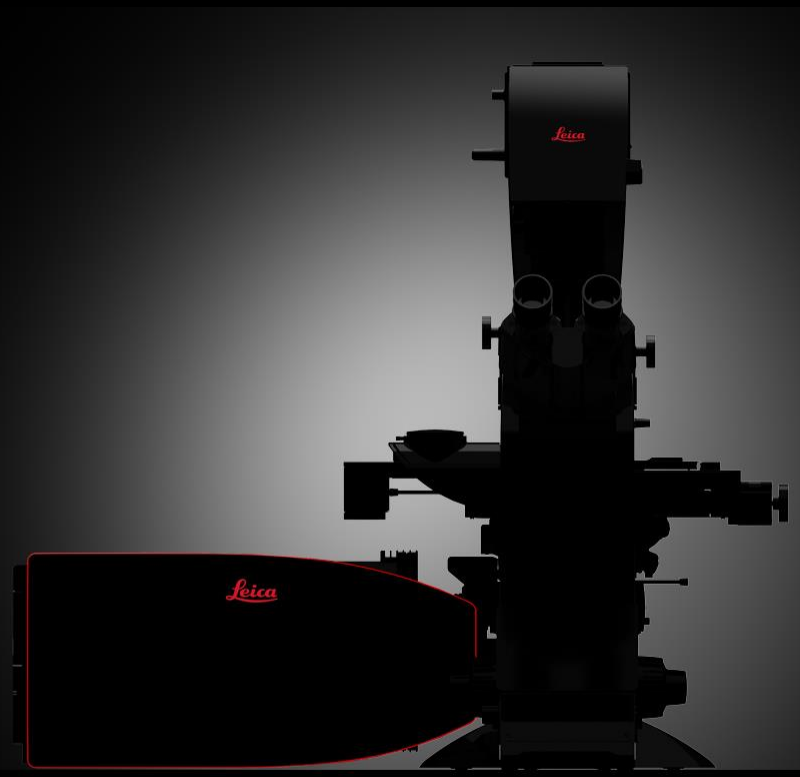
Leica
MICROSYSTEMS

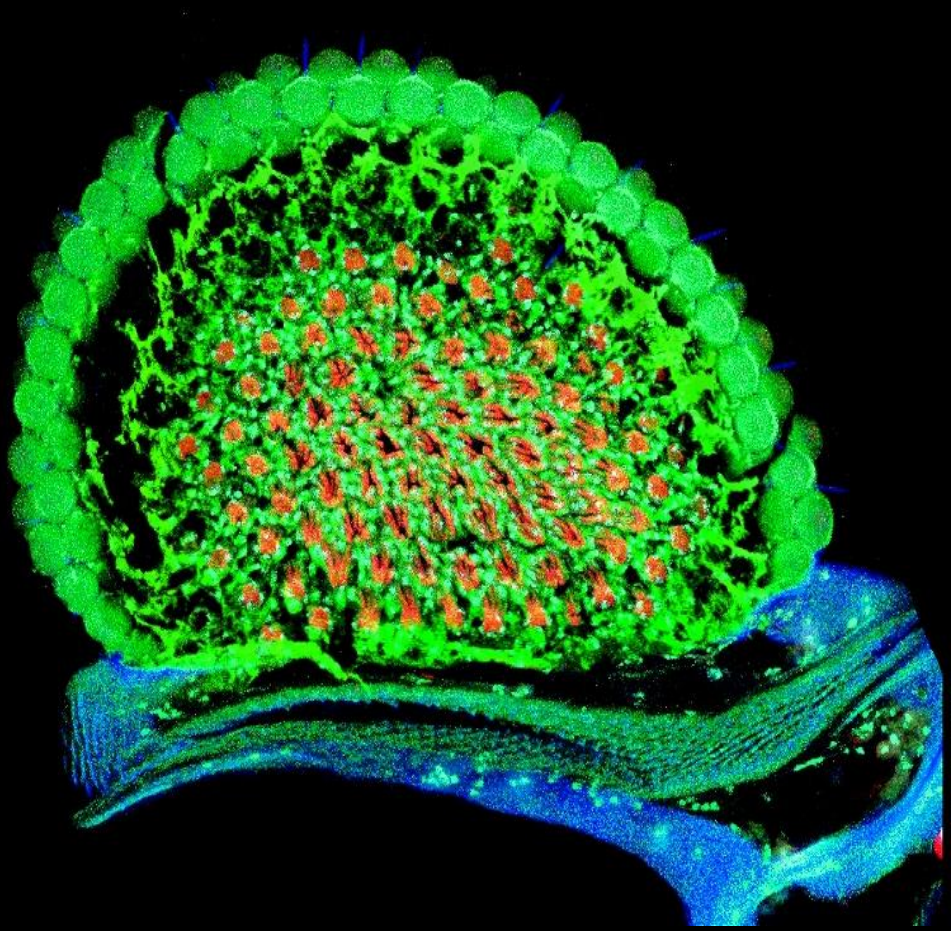


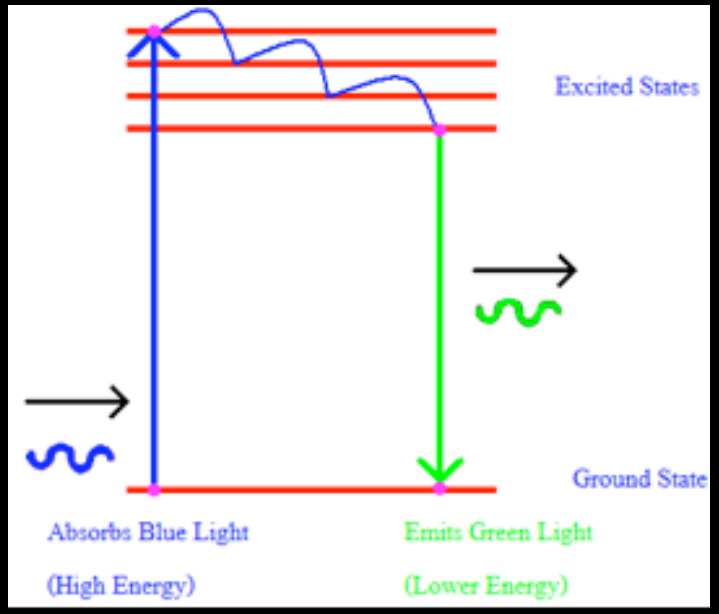
STELLARIS

Leica New Confocal Platform

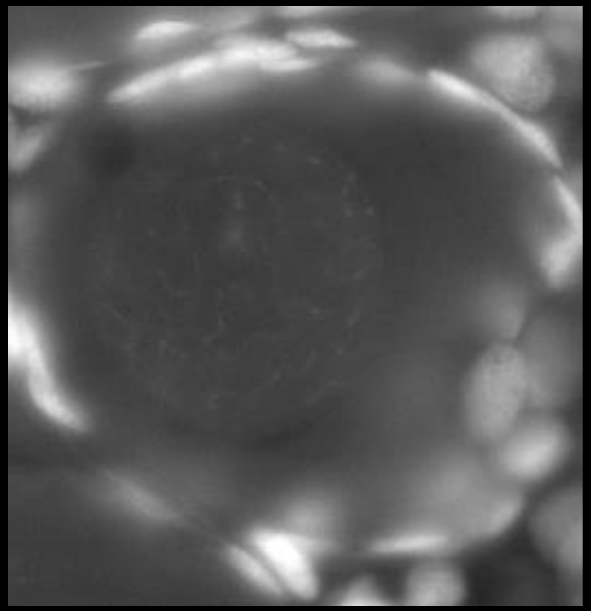
劉思嫻
美嘉儀器股份有限公司
www.major.com.tw

Confocal Image -- Fluorescence





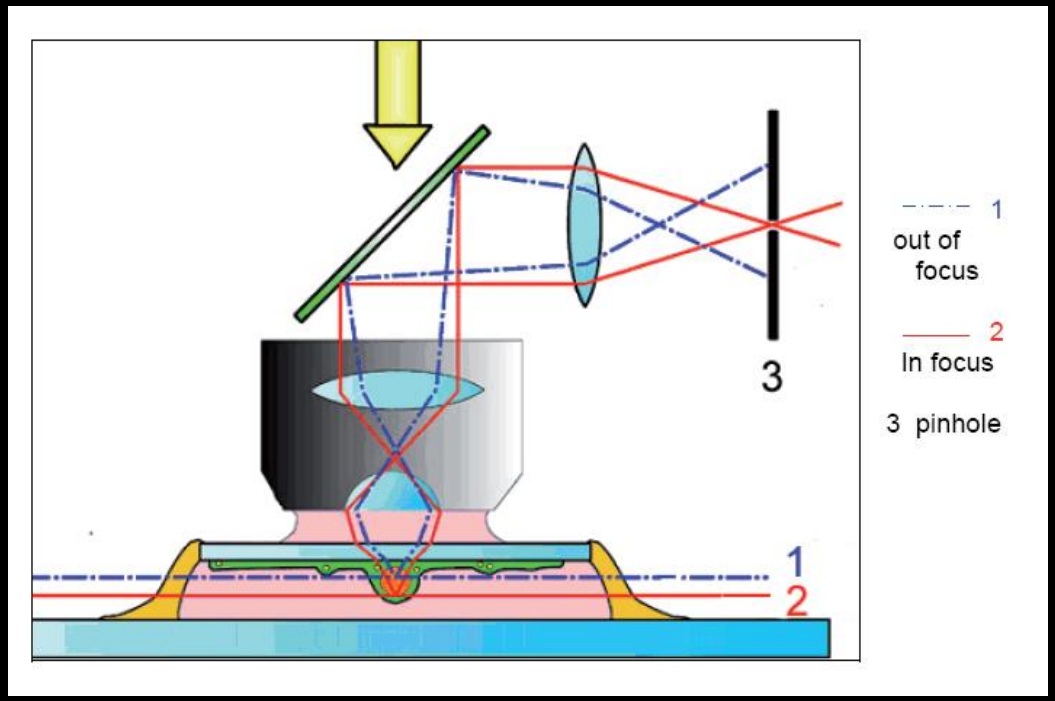
Fluorescence Microscope → Confocal System



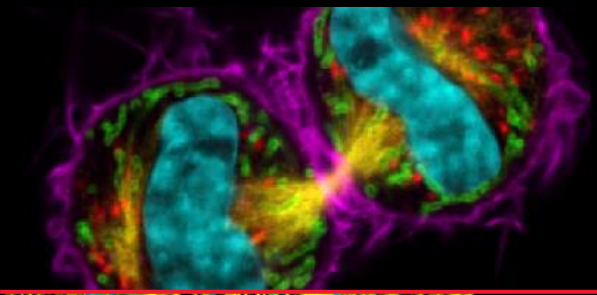
Confocal Microscopes can optically remove all information that is from outside the depth of focus.

The consequence is a sharp optical section.

Conventional Microscope → Confocal ?

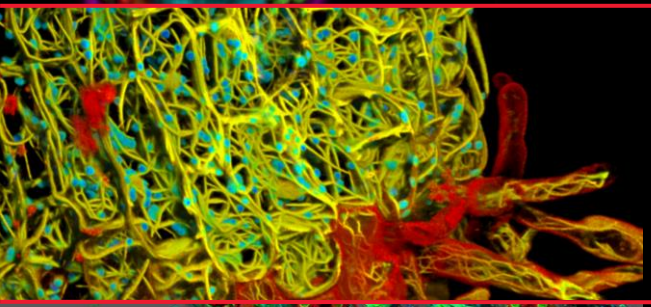


STELLARIS: Three Key Innovative Attributes



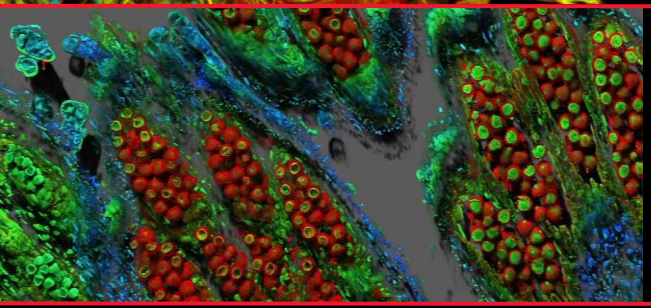
POWER

SEE MORE



POTENTIAL

DISCOVER MORE



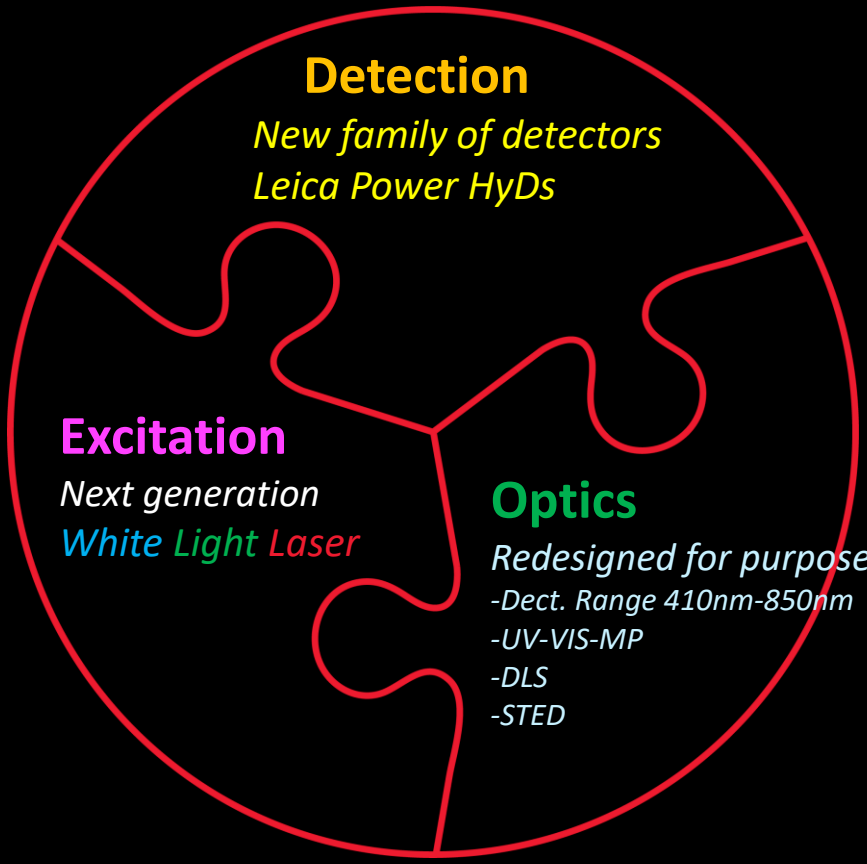
PRODUCTIVITY

DO MORE

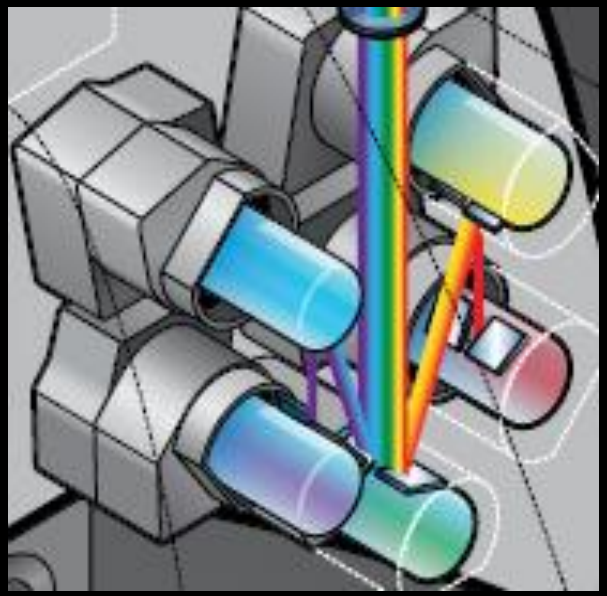
Leica

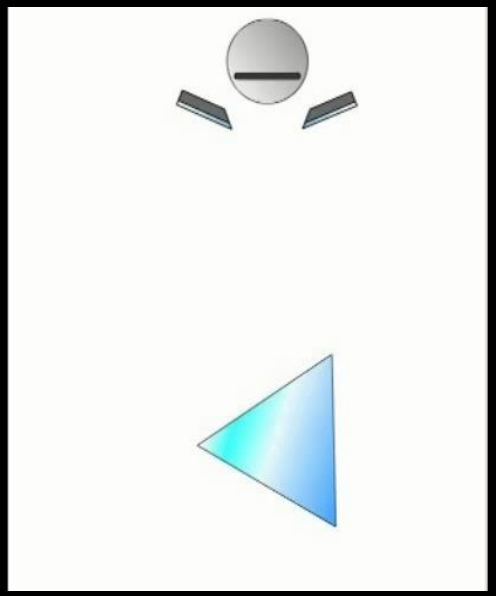
What is Power of Confocal Microscope?

Three key points:
More Sensitivities, More Flexibilities, More Modularizations



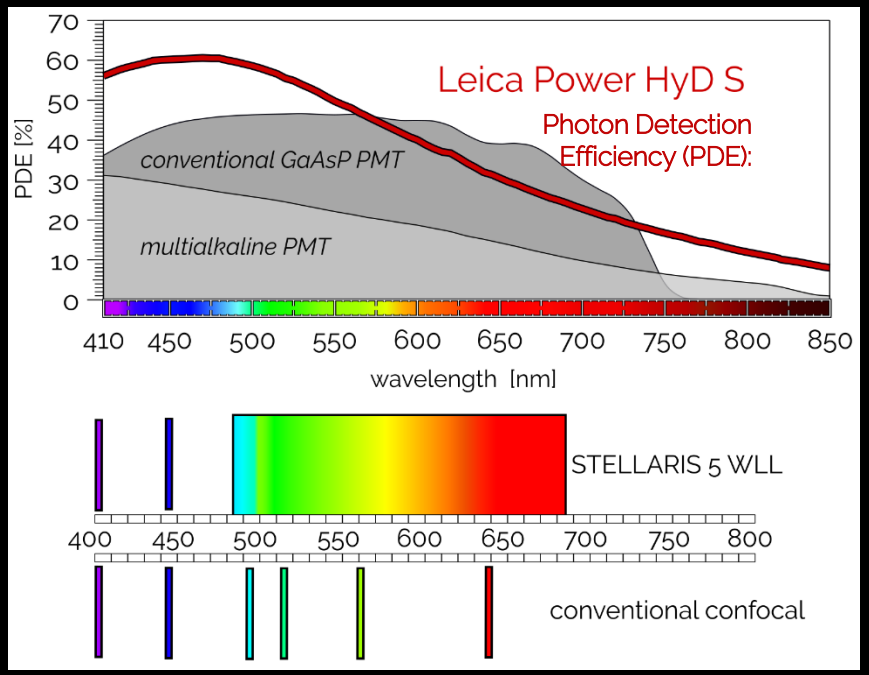
Spectral Imaging Detector -- Fit For Purpose

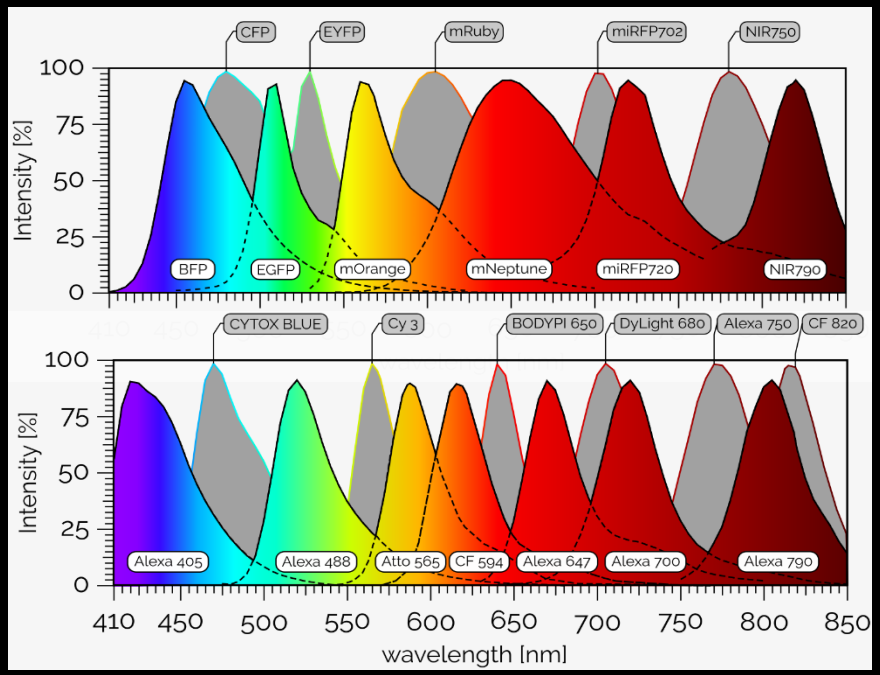




Enhanced Spectral Freedom: STELLARIS

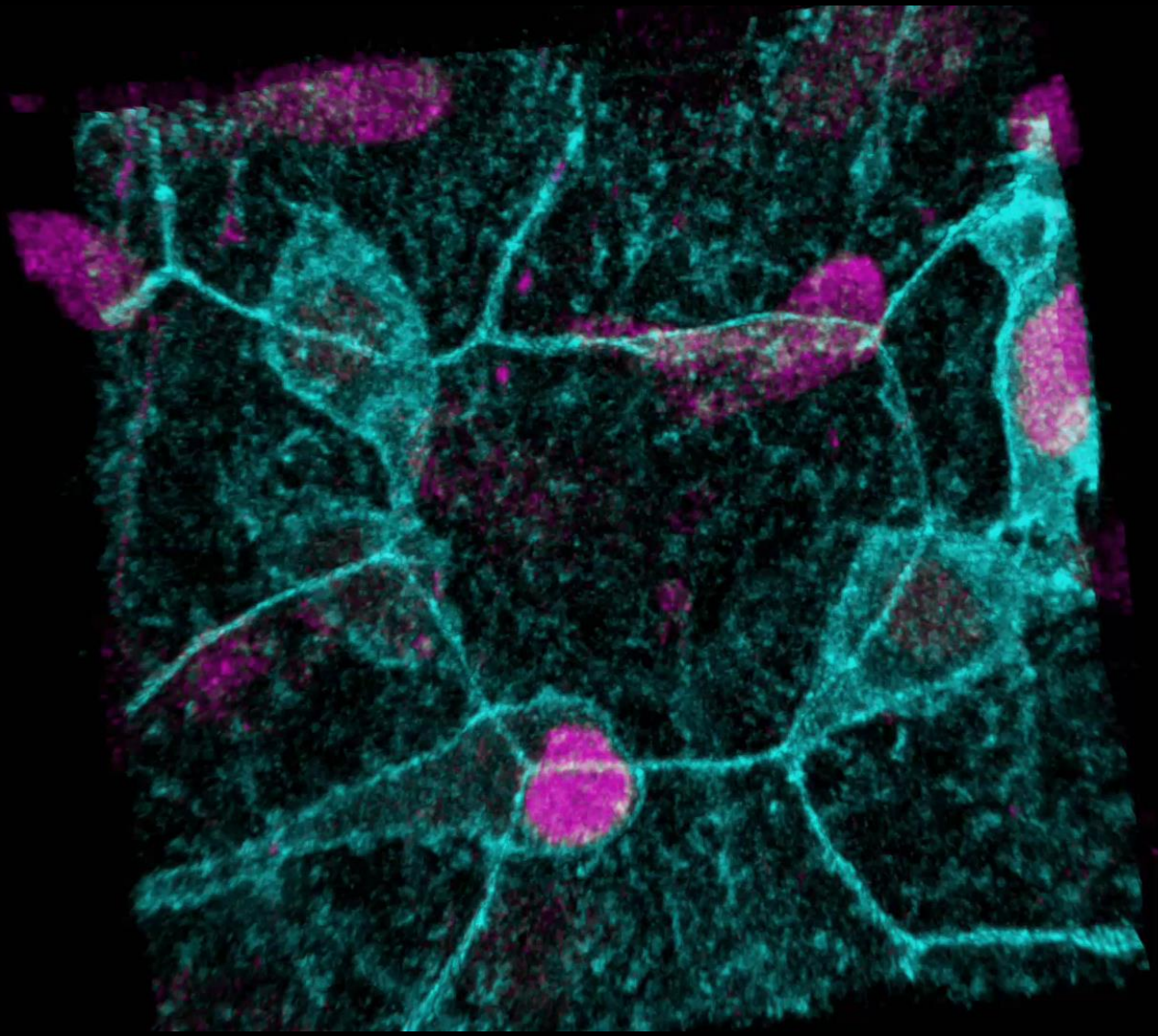
The Power HyD S Is The New All-rounder Detector For Confocal Applications





- No more PMT or GaAsP detectors
- Detection range: 410nm-------850nm
  (normal confocal: 400nm-750nm)

Gentle Live-Cell Imaging



- Perform imaging for longer periods, since both excitation as well as detection are optimally tuned
- Preserve sample integrity with efficient signal acquisition, at the lowest needed power

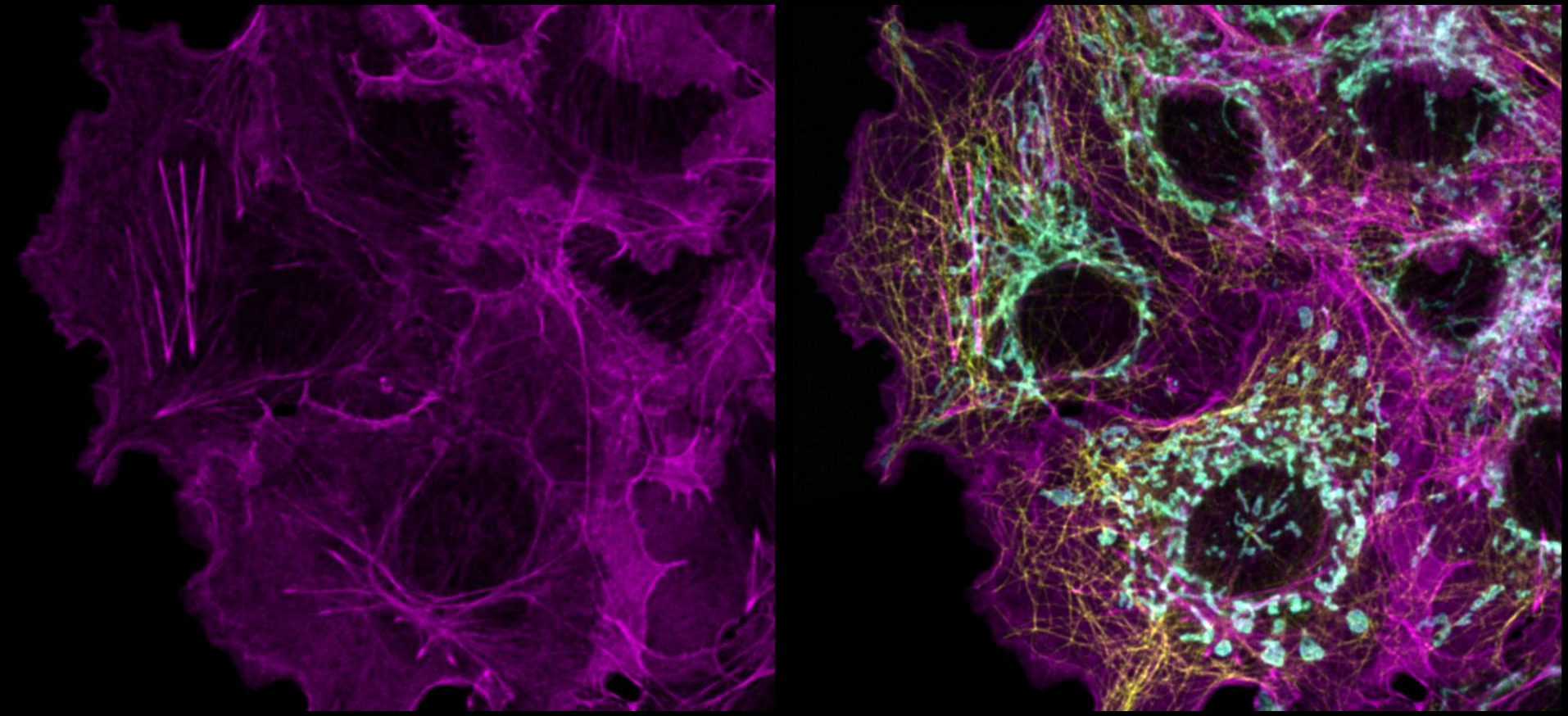
Zebrafish posterior lateral line primordium migration. Cyan: Membranes, GFP, Magenta: Nuclei, tdTomato
Sample Courtesy: Jonas Hartmann, Gilmour Group, EMBL Heidelberg.

Leica

STELLARIS Delivers Expanded Multicolor Flexibility

Traditional Confocal

STELLARIS

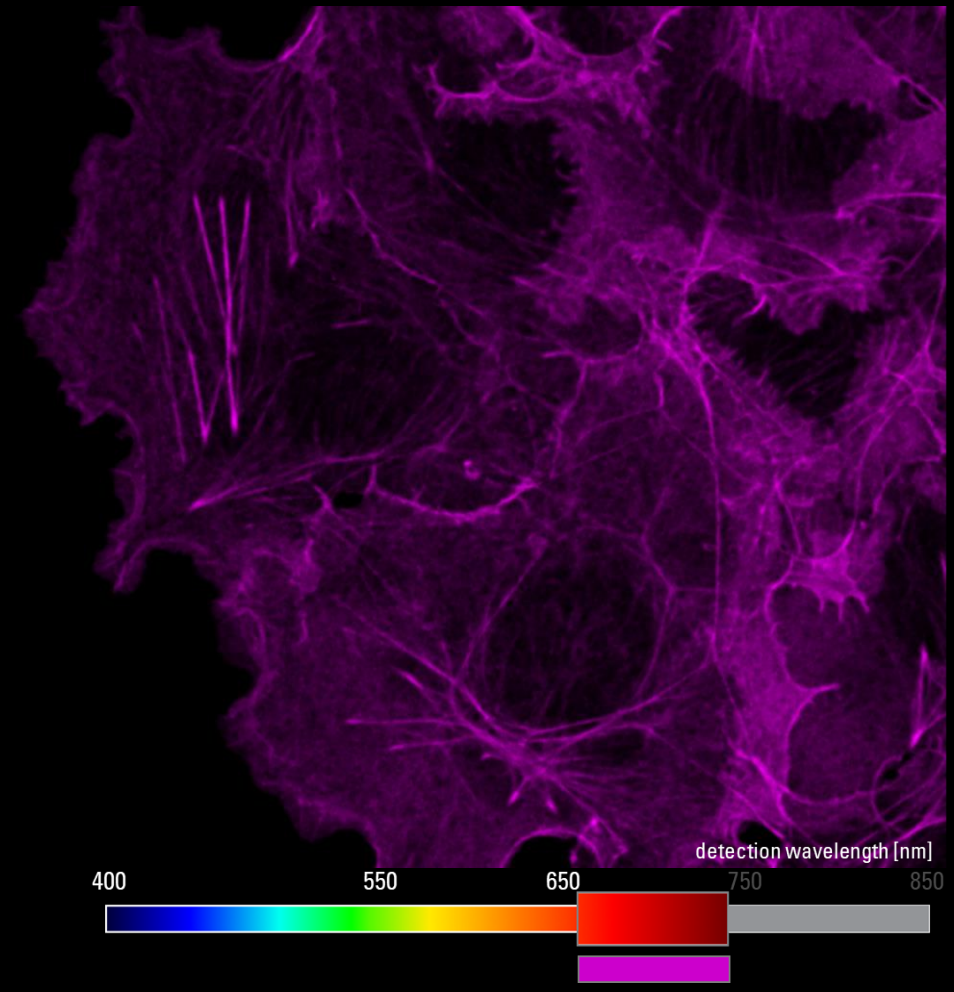


COS7 cells. Actin (magenta, SiR-Actin 657-740 nm), Mitochondria (cyan, AF750 760-790 nm), Microtubules (yellow, AF790 810-850 nm)
Sample Courtesy: Jana Döhner, Urs Ziegler, University of Zurich

Leica

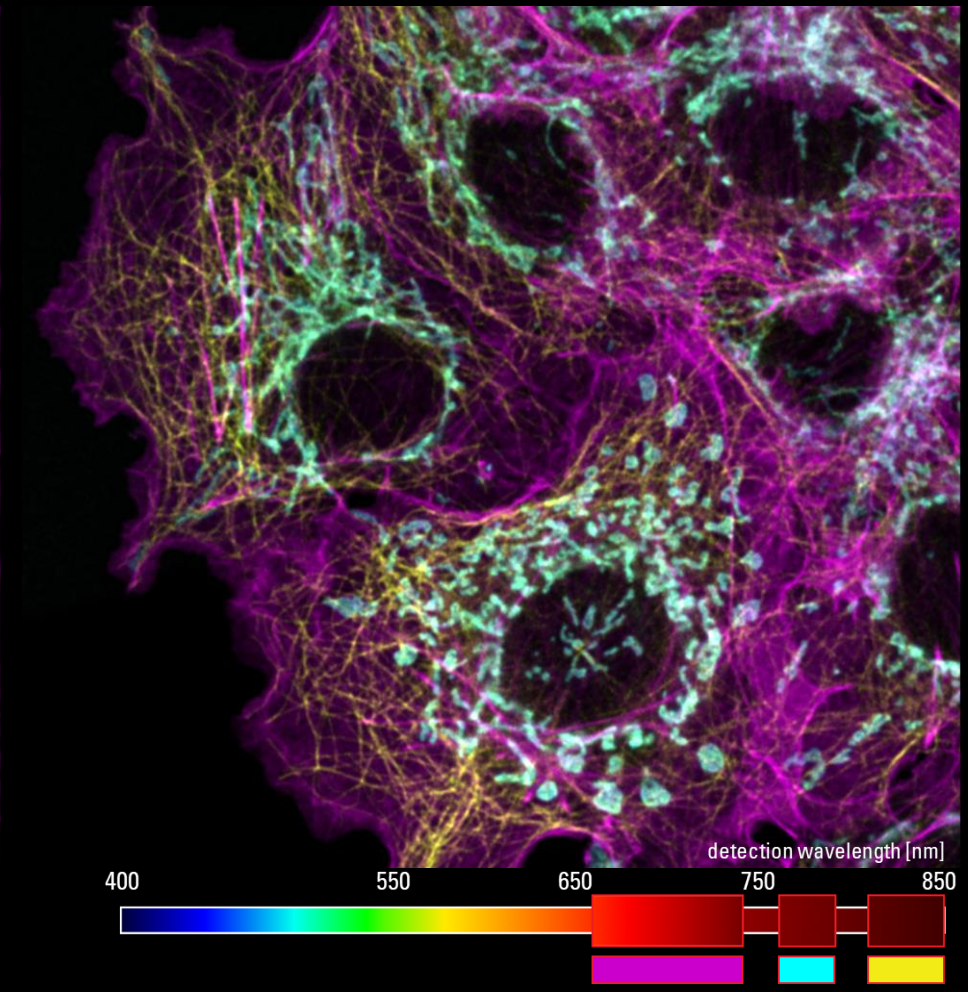
STELLARIS Delivers Expanded Multicolor Flexibility

Traditional Confocal



1 channel

STELLARIS



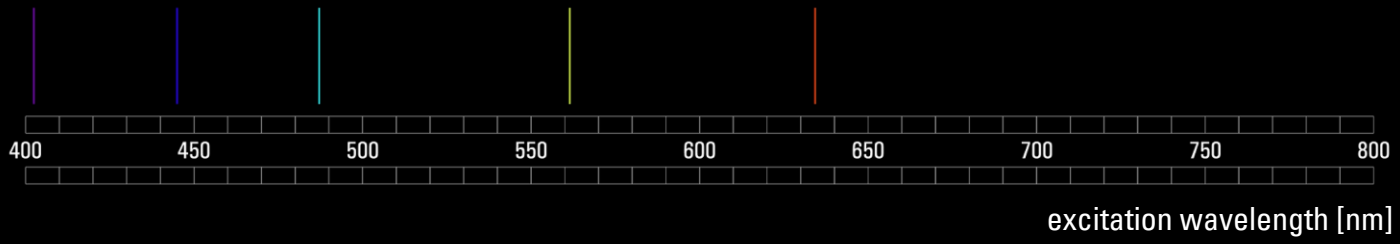
3 channels extended to NIR

COS7 cells. Actin (magenta, SiR-Actin 657-740 nm), Mitochondria (cyan, AF750 760-790 nm), Microtubules (yellow, AF790 810-850 nm)
Sample Courtesy: Jana Döhner, Urs Ziegler, University of Zurich

Leica

The Second Key Innovation: The next-generation White Light Lasers

Traditional Confocal

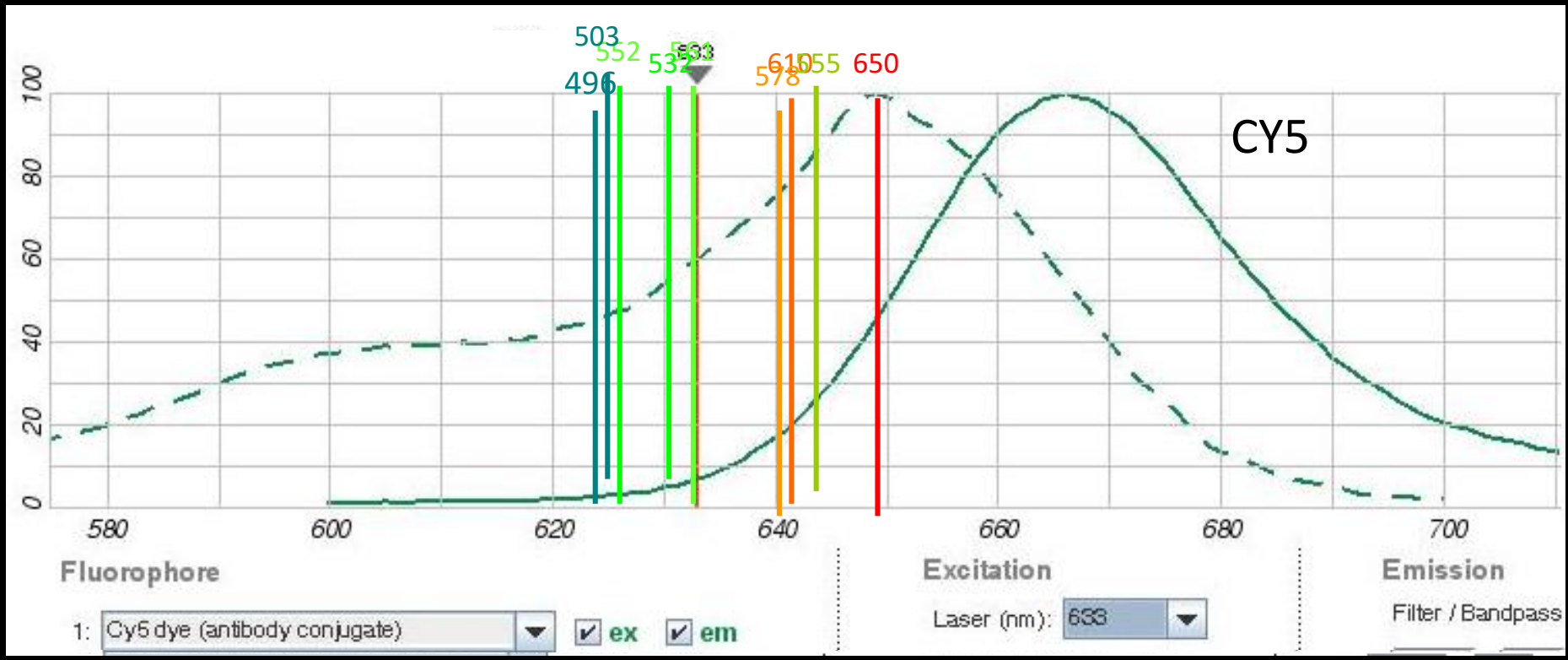


STELLARIS 8

- Experience complete spectral freedom with excitation perfectly matched to the fluorochrome
- Less complexity, more flexibility: a single laser to do the work of many. Up to 8 single excitation lines from 440 nm to 790 nm can be used simultaneously
- New optics design: detection range from 410nm to 850nm

Excitation wavelength

458nm, 476nm, 488nm, 496nm, 514nm, 543nm, 561nm, *594nm*, 633nm



LEICA STELLARIS Confocal Microscope

Excitation wavelength

440nm - 790nm, 351 ex. Lines, no limitation

The Red Extended Benefits Of Our Next Generation WLLs

- Excite each fluorophore optimally at its excitation peak
- Enhance multiplexing capabilities by adding up to 3 more fluorophores in the NIR range

Some >685 nm excitable dyes:

ATTO 740

CF680

CellBrite NIR680

CF750

BioTracker NIR750

Alexa 700

CellBrite NIR700

ATTO 700

CellBrite NIR750

Alexa 750

CF700

MitoView720

CellBrite NIR770

Alexa 680

ATTO 680

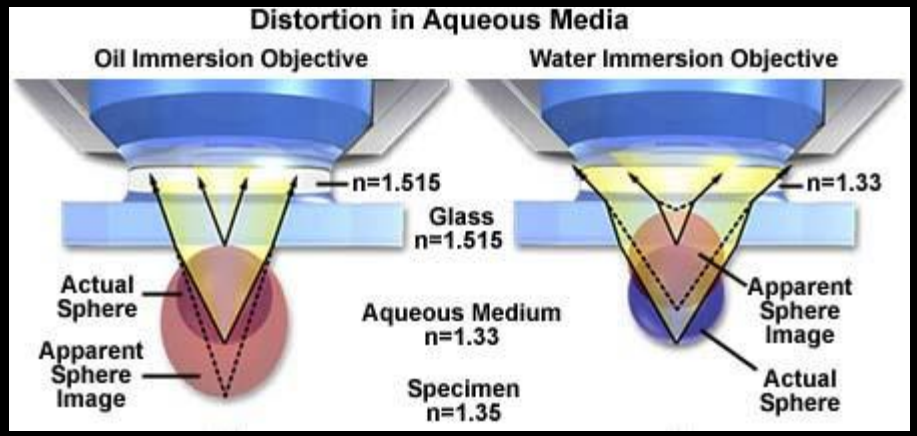
ATTO 725

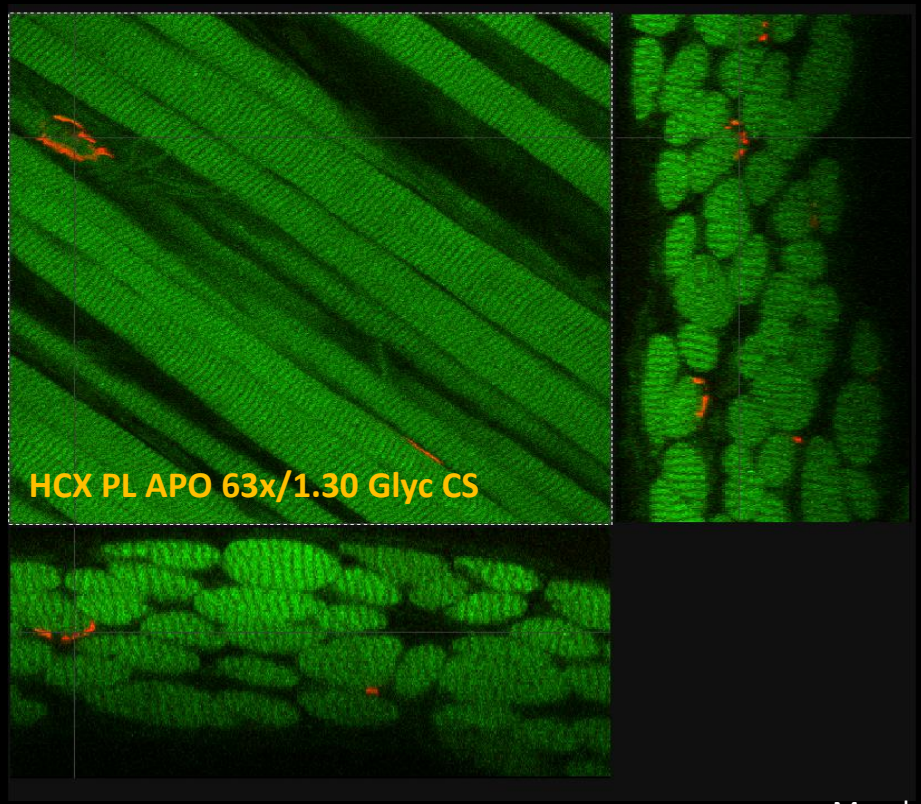
New Objectives

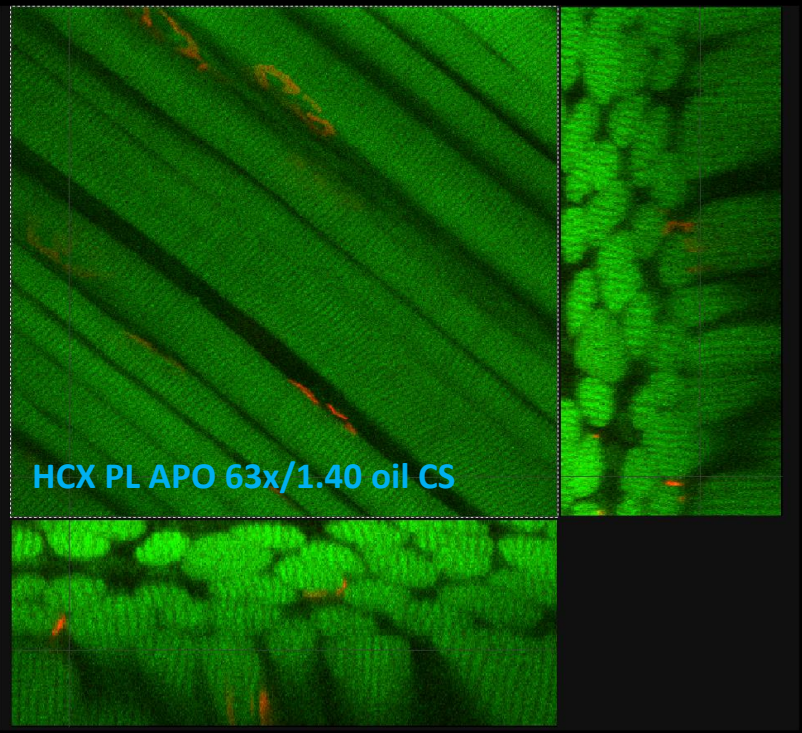
20X/0.75 IMM
Water, Glyc,Oil

40X/1.25 Glyc

- Reflective Index Match -



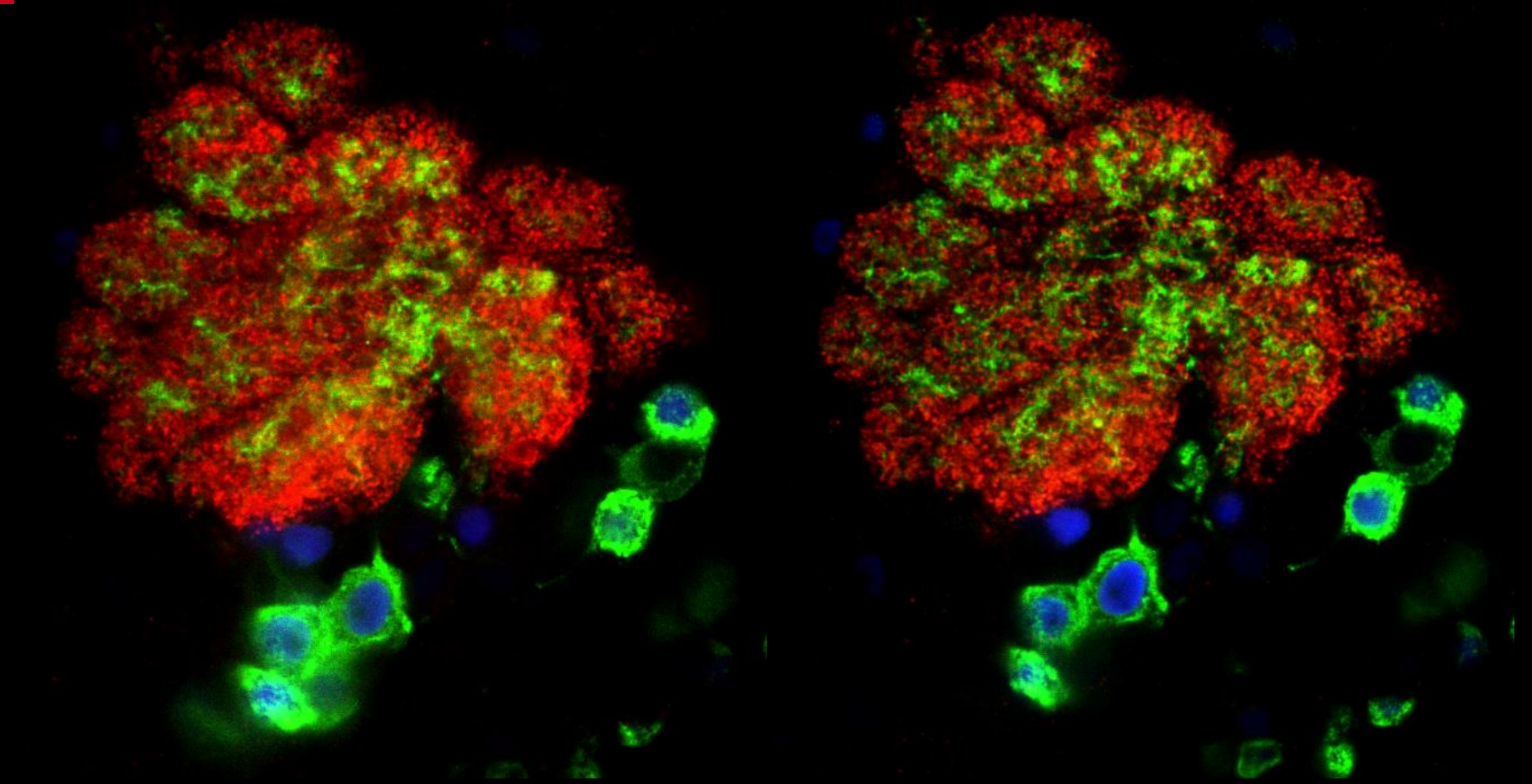




Moscle tissue embedded in glycerol/water (80%/20%), recorded stack: 100um

HCX PL APO 63x/1.40 oil CS

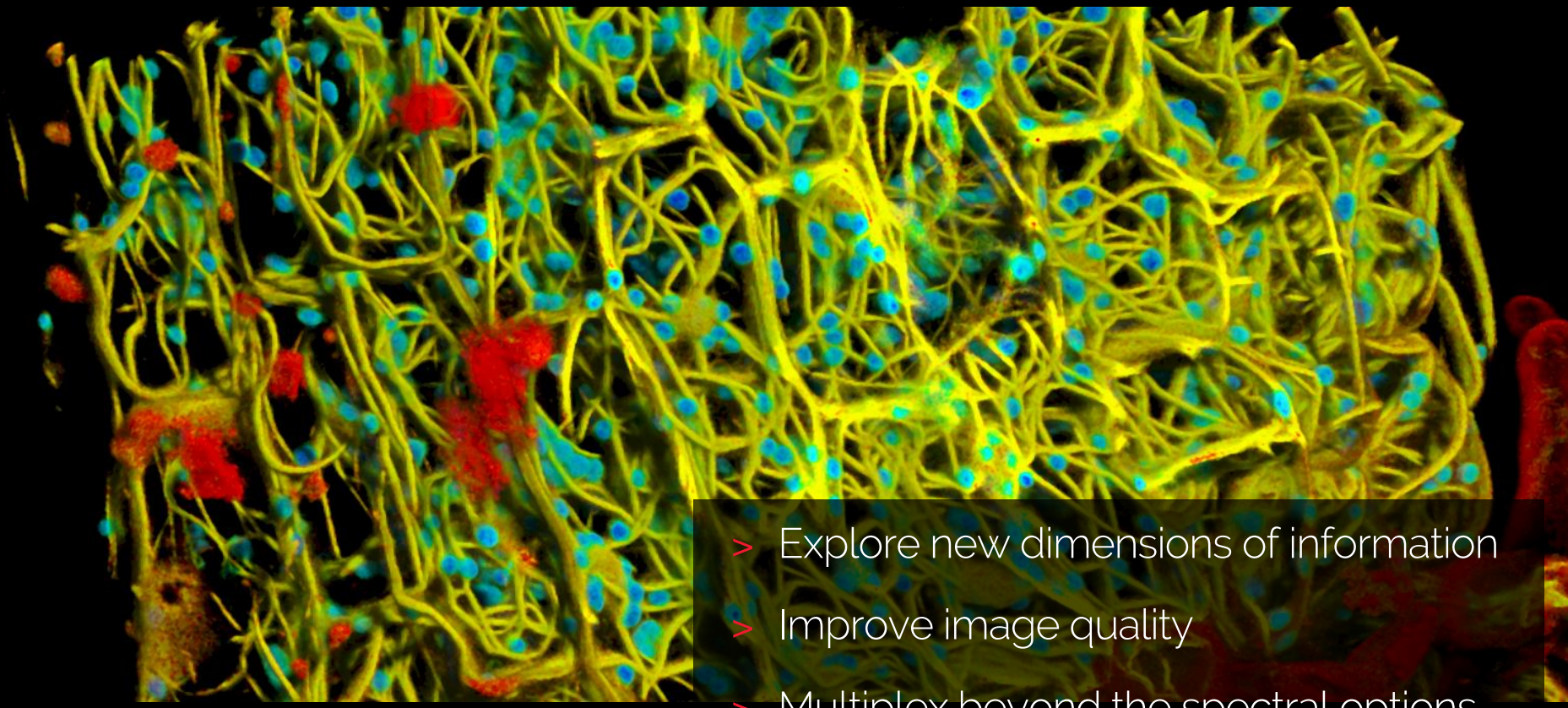
HCX PL APO 63x/1.30 Glyc CS



* Sample prepared by Dr. Ya-Hui Cuou

POTENTIAL

DISCOVER MORE



- Explore new dimensions of information
- Improve image quality
- Multiplex beyond the spectral options

Leica

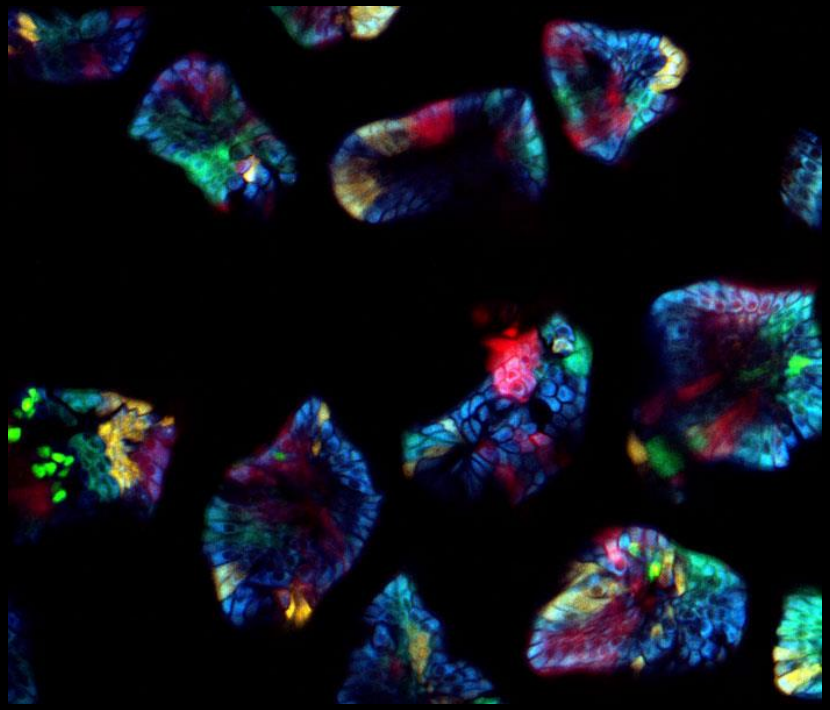
The Technology Behind STELLARIS Potential

TauSense™

> Set of tools based on fluorescence lifetime

Leica STELLARIS首度將時脈頻譜技術 TauSense technology 整合於共軛焦顯微平台上

Fluorescence imaging focus on spectral contrast….



Confetti Mouse Small Intestine. CFP, GFP, YFP and RFP. Acquired with SP8 DIVE
Sample courtesy of Jacco van Rheenen, University of Utrecht, the Netherlands

… fluorescence contains much more information

Leica

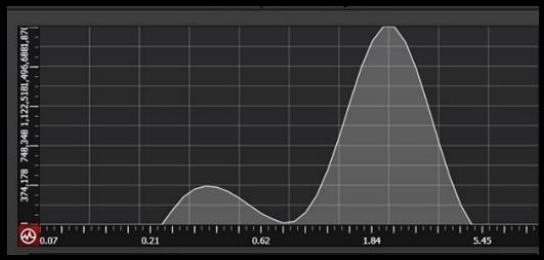
時脈頻譜(TauSense)技術的應用概念來自螢光生命週期影像技術

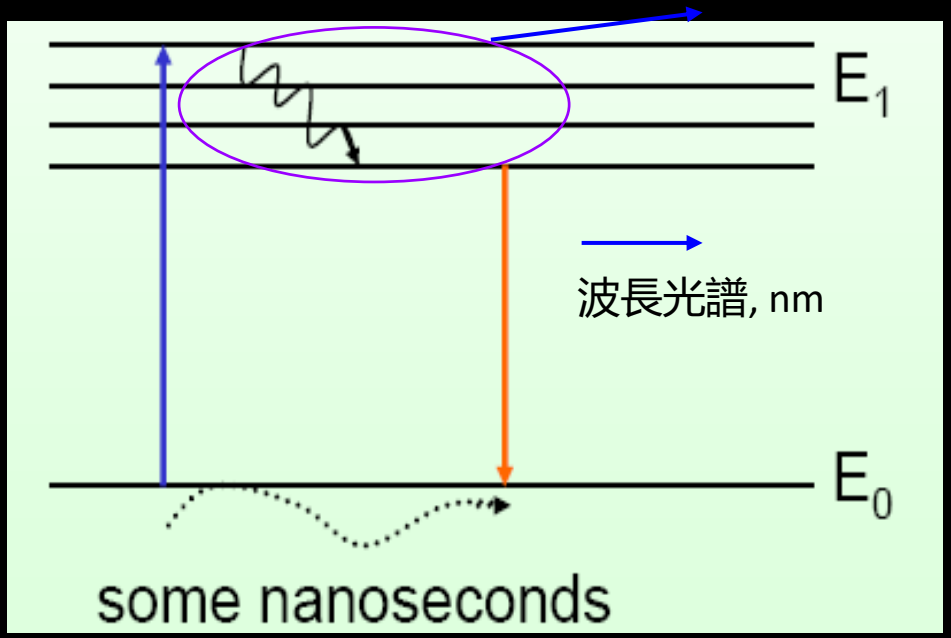
Fluorescence Lifetime Imaging Microscopy (FLIM)

Fluorescence lifetime

Average time that molecules stay in their excited state

生命週期（時脈頻譜, ns）





Fluorophore	Ex. Max.	Em. Max.
AF 488	494nm	519nm
GFP	498nm	516nm

(X)

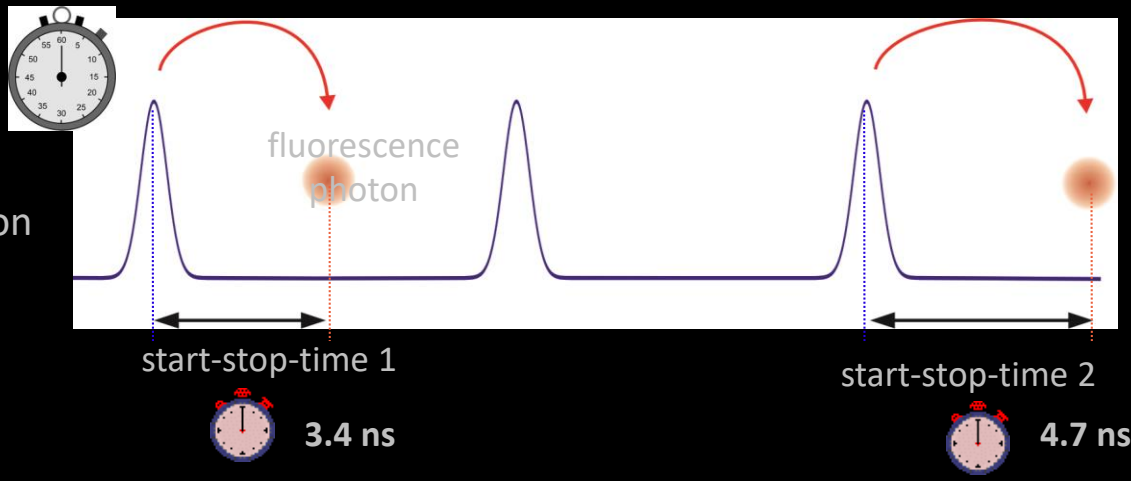
Fluorophore	Ex. Max.	Em. Max.
Fluo3 w. Ca2+	490nm	520nm
Fluo3 w/o Ca2+	490nm	520nm

How to Measure the Time?

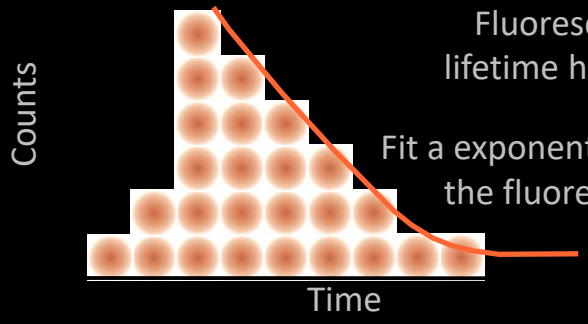
In principle with a stop watch:

1. Start the clock with a laser pulse.
2. Stop the clock with the first photon that arrives at the detector.
3. Reset the clock and wait for next start signal.

It is a statistical process!



- Repeat this time measurement very often and count "how many photons have arrived after what time"
- Sort the photons within a histogram into time bins according to their arrival times



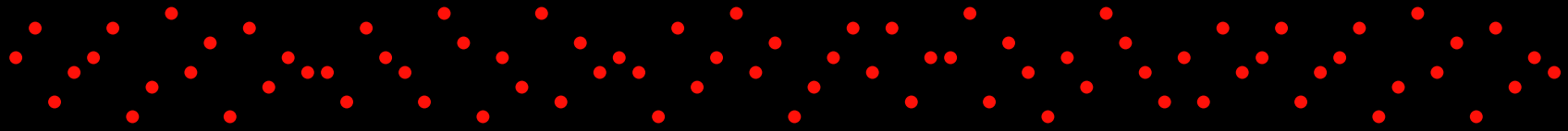
Fluorescence lifetime histogram:

Fit a exponential decay to get the fluorescence lifetime

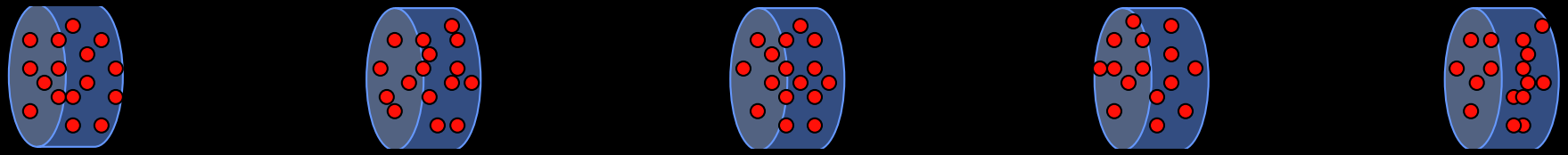
What we need for TauSense or FLIM?

- A pulsed laser

Continuous laser



Pulsed laser



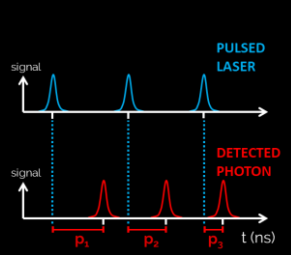
Leica STELLARIS white light laser is pulsed laser 440nm-790nm

- Photon counting detector

Normal PMT or GaAsP is not suitable for photon counting
Leica STELLARIS power HyD detectors are photon counting detectors

The Technology Behind TauSense

- Fluorescence Intensity ($N_{photons}$)
- Photon Arrival Time (ns)

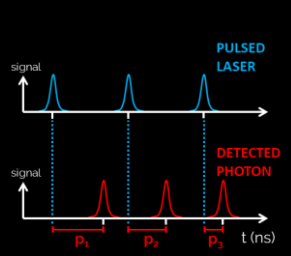


1) FPGA
2) Pixel-by-pixel
3) On the fly

- Fluorescence Intensity ($N_{photons}$)
- Average Photon Arrival Times (AAT, ns)

The Technology Behind TauSense

- Fluorescence Intensity ($N_{photons}$)
- Photon Arrival Time (ns)



1) FPGA
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- Fluorescence Intensity ($N_{photons}$)
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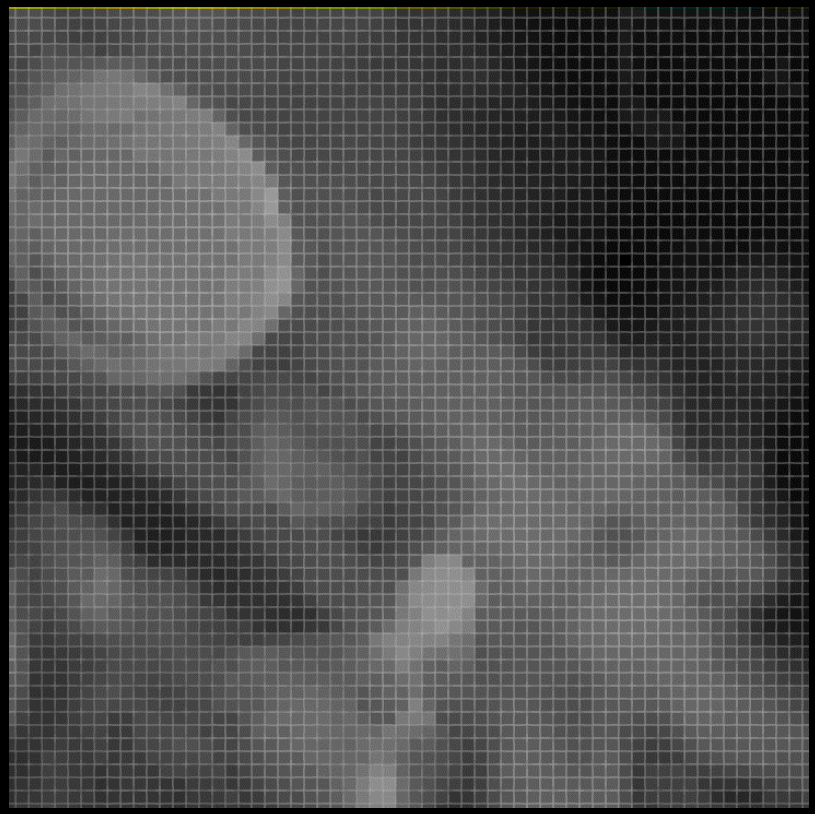
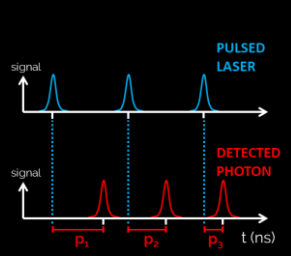


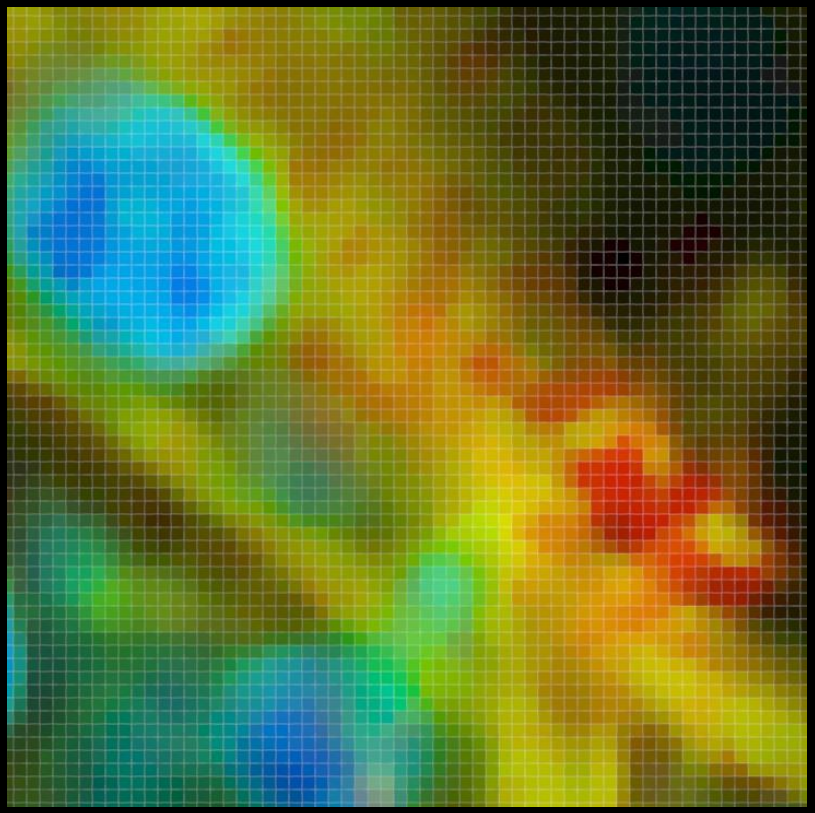
The Technology Behind TauSense

- Fluorescence Intensity ($N_{photons}$)
- Photon Arrival Time (ns)



1) FPGA
2) Pixel-by-pixel
3) On the fly

- Fluorescence Intensity ($N_{photons}$)
- Average Photon Arrival Times (AAT, ns)



Alexa Dyes

Alexa Fluor Dye *	Ex (nm)	Em (nm)	τ (ns) ‡
Alexa Fluor 488	496	519	4.1 §
Alexa Fluor 532	532	553	2.5
Alexa Fluor 546	556	573	4.1
Alexa Fluor 555	555	565	0.3
Alexa Fluor 568	578	603	3.6 §
Alexa Fluor 594	590	617	3.9 §
Alexa Fluor 647	650	665	1.0
Alexa Fluor 660	663	690	1.2 **
Alexa Fluor 680	679	702	1.2
Alexa Fluor 700	702	723	1.0
Alexa Fluor 750	749	775	0.7

Measurements were made on free succinimidyl ester derivatives in aqueous solutions. † For Alexa Fluor 488, Alexa Fluor 532, Alexa Fluor 546, Alexa Fluor 555, Alexa Fluor 568, Alexa Fluor 594 and Alexa Fluor 647 dyes, QY measurements were made in PBS (50 mM potassium phosphate, 150 mM NaCl, pH 7.2) at 22°C relative to fluorescein in 0.01 M NaOH (QY = 0.92). For Alexa Fluor 660, Alexa Fluor 680, Alexa Fluor 700 and Alexa Fluor 750 dyes, QY measurements were made in PBS (50 mM potassium phosphate, 150 mM NaCl, pH 7.2) at 22°C relative to Alexa Fluor 647 succinimidyl ester in PBS (QY = 0.33). ‡ Except for the footnoted values, lifetime measurements were made in water at 22°C, data provided by ISS Inc. (Champaign, IL). § Lifetime measurements were provided by the SPEX Fluorescence Group, Horiba Jobin Yvon Inc. ** Lifetime measurement was made in pH 7.5 buffer at 20°C by Pierre-Alain Muller, Max Planck Institute for Biophysical Chemistry, Göttingen.

Atto Dyes

Dyes	Ex (nm)	Em (nm)	τ (ns)
Atto 465	453	508	2.2
Atto 488	501	523	3.2
Atto 495	495	527	2.4
Atto 514	511	533	3.0
Atto 520	516	538	3.8
Atto 532	532	553	3.8
Atto Rho6G	535	560	4.1
Atto 550	554	576	3.2
Atto 565	563	592	3.4
Atto Rho3B	565	592	1.5
Atto Rho11	571	595	4.0
Atto Rho12	576	601	4.0
Atto Thio12	579	609	2.0
Atto Rho101	586	610	4.2

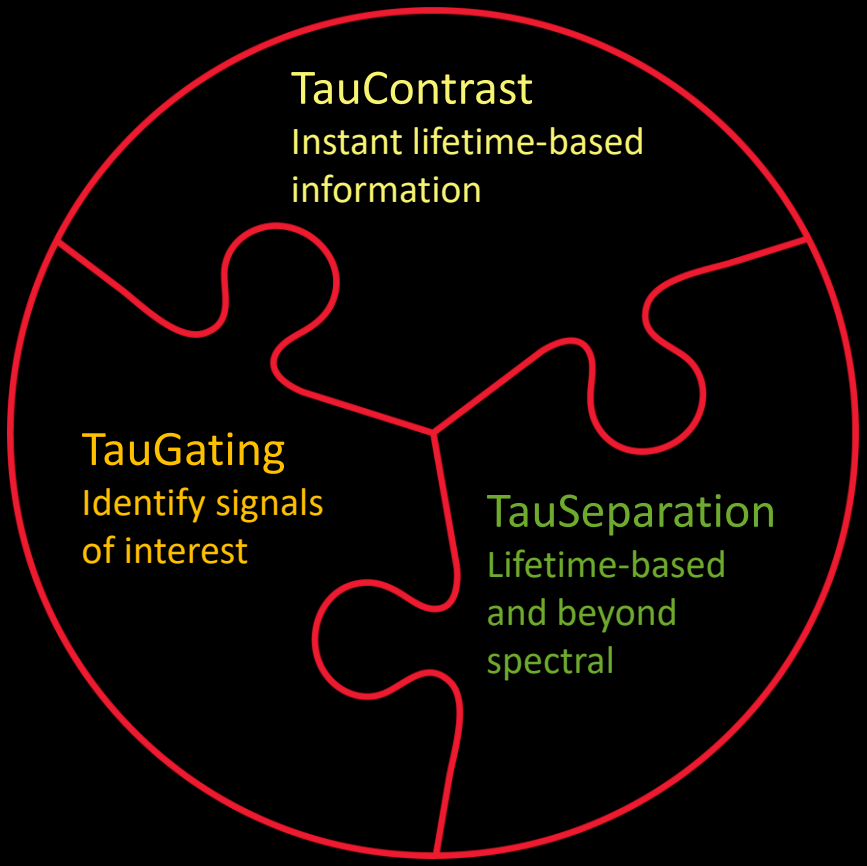
Dyes	Ex(nm)	Em(nm)	τ (ns)
Atto 590	594	624	3.7
Atto 594	601	627	3.5
Atto Rho13	600	625	3.9
Atto 610	615	634	3.3
Atto 620	619	643	2.9
Atto Rho14	625	646	3.7
Atto 633	629	657	3.2
Atto 647	645	669	2.3
Atto 647N	644	669	3.4
Atto 655	663	684	1.9
Atto Oxa12	663	684	1.8
Atto 665	663	684	2.9
Atto 680	680	700	1.8
Atto 700	700	719	1.5
Atto 725	729	752	0.5
Atto 740	740	764	0.6

Dye	Ex (nm)	Em (nm)	τ (ns)
Cy3	548	562	0.3
Cy3.5	581	507	2.6
Cy5	646	664	1.0
Cy5.5	675	695	1.0
FITC	494	518	4.1
Oregon Green 488	493	520	4.1
Oregon Green 500	402	522	2.18
Rhodamine 6G	525	555	4.08
Rhodamin B	562	583	1.68
Texas Red	589	615	4.2
TOTO-1	514	533	2.2

Fluorescent Protein

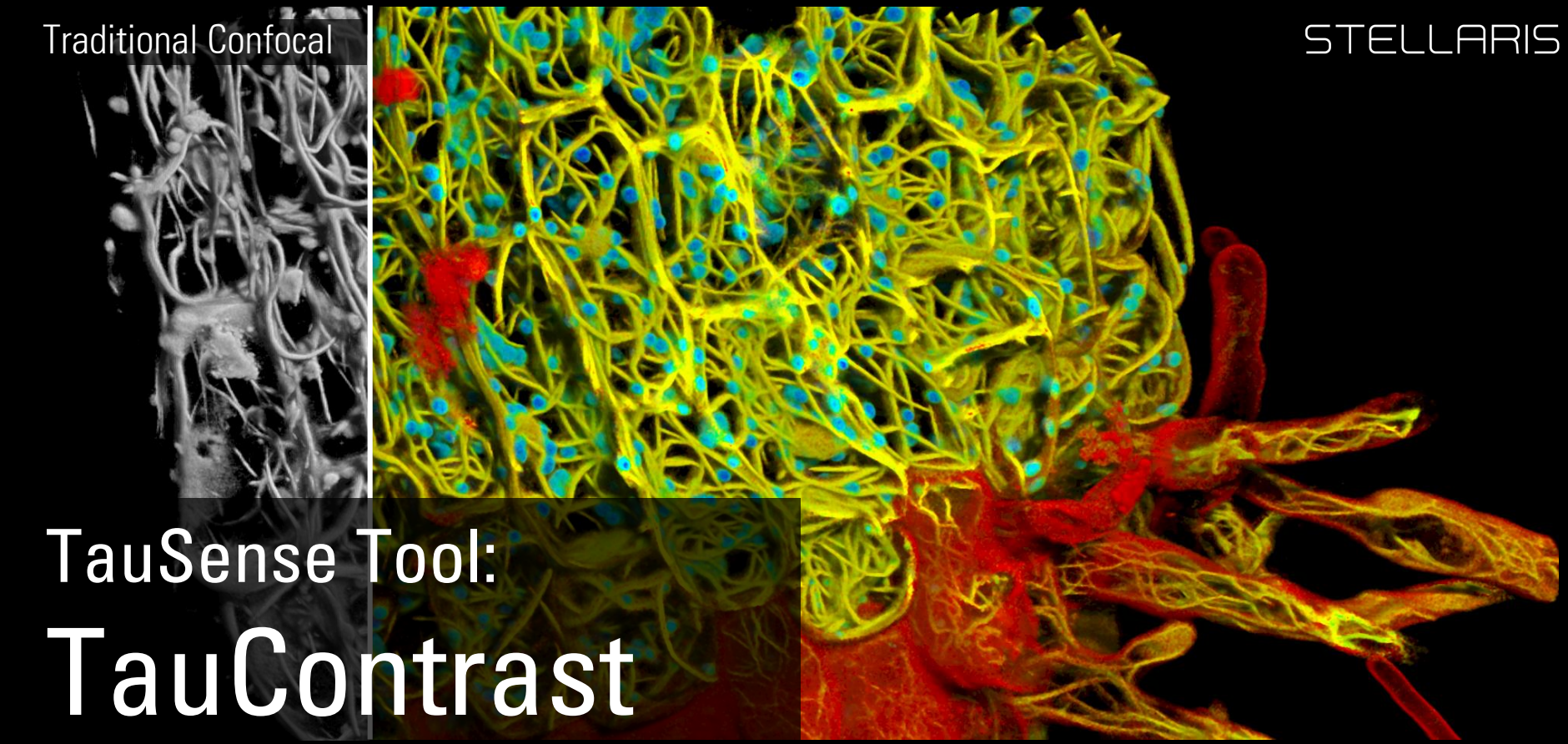
Fluorescent Protein	Ex (nm)	Em (nm)	τ (ns)
ECFP	434	477	3.0
EGFP	488	507	2.6
EYFP	513	527	3.1
mRuby	558	605	2.6
mScarlet	569	594	3.9
mCherry	587	610	1.4
mKate2	588	633	2.5

STELLARIS: Ready to Discover with TauSense（時脈頻譜技術）



ALL Leica STELLARIS series confocal with WLL offer TauSense technology (lifetime-based information) to discover more

Explore A New Dimension Of Information



TauSense Tool:

TauContrast

Leica

Explore New Dimensions Of Information

Root-hypocotyl-junction of Arabidopsis thaliana (Era et al. Plant Cell Physiol., 2009). Chlorophyll, Life-Act Venus, IProp. Sample courtesy: Dr. Krebs, COS, University of Heidelberg.

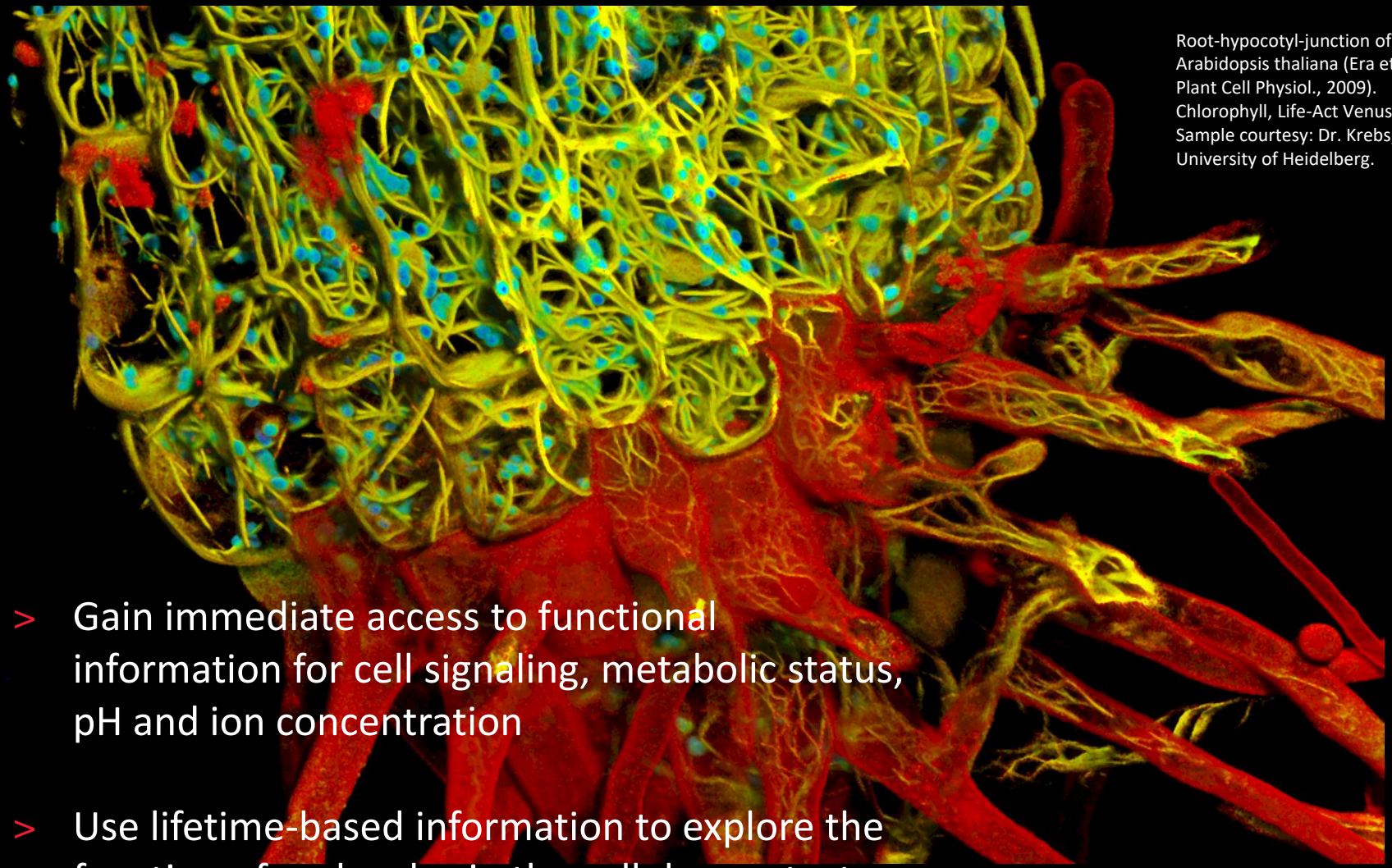
… go beyond today's limits

… see the unseen

… acquire more accurate and reliable data to prove your hypothesis

Leica

Explore New Dimensions Of Information - TauContrast



Root-hypocotyl-junction of Arabidopsis thaliana (Era et al. Plant Cell Physiol., 2009). Chlorophyll, Life-Act Venus, IProp. Sample courtesy: Dr. Krebs, COS, University of Heidelberg.

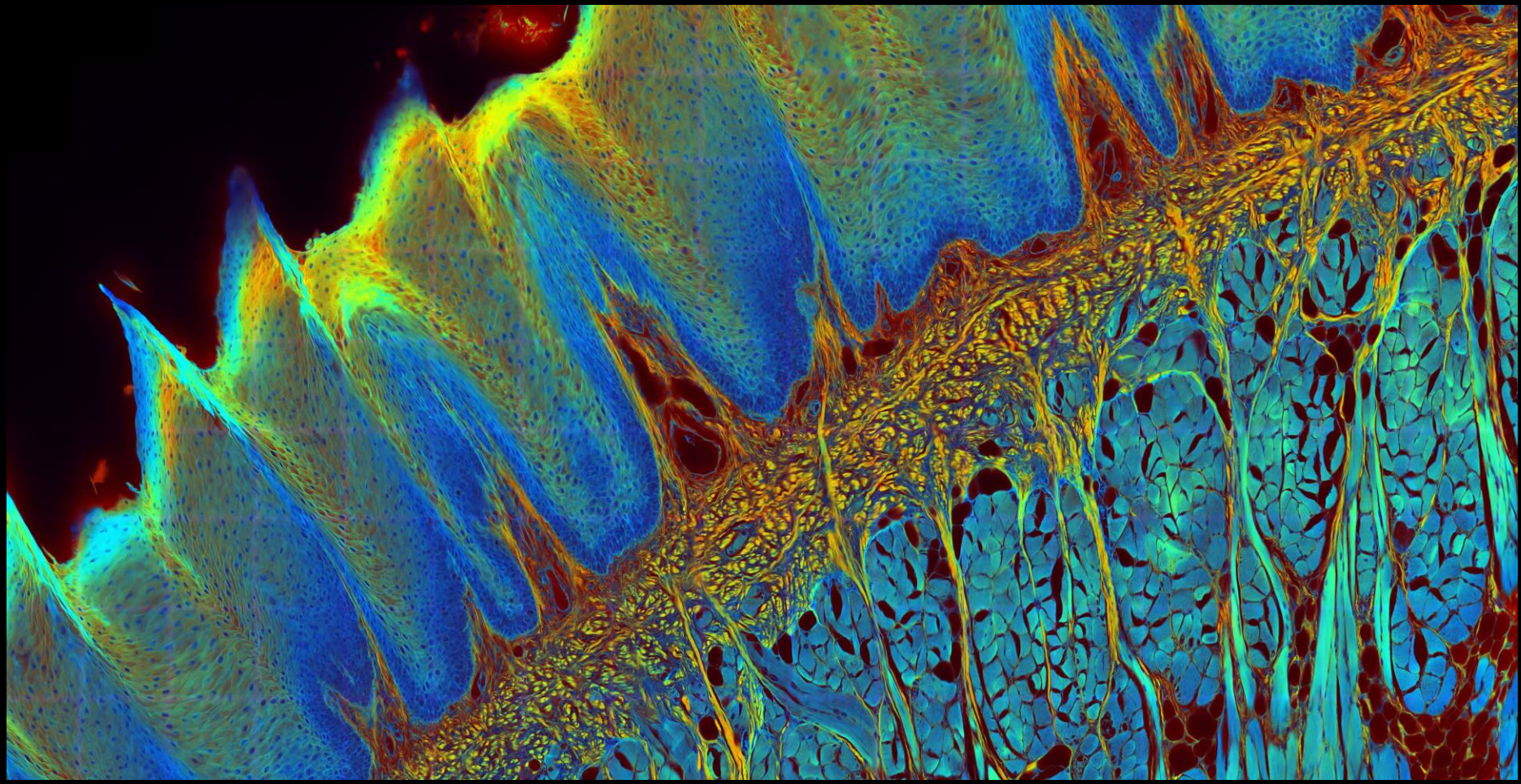
- Gain immediate access to functional information for cell signaling, metabolic status, pH and ion concentration
- Use lifetime-based information to explore the function of molecules in the cellular context

TO GET CLOSER TO THE TRUTH

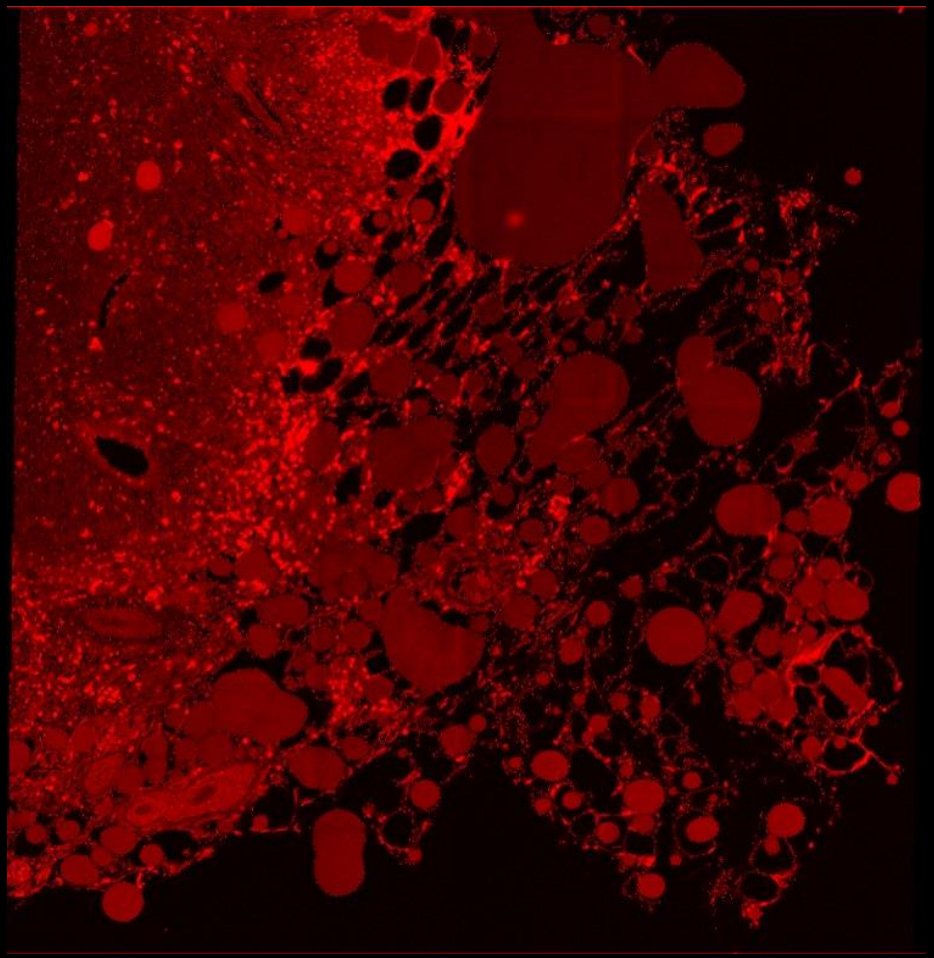
Leica

Explore New Dimensions Of Information - TauContrast

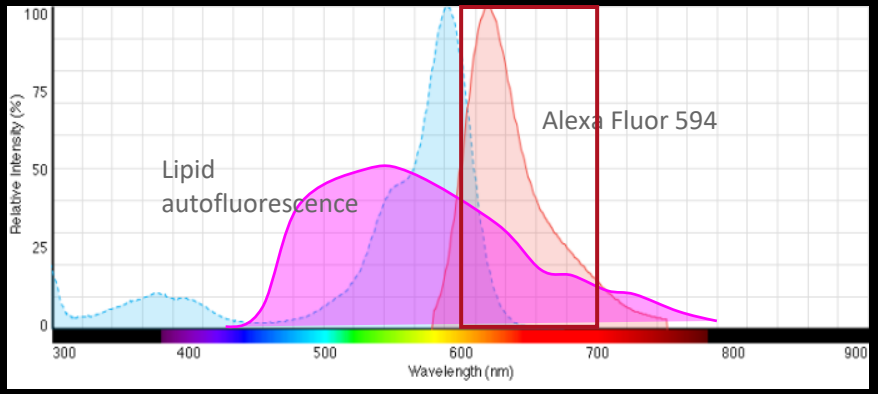
Histological section of a rabbit tongue (Filiform Papillae)



TauContrast – autofluorescence- T cell



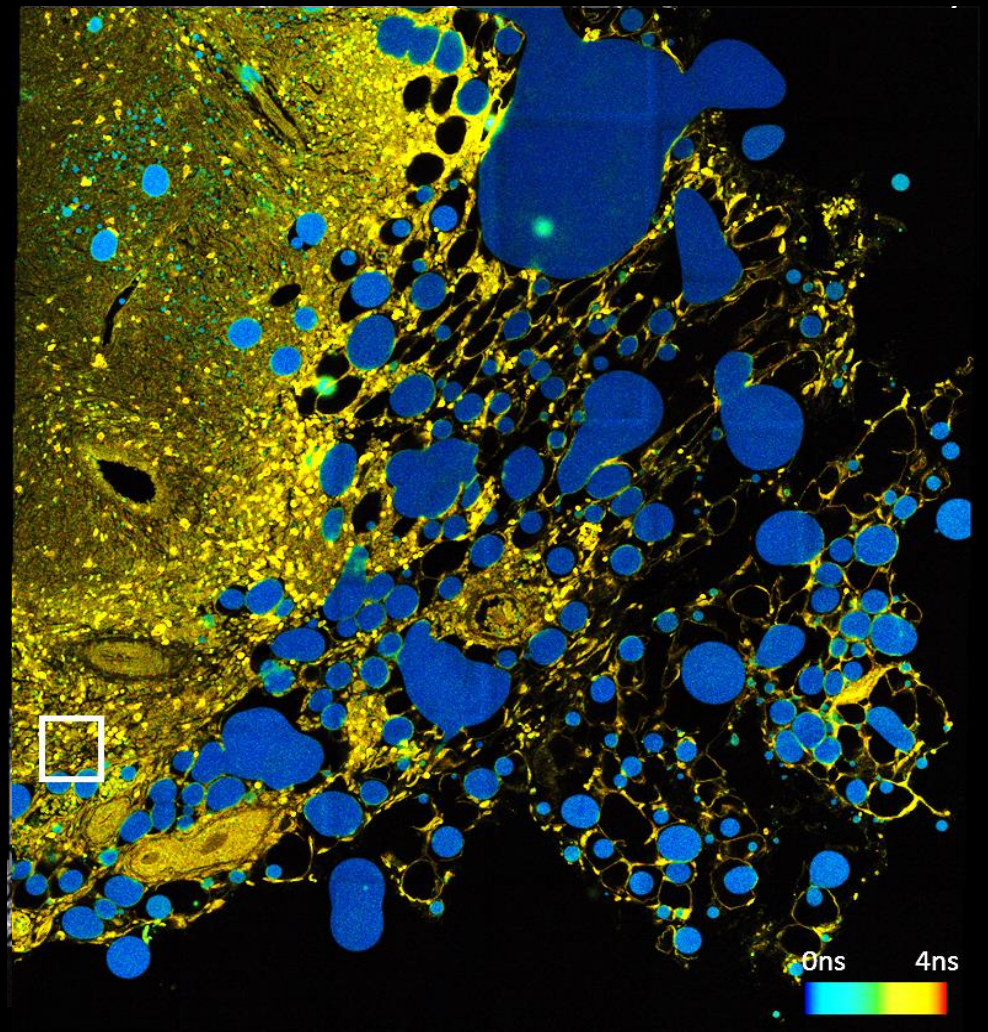
Excitation : 588nm, detection : 615-705nm



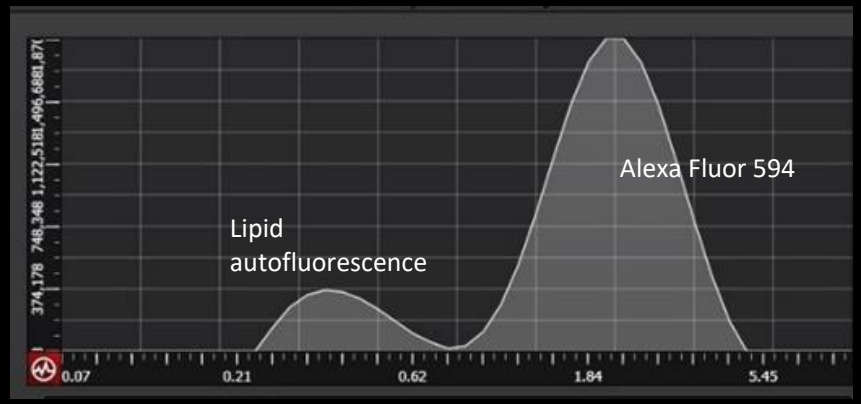
Subcutaneous BRAF600E mouse melanoma with surrounding fat tissue. Cytotoxic T cells stained with anti-CD8a Alexa Fluor 594. Tissue shows high autofluorescence which interferes with Alexa Fluor 594 signal. Fluorescence lifetime information (fast FLIM) enables to distinguish CD8a+ T cells (longer lifetime, yellow) from the autofluroescence of the lipid droplets (short lifetime in blue) and of other cells (green in Fast FLIM images)

Courtesy of Dr. Jan Boettcher, Institute of Molecular Immunology, TU Munich

TauContrast – autofluorescence- T cell



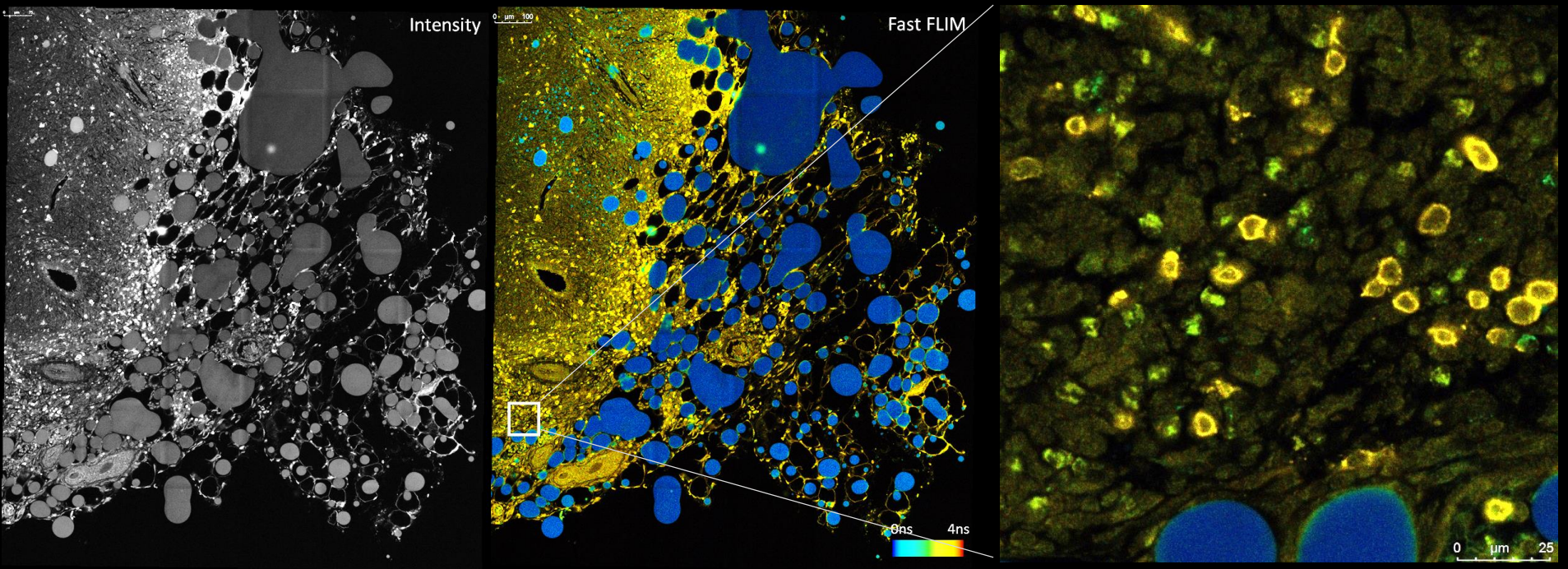
Excitation : 588nm, detection : 615-705nm



Subcutaneous BRAF600E mouse melanoma with surrounding fat tissue. Cytotoxic T cells stained with anti-CD8a Alexa Fluor 594. Tissue shows high autofluorescence which interferes with Alexa Fluor 594 signal. Fluorescence lifetime information (fast FLIM) enables to distinguish CD8a+ T cells (longer lifetime, yellow) from the autofluroescence of the lipid droplets (short lifetime in blue) and of other cells (green in Fast FLIM images)

Courtesy of Dr. Jan Boettcher, Institute of Molecular Immunology, TU Munich

TauContrast – autofluorescence- T cell



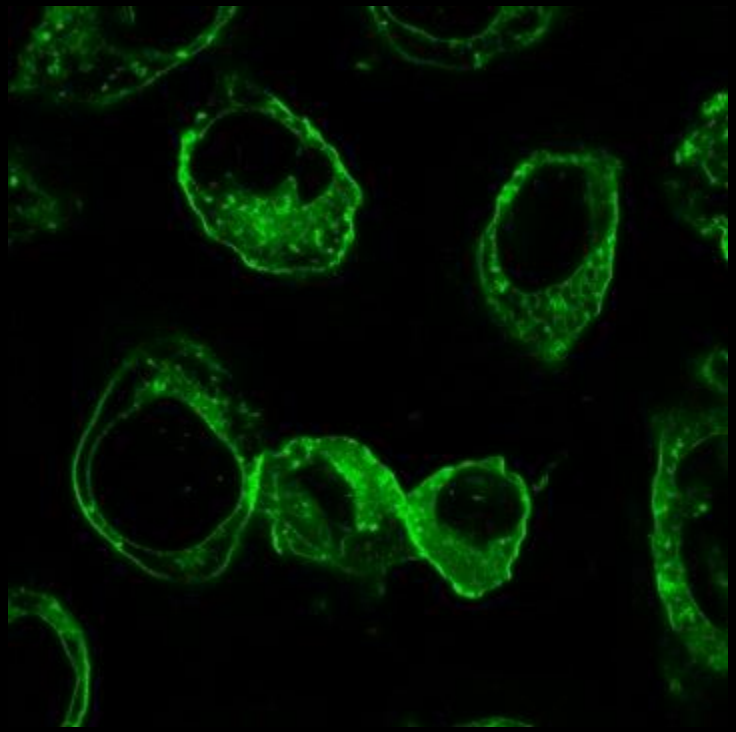
Subcutaneous BRAF600E mouse melanoma with surrounding fat tissue. Cytotoxic T cells stained with anti-CD8a Alexa Fluor 594. Tissue shows high autofluorescence which interferes with Alexa Fluor 594 signal. Fluorescence lifetime information (fast FLIM) enables to distinguish CD8a+ T cells (longer lifetime, yellow) from the autofluroescence of the lipid droplets (short lifetime in blue) and of other cells (green in Fast FLIM images)

Courtesy of Dr. Jan Boettcher, Institute of Molecular Immunology, TU Munich

Membrane tension visualization with FlipperTR®

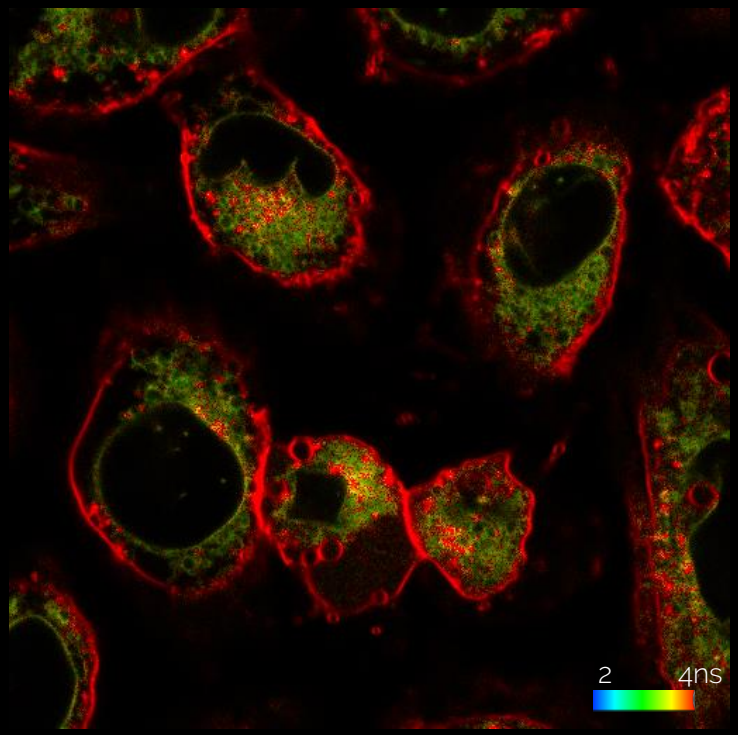
Excitation : 488nm, detection : 575-625nm

Intensity



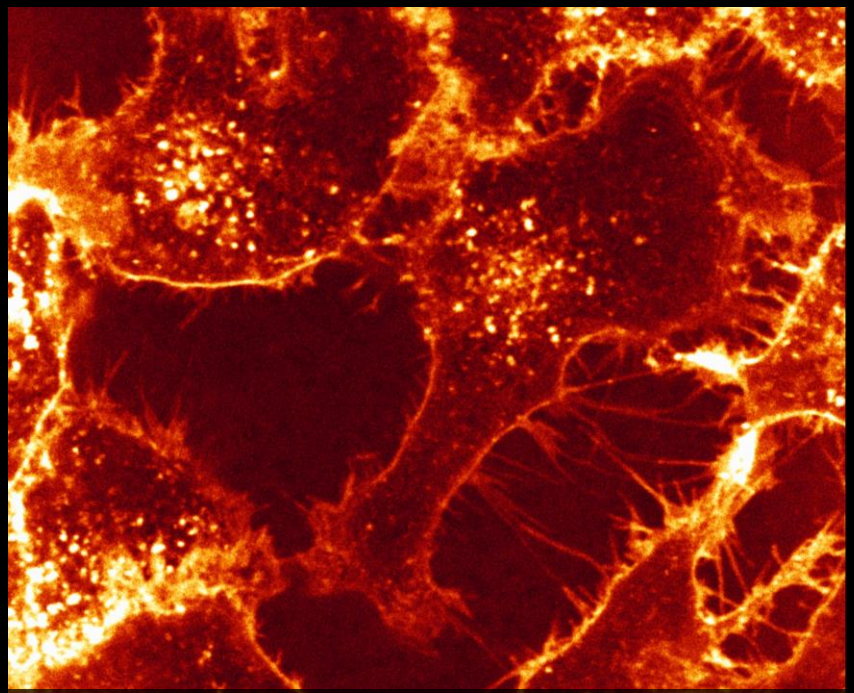
U2OS cells labeled with Flipper TR,

Tau Contrast

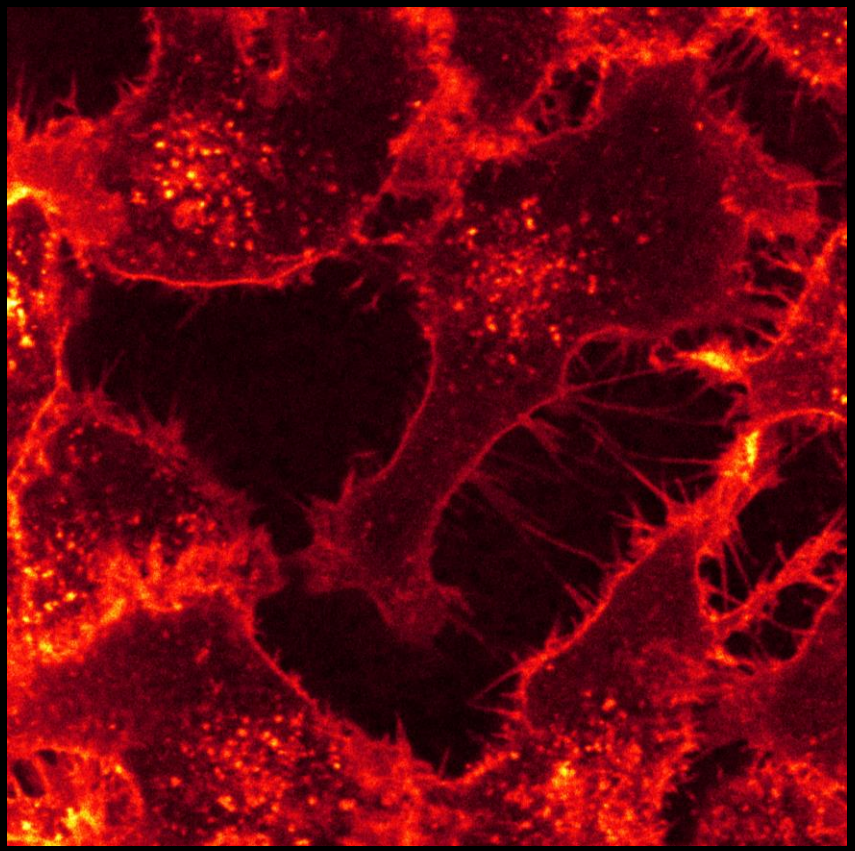


Improve Image Quality

Traditional Confocal



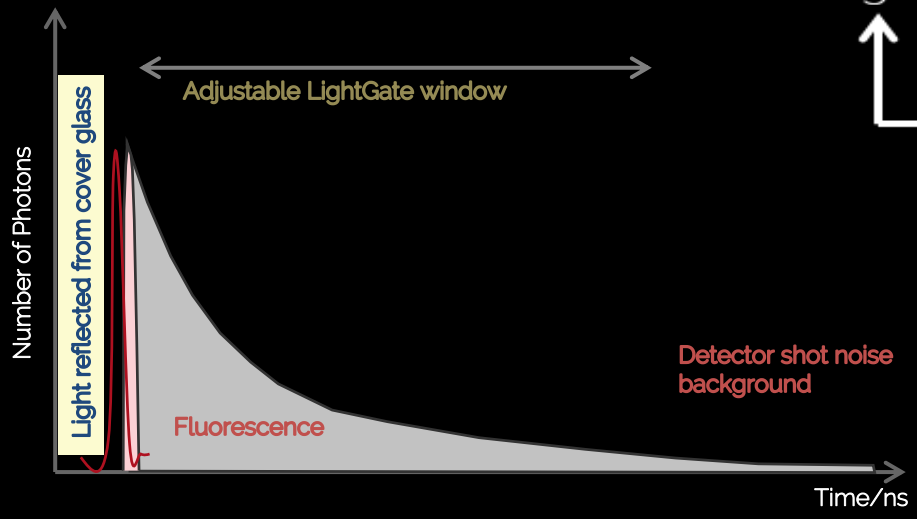
STELLARIS

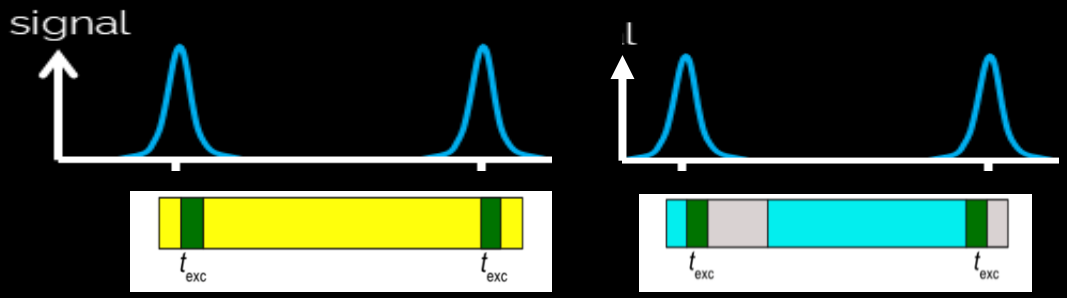


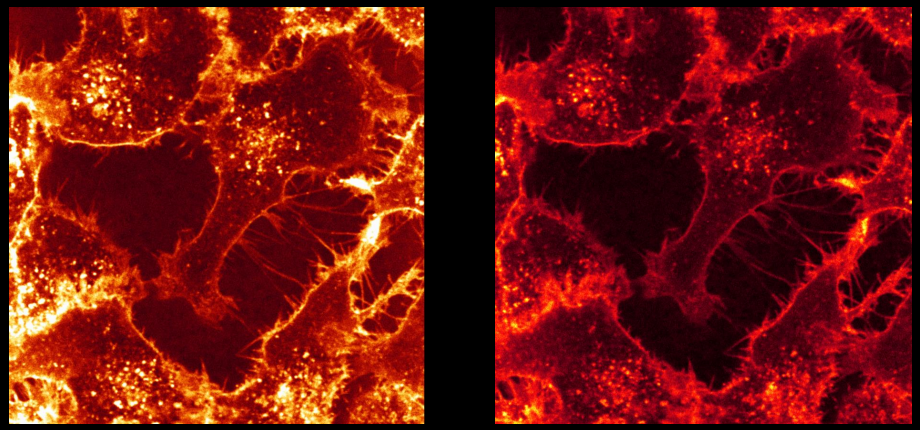
TauSense Tool:

TauGating

The Technology Behind TauGating



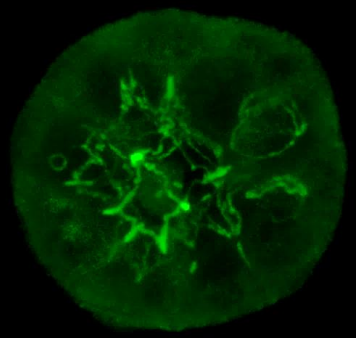




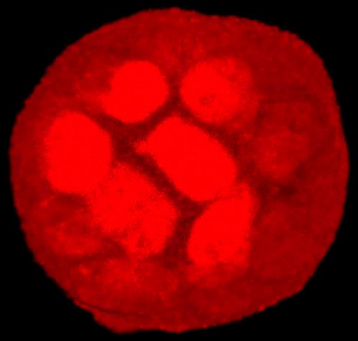
- Digital Gate Channels (Intensity, $N_{photons}$)

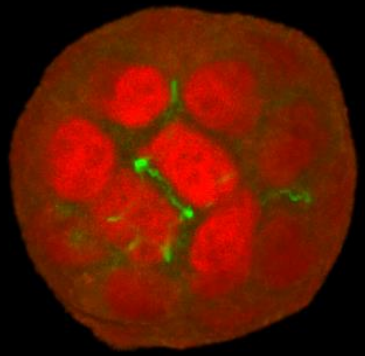
TauGating - Improve Image Quality

Traditional Confocal

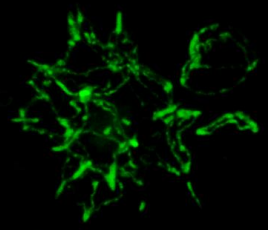


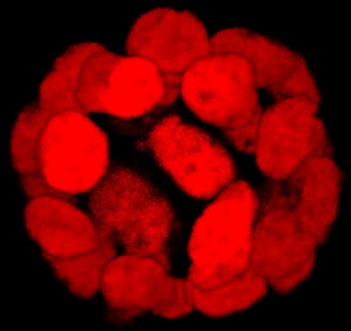
3D cell culture

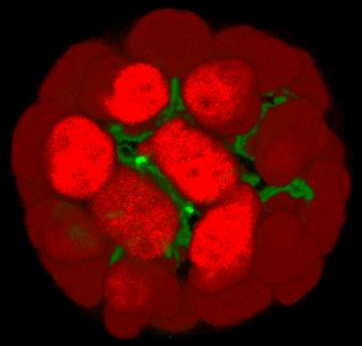




STELLARIS TauGating



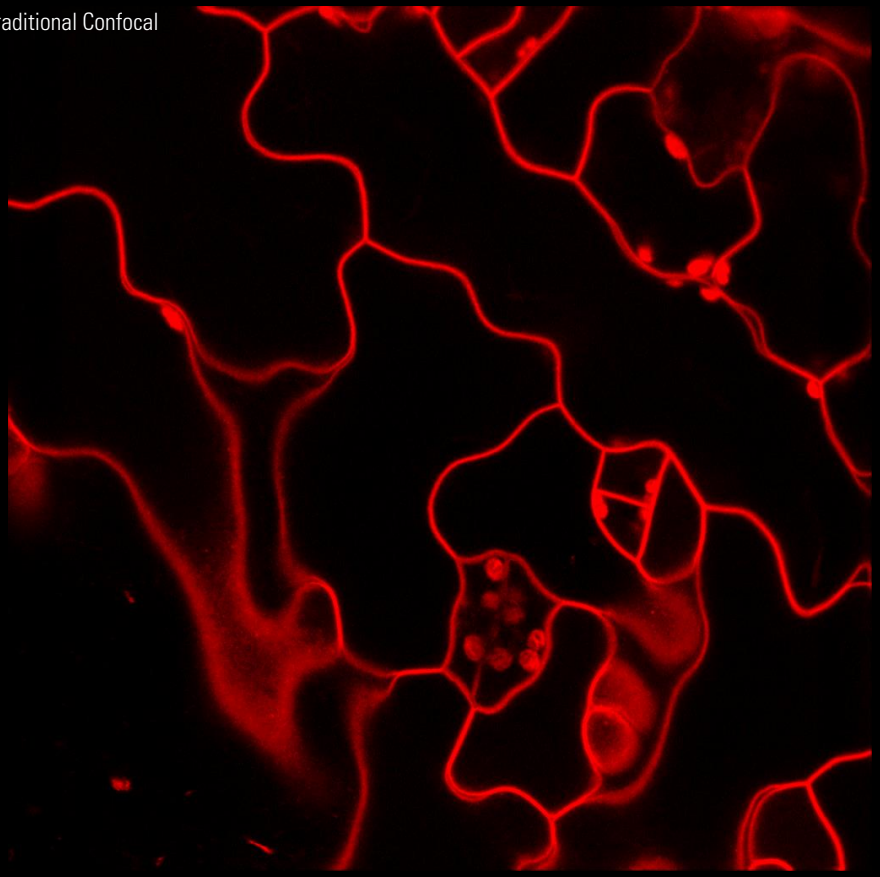


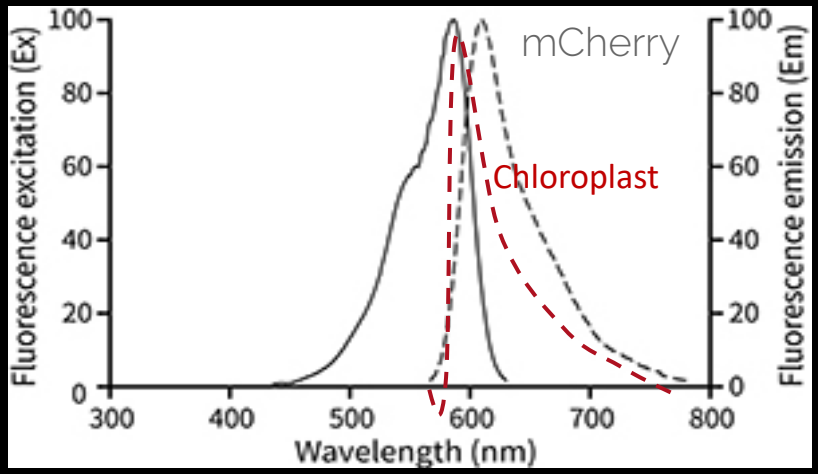


Leica

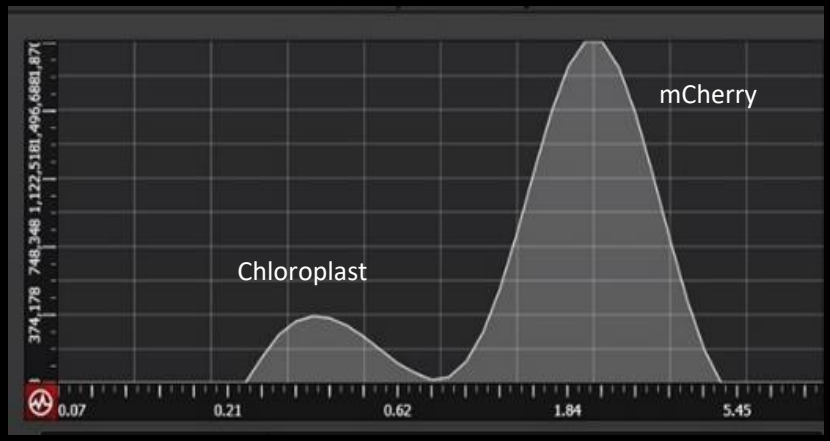
mCherry + Chloroplast

Traditional Confocal





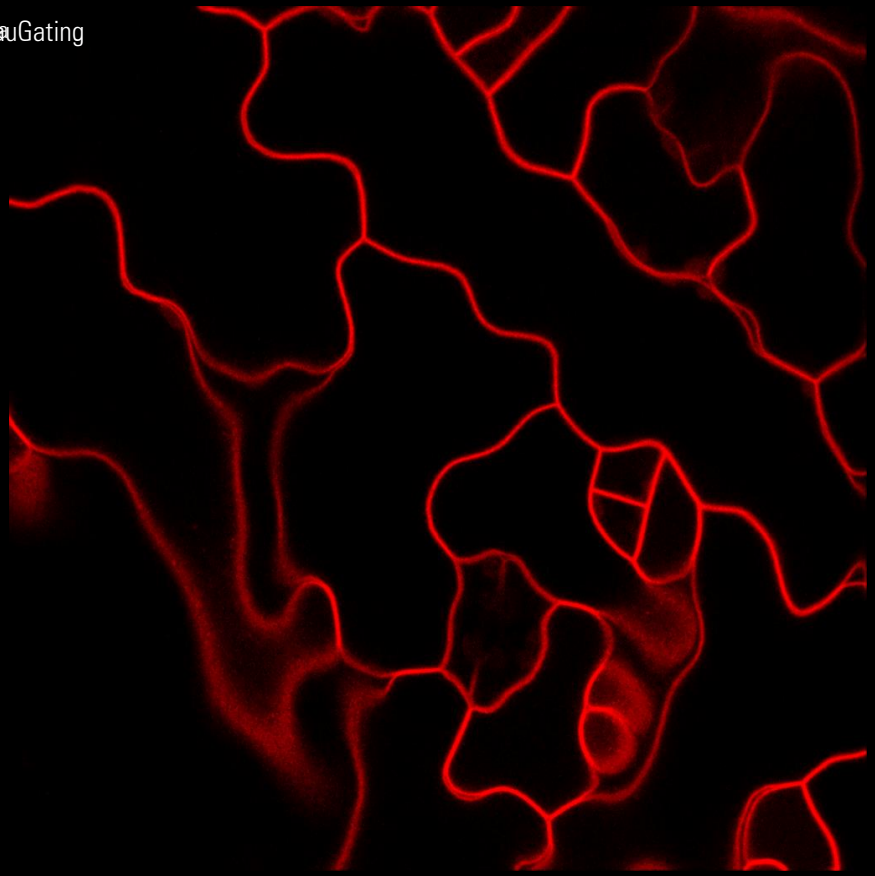
Photon counts

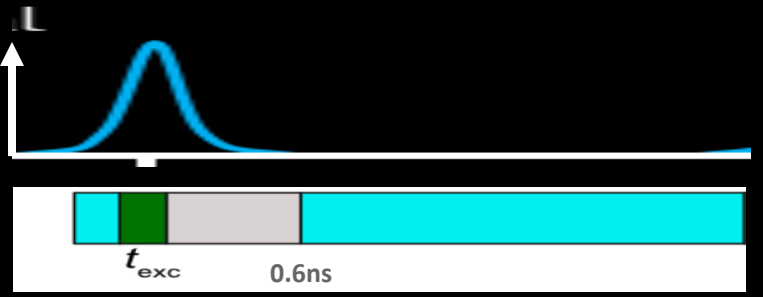


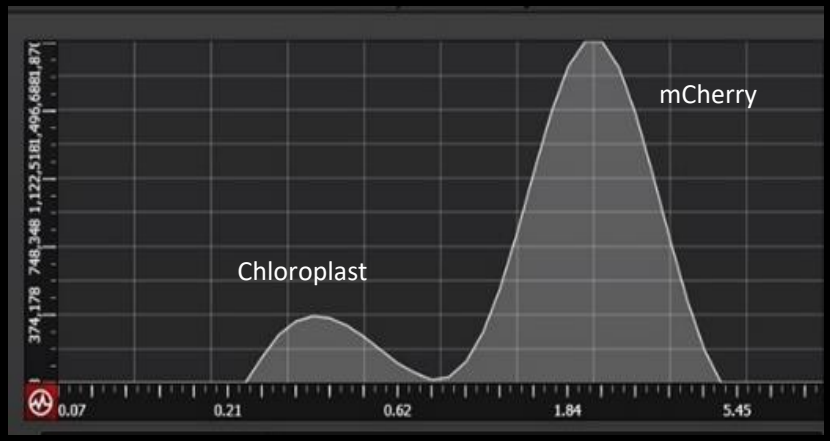
Average arrival time, ns

mCherry + Chloroplast

TauGating

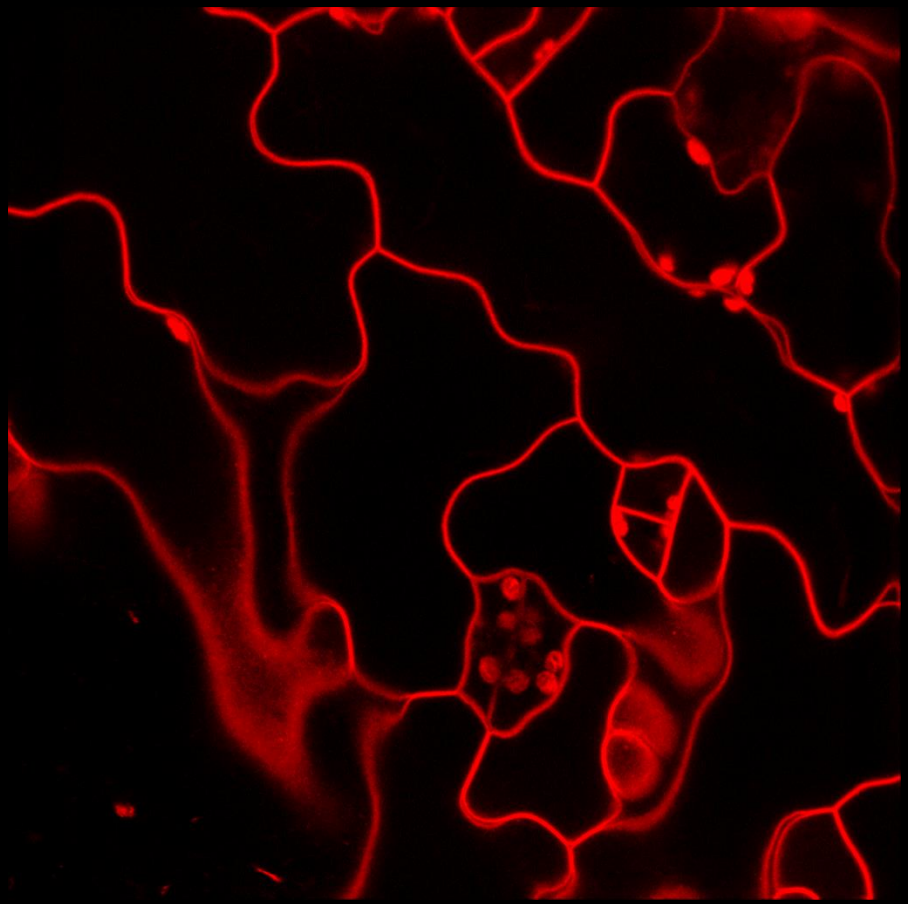




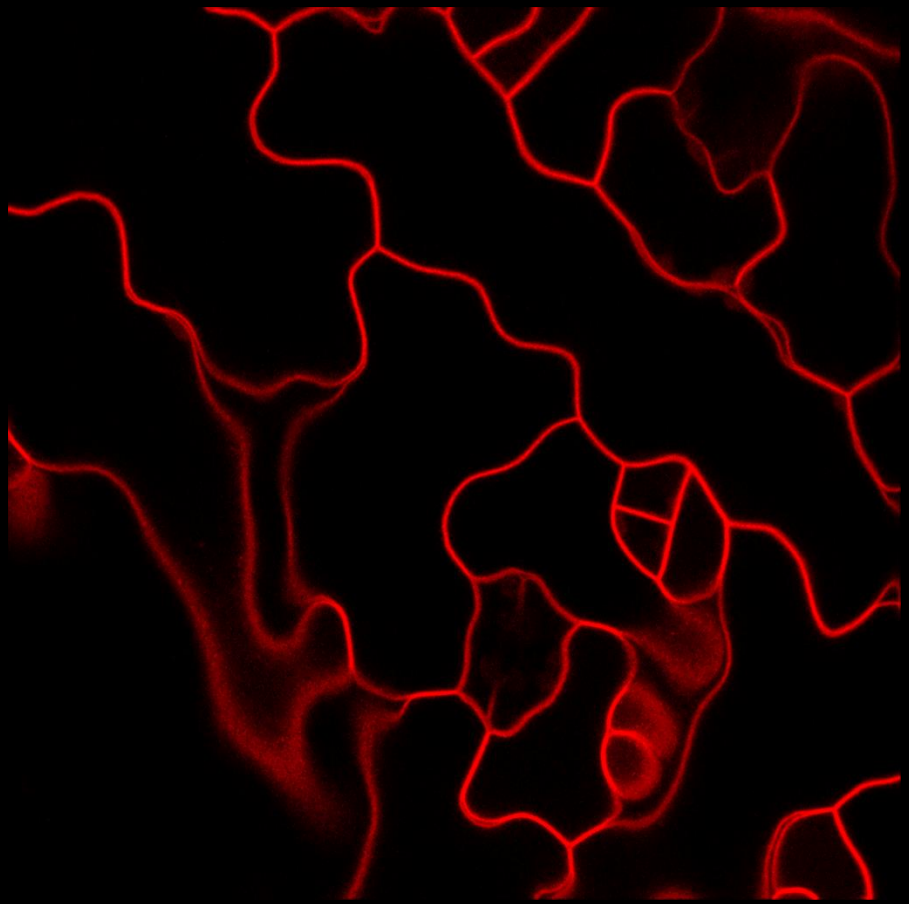


TauGating - Improve Image Quality

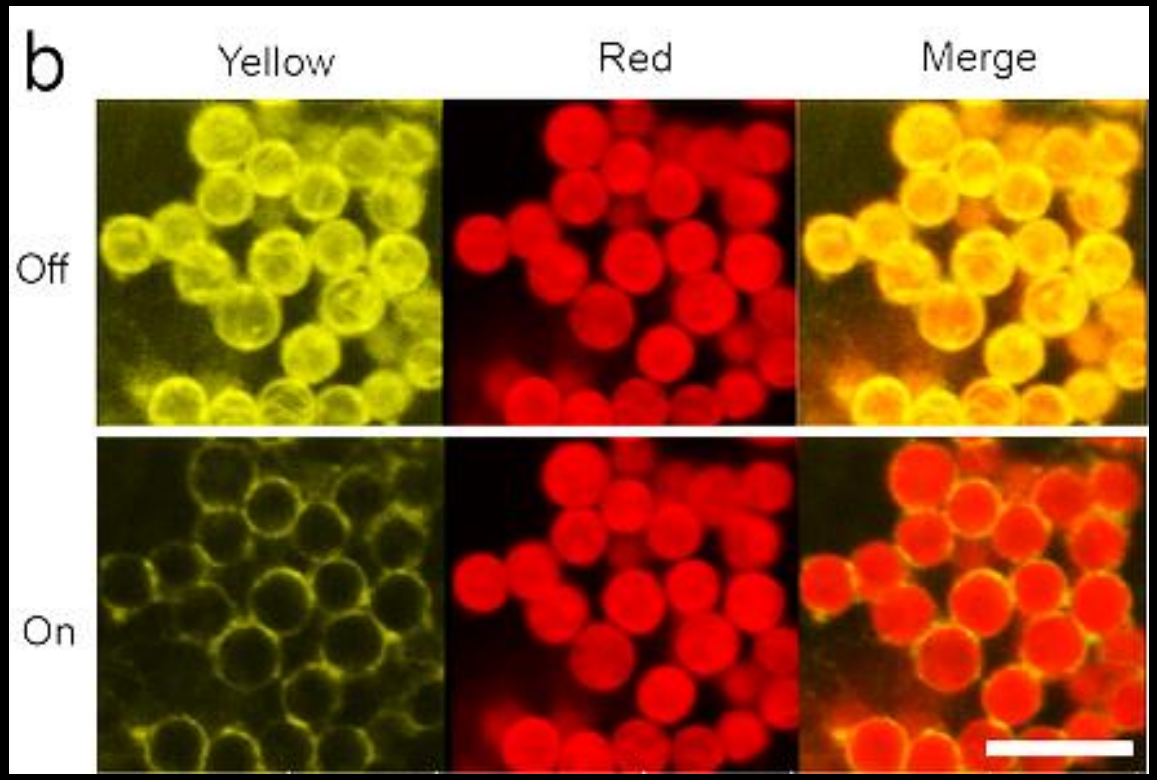
Traditional Confocal



Tau gating



Fluorescence imaging of Mpphot-Citrine on the chloroplast periphery of transgenic Marchantia polymorpha.

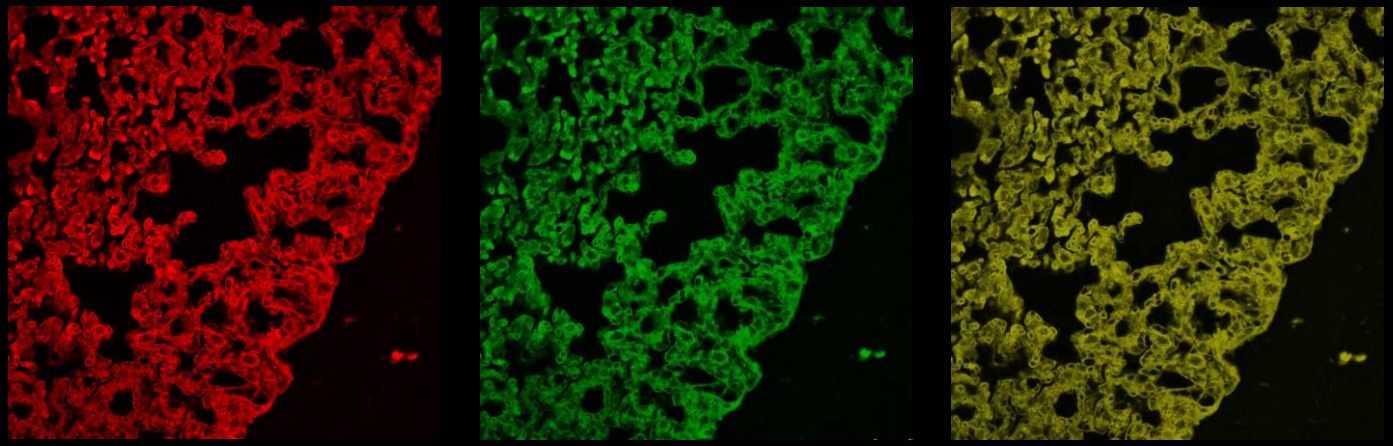


Kodama Y (2016) Time Gating of Chloroplast Autofluorescence Allows Clearer Fluorescence Imaging In Planta. PLOS ONE 11(3): e0152484. https://doi.org/10.1371/journal.pone.0152484
https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0152484

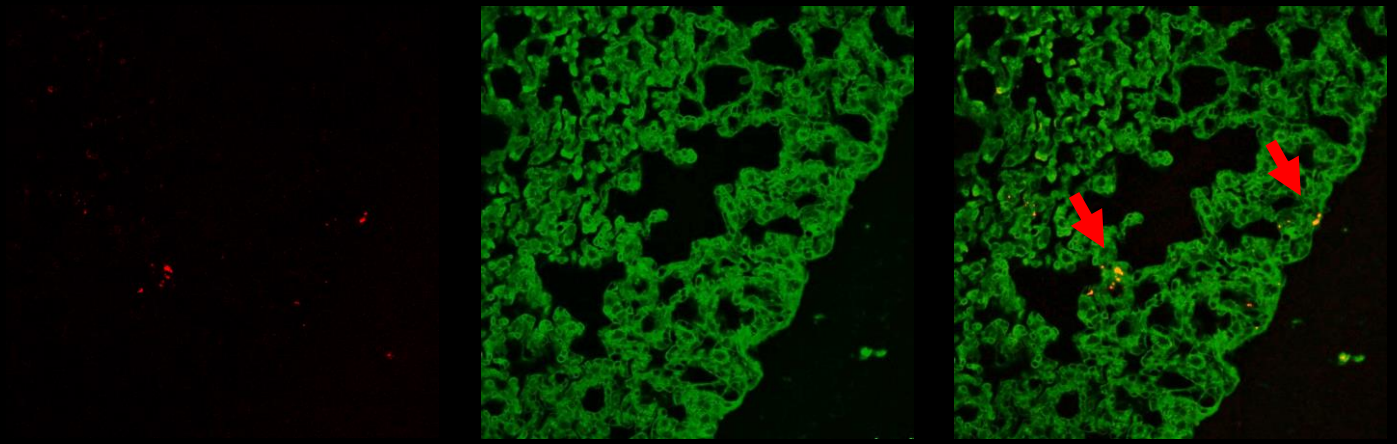
TauGating - Improve Image Quality

Tissue & nano diamond

Traditional Confocal

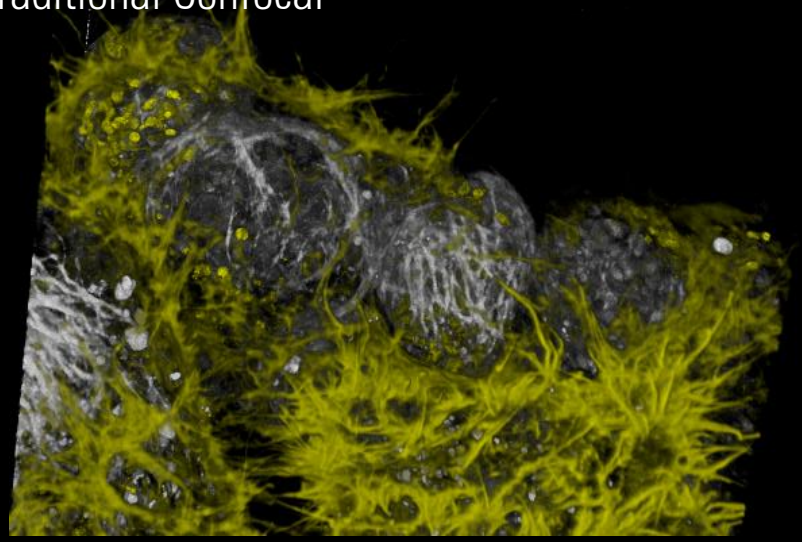


Tau gating

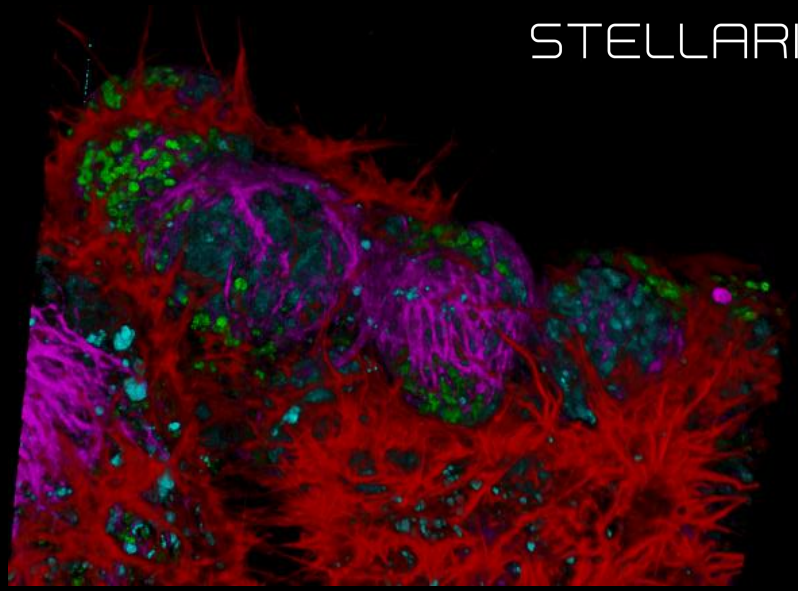


Multiplex Beyond The Spectral Options

Traditional Confocal



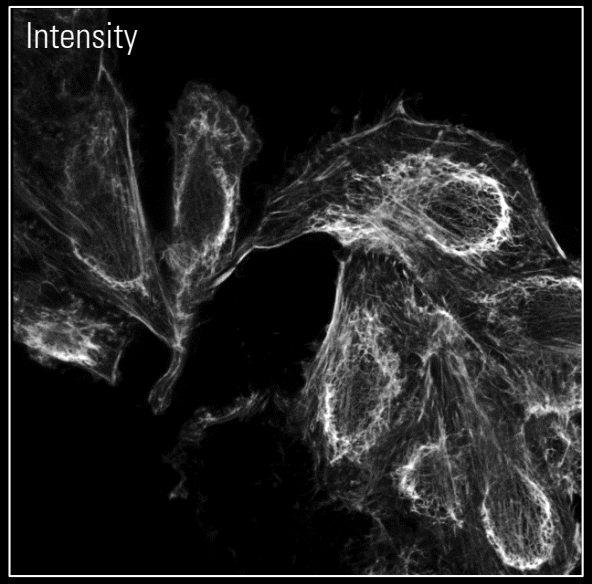
STELLARIS



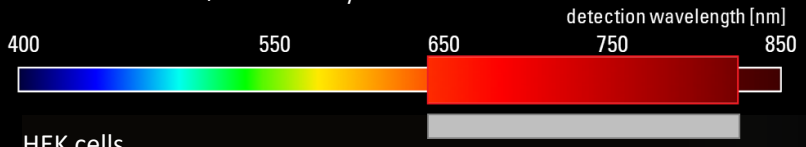
TauSense Tool:

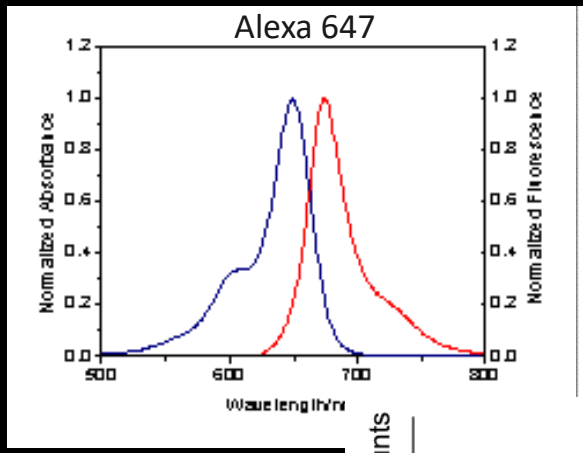
TauSeparation

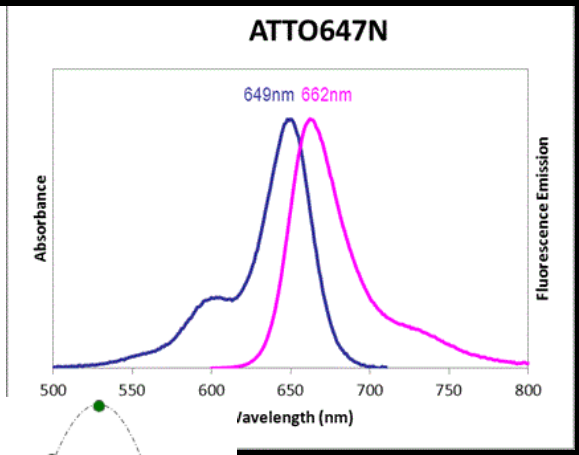
Species Separation using TauSense

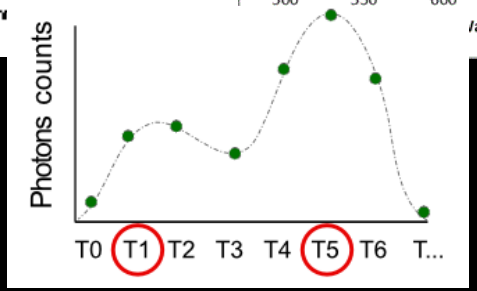


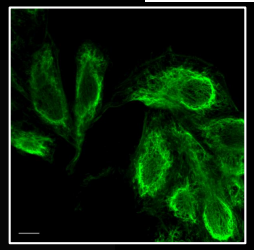
1 detector, 1 intensity channel

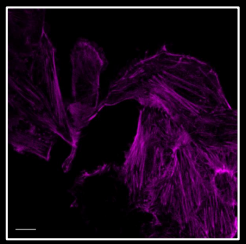












HEK cells.

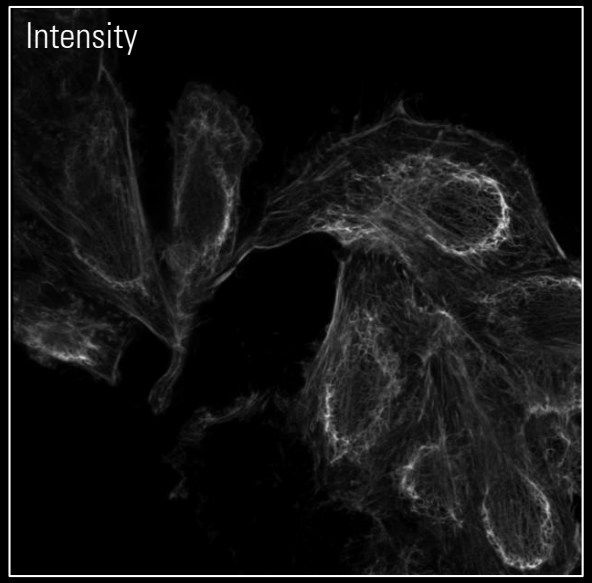
Vimentin (Alexa 647 IF), actin (ATTO647N-phalloidin)

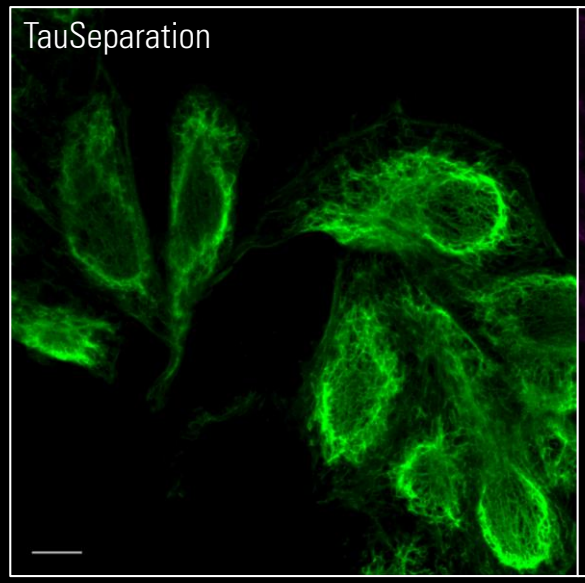
Sample Courtesy: Sebastian Hänsch,

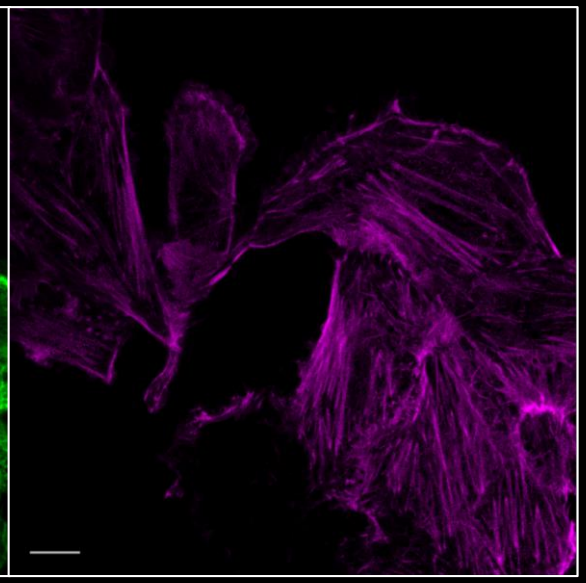
Stephanie Weidtkamp-Peters, CAI, Düsseldorf.

Application Example

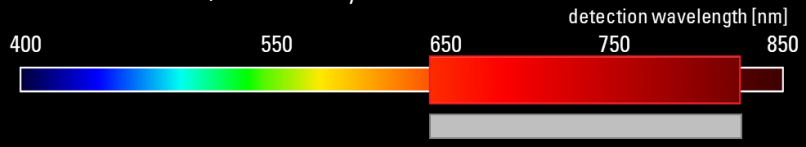
Species Separation using TauSense



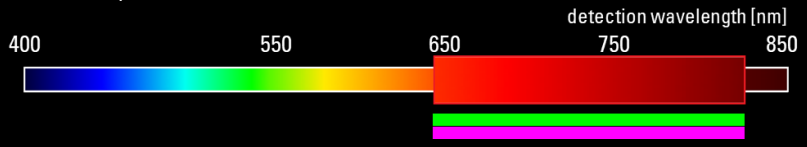




1 detector, 1 intensity channel



1 detector, 2 lifetime-based channels

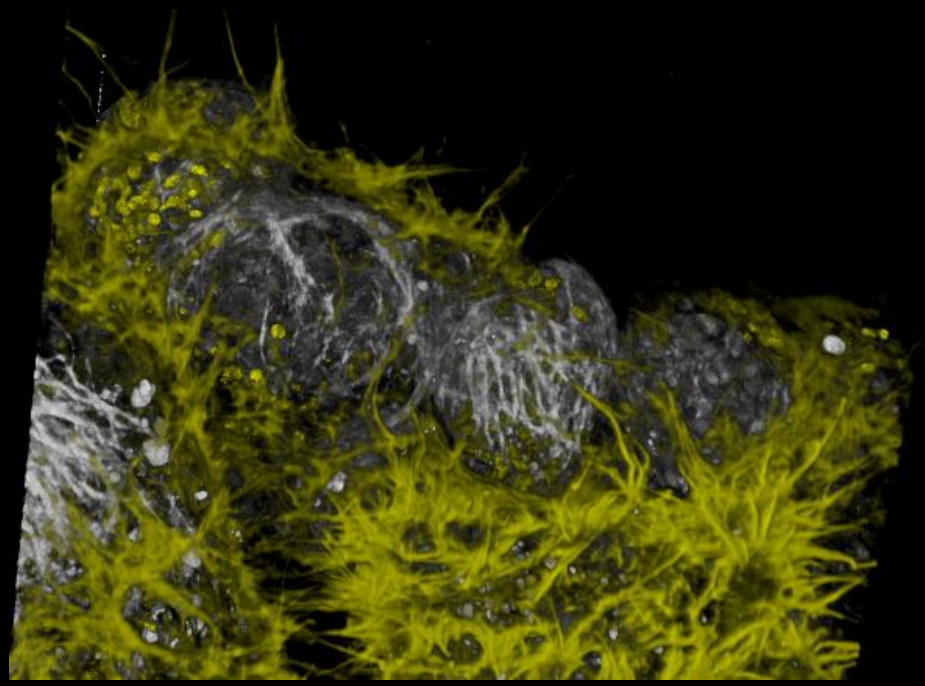


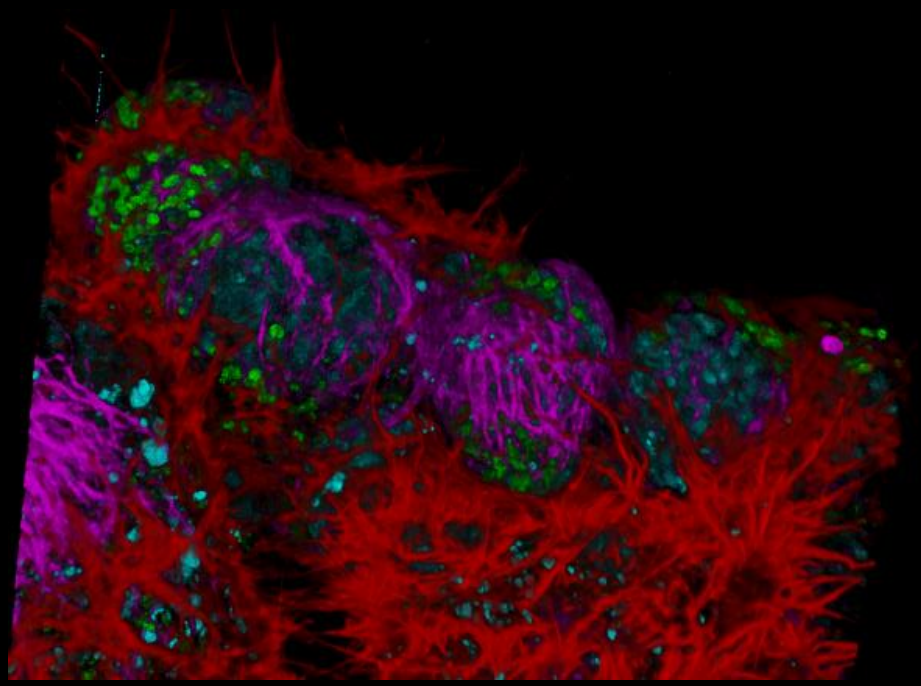
HEK cells. Vimentin (left: gray, Alexa 647 IF), actin (left: gray, ATTO647N-phalloidin). TauSeparation separates the signals coming to the detector according to the lifetime components distribution generated online at the FPGA level (right: green, Vimentin; right: magenta, Actin). Scale bar 10µm.
Sample Courtesy: Sebastian Hänsch, Stephanie Weidtkamp-Peters, CAI, Düsseldorf.

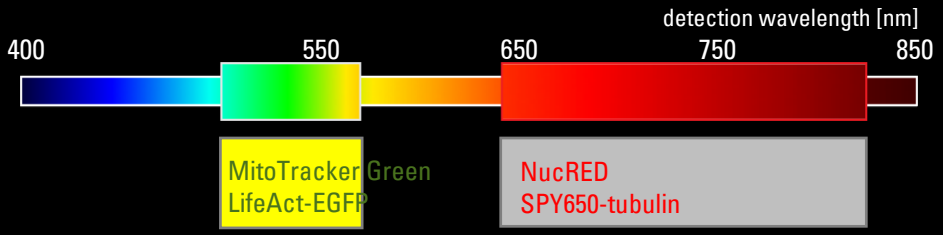
Separate Species Beyond The Spectral Options

Traditional Confocal

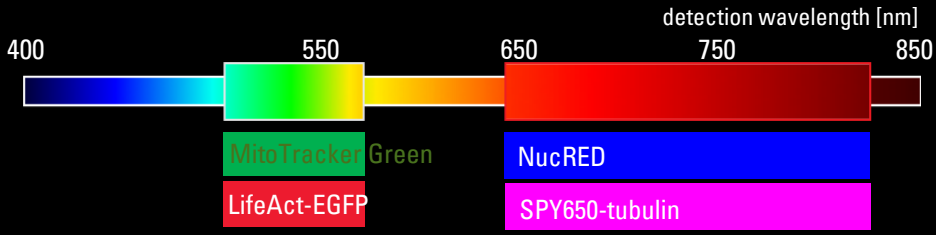
STELLARIS







2 detectors, 2 intensity chnnels



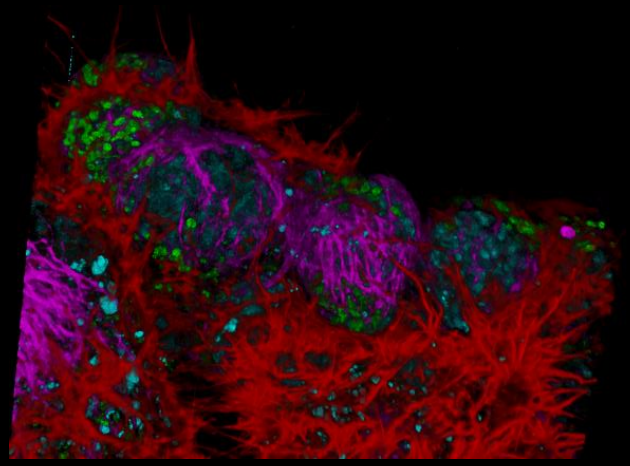
2 detectors, 4 lifetime-based channels

NE-115 cells. LifeAct-mNeonGreen (left: yellow, right: red), MitoTracker Green (left: yellow, right: green), NUC Red (left: gray, right: blue), and SiR-tubulin (left: gray, right: magenta).
Courtesy: Max Heydasch, University of Bern and Spirochrome

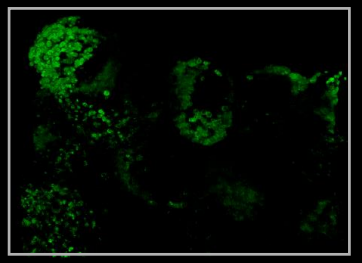
Leica

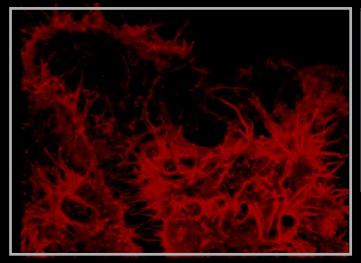
Separate Species Beyond The Spectral Options

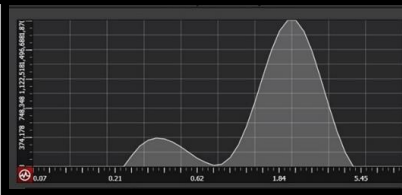
Tau Separation



NE-115 cells.
LifeAct-mNeonGreen(left: yellow, right: red),
MitoTracker Green (left: yellow, right: green), NUC Red (left: gray, right: blue), and SiR-tubulin (left: gray, right: magenta).
Courtesy: Max Heydasch, University of Bern and Spirochrome

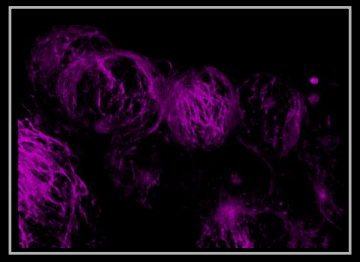


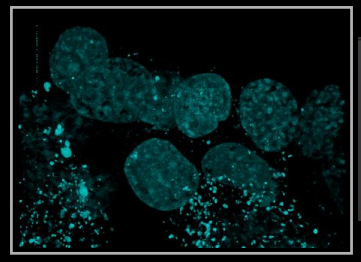


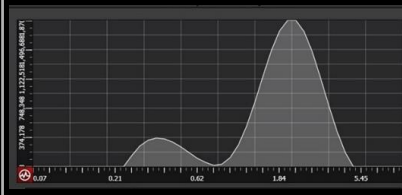


Detector 1

Lifetime-based Channel 1 Lifetime-based Channel 2



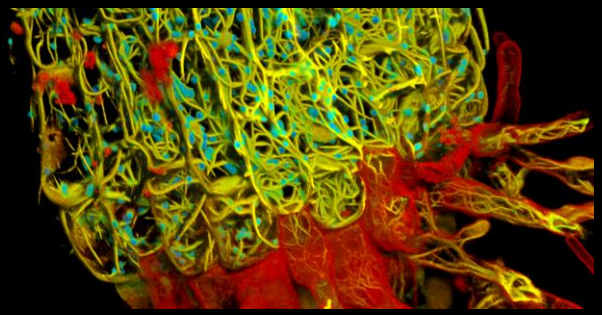




Detector 2

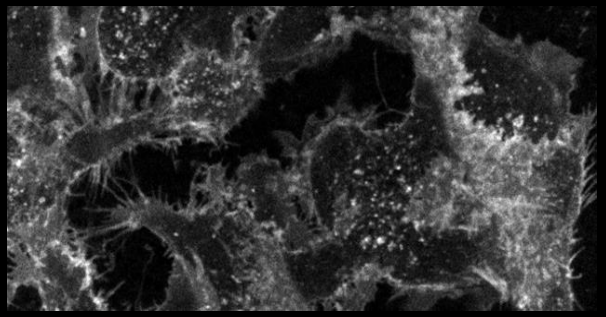
Lifetime-based Channel 3 Lifetime-based Channel 4

What is TauSense Good For?



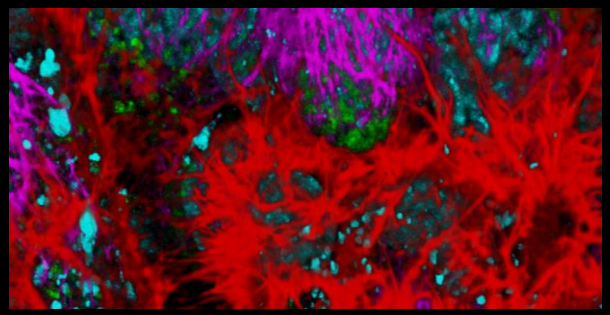
TauContrast

- Qualitative / Semi-quantitative information
- Is there a change in microenvironment? Is FRET happening?
- Changes over time (x-fold ↑↓ compared to baseline)



TauGating

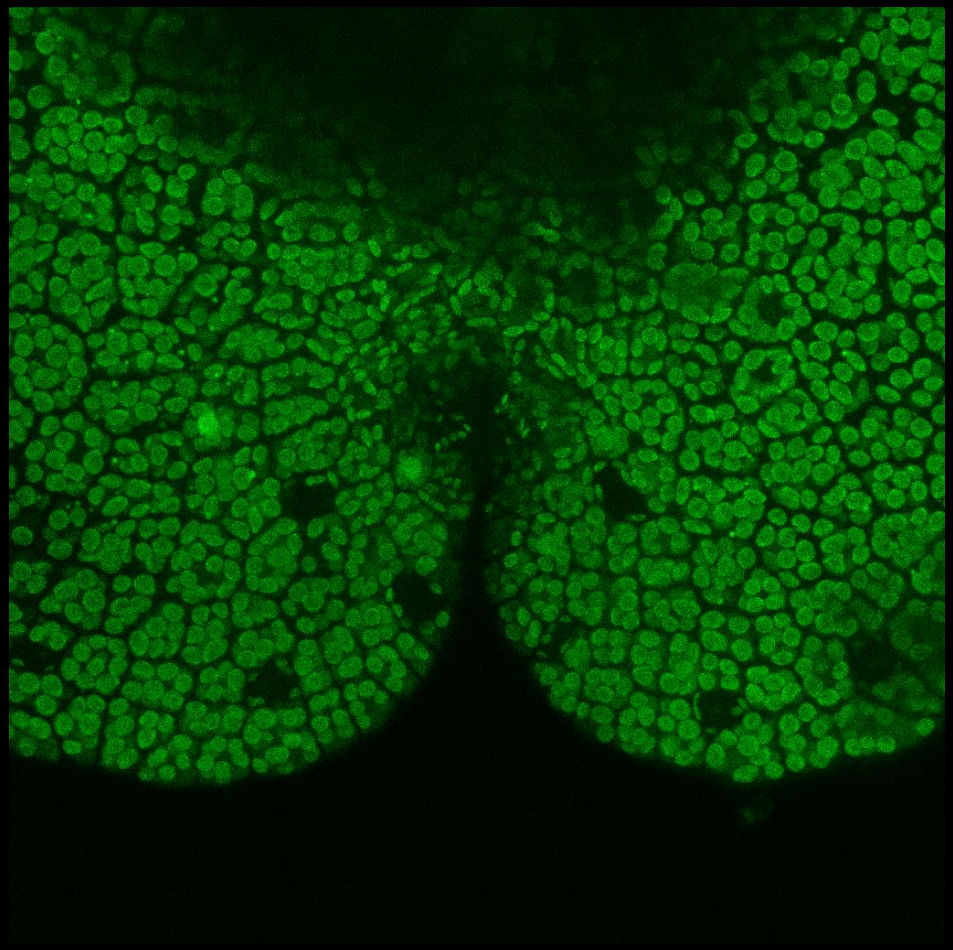
- Explore sample with gates
- Remove reflections
- Remove unwanted fluorescence contributions



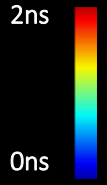
TauSeparation

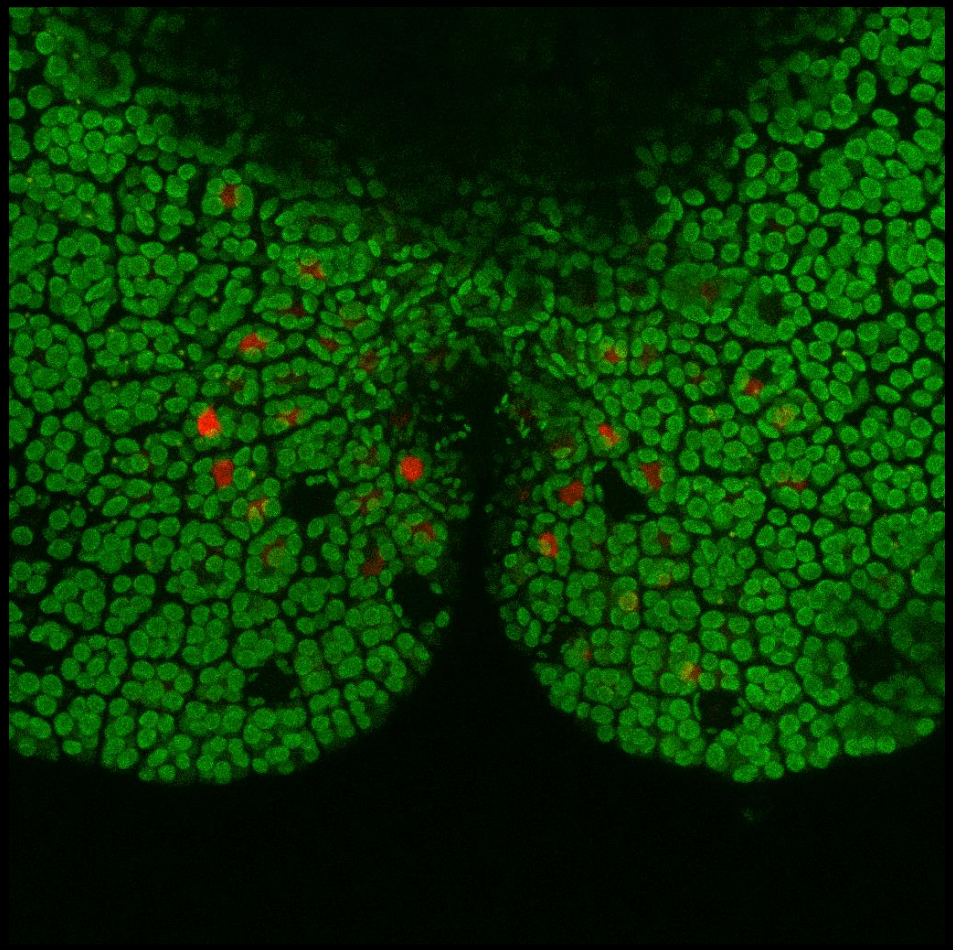
- Separate species with different lifetimes

Traditional Confocal - intensity



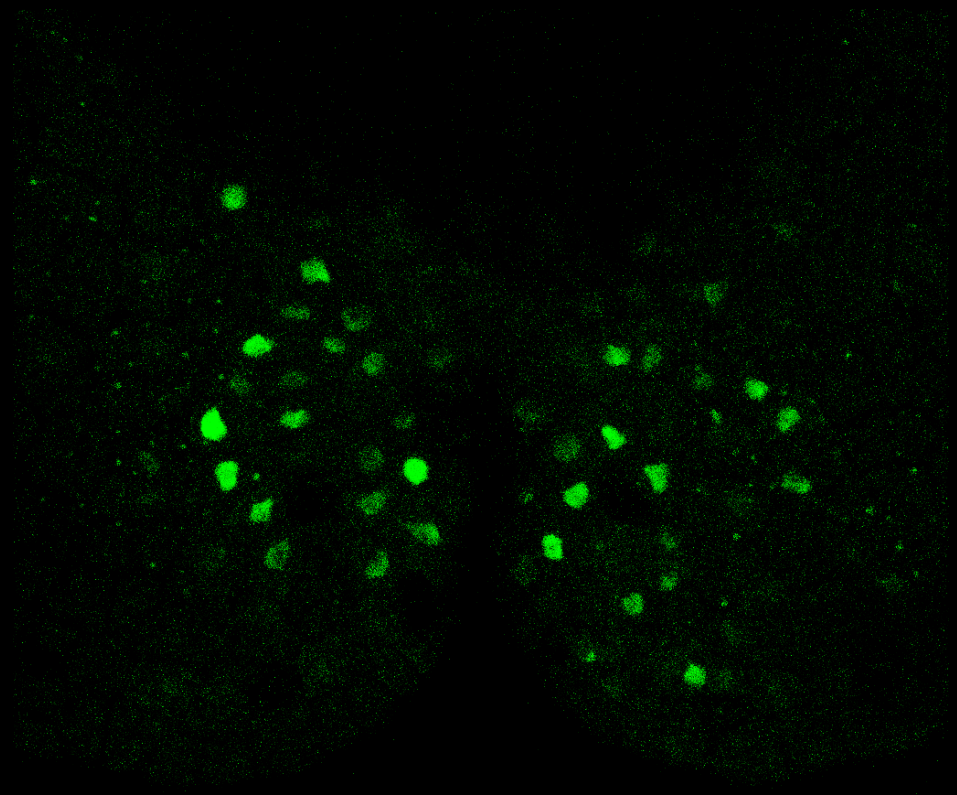
Tau Contrast



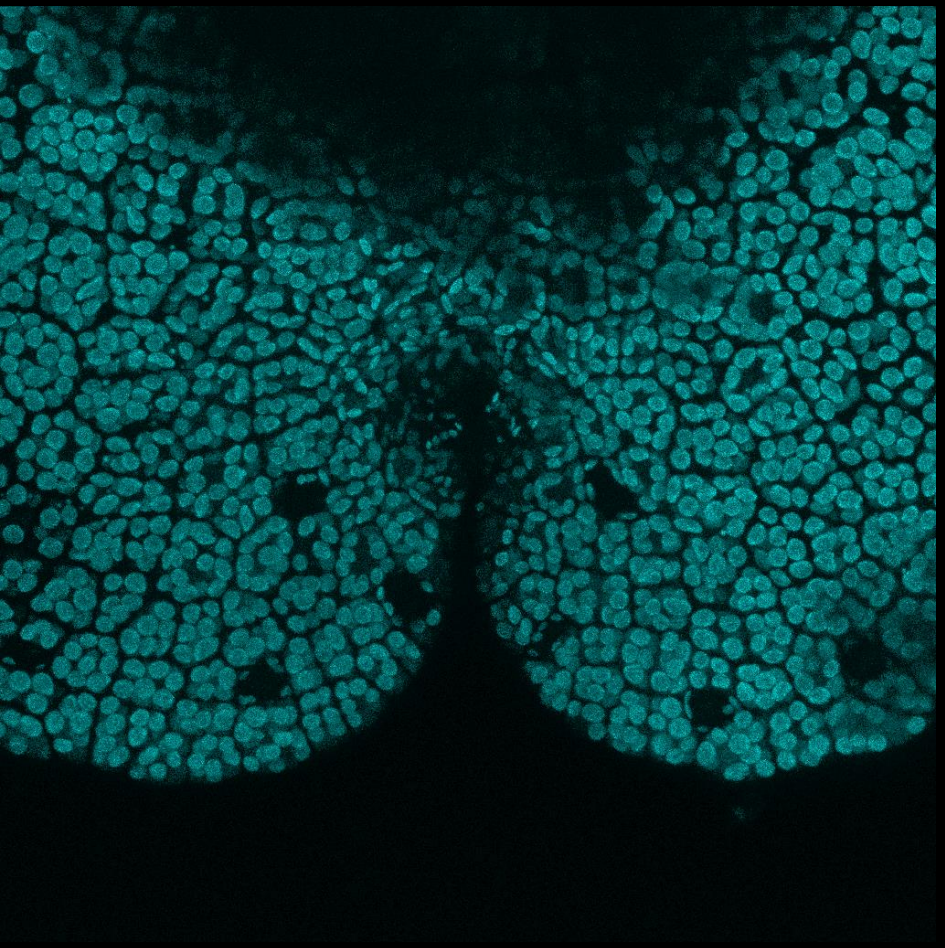


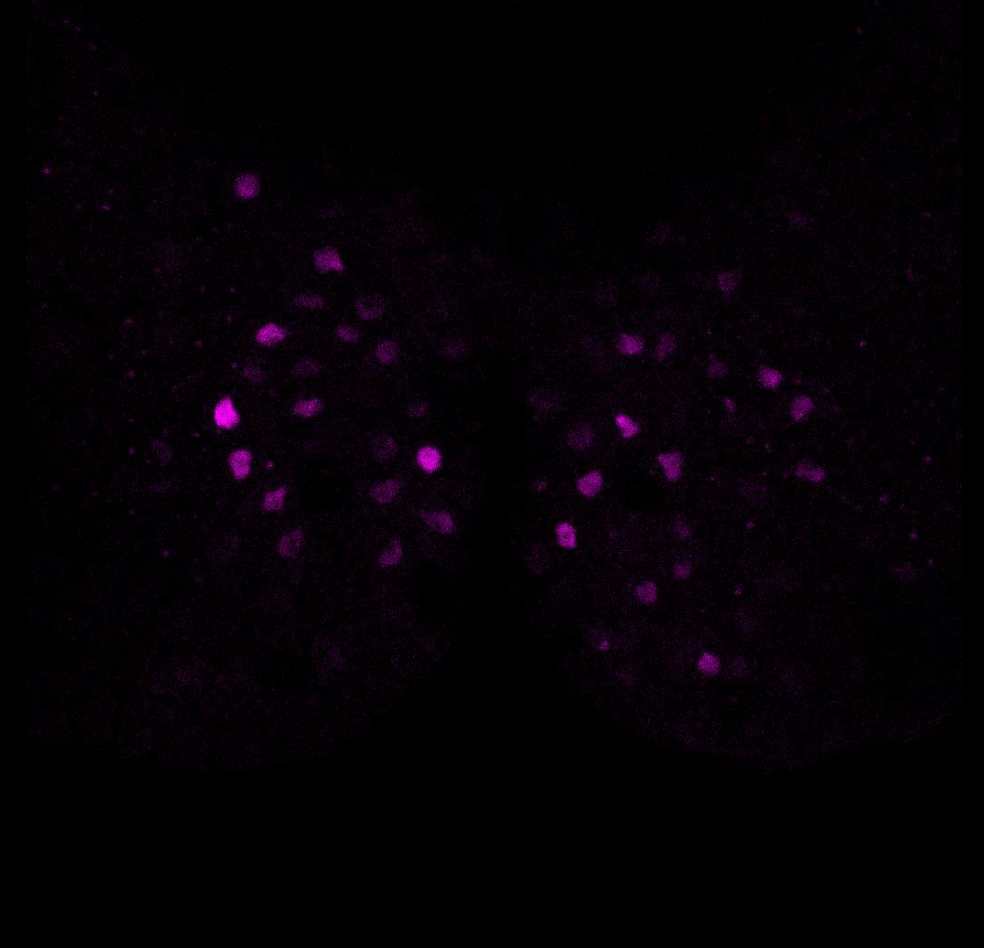
Tau Gating

Gating : 0.5-6 ns



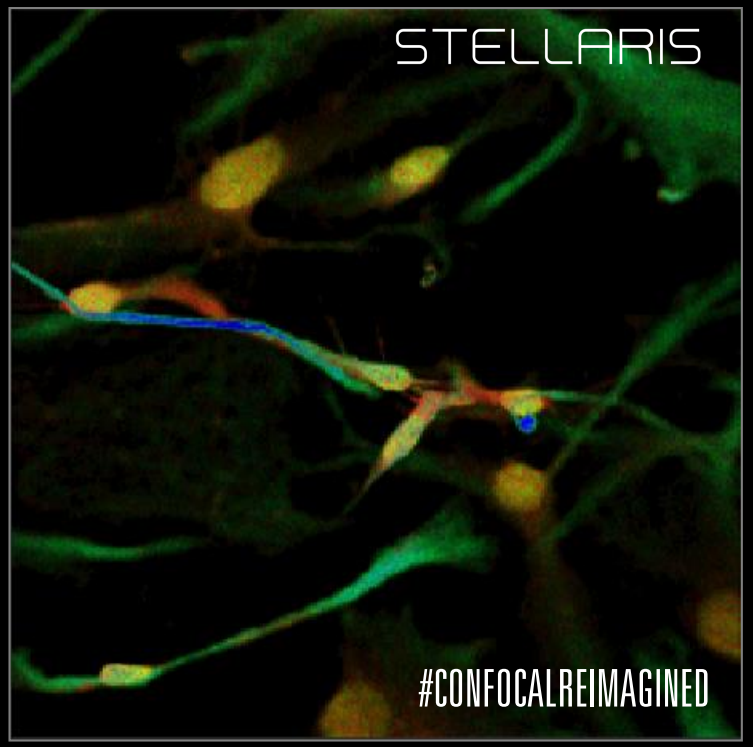
Tau Seperation





Tau sense tool – One Click

TauContrast

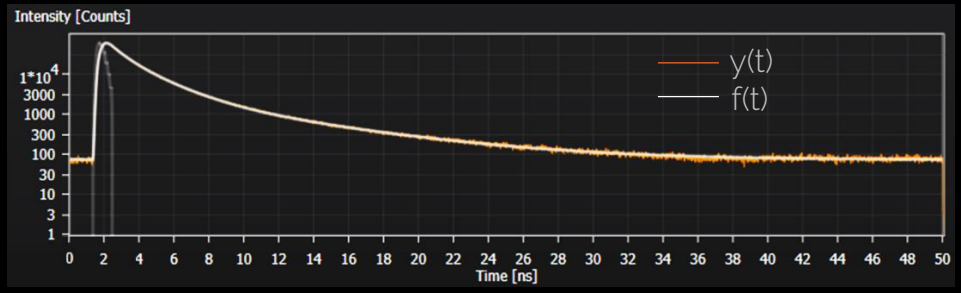


FALCON: FAst Lifetime CONtrast

Fit Model

$$f(t) = \int_0^t IRF(x) \cdot \left\{ \sum_{i=0}^{n-1} A(n) \cdot e^{-\frac{x}{\tau(n)}} + B \right\} dx$$

$y(t)$ - Experimental data
$f(t)$ - Theoretical curve
$IRF(t)$ - Instrument Response Function
$A(n)$ - Amplitude of n-th component
$\tau(n)$ - Decay time of n-th component
B - Background



Maximum-Likelihood Estimator (Poissonian distribution)

Minimizes: $\chi^2_{mle} = 2 \cdot \sum_{i=1}^{N} f(i) - y(i) - 2 \cdot \sum_{i=1}^{N} y(i) \cdot ln(f(i)/y(i))$

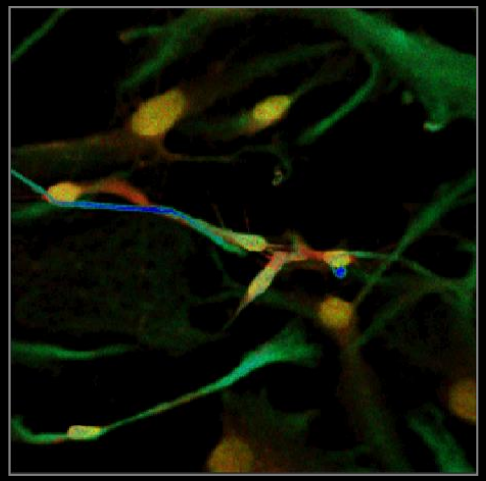
FALCON: Synergies and integrated workflows

- Component Separation

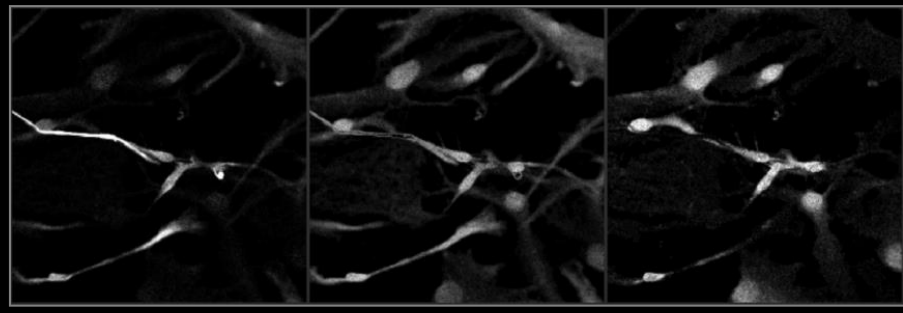
Parameters to fit

Parameter	Fit
Decay Time 1	
Decay Time 2	
Decay Time 3	
Amplitude 1	✓
Amplitude 2	✓
Amplitude 3	✓
Tail Offset	✓
IRF Background	
IRF Shift	

FLIM Image Fit
3 Components



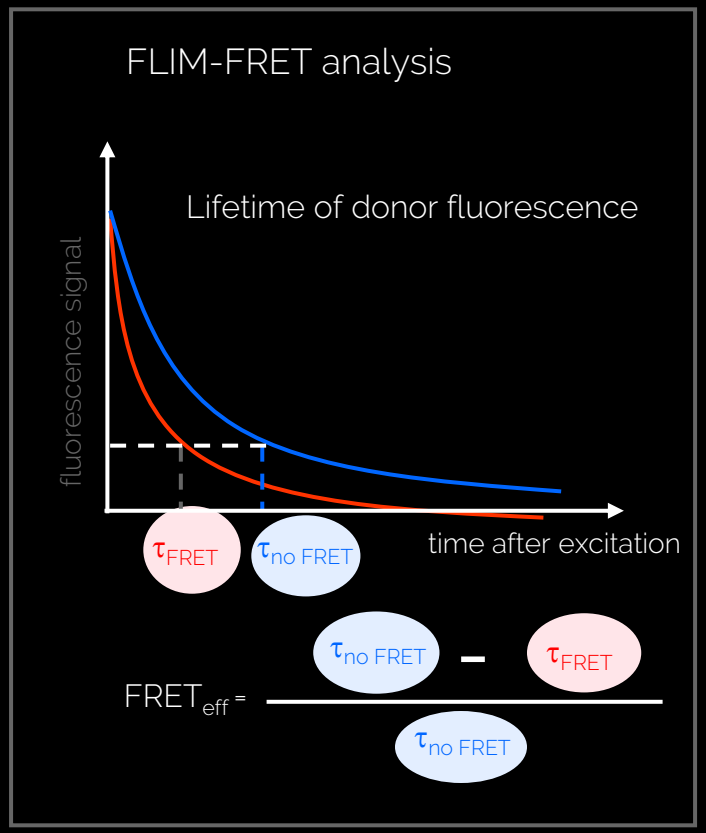
0.75 ns **1.84 ns** **6.30 ns**



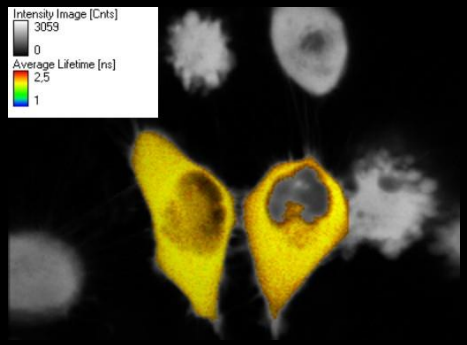
FLIM-FRET?

- **Donor lifetime shortens**

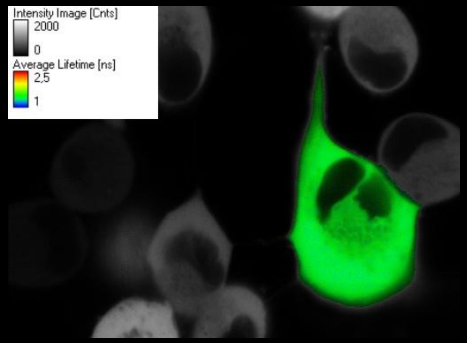
  FRET efficiency is calculated from the difference between arising fast component in donor lifetime in the presence of the acceptor and original lifetime in the absence of the acceptor



FLIM-FRET (CFP-YFP) in live cells



Donor only (CFP)



FRET pair (CFP-YFP tandem)

Donor lifetime images of FRET and control cells:

Sample: RBKB78 cells transfected with a CFP donor only or CFP-YFP fusion.

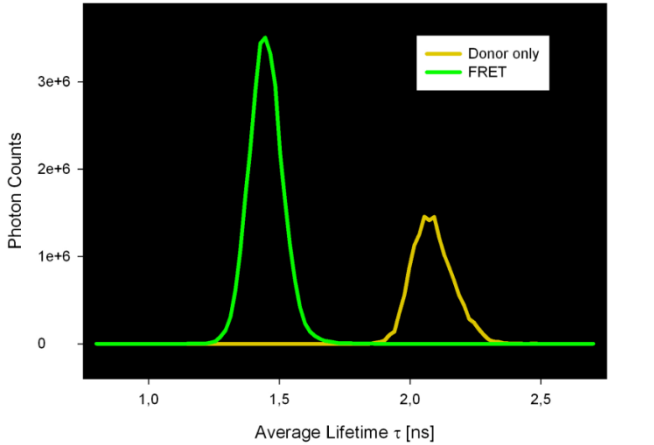
Data Acquisition: The detection band was set between 445-495 nm. Excitation @ 405 nm

Data Analysis: The coloured region has been used for analysis. Colours represent intensity modulated fluorescence lifetimes.

Result: In the presence of acceptor the donor lifetime is decreased.

Courtesy: G. Hams, University of Würzburg

FLIM-FRET (CFP-YFP) in live cells: Quantitative data analysis



Fluorescence lifetime distribution histogram of **donor only (yellow)** and **FRET (green)** samples using average lifetimes.
There is a clear shift of 0.7 ns towards shorter lifetimes in the FRET sample.

From lifetime distribution histograms one obtains:

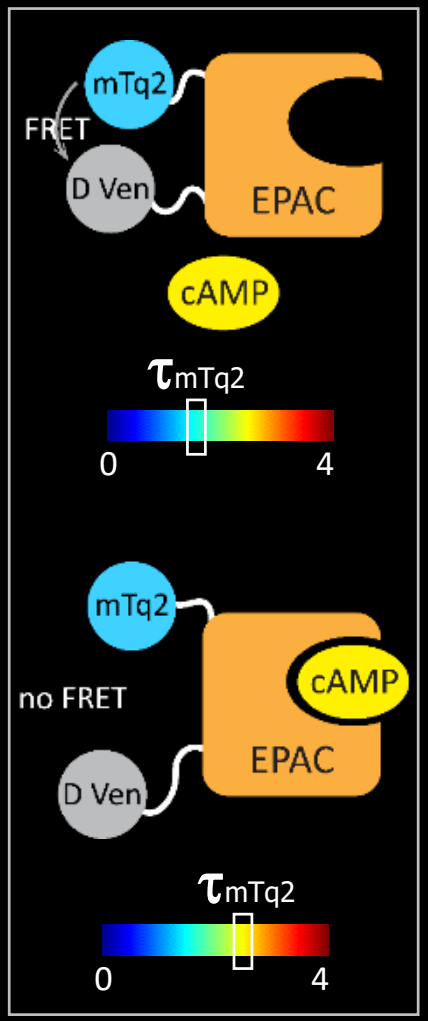
- average lifetime of the donor is: 2.1 ns.
- donor lifetime of the FRET construct is: 1.4 ns.
- FRET efficiency is: E = 30%.

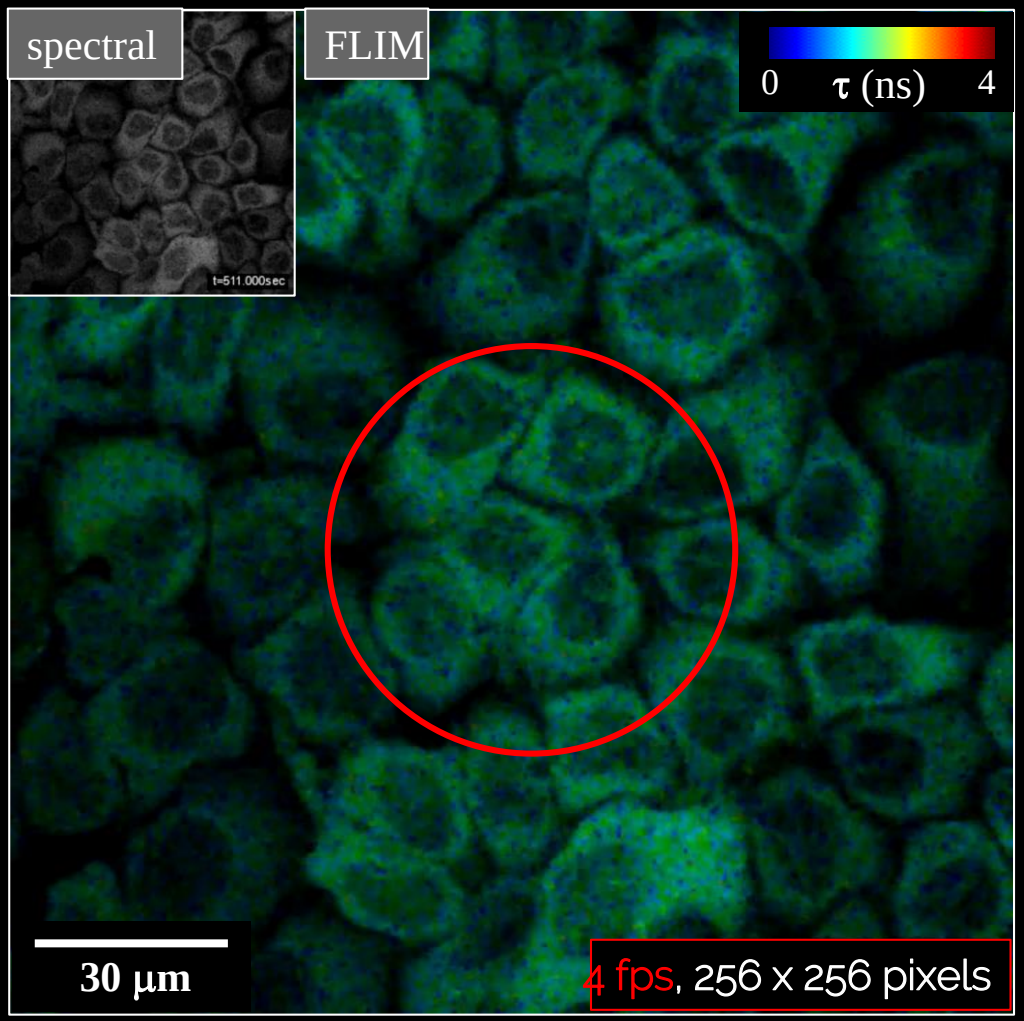
Computation of FRET Efficiency:

$$E = 1 - \frac{\tau_{quench}}{\tau}$$

Key application: Molecular interaction

cAMP signalling on-the-fly with a FLIM-FRET sensor



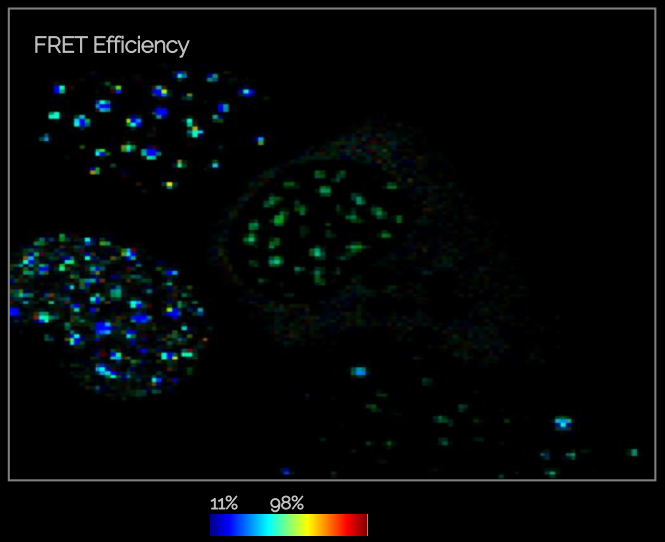


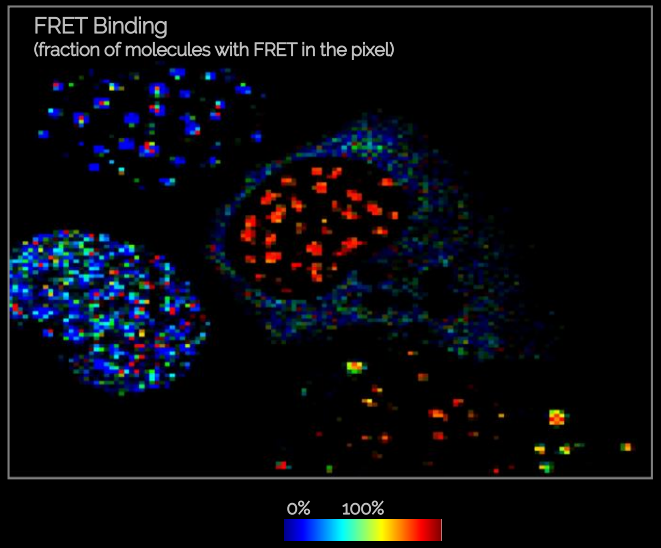
Caged cAMP in HeLa cells expressing EPAC mT2-dVenus FRET sensor.
EPAC response to UV-mediated cAMP uncaging.
Courtesy Kees Jalink, Bram van den Broek, NKI Amsterdam.

- FLIM-FRET

Parameters to fit

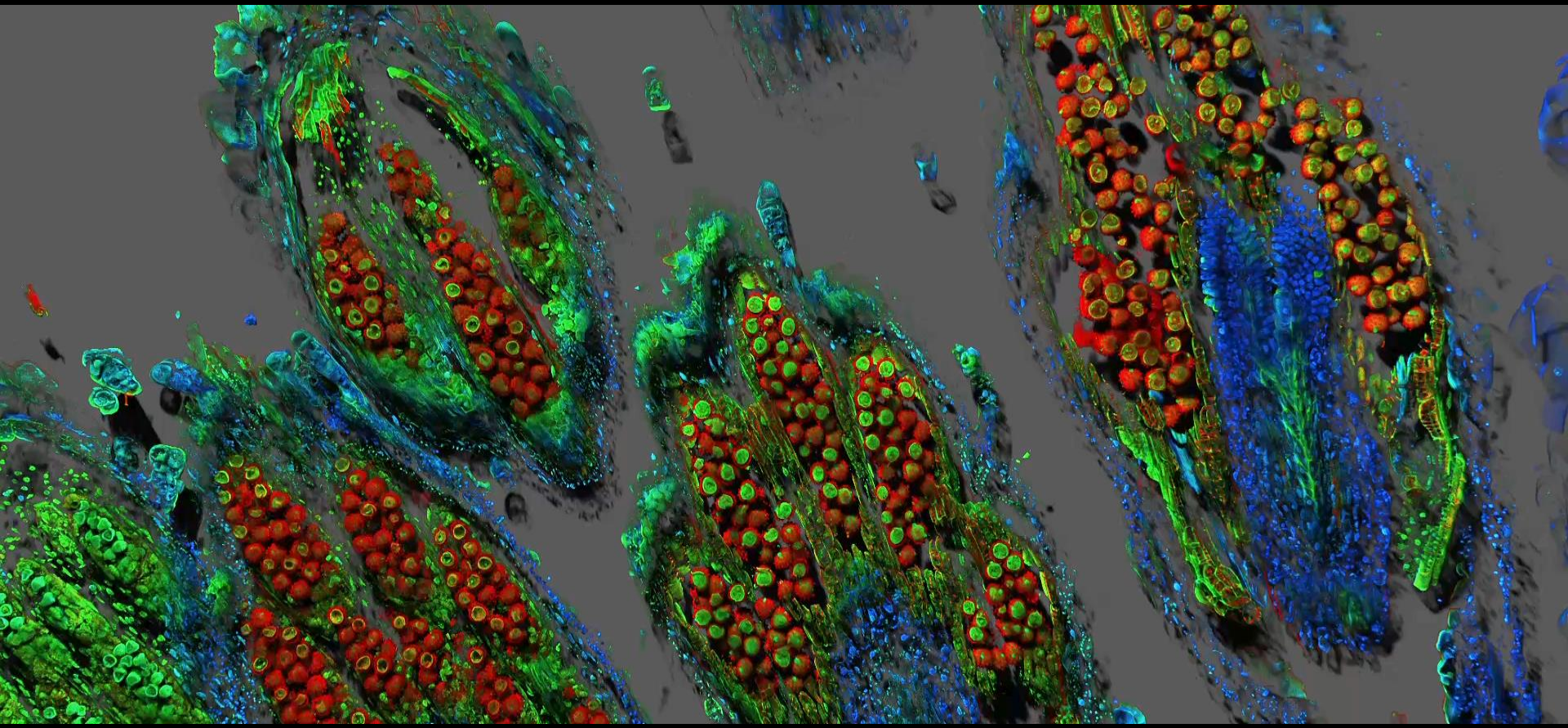
Parameter	Fit
Unquenched Donor Lifetime	
Unquenched Donor Amplitude	✓
Quenched Donor Lifetime	✓
Quenched Donor Amplitude	✓
Tail Offset	✓
IRF Background	
IRF Shift	



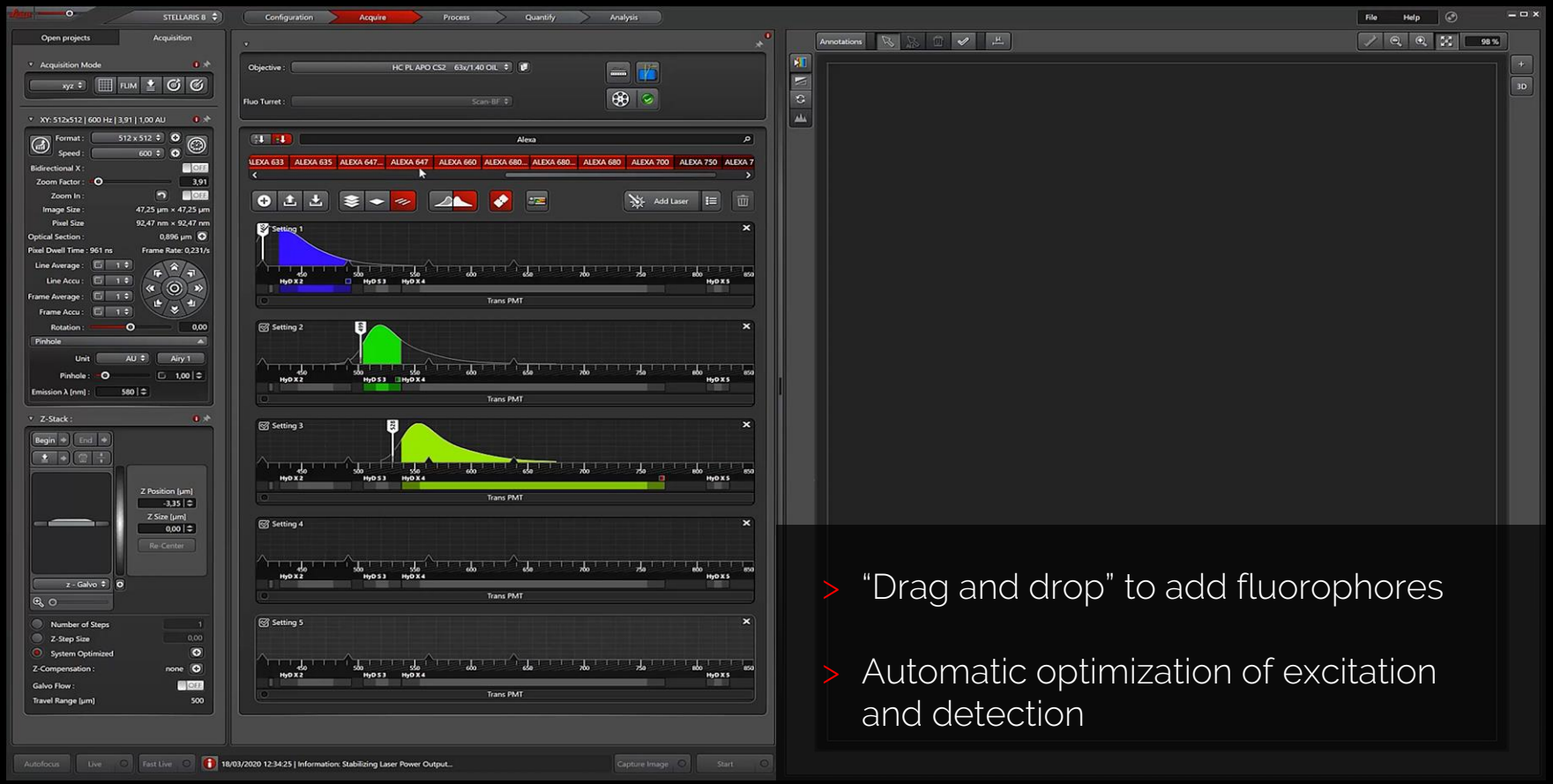


PRODUCTIVITY

DO MORE



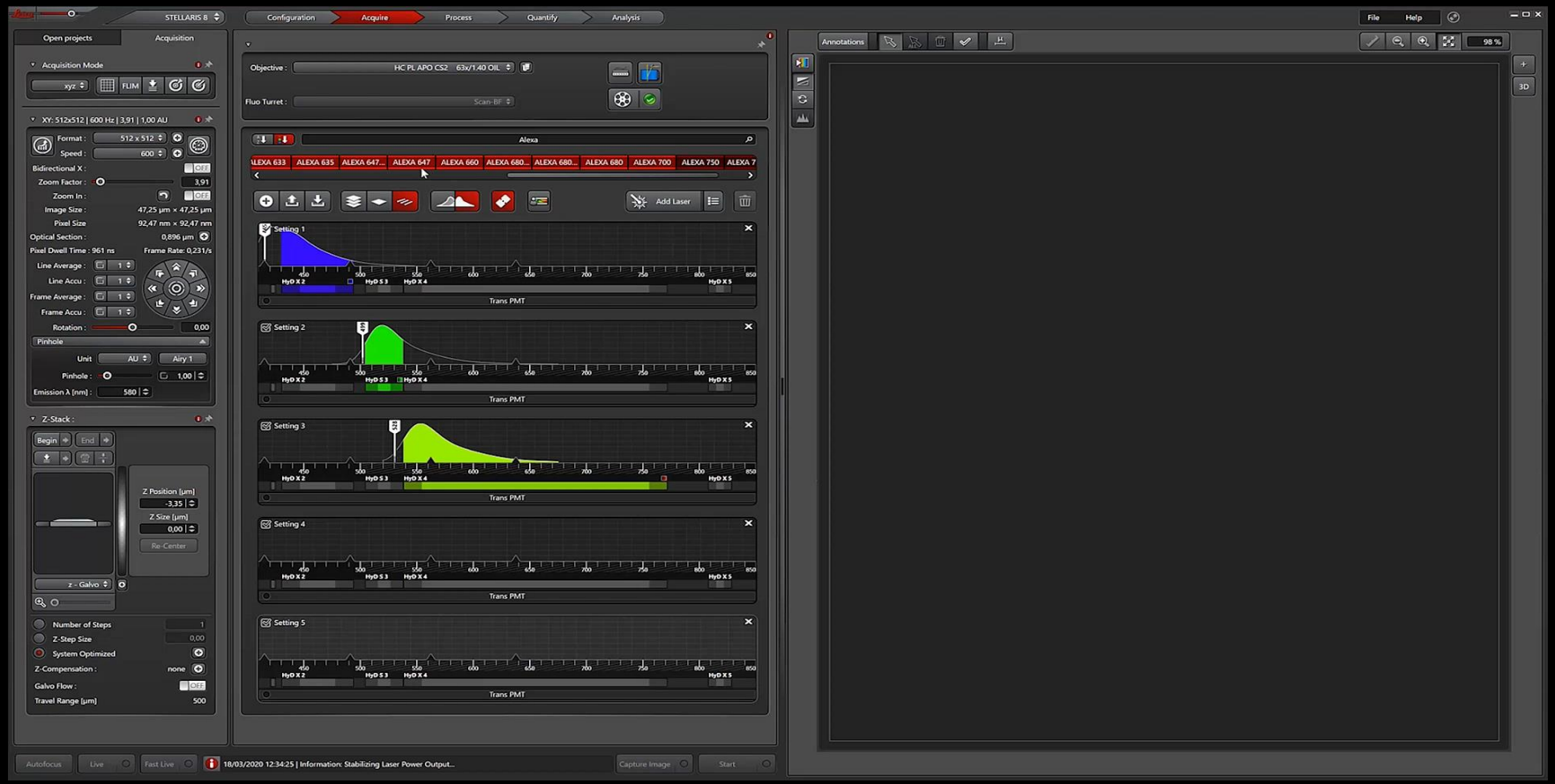
Simple, Even For Complex Experiments



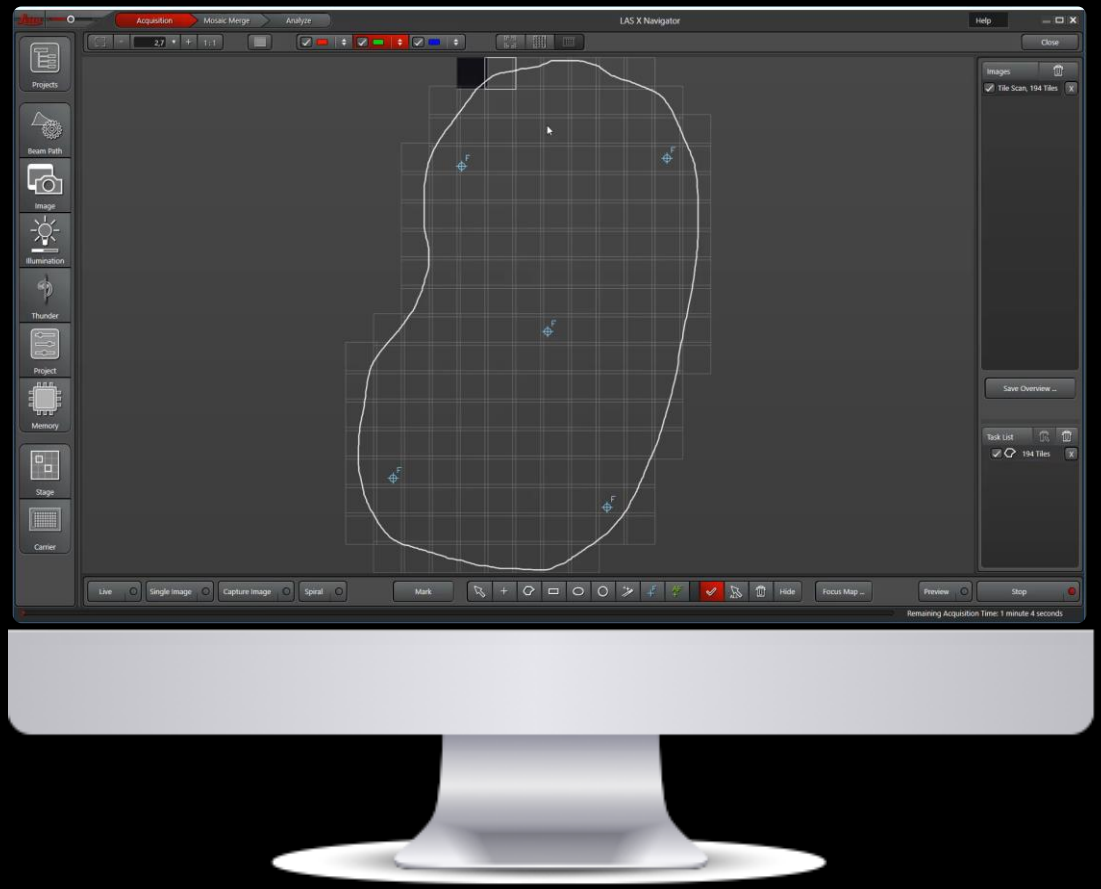
> “Drag and drop” to add fluorophores

> Automatic optimization of excitation and detection

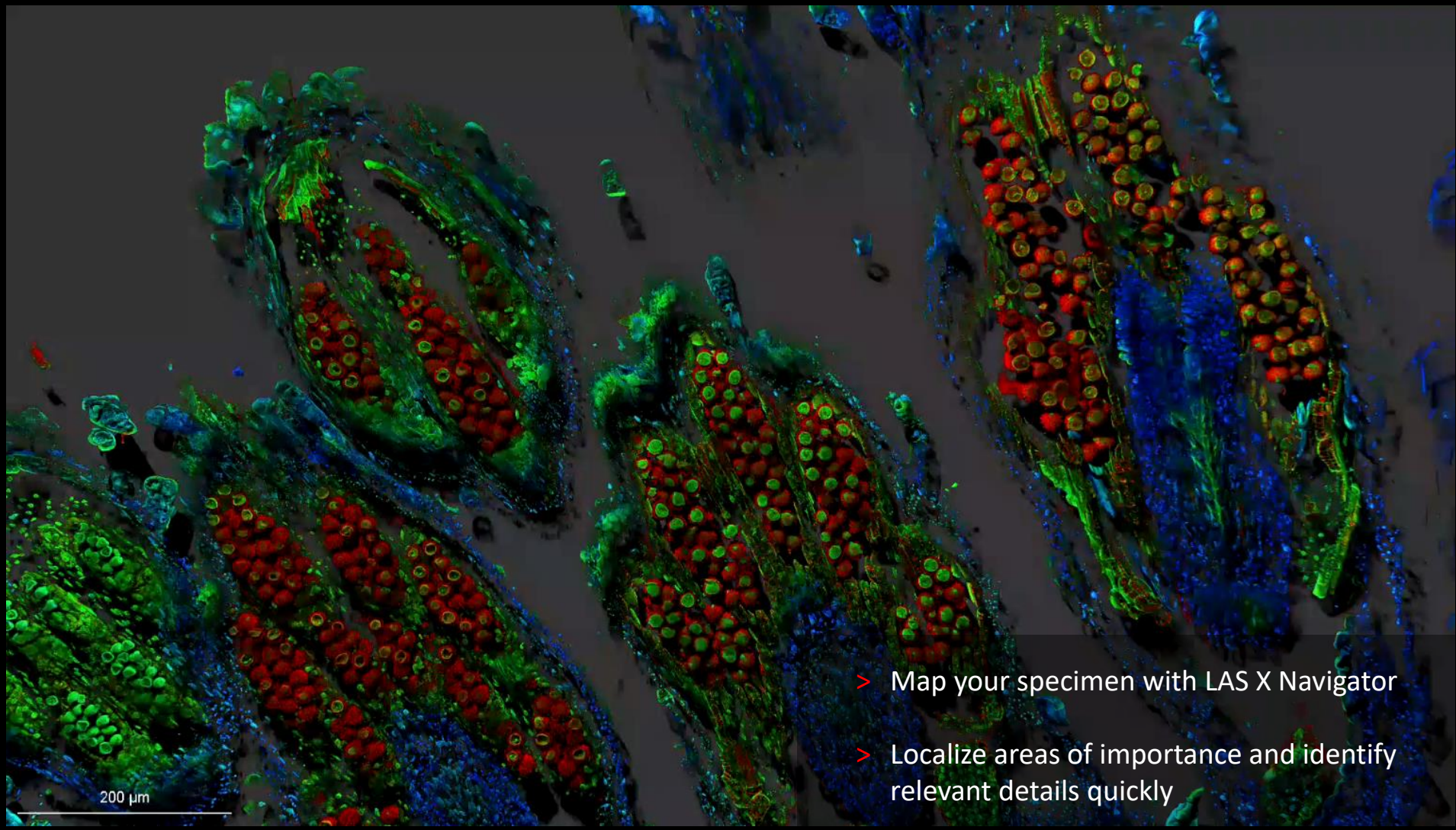
Simple, Even For Complex Experiments – Image Compass



LAS X software - Navigator



Relevant Details Instantly Identified



> Map your specimen with LAS X Navigator

> Localize areas of importance and identify relevant details quickly

Daisy pollen. Image acquired with TauContrast and LAS X Navigator.

Leica

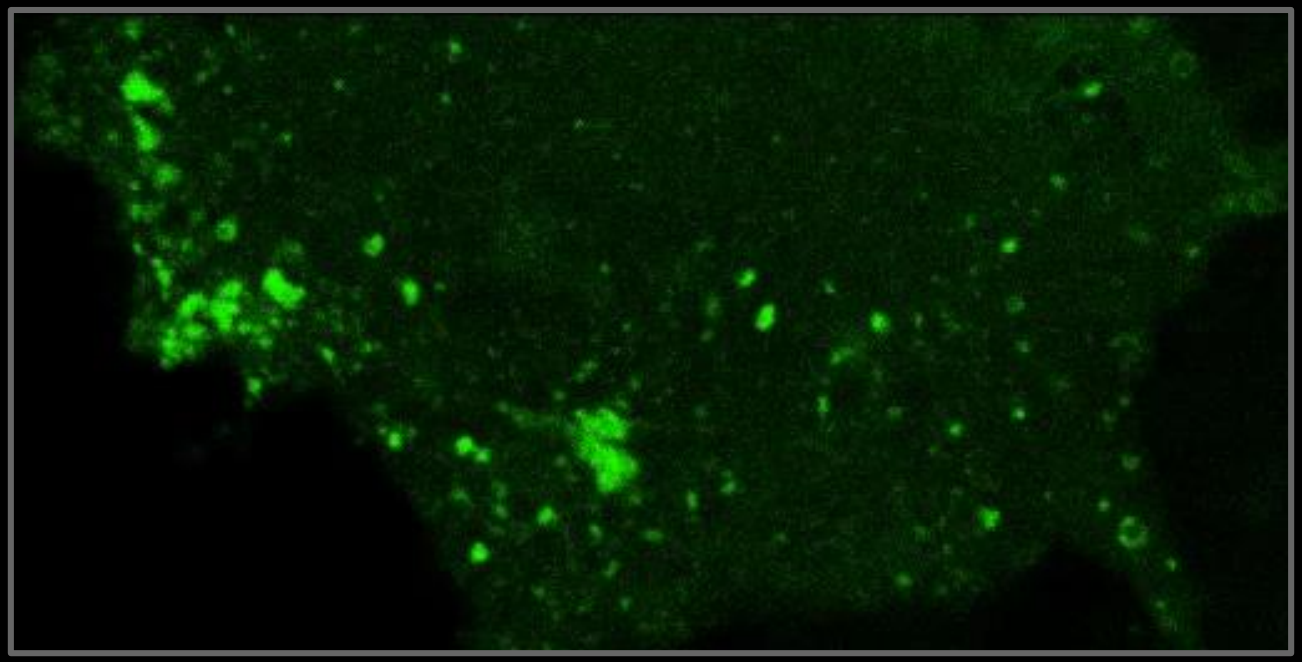
Tandem Scanner

- Tandem Scanner with 8 kHz

- Fits in well with
  - SuperZ
  - Galvoflow
  - Power HyD

Scan format	8 kHz [fps]
512 x 512	28
512 x 32	145
512 x 16	286

50 fps, Resonant Scanner



Hrab5a_GFP, vesicle dynamics in live cell imaging, Sample Courtesy of Dr. Sandra Ritz, Microscopy & Histology Core Facility, Institute of Molecular Biology gGmbH (IMB), Mainz, Germany. Transfection of EGFP by Marino Zerial

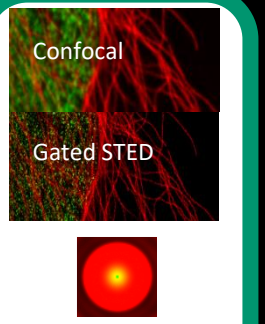
LEICA STELLARIS

Nano — Macro

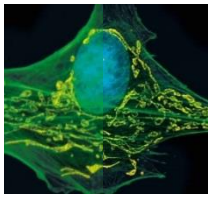
50 nm | 100 nm | 200 nm | 300 nm | ~ um

30 nm | 140 nm | 180 nm

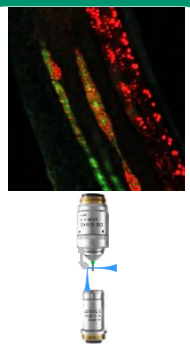
775STED+gSTED	Confocal SR	Confocal microscopy	Digital light sheet



775 pulsed STED



Lightning



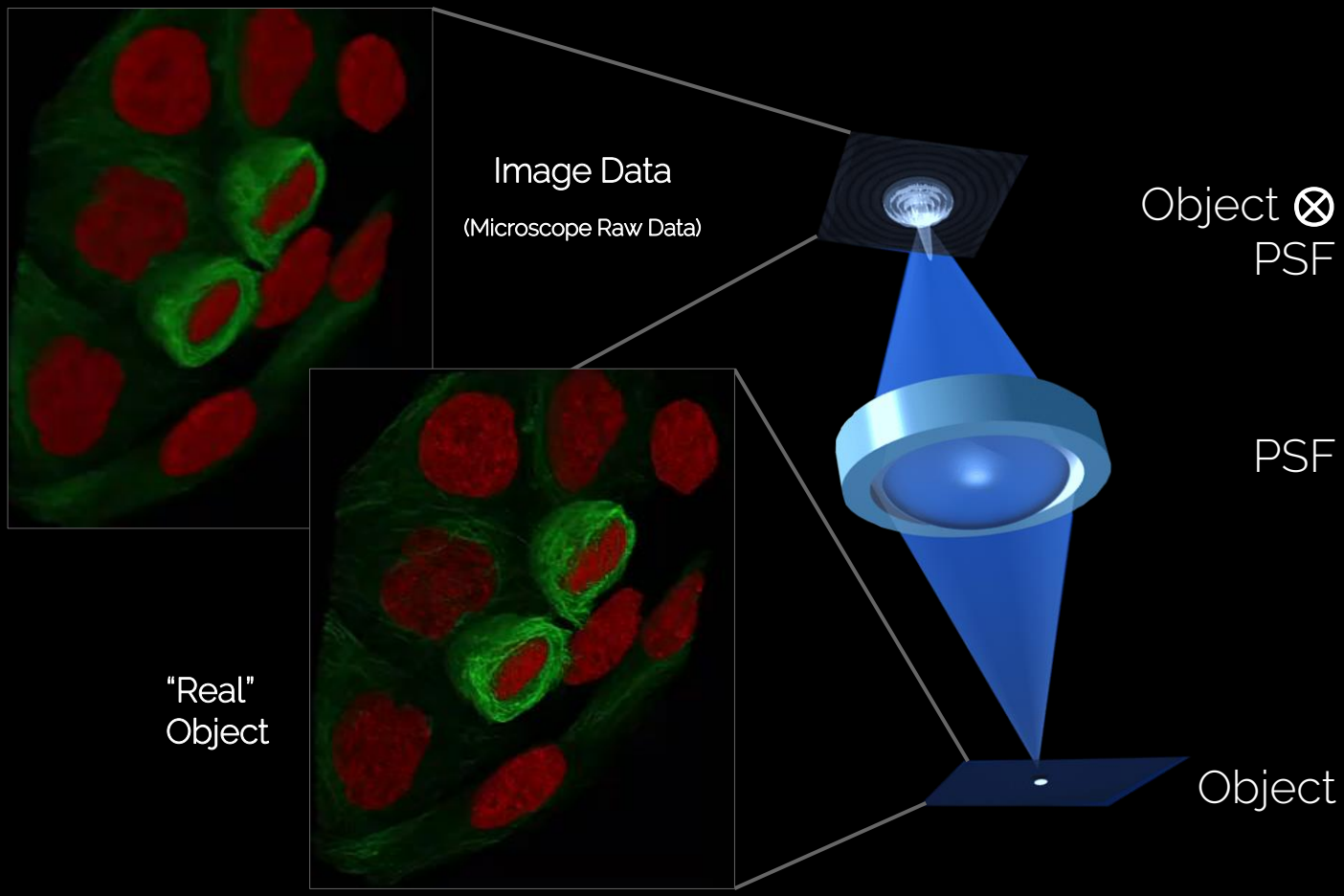
DLS

STELLARIS

LIGHTNING

Image Information Extraction

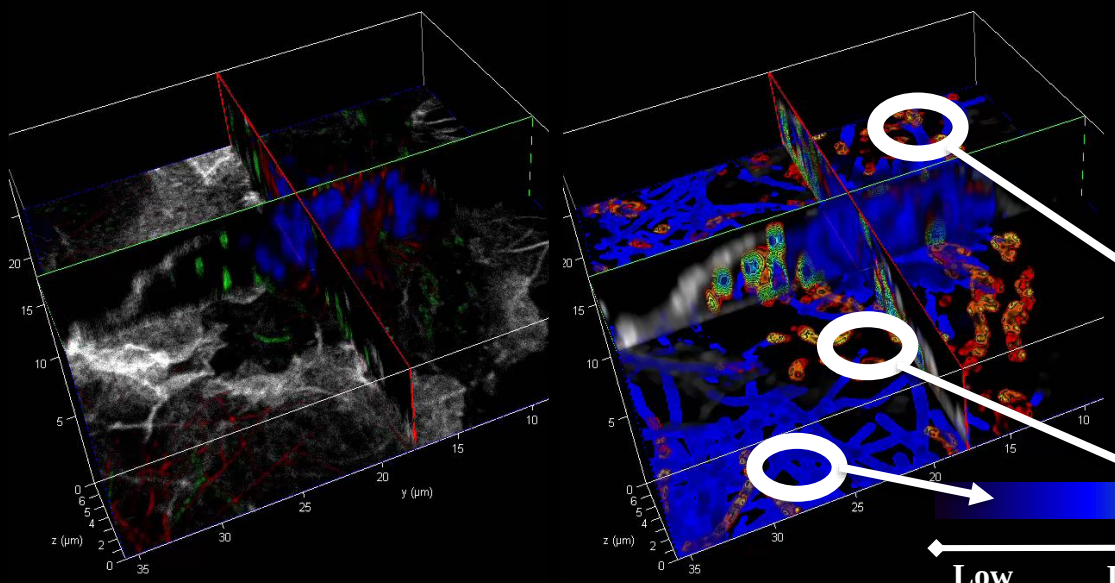
Microscopy Image Formation



Raw data

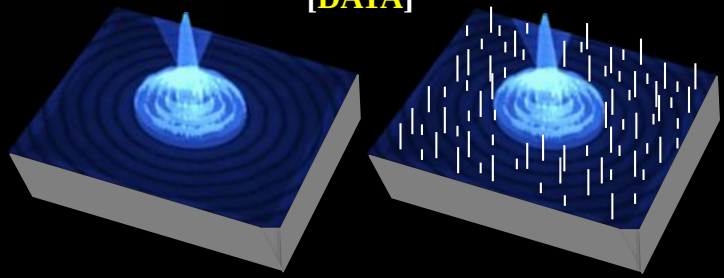
Decision Mask

Position dependant image quality



Low IMAGE QUALITY High

[DATA]

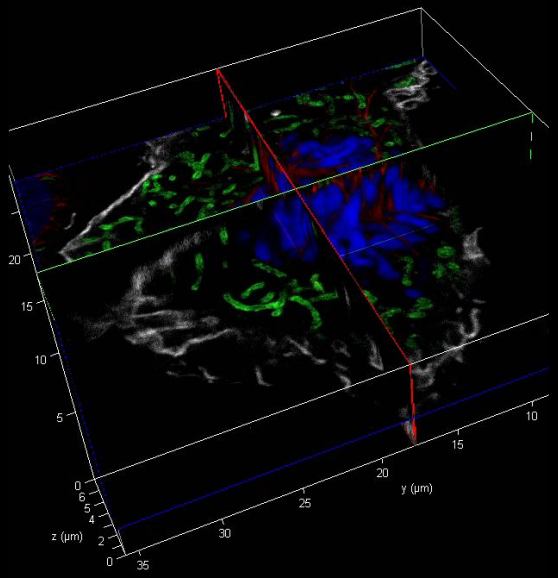


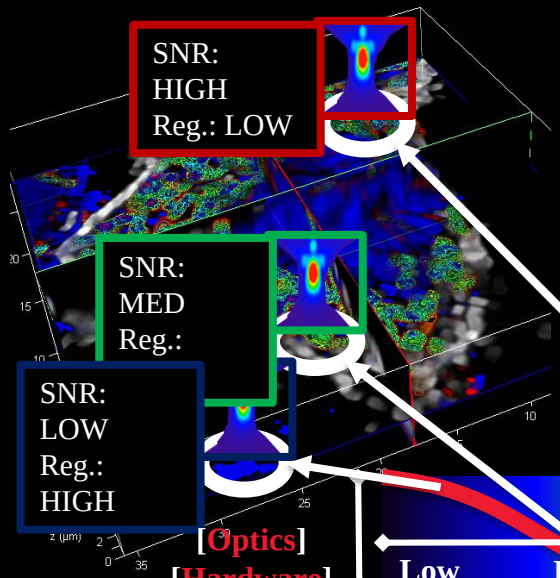
GATTA cells

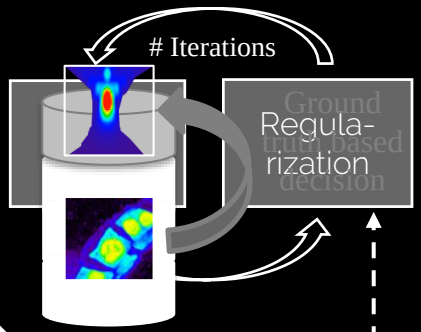
Raw data

Decision Mask

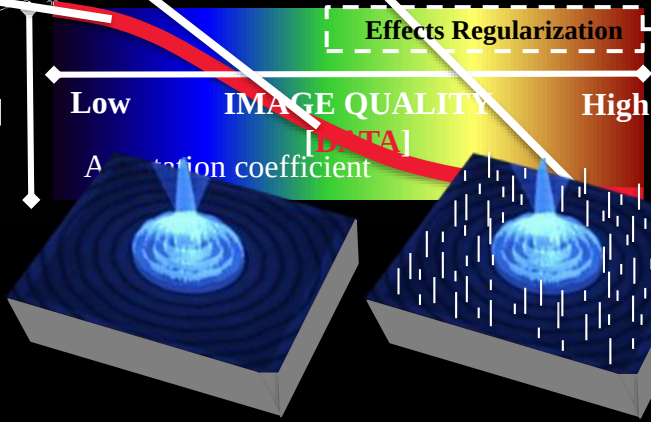
Adaptive Deconvolution





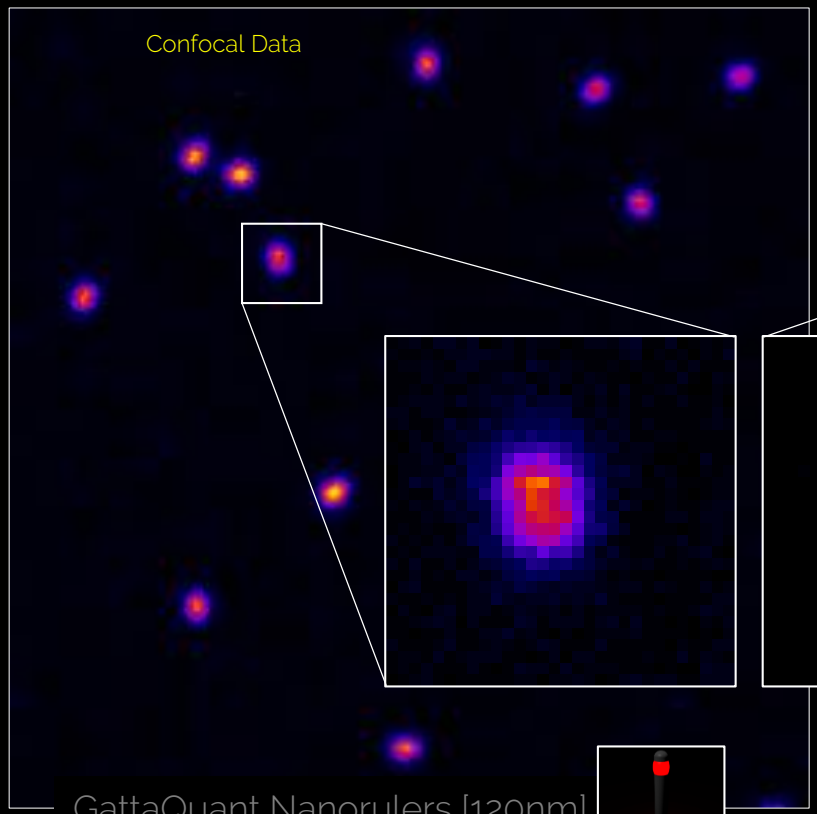


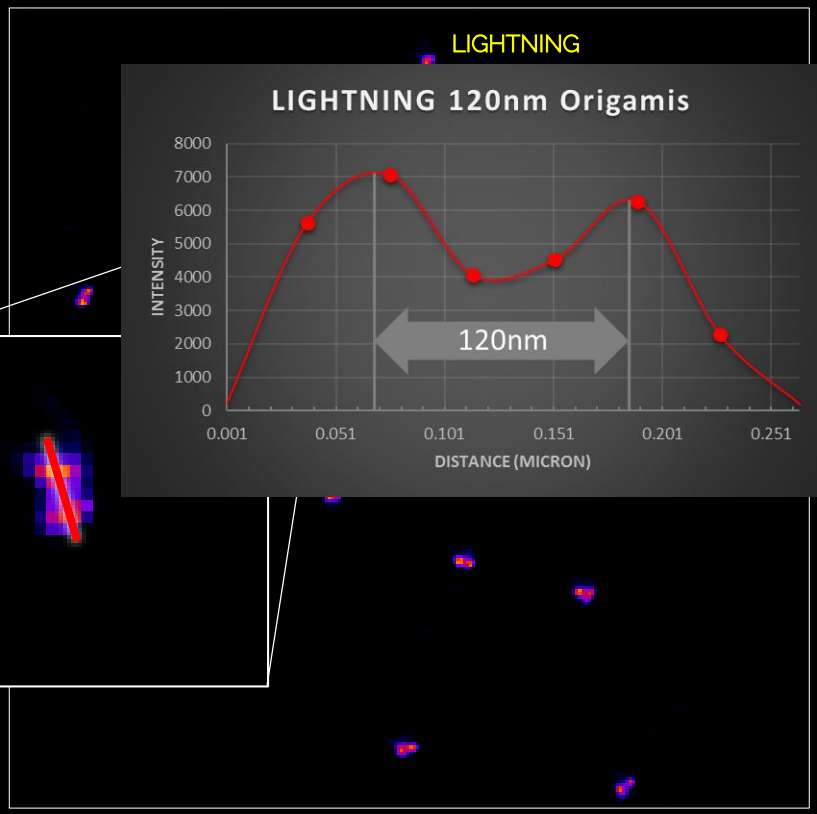
[Optics]
[Hardware]
[Modality]



Gatta cells

Accessing Super-Resolution



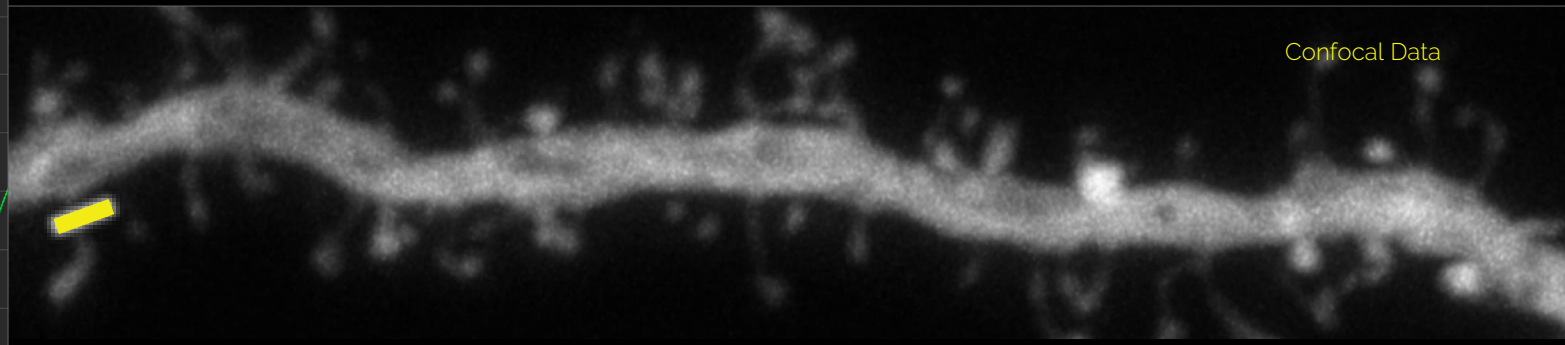


GattaQuant Nanorulers [120nm]

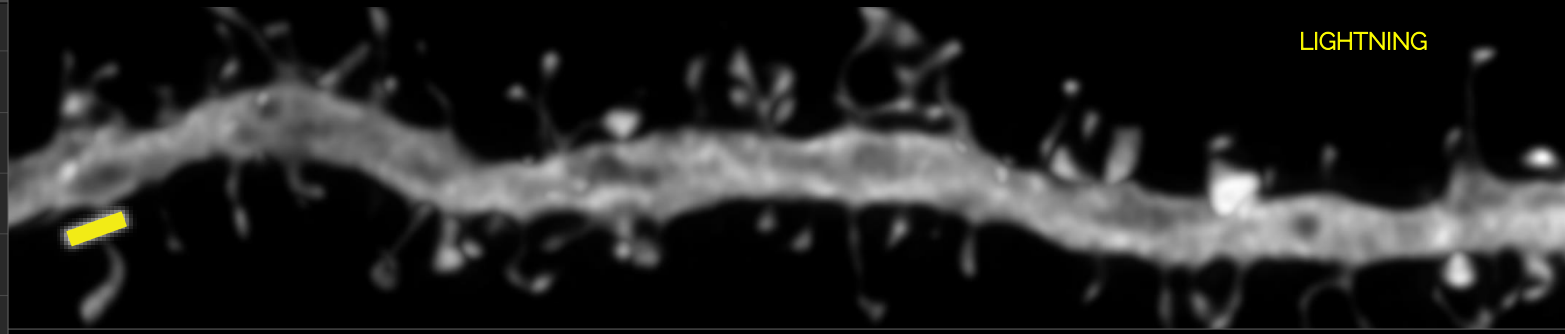
Accessing Structural Information

MAX projection

Confocal Data

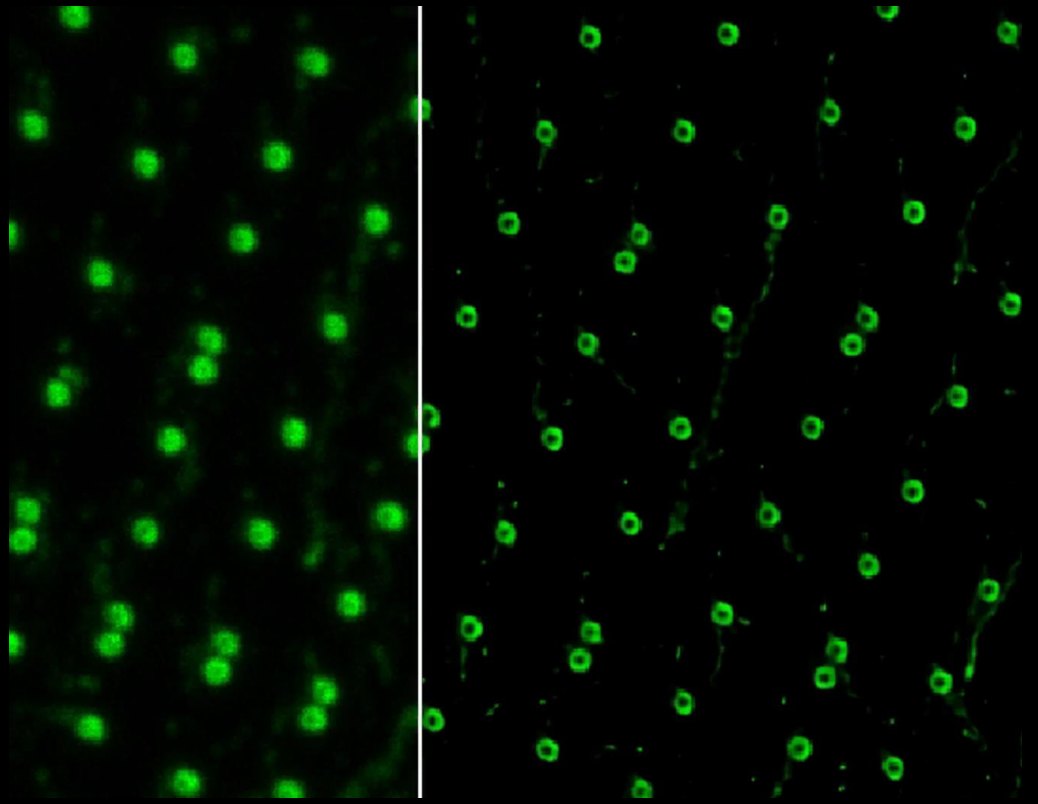


LIGHTNING



Sample: courtesy of Dani Dumitriu, PhD, MD Neuroscience, Icahn School of Medicine, New York, US

LIGHTNING: Accessing The True Nature Of Image Data

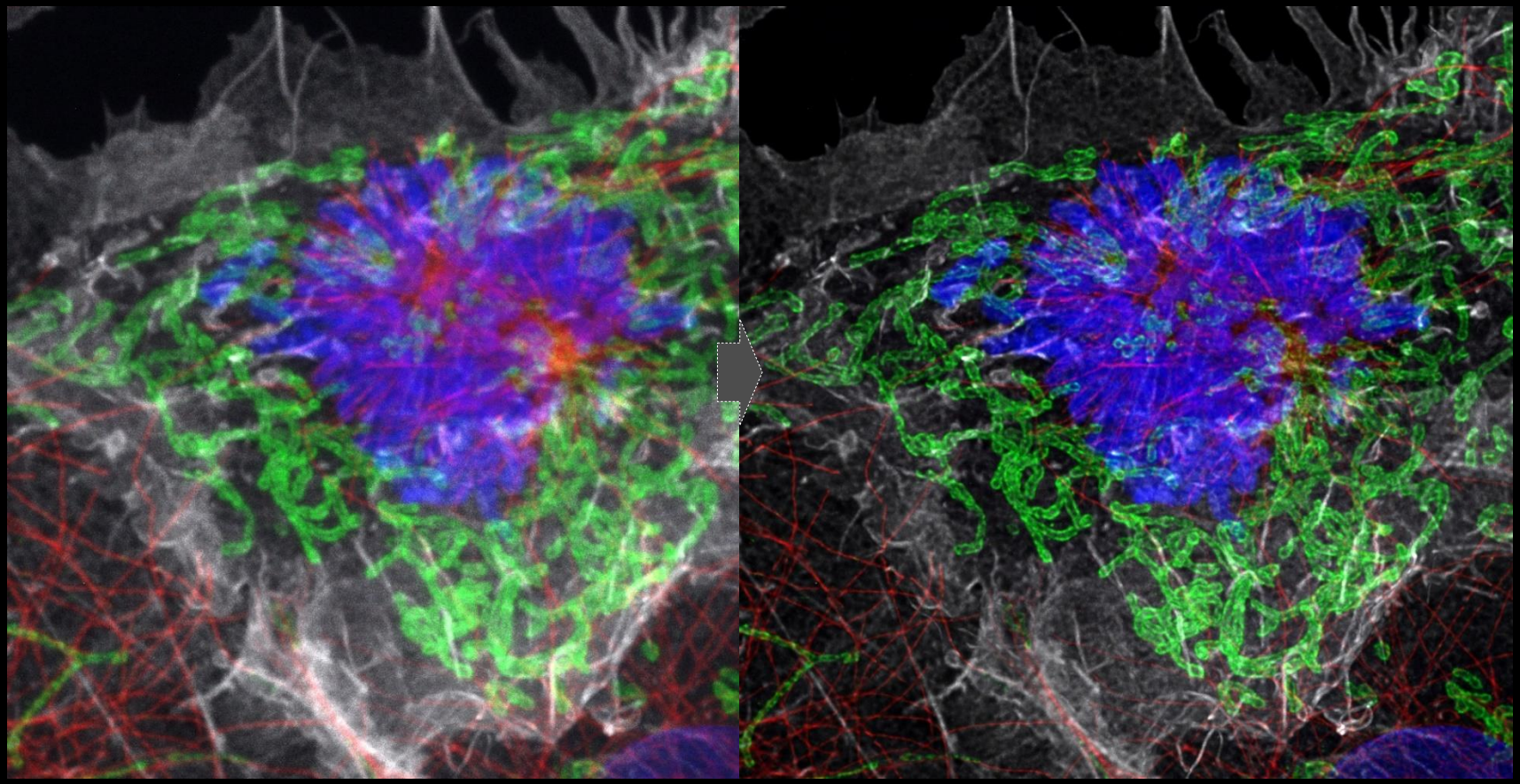


Confocal | MP | gated STED

Including every imaging modality

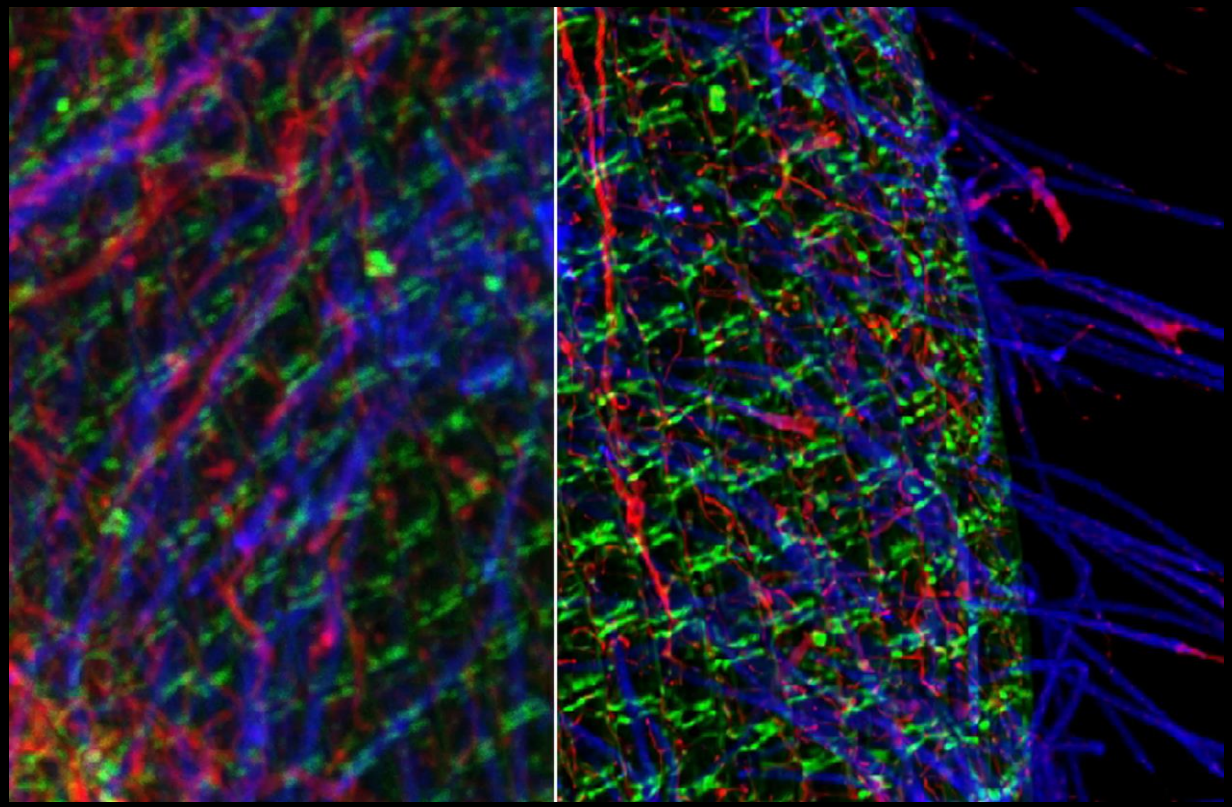
LIGHTNING: Adaptive Multicolor Super-Resolution

Adaptive Deconvolution



Gatta cells

LIGHTNING: Adaptive Multicolor Super-Resolution



Confocal | MP | gated STED

Including every imaging modality

Key Features of Lightning

-Leica Confocal Superresolution Microscopy-

- dxy~120nm *vs* dxy cofocal~180nm;
  dz~350*nm vs* dz confocal~lambda(500nm)
  4x contrast better

- **Simultaneously multicolor confocal superresolution**

- **As same speed as confocal scan**

- High-speed multicolor live imaging

- Std. application for STELLARIS

STELLARIS

Leica