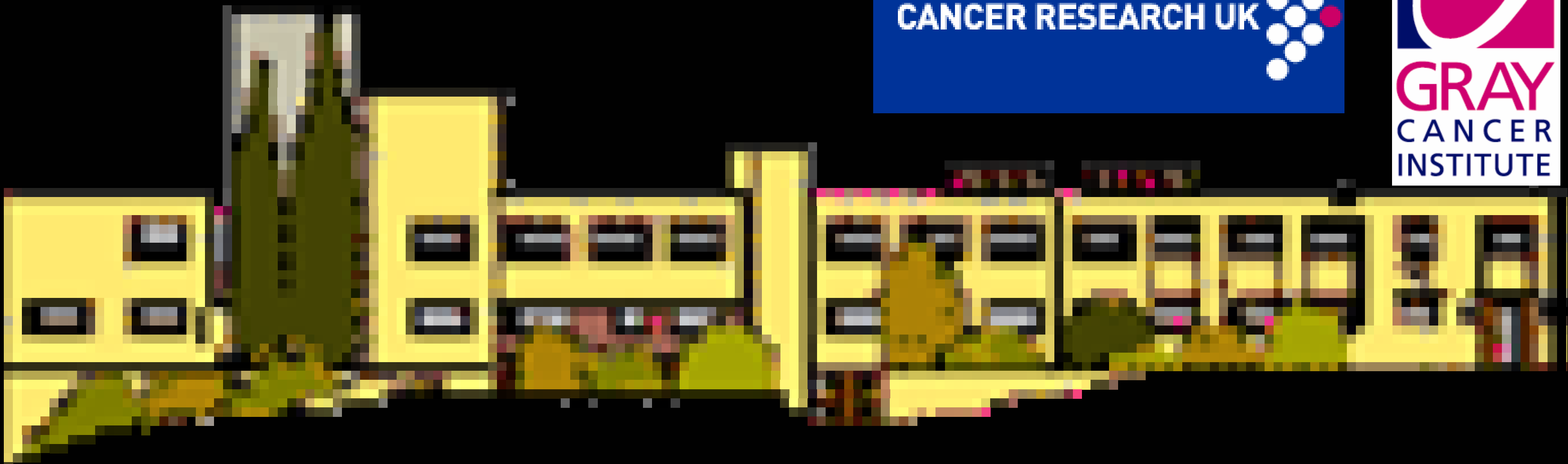
Cellion meeting

21st April 2004

Techniques for visualising and analysing cells and their responses to micro-irradiation

B. Vojnovic, GCI Advanced Technology Development Group



Cell imaging techniques for use with microbeams

Presentation outline

- Fluorescence / non-fluorescence imaging
- Low-light level imaging
- Cell finding
- Potential methods of imaging at depth
- Time-resolved and non-linear excitation methods

Fluorescence / non-fluorescence imaging

- Fluorescence imaging 'straightforward'
  - cell toxicity potential problem – but often not a problem
  - can be highly specific
  - readily adaptable to study cell signalling responses
  - rugged, good signal-noise ratios
  - is preferred for 'routine' work

- Non-fluorescence imaging definitely NOT 'straightforward'
  - cell toxicity limited to photo-toxicity – i.e. not a problem
  - transillumination methods available to segment cell features

    …..but difficult to implement in practice

    …..microbeam 'delivery' systems in the way
  - epi-illumination requirement
  - cell substrate optical quality is limiting
  - evanescent wave methods possible but of limited use

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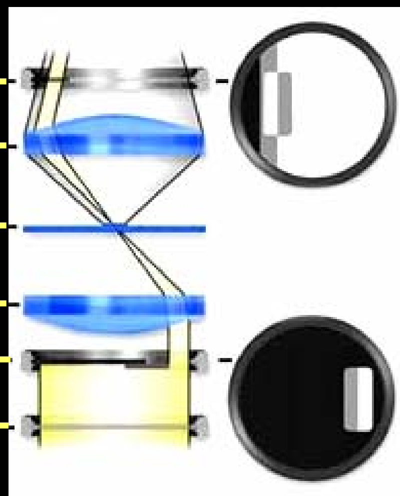
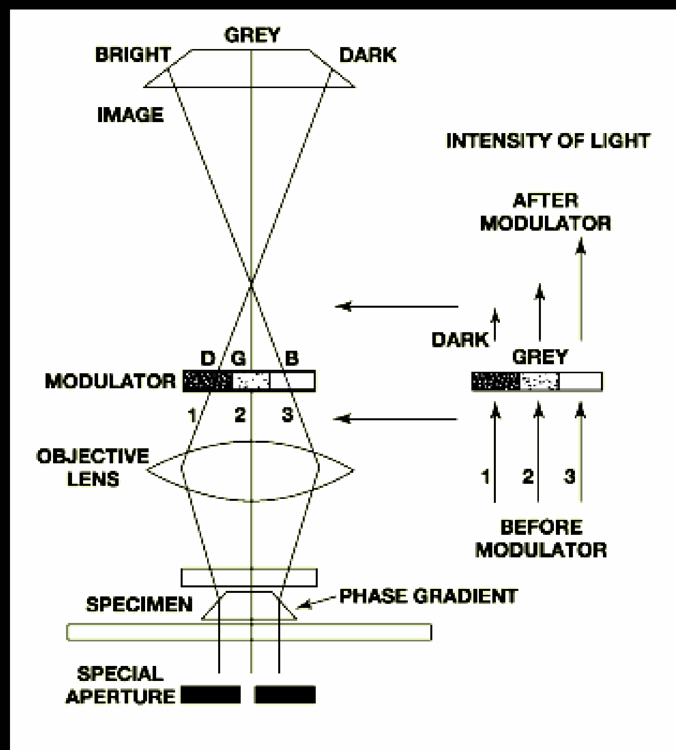
Non-fluorescence imaging

- Dark–field / phase contrast methods
  - transillumination / annular illumination possible
  - poor for segmentation of cellular compartments
- Contrast generation from optical path differences
  - DIC, Hoffman….possible but ‘location’ of features potentially incorrect, image processing hard, slow
  - require transillumination
- Interferometric methods
  - potentially excellent but extremely difficult to implement in practice
- Polarisation microscopy
  - potentially excellent but difficult to interpret routinely
- AFM / ion electrode microscopy
  - ‘top’ surface – membrane imaging
  - recent speed improvements

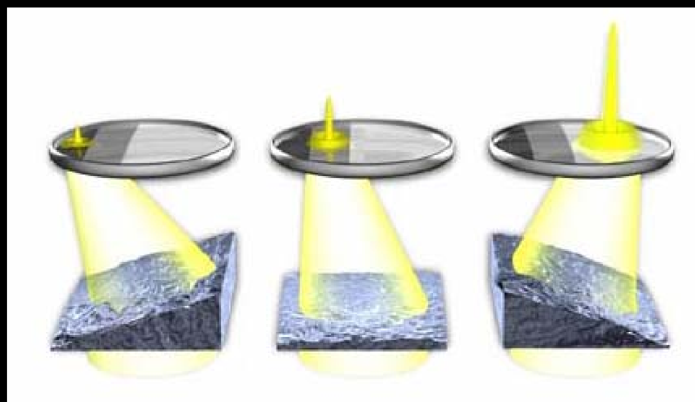
Hoffman modulation contrast

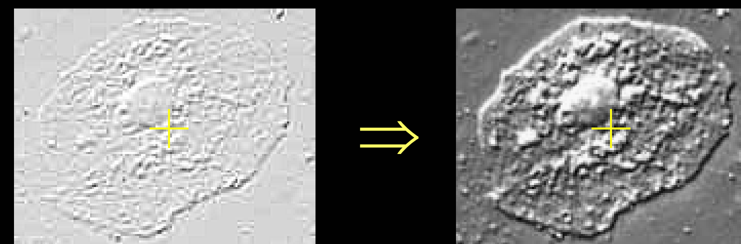
modulator
objective
sample
condenser
slit plate
polariser





Δ Phase $\Rightarrow$ Δ amplitude





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Imaging and cell finding

- Minimise dye concentration and illumination
  - Use emerging red fluorescent dyes
  - Use low power, high n.a. objectives
  - Use of fast shutter, sensitive camera
  - High speed image transfer
  - CCD's: 2-10 e^{-} $pixel^{-1}$ noise, depends on readout speed
  - Intensified cameras – fast but inherent image distortion
- Image processing techniques 'on the fly'
  - Autofocus
  - Cell recognition, size filtering
  - Cell 'separation'
- Transparent image processing to hardware interface

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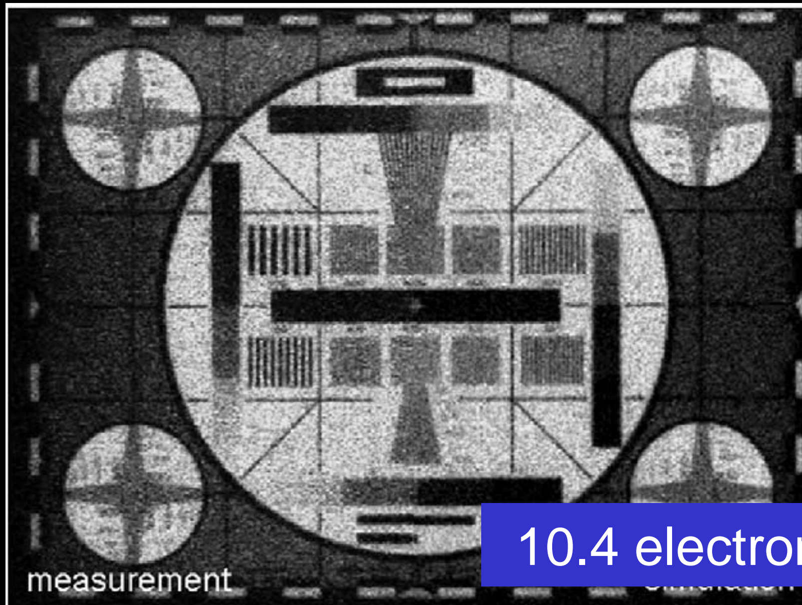
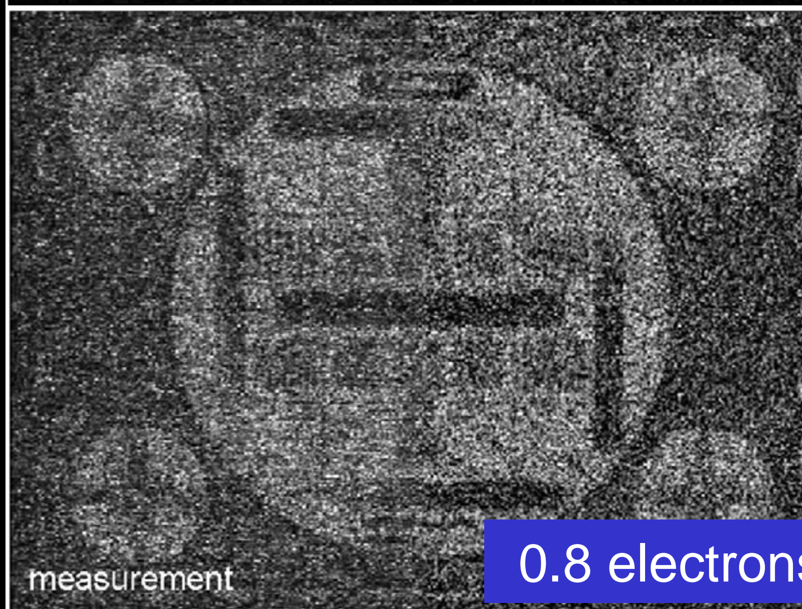
e2v technologies EMCCD

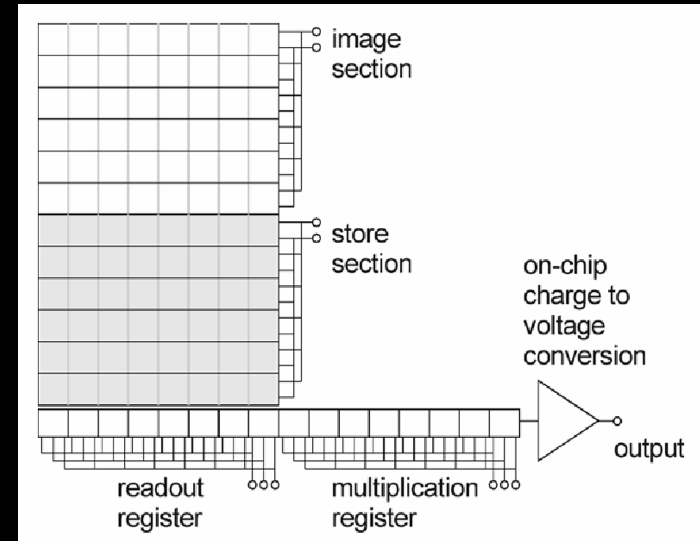
Electron multiplication – typ. x1000



10.4 electrons pixel^{-1}



0.8 electrons pixel^{-1}



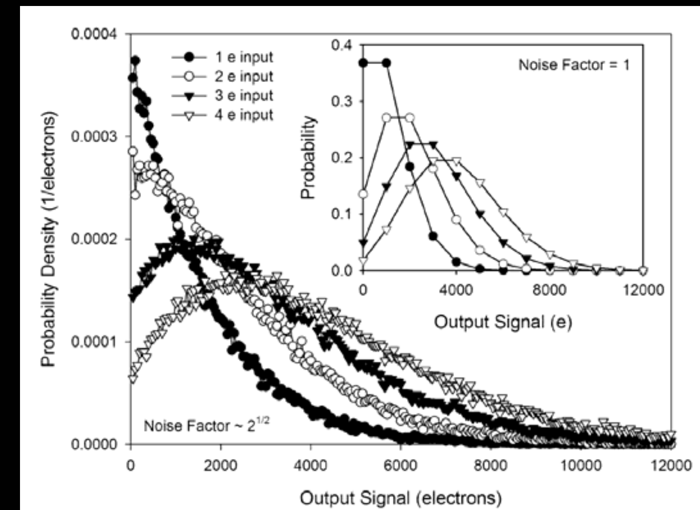


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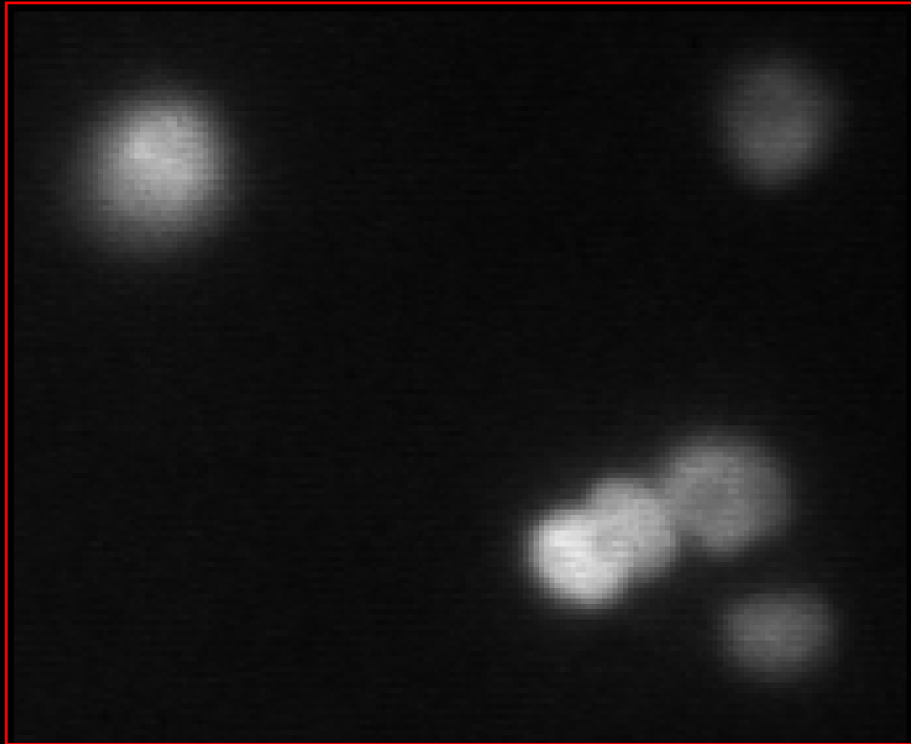
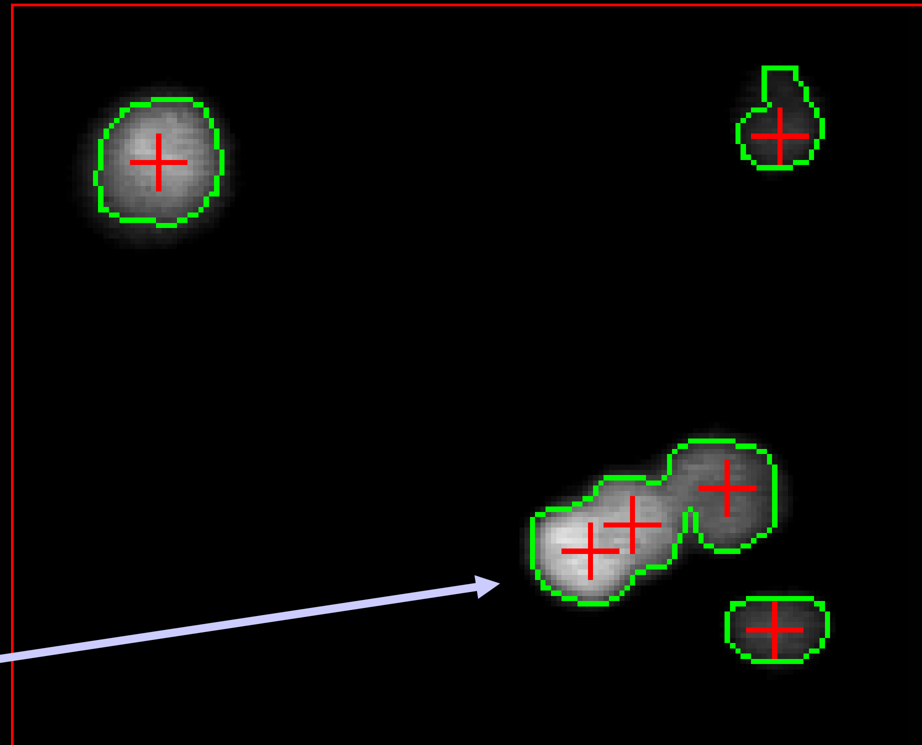
Image processing: counting touching objects



Raw image

- a single nucleus has 1 point as far as possible from the cell's edge.
- two or more touching cells have >2 such points, one for each cell.



Processed image

3 points furthest 'inland' are found on the image of 3 cells

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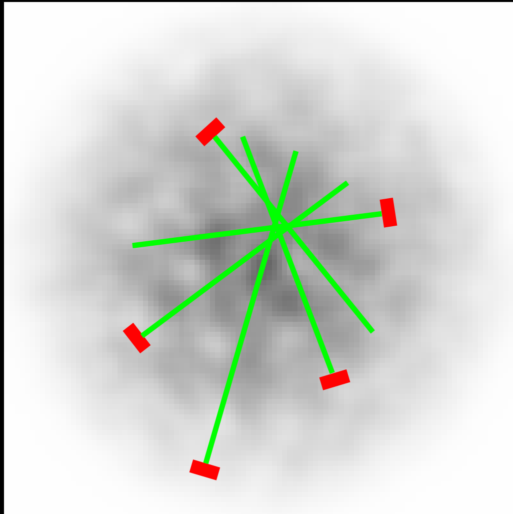
Image processing – cell finding

Compact Hough Transform

Search outwards
Local area mapping

- Independent of intensity
- Independent of shape
- Overlapping cells identified



Cell imaging / cell finding

(Normalised display)

2942

0

Automatic Cell Finding

Objective 3 x10/0.25 1.180 px/um

Field of View 1139 x 868 um

Estimated cell diameter 20 um

Measured mean diameter 20.0 um

Experiment

Title Cell Type 1

Dish Dish 1

Region of Dish

10 x 10 mm 5 x 5 mm 2.5 x 2.5 mm

Scan Options

Excel File test3.xls

Analyse Cells Show Processing

Save Nothing View All

Set Criteria Bit Mode 12

Start scan Pause scan Stop scan

Frame 35 of 35

Cell 26

Review/Revisit

Acquisition

Camera Settings

Gain Black Level

Exposure Bit Mode Binning

0.1000 sec 12 None

Capture Mode

Live Freeze Snap

Grid 2 x 2 Green

Acq active

Background Correction

Acquire Black Acq Norm Ref Process

Display Black Disp Norm Ref

Microscope Settings

Details False Colour on off

Cube 2 460/505/515-555

Objective 3 x10/0.25 1.180 px/um

Display

Histogram LUT Normal Profile

File Management

Store Recall

Filename

Snaps 64

Retries 0 Test Close

Joystick

0.200 Speed mm/sec

XY Z

Motorised stage control

Define Scan

Define Sequence

DATUM Position

Set: Set All, Set XY, Set Z

Go to: GoTo XYZ 0, GoTo XY 0, GoTo Z 0

Go to XYZ

Move XYZ by

Move to X um 25000.0 Go

Move to Y um 25000.0 Go

Move to Z um 0.0 Go

X increment um -200.0 Go

Y increment um -500.0 Go

Z increment um 0.0 Go

Position ?

X Position um 1399.6

Y Position um -4660.0

Z Position um -29.3

On

Joystick

Speed mm/sec 10.000

Active

Initialise

Abort Init

Moving

Abort Move

Z Test

Calibrate

About

Close

Focus Indicator

Worse Better 53

0 100 200 300 400 500 600 700 800 900 1000 Max 1000

On Off

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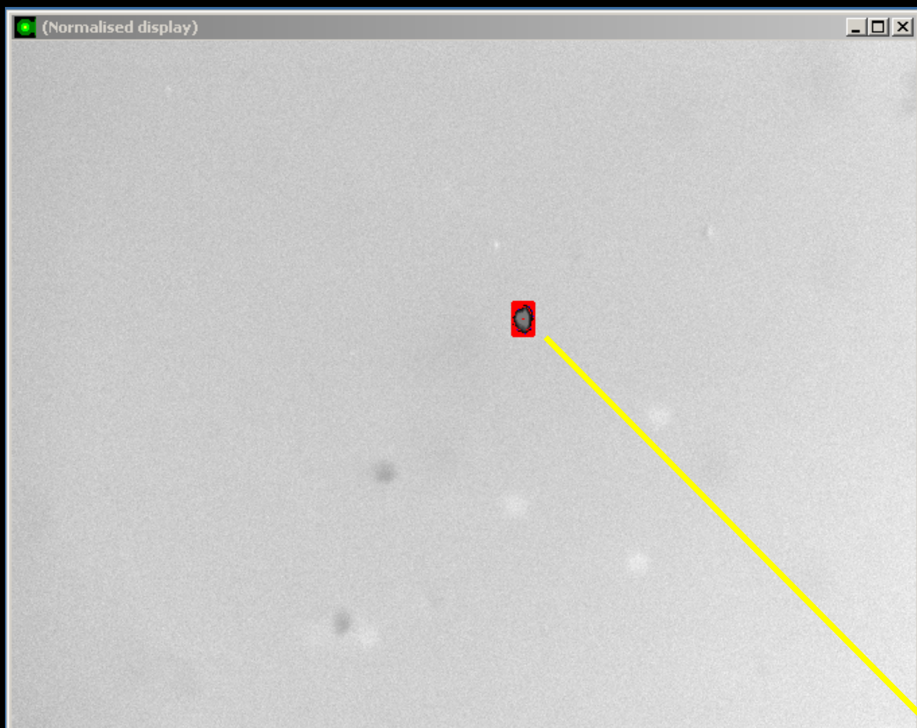

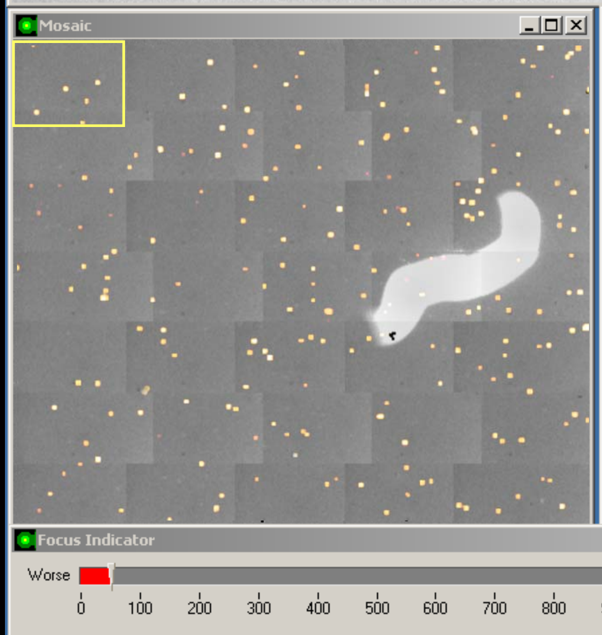

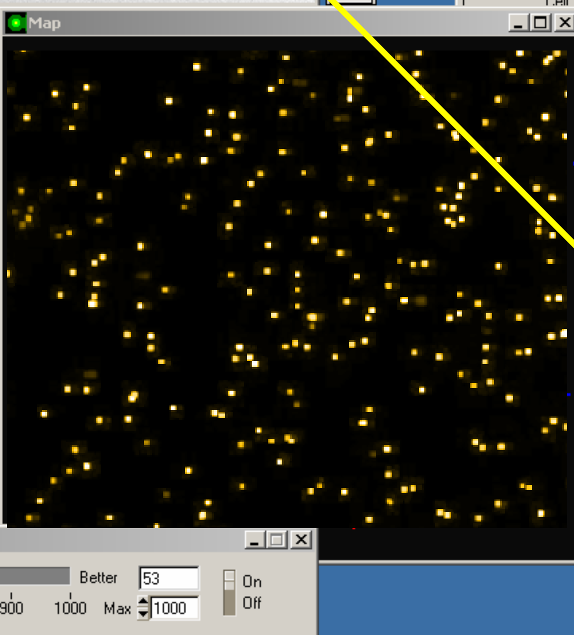

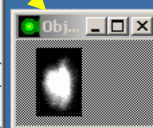

Automatic Cell Finding
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Field of View 1139 x 868 um
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Experiment
Title Cell Type 1
Dish Dish 1
Region of Dish
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Scan Options
Excel File test3.xls
Analyse Cells Show Processing
Save Nothing View All
Set Criteria Bit Mode 12
Start scan Pause scan Stop scan
Frame 35 of 35
Cell 26
Recall Revisit
Recall Set Cursor
Close

Acquisition
Camera Settings
Gain Black Level
Exposure Bit Mode Binning
0.1000 sec 12 None
Capture Mode
Live Freeze Snap
Grid 2 x 2 Green
Acq active
Background Correction
Acquire Black Acq Norm Ref Process
Display Black Disp Norm Ref
Microscope Settings
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Cube 2 460/505/515-555
Objective 3 x10/0.25 1.180 px/um
Display
Histogram LUT Normal Profile
File Management
Store Recall
Filename
Snaps 64
Retries 0 Test Close

Joystick
0.200 Speed mm/sec
XY Z

Tools
I:
X:
Y:

Focus Indicator
Worse Better 53 On Off
0 100 200 300 400 500 600 700 800 900 1000 Max 1000

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Imaging at depth

- Optical 'slices' – steady-state illumination
  - deconvolution
  - confocal – point scanning or Nipkow disc
  - structured light imaging
  - extended focus imaging / phase plates
  - extended focus imaging / spatial light modulation
- Optical 'slices' – non-linear excitation
  - two-photon excitation
  - point-spread function engineering
  - second harmonic generation
- Tomographic imaging
  - 'contact' imaging / sample rotation
  - multiple beam tomography

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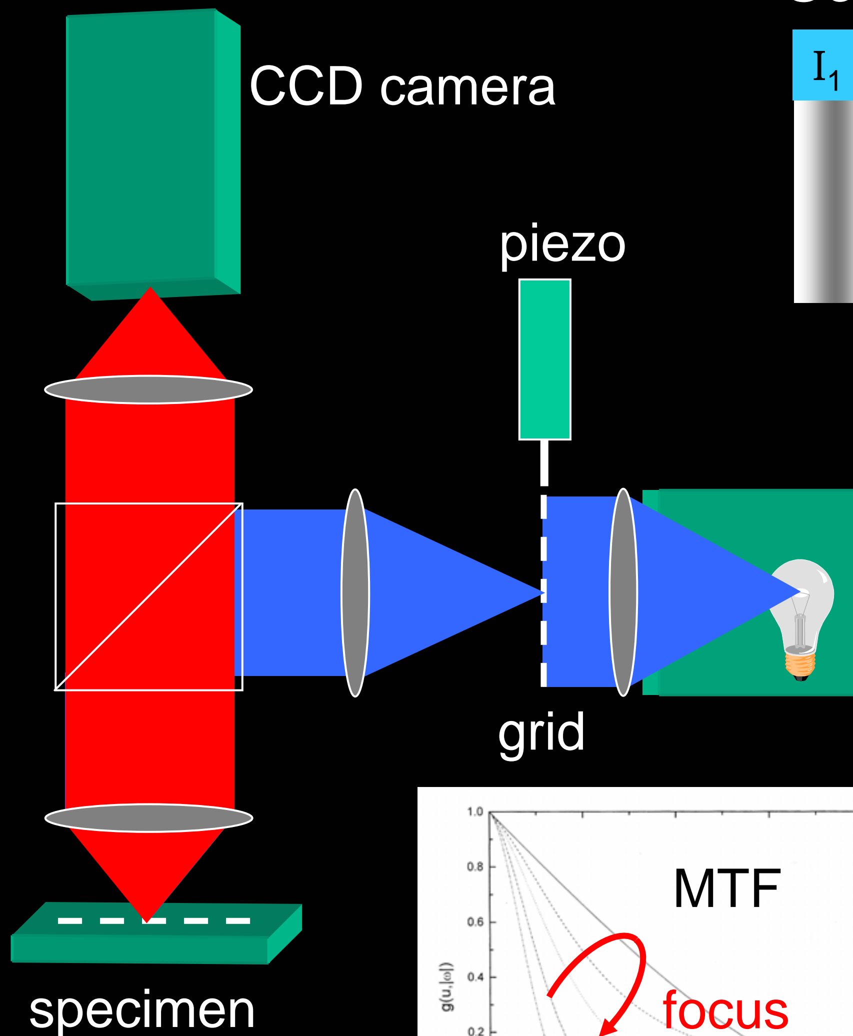
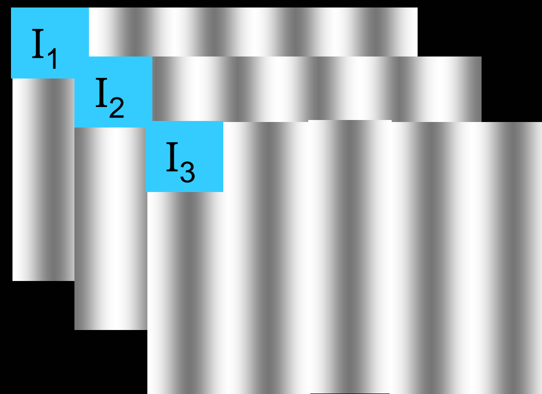
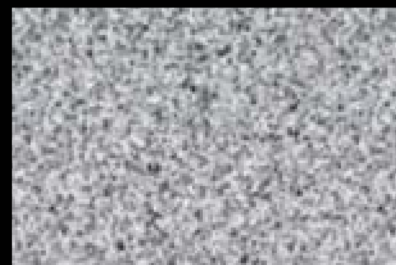
Fast optical sectioning – Wilson *et al* (Oxford)



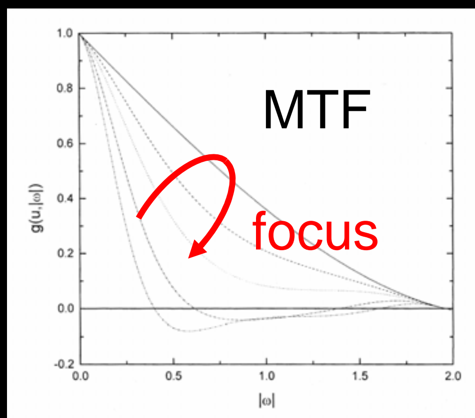
⇨ Capture images at 3 grid phases



$$I_p = \left[(I_1 - I_2)^2 + (I_1 - I_3)^2 + (I_2 - I_3)^2 \right]^{1/2}$$



Optically sectioned image



- Conventional image also available

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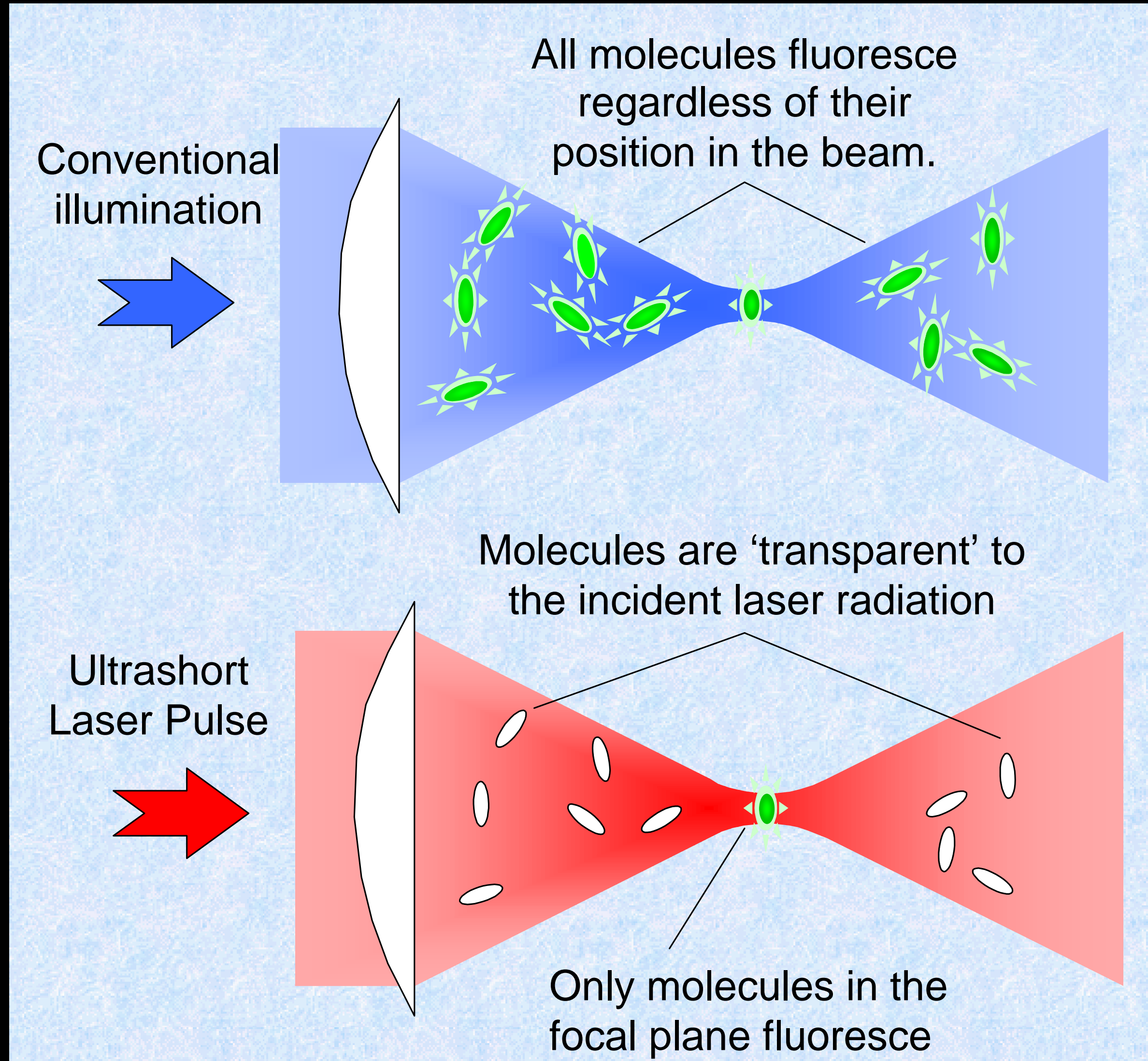
Multi-photon excitation – principle



An ultrashort laser pulse is used for excitation – very high peak power, very low average power, …100 fs in 10 ns…

Only a single voxel is excited at any one time – photon density appropriate only at focus

This excitation point is scanned in x,y and z to build up image

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Multiphoton microscope

- Scanning confocal system – BioRad 1024MP
- Inverted microscope adapted / optimised for *in vitro, in vivo.*
- Femtosecond multi-photon system.
- External detectors, time-resolved detection (TCSPC).

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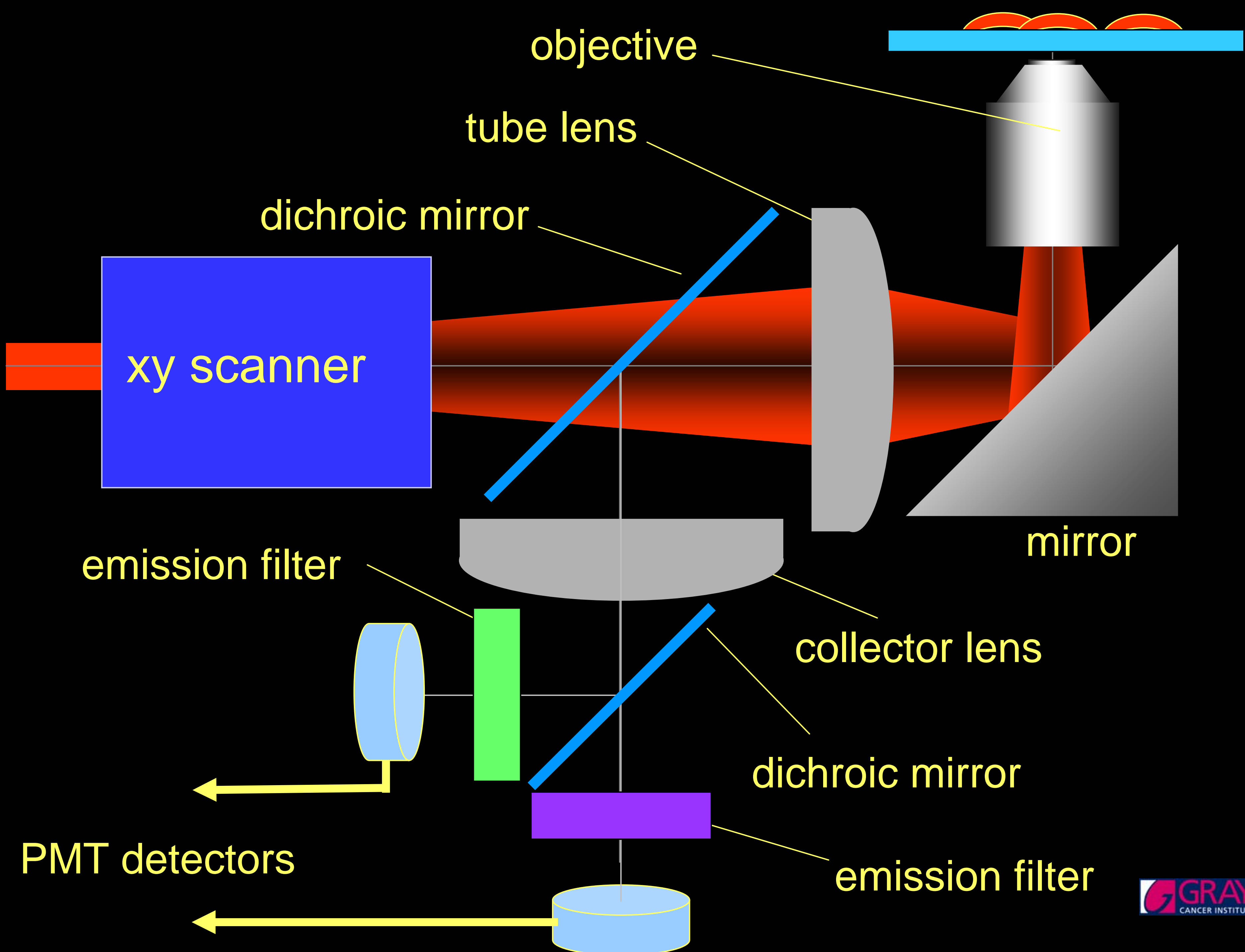
objective
tube lens
dichroic mirror
xy scanner
mirror
emission filter
collector lens
dichroic mirror
PMT detectors
emission filter
GRAY
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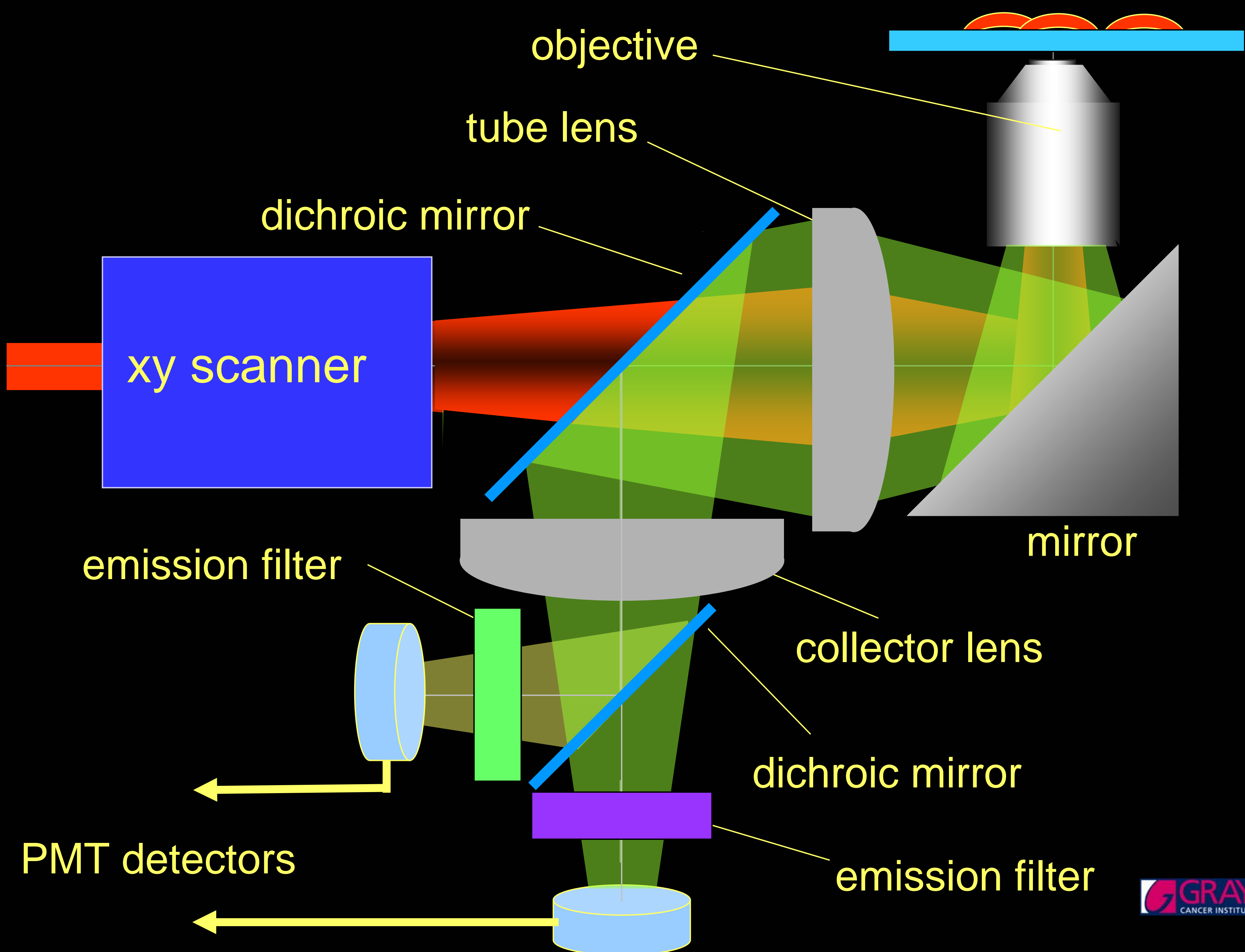
objective
tube lens
dichroic mirror
xy scanner
mirror
emission filter
collector lens
dichroic mirror
PMT detectors
emission filter
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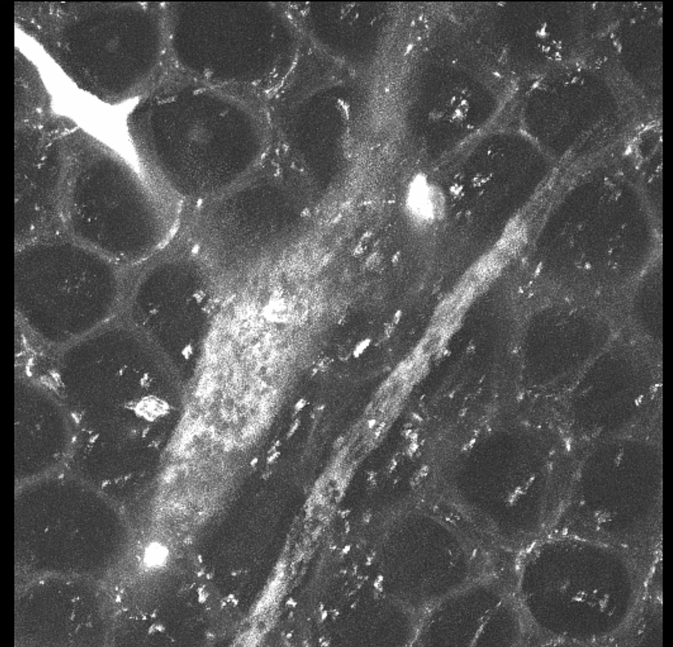
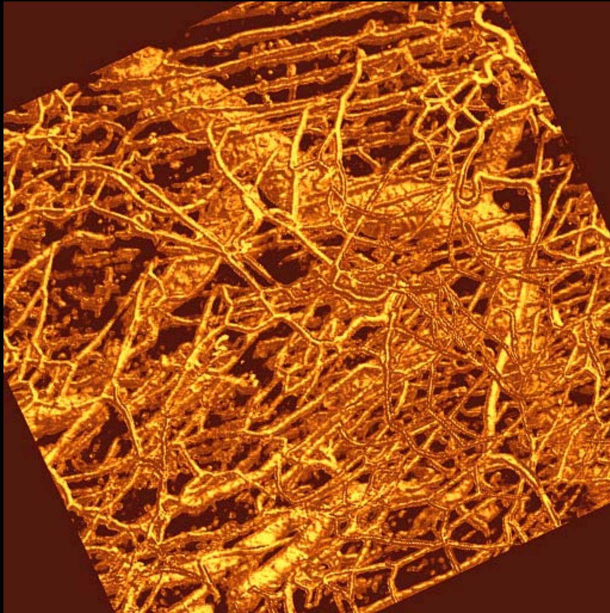
Ex vivo imaging - rat gut

Image sequence of successive optically-sectioned layers

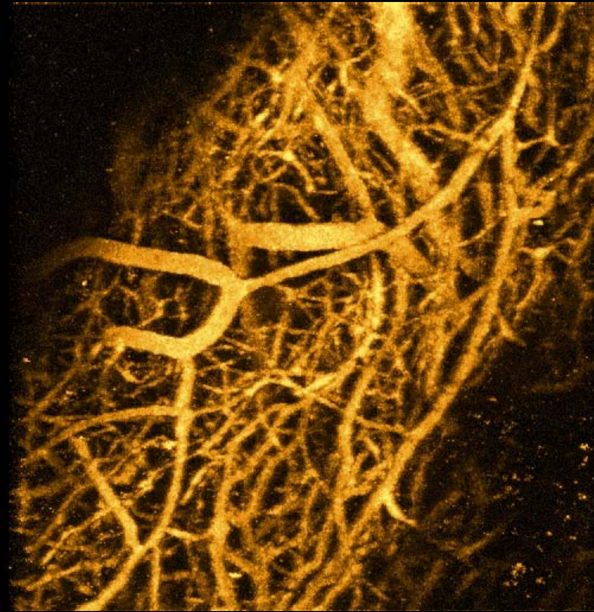
With MP excitation, it is possible to image deeply into specimens, (>>100 microns)



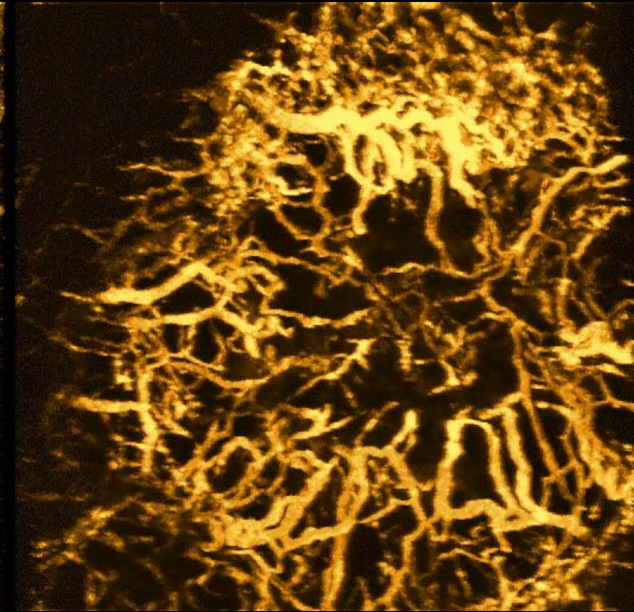
Two-photon fluorescence excitation - *in vivo*



muscle vasculature
CBA mouse



P22 fibrosarcoma
BD9 rat



HT29 human colon carcinoma
SCID mouse

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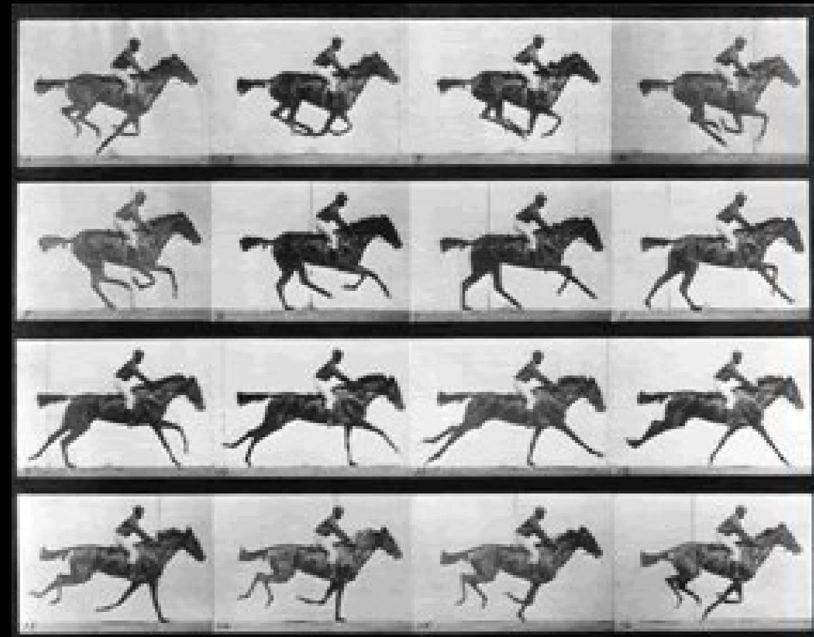
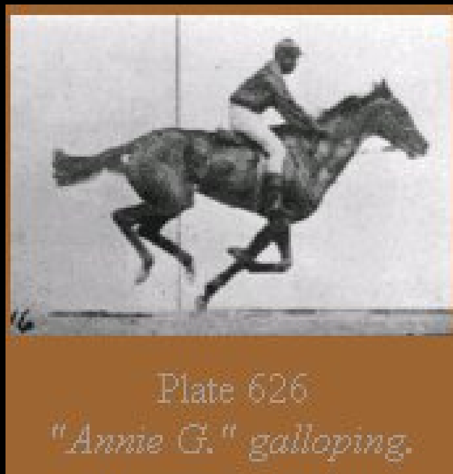
Time-resolved imaging





June 15th 1878
Eadweard Muybridge

Full-frame, time-gated imaging

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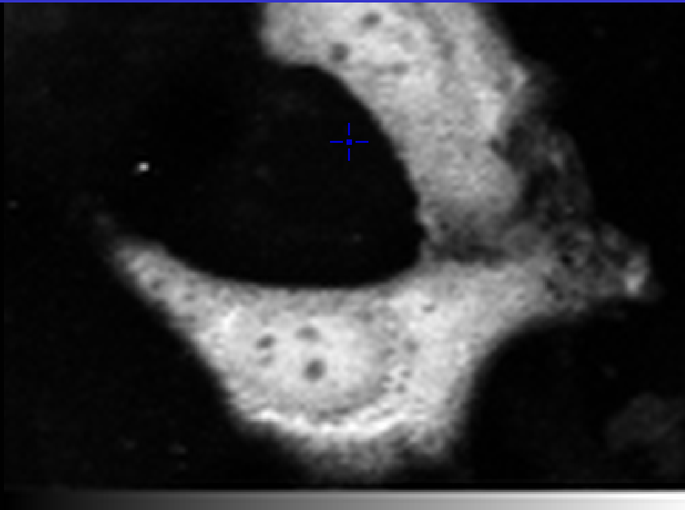
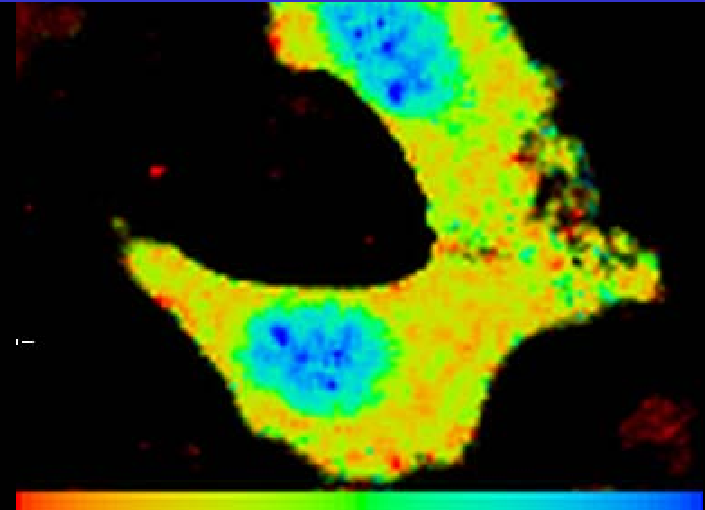
Fluorescence lifetime imaging - FLIM

Analysis of the excited state lifetime of a population of fluorescent probe molecules + Spatially resolved acquisition of data

Informs on molecular environment



Intensity image



Lifetime image

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Fluorescence lifetime measurement

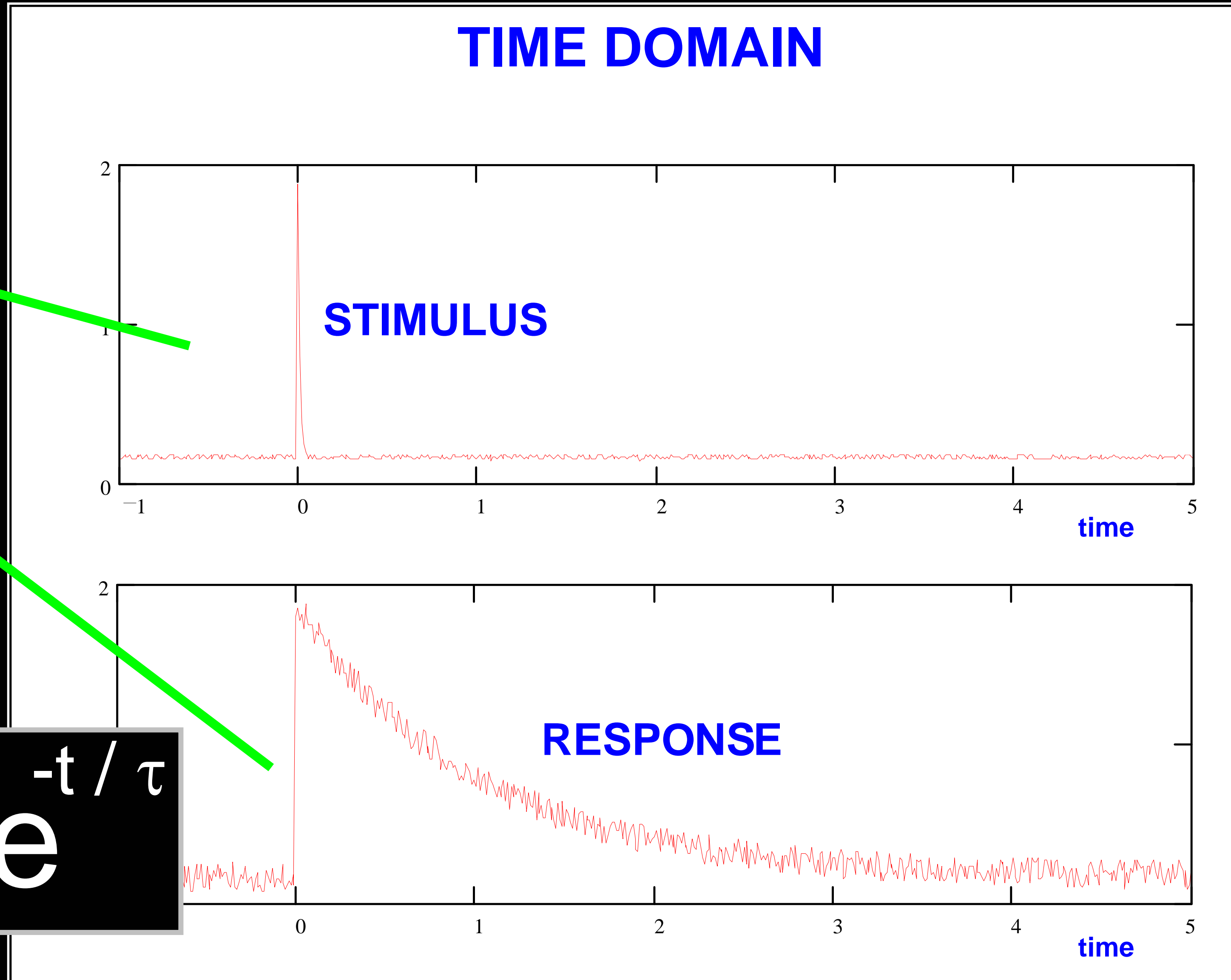
short pulse

Excitation

Emission

kinetics analysed directly

$$I(t) = I_0 e^{-t/\tau}$$



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Fluorescence Lifetime measurement

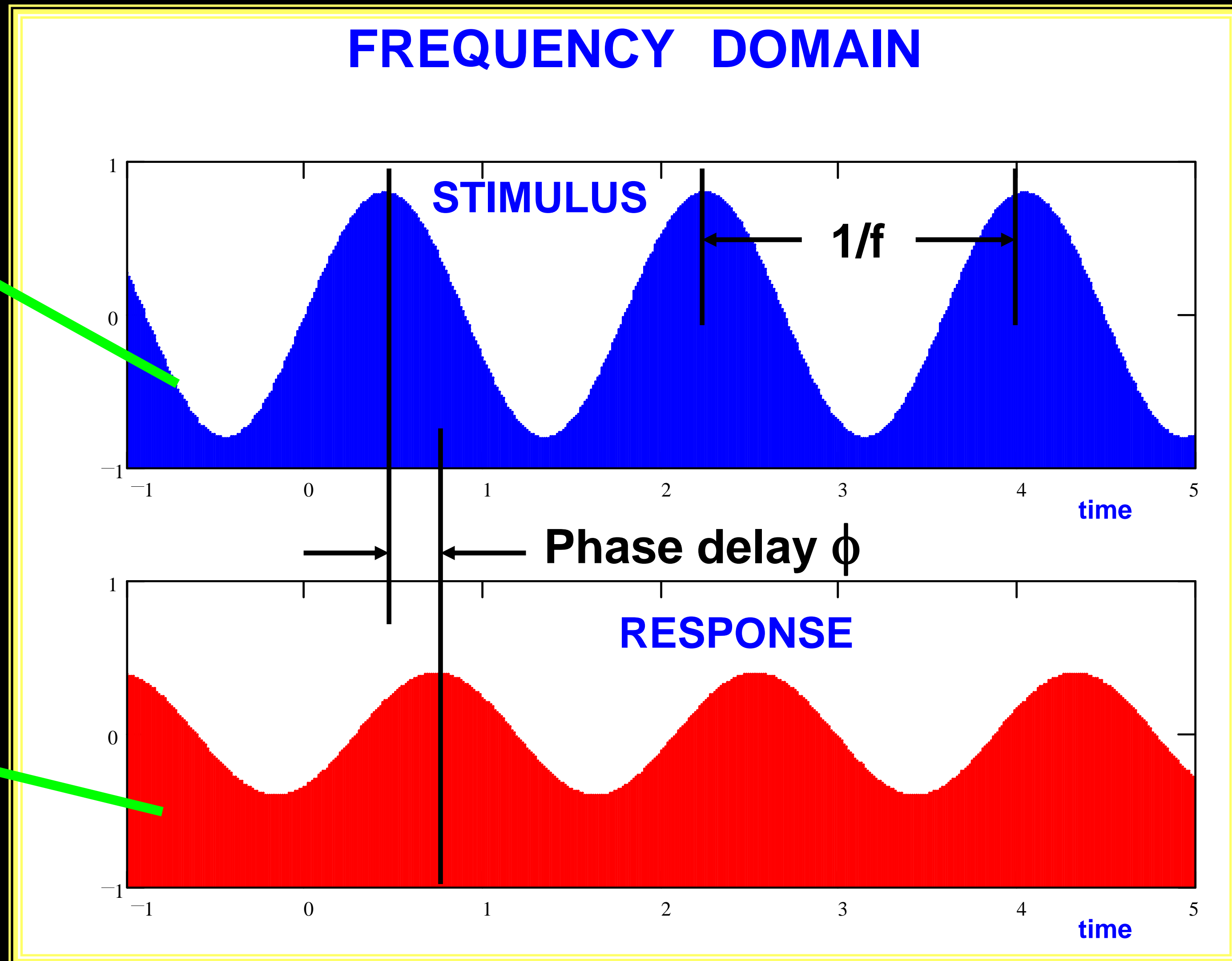
Excitation

Frequency (f) chosen to result in phase shift without significant amplitude drop

in

Emission

$\tau = \tan(\phi)/2\pi f$



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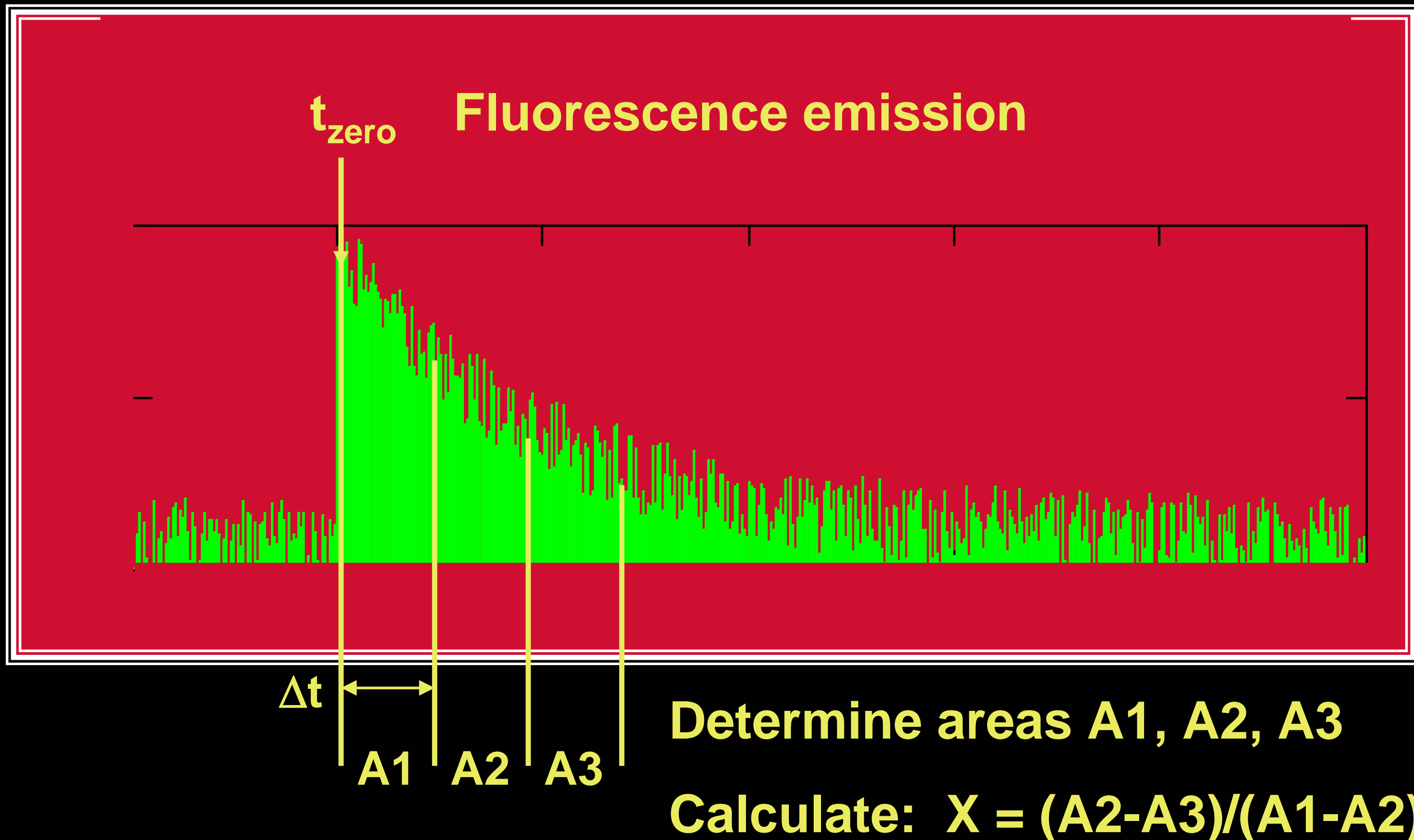
Lifetime determination from three areas



Determine areas A1, A2, A3

Calculate: $X = (A2-A3)/(A1-A2)$

Decay time: $\tau = \Delta t / -\ln(X)$

Initial value: $I = (A1-A2) \ln(X) / \Delta t(1-X)^2$

Baseline: $B = (A1 - ((A1-A2) / (1 - e^{-(\Delta t/\tau)})) / \Delta t) - I$

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Fluorescence Lifetime Imaging

Frequency-Domain:

- Complex instrumentation ⇨ not commercially available
- Can be error prone with multi-component decays
- Complex analysis (relatively…)

Time-domain (TCSPC):

- Direct measurement ⇨ conceptually simple
- Photon efficient, digital method
- Fast (3-4 x 10^6 photons sec^{-1}) ?
- High precision ~40 ps instrument response possible
- Readily combined with inherent 3D sectioning of MPM

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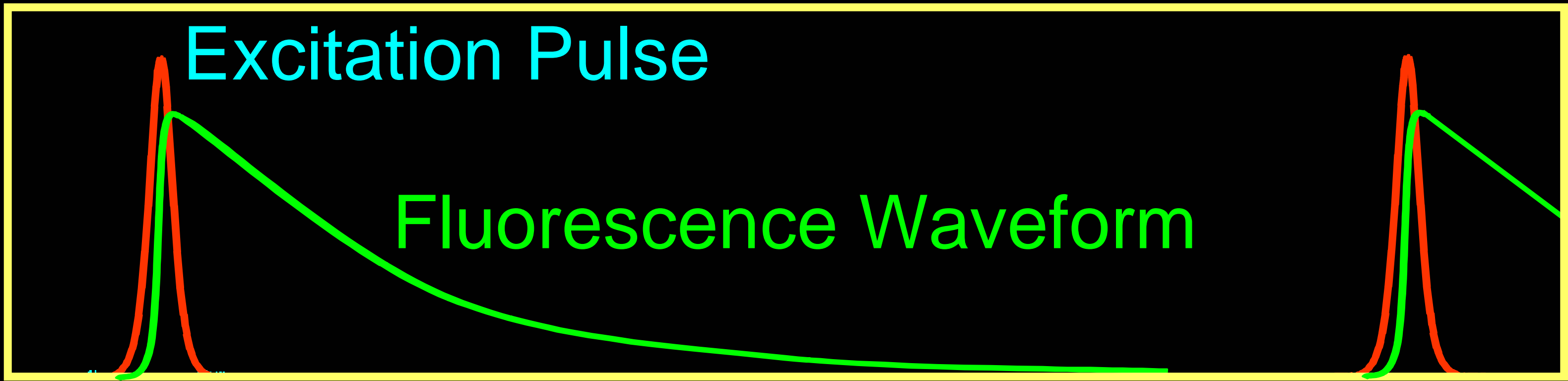
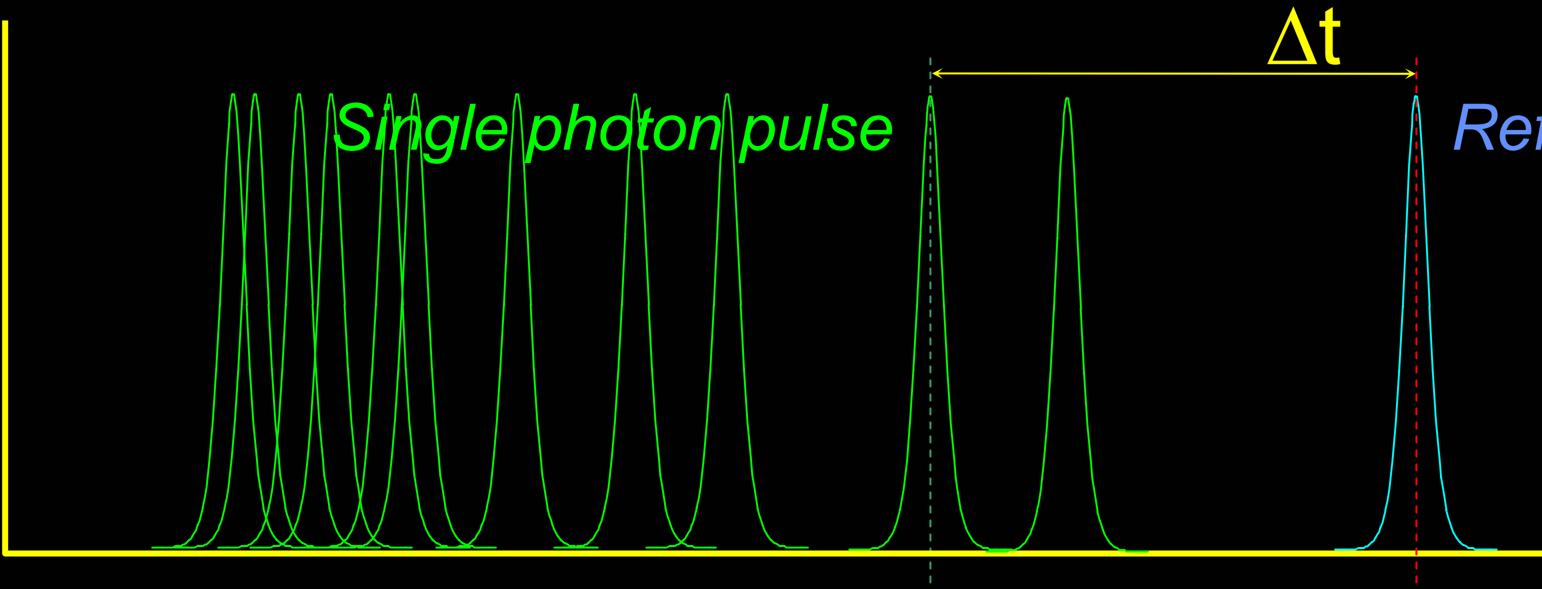
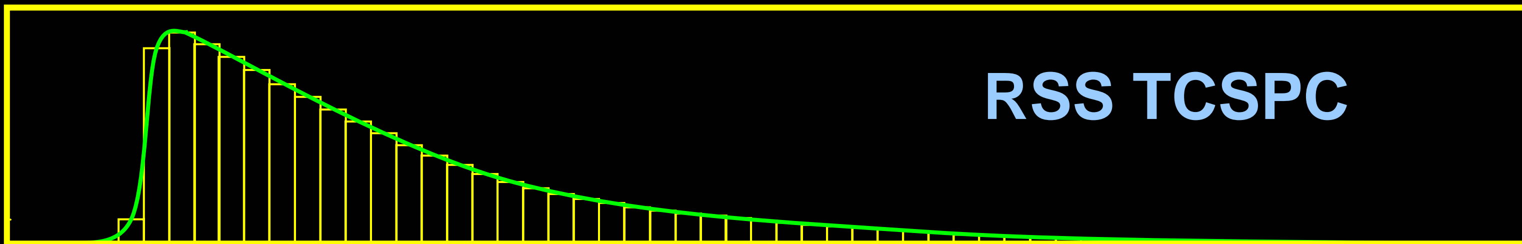


Intensity

Time



Intensity



Events

time delay (1/repetition rate - Δt)

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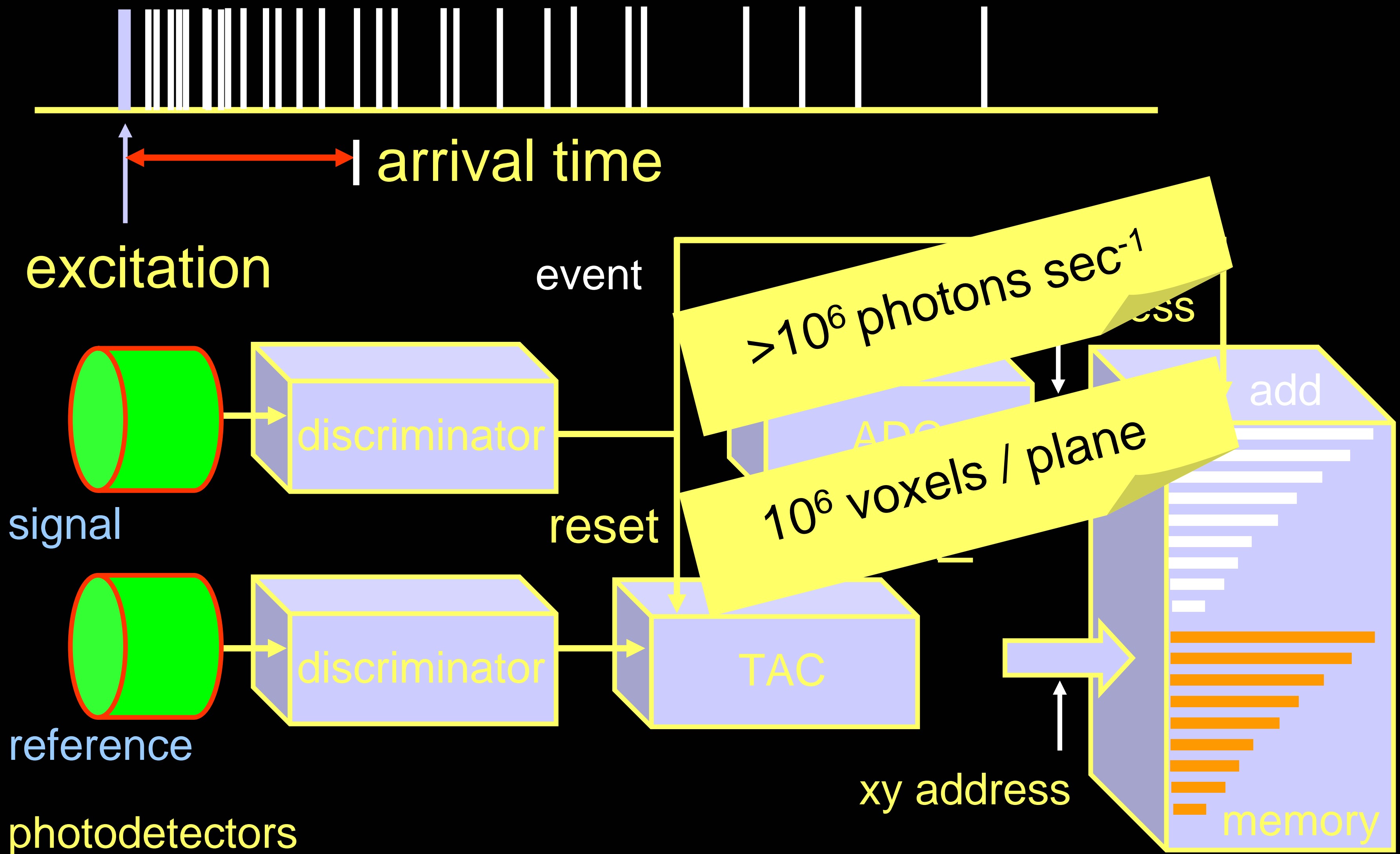
Time-correlated single photon counting



FLIM using TCSPC

- Extremely accurate
- High spatial resolution easily achieved – no image distortion device
- Every detected photon is used – limit set by objective n.a. and detector quantum efficiency
- Photon counting rates typ. 3 MHz
  - ⇨ relatively slow, BUT
  - ⇨ in case on MP excitation, only ‘thin’ optical slice is excited, hence low output rate anyway…
  - ⇨ particularly suited to live cell imaging, low toxicity
- Not appropriate to IMAGING dynamic events
- ‘Line’ or ‘point’ excitation much faster

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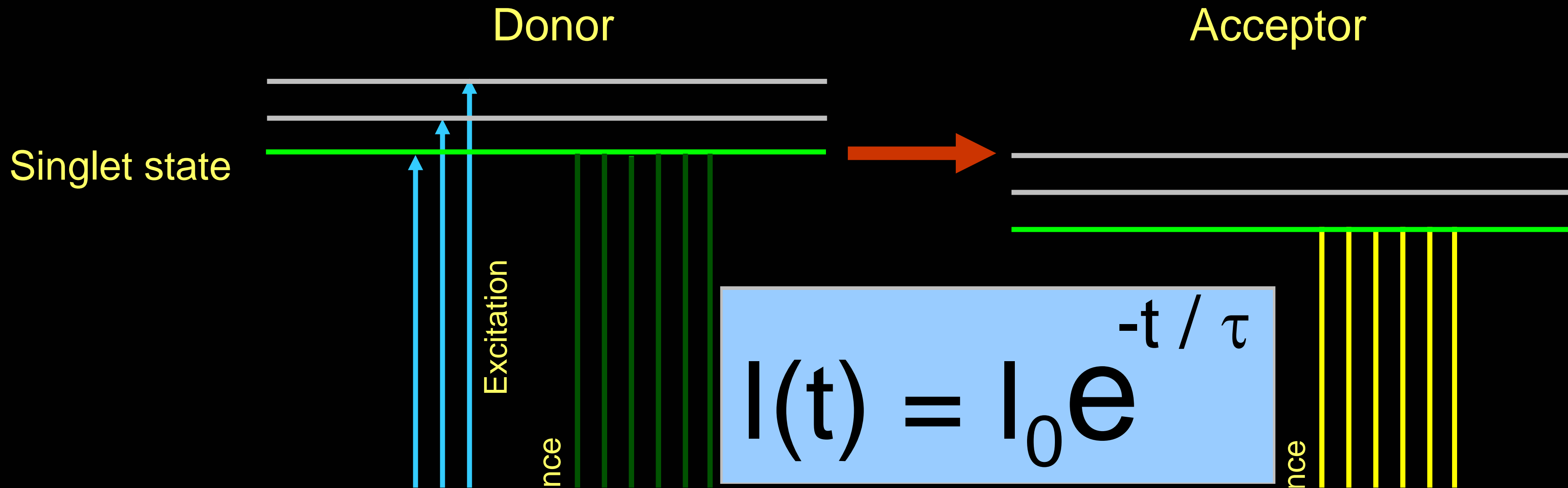
Förster Resonance Energy Transfer (FRET)



Branching of the potential energy surface of the excited state…
Singlet state lifetime **reduced** in presence of the acceptor

Far-field imaging capable of determining distances associated with **near-field** techniques

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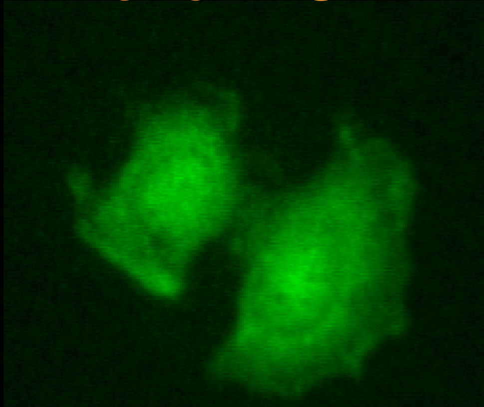
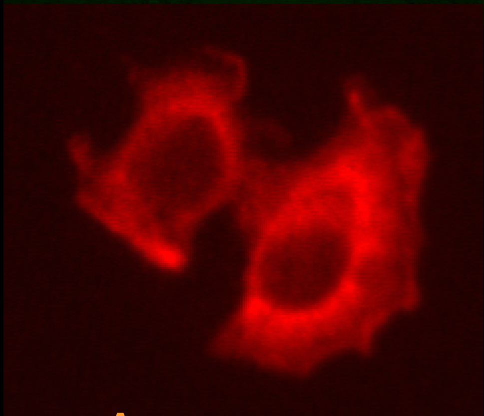
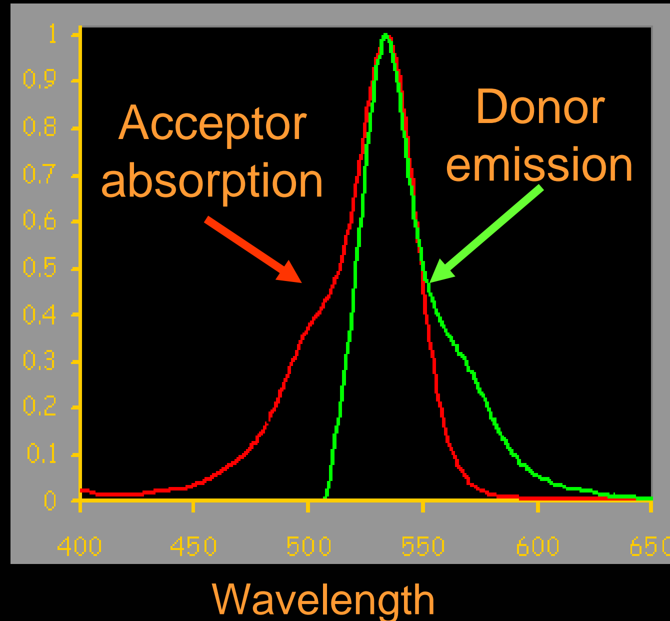
MCF-7 cells
Donor EGFP





Acceptor
(Alexa fluor 532)



Wavelength

- Donor emission and acceptor excitation spectra should overlap
- FRET occurs only when interaction distances are small (typ. <10 nm) and dipole alignment is present

$$Eff. = 1 - \frac{\tau}{\tau_D} = \frac{R_0^6}{R_0^6 + r^6}$$

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$$Eff. = 1 - \frac{\tau}{\tau_D} = \frac{R_0^6}{R_0^6 + r^6}$$

Population of FRET-ing species

= A1 / (A2+A1)

A1 = rel. ampl. of quenched donor lifetime

A2 = rel. ampl. of control donor lifetime

$$R_0 = \frac{0.211}{\left(\kappa^2 J(\lambda) n^{-4} Q\right)^6} \text{ Å}$$

$$J(\lambda) = \frac{\int_0^\infty F(\lambda)\varepsilon(\lambda)\lambda^4 d\lambda}{\int_0^\infty F(\lambda) d\lambda} \text{ M}^{-1}\text{ cm}^{-1}\text{ (nm)}^4$$

κ = relative dipole orientation
J = spectral overlap integral
n = refractive index
Q = donor quantum efficiency

F = fluorescence intensity
ε = extinction coefficient of acceptor
λ = wavelength

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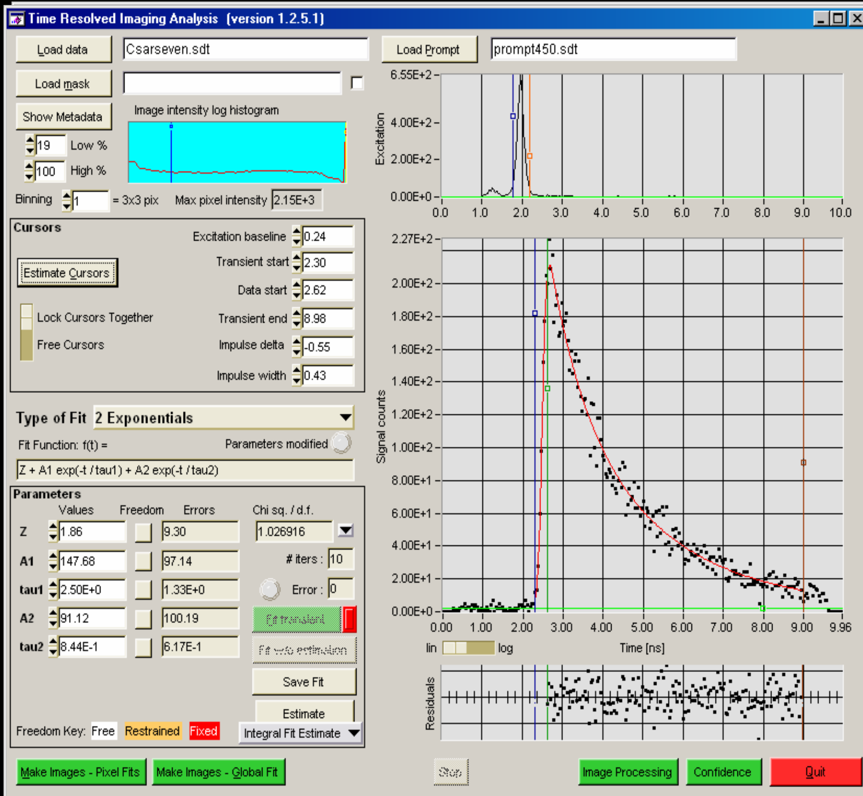

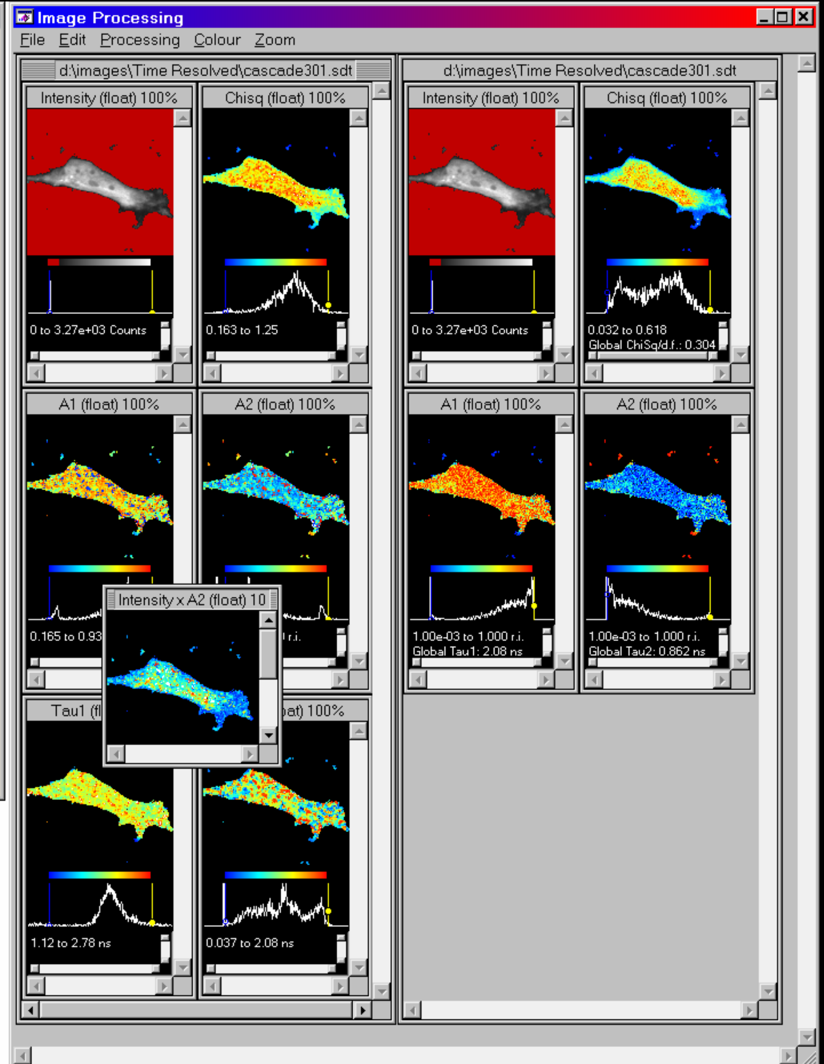

Multiple area, NLLS single, multi-exponential & distributed fitting
Global analysis, error map generation, support plane analysis
Image arithmetic functions to derive populations, efficiencies
Flexible image display / on-line comparison

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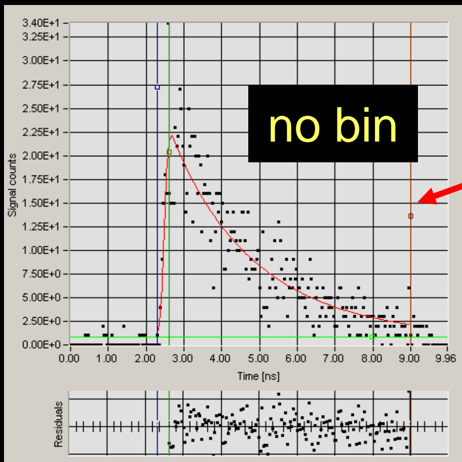
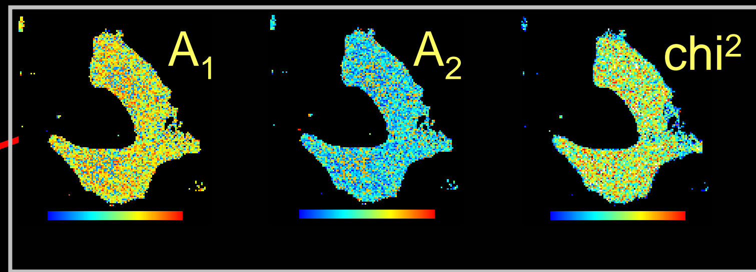
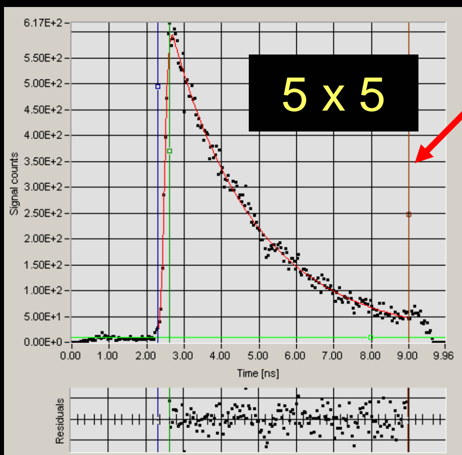
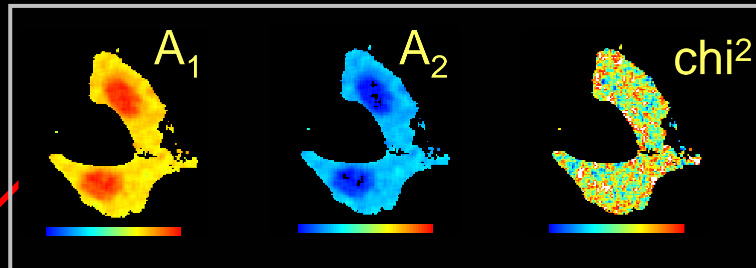
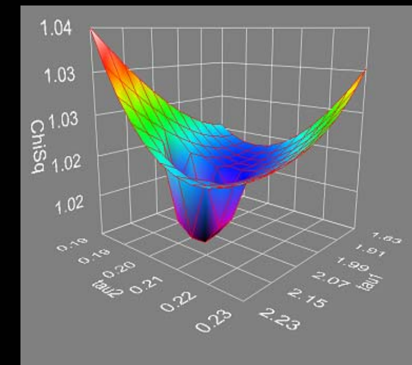




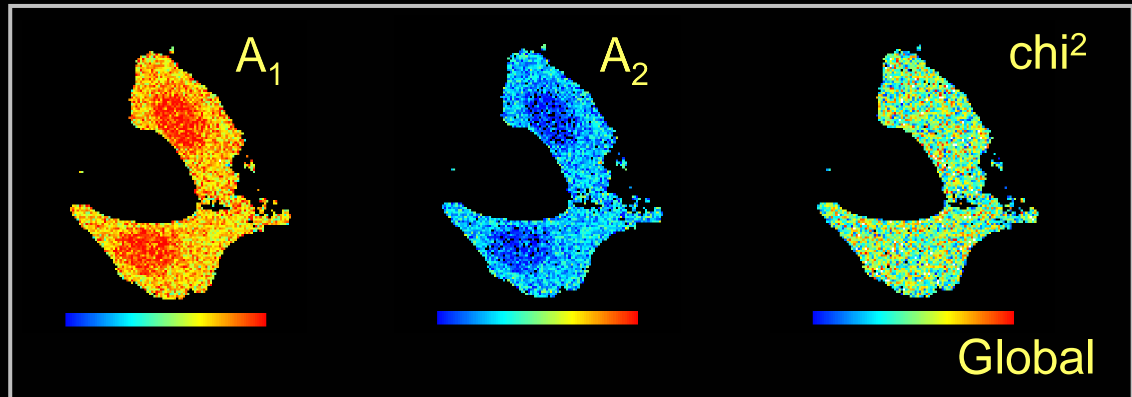






Global analysis

– ratios of two lifetime components at each pixel



τ_1 = 0.65 ns τ_2 = 2.31 ns

1.7 τ_{ave} 2.4 ns

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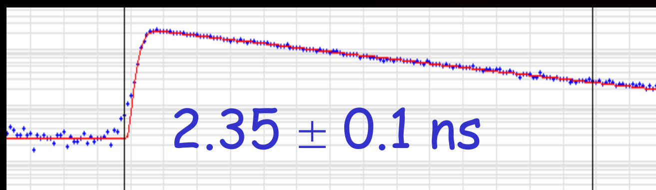
p65 NfκB–IKK interaction

H_2O_2 100 μM 5 minutes

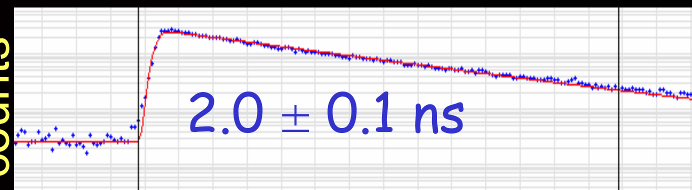
Nucleus



Photon counts

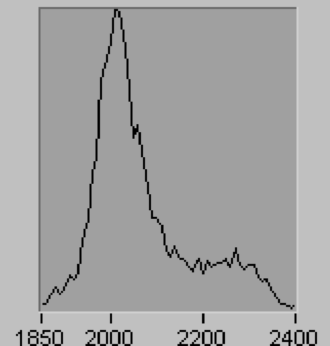
Time (ns)

Cytoplasm



Photon counts

Time (ns)



FRET efficiency

0.23

0

1.8

Lifetime (ns)

2.4

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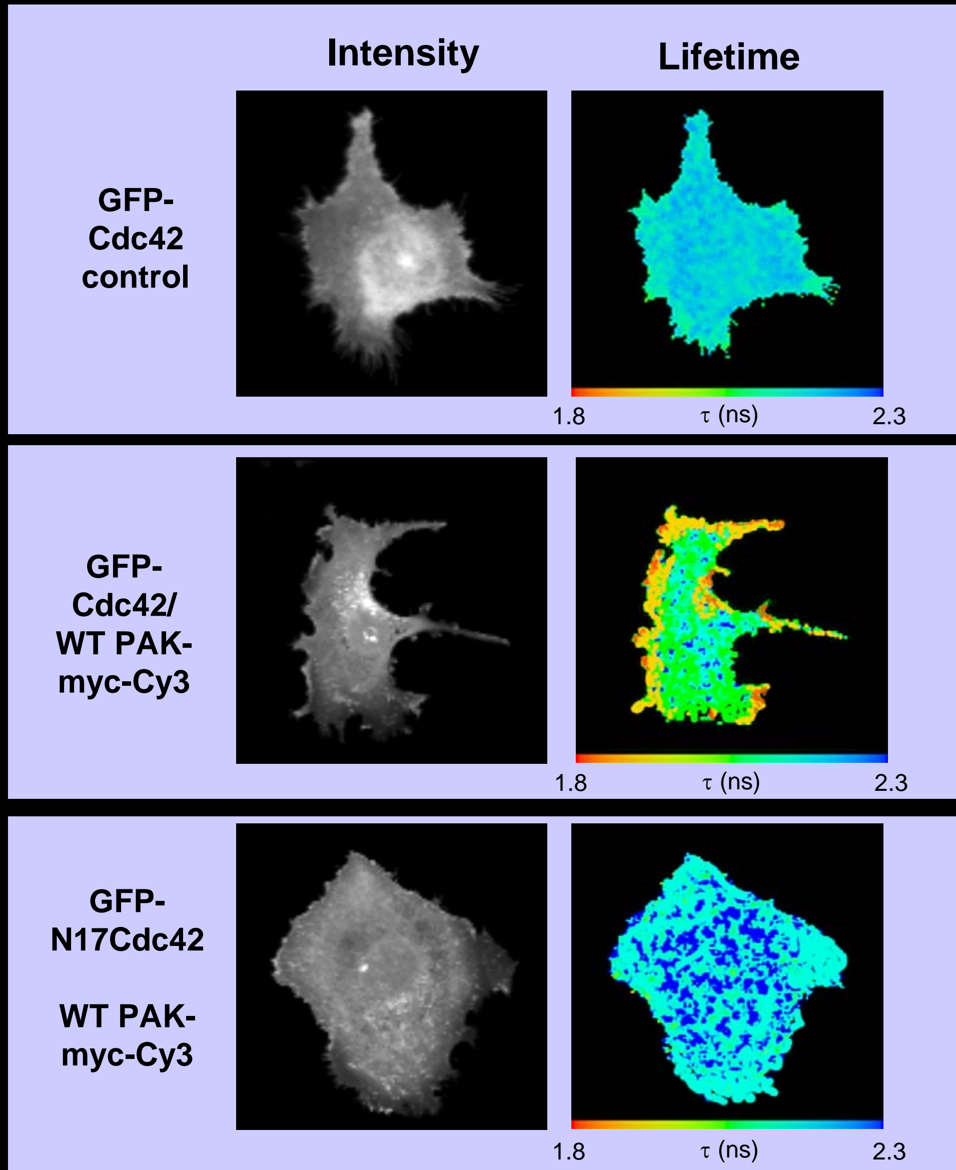

MDA MB 231 breast carcinoma cells microinjected with plasmids encoding GFP-Cdc42 (WT or N17 dominant negative variants) and PAK1-myc.

Cells fixed and stained using an anti-myc-Cy3 conjugated antibody.

Top panels: GFP control lifetime (typically 2.2 ns) in the absence of Cy-3 antibody.

Middle panels: drop in GFP lifetime in the presence of WT Cdc42-myc-Cy3 plus epidermal growth factor (EGF) to stimulate Cdc42 activation. Localisation of interaction between WT GFP-Cdc42 and PAK-myc-Cy3 in the presence of EGF clearly seen at cell periphery & in membrane protrusions.

Bottom panels: N17Cdc42 dominant negative control has an observed lifetime comparable to control values and, therefore, no interaction with PAK1.

In collaboration with M. Parsons, T. Ng
King's College, London

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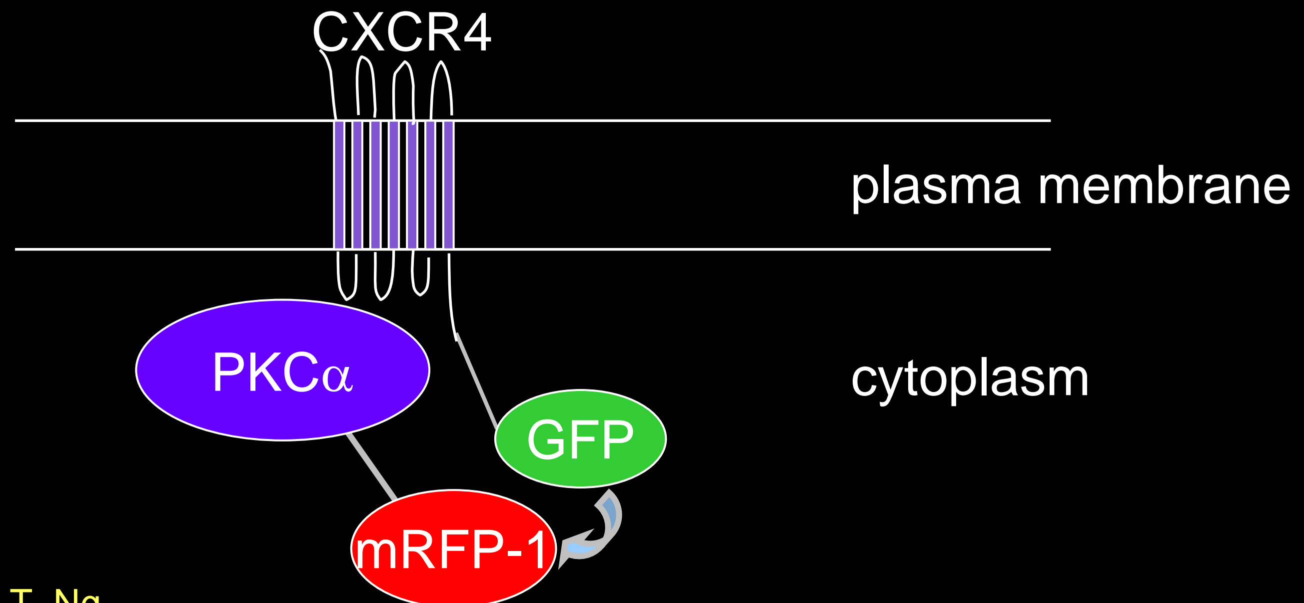
CXCR4 function – GFP : mRFP-1 FRET

- CXCR4 is a membrane receptor which binds to the soluble chemoattractant SDF-1
- CXCR4 is highly expressed in malignant breast cancer tissue
- SDF-1 is expressed in organs where breast cancer metastases are frequently found (bone marrow, lymph node, lung, liver)
- Also involved in development of the immune, circulatory and central nervous systems; is a co-receptor for HIV-1
- Function of CXCR4 is dependent on its interaction with PKC



In collaboration with M. Peter & T. Ng
King's College, London; R. Tsien

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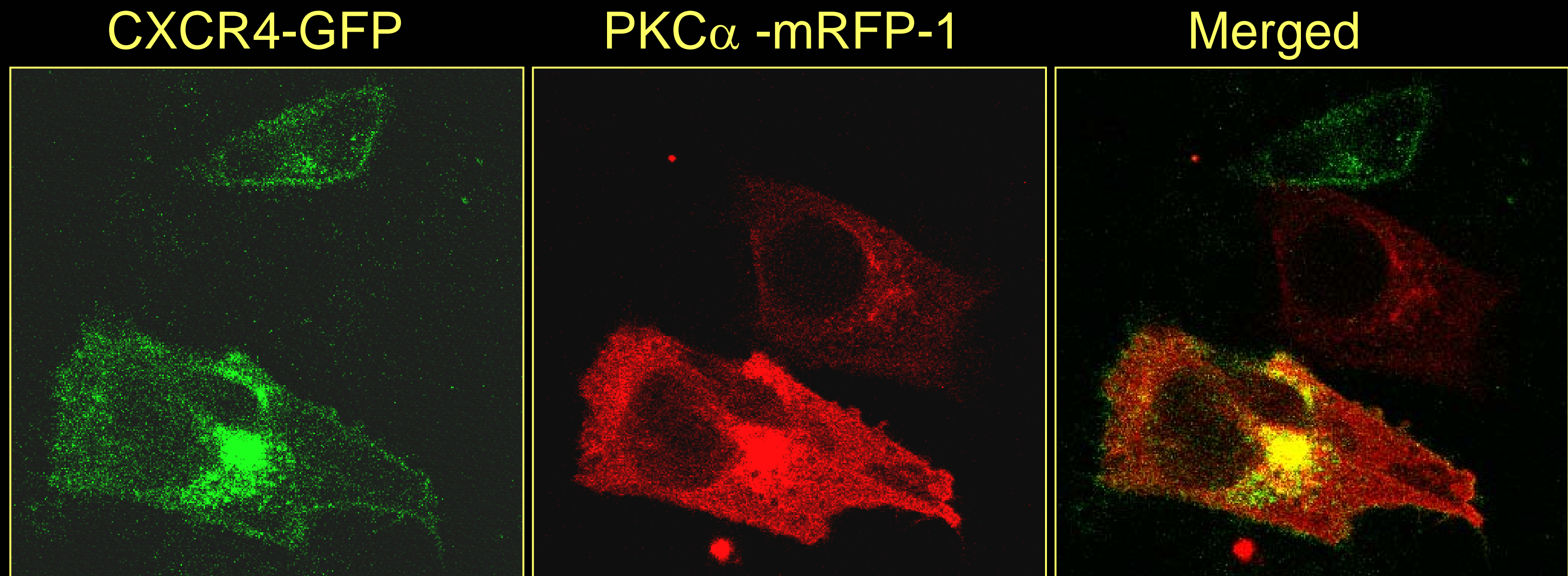
Imaging CXCR4-GFP and PKCα - mRFP-1

MDA-MB-231-CXCR4-GFP cells transiently transfected with PKCα (aa1-337)-mRFP-1



- Fast maturation
- Monomeric
- Spectrally distinct

In collaboration with M. Peter & T. Ng
King's College, London; R. Tsien

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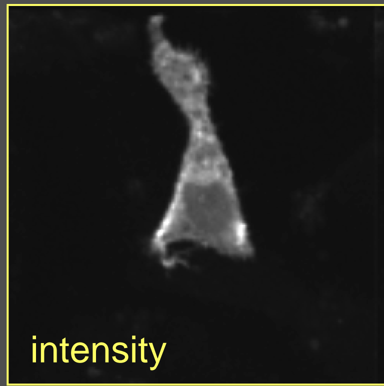
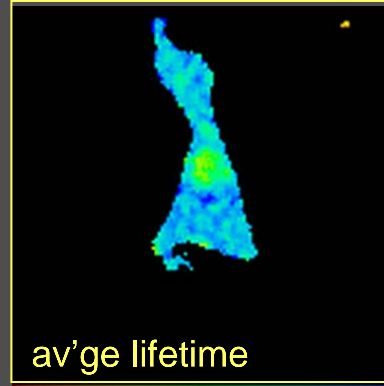
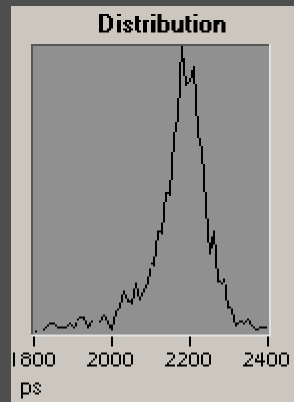
CXCR4-GFP alone

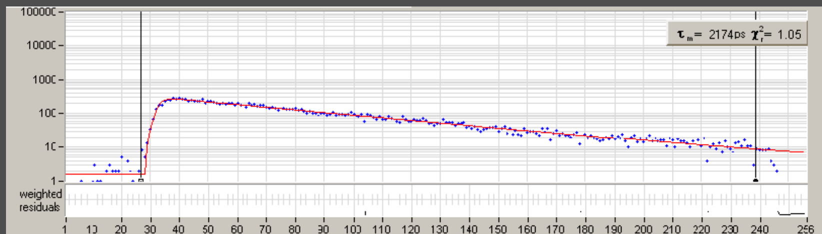




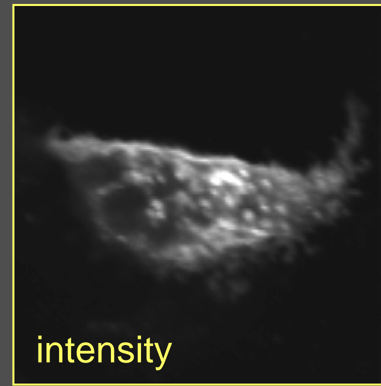
1.8 τ(ns) 2.3

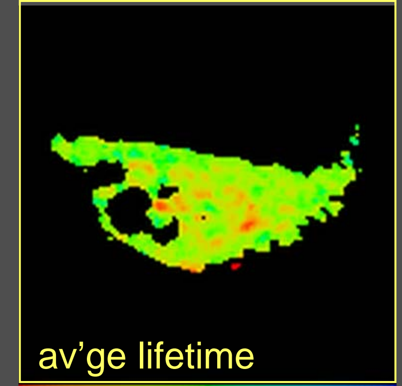


In collaboration with M. Peter, M. Hughes & T. Ng King's College, London

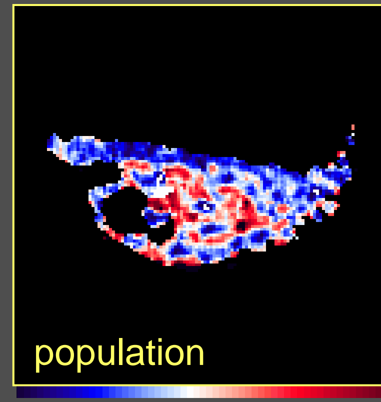


CXCR4-GFP + PKCα-mRFP-1 vesicle interaction

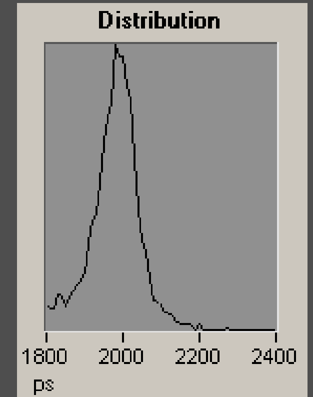


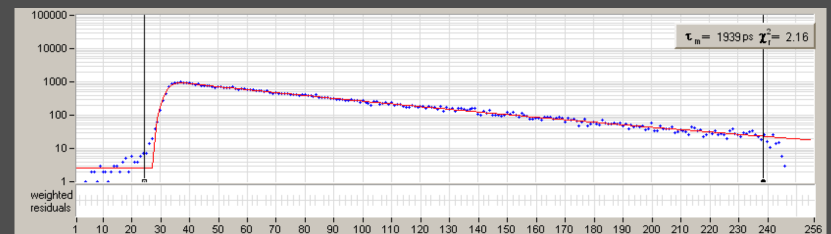


1.8 τ(ns) 2.3



1% FRET 46%





FRET couples: Summary

GFP ⇨ Cy3-IgG labelling offers a robust platform for FRET Imaging
+ Mature technique (5+ years experience in team)
-Large separation
-Live imaging is challenging
Most suited to intramolecular FRET

GFP ⇨ REACh offers advantages over CFP ⇨ YFP and GFP ⇨YFP
+ No bleed through of acceptor fluorescence
+ Live cell imaging
+/- Large R_0
- Uncertainty over acceptor +ve
Most suited to inter-molecular FRET

GFP ⇨ mRFP offers most exciting alternative to GFP ⇨ Cy3
+ No bleed through of acceptor fluorescence
+ Live cell imaging
- Low acceptor fluorescence quantum yield
- Not widely available and immature technology
Most suited to “in vivo” FRET

Summary

- Non-fluorescence imaging is challenging – and is likely to remain so…
- Camera-based fluorescence systems ⇨ straightforward
- Fluorescence image processing ⇨ rugged segmentation methods
- Fluoresence lifetime imaging informs on molecular environment
- Imaging at depth, in 3D
  - ⇨ Structured light methods may have applications
  - ⇨ MP methods mature but still costly
  - ⇨ MP methods provide FLIM
- FLIM / FRET methods are state-of-the-art techniques with wide applications in cell signalling, protein interactions and conformation
- The goal is to unravel cell signalling responses, bystander effects
- Combine technologies on one platform

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AG Michette *
P Charalambous *
S Pfauntsch *

FLIM/FRET
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PR Barber
RJ Locke
RG Newman
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T Ng*
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S Pfauntsch*
N Edme *
M Keppler *
M Peter *

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