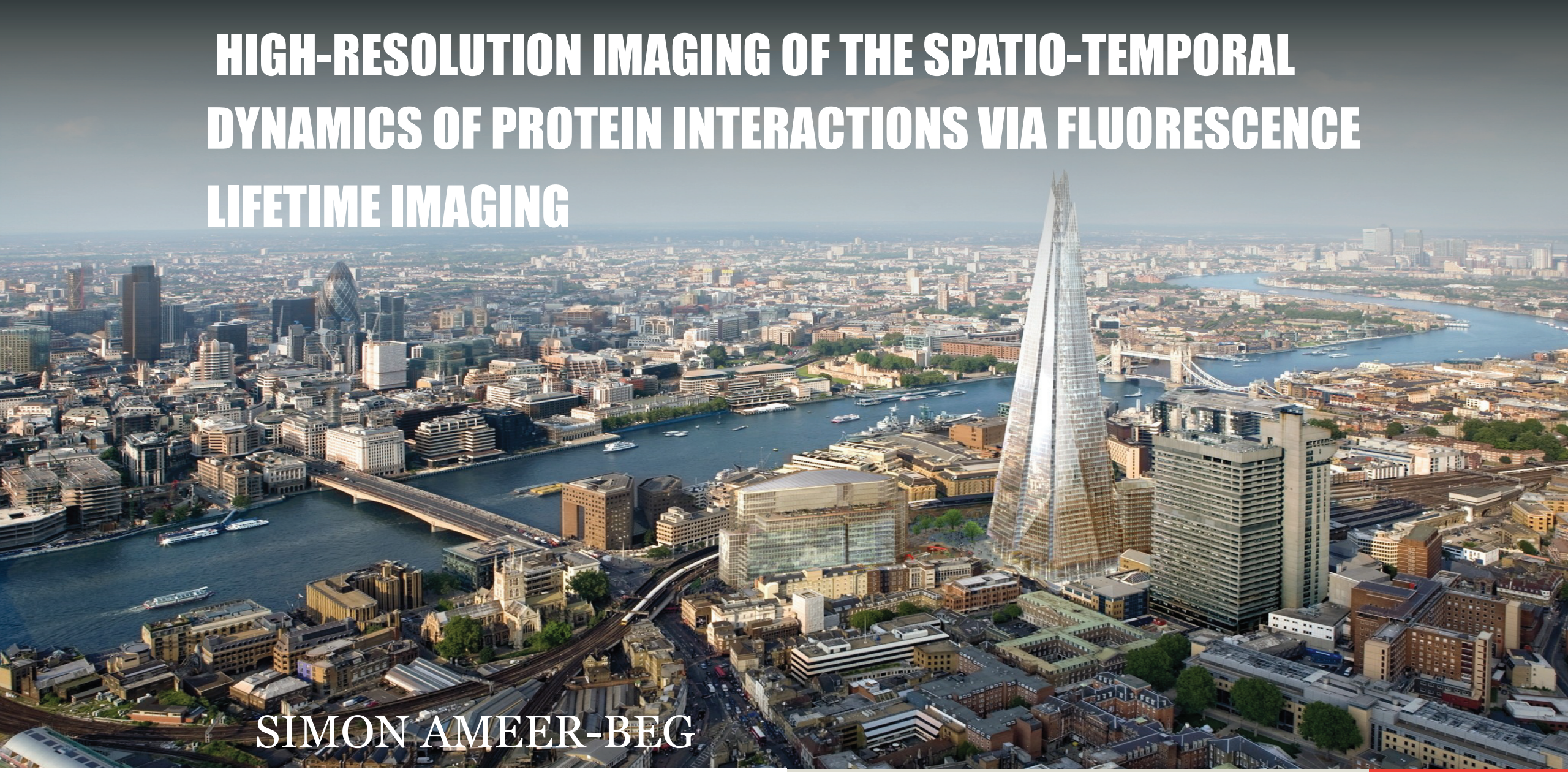
HIGH-RESOLUTION IMAGING OF THE SPATIO-TEMPORAL DYNAMICS OF PROTEIN INTERACTIONS VIA FLUORESCENCE LIFETIME IMAGING



SIMON AMEER-BEG

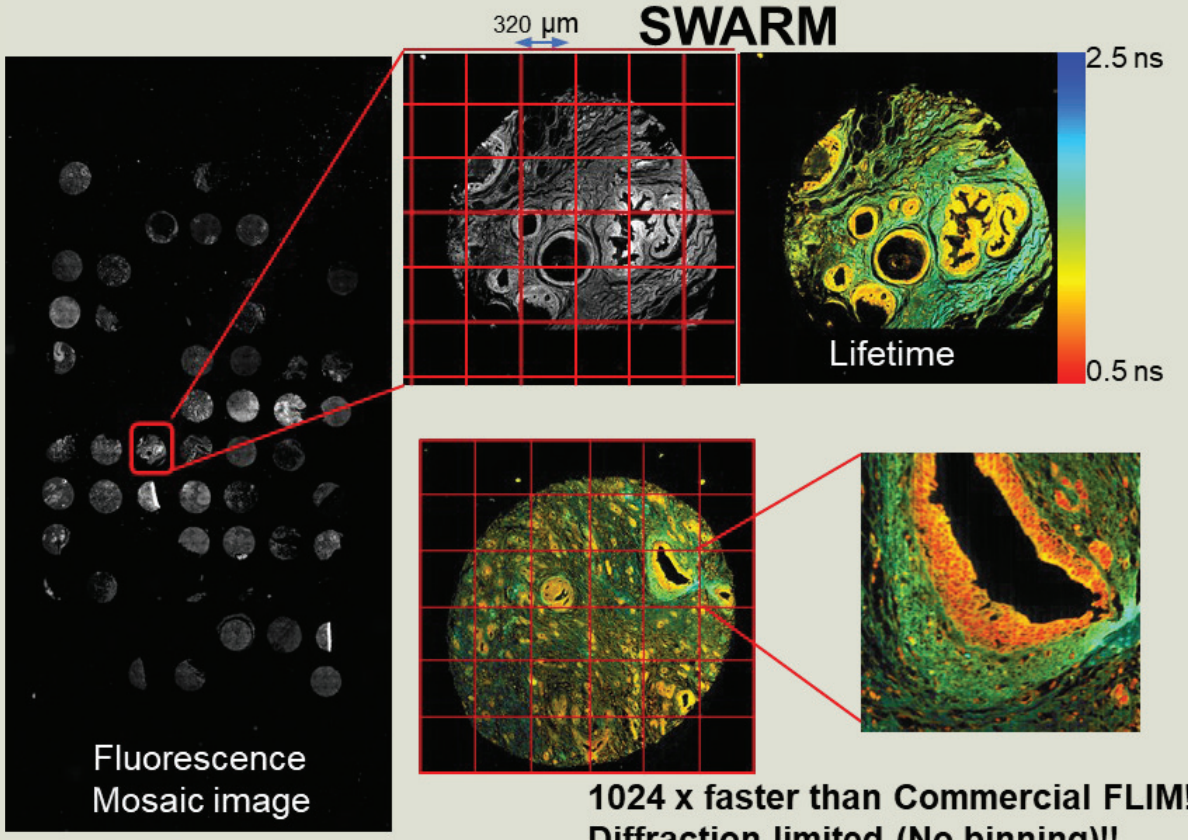
10/06/2020

Richard Dimbleby Cancer research Labs
Optical Oncology Group
School Of Cancer and Pharmaceutical Sciences
Randall Centre of Cell and Molecular Biophysics

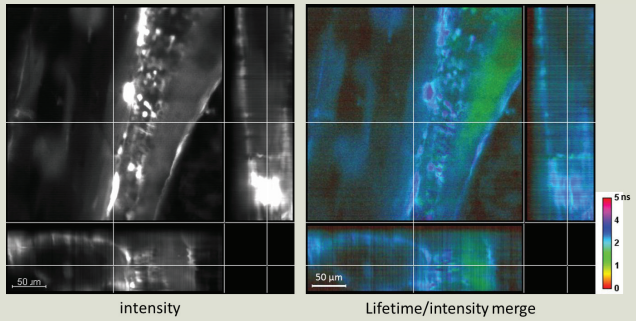
KING'S College LONDON

Functional Histology and Pre-Clinical

- ***Tissues***
- FLIM - Histology
- Multiphoton Microscopy
- Organoids
- Light-Sheet FLIM
- High Content Screening
- Bioengineering

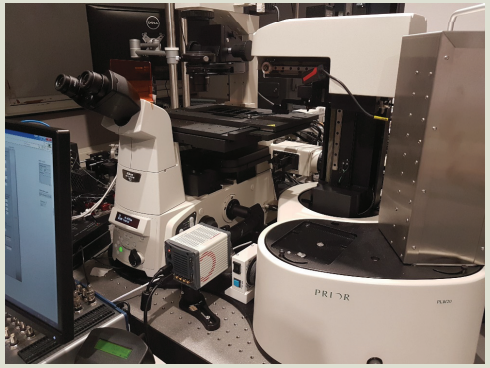


Frequency domain DSL-FLIM



1024 x faster than Commercial FLIM!
Diffraction limited (No binning)!!

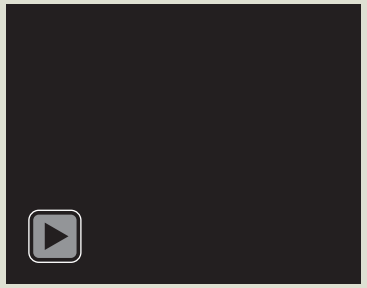
Semi-Autonomous Microscopy

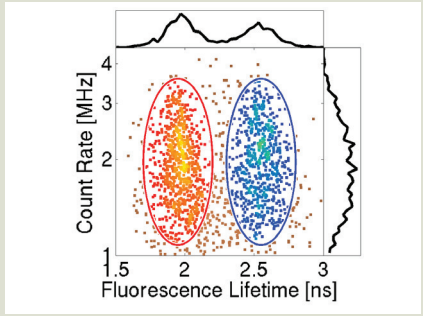


3D Printing

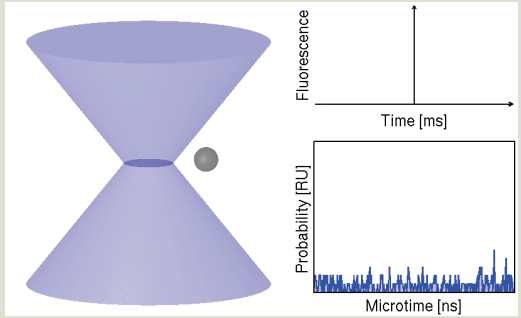
Single Cell Functional Imaging

- Optical Proteomics
- Multifocal Fluorescence Lifetime Imaging
- Rare-cell analysis
- MicroFLiC
- Optical Trapping
- High Content Screening

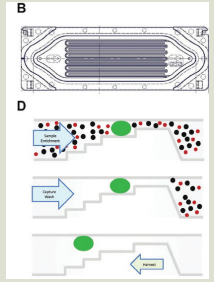




MicroFLiC

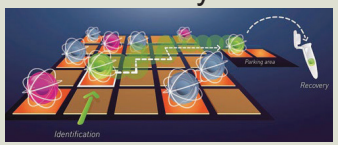


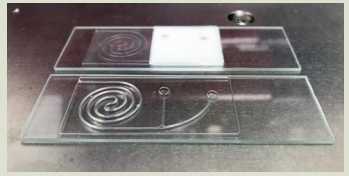
CTC's

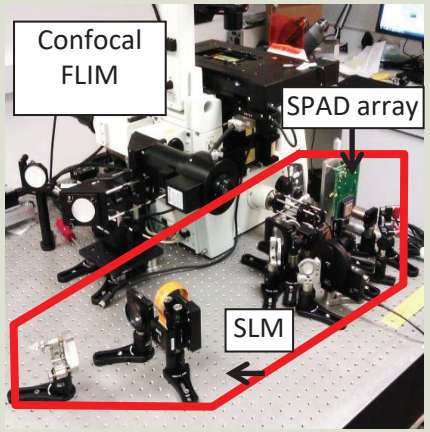


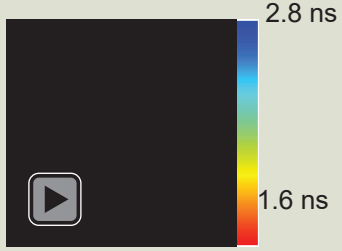
Parsortex

DEPArray



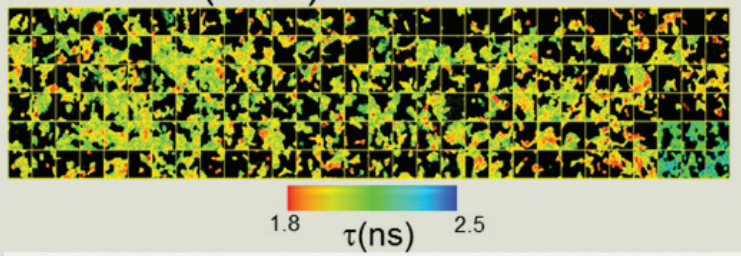


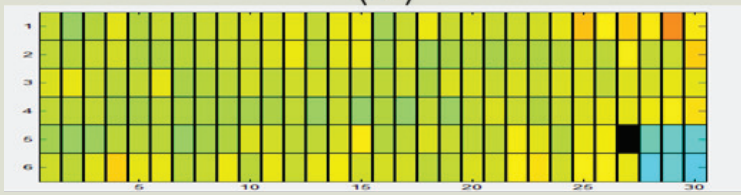




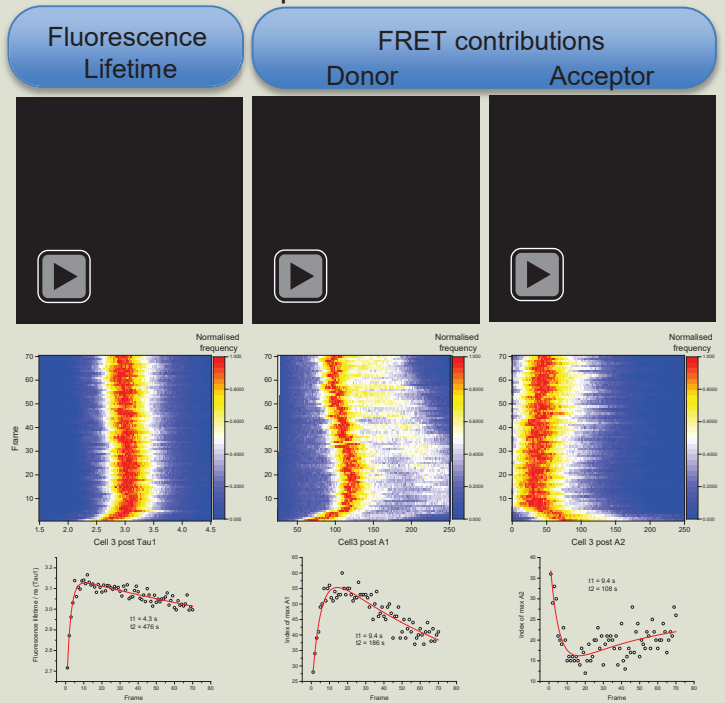
Fascin-GFP+Lifeact-RFP
+ 4 µM smifh2 (inhibitor)

Donor (GFP) FLIM – 96-well





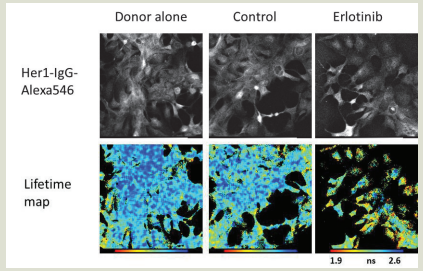
mTurq2-EPAC-dVenus

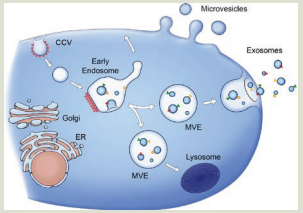


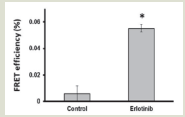
Exosomes and Single Molecules

Single-molecule Imaging

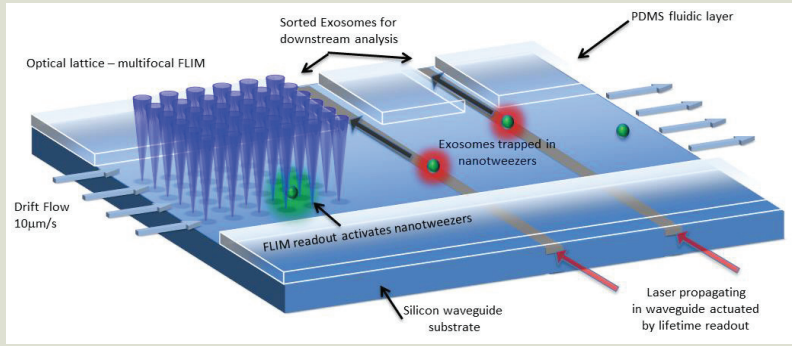
- Dynamic FRET
- SPT – receptor dynamics
- Functional STORM/PALM
- Exosome Imaging and tracking

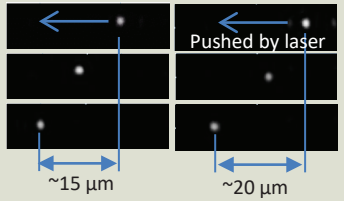


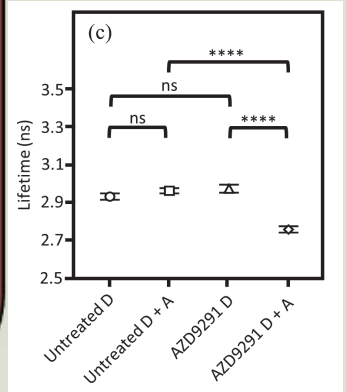




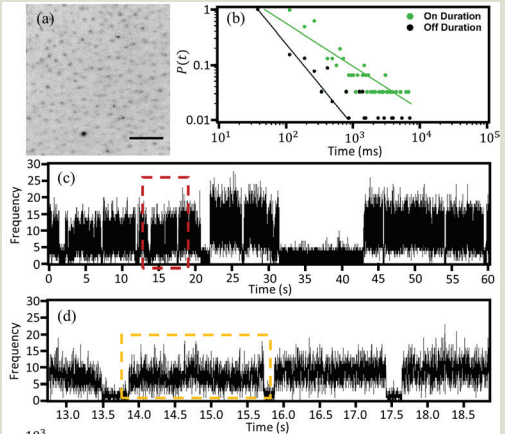
Exosome Trapping/Measuring with optical waveguides

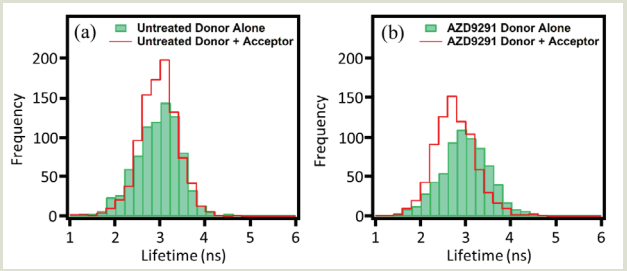




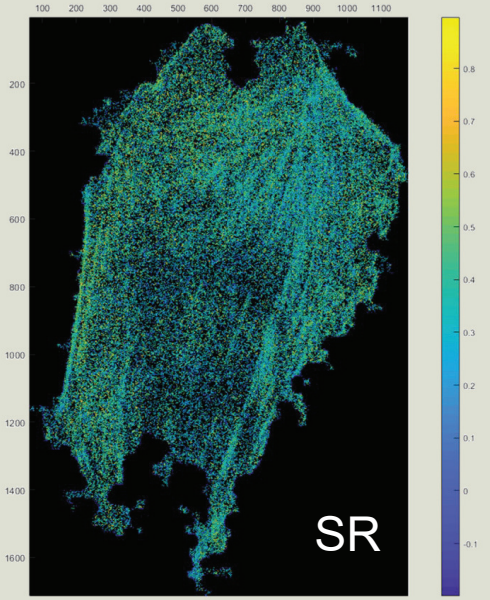


SM-tracking surface receptors

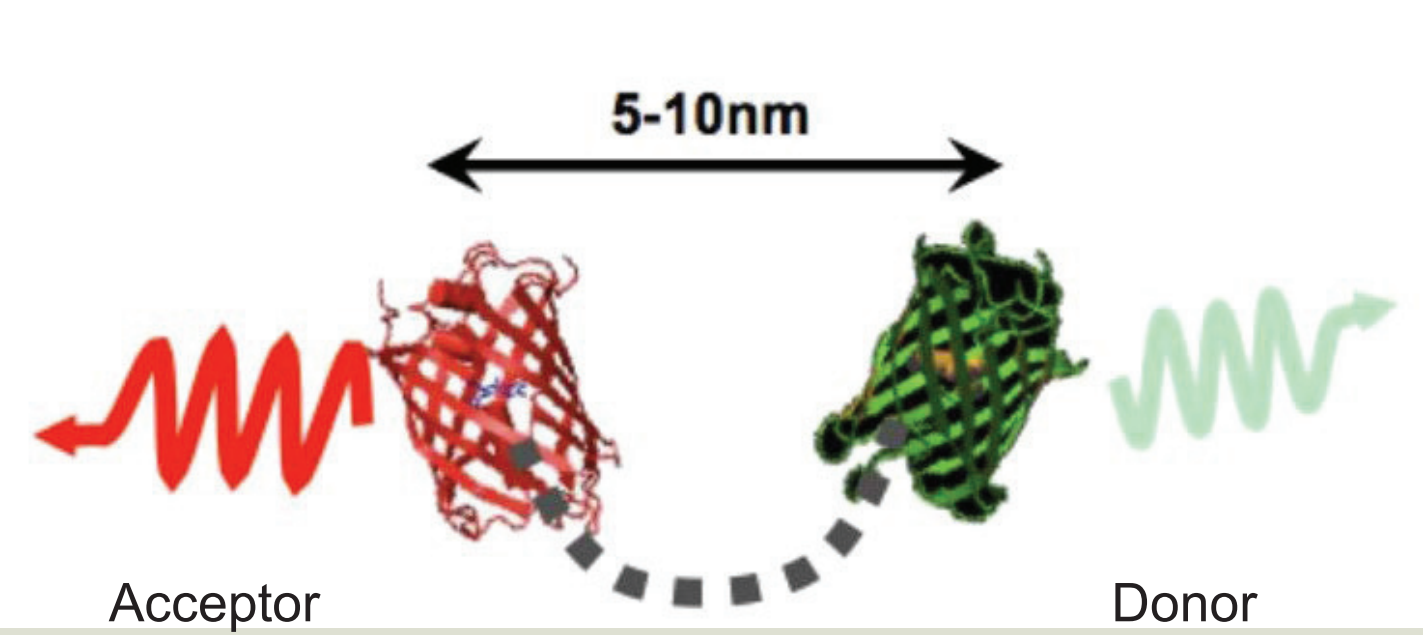




A-STORM (Lifeact-mEOS2)



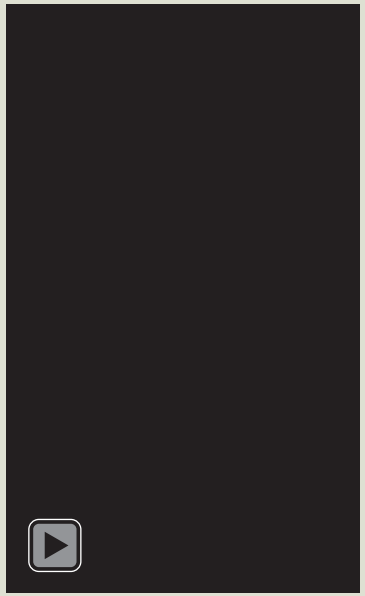
Förster Resonant Energy Transfer (FRET)



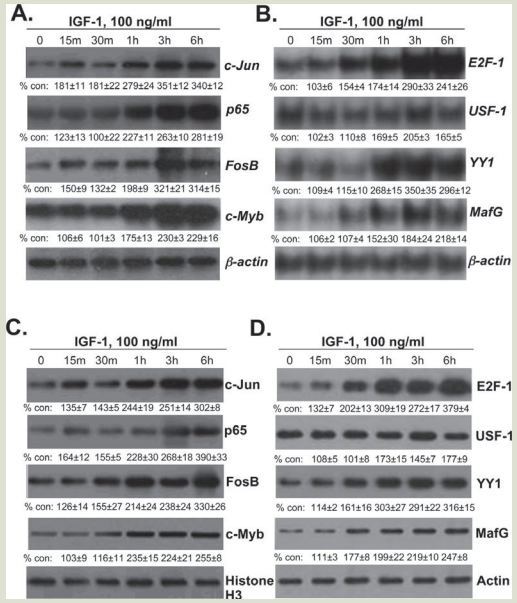
Matthew Collett

Why image protein-protein interactions?

Imaging

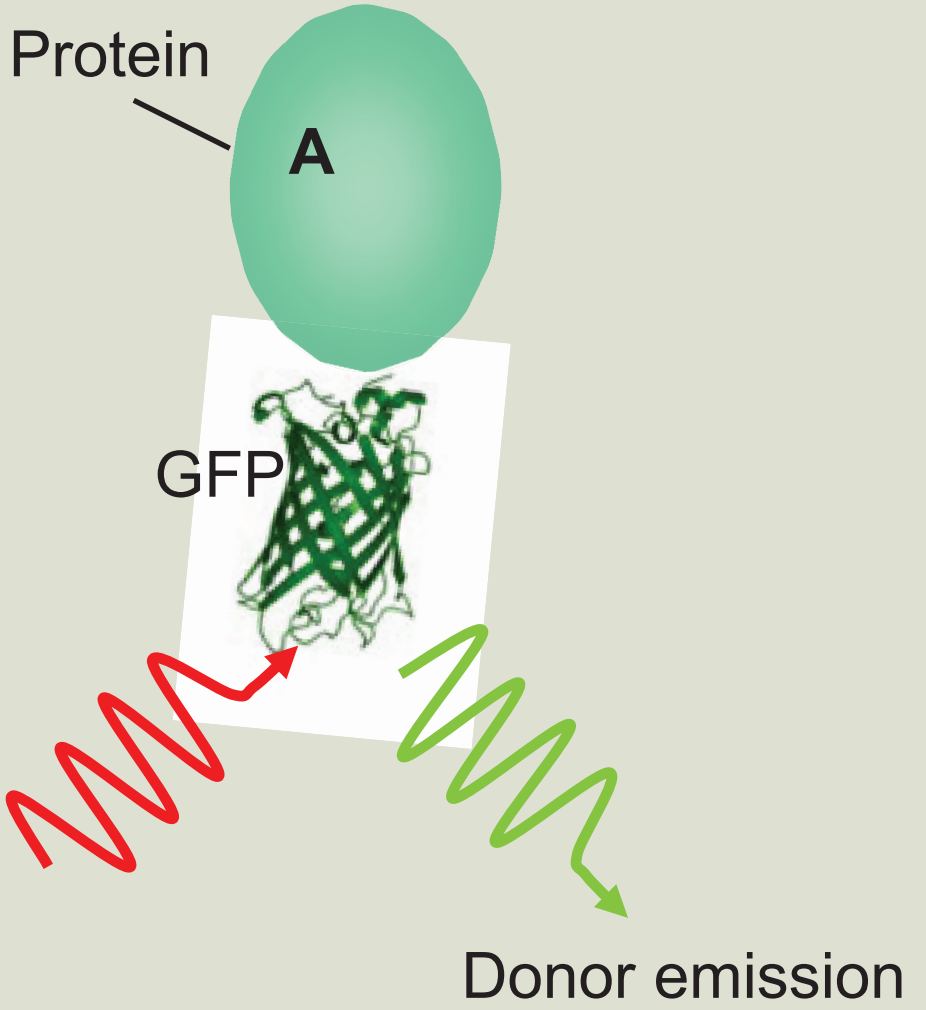


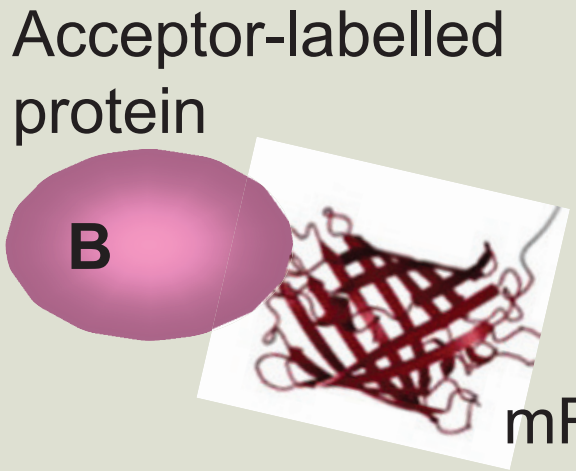
Biochemistry

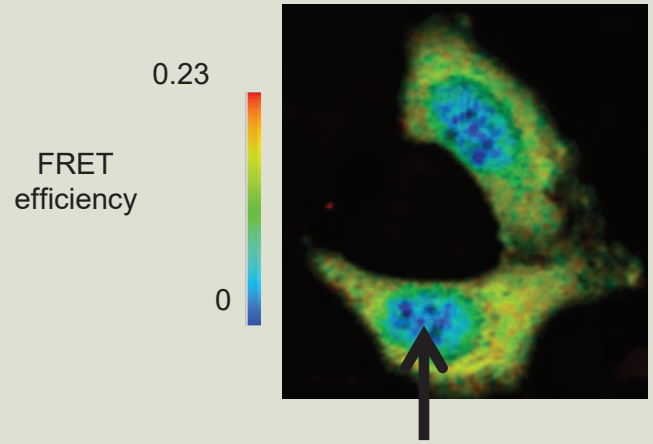


Why not?

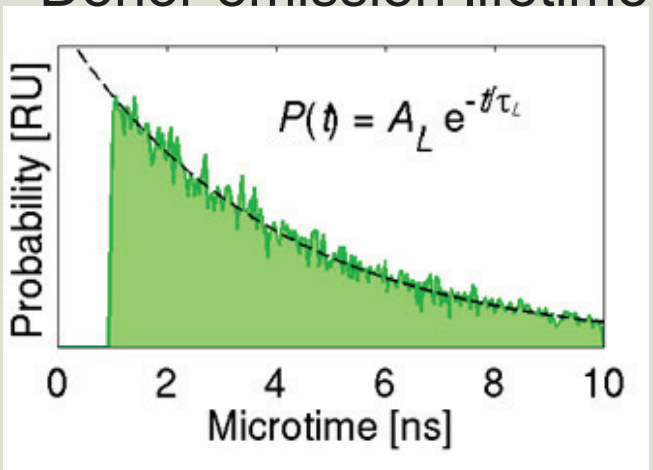
Protein-protein interactions



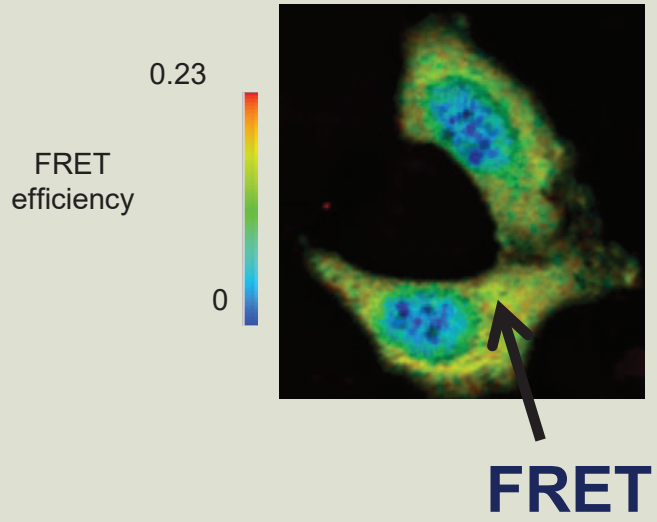


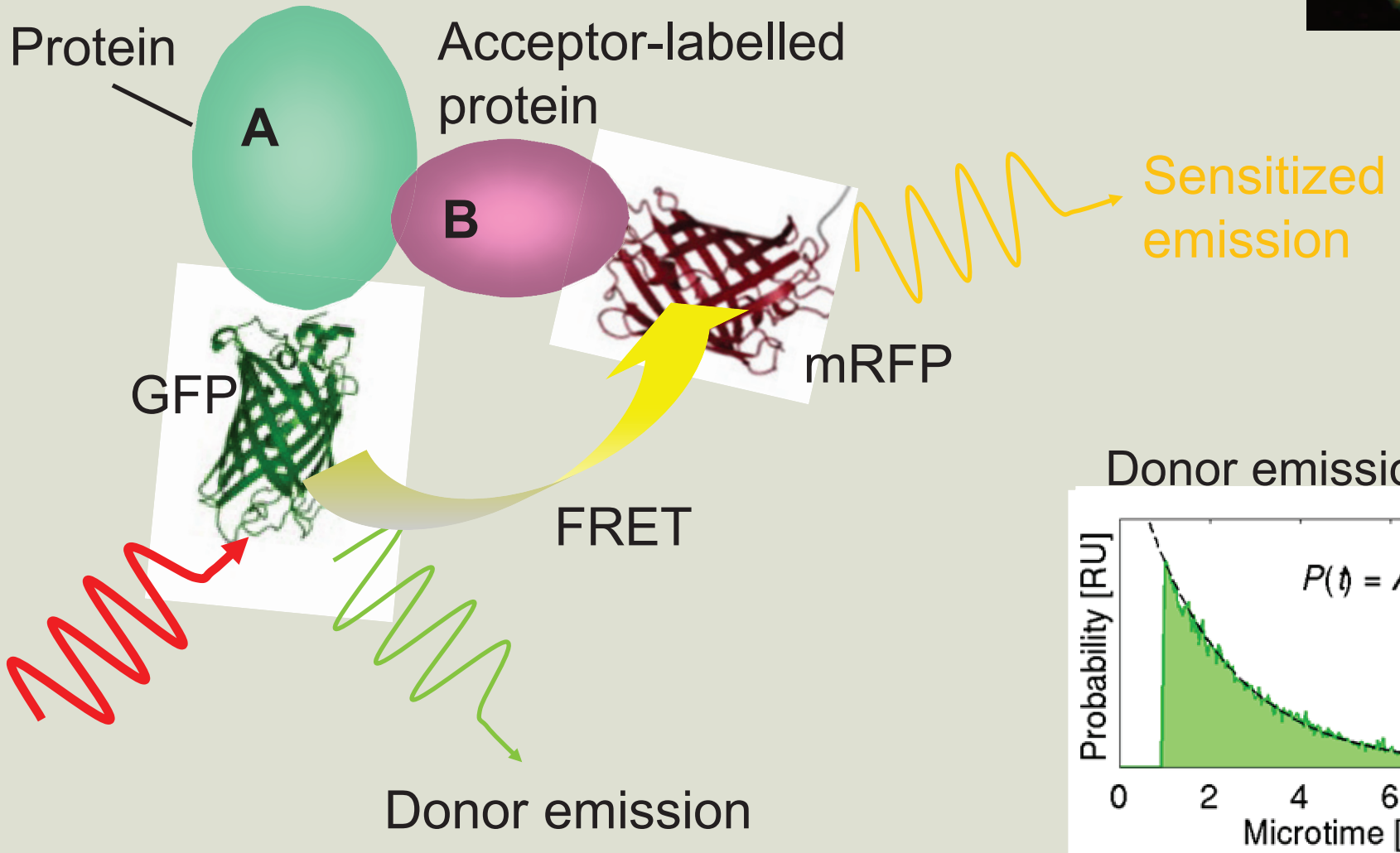


Donor emission lifetime



Protein-protein interactions

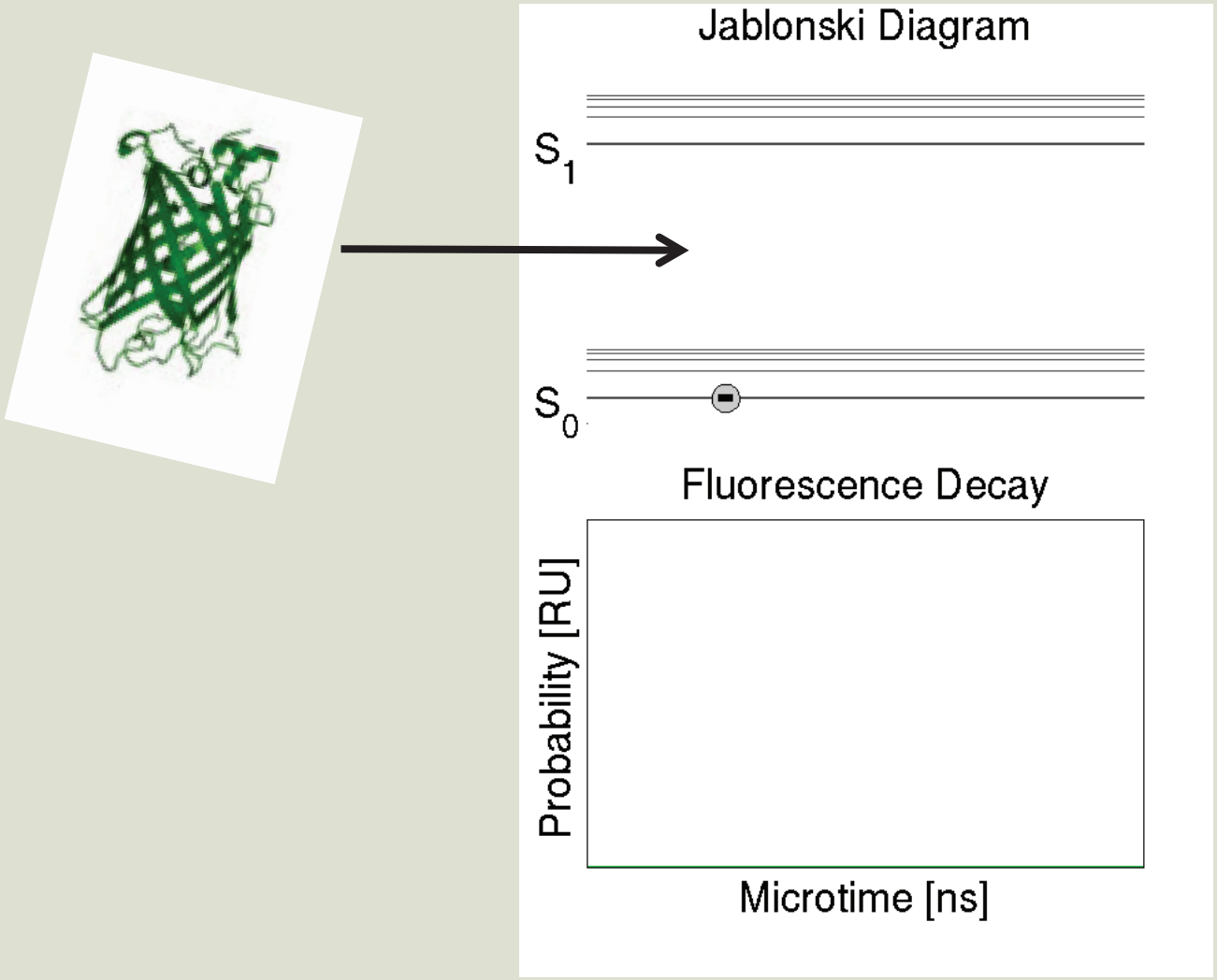




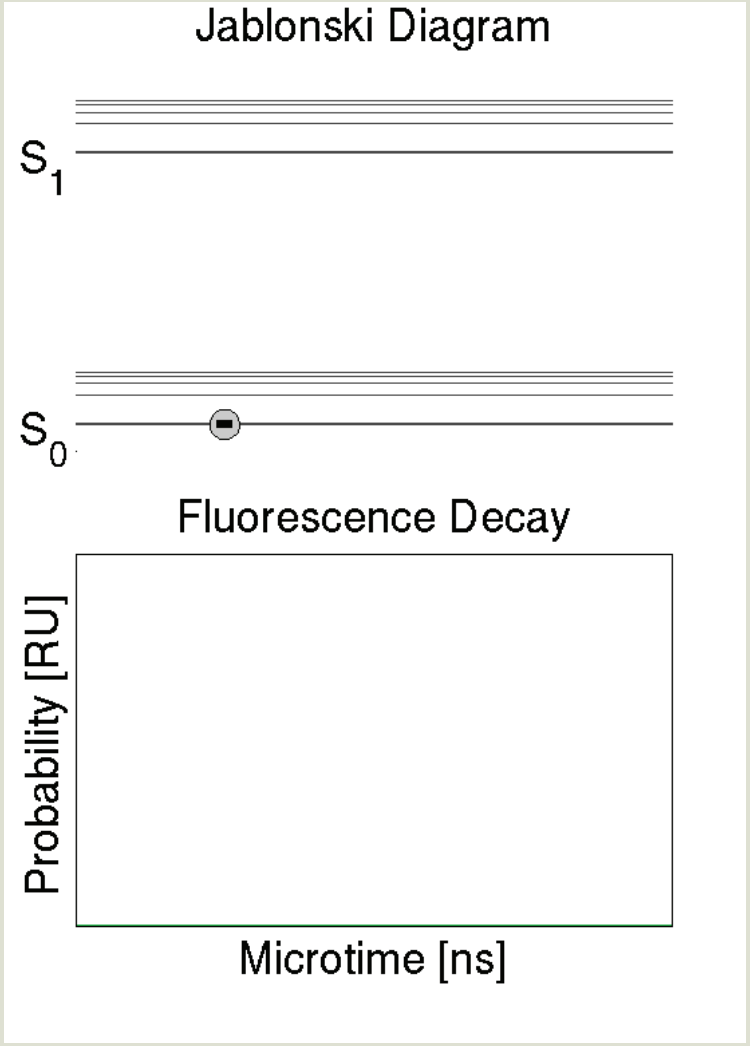
Donor emission lifetime

Fluorescence Lifetime & TCSPC

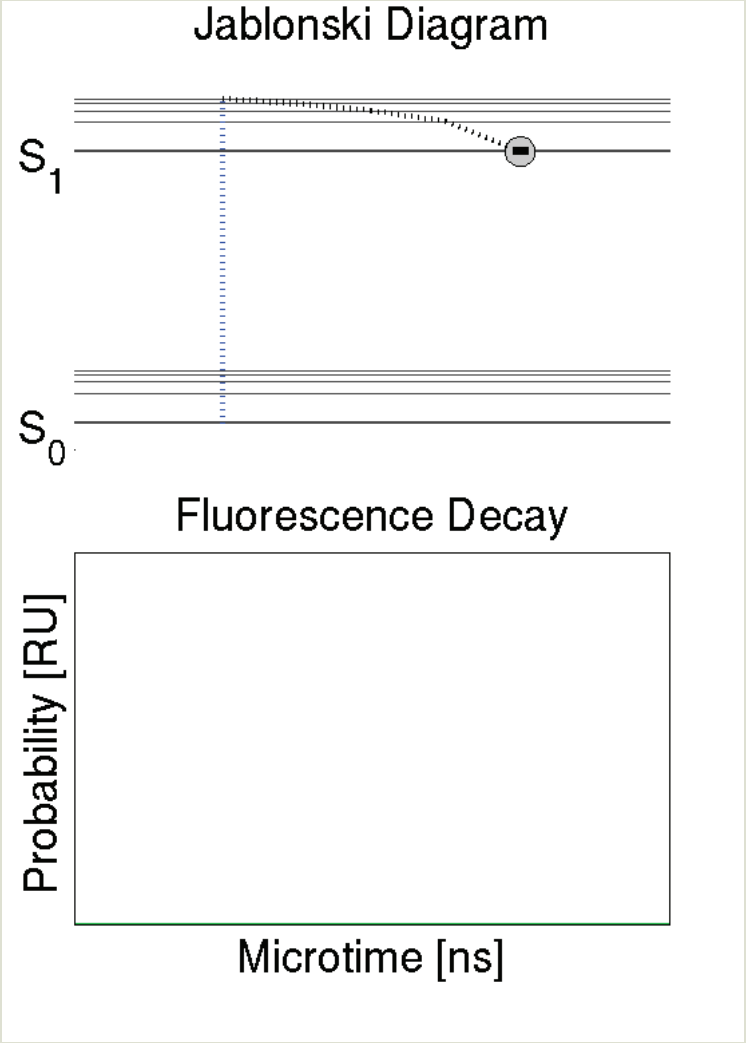
Fluorescence Lifetime



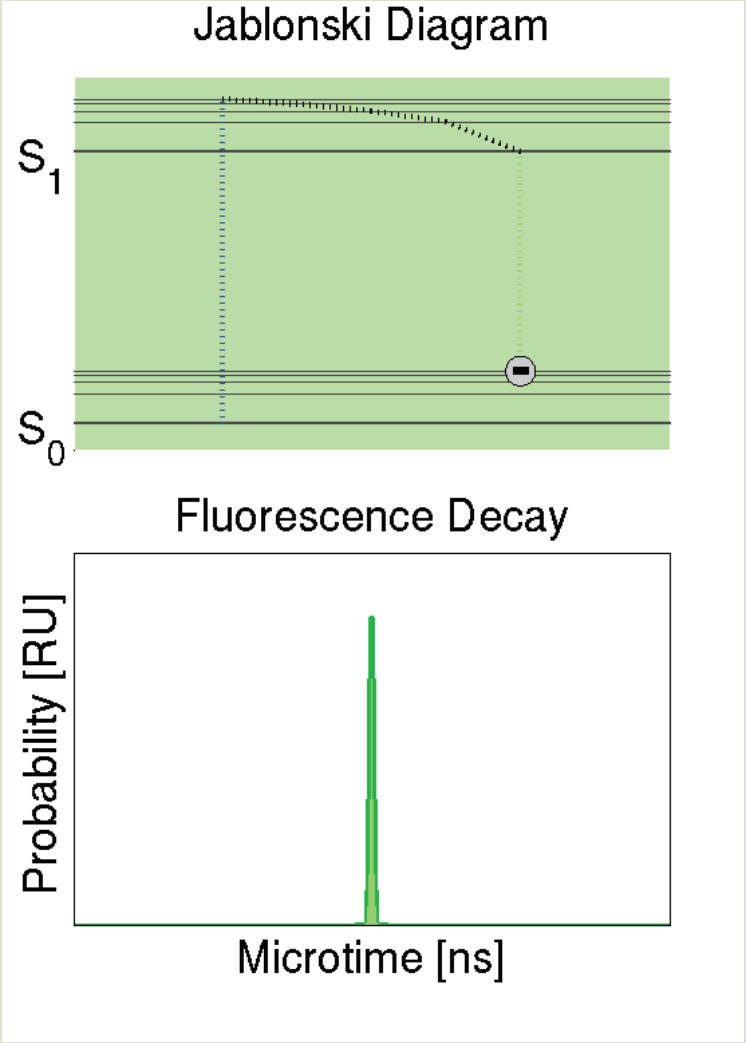
Fluorescence Lifetime



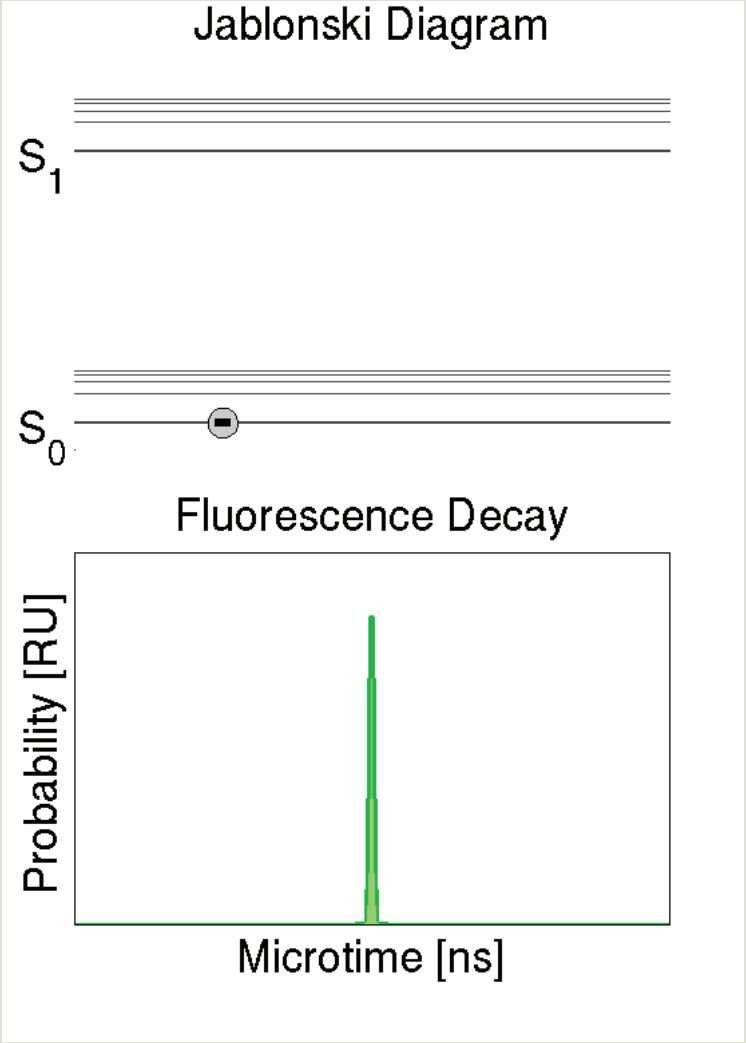
Fluorescence Lifetime



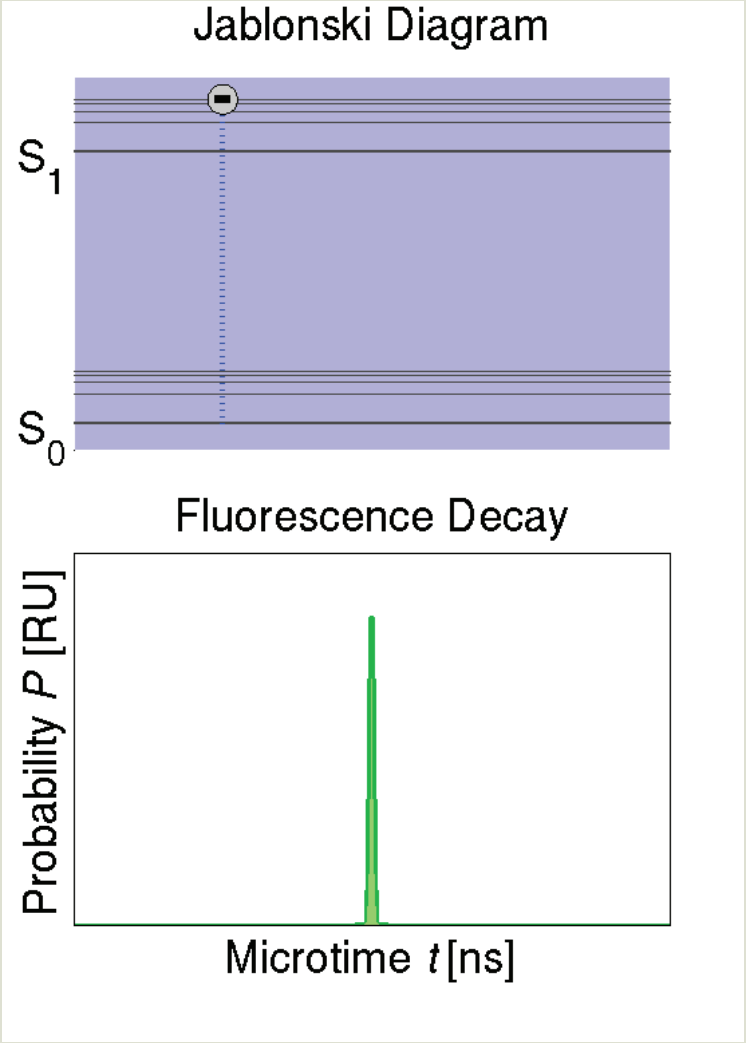
Fluorescence Lifetime



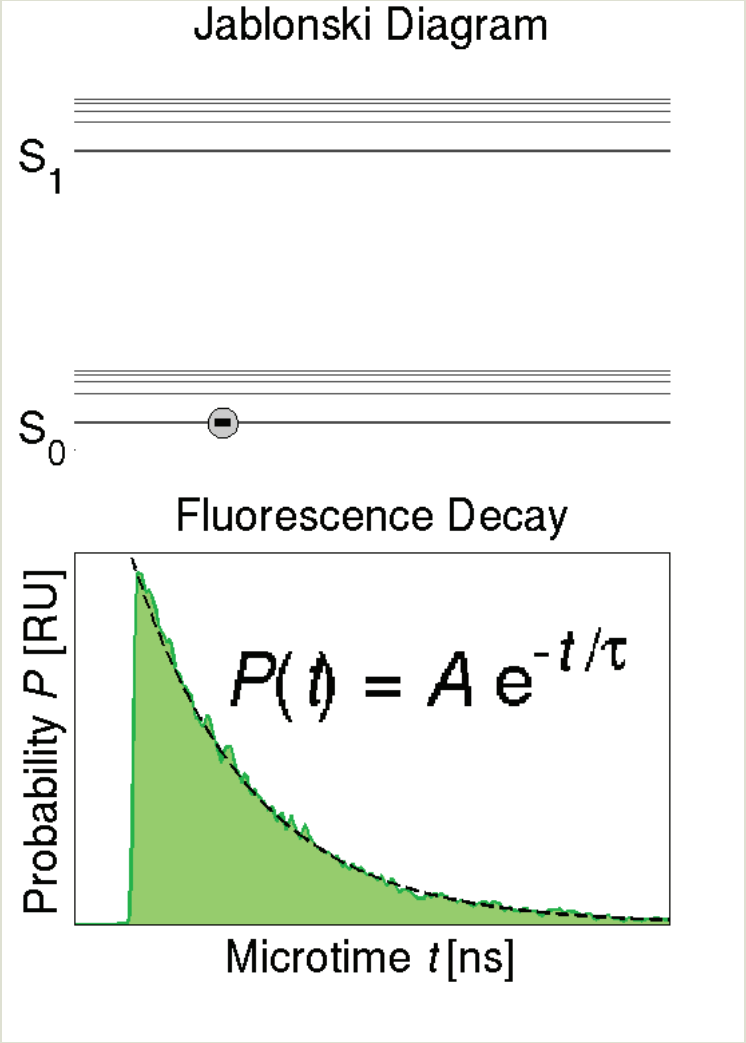
Fluorescence Lifetime



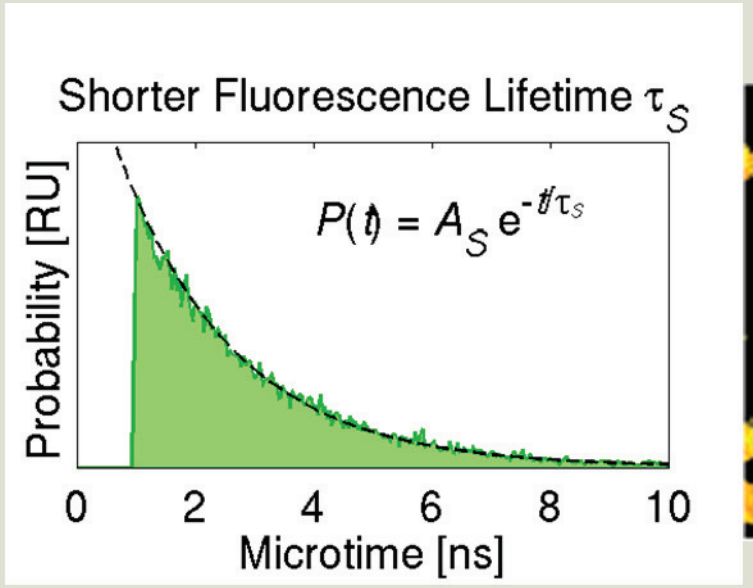
Fluorescence Lifetime

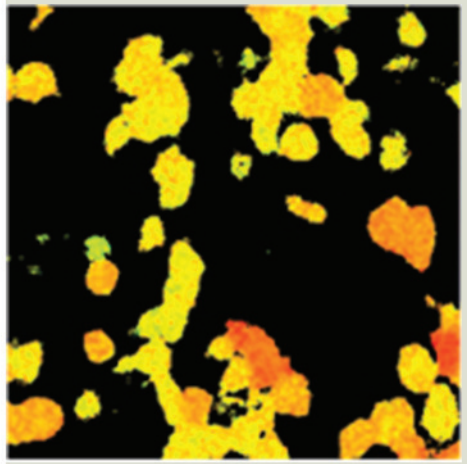


Fluorescence Lifetime

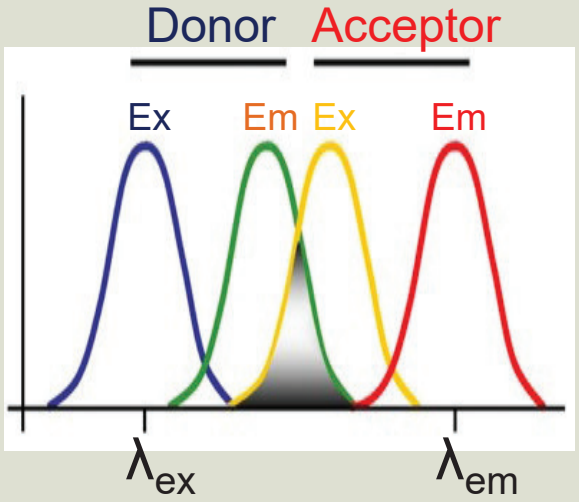


FRET Basics

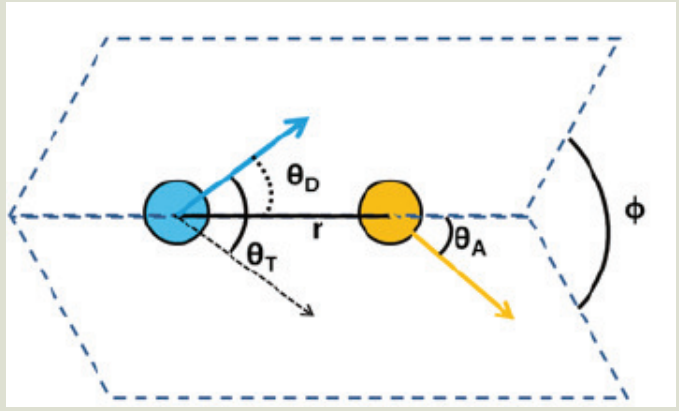




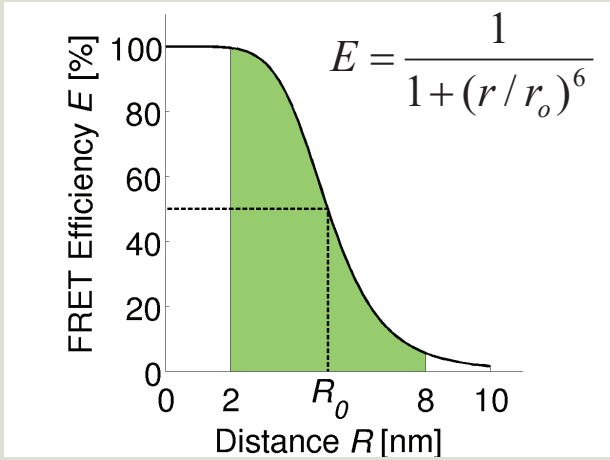
SPECTRAL OVERLAP



RELATIVE ORIENTATION

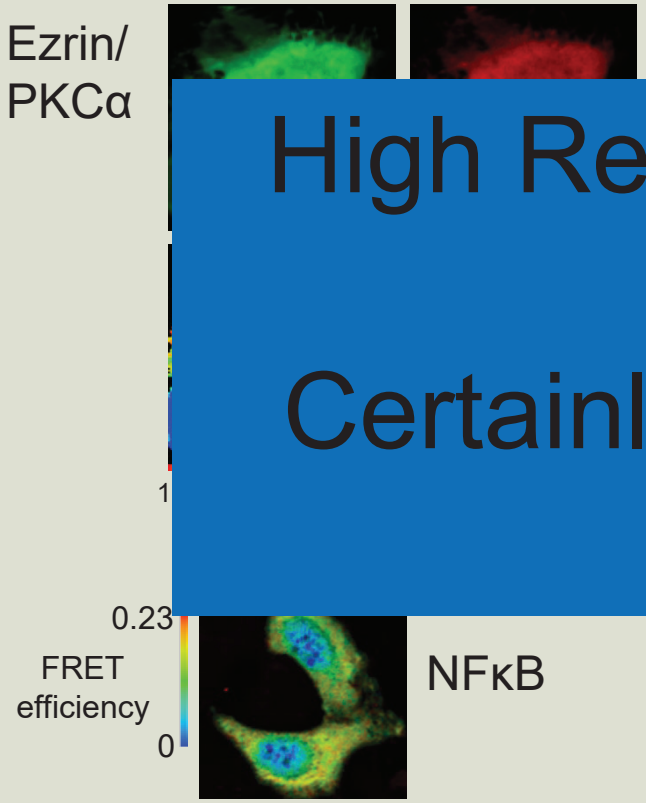


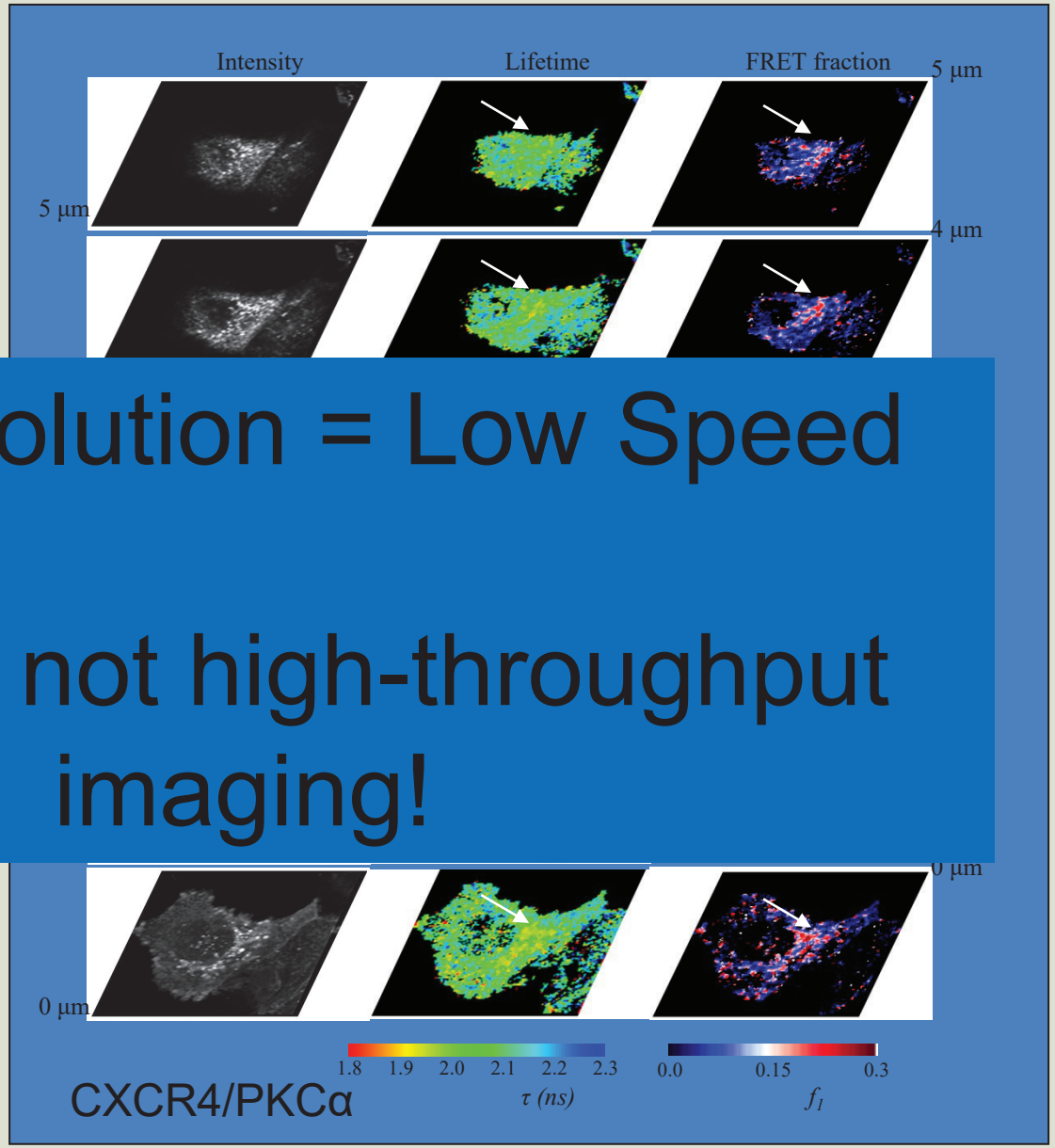
DISTANCE



The Story so far...

Quantitative FRET:
Multi-photon FLIM (TCSPC)





High Resolution = Low Speed

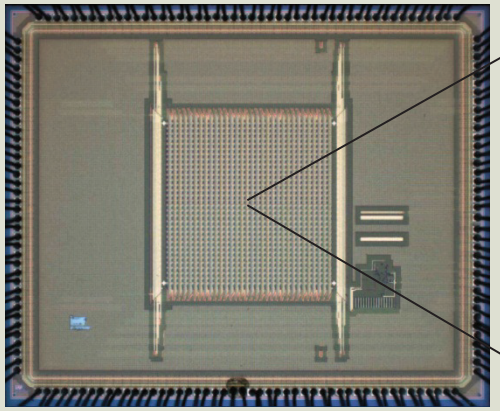
Certainly not high-throughput imaging!

How can we speed up TCSPC-FLIM?

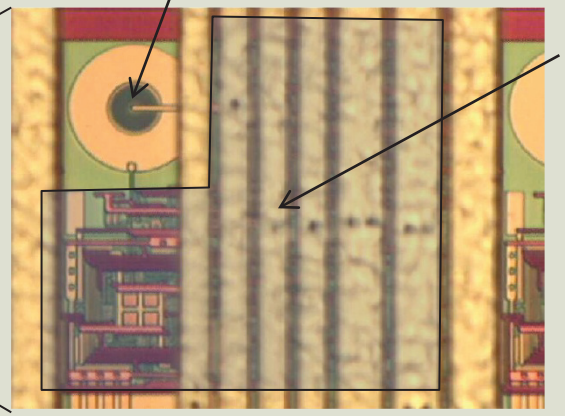
Massively Parallelised Fluorescence Lifetime Imaging

The MegaFrame Chip

Robert Henderson
University of Edinburgh
Scotland



32x32 SPAD array

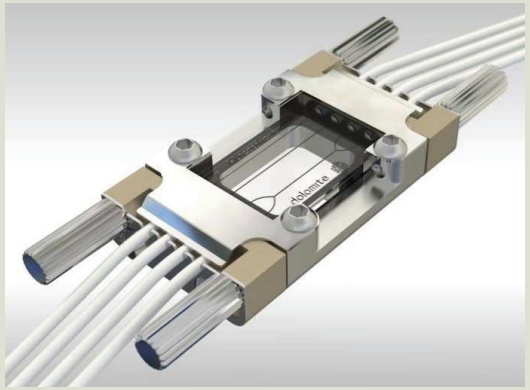


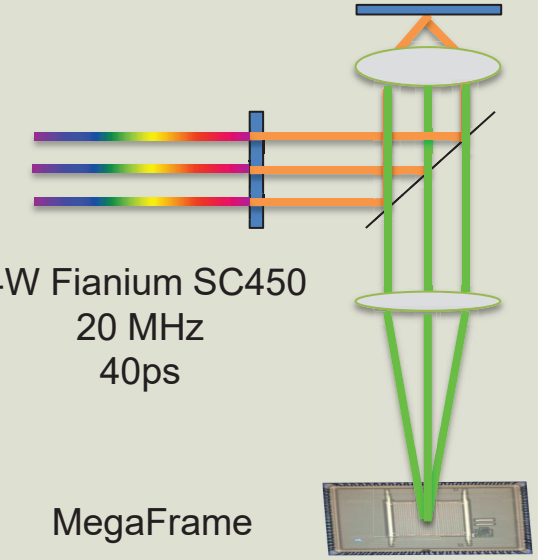
Individual SPAD

- 32×32 10-bit time-domain counter (TDC) array (~55ps) with integrated low dark-count SPAD.
- Has a quantum efficiency of 28% at 500nm. (Hybrid PMT – 45%: PMT – comparable)
- Operates in time correlated (lifetime) or time-uncorrelated modes (intensity).
- Each on-pixel TDC generates raw arrival time data, which are post-processed either on chip or on a PC.
- Small size of the SPAD active area (6 μm diameter, ~28 μm^2) and low fill factor (0.011) are a significant disadvantages for collection efficiency

Richardson, J.; Walker, R.; Grant, L.; Stoppa, D.; Borghetti, F.; Charbon, E.; Gersbach, M.; Henderson, R. A 32x32 50ps resolution 10bit time to digital converter array in 130nm CMOS for time correlated imaging, Proc. IEEE Custom Integrated Circuits Conf. (CICC),. 2009, pp. 77-80.

Video Rate Wide-field FLIM





4W Fianium SC450
20 MHz
40ps

MegaFrame

Dilute solution of 10μm fluorescent beads (G1000, Duke Scientific, USA) was used to simulate cells flowing through the system

Microfluidic Imaging flow cytometry

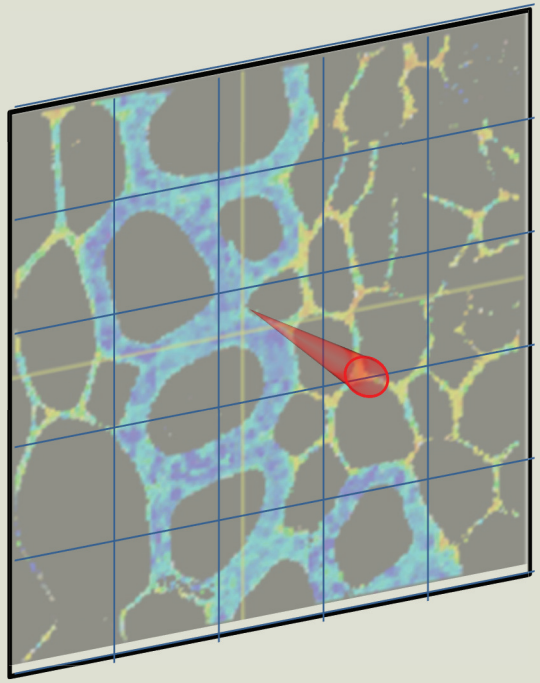
On-chip CMM Fluorescence lifetime calculation

Lifetime calculated every 1000 photons.
Images Streamed at 50 fps

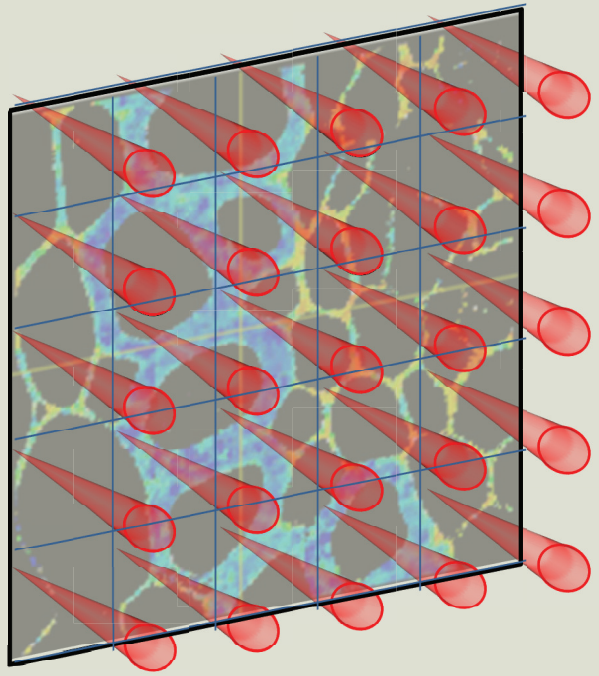
Problem:
1.11% Fill Factor

Solution: Multifocal Beam Scanning

Single point scanning

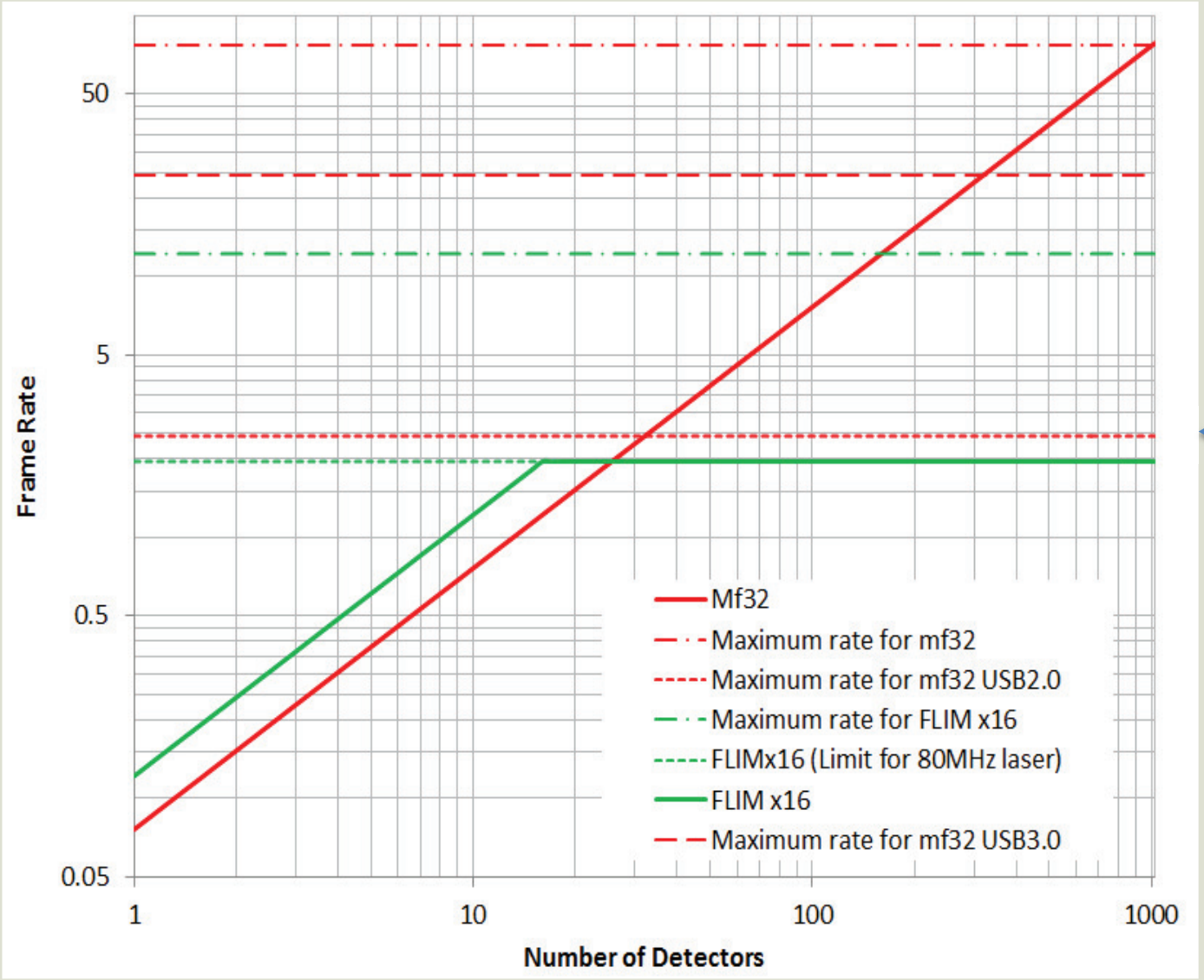


Multi-beam scanning



- An 8x8 array this would improve acquisition time by a factor of 64.
- A conventional 4 minute acquisition would take 3.75 seconds!!

FLIM rates for 256x256 pixel image (100 photons)

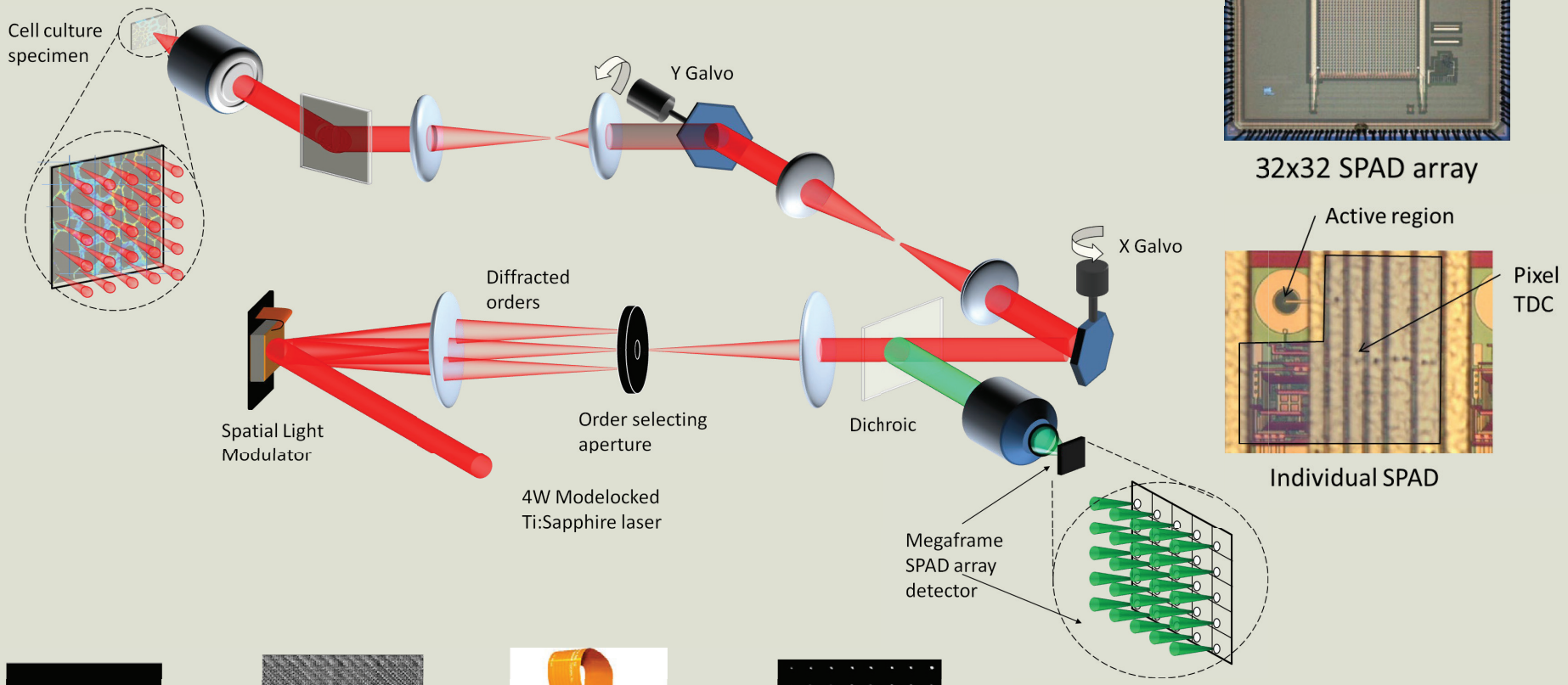


78 Hz
Without data compression

~ 2 Hz

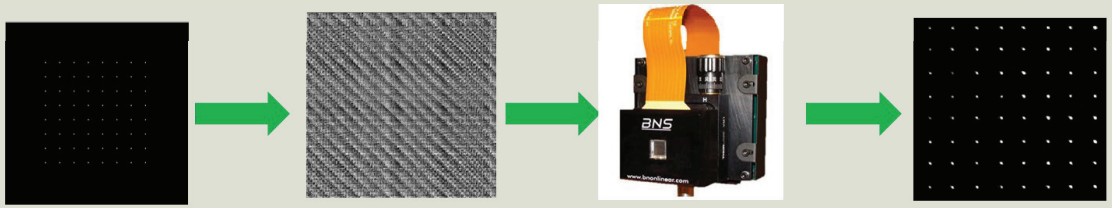
Assuming 500 kHz
Per pixel

Multifocal Multiphoton FLIM



32x32 SPAD array

Individual SPAD

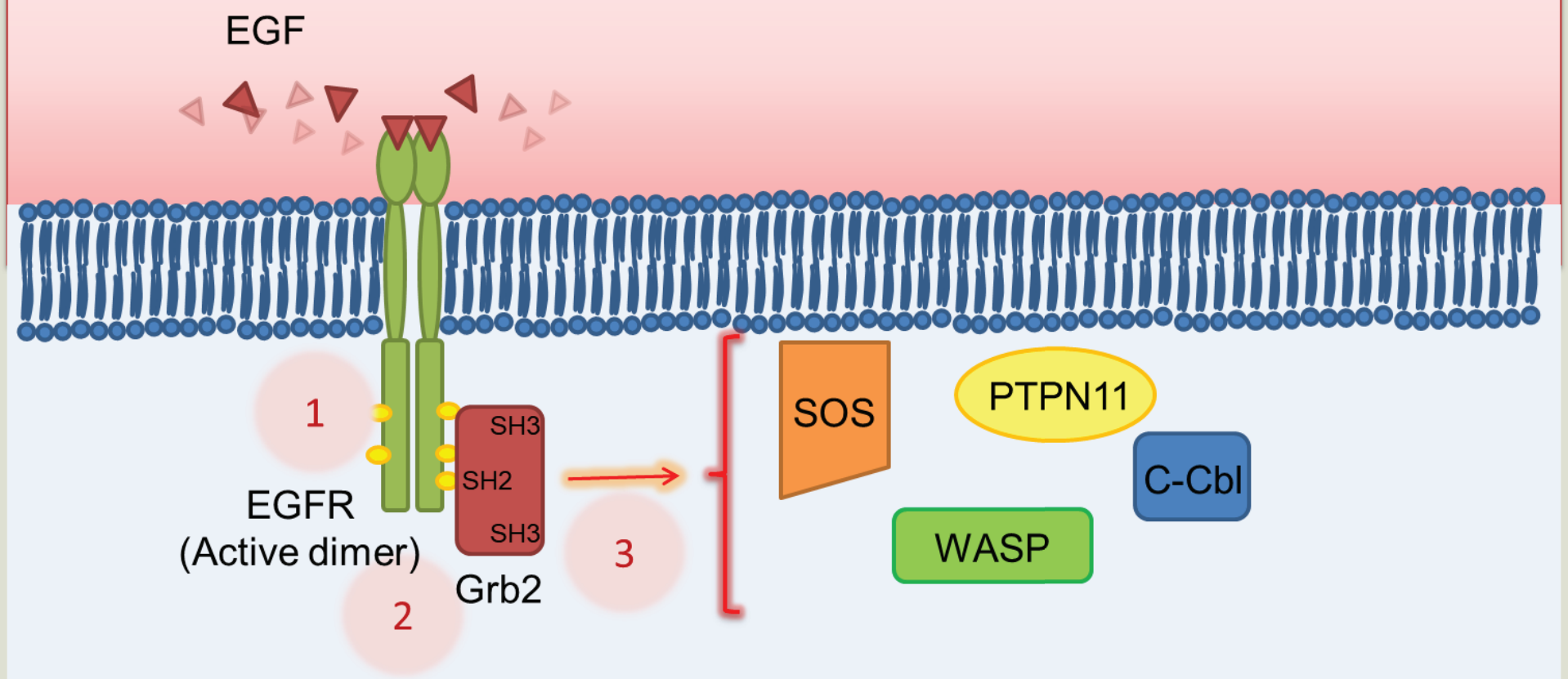


Poland, S.P. *et al.* (2015) A High Speed Multifocal Multiphoton Fluorescence Lifetime Imaging Microscope For Live-cell FRET Imaging, Biomedical Optics Express, Biomed.Opt. Exp., Vol. 6, Issue 2, pp. 277-296.

Imaging Receptor Dynamics

Poland, S.P. *et al.* (2015) A High Speed Multifocal Multiphoton Fluorescence Lifetime Imaging Microscope For Live-cell FRET Imaging, Biomedical Optics Express, Biomed.Opt. Exp., Vol. 6, Issue 2, pp. 277-296.

Ligand-dependent EGFR activation and subsequent recruitment of Grb2 initiates a diverse array of downstream signalling cascades

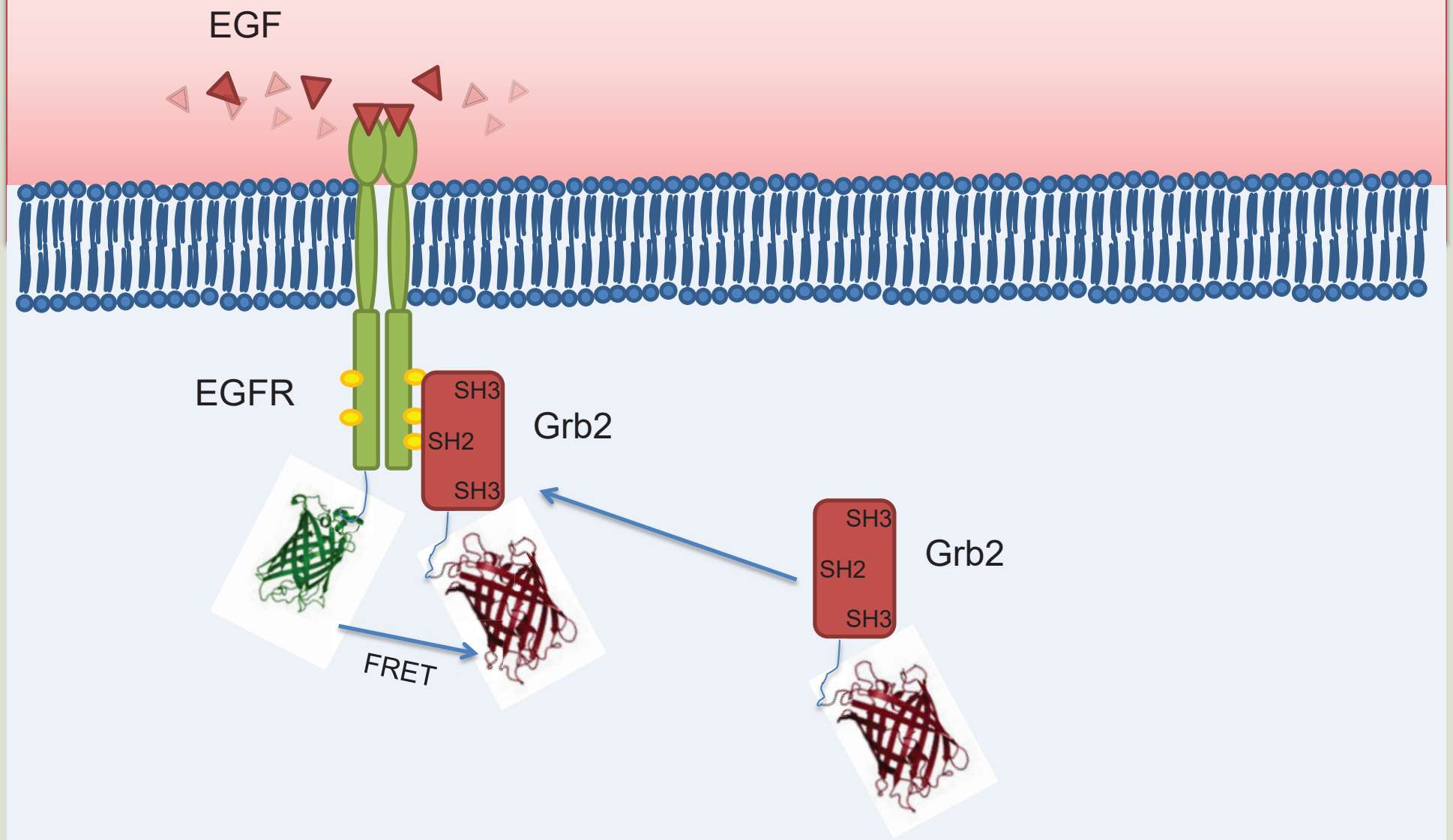


1. Ligand-induced receptor dimerization and auto/trans-phosphorylation on tyrosine

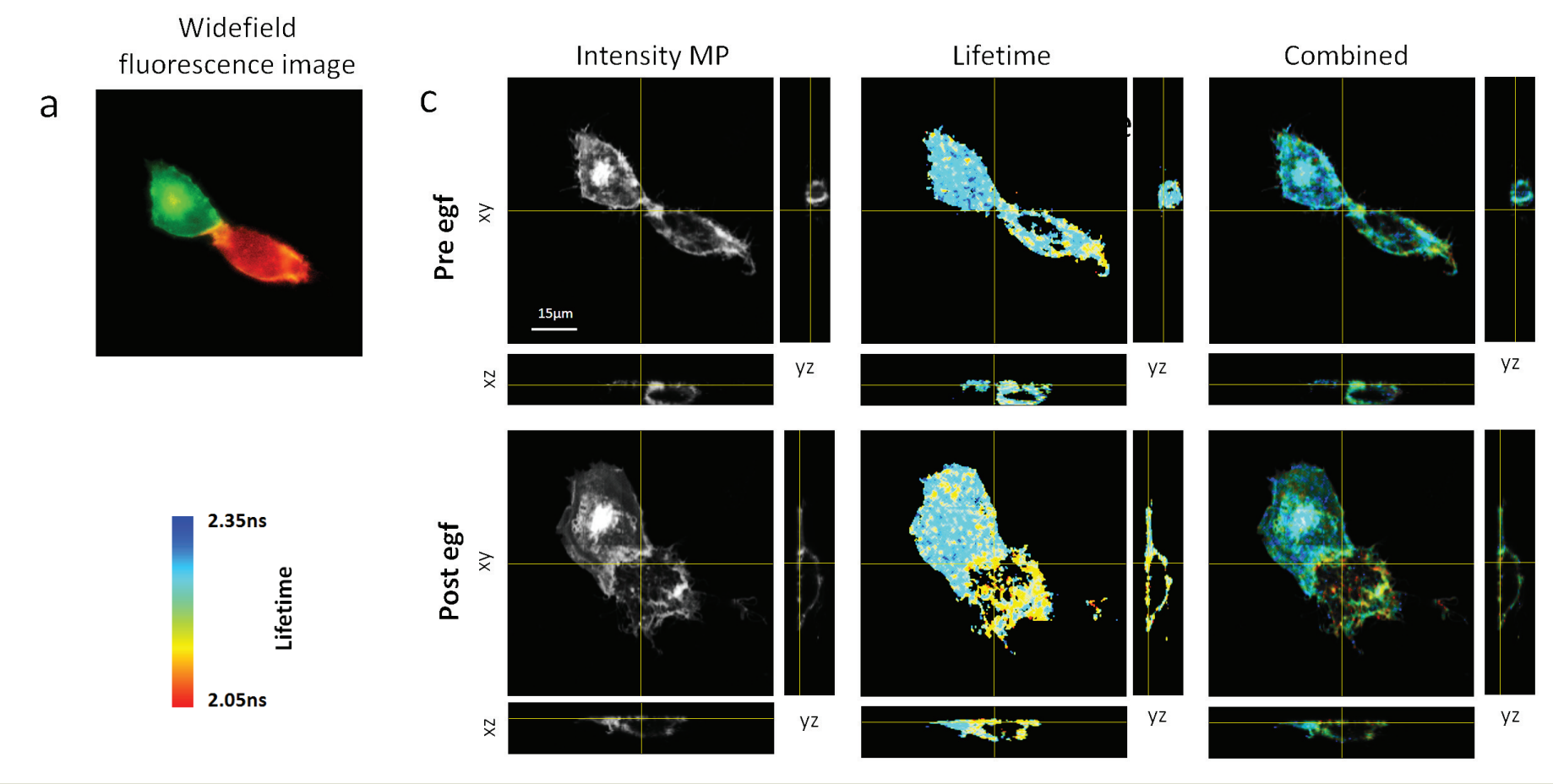
2. Recruitment of the adaptor protein Grb2 (either directly or via Shc) through SH2 domains.

3. Recruitment of a wide array of signalling partners: Phosphatases, ubiquitin ligases, exchange factors, cytoskeletal proteins

Ligand-dependent EGFR activation and subsequent recruitment of Grb2 initiates a diverse array of downstream signalling cascades



EGFR Grb2 Interaction



EGFR Grb2 Interaction

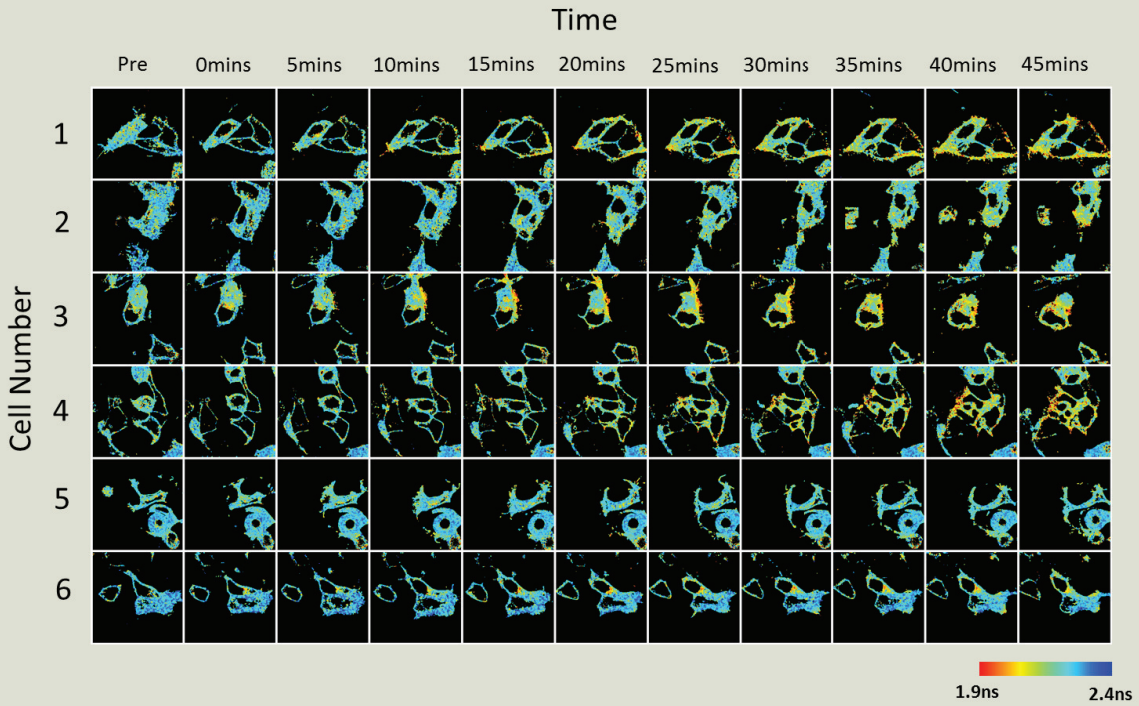
Intensity Lifetime Composite

2.05ns 2.35ns

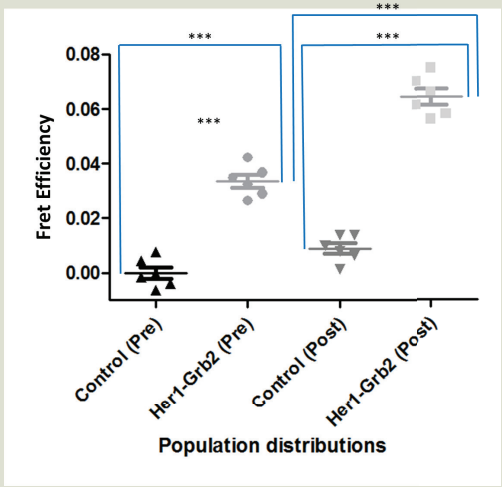
•Live-cell time lapse video of MCF-7 cells coexpressing EGFR-EGFP and Grb2-mCherry with 10 s frames

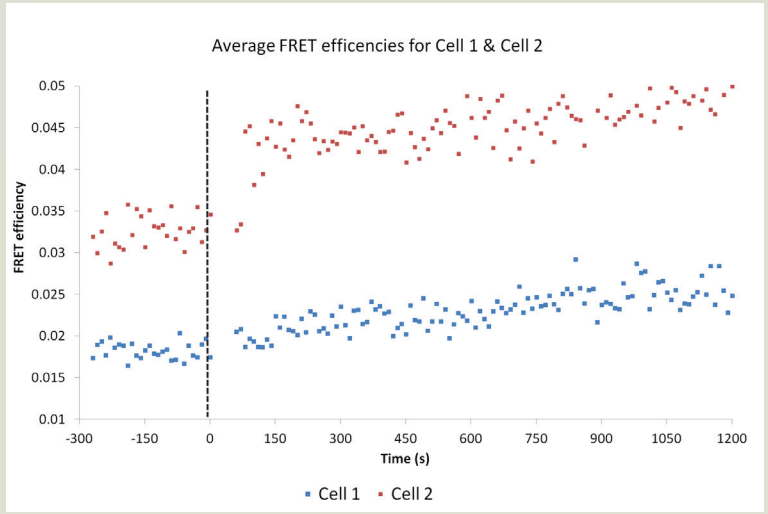
•+/- EGF addition

Multi-cell analysis – time lapse



Time lapse data sets

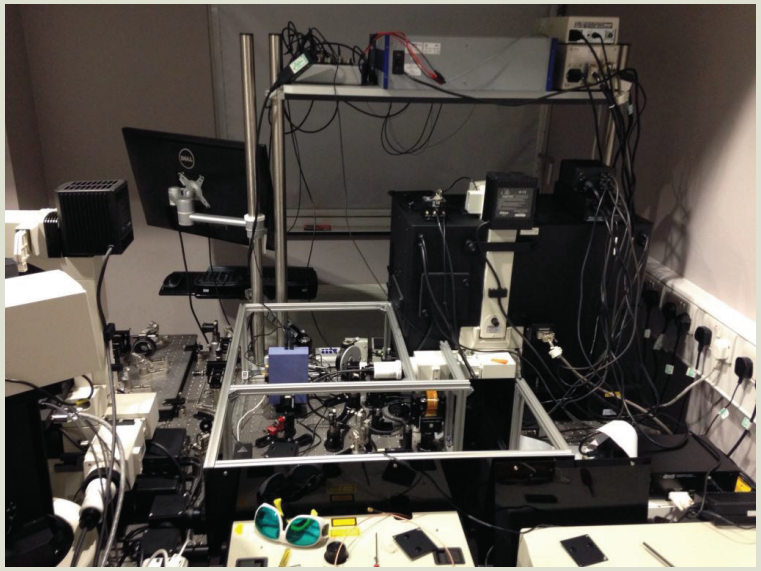


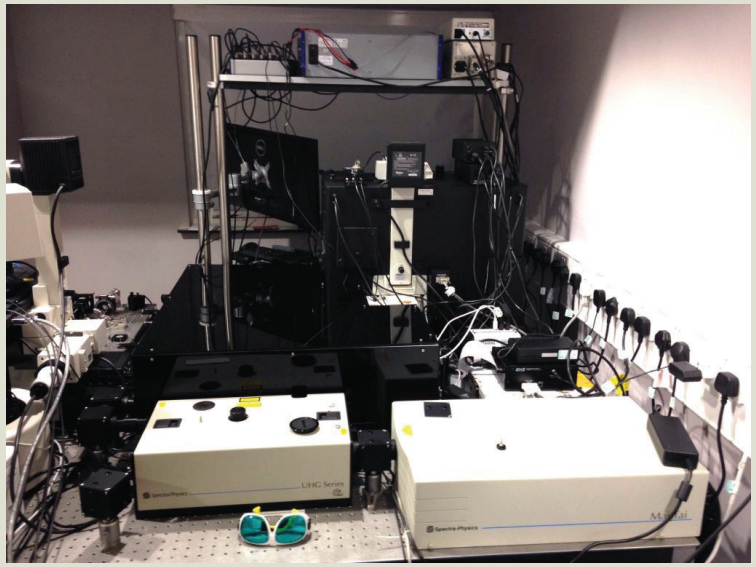


$$E = 1 - \frac{\tau_{DA}}{\tau_D} = \frac{R_o^{\,6}}{R_o^{\,6} + r^6}$$

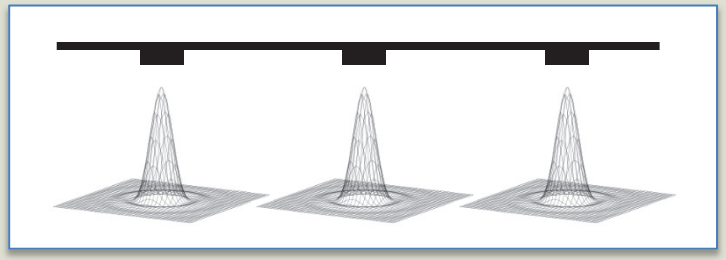
- On addition of EGF ligand FRET efficiency increases to 6.1 ± 0.9 %.

Confocal multifocal FLIM – Evolution





Each SPAD is a "confocal pinhole"
Active area ~ 1 Airy unit
plus
the 50 µm spacing is an advantage-
minimises cross-talk



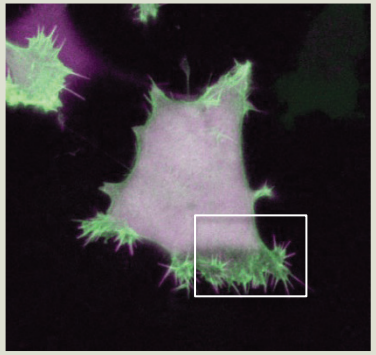
- 2nd generation design
- Smaller footprint

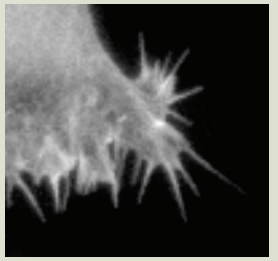
Advantages over multiphoton setup
- Lower laser power – more beams
- More beams means faster!

Fascin

Fascin is an actin-binding protein that regulates the parallel bundling of actin filaments. We are interested in fascin in filopodia (extending beyond the cell edge) ~ 250 nm diameter, μm length.

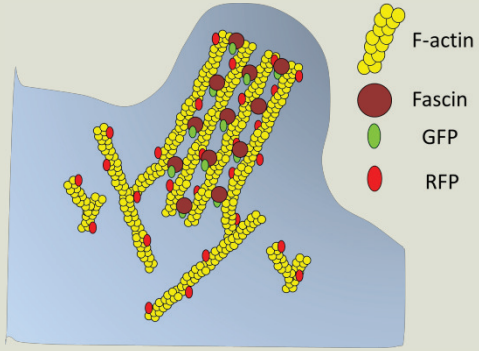
Fascin expression is very low or absent in normal adult epithelia. Dramatic up-regulation has been reported in all forms of human carcinomas studied to date.

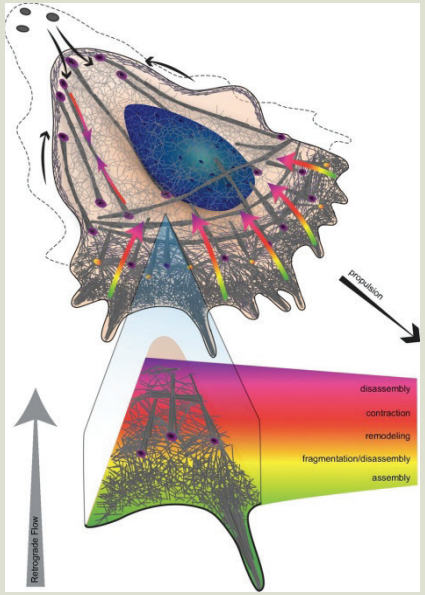




Credit: Prof. Maddy Parsons

Maddy Parsons
KCL,
London





After Vignjevic, D. et al. *J. Cell Bio.* **174**:863-875 (2006).

Loss of fascin function in a range of cell types results in reduced assembly of actin protrusions, more stable focal adhesions and reduced migration and invasion *in vivo.*

QUESTIONS

- **What are the kinetics and localisation of fascin-actin binding in dynamic filopodia?**
- **Does the efficiency of fascin-actin binding predict filopodia stability?**

Confocal multibeam FLIM: fascin-actin interaction

Fascin is an actin-binding protein that regulates the parallel bundling of actin filaments. We are interested in fascin in filopodia (extending beyond the cell edge) ~ 250 nm diameter, µm length.

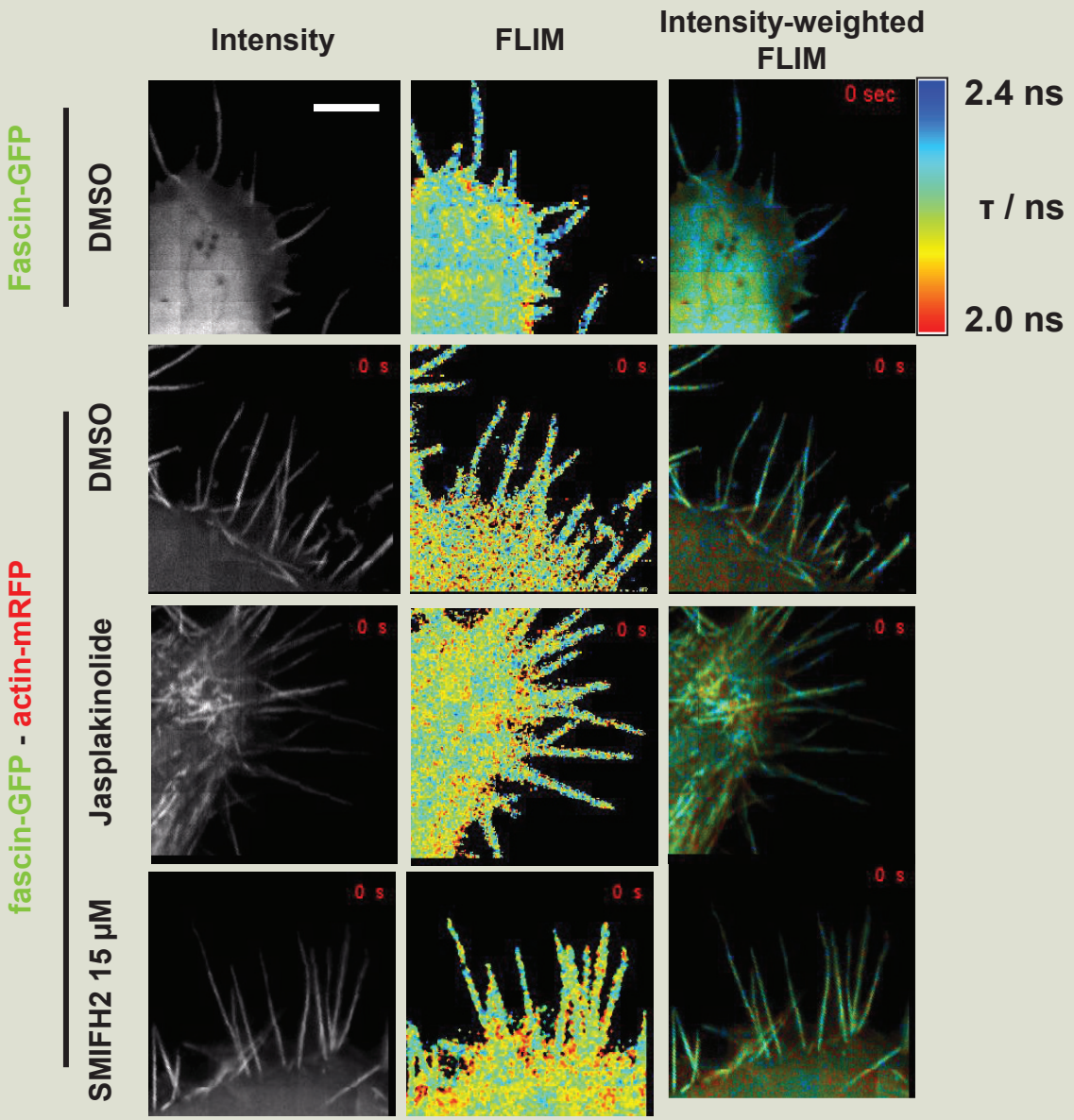
Dramatic up-regulation has been reported in all forms of human carcinomas studied to date.

Fascin knockdown HeLa expressing fascin-GFP and actin-mRFP.

Imaging conditions:

- 8x8 beamlets at 480 nm
- 256 x 256 pixel images
- 37 °C
- 16 µm x 16 µm
- 3 s per frame
- Scale bar 5 µm

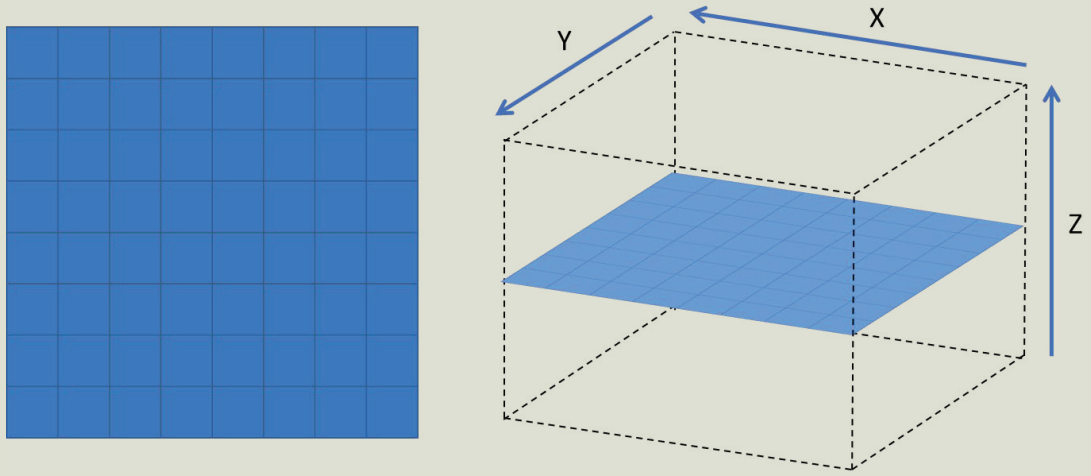
[1]Li et al. *J. Biomed. Opt* (2010)
[2]Poland et al. *Biomed. Opt. Exp.* (2016)
[3]Pfisterer *et al.* J Cell Biol. (2020)



Volumetric imaging technique to multifocal

Volumetric imaging for multifocal FLIM

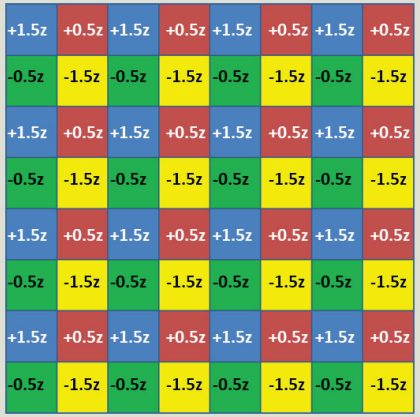
• In normal operation beamlets are generated by the Spatial light modulator along x and y within a single plane in z.

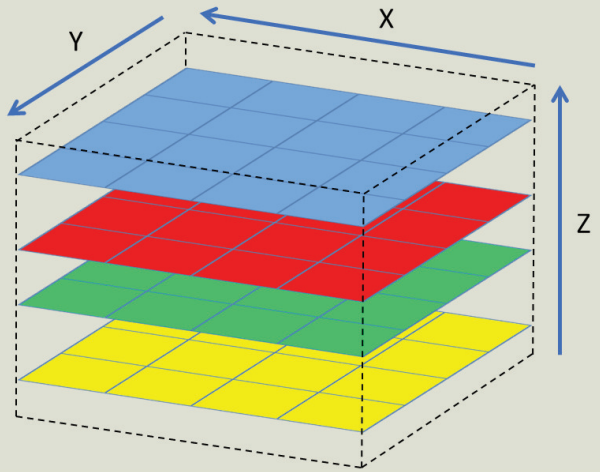


S. P. Poland, et al. "A multifocal multiphoton volumetric imaging approach for high speed time-resolved FRET imaging in vivo." Optics letters (2018) *In Press*

Volumetric imaging for multifocal FLIM

Defocus added to each beamlet generates
64 beamlets = 16 beamlets in 4 planes.





Z= -1.5 μm Z= -0.5 μm Z= +0.5 μm Z= +1.5 μm

- Acquisition of HeLa cells containing EGFP-Fascin (10 s/frame)

S. P. Poland, et al. "A multifocal multiphoton volumetric imaging approach for high speed time-resolved FRET imaging in vivo." Optics letters (2018)

Volumetric imaging for multifocal FLIM

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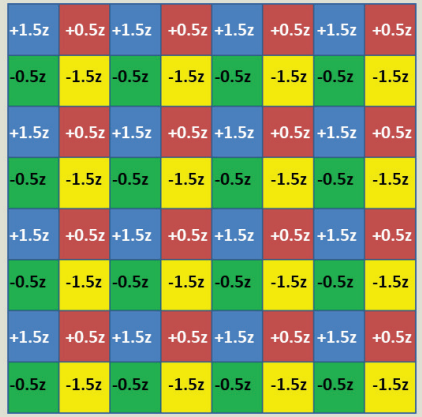
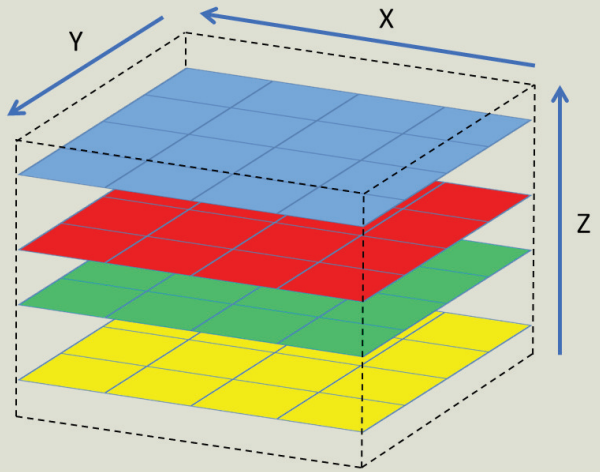
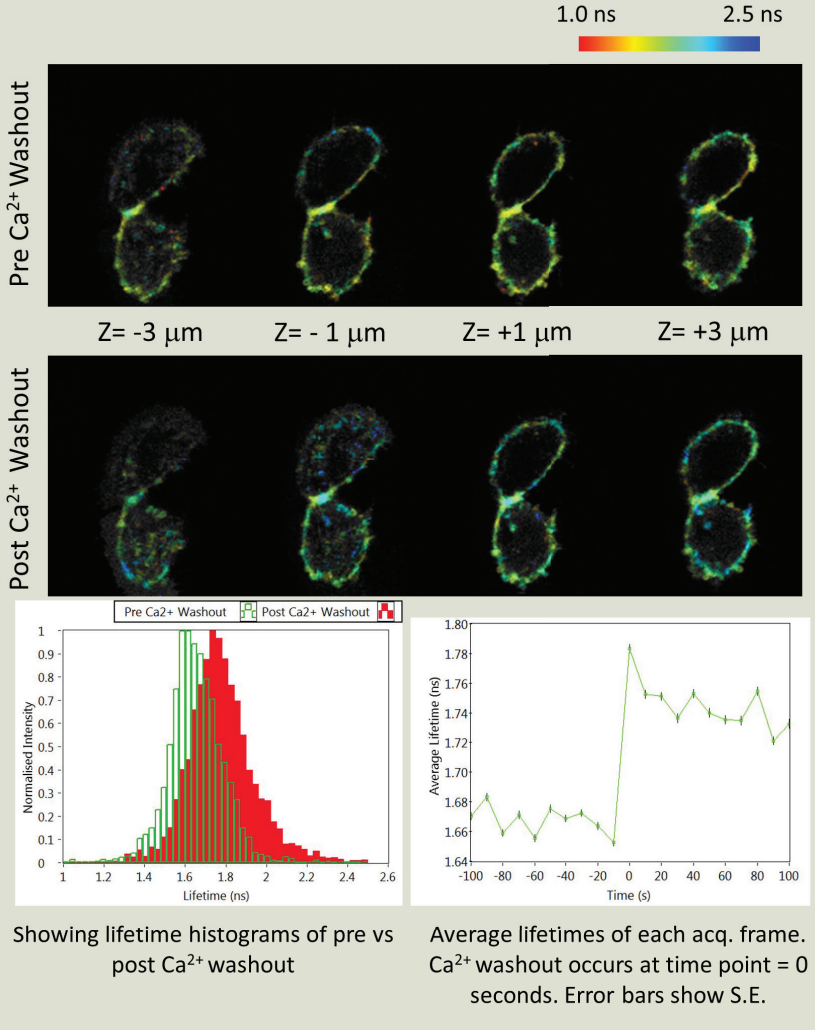




Z= -1.5 μm Z= -0.5 μm Z= +0.5 μm Z= +1.5 μm

• Acquisition of HeLa cells containing EGFP-Fascin (10 s/frame)



Showing lifetime histograms of pre vs post Ca^{2+} washout

Average lifetimes of each acq. frame. Ca^{2+} washout occurs at time point = 0 seconds. Error bars show S.E.

• Live MCF7 cells expressing RhoA GTPase mTFP/Venus FRET biosensor.
• Ca^{2+} Washout induces cell-cell dissociation
  - disengagement of cadherin receptors

S. P. Poland, et al. "A multifocal multiphoton volumetric imaging approach for high speed time-resolved FRET imaging in vivo." Optics letters (2018)

Limitations to our acquisition size (and speed)

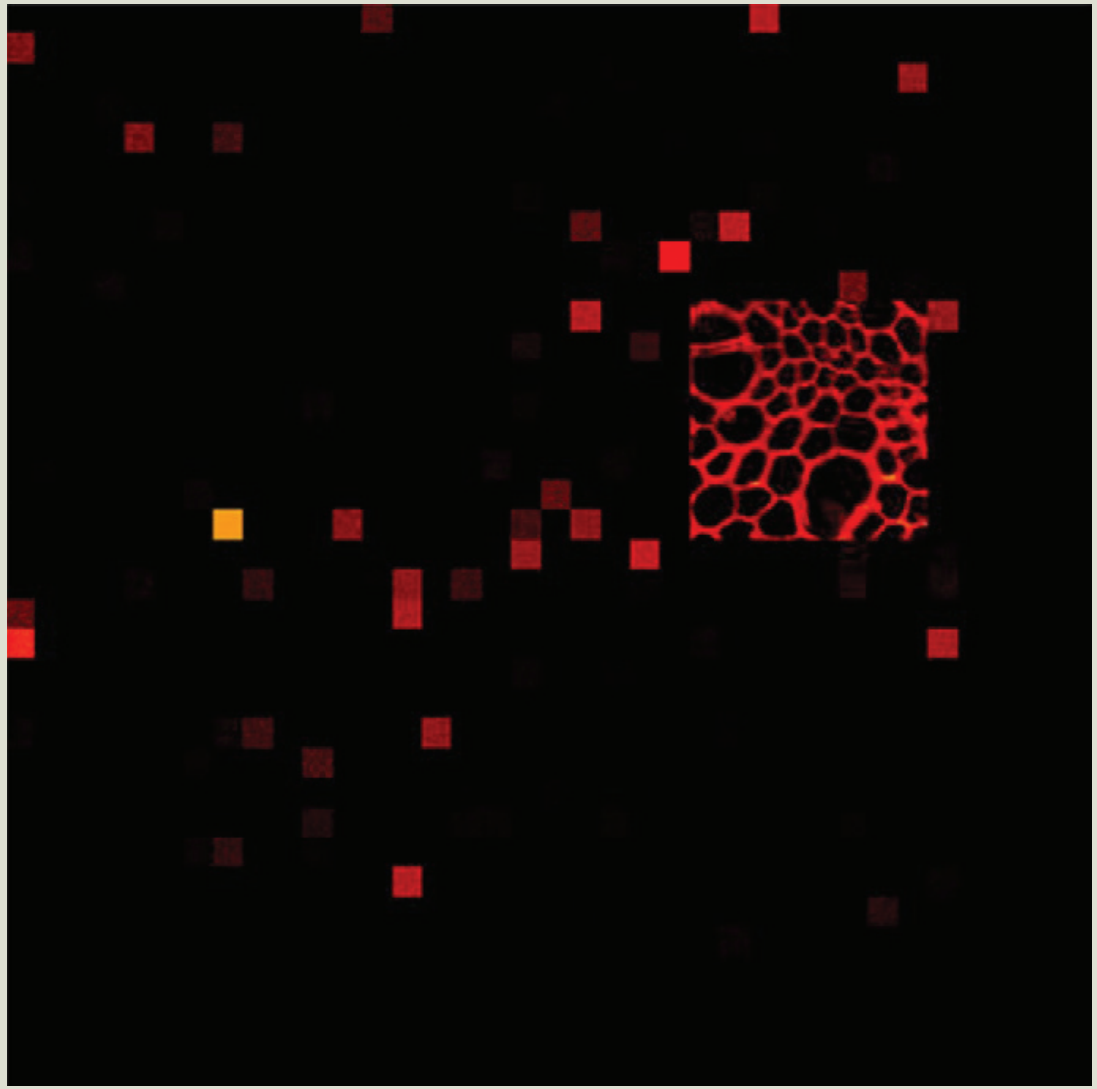
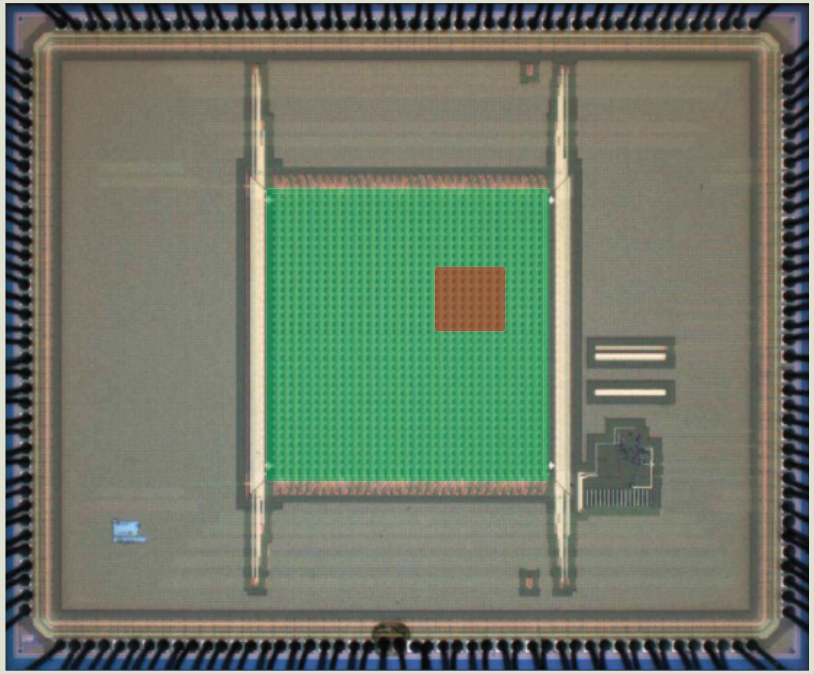


Image showing region of the megaframe chip used when imaging with 8x8 beamlets

Until recently the multiphoton system could only utilize 8 x 8 detectors 64/1024 = 1/16 of the total Megaframe chip



Megaframe MF32 camera
(Illuminated region shown in red)

Tony Ng
KCL and UCL,
London

SWARM –
SWept ARray Microscopy

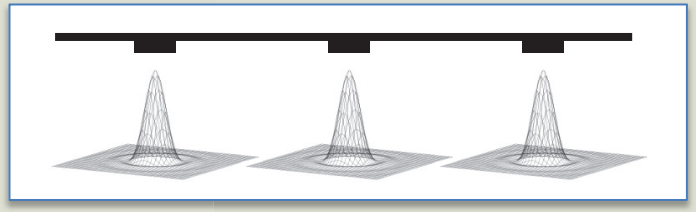
Simon Poland
CRUK Centre Fellow
KCL, London

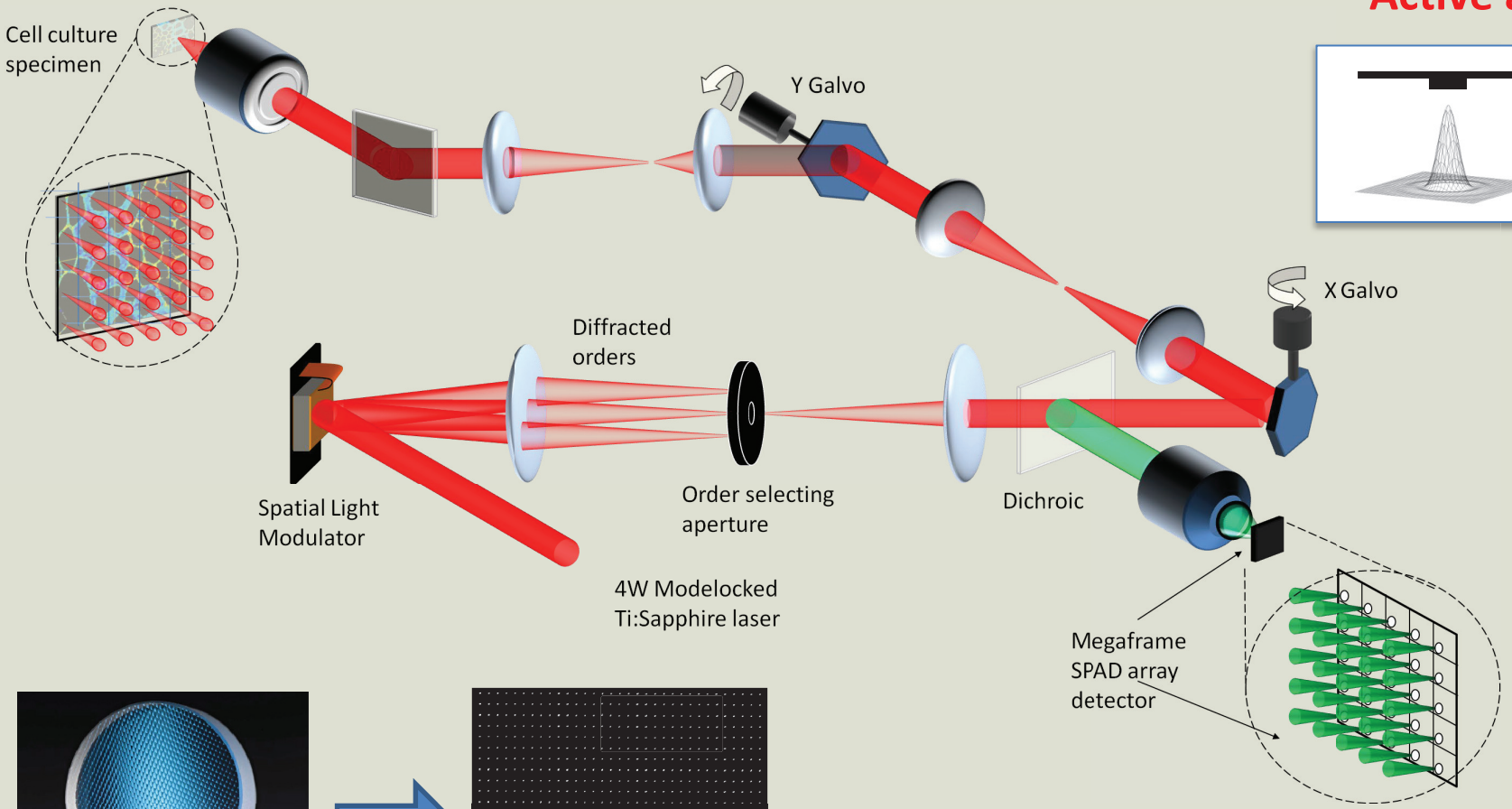
Robert Henderson
U of Edinburgh
Scotland

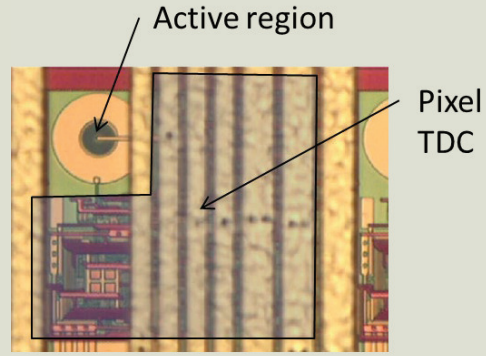
Paul Barber
UCL and KCL
London

Limitations to current set up

Each SPAD is a "confocal pinhole"
Active area ~ 1 Airy unit

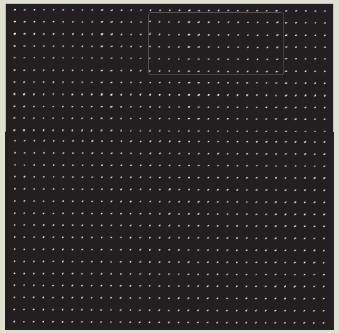






Individual SPAD

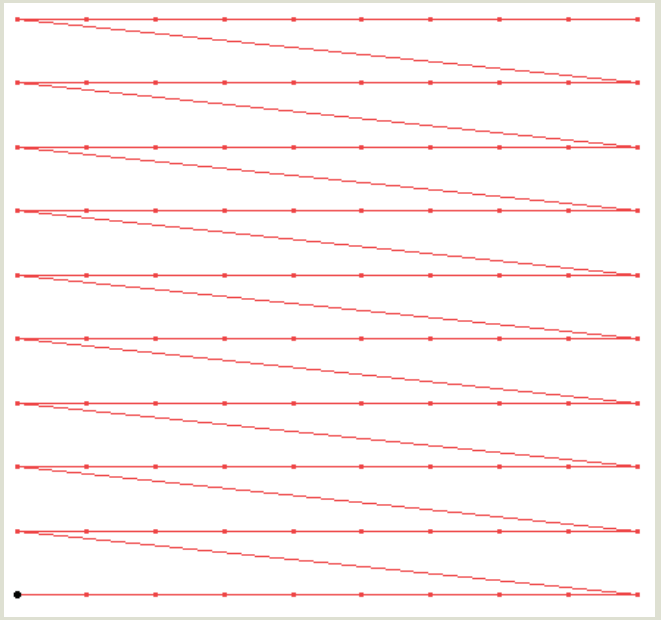
Custom designed Diffractive Optical Element



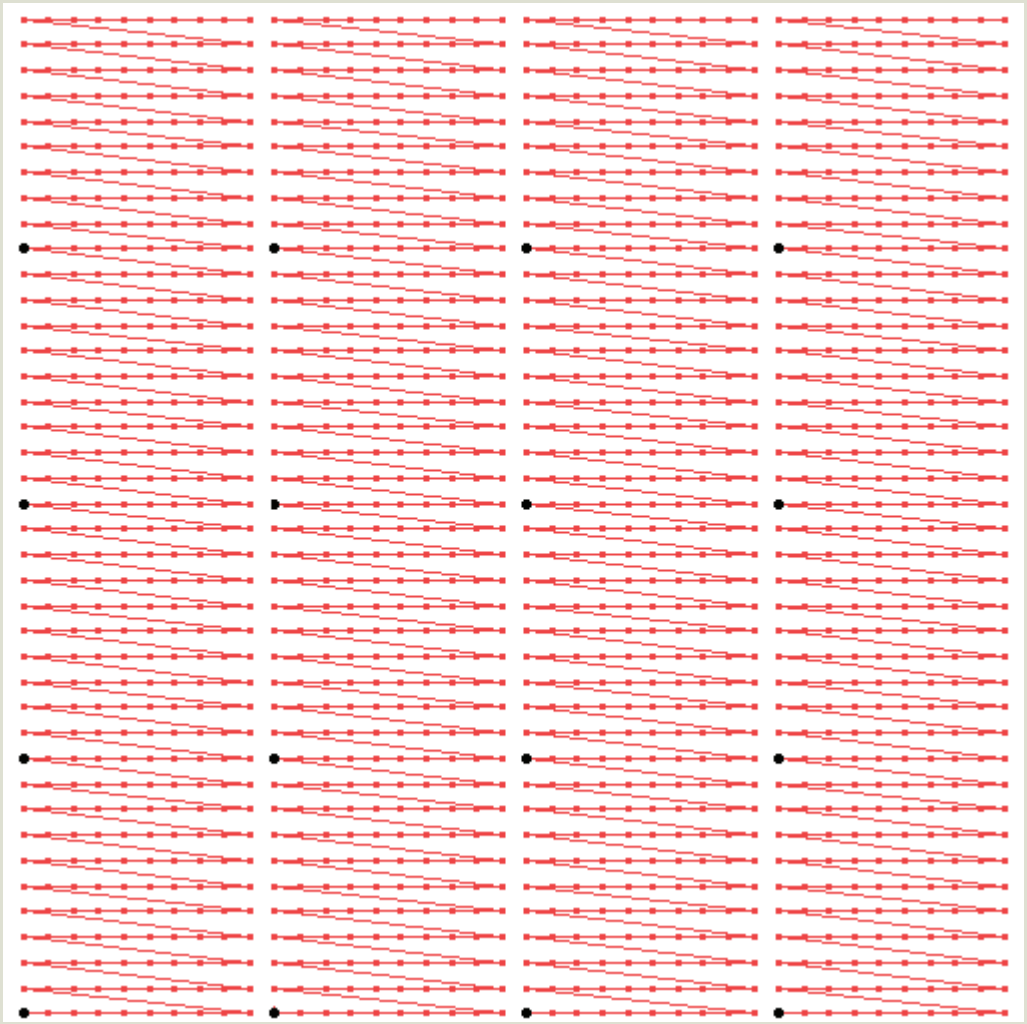
Beamlet array pattern

- >70% optical efficiency
- Single photon excitation instead of multiphoton
- Larger number of beamlets generated

Tiled raster scanning

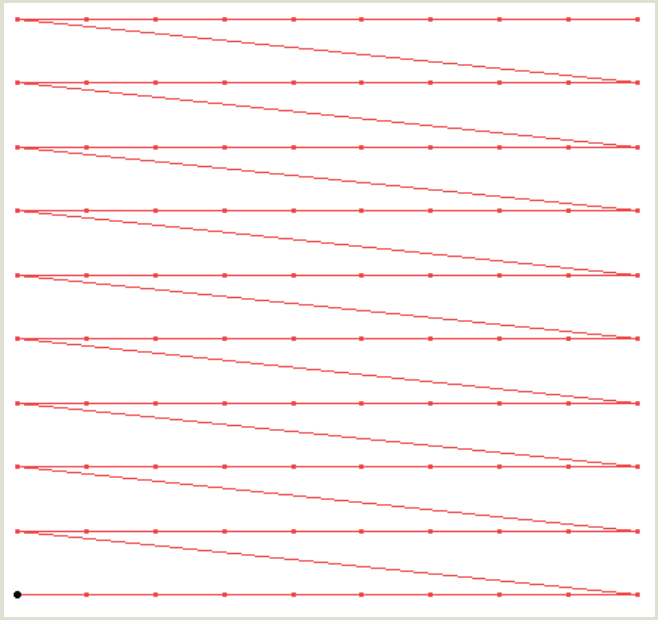


Single beam 2D raster scan
10x10 points



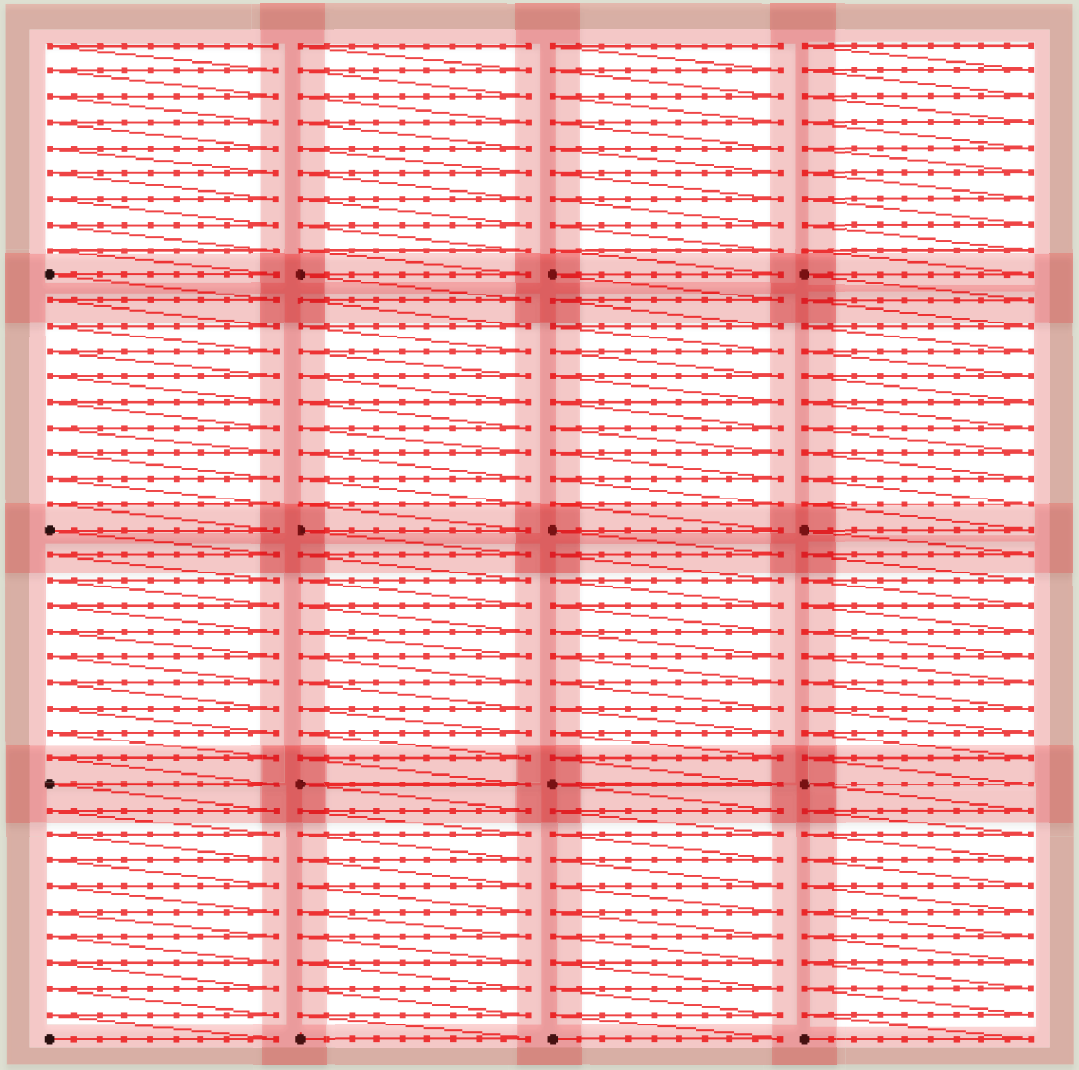
16 beams 2D raster scan
10x10 points total image size = 40x40

Tiled raster scanning



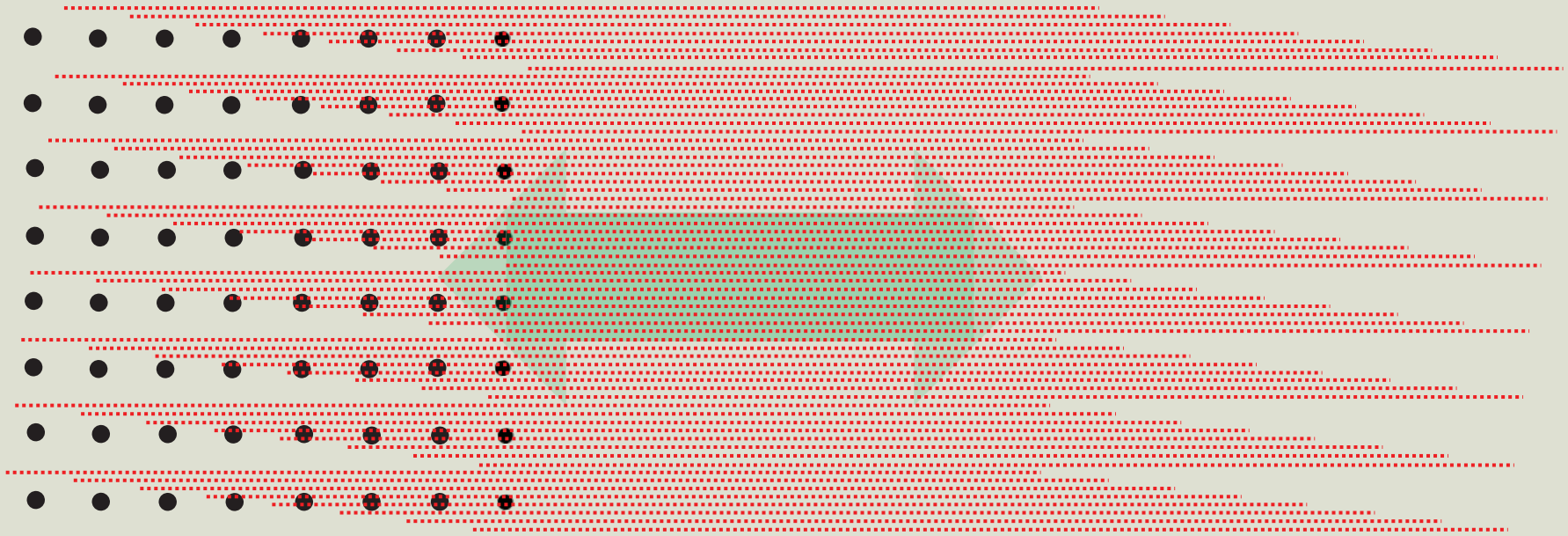
Single beam 2D raster scan
10x10 points

Over scan introduces
Fixed-pattern
–may cause local
photobleaching etc



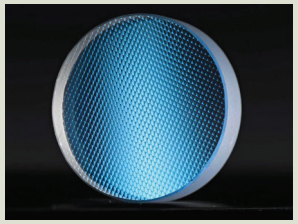
SWept ARray Microscopy (SWARM)

Careful choice of magnification between DOE and Object Plane
Enables Nyquist sampling of the sample

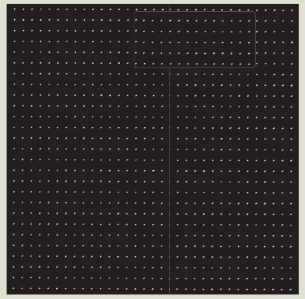


64beams 1D swept array scan
100 points total image size = 64x100

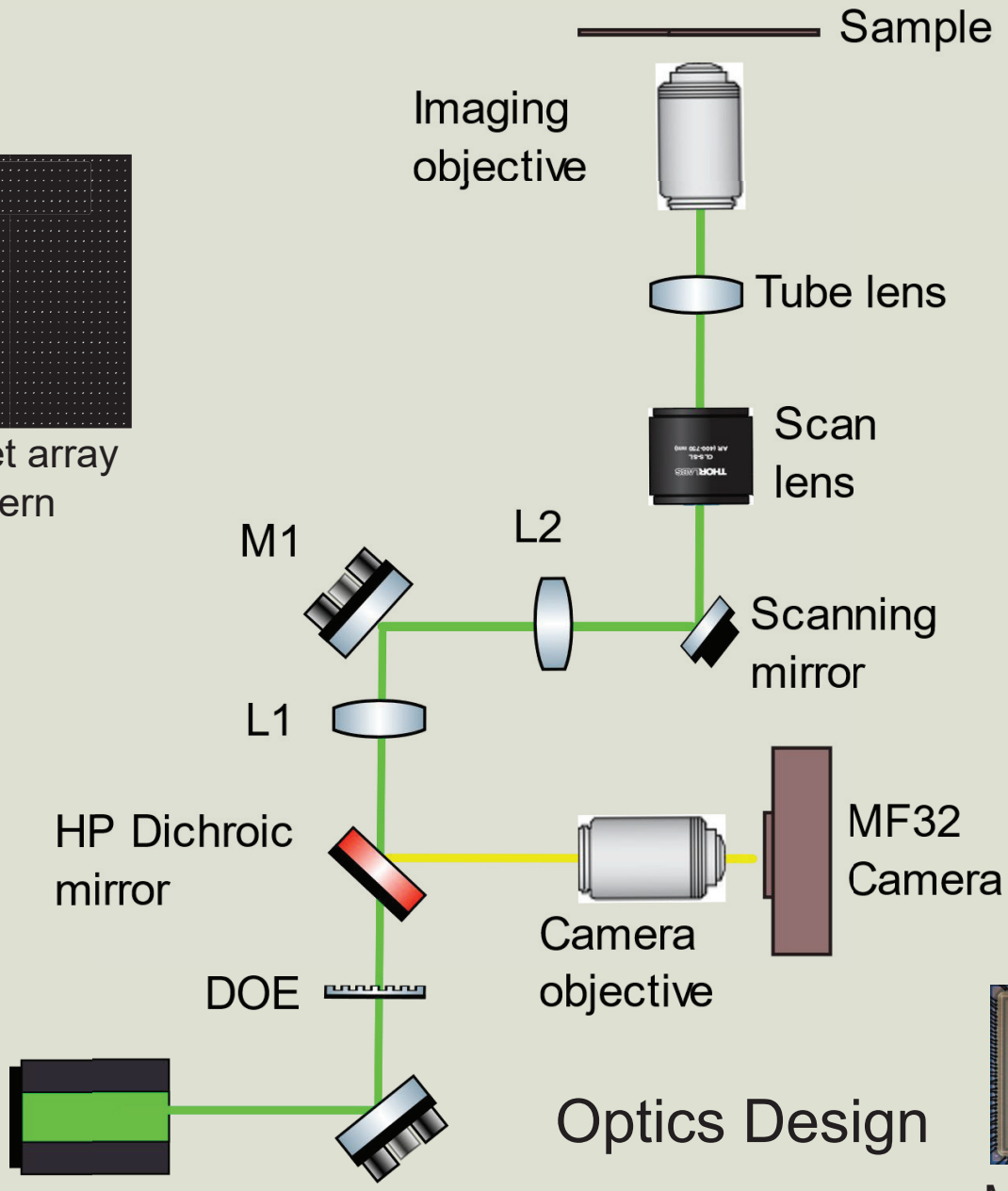
Evolution – Confocal (1024 beamlets)



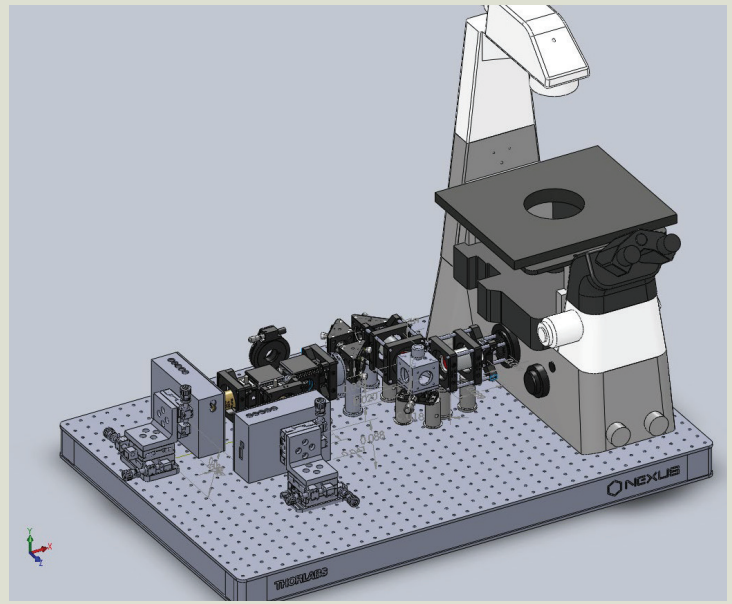
Custom designed DOE



Beamlet array pattern



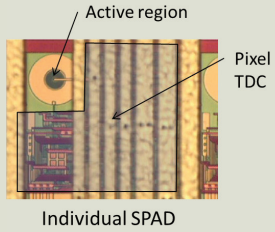
Optics Design

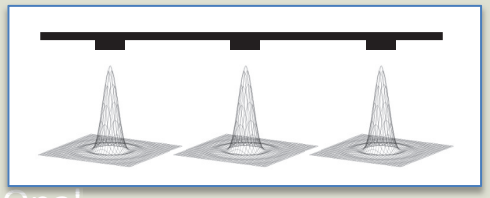


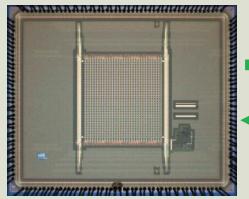
3D schematic of the setup
(small physical footprint)

Spark Antares 532nm, 10ps pulsed laser (300mW)

Each SPAD is a "confocal pinhole"
Active area ~ 1 Airy unit







Megaframe

Opal Kelly FPGA XEM 6310

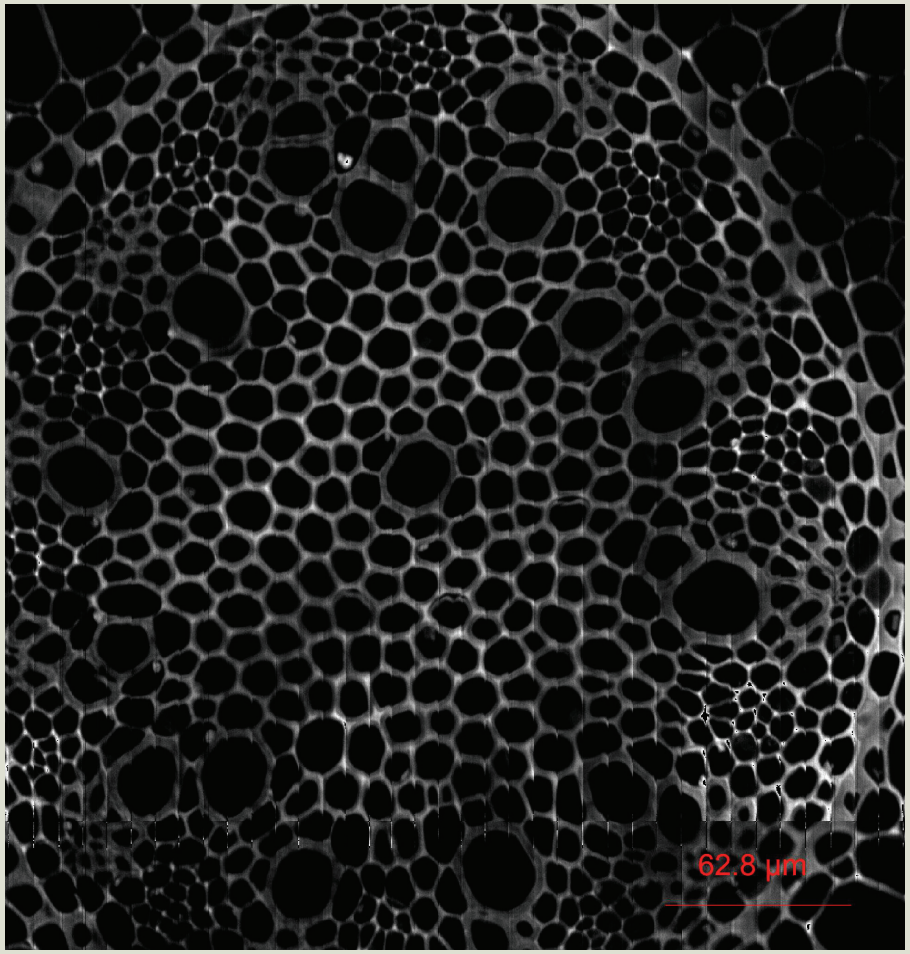
USB 3

Max count rate 250Mcounts/s

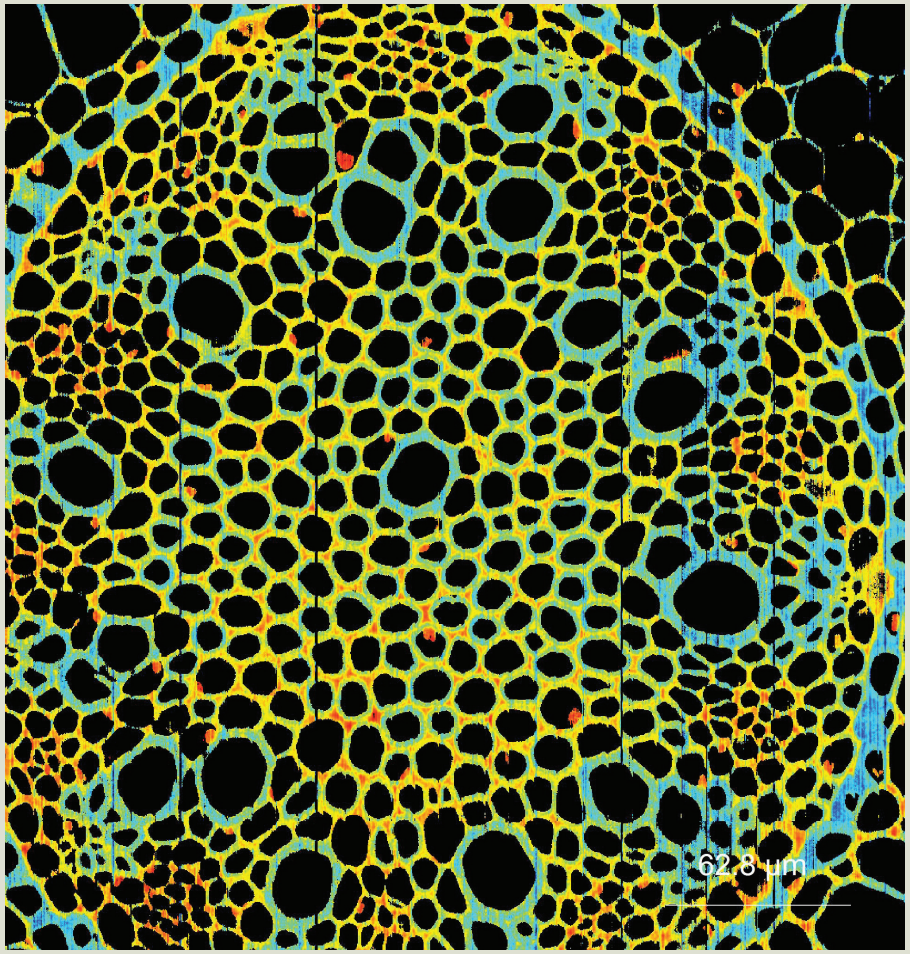
Computer

SWARM image acquisition 500ms (1024 points per detector)

Convallaria (Lily of the valley)



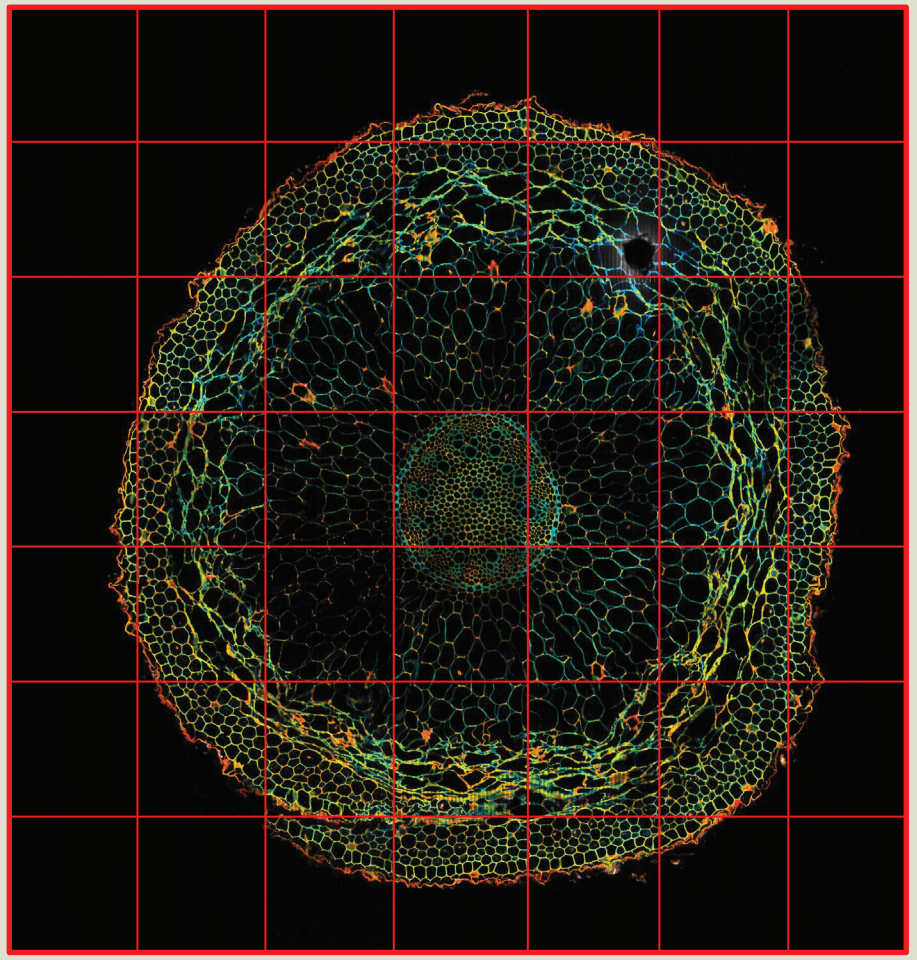
Intensity image



Lifetime Image

Convallaria (Lily of the valley)

- SWARM lifetime composite image composed of a mosaic of 49 individual images (total acquisition time 5 x 49 = 245 seconds
- Image size = 2 x 2 mm
- (7168 x 7168 pixels)
- In comparison the time taken to acquire a conventional FLIM image of 256 x 256 pixels = 5 minutes



Lifetime Composite

Automated Histological FLIM HCS

Analysis of Tissue Micro Arrays (TMA)

Slide scan and wellplate loading

↓

Wellplate hotel Scan & slide populate

↓

Widefield slide mosaic scan

↓

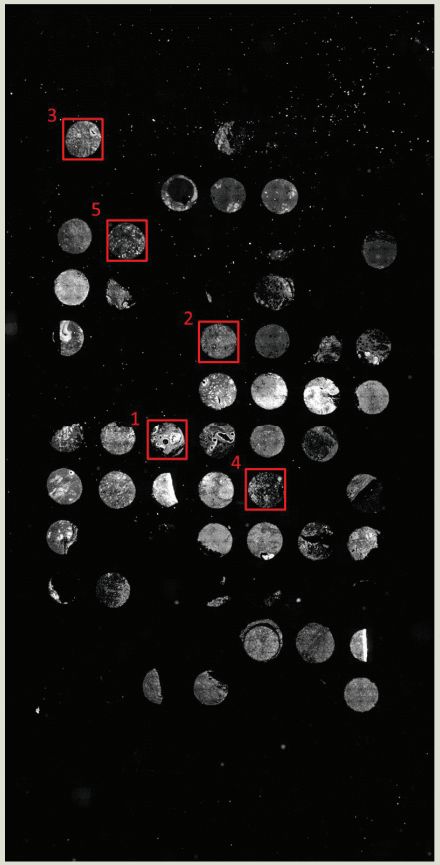
Image analysis & core discrimination tool (GUI)

↓

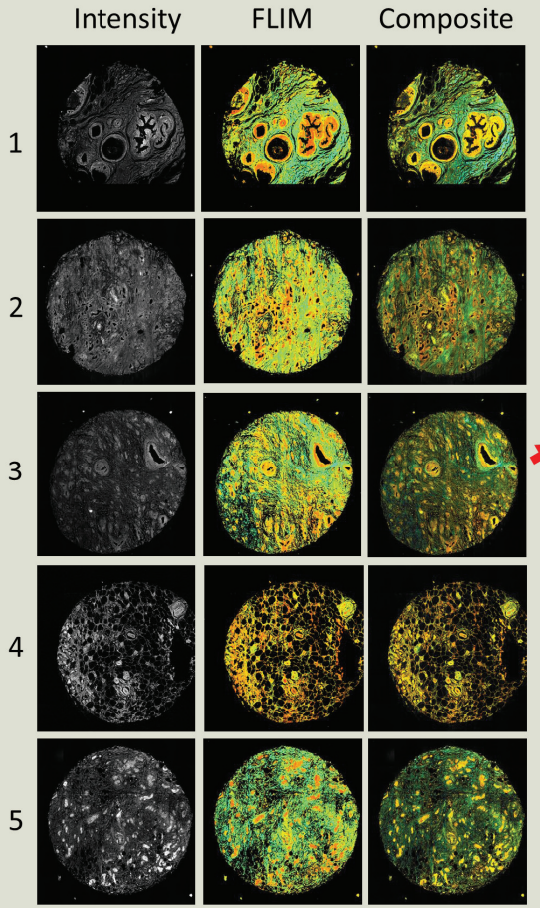
Multibeam FLIM acquisition for each core

↓

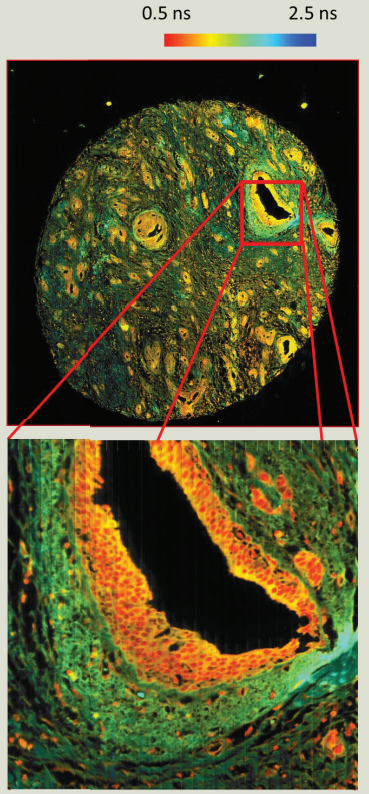
Data sorting and analysis



Widefield Fluorescence Mosaic image of TMA

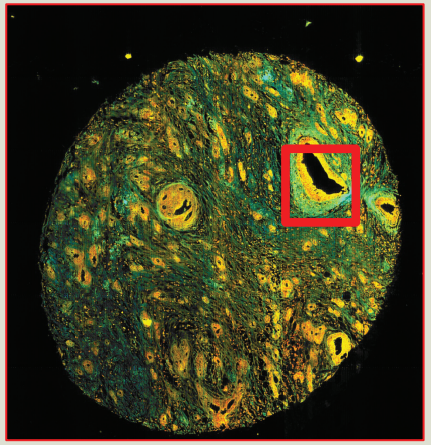


FLIM mosaics composed of 6 x 6 individual images (Total image size = 1920µm x 1920µm).

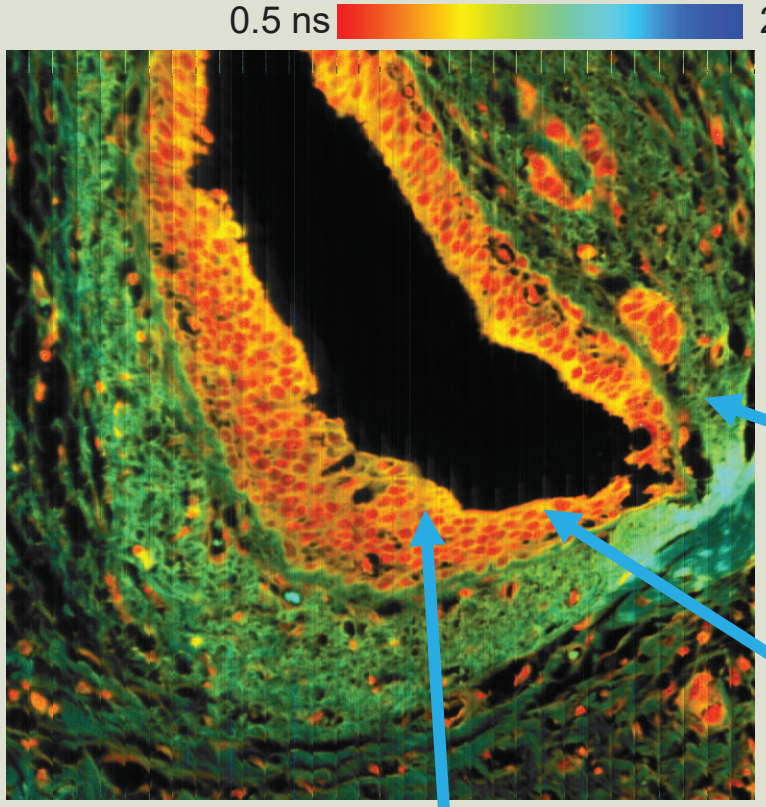


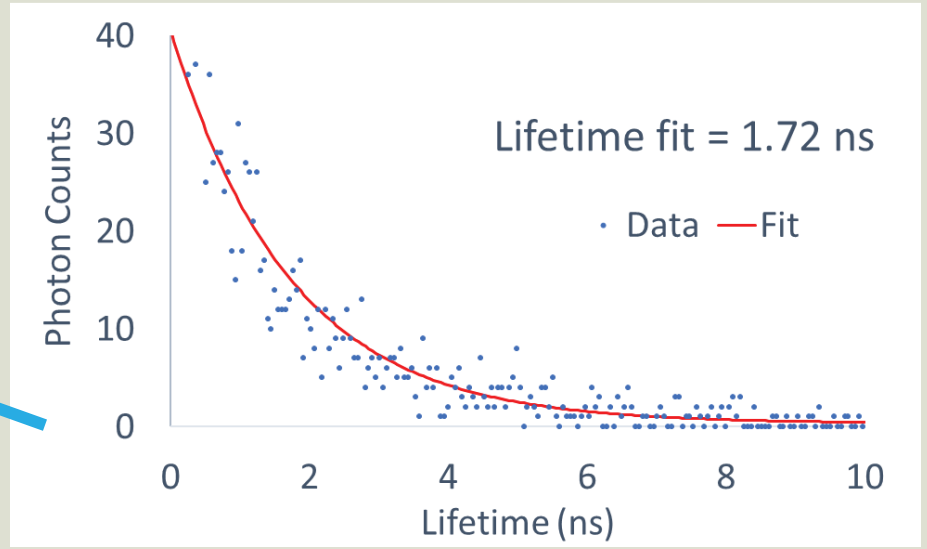
composite single image (320µm x 320µm)

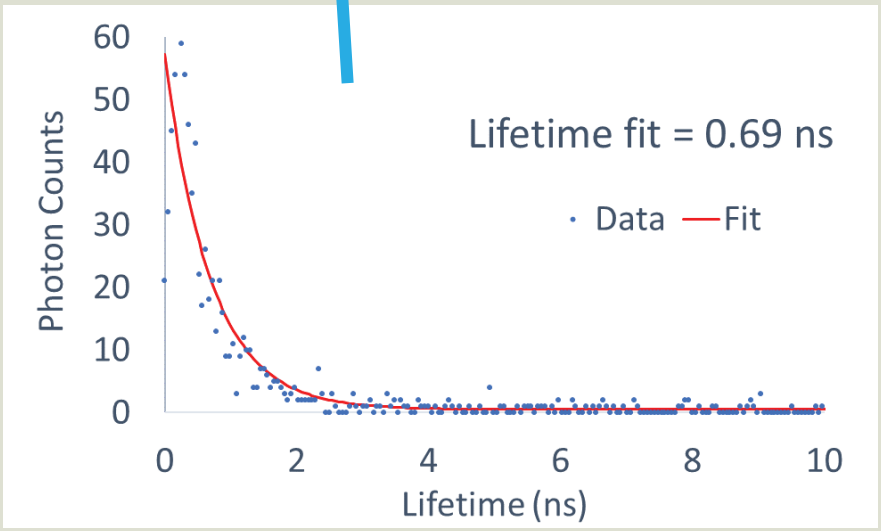
Individual 1024 x 1024 datasets acquired in 10 s

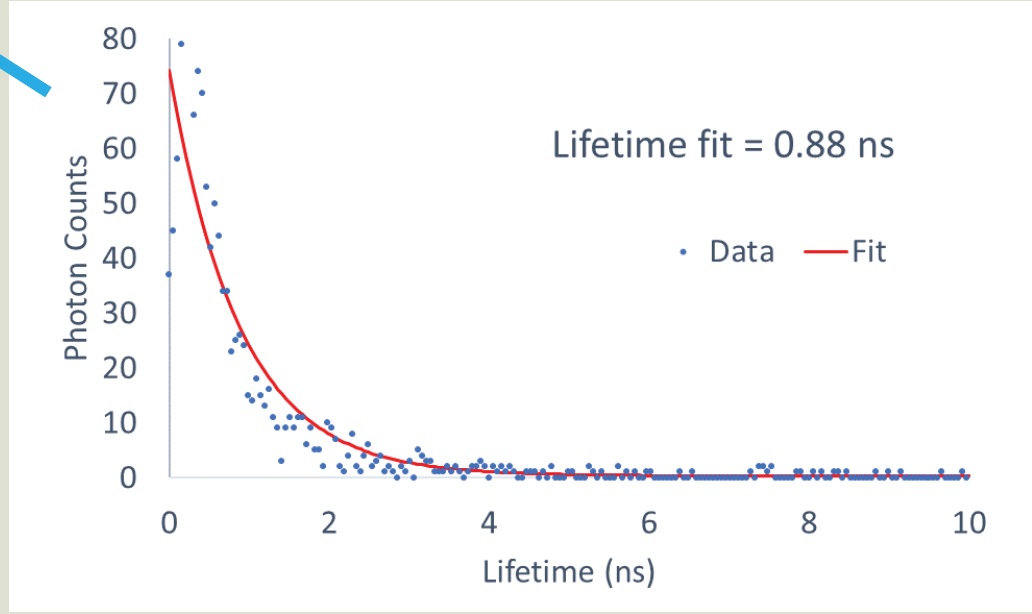


SWARM mosaic composite image





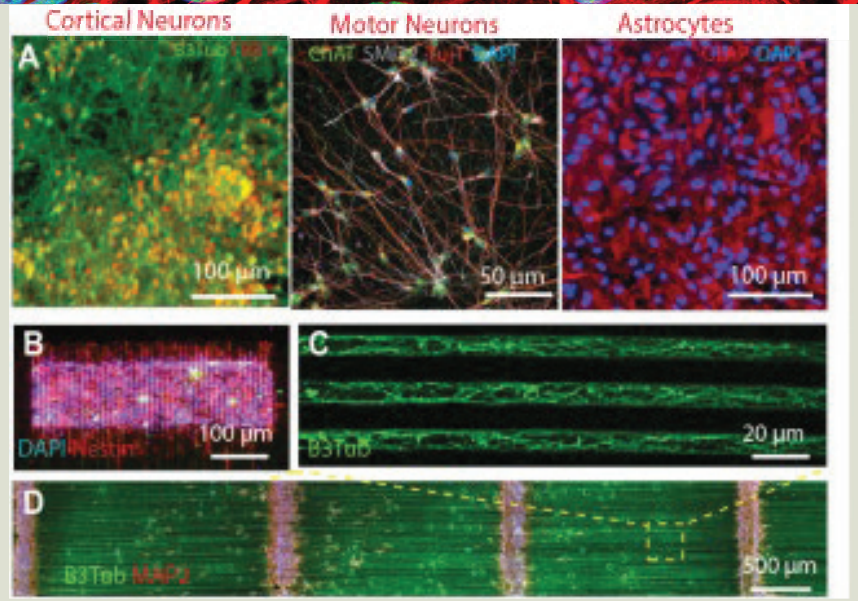


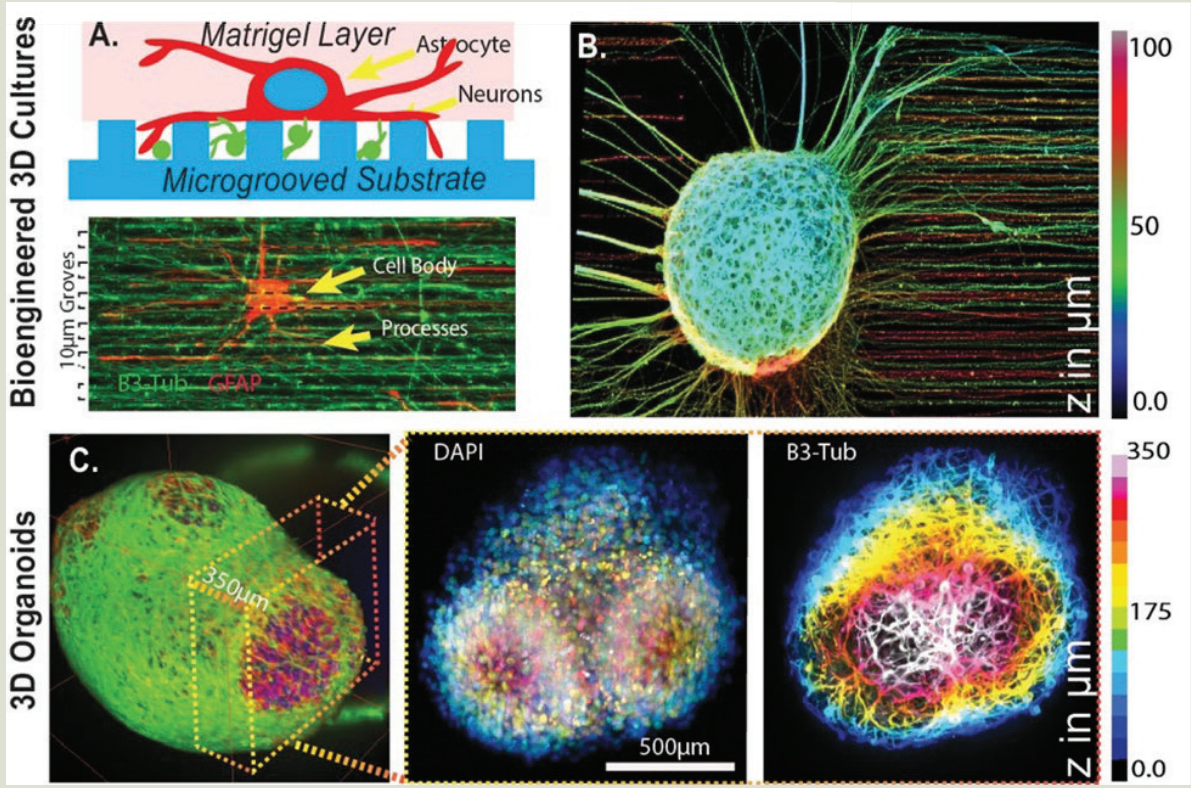


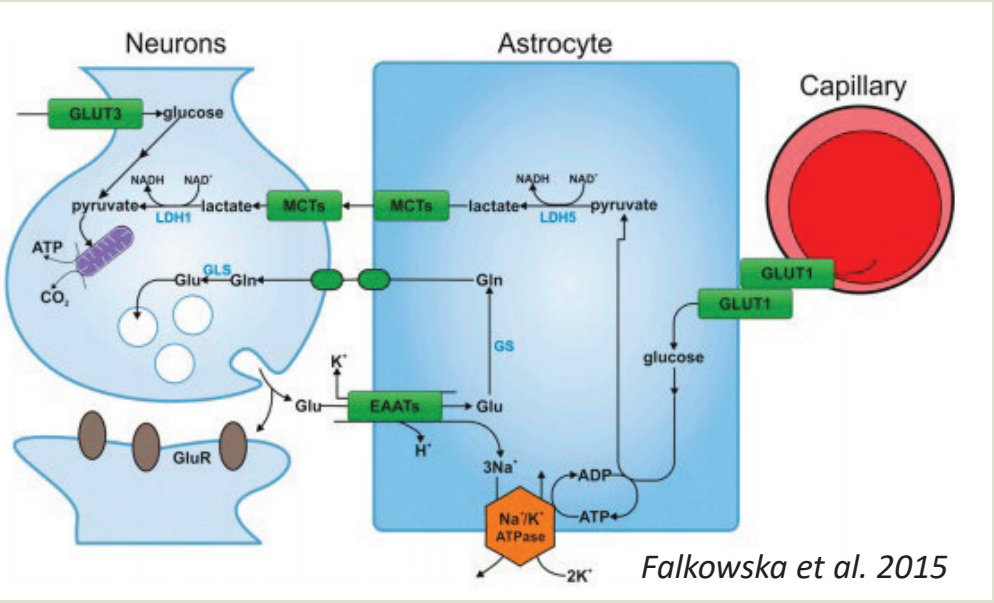
- Acquiring over 1000 photons per pixel after 5 seconds acquisition
- **No binning to perform lifetime fit!**

Serio Lab: Applications in Neuroscience

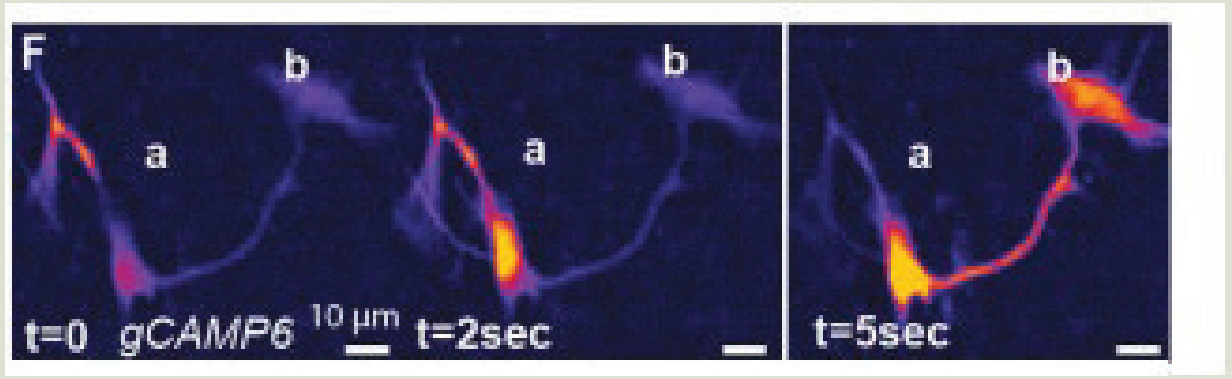
Andrea Serio
Crick Institute,
London







Falkowska et al. 2015



PercevalHR – Sensing ATP/ADP

- Competitive binding of high affinity GlnK1 site reports ATP/ADP ratio.
- Ratiometric Sensor allows for tunability of bound states.

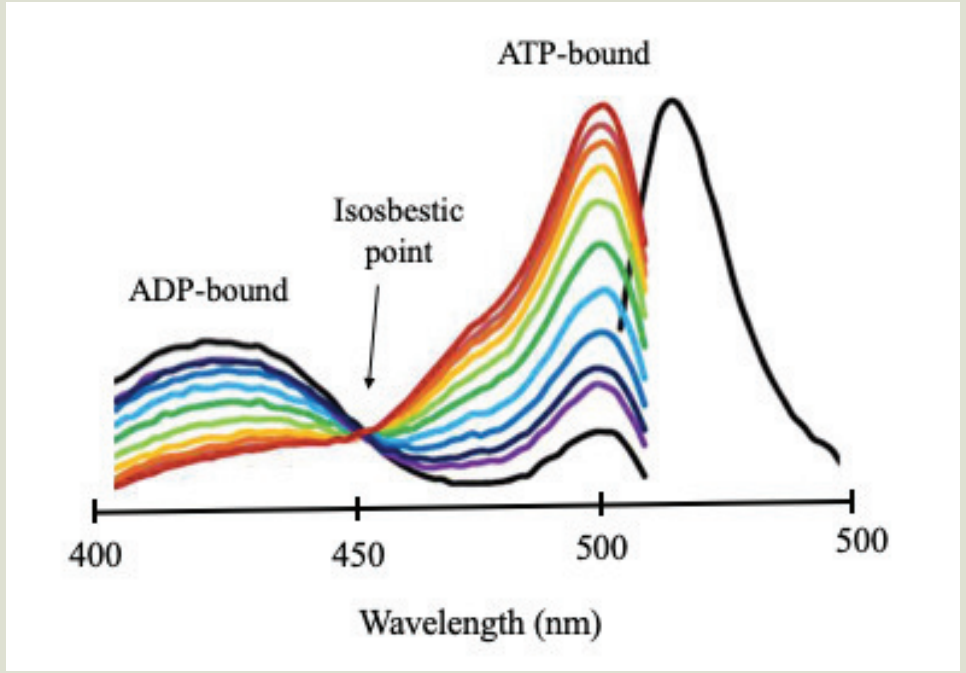


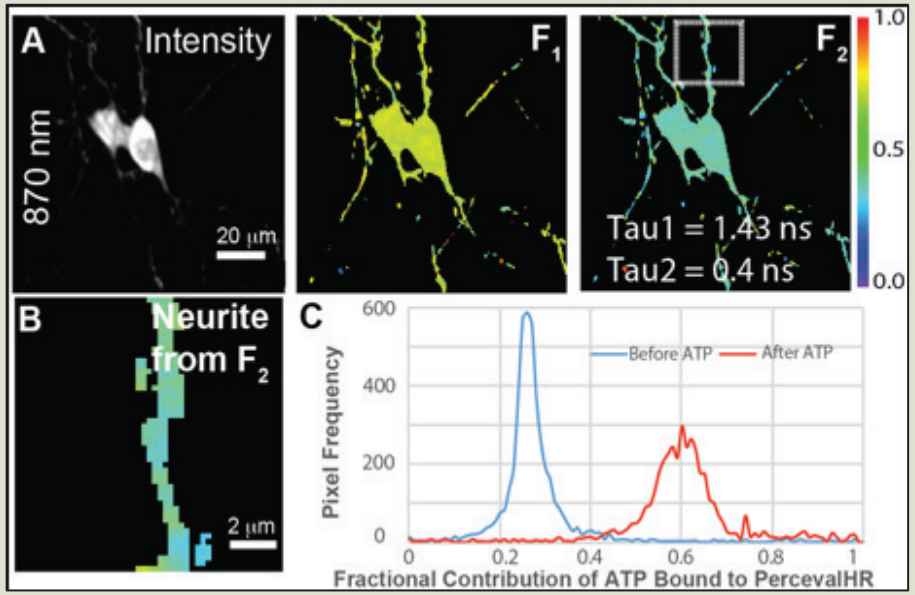
Figure 1. P-HR Composition and Spectral Properties[1]

Tantama, M., Martínez-François, J. R., Mongeon, R., & Yellen, G. (2013). Imaging energy status in live cells with a fluorescent biosensor of the intracellular ATP-to-ADP ratio. *Nature Communications, 4*(1), 2550. doi:10.1038/ncomms3550

Visualise metabolism and signalling with advanced microscopy

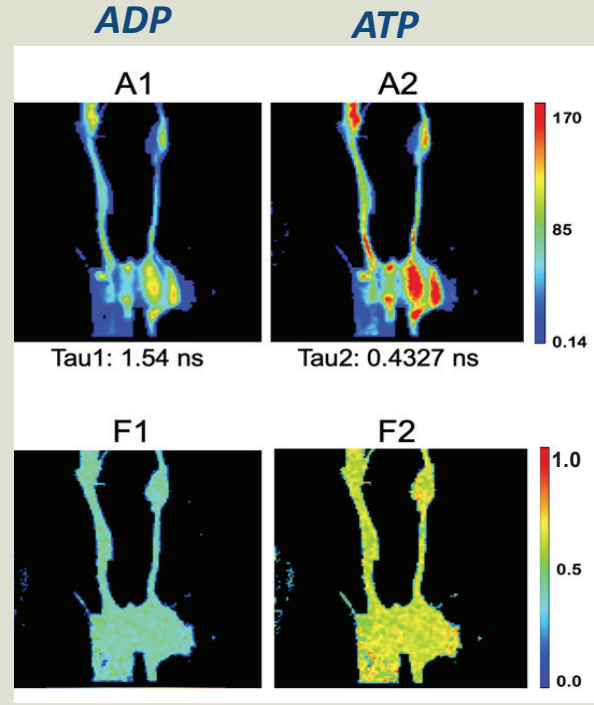
Neurite stimulated with ATP

Before ATP stimulation



Increase in 'heat map' signal of ATP lifetime (F2) due to increased fractional contribution of ATP bound to PercevalHR

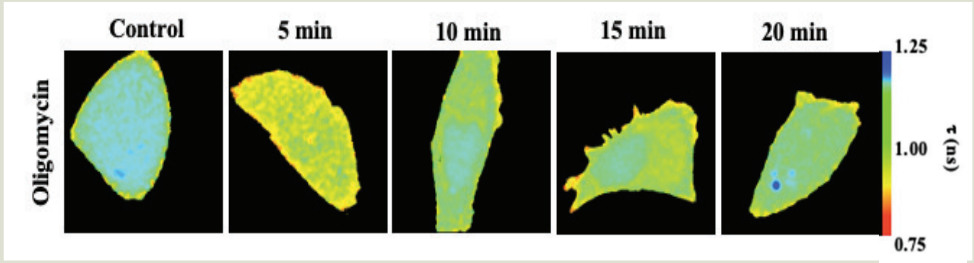
Astrocyte in grooves at baseline



Fluorescence lifetimes produced via FLIM with PercevalHR ATP:ADP ratio sensor construct

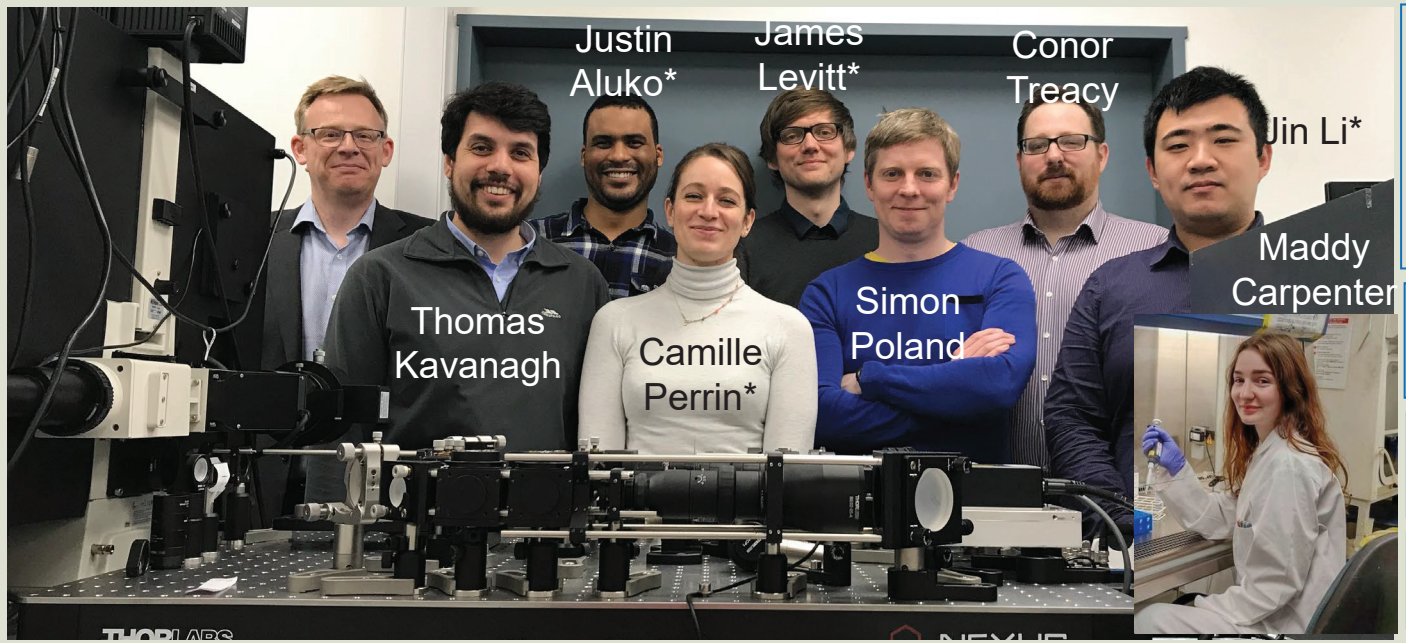
Optimise FLIM imaging protocol:

- Investigate the impact of **metabolic inhibitors** on fluorescent lifetimes



Acknowledgements

BBSRC 20 Years of Pioneering Great British Bioscience — ACTIVATING IMPACT — WINNER — FOSTERING INNOVATION 2014



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•M-Squared Life
•Carl Zeiss GmBh
•UCB-CellTech
•MSquared Life

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MRC Medical Research Council

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*Former group members