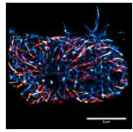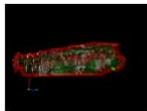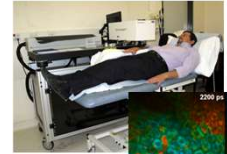# Multidimensional fluorescence imaging

Christopher Dunsby   Paul French

James McGinty   Mark Neil

(Photonics Group) LIGHT,
Physics Department
Imperial College London
www.imperial.ac.uk/research/photonics
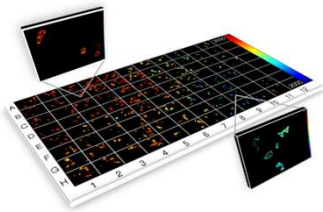
& quantitative phase

Cell Biology   3D, in vivo models   Clinical imaging

1

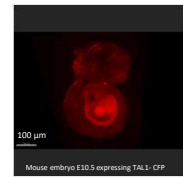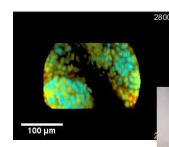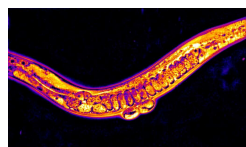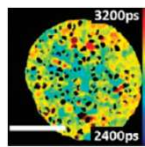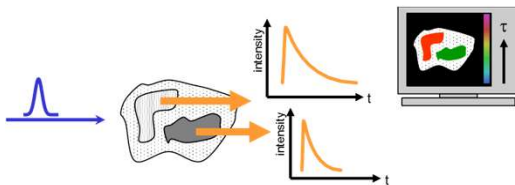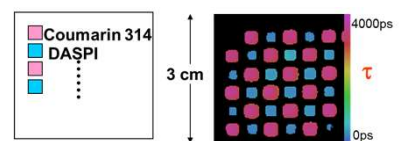# Quantitative imaging in biological tissue, 3D cell cultures, …

Challenges:
- *unknown [fluorophore]*
- *sample scattering/absorption*
- *background (auto)fluorescence*

} *heterogeneity*

Coumarin 314
DASPI

3 cm

τ

4000ps

0ps

Fluorescence lifetime imaging (FLIM)

Contrasting different fluorophore species

Unlabelled frozen section rat tail

collagen bundles
fatty tissue
hair follicle
dermis

3010 ps
1170 ps

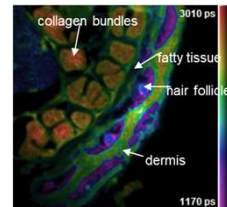- *inherently ratiometric - in single spectral channel*
- *insensitive to attenuation (scattering, absorption)*
- *background (auto)fluorescence can be quantified*
- *quantitative FRET readouts*
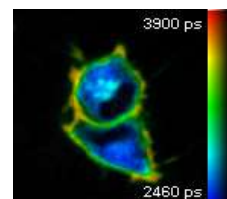 (e.g. protein interactions, drug-target engagement, …)

Di-4-ANEPPDHQ membrane dye fluorescence lifetime senses order in lipid bilayer

3900 ps
2460 ps

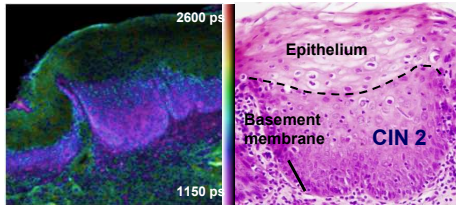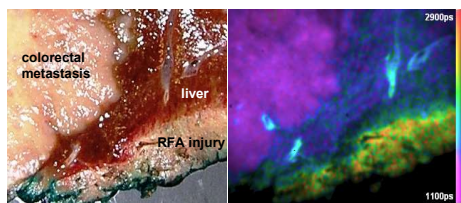**Contrasting different fluorophore environments**

2

FLIM for label-free quantitative imaging & metrology in biological tissue

Wide-field FLIM

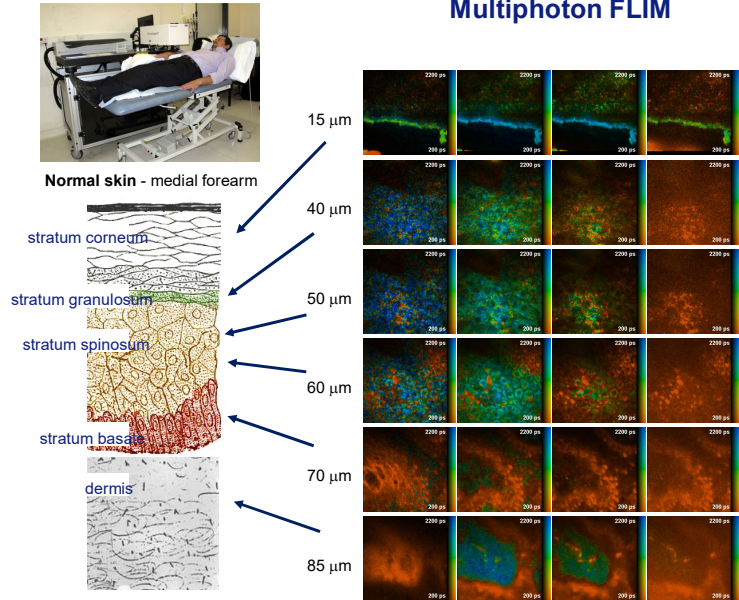Fresh section human cervix (740 nm excitation)

Elson et al, Reviews in Fluorescence 2006

Colour photograph and FLIM images of fresh human liver tissue containing metastatic colorectal cancer and region of RFA damage

Multiphoton FLIM

Normal skin - medial forearm

3

Fluorescence mapping of biomolecular interactions $\Rightarrow$ FRET

intermolecular FRET

e.g. change in conformation

Donor lifetime shortens

intramolecular FRET biosensor

$$I = I_0 \left(\beta e^{-\frac{t}{\tau_{DA}}} + (1-\beta) e^{-\frac{t}{\tau_D}} \right)$$

$$Kd = \frac{[D_{free}][A_{free}]}{[DA_{FRET}]}$$

GFP-mChFP PARP1 FRET biosensor

background fluorescence → FLIM
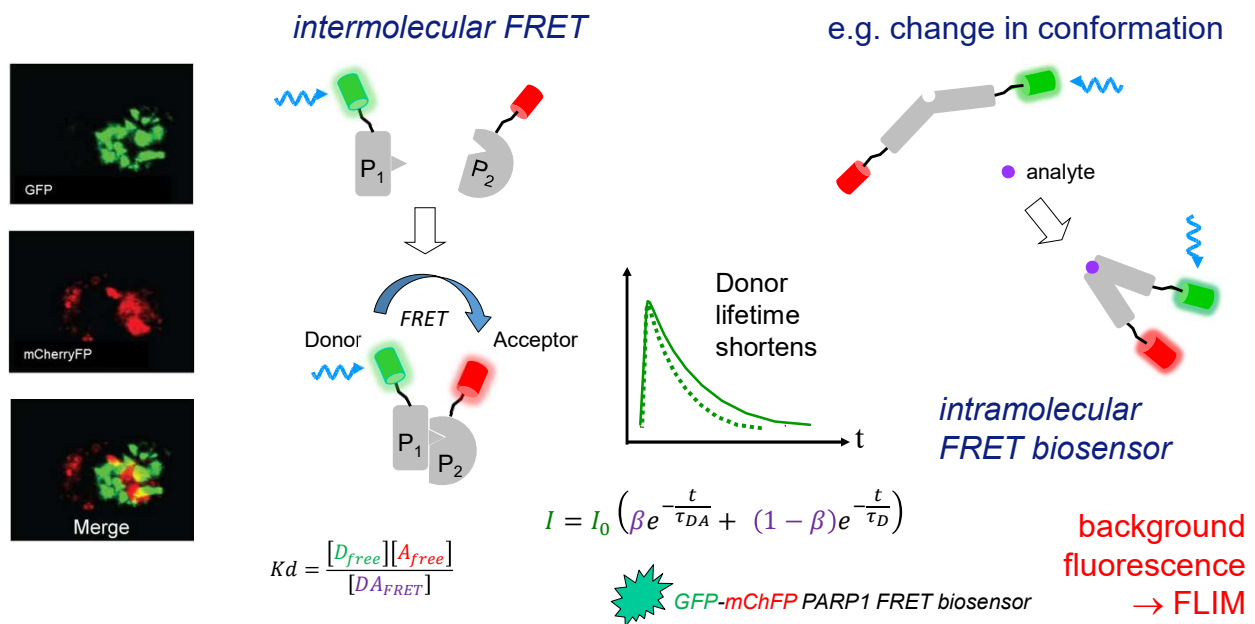
4

openFLIM HCA: automated multiwell plate FLIM microscopy

Motorised microscope frame
(Olympus IX-81 ZDC)

+ *spinning Nipkow disc scanner*
+ *wide-field time-gating*
+ *supercontinuum excitation source*
+ *FLIM/segmentation analysis*

open-source automated image
data acquisition
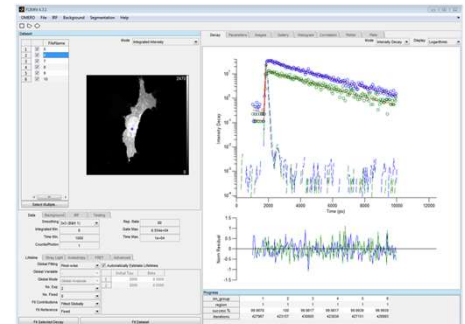
open-source automated image
data analysis

https://www.imperial.ac.uk/photonics/research/biophotonics/

http://downloads.openmicroscopy.org/latest/flimfit

Görlitz et al. JoVE (2017)

5

FLIM HCA of signalling networks using FRET

Mapping & quantifying protein interactions

RASSF protein family
– roles in cancer
– promoting apoptosis through
   RASSF-MST1/2 interaction
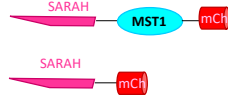

Scrace & O'Neill Mol, Biol Intl 2012
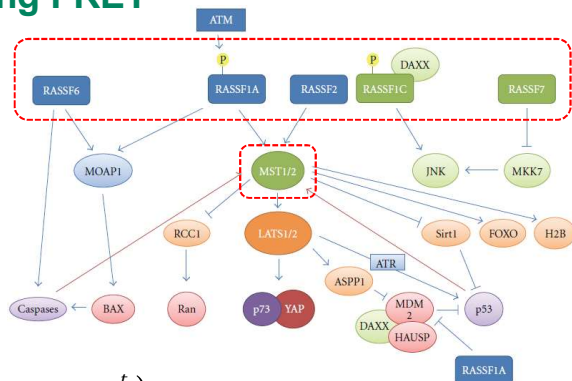
RASSF 1 & RASSF 5
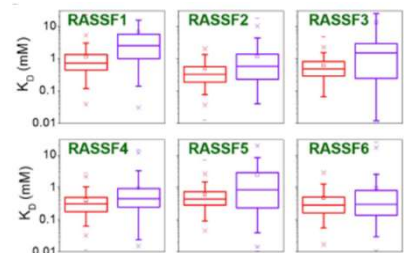
RASSF 2, RASSF 3, RASSF 4 & RASSF 6

RASSF 7 - RASSF 10

$$I = I_0 \left(\beta e^{-\frac{t}{\tau_{DA}}} + (1-\beta)e^{-\frac{t}{\tau_D}} \right)$$

$$Kd = \frac{[D_{free}][A_{free}]}{[DA_{FRET}]}$$
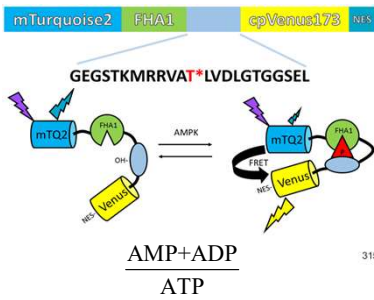
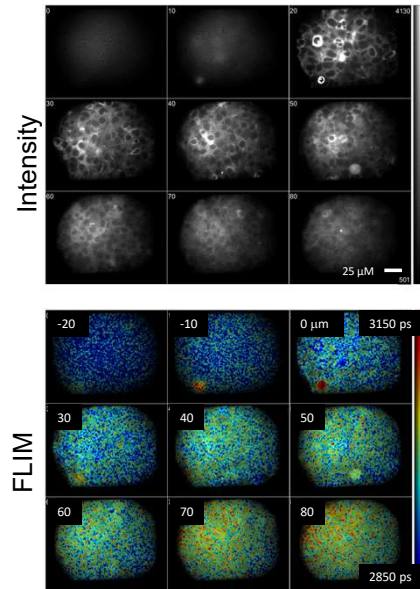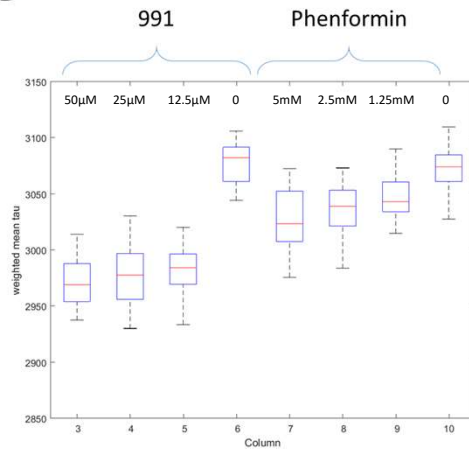*Margineanu et al, Sci. Rep. 2016*

6

Automated multiwell plate FLIM/FRET of AMPK biosensor

Adapted from AMPKAR sensor
Tsou et al, Cell Metab. (2011)
→ ***Chennell et al., Sensors (2016)***

Dose response of AMPK activity read out by T2-AMPKAR FRET biosensor in spheroids of HEK cells

7

Confocal FLIM endomicroscope

Hugh Sparks, Hiroshi Kondo
Photonics/Sahai lab
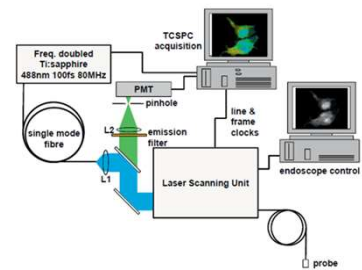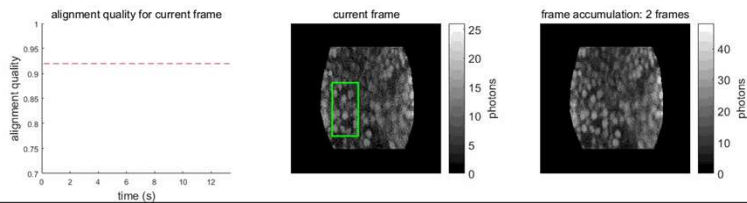
Kennedy et al., J Biophotonics.(2009)

In vivo image acquisition and registration
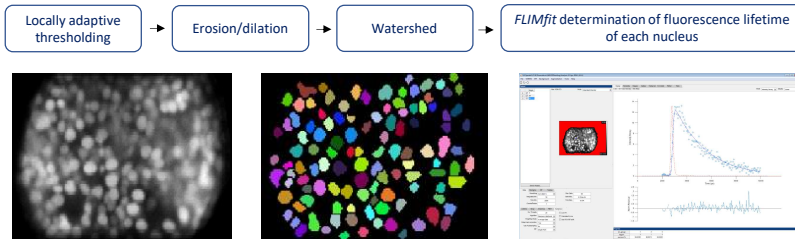
Multiphoton FLIM microscopy

Sahai lab

Image processing

Locally adaptive thresholding → Erosion/dilation → Watershed → *FLIMfit* determination of fluorescence lifetime of each nucleus
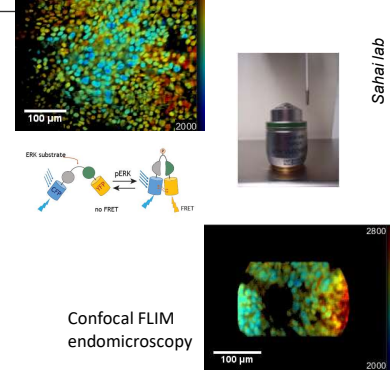
Confocal FLIM endomicroscopy

8

In vivo TCSPC FLIM/FRET confocal endomicroscopy of Doxorubicin binding DNA

in vivo comparison of target engagement between intraperitoneal and intravenous injection of DOX in IGROV-1 H1-GFP labeled tumors in mouse model

No DOX binding

DOX binding DNA

$$\Longrightarrow \text{FRET}$$
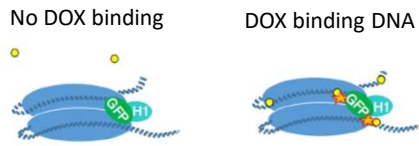
$$I = I_0 \left(\beta e^{-\frac{t}{\tau_D}} + (1 - \beta) e^{-\frac{t}{\tau_{DA}}} \right)$$
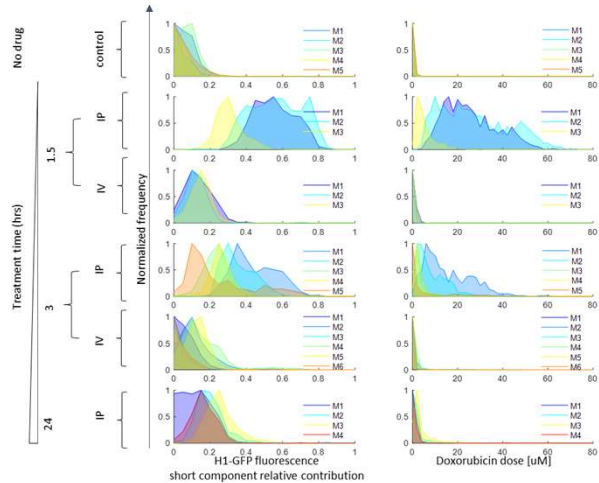
Unbound GFP — DOX-bound GFP

$\Rightarrow$ distinguish drug engagement and drug resistance

$\Rightarrow$ compare different drug delivery approaches

$\Rightarrow$ resolve single cell behaviour in vivo

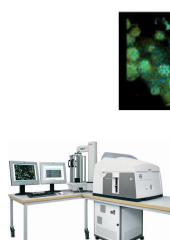*Sparks et al, Nat Comm 2018*

9

Accelerating our ability to understand and target complexity and heterogeneity in cancer
through automated imaging of 3D cancer models including patient-derived organoids

www.mach3cancer.org

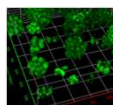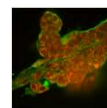Throughput

FLIM HCA
Automated spinning disc, FLIM

Crick/Imperial

OPM HCA
Automated high speed 3D (light-sheet)

All partners

M³M HCA
Automated multiphoton multiwell FLIM

Crick/Imperial

preclinical, clinical trials

Physiology

10

dOPM HCA system at Crick
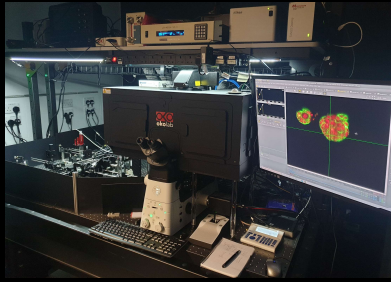
Timelapse sub-cellular imaging:
50 organoids imaged in 3D every 15 minutes over 7 days
Maximum Intensity Projections (MIP)

155.500 hours

1000 µm

Green GFP-Actin Yellow tdTomato-membrane Magenta SPY650-DNA
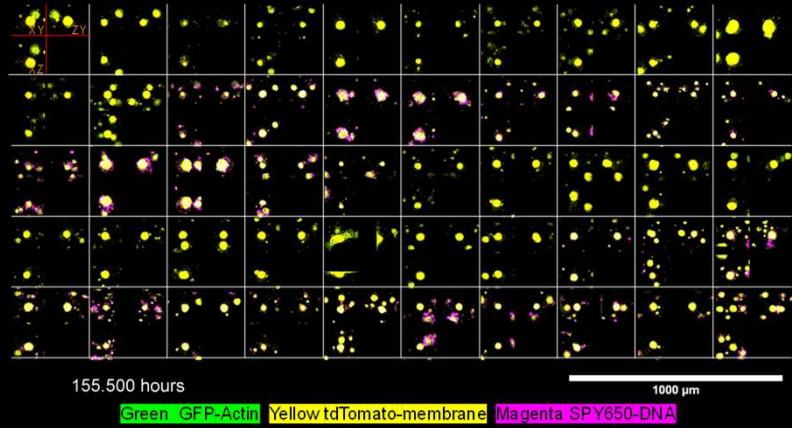
www.mach3cancer.org

11

Multibeam Multiphoton Multiwell plate Microscope (M³M HCA)
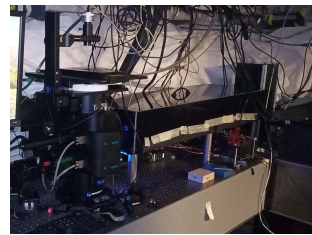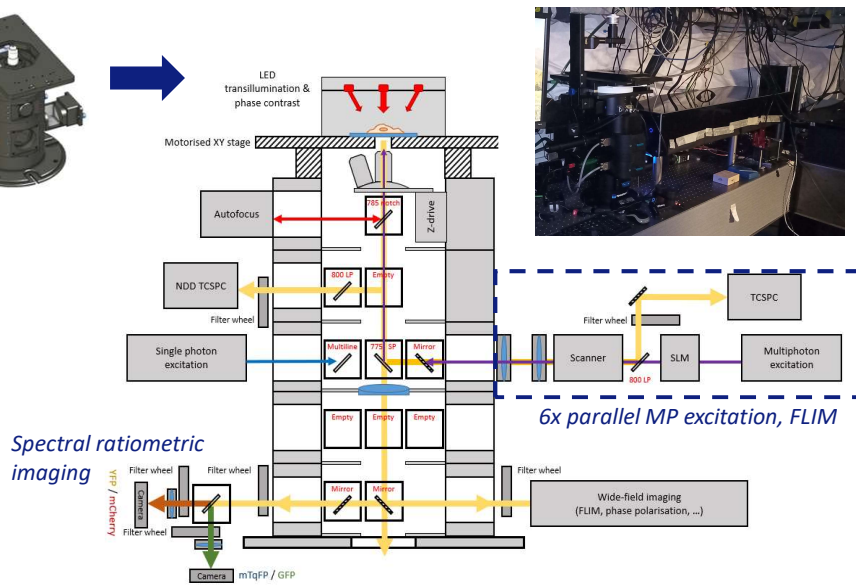
Multiphoton FLIM
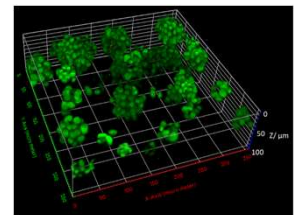
Spheroids cultured from mouse cells in BME with IRFP670 labelling histones
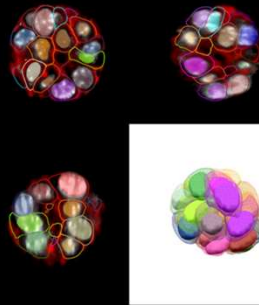
Mouse lung tissue actin (AF633) and nuclei (CFP)

Spectral ratiometric imaging

6x parallel MP excitation, FLIM

12

Optical Projection Tomography

Sharpe et al, Science (2002)

(OPT system from ~£10k)

ex vivo mouse embryo

neurofilament labelled with
Alexa-488 conjugated antibody

fixed and cleared in BABB

Live zebrafish

Day 40

3-D β-cell mass assay (Guy Rutter)
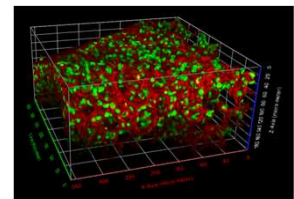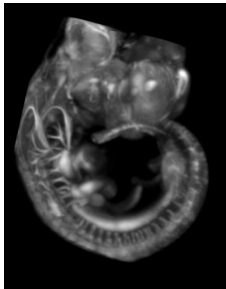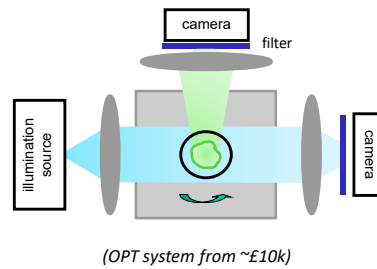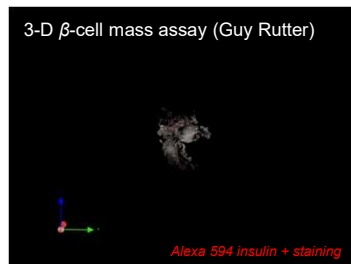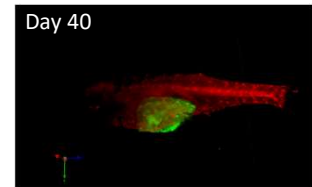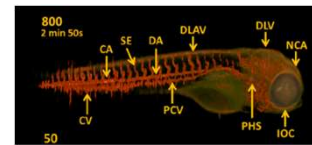
Alexa 594 insulin + staining

cleared with CLARITY

Brain (Eric Sahai)

GFP – tumour cells
Alexa 555 – endothelial cells
Alexa 647 – activated astrocytes
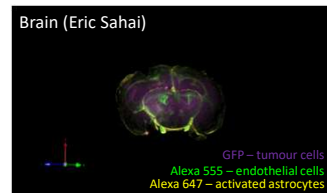
Low-cost OPT
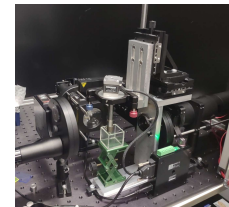
13

Accessible, robust, low-cost SMLM

Kwakwa, K. et al, J Biophotonics (2016)

easySTORM

~€300 multimode diode lasers

reflective fibre collimator (RFC)

Multimode optical fibre

optical autofocus   x-y   stage   sample

TIRF⇔ **epi**

microscope frame
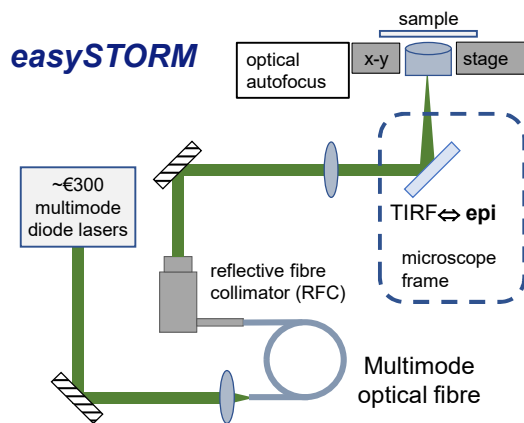
MMF → low-cost, high power lasers,

  ~125 x 125 µm² FOV

HPC parallelised SMLM data processing

Munro et al, J Microscopy 2019

easySTORM   wide-field

easySTORM

120 µm
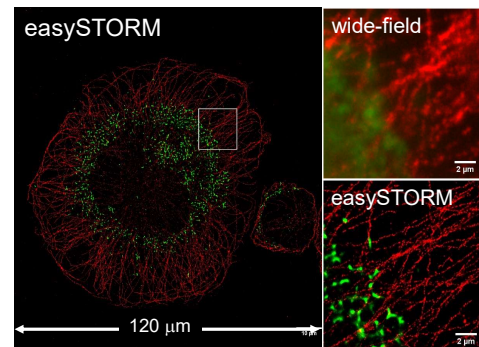
α-tubulin labelled with AF647 in THP-1 cells with AF488-labelled S. pneumoniae

easySTORM at IIT Guwahati (<~£5k upgrade of standard fluorescence microscope)

IIT Guwahati
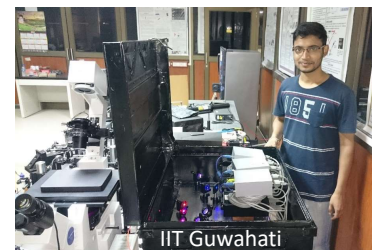
locally sustainable

www.openscopes.com

14

easySTORM → histological analysis of human glomerular disease

Frozen section presenting Membranous Glomerulonephritis

Basement membrane (laminin, green – Alexa Fluor 555), immunoglobulin G deposits (IgG, red –iFluor 647)

"histoSTORM"

replace diagnostic EM?
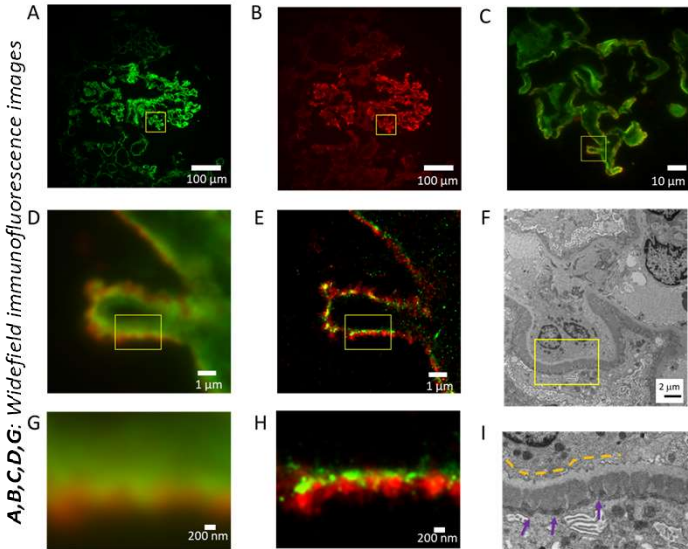
Glomerular Basement Membrane (Laminin-iFluor 647) **FFPE section** presenting Minimal Change Disease
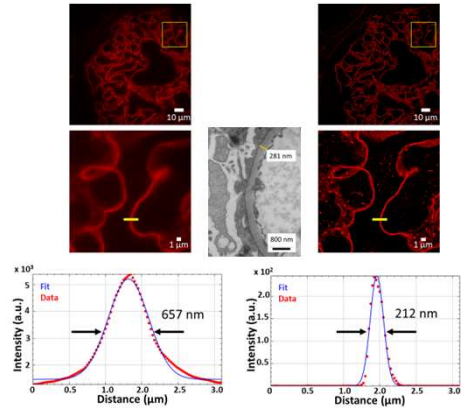
Garcia et al, J. pathology: Clinical Research 2021

15

Automated multiwell plate *easySTORM*
WM2664 melanoma cells arrayed in 6x4 wells of a 96-well plate

F-actin labelled with ifluor647

Paxillin labelled with AF488

FOV ~125 x125 µm   FOV ~12.5 x12.5 µm   *with Chris Bakal, ICR*

16

Cluster analysis of *easySTORM* SMLM data of chromatin compaction
Trichostatin-A treatment ⇒ smaller nucleosome clusters

(OvCar4 cells, labelled with anti-H3-AF647)

Ricci et al., Cell (2015)
http://dx.doi.org/10.1016/j.cell.2015.01.054

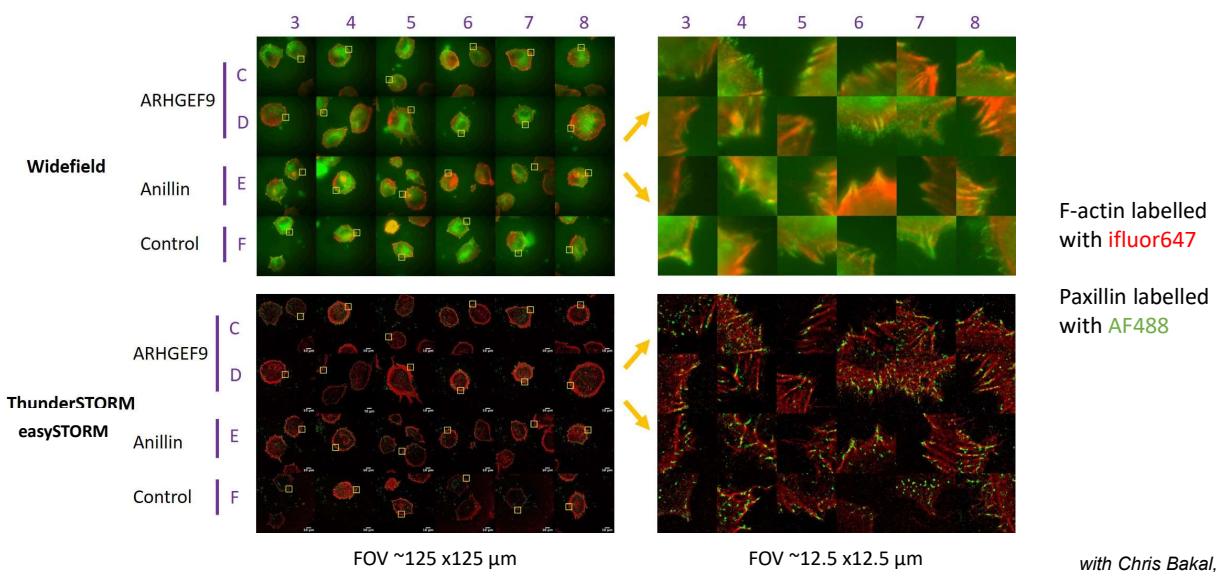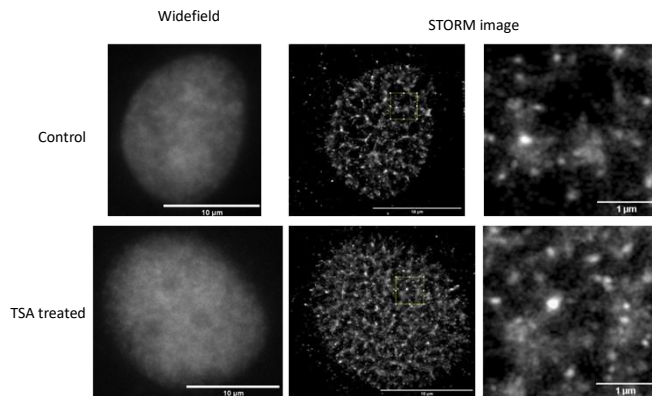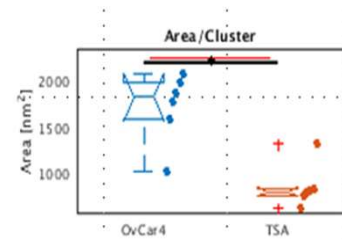⇒ *high throughput SMLM analysis of chromatin ultrastructure to screen therapeutic responses ...*

17

openFrame microscopes

Imperial College London

CAD

Epifluorescence

easySTORM

(A)
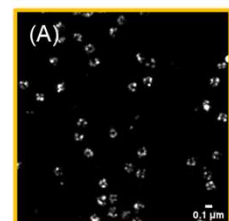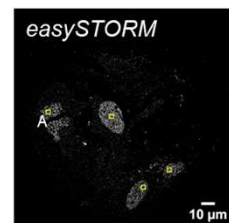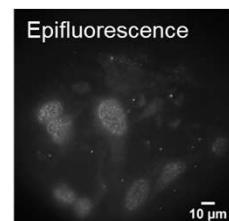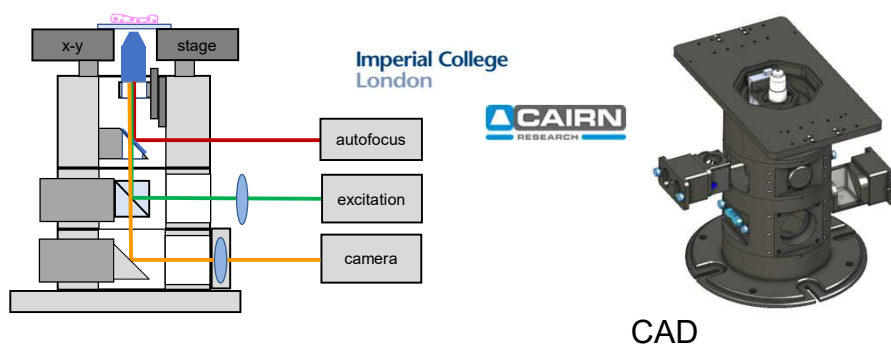
Open source microscopy & HCA
- CAD files of basic *openFrame* components to be shared openly & available at ~low cost
- Image acquisition software (e.g., μ*Manager* plug-ins) to be shared

  ⇒ academia, industry, low-resource settings, teaching ...

https://www.imperial.ac.uk/photonics/research/biophotonics/ or *www.openScopes.com*

18

VP-CLEM-KIT: a pipeline for democratising volumetric visual proteomics

Lucy Collinson, Paul French, Ricardo Henriques

Chan Zuckerberg Initiative ⊕

tomoSTORM *(i2i)*

STORM of FP in resin-embedded tissue sections (volumetric imaging at ~50 nm resolution)

19

Imperial College London

Multidimensional microscopy across the scales

THE FRANCIS CRICK INSTITUTE

Multiwell plate FLIM, FRET

Dominic Alibhai
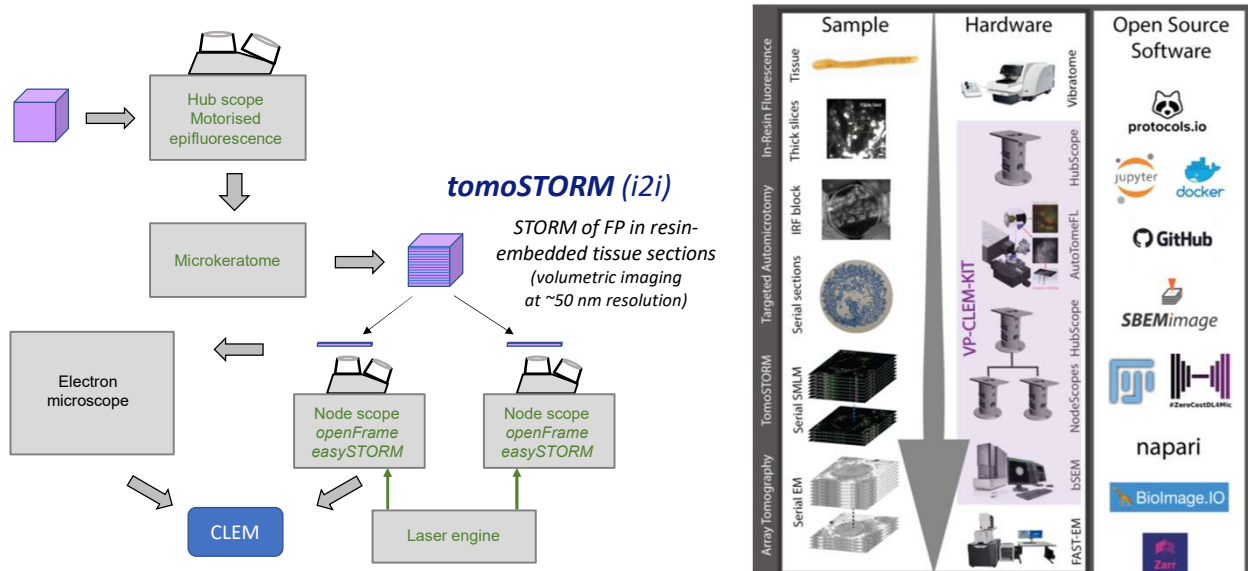*George Chennell*
David Grant
Douglas Kelly
Sean Warren
Romain Laine
Frederik Görlitz
Wenjun Guo
Edwin Garcia Castano
Sunil Kumar
Yuriy Alexandrov
Hugh Sparks
Anca Margineanu
Bill Flanagan
Jonathan Lightley
Dan Marks
Ian Munro
Clifford Talbot
Ewan McGhee

David Carling
Alex Sardini
Ed Tate
Chris Dunsby
Paul French
James McGinty
Mark Neil
Matilda Katan (UCL)
Michael Howell (Crick)
Alix Le Marois, Colin Radcliffe,
Erik Sahai (Crick)
Peter Thorpe (Crick/QMUL)
Beverley Isherwood (AZ)
Martin Ruediger (GSK)
Ted Murray (*Pfizer*)
Frank Stuhmeier (*Pfizer*)

Biology, Chemistry, ICB,
Mathematics, Medicine,
Physics

Institute of Chemical Biology

easySTORM

Jonathan Lightley
Riccardo Wysoczanski,
Edwin Garcia Castano
Frederik Görlitz, Stina Guldbrand,
Sunil Kumar, *Kwasi Kwakwa*
Yuriy Alexandrov
Peter Barnes, Louise Donnelly
Michael Osborne, Candice Roufosse
Arinbjorn Kolbeinsson
Seth Flaxman
Paul French, Chris Dunsby, Mark Neil
Dumisile Lumkwana, Martin Jones,
Lucy Collinson, (Crick)
Victoria Bousgouni, Lucas Dent,
Chris Bakal (ICR)

OPT

Connor Darling, Sam Davis,
Sunil Kumar, Yuriy Alexandrov
Paul French, James McGinty,
Ajay Bhargava, Clara Collart,
Luca Guglielmi, (Crick)
Caroline Hill, Erik Sahai, Jim Smith

pDPC

Bill Flanagan
Huihui Liu, Dan Marks
Karishma Valand, Matt De Vries,
Sunil Kumar, Yuriy Alexandrov
Chris Dunsby, Paul French

openFrame

Simon Johnson, Martin Kehoe
Chris Dunsby, Paul French,
James McGinty, Mark Neil
Callum Hollick, Elliot Steele
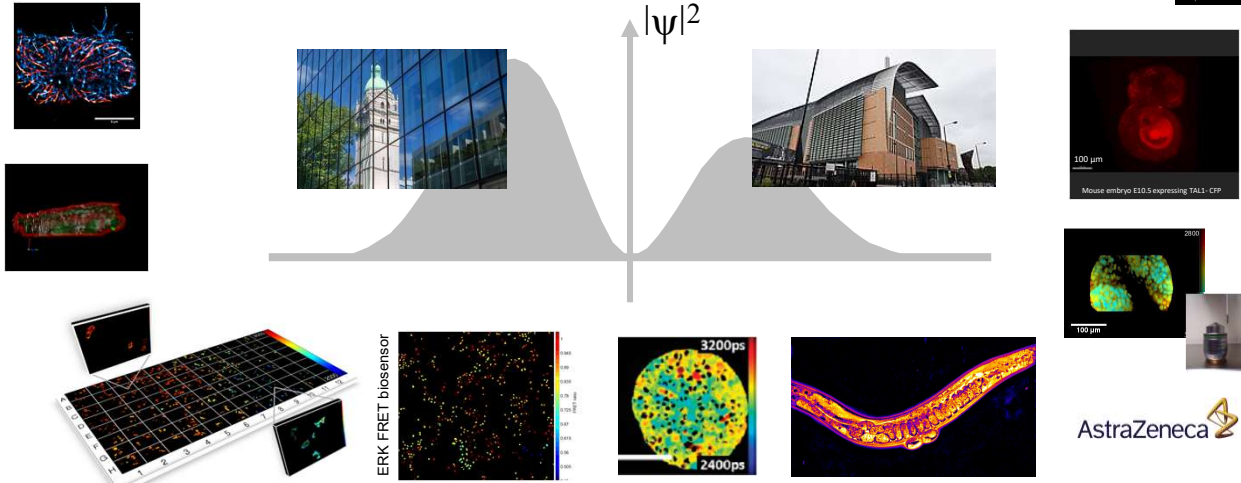Jeremy Graham

CAIRN RESEARCH

OPM

Hugh Sparks, *Nathan Curry, Nils Gustafson,* Liuba Dvinskikh,
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Thank you

$|\psi|^2$

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open HCA: automated multiwell plate microscopy

Automated FLIM/FRET to assay protein interactions

homoFRET
Z' > 0.5

heteroFRET
Z' > 0.5

■ Homo-FRET
■ Hetero-FRET
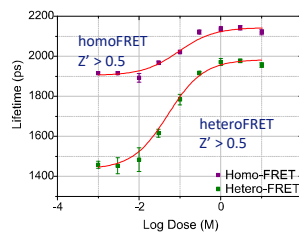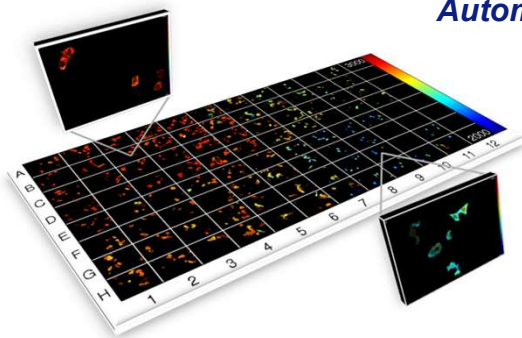
FLIM/FRET screen

Wenjun Guo
Photonics/
Thorpe lab

Microtubule

GFP   Rdh54p-
mCherryFP
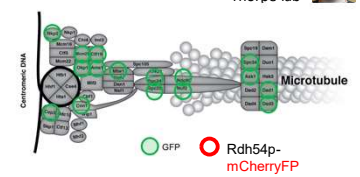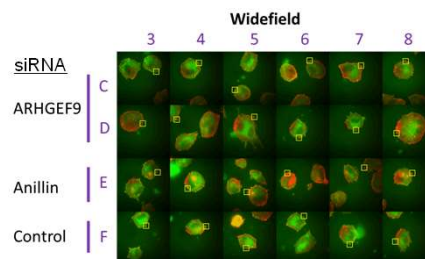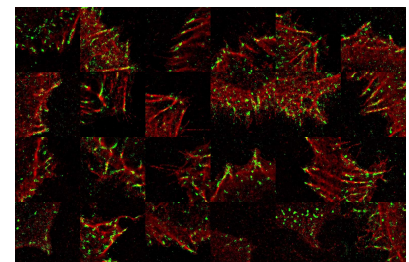
Automated multiwell plate STORM for super-resolved HCA

Focal adhesions and F-actin in WM2664 melanoma cells
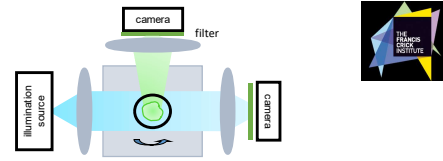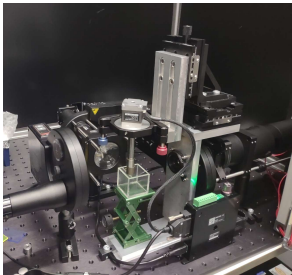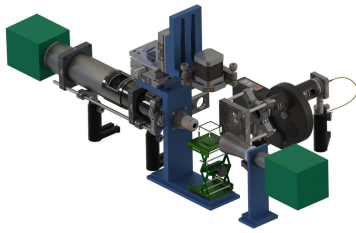
Lightley et al, J Microscopy 2021

Widefield

siRNA

ARHGEF9

Anillin

Control

Paxillin AF488 & F-actin Phalloidin ifluor647N

openScopes - modular OPT

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Relatively high throughput volumetric imaging

Role of Smad4 in zebrafish embryo morphogenesis
- essential for BMP signalling (inhibited by DMH1)

3D imaging & analysis pipeline $\Rightarrow$ BMP "morphospace"

Guglielmi et al, Nature Comm (2021)