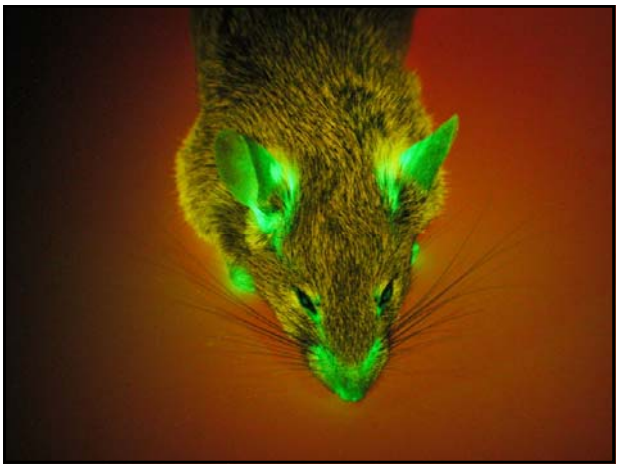


CONFOCAL

- **A.** *adj*. Having the same focus or foci.

1867 THOMSON & TAIT *Nat. Phil.* §494 *Any two confocal homogeneous solid ellipsoids of equal masses produce equal attraction through all space external to both.*

- **Achieved via narrow beam of light and PINHOLE**

A biased and brief history of confocals

- **1957** Minsky filed patent (see http://web.media.mit.edu/~minsky/papers/ConfocalMemoir.html)
- **1960** Maimain developed first working laser
- **1972** Davidovits & Egger patent for scanning laser
- **Mid-1980's** First CLSM & application to biological material Åslund?? Amos??
- **1990** Denk 2-photon

Types

- Spinning or Nipkow disk WHOLE PICTURE AT ONCE e.g. Perkins Elmer
- Scanning PIXEL BY PIXEL, LINE BY LINE e.g. Zeiss, Leica, Nikon, BioRad (R.I.P)

Laser Confocal Microscopy LCM

- Ions e.g. Ca^{2+}, H^{+}, Cl^{-}
- Membrane potential
- Localise labels
- Metabolism

NAD(P)H/Flavins

Single photon versus multiphoton

- If 1 photon of 490 nm can excite a fluorophore, then 2 photons of 980 nm arriving within 10^{-15} s can do the same.
- Pentrate deeper 100 µm vs 10µm
- More localised, greater resolution
- Less noise
- Size, space, cost more
- See Nat Methods. 2005 2: 932-940

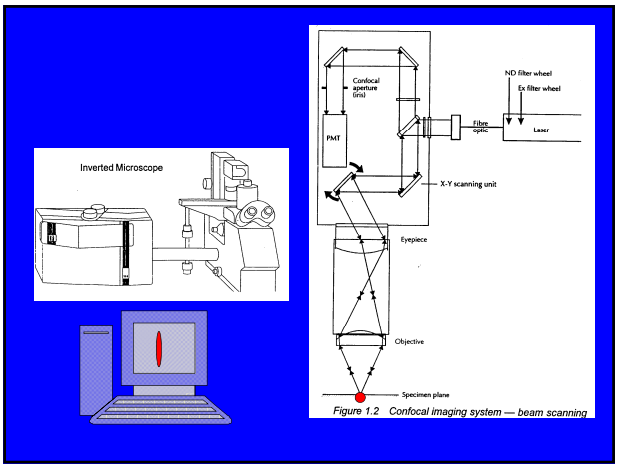
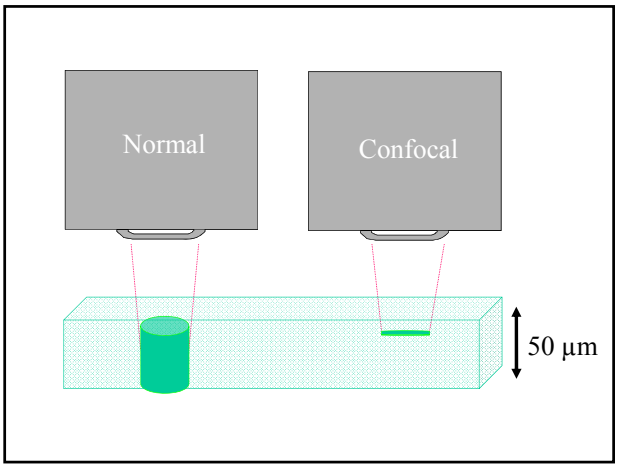


Figure 1.2 Confocal imaging system — beam scanning



Pluses of Confocal Microscope

- Small confocal plane = more detail
  0.5 µm vs 50 µm
- Localise areas of interest
  RyR, IP_3 receptors
- Intracellular release or movement
  Ca^{2+}, vesicles
- Dynamic changes
  Ca^{2+}-induced Membrane fusion,
  Ca^{2+} exit or entry ER/mitochondria

Limitations of Confocal Microscopy

- Events over time or survey the cell?
  In most, scan time per line c. 2ms
  Frame rate now >1000/s
- Tissue depth
  Quality of image gets worse.
- Calibration problems
  e.g. Relative changes or real $[Ca^{2+}]$?

Minuses of Confocal Microscope

- **Expense (equipment, dyes, TIME)**
- **Seductive**
- **Analysis**

THE PERFECT EXPERIMENT

- No change in autofluorescence
- Dye loads where you want
- No loss of dye
- No bleaching
- Structures stay in focus

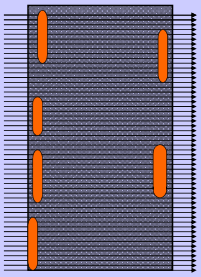
Autofluorescence

- Always some
- Depends on excitation & emission λ
  NAD(P)H Ex 360 – 400; Em 440 - 500
  Flavins Ex 380 - 490; Em 520 – 560
- NAD(P)H about 50 – 100x Flavins
- Major interference with CFP and GFP

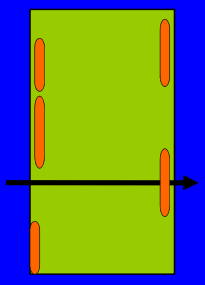
Peculiarities of dyes in cells

- Binding to proteins or metals (most dyes)
- Ejection or breakdown (e.g. indo-1)
- Bleaching or dyes (OG-BAPTA 488)
- Toxicity, formaldehyde production
  excess dye (Zn^{2+} binding?)
- Enters into compartments (indo-1, rhod-2)

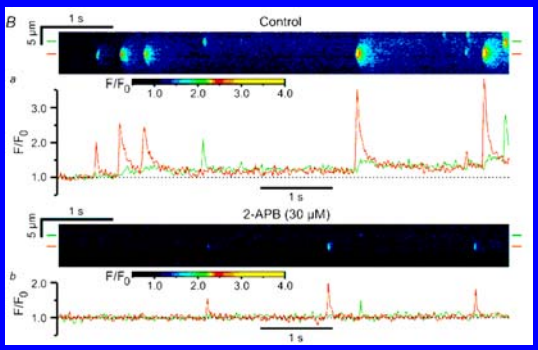
XY scanning to localise nuclei



XT scanning for dynamic changes in mitochondrial Ca^{2+}

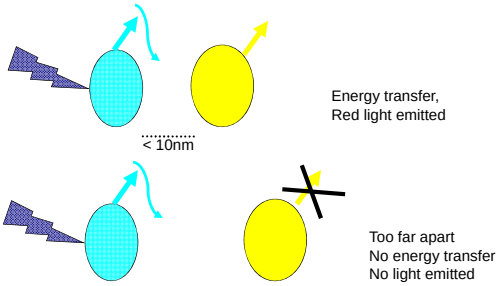


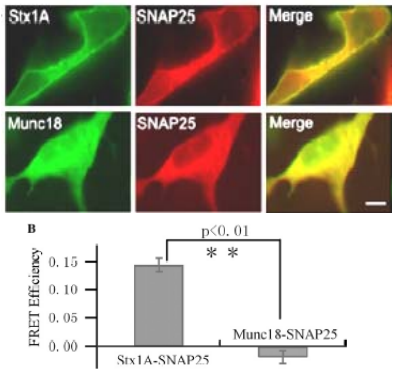
Line scan sees Ca^{2+} sparks and puffs



DV Gordienko & TB Bolton, Journal of Physiology (2002), 542, 743-762

Fluorescence **R**esonance **E**nergy **T**ransfer (**FRET**∝ (inter-molecule distance)^{6})





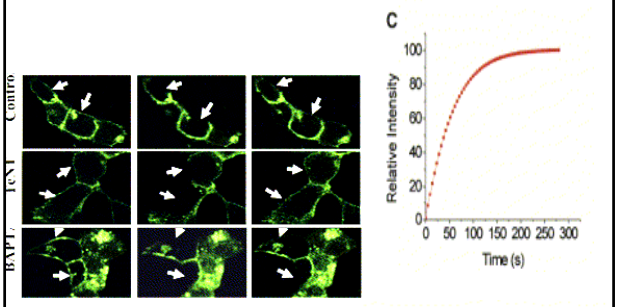
Yang et al., (2005) Biochem Biophys Res Commun. 330, 914-920

Fluorescence Recovery After Photobleaching (FRAP)

PRINCIPLE

- Bleach photosensitive molecule.
- No longer able to fluoresce
- Measure recovery.
- Trace diffusion in cells, movement of proteins

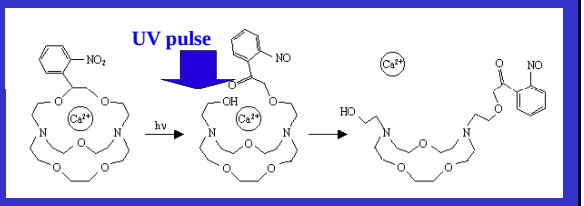
Trafficking of TRPC3 to the plasma membrane in HEK cells



$t_{1/2}$ of flourescence recovery (secs)	
Control	38.9±5 (n=15) *
TeNT	68.5±7 (n=6)
BAPTA	836±150 (n=4)
BFA	not detectable (n=5)

Singh et al. Mol Cell. 2004 15:635-646.

UNCAGING caged compounds



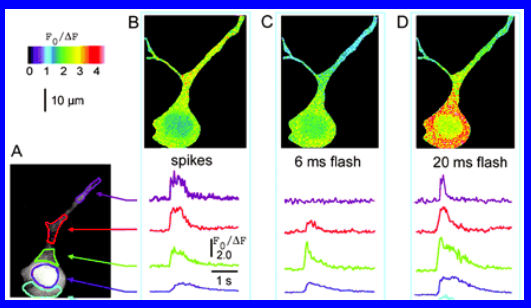
http://www.fis.unipr.it/~viappian/pracady/cagedcalcium.htm

What does IP_3 do in prefrontal cortex neurones?

Load with fura-2 & caged IP_3.
Homemade 2-photon, video rate aquisition
100 fs pulses @ 780 nm to excite fura-2
10 ms pulses of uv light to release IP_3 from cage
Collect < 650 nm

Stutzmann et al., Journal of Neuroscience, 2003, 23(3):758

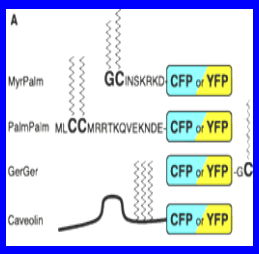
2-photon detection of IP3 induced rise in $[Ca^{2+}]$

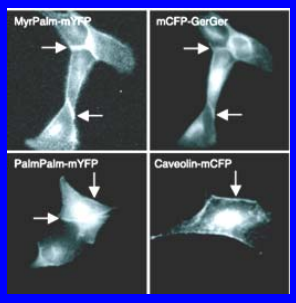


Useful links for confocal users

- http://rsb.info.nih.gov/ij
- http://www.tsienlab.ucsd.edu/
- http://www.invitrogen.com/site/us/en/home/support/Tutorials.html

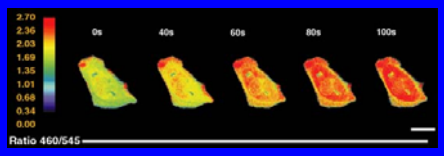
Seeing membrane proteins with FRET





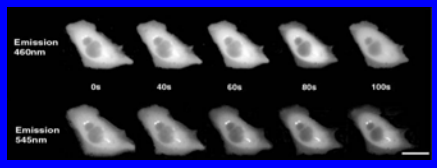
DA Zacharias, JD Violin, AC Newton, and RY Tsien , (Science 2002) 296: 913-916.

FRET follows cAMP activation of PKA



Blue

Green



Zaccolo et al., Nature Cell Biology (2000) 2, 25-29

Bleaching may be unequal

Excite 380 nm