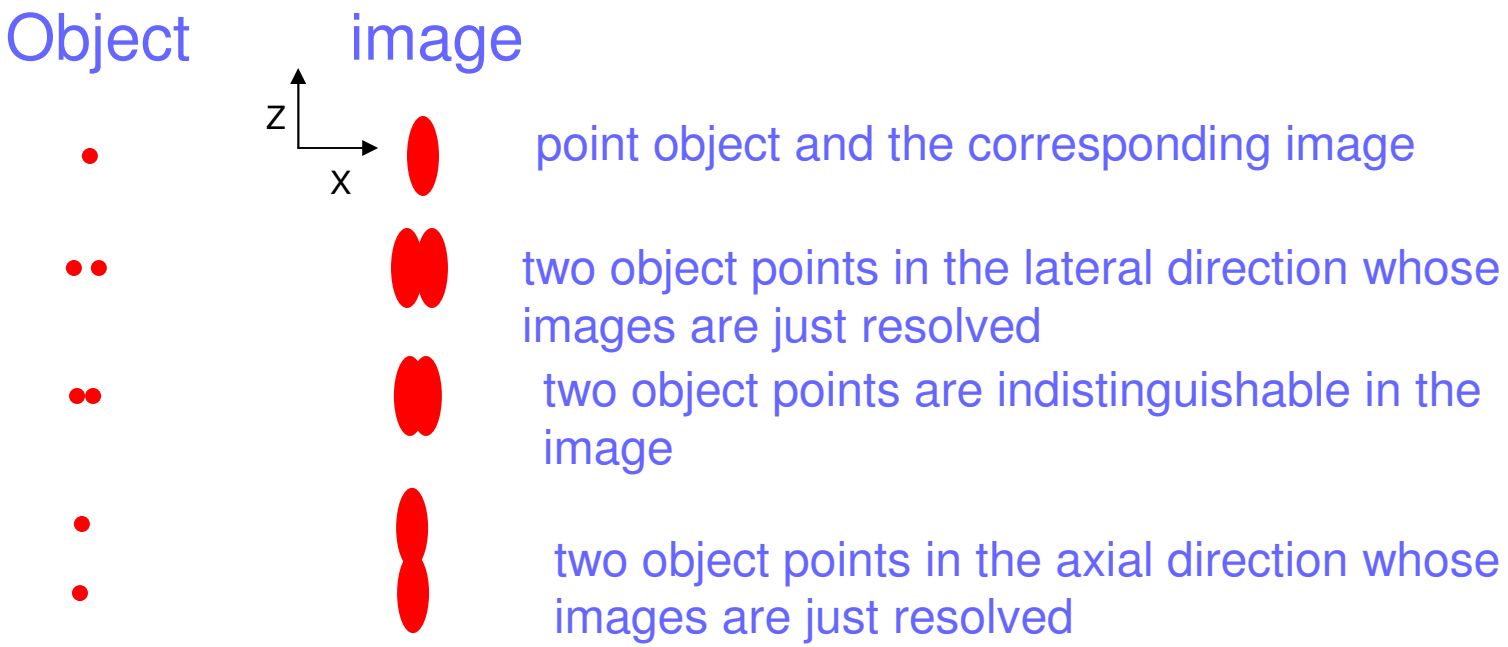
Contents

A simple microscope



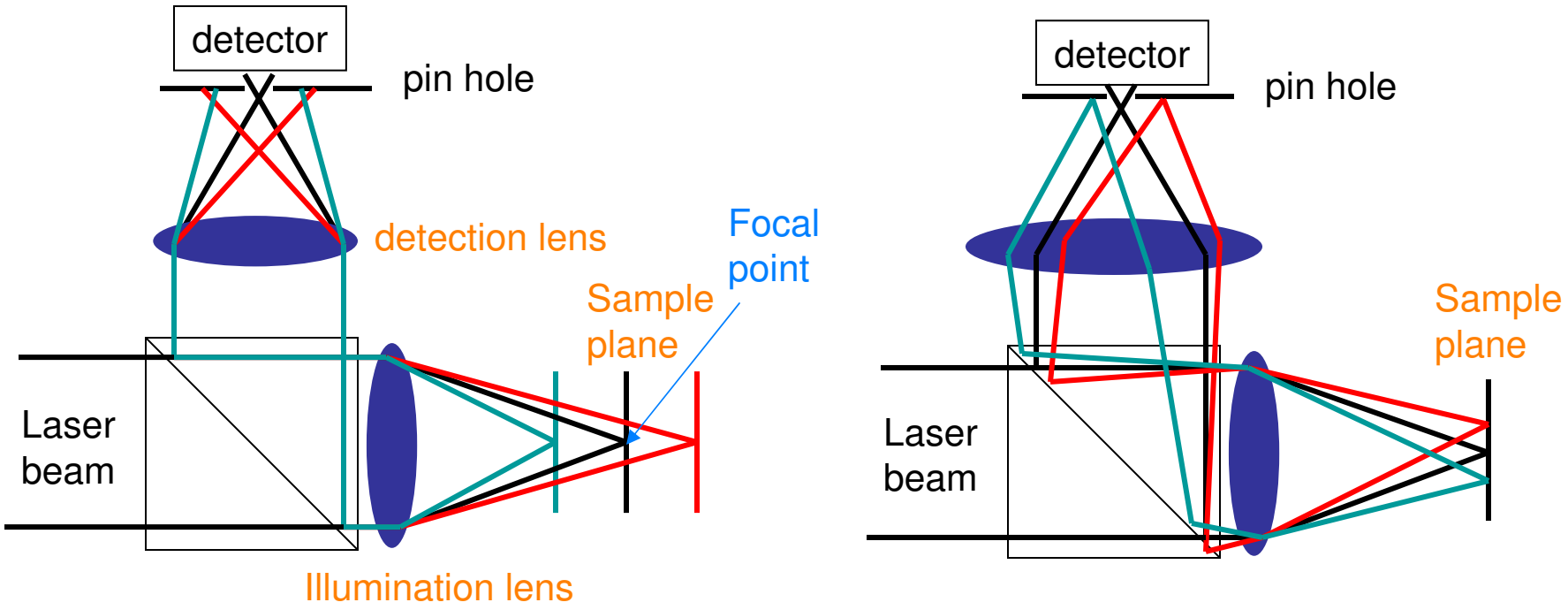
- Collimated beam of wavelength λ is focused by L1 to a diffraction limited volume
- The illumination volume depends on λ, focal length and diameter of the illumination lens
- A point object is imaged into a diffraction limited volume in the image space

Resolution of a microscope



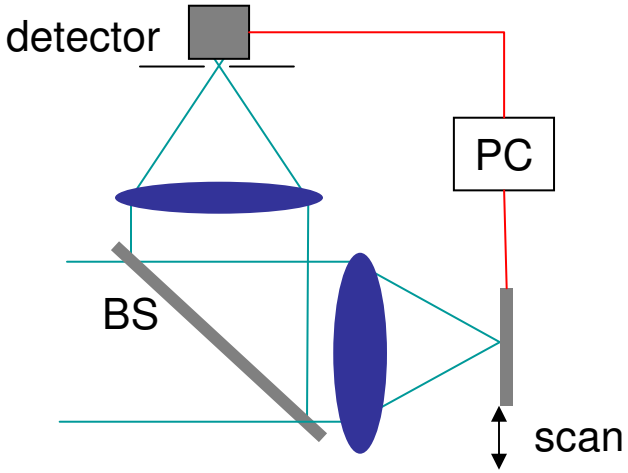
- Resolution: minimum separation between two point objects whose images are just resolved
  - Contributions from diffractions due to the illumination and detection lenses
- Axial resolution is worse than lateral resolution

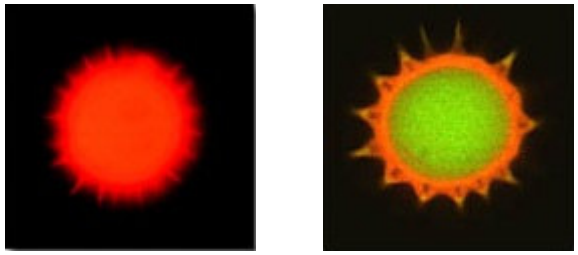
Optical sectioning with a confocal microscope



- Confocal arrangement of focal point and pinhole blocks light from out of focus planes or points away from the optic axis
- The detector receives light mostly from the focal point
  - Image, free of out of focus blur, of a point object located at the focal point

Optical sectioning with a confocal microscope



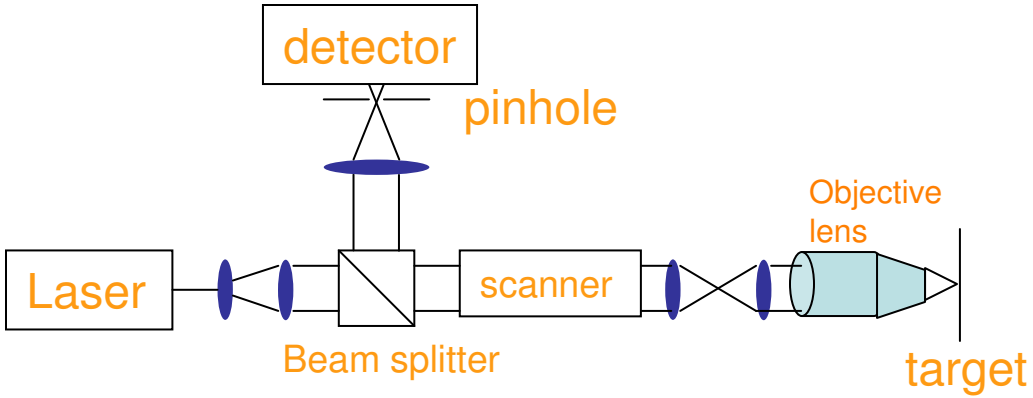


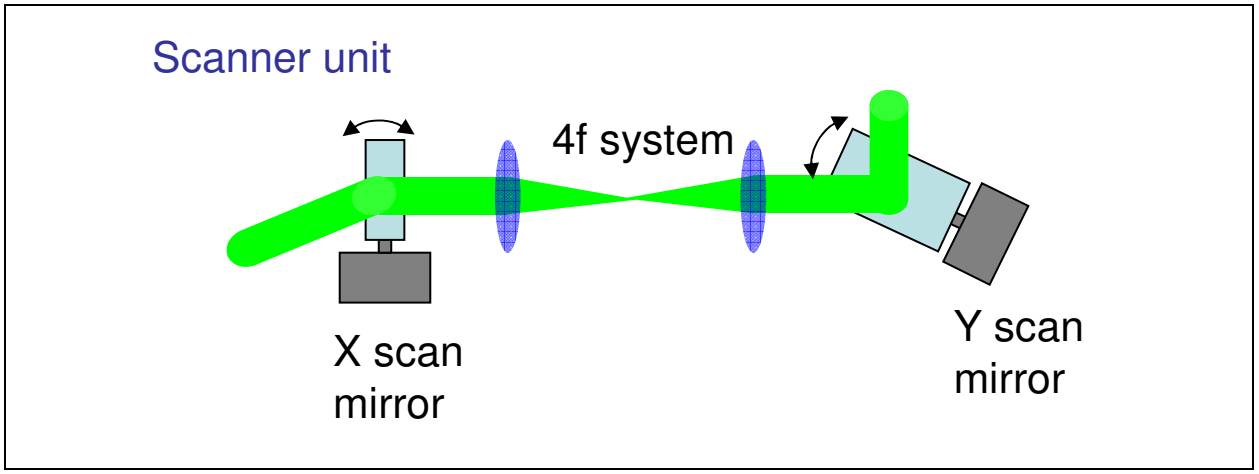
Wide field image Confocal image

(Source :www.olympusfluoview.com)

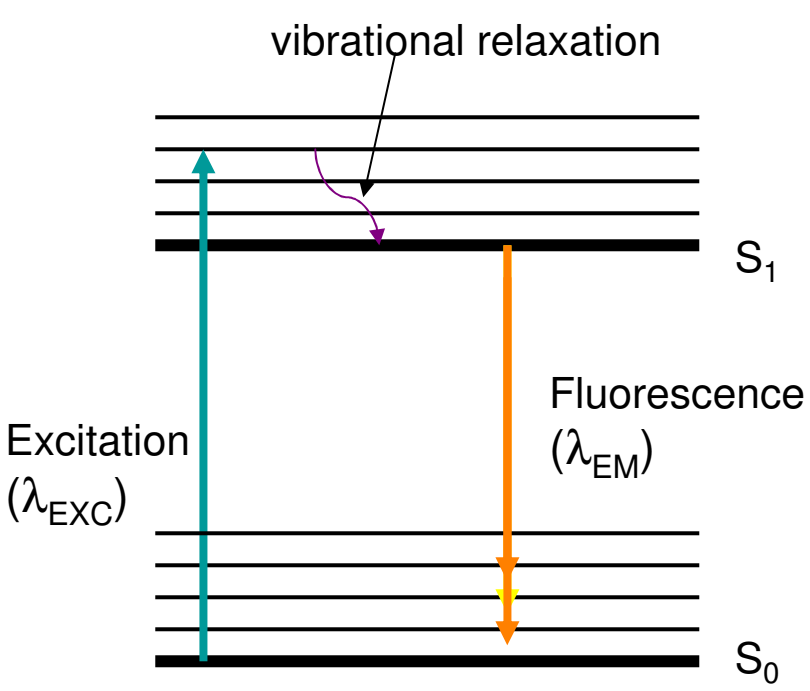
- Either the sample holding stage or the illumination spot is scanned
  - Scanning is controlled by a PC
- For each object point at the illumination spot, the detector signal is stored in the PC
- Results in an optically sectioned image (image corresponds to a sharply defined object plane, devoid of out of focus blur) of the sample
- Much better axial and marginally better lateral resolutions than a conventional (wide field) microscope
- Best resolution: lateral=~$\lambda/2$, axial=~λ

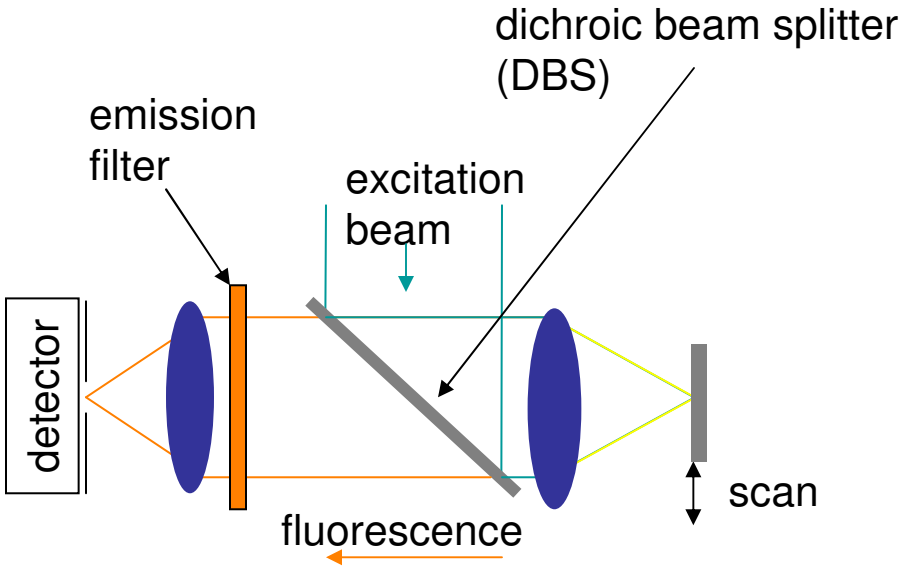
A beam scanning confocal setup





Confocal fluorescence microscope

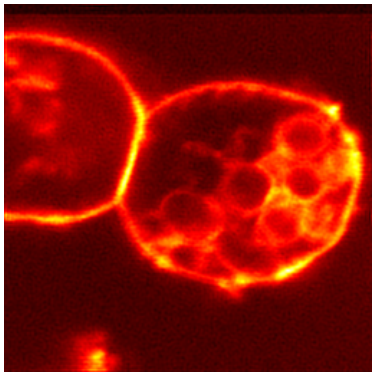




- Molecules (fluorophores) are excited with a laser beam of wavelength (λ_{EXC}), which than undergo a series of spontaneous emissions called fluorescence at the mean wavelength (λ_{EM})
- DBS: reflects λ_{EXC} and transmits λ_{EM}
- Emission filter : blocks reflected light from the sample at λ_{EXC}

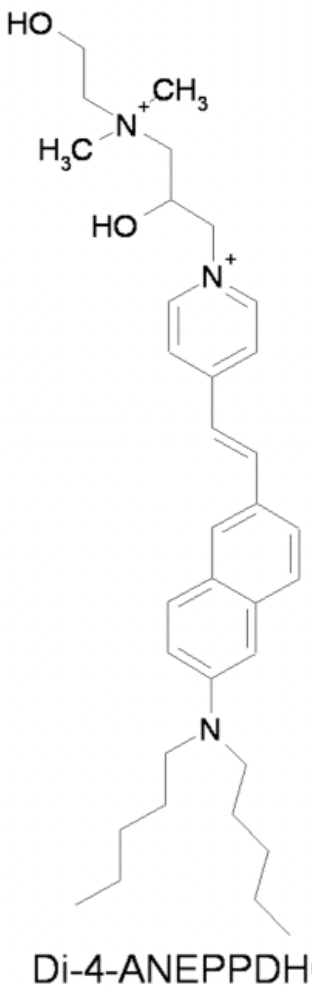
Confocal fluorescence imaging

- The target molecules are tagged with fluorescent probes or fluorophores
- Confocal detection of the fluorescent light in a beam scanning or stage scanning set up
- Fluorescence image provides information about the physical and chemical environment and orientation of the fluorophores and hence of the attached target molecules
- Best resolution working in the UV-visible range (lateral >200 nm, axial >500 nm)
  - Not enough for visualising light-matter interaction at nanoscale



Confocal fluorescence image of human T cells
(source: PhD thesis, B R Boruah)

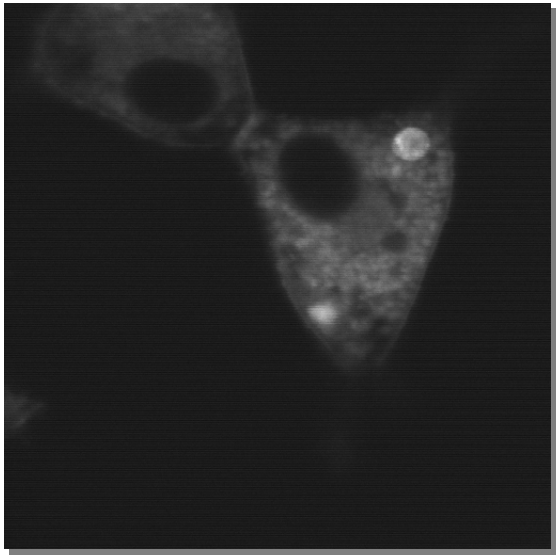
Confocal fluorescence imaging



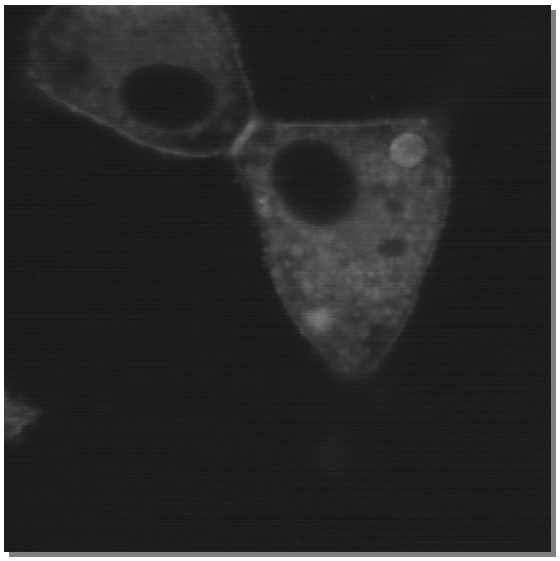
HEK293 cells stained with Di-4-ANEPPDHQ, a membrane specific and lipid activated fluorophore, which orients in the membrane,normal to the surface

532nm illumination, 60x 1.2NA olympus water immersion lens

(source: PhD thesis, B R Boruah)

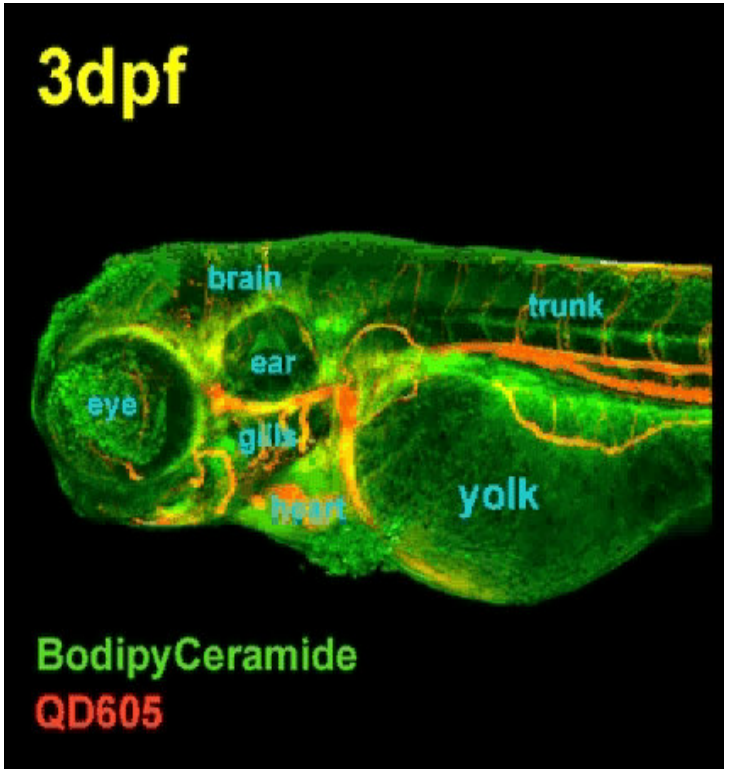


X



Y

Confocal fluorescence imaging

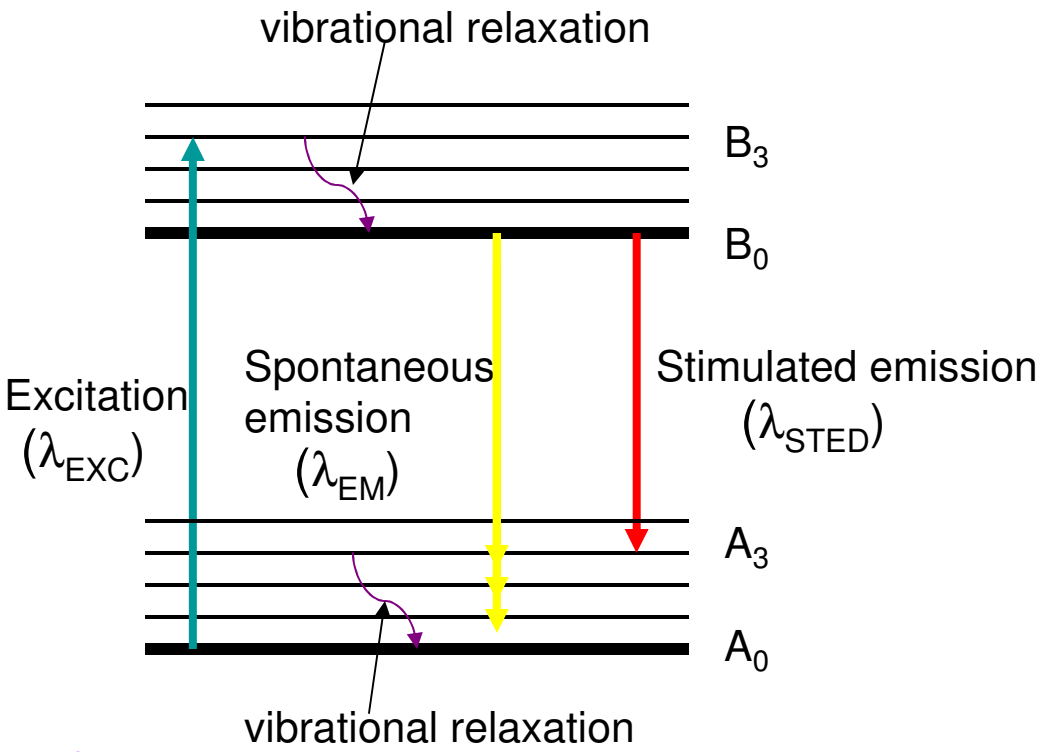


- Zebra fish embryo blood vessels are injected with fluorescence active quantum dots
- Head: 350 μm thick and 71 images, each of 5 μm apart
- trunk: 160 μm thick and 80 images, each of 2 μm apart

3D animation of zebra fish head and trunk

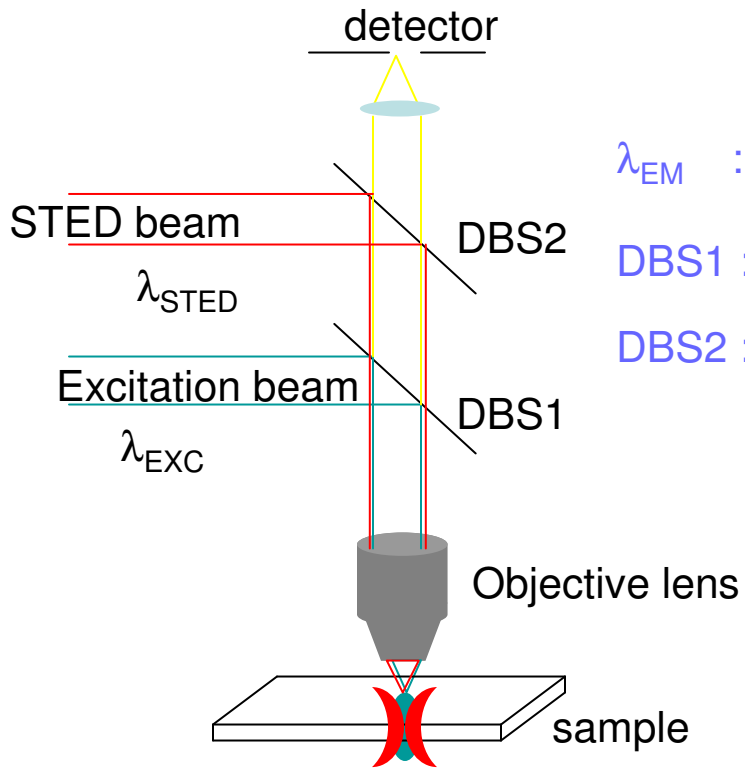
Source: www.helmholtz-muenchen.de

Stimulated emission depletion (STED)



- Laser beam (λ_{EXC}) excites a molecule to the upper electronic state
- Another laser beam, called STED beam, at (λ_{STED}) shines on the excited molecule
  - Stimulates it to undergo emission at (λ_{STED})
  - No emission at (λ_{EM}) i.e. No fluorescence from the excited molecule

STED in a confocal fluorescence microscope



λ_{EM} :mean emission wavelength

DBS1 : reflects λ_{EXC} and transmits wavelengths $>\lambda_{EXC}$

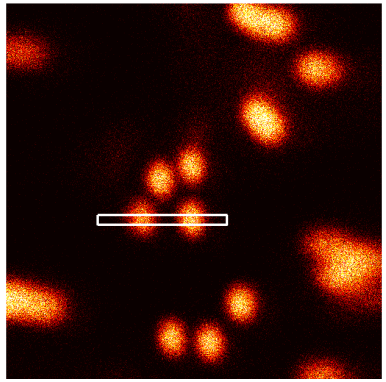
DBS2 : reflects λ_{STED} and transmits wavelengths $<\lambda_{STED}$

- Both excitation and STED beams are pulses following one another, usually derived from the same femto second laser
- Image is formed by scanning the stage or by scanning the beams

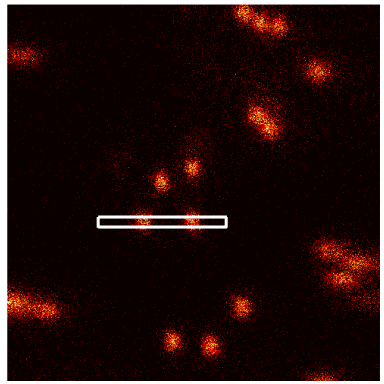
Applications of STED microscopy

Nanoscale imaging of fluorescent beads

Confocal image



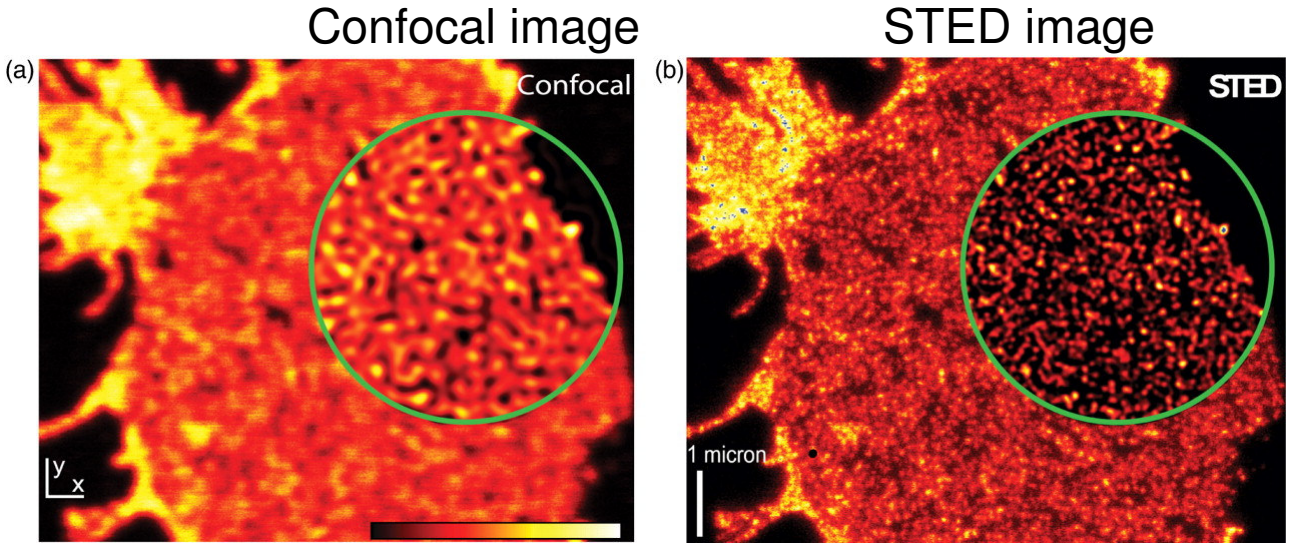
STED image



XY plane images of 200 nm fluorescent beads
(source: PhD thesis, B R Boruah, Imperial College London)

Applications of STED microscopy

In biological science



- Reveals nanopattern in the in SNAP-25 protein found in the plasma membrane of mamalian cells (source: Briefings in functional genomics and proteomics, Vol 5, No 4, 289-301)

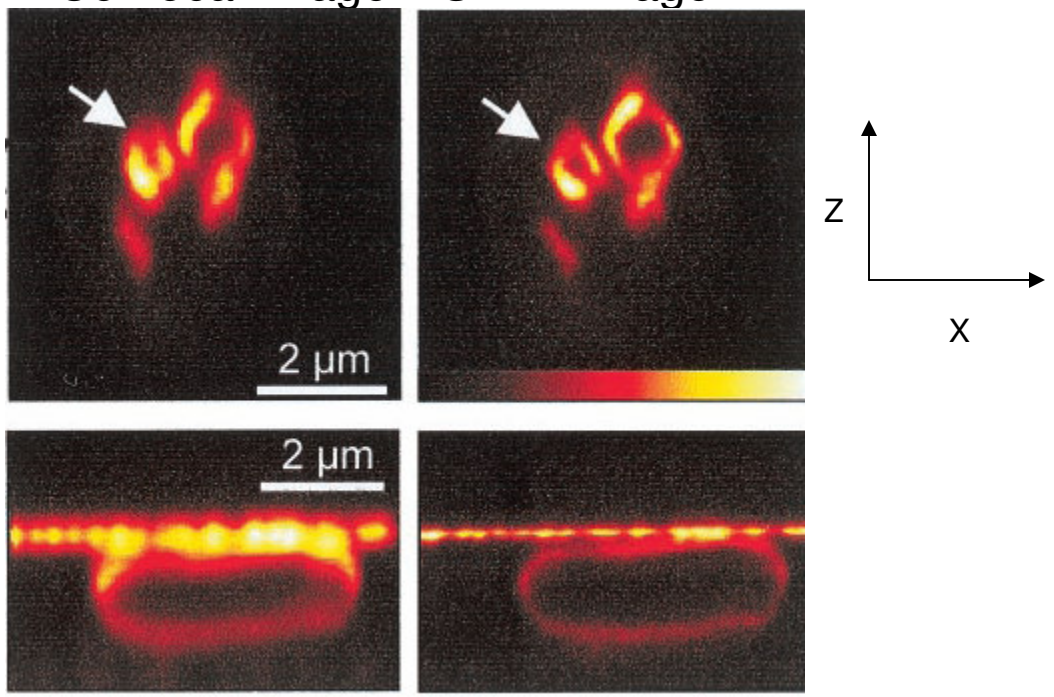
Applications of STED microscopy

Nanoscale imaging of live cells

Confocal image STED image

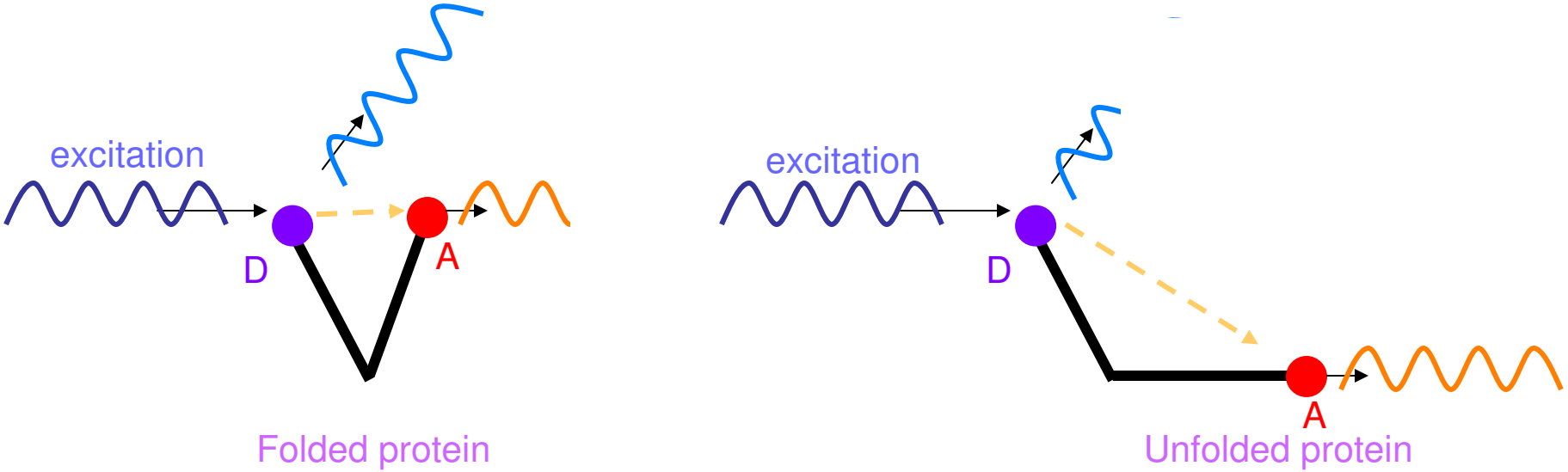
Live yeast cells

Live E-coli bacteria



(source: PNAS, 97, 15, 2000, 8206-8210)

Fluorescence resonance energy transfer



Folded protein

Unfolded protein

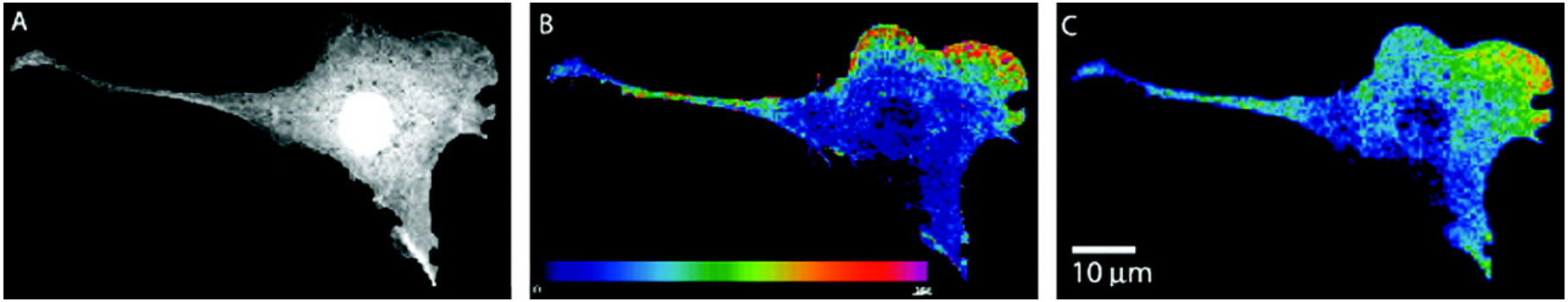
D : donor fluorescent molecule

A : acceptor fluorescent molecule

– → : fluorescence resonance energy transfer (FRET)

- Energy transfer from D to A when they are close by
  - Fluorescence from A
- No energy transfer from D to A when they are far apart
  - No fluorescence from A

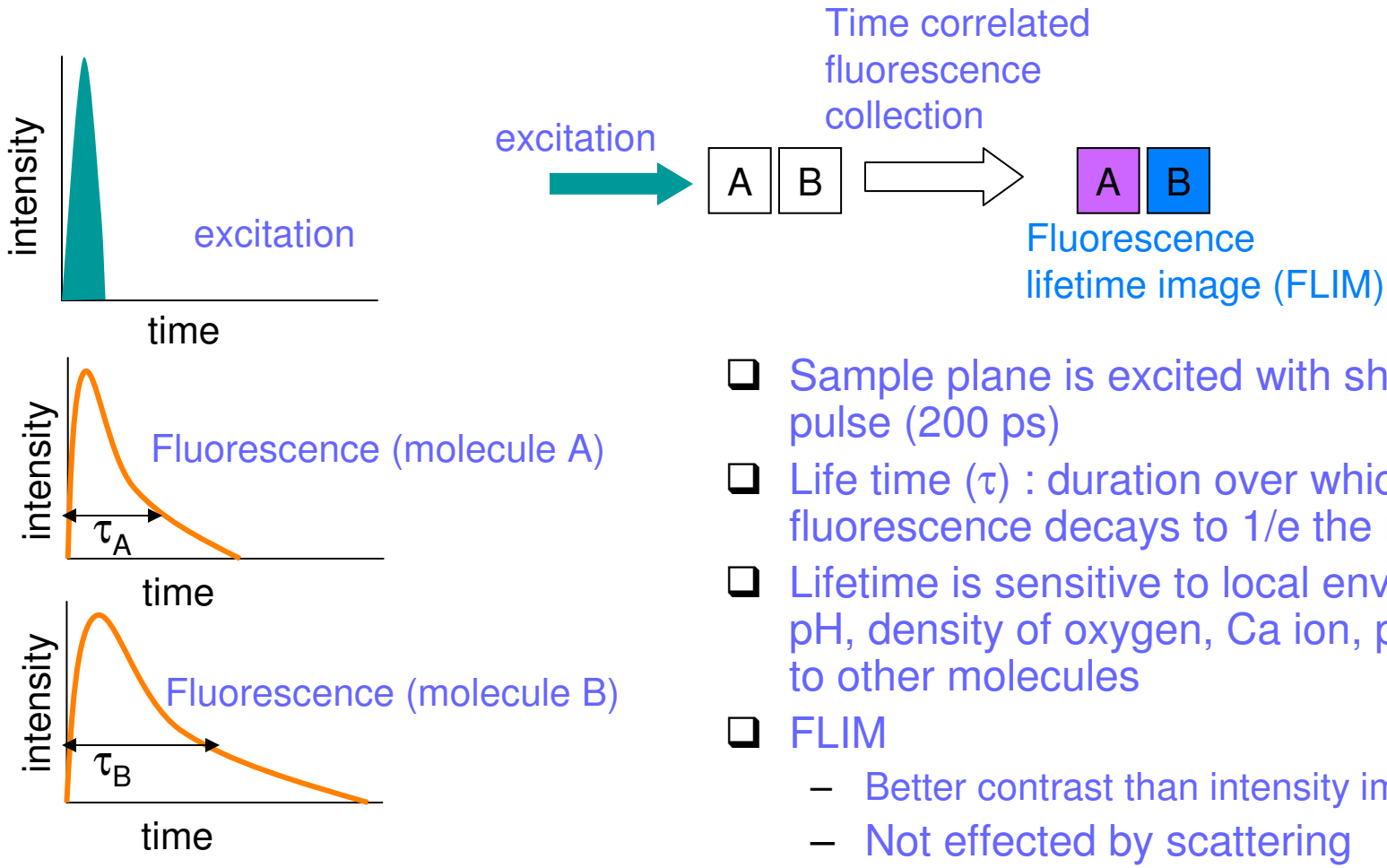
Cell protein localizations with confocal FRET



Integrins induce local Rac–effector coupling.
Donor (A), uncorrected FRET (B), and corrected FRET (C)

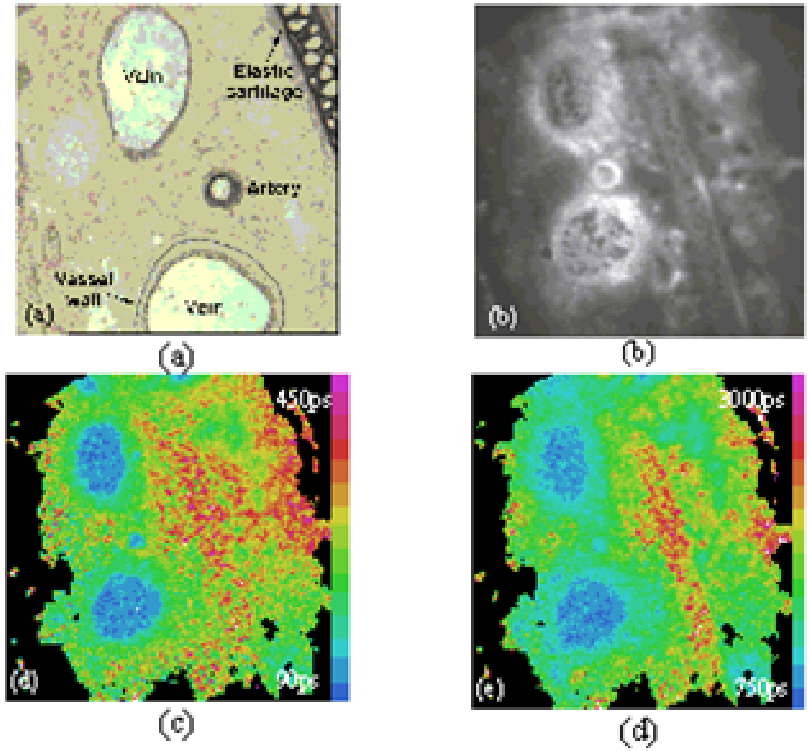
Sekar, Periasamy J. Cell Biol. 2008:160:629-633

Fluorescence lifetime imaging



- Sample plane is excited with short laser pulse (200 ps)
- Life time (τ) : duration over which the fluorescence decays to 1/e the maximum
- Lifetime is sensitive to local environment: pH, density of oxygen, Ca ion, proximity to other molecules
- FLIM
  - Better contrast than intensity imaging
  - Not effected by scattering

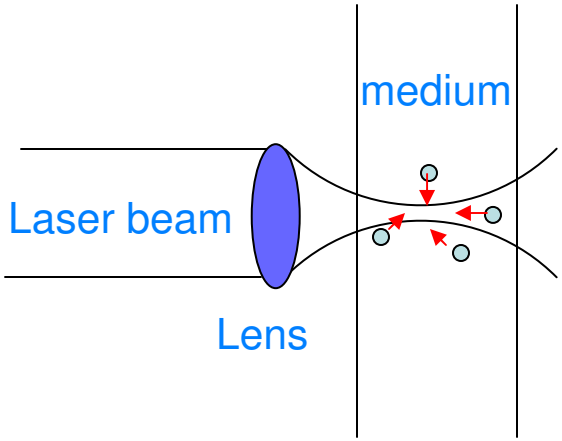
Confocal microscopy with lifetime imaging



Source: Photonics group, Imperial College London

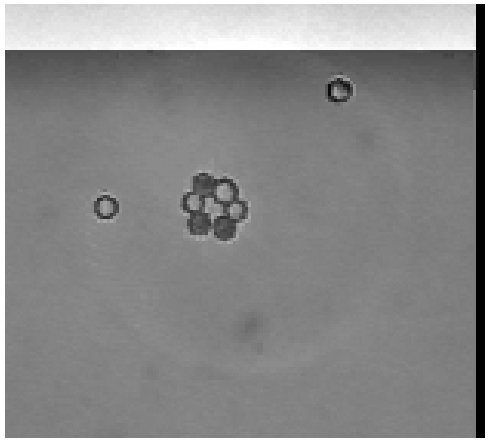
Images of rat's ear showing two veins, an artery ,and an elastic cartilage. (a) Microscopic image, (b) fluorescence image (c) fast FLIM image, (d) slow FLIM image

Optical trapping



← Pico newton level force

○ Particles in a medium (say liquid)

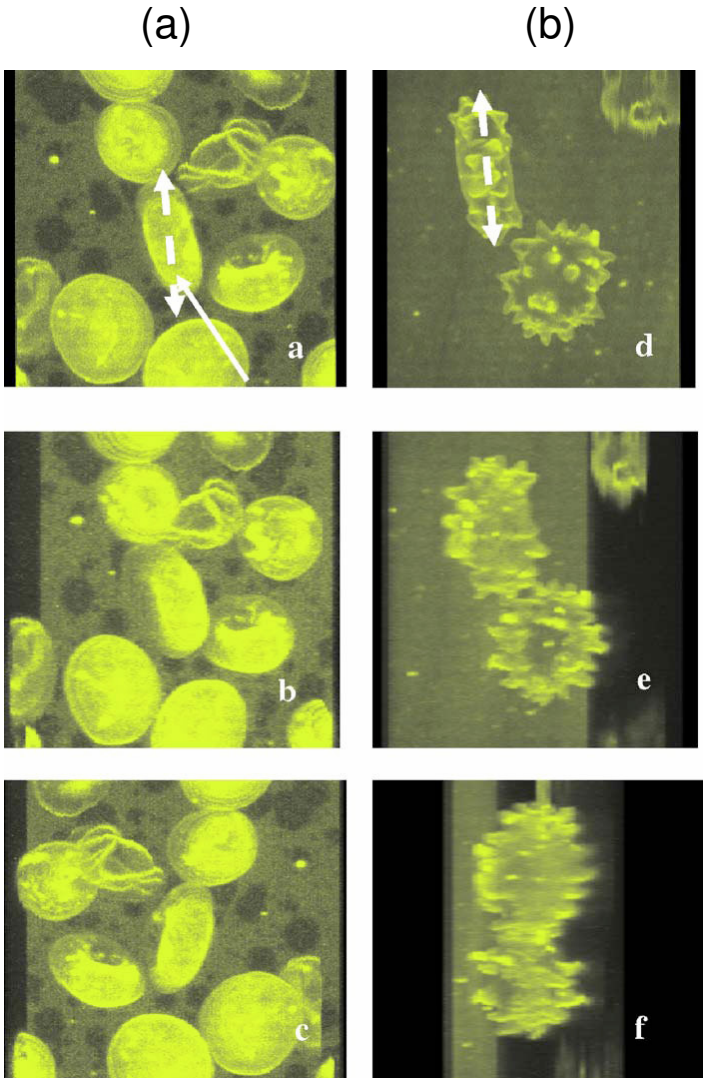


Manipulation of trapped micro-beads

A. Jesacher, et al., Optics Express, 2006

- Laser beam (≈100mW) is focused tightly by a lens into a medium
- Particle in the medium having contrast in the refractive index will experience pico newton magnitude force towards the focus point
- changing the direction of the laser beam will change in shift in focal spot along with the trapped particle

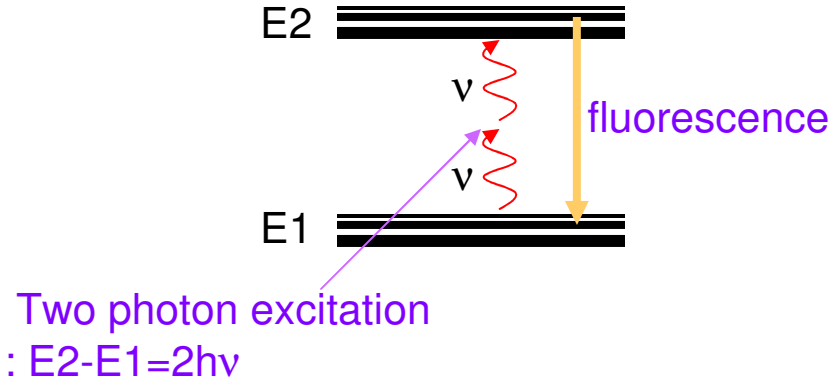
Confocal fluorescence microscopy with optical trapping

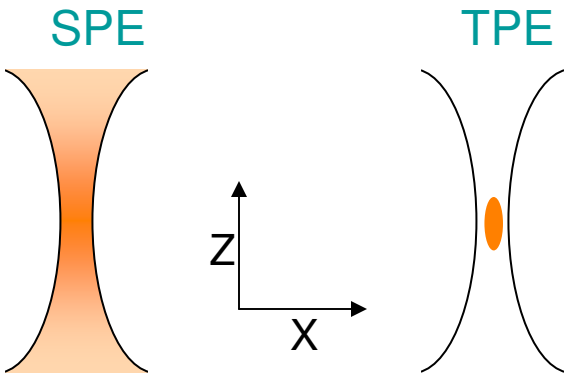


- RBC cell in (a) isotoinic buffer (b) hypertonic buffer
- Cells with arrow mark are trapped
- Confocal images from various view angles
  - No change in shape of the trapped cell

K Mohanty, et al., JBO, 2007

Two Photon excitation (TPE)

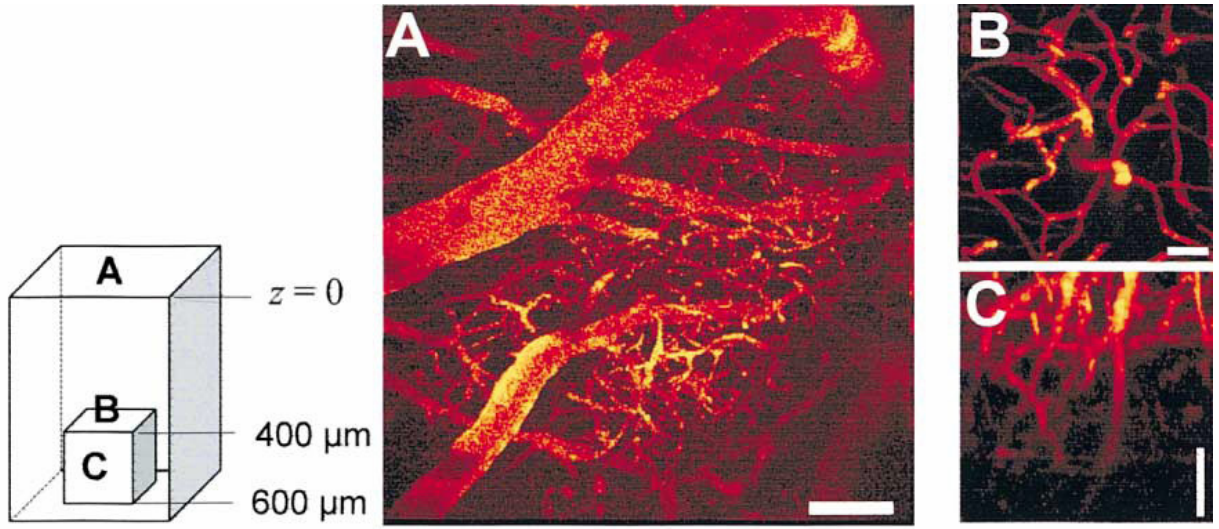




Excited molecules in the sample

- the time interval between arrivals of the two photons of frequency ν at the site of the molecule $<10^{-16}$ sec
- the molecules sees as if there is a single photon of frequency 2ν
- Excitation probability is proportional to $(\text{intensity})^2$
- Fluorescence emission is only from a small region near the focus unlike in single photon excitation

Deep tissue imaging using two photon excitation microscopy

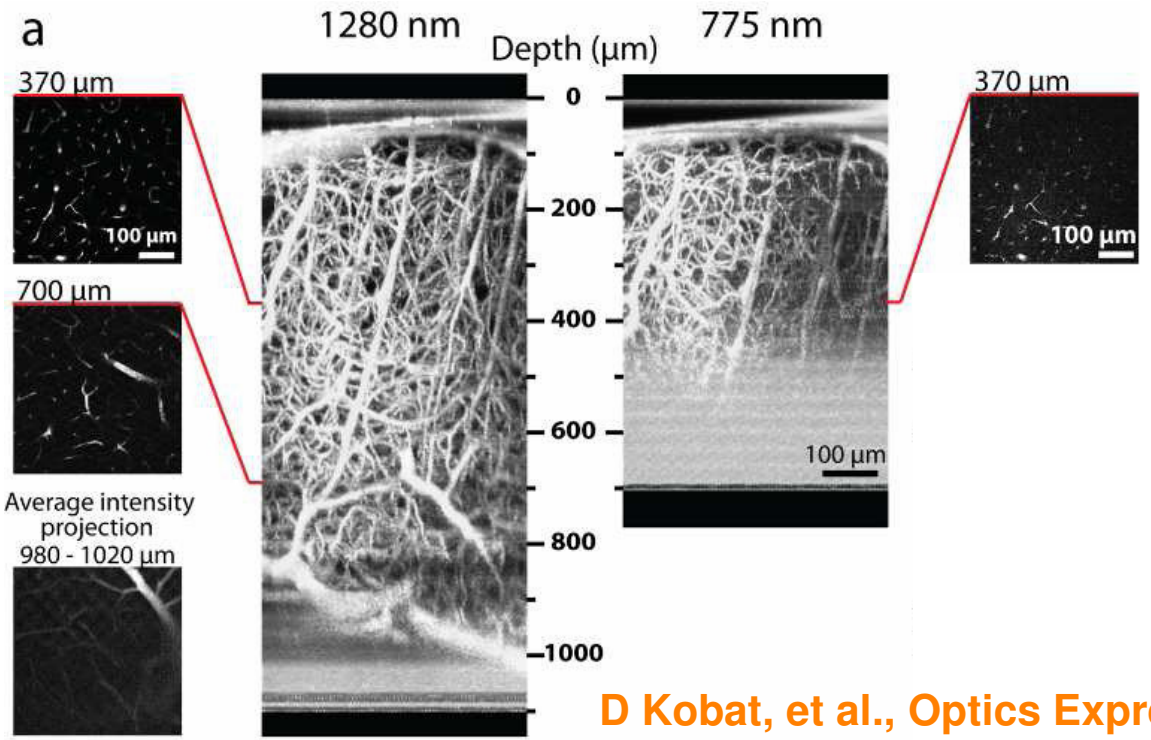


In vivo imaging of brain tissue of an anesthetized rat (up to a depth of 600 µm)

M. Oheim et al, Journal of Neuroscience Methods 111 (2001)

- Excitation wavelength is twice that of single photon excitation
  - Less scattering (larger the wavelength smaller is the scattering)
  - Excitation beam enters deep into the sample (upto 1 mm)
- Less amount of photo damage
- In vivo imaging of live tissues

Deep tissue imaging using two photon excitation microscopy



D Kobat, et al., Optics Express, August 2009

- TPE image of mouse brain shows high contrast blood vessels
  - Upto a depth of 500 µm when excited with 775 nm
  - Upto a depth of 1 mm when excited with 1280 nm

Conclusion

- confocal fluorescence microscopy is a powerful tool to get a high contrast image of a thin slice of the sample in a non-invasive way
  - Has number of application in biology (and the number is growing every day)
- Confocal fluorescence microscopy using stimulated emission depletion provides nanoscale imaging
- Confocal fluorescence microscopy can be combined with other techniques such as FRET, FLIM, optical trapping etc. to reveal further information from the sample
- Two photon excitation instead of single photon excitation provides high contrast image upto a depth of 1 mm
  - Useful for imaging in cellular environment

Thank You