FLIM, Spectral FLIM, Phasors

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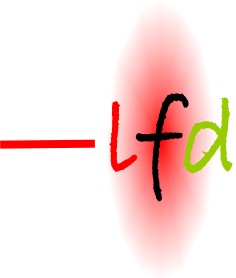


http://www.lfd.uci.edu/

Why do FLIM?

FLIM is used for :

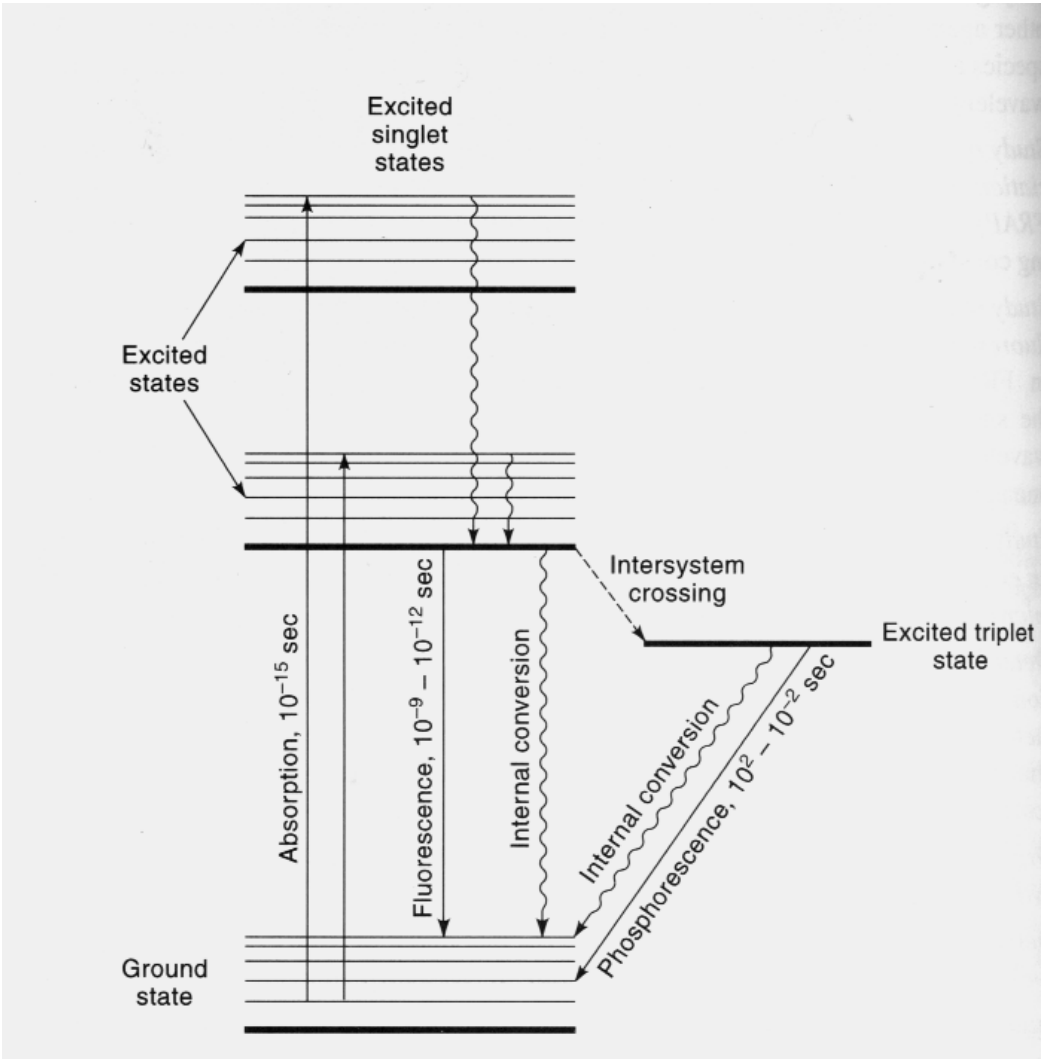
- **FRET**
- **Intracellular mapping of Ion concentration and pH imaging**
- **Biochemical reactions (oxidation/reduction) processes**
  - **NAD and NADH**
- **Long lifetime imaging (phosphorescence).**
  - **For example O_2 concentration in the cell or in tissues**

Time Resolved Fluorescence

- What's happening during the time of the fluorescence emission?

- Fluorescence Lifetime

Fluorescence Quantum Yield φ: important for dyes
Ratio of the rate of fluorescence and the sum of the rates that depopulate the excited state



Quantum Yield:

Can be expressed as the lifetime

$$\phi = \frac{k_f}{k_f + k_{isc} + k_{nonrad}}$$

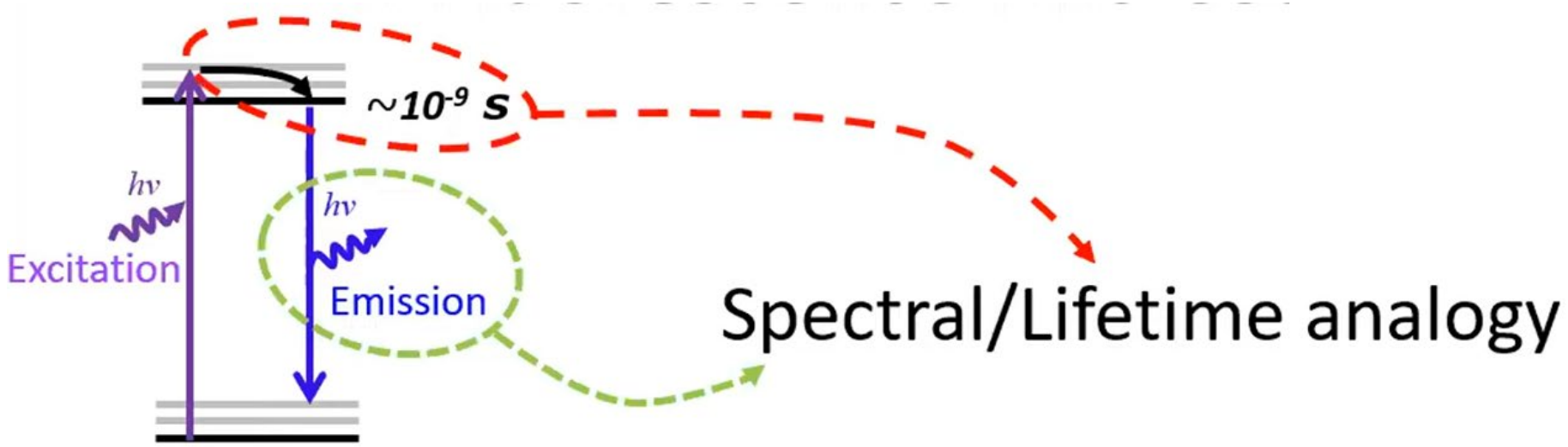
$$\tau_0^{-1} = k_f$$

Natural lifetime:
Inverse of the fluorescence emission rate

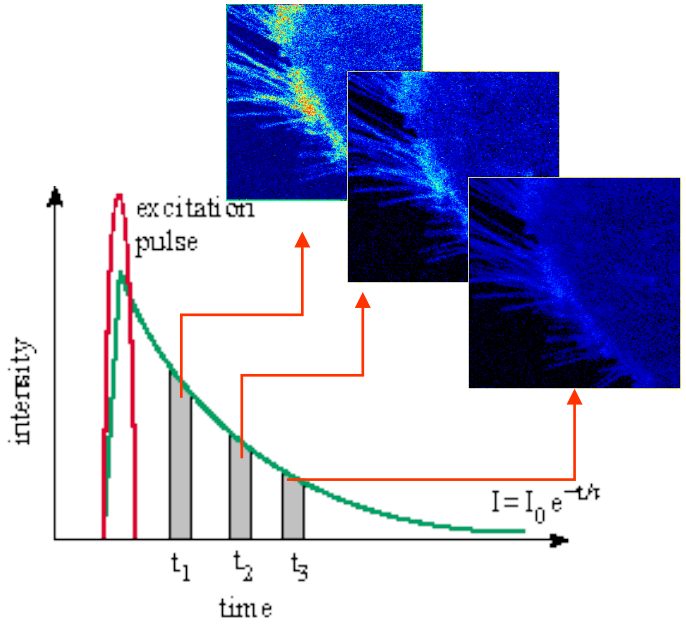
$$\tau^{-1} = k_f + k_{isc}$$

Measured lifetime is sum of
Rates of natural lifetime
and non radiative decay paths

Fluorescence Lifetime Imaging Microscopy (FLIM)



How to calculate the components g and s of a phasor from the time decay?



A sample is flashed many times by a short duration laser source

The interval between the excitation flashes, and 1st excited photon is measured

Measuring fluorescence lifetime using a field programmable gate array (FPGA)

- Our approach uses serial detectors in the photon counting mode, and the digital heterodyning method to acquire data which is directly analyzed in the frequency domain.
- the sampling windows slide through the entire period of the emission response due to the slight difference in frequencies, for a total of 256 steps

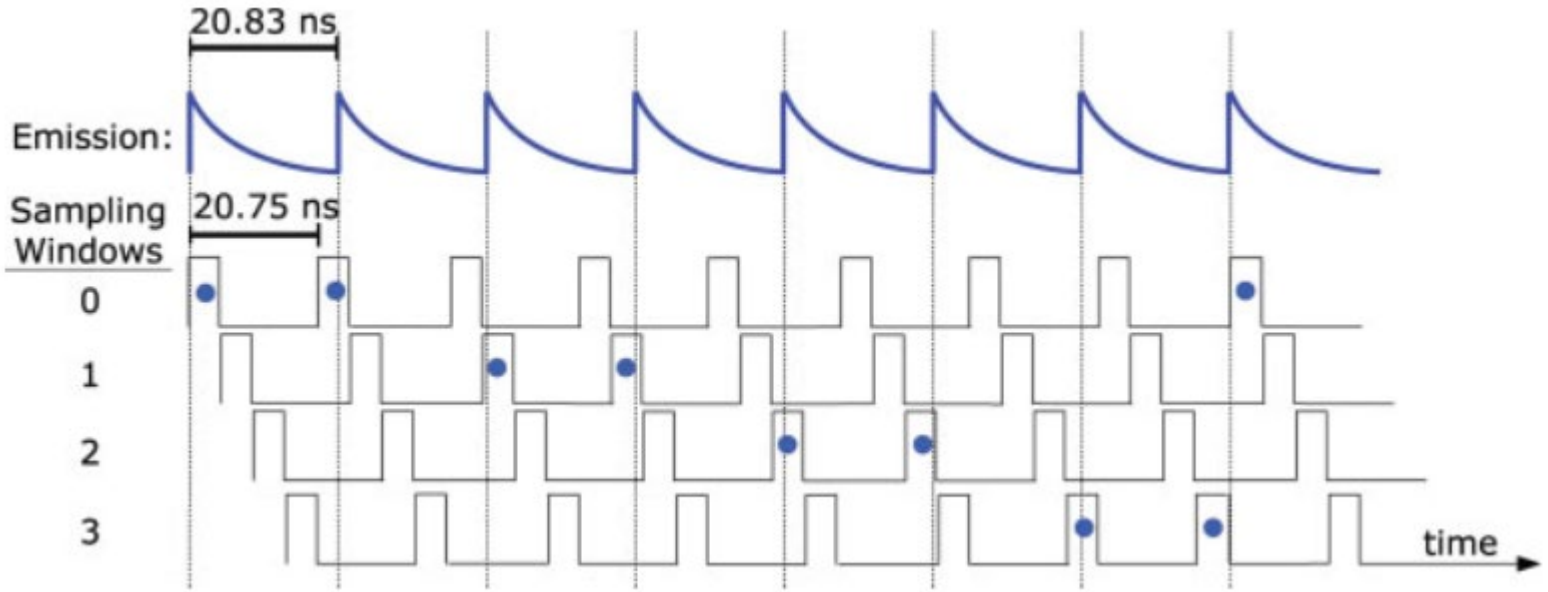
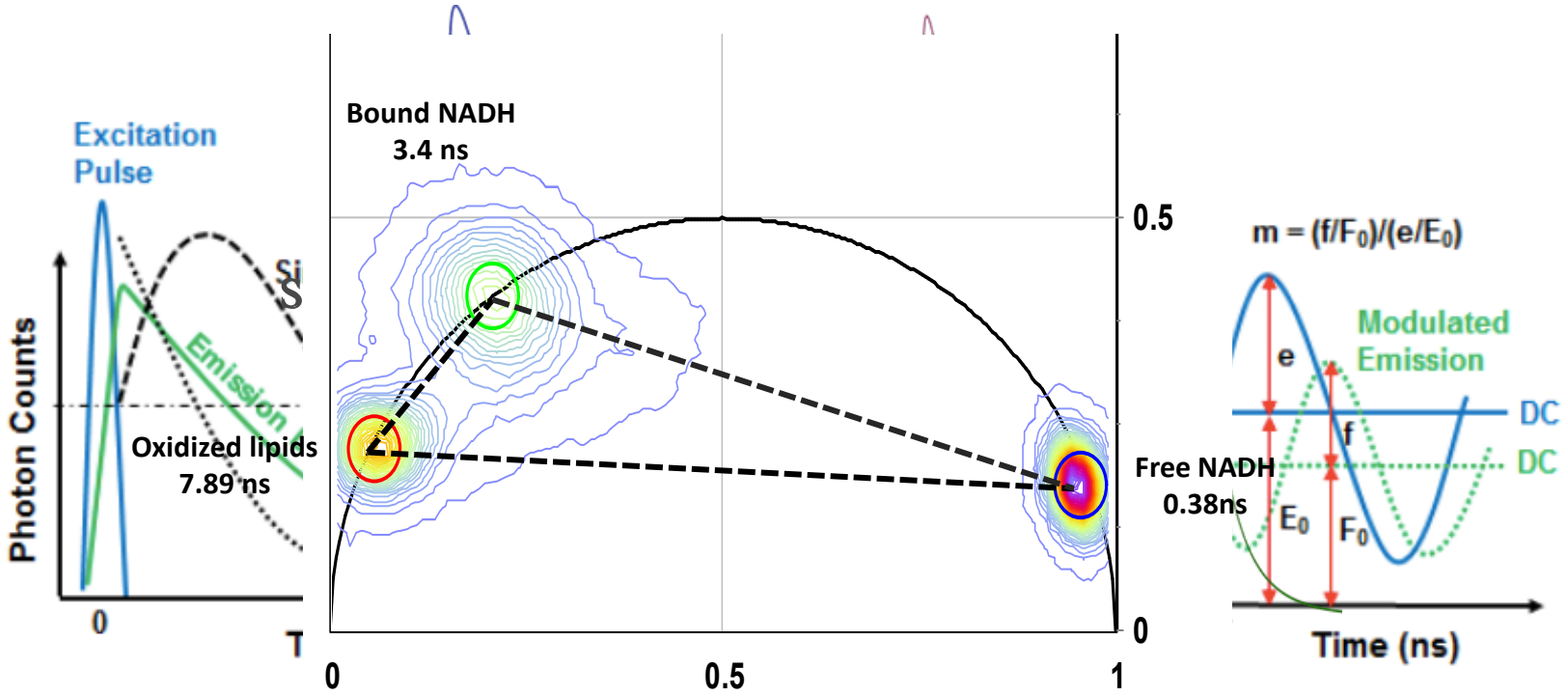


illustration of the digital heterodyning principle
Colyer et al.

Colyer RA, Lee CY, Gratton E. Microsc Res Tech. 2008; 71(3): 201-213.

Phasor - A Graphic Representation of the Raw FLIM Data



$$g_i(\omega) = \int_0^\infty I(t)\cos(\omega t)dt \,/\, \int_0^\infty I(t)dt$$

$$s_i(\omega) = \int_0^\infty I(t)\sin(\omega t)dt \,/\, \int_0^\infty I(t)dt$$

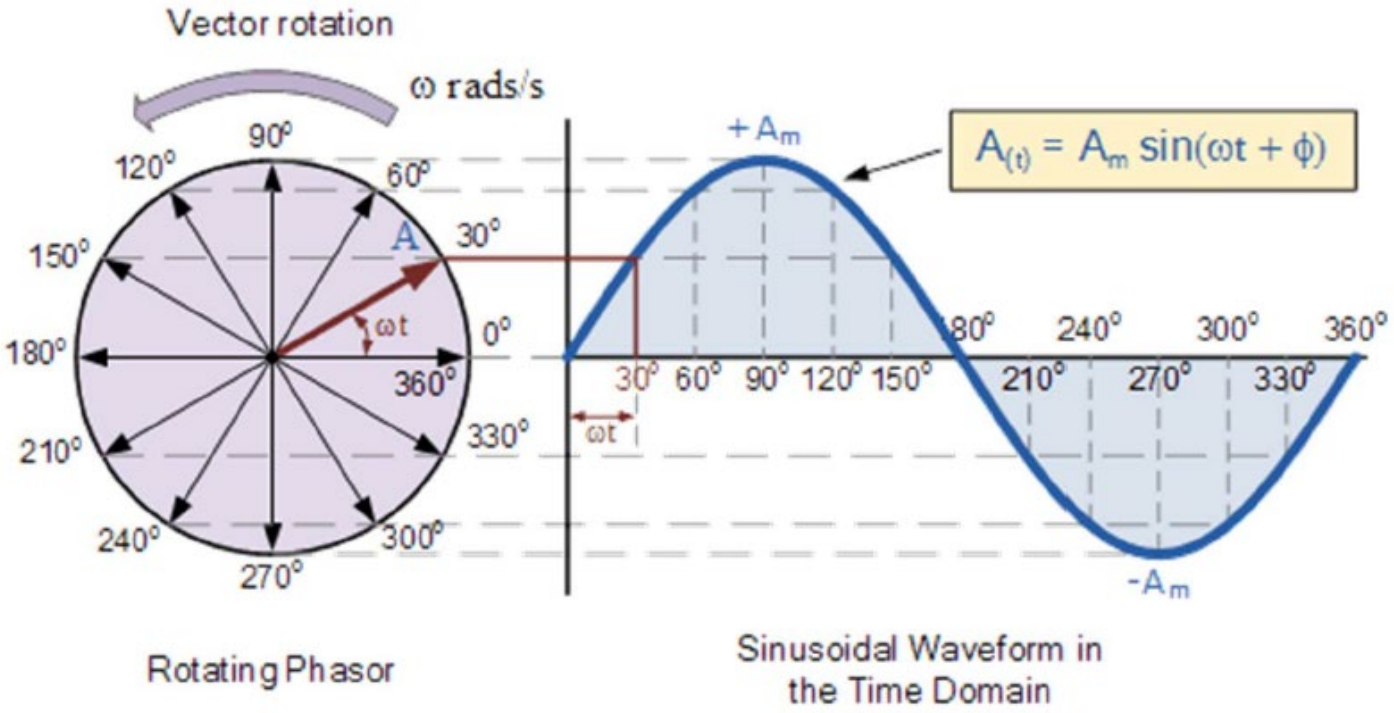
$$g(\omega) = m\cos(\varphi) \quad s(\omega) = m\sin(\varphi)$$

Digman M.A. et al Biophys J. 2008; 94(2): L14-16. PMC2157251

The Phasors

- The phasor representations have been used since the late 19th century in electrical engineering applications (the word phasor comes from “phase vector”)

For example, phasors are used to describe alternating current (AC) circuits



The Phasors

Resolution of the Fluorescence Lifetimes in a Heterogeneous System by Phase and Modulation Measurements

Gregorio Weber

Department of Biochemistry, School of Chemical Sciences, University of Illinois, Urbana, Illinois 61801 (Received: August 12, 1980)

A closed-form procedure is described for the determination of the decay constants and the relative contributing intensities of the N independent components of a heterogeneous fluorescence emission employing measurements of the phase shift and relative modulation of the total fluorescence at N appropriate harmonic excitation frequencies. At each frequency the phase and modulation measurements yield the real part of the Fourier transform of the fluorescence impulse response, G, and its imaginary part, S. It is shown that the moments of a distribution of the lifetimes are linear combinations of the Gs (zero and even moments) or the Ss (odd moments), and the rule for the construction of the coefficients of G and S in these linear combinations is derived. The classical de Prony method is used to obtain the lifetimes and fractional contributions of the components from the moments. For binary and ternary mixtures the numerical computations required are trivial. In the present state of the art, the lifetimes of the components of a binary mixture should be derivable with a loss in precision somewhat smaller than 1 order of magnitude with respect to the overall measured lifetimes.

$$G_r = M_r \cos \Phi_r = [(1 + (\omega_r \tau_r^P)^2)(1 + (\omega_r \tau_r^M)^2)]^{-1/2} \quad (9)$$

$$S_r = M_r \sin \Phi_r = G_r \omega_r \tau_r^P \quad (10)$$

Frequency domain

$$G(\omega) = \int_0^\infty I(t) \cos \omega t \, dt$$

$$S(\omega) = \int_0^\infty I(t) \sin \omega t \, dt \quad (55)$$

Time domain

G. Weber J. Phys. Chem., 85 (1981), pp. 949-953

D.M. Jameson, E. Gratton, R.D. Hall
Appl. Spectrosc. Rev., 20 (1984)

The Phasors

- The phasor approach in fluorescence has been around since 80's but was dormant until the last decade until it was applied to FLIM *although with different names

Tom Jovin
Andrew Clayton
Quentin Hanley

→ AB Plots

Q.S. Hanley, A.H.A. Clayton J. Microsc., 218 (2005), pp. 62-67

Bob Clegg

→ Polar plots

Redford, G.I., Clegg, R.M.. *J Fluoresc* **15**, 805–815 (2005).

Enrico Gratton
Michelle Digman

→ Phasor Plots

M.A. Digman, V.R. Caiolfa, M. Zamai, E. Gratton Biophys. J., 94 (2008), pp. 14-16

Book1 - Microsoft Excel

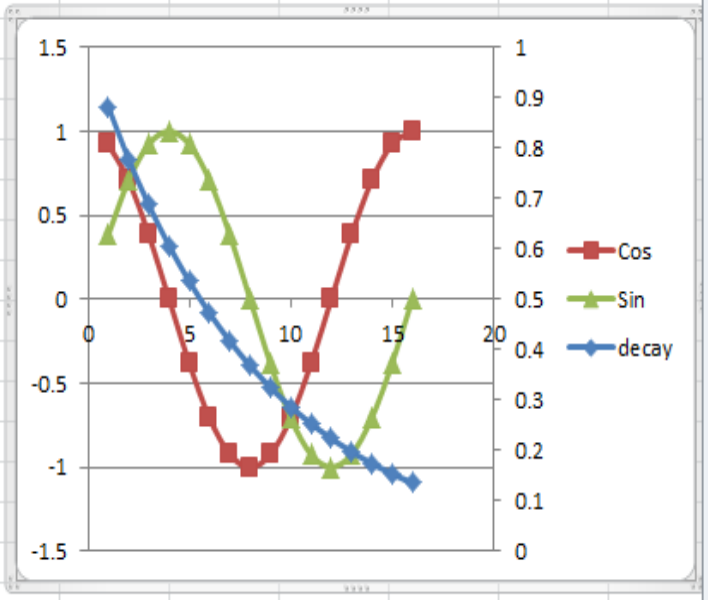
Chart Tools

File | Home | Insert | Page Layout | Formulas | Data | Review | View | Add-Ins | Design | Layout | Format

Change Chart Type | Save As Template | Type | Switch Row/Column | Select Data | Data | Chart Layouts | Chart Styles | Move Chart | Location

Chart 1

	A	B	C	D	E	F	G	H
1								
2		t	I(t)	cos(2pif)	sin(2pif)		i(t)Cos(2pif)	i(t)*sin(2*pi*f)
3		1	0.882497	0.923879659	0.382683126		0.815320938	0.337716673
4		2	0.778801	0.70710725	0.707106312		0.55069568	0.55069495
5		3	0.687289	0.382684352	0.923879152		0.263014852	0.634972236
6		4	0.606531	1.32679E-06	1		8.04742E-07	0.60653066
7		5	0.535261	-0.3826819	0.923880167		-0.204834861	0.494517418
8		6	0.472367	-0.707105374	0.707108188		-0.334012928	0.334014257
9		7	0.416862	-0.923878644	0.382685578		-0.385129917	0.159527083
10		8	0.367879	-1	2.65359E-06		-0.367879441	9.76201E-07
11		9	0.324652	-0.923880675	-0.382680674		-0.299940141	-0.124238225
12		10	0.286505	-0.707109127	-0.707104436		-0.202590157	-0.202588813
13		11	0.25284	-0.382686803	-0.923878136		-0.096758377	-0.233592975
14		12	0.22313	-3.98038E-06	-1		-8.88144E-07	-0.22313016
15		13	0.196912	0.382679449	-0.923881183		0.075354051	-0.181922991
16		14	0.173774	0.707103498	-0.707110065		0.122876163	-0.122877304
17		15	0.153355	0.923877628	-0.382688029		0.141681223	-0.05868711
18		16	0.135335	1	-5.30718E-06		0.135335283	-7.18249E-07
19								
20		Sum	6.49399				0.213132286	1.970935956
21						g and s	0.032819929	0.303501541

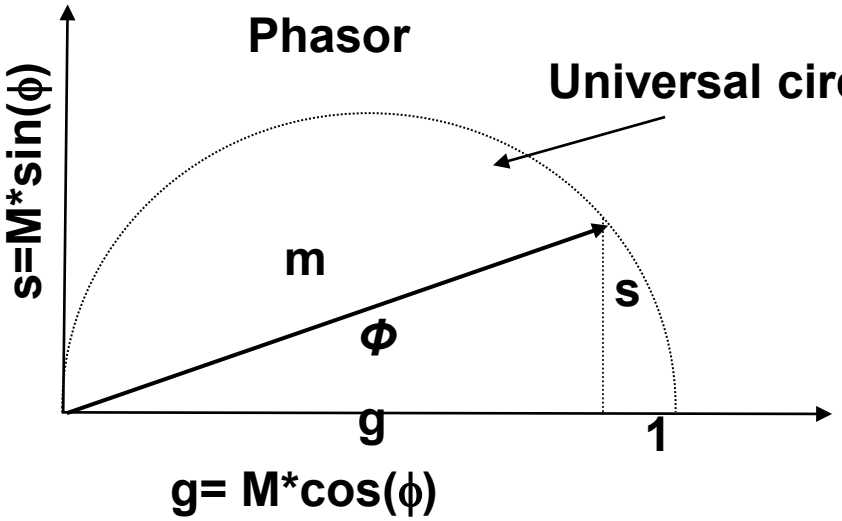


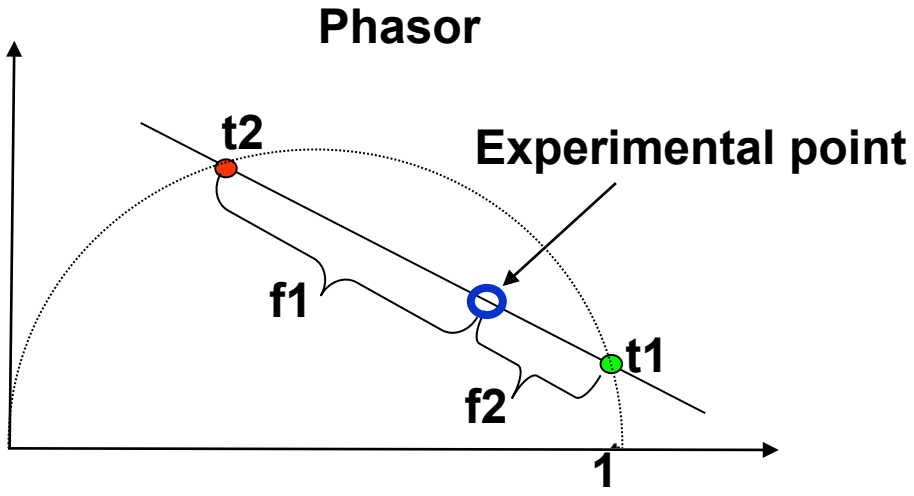
Sheet1 | Sheet2 | Sheet3

Ready

100%

The algebra of phasors

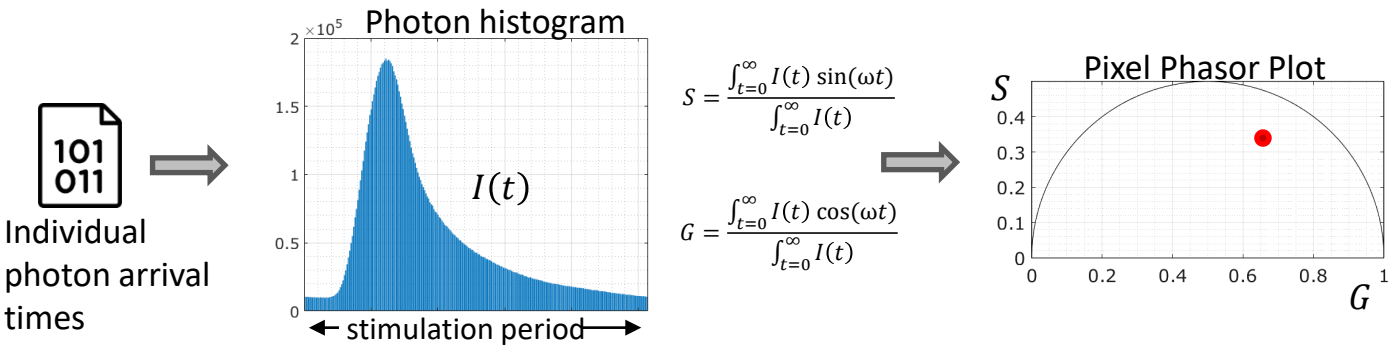


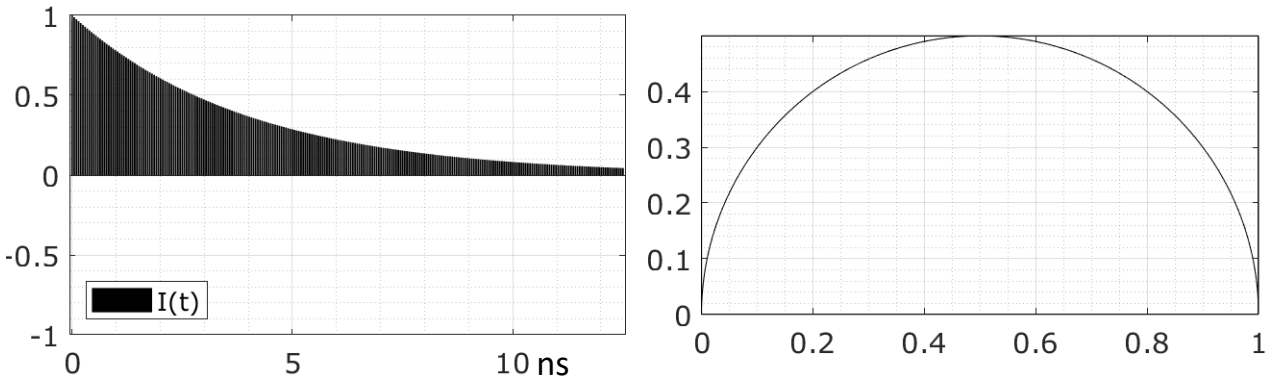


Simple rules to the Phasor plot:

1) All single exponential lifetimes lie on the “universal circle”
2) Multi-exponential lifetimes are a linear combination of their components
3) The ratio of the linear combination determines the fraction of the components

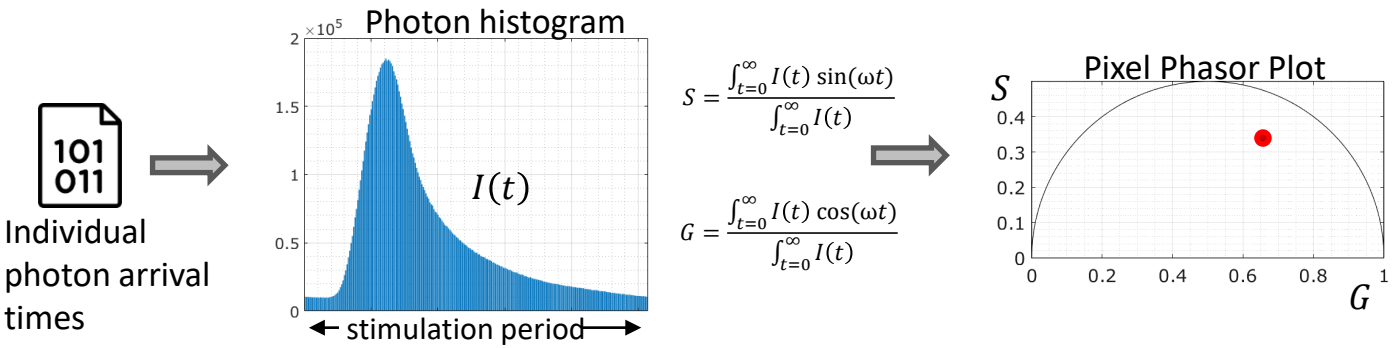
The Phasor Transform in Lifetime Microscopy

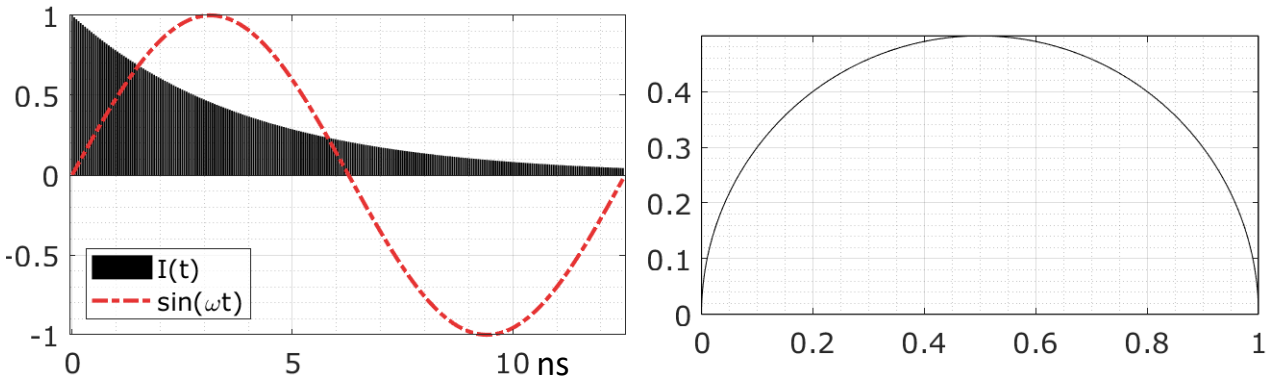




Slide credit: Alex Vallmitjana

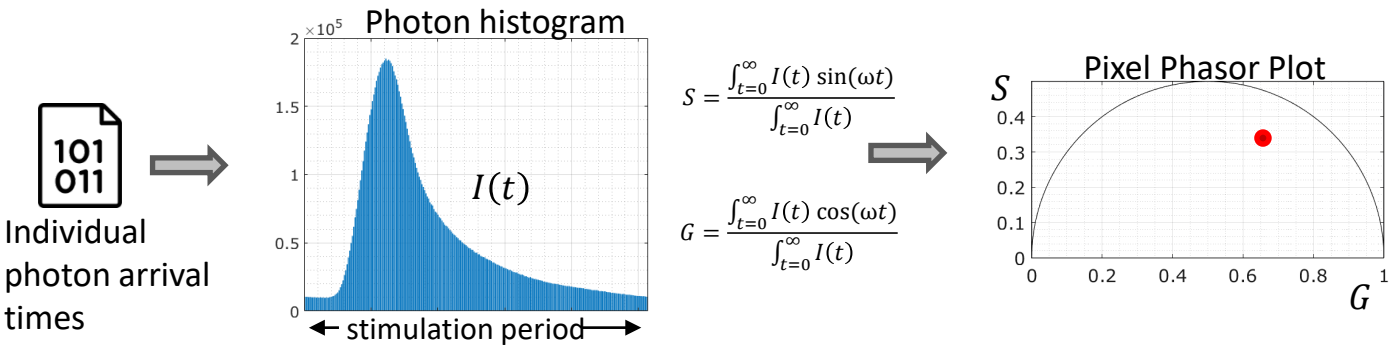
The Phasor Transform in Lifetime Microscopy

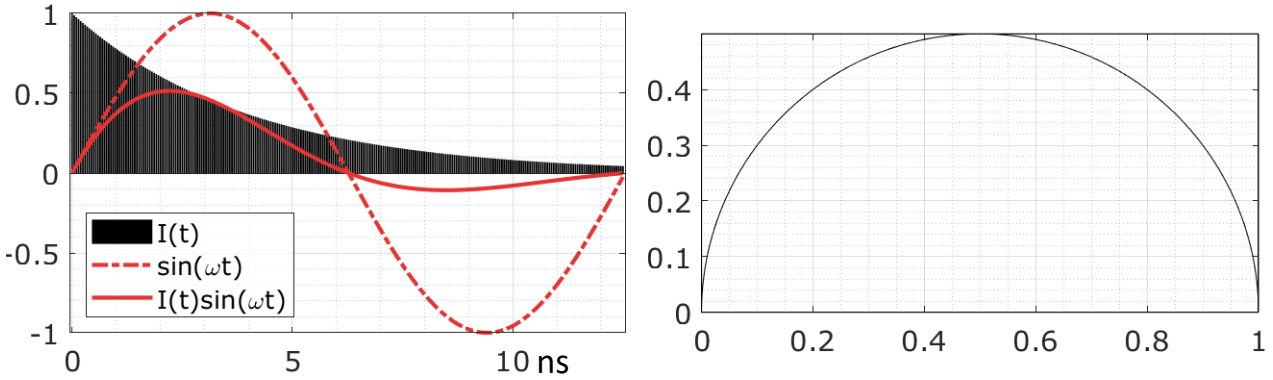




Slide credit: Alex Vallmitjana

The Phasor Transform in Lifetime Microscopy





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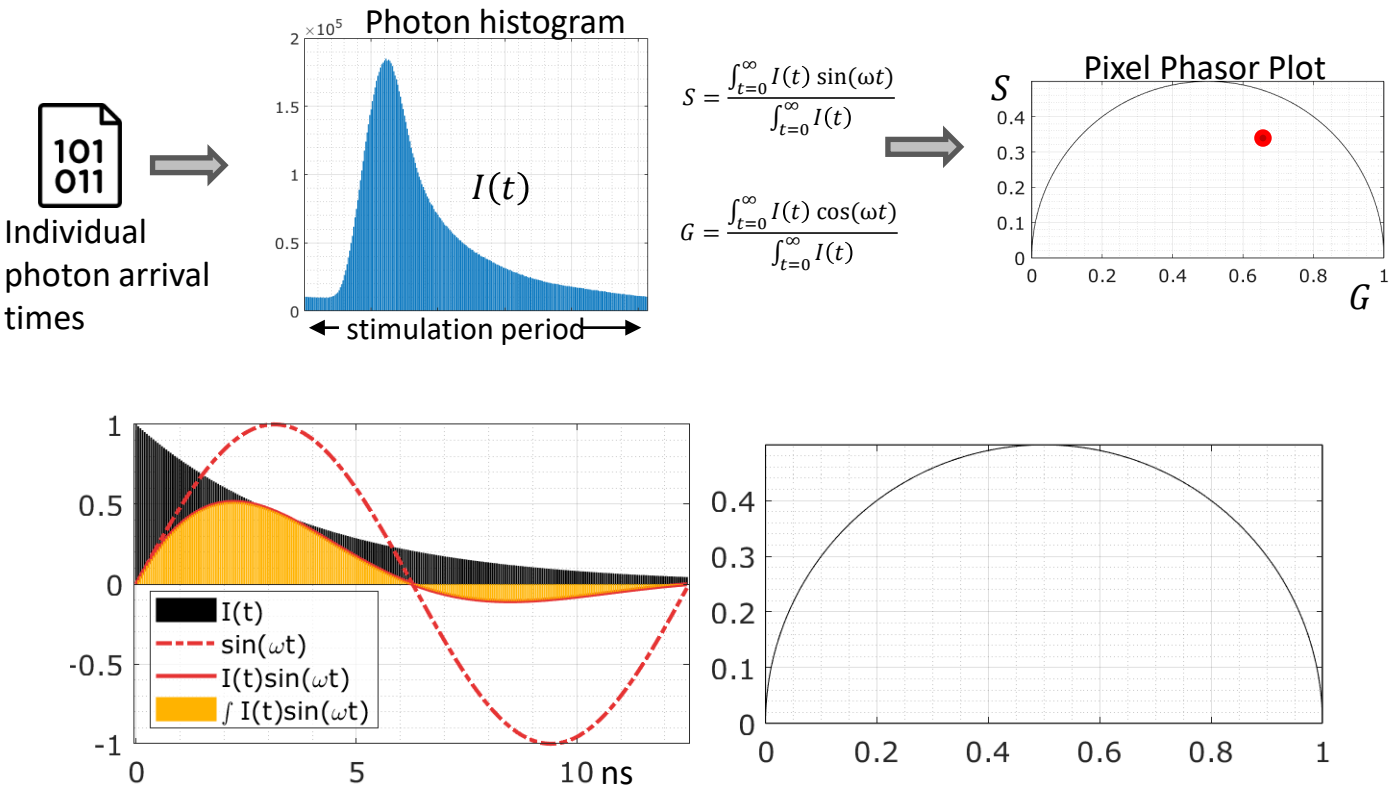

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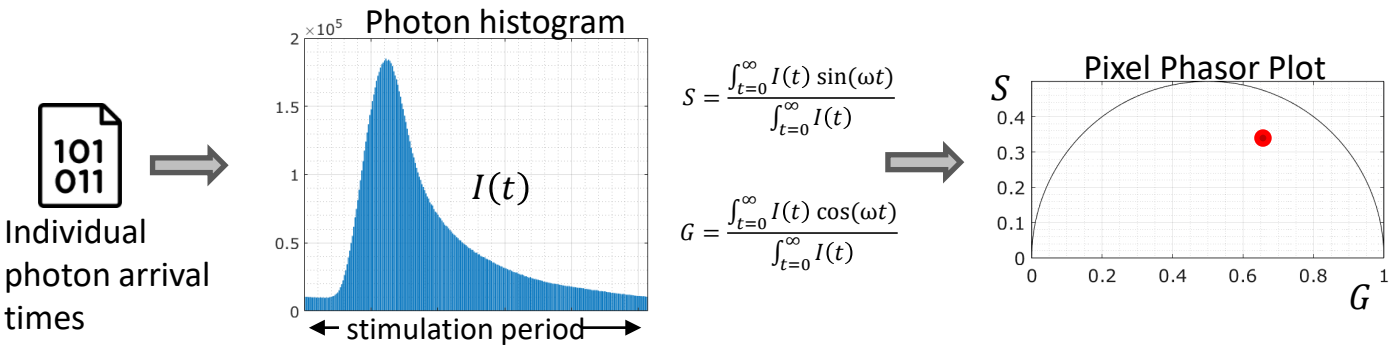

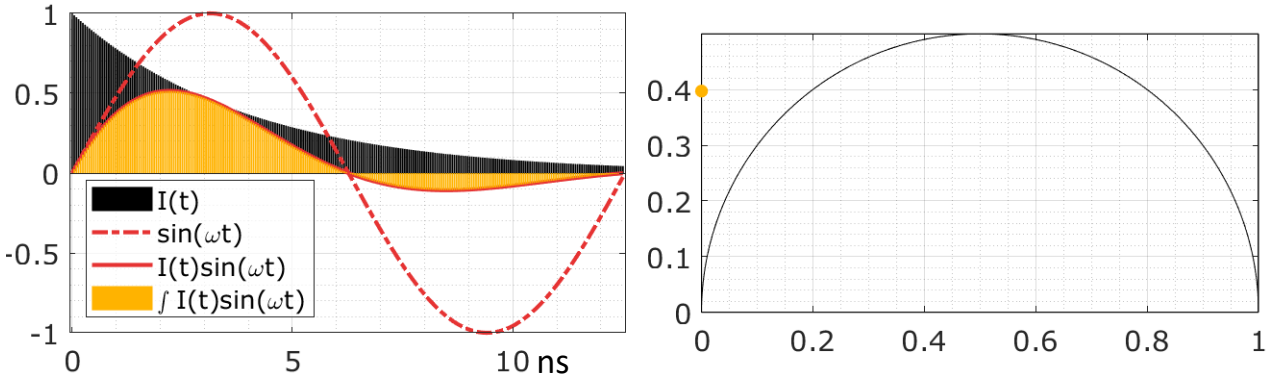

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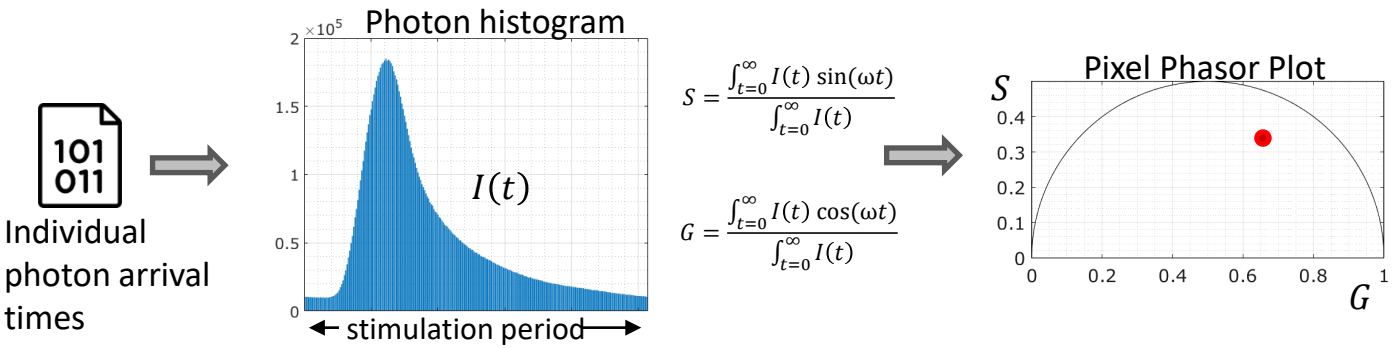

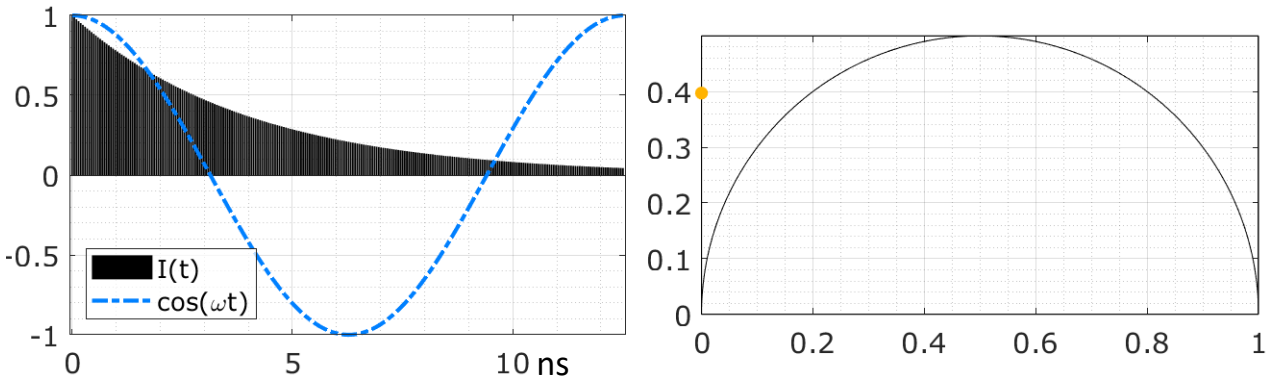

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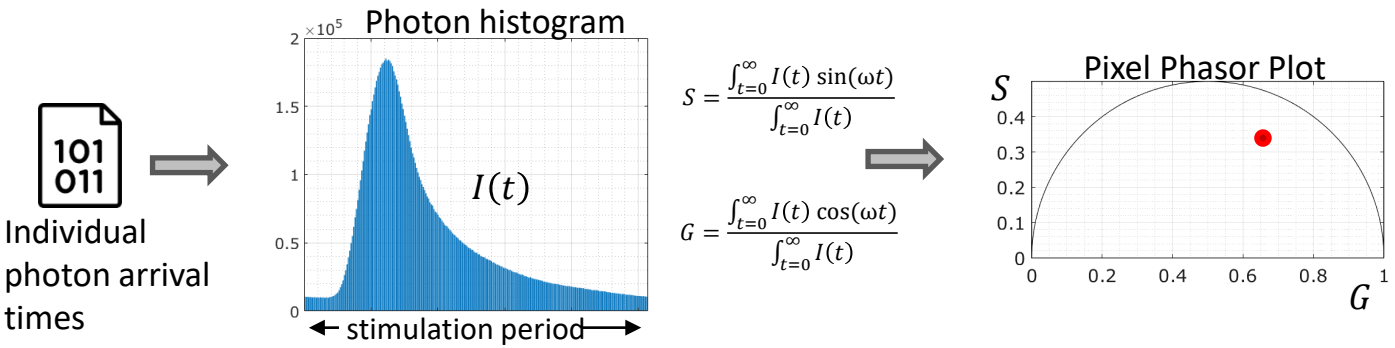

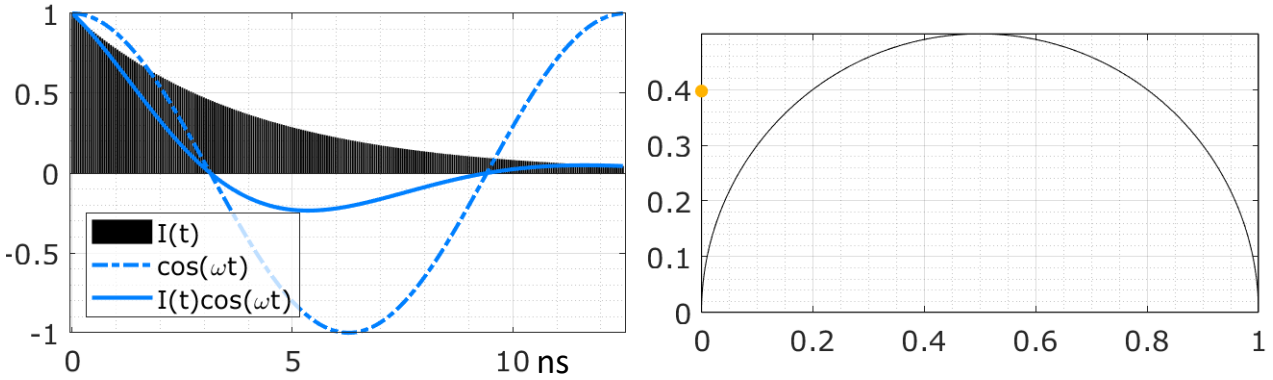

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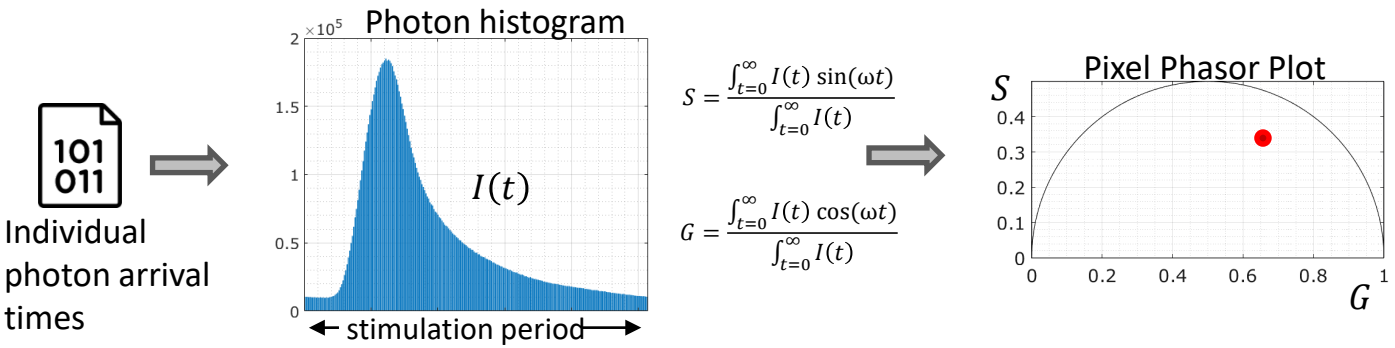

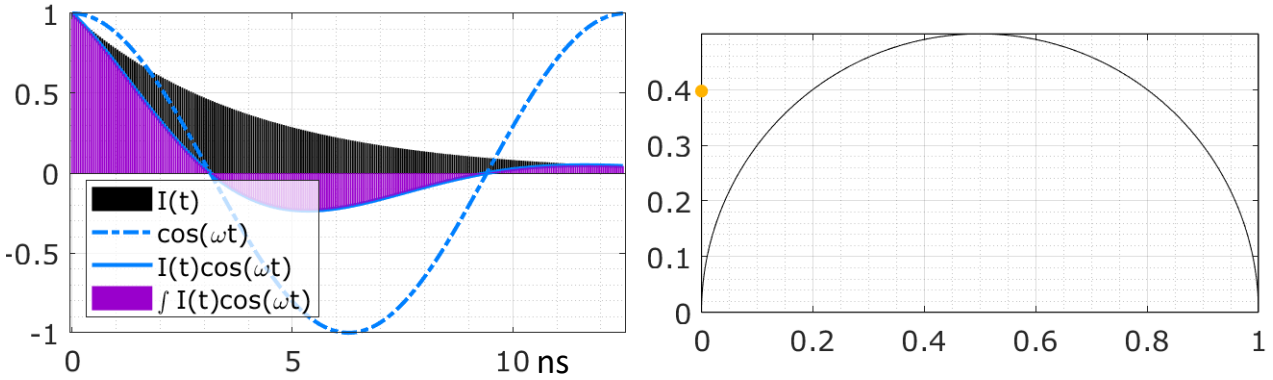

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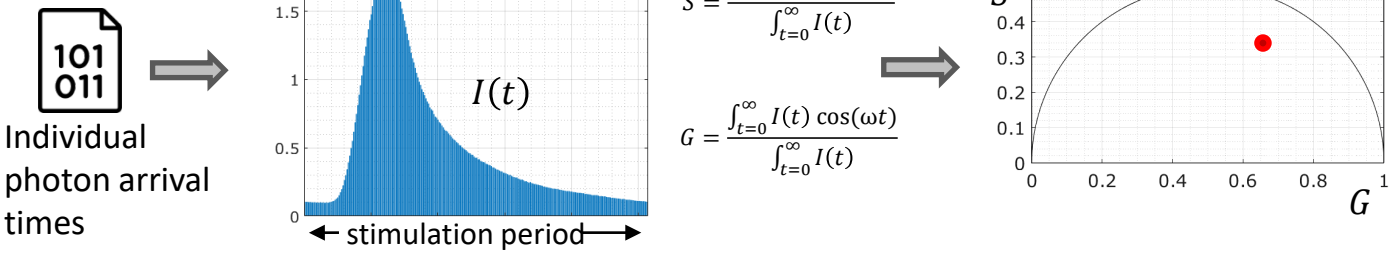

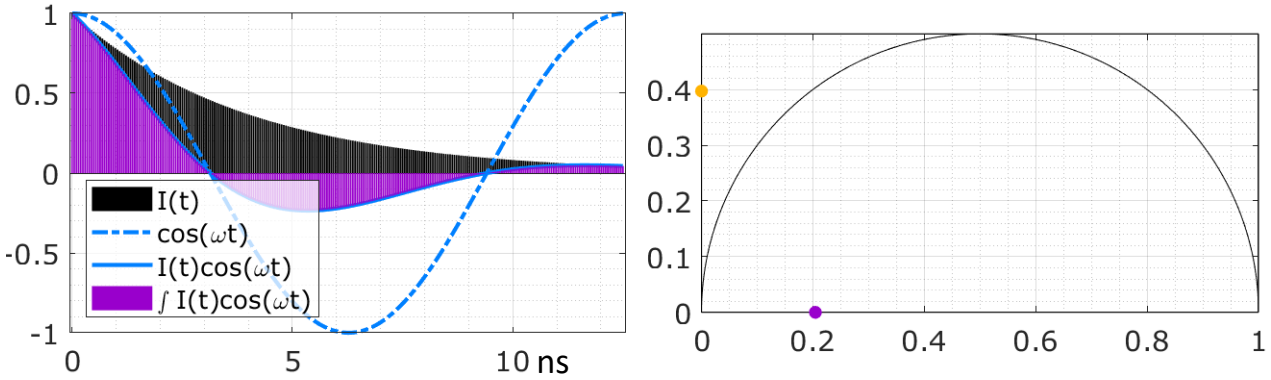

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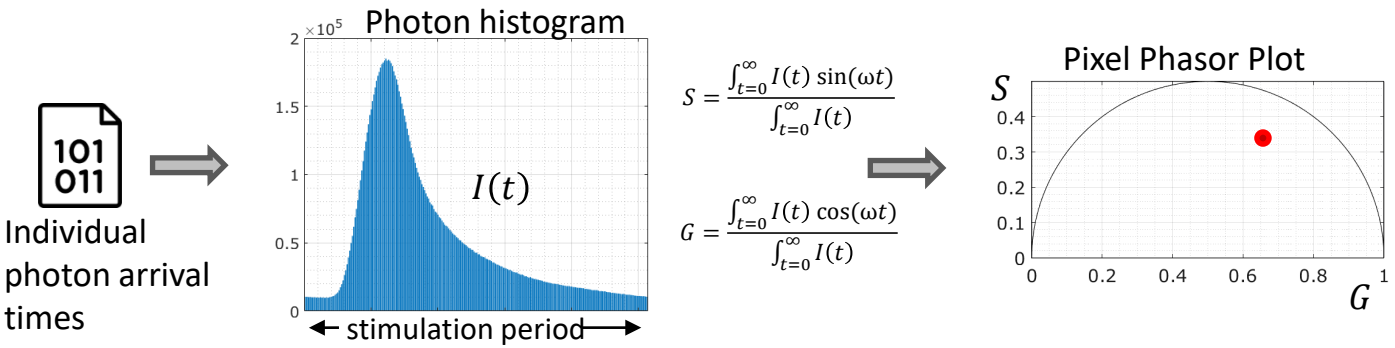

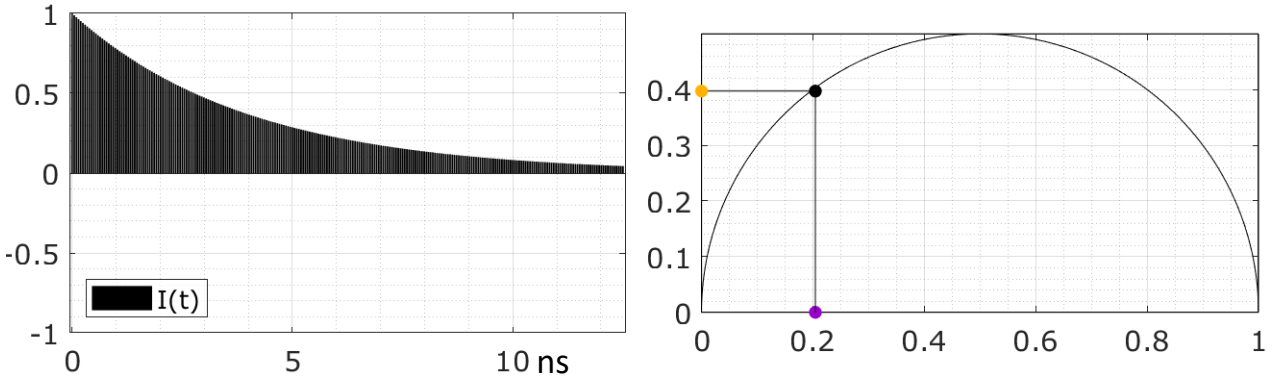

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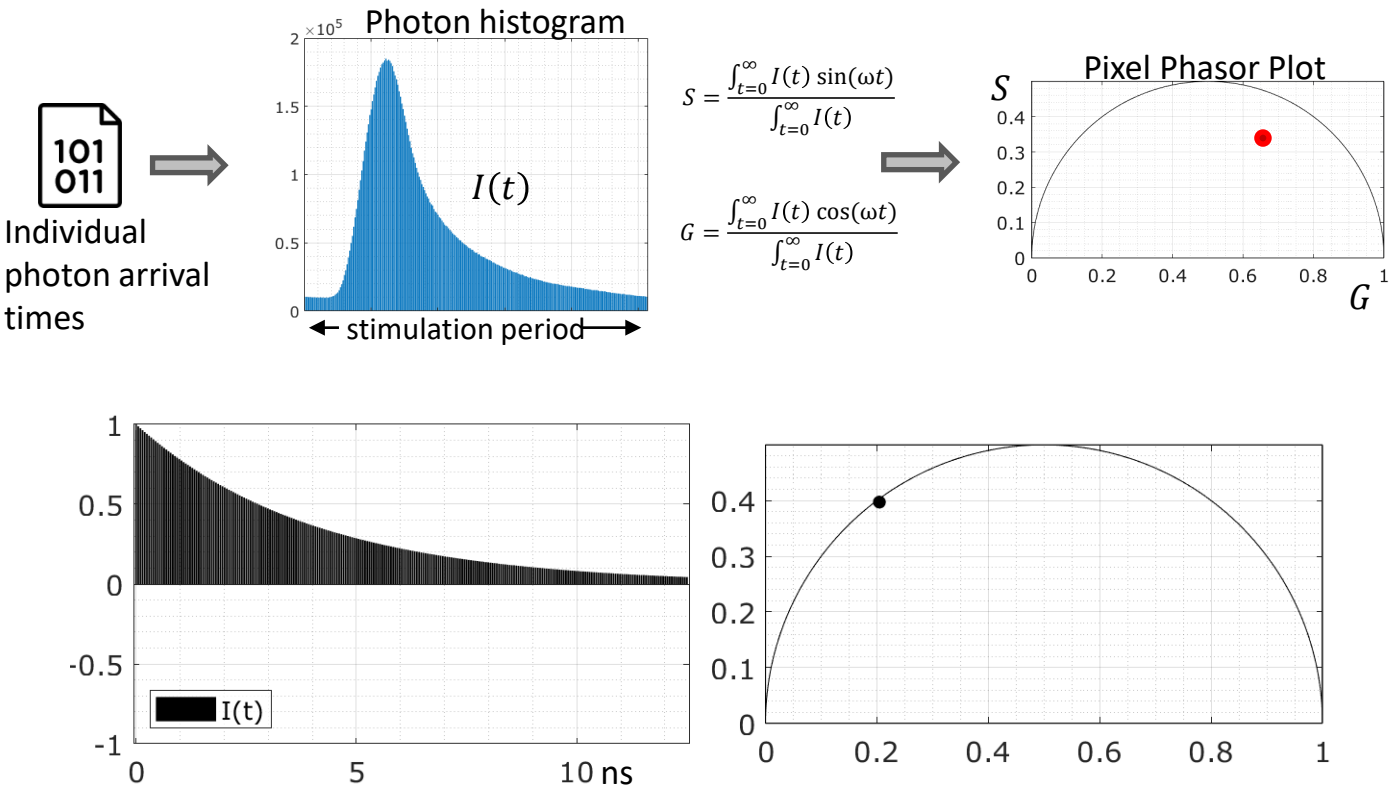
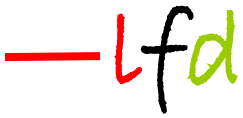
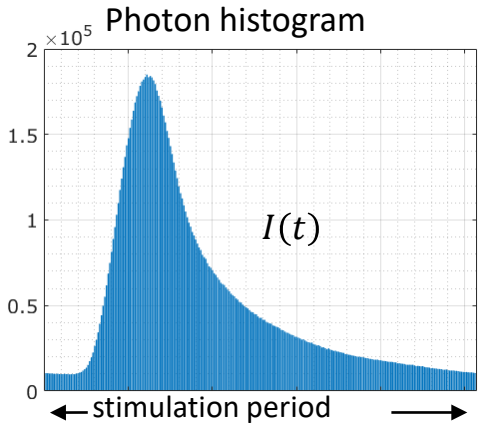


Slide credit: Alex Vallmitjana

The Phasor Transform in Lifetime Microscopy

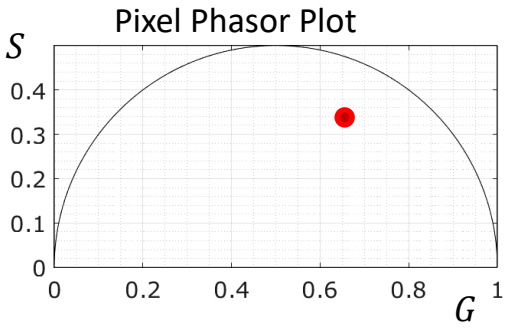


Individual photon arrival times



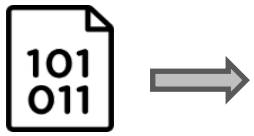
$$S = \frac{\int_{t=0}^{\infty} I(t)\sin(\omega n t)}{\int_{t=0}^{\infty} I(t)}$$

$$G = \frac{\int_{t=0}^{\infty} I(t)\cos(\omega n t)}{\int_{t=0}^{\infty} I(t)}$$

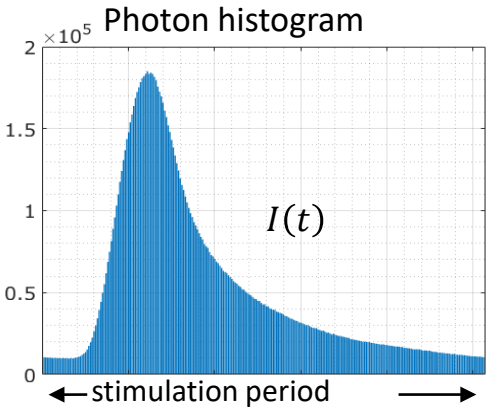


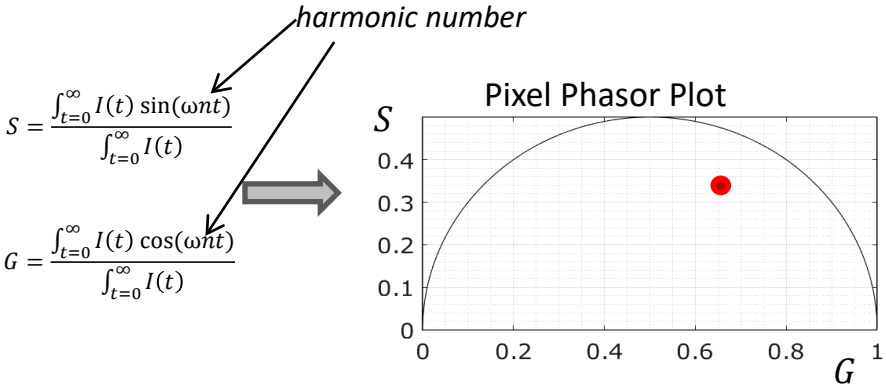
Slide credit: Alex Vallmitjana

The Phasor Transform in Lifetime Microscopy



Individual photon arrival times

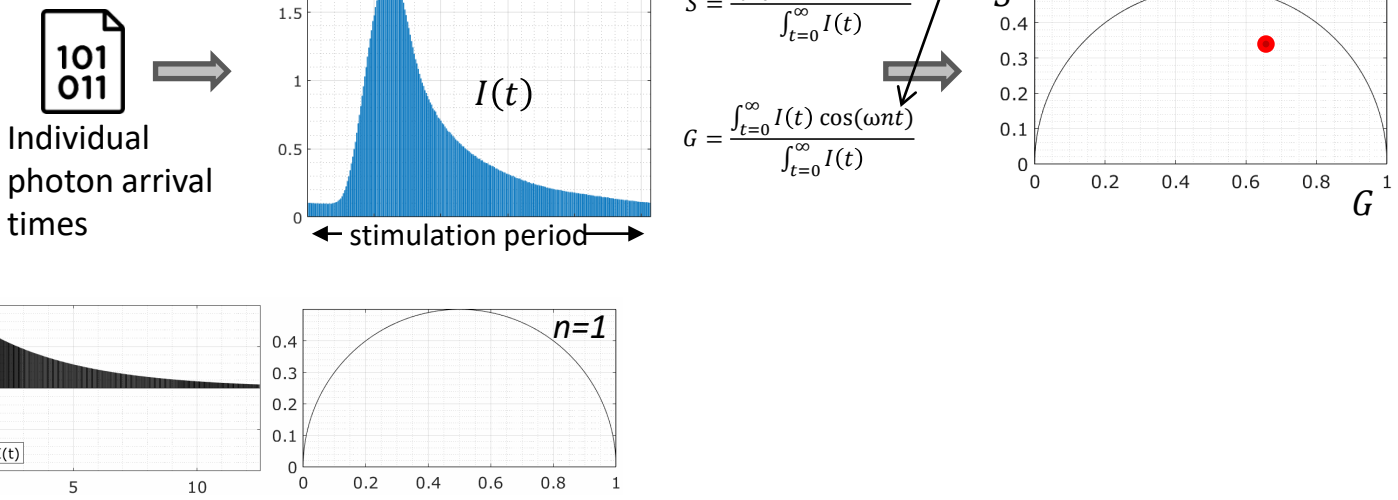




Slide credit: Alex Vallmitjana

$\omega=2\pi f$ where f is the linear modulation frequency

The Phasor Transform in Lifetime Microscopy



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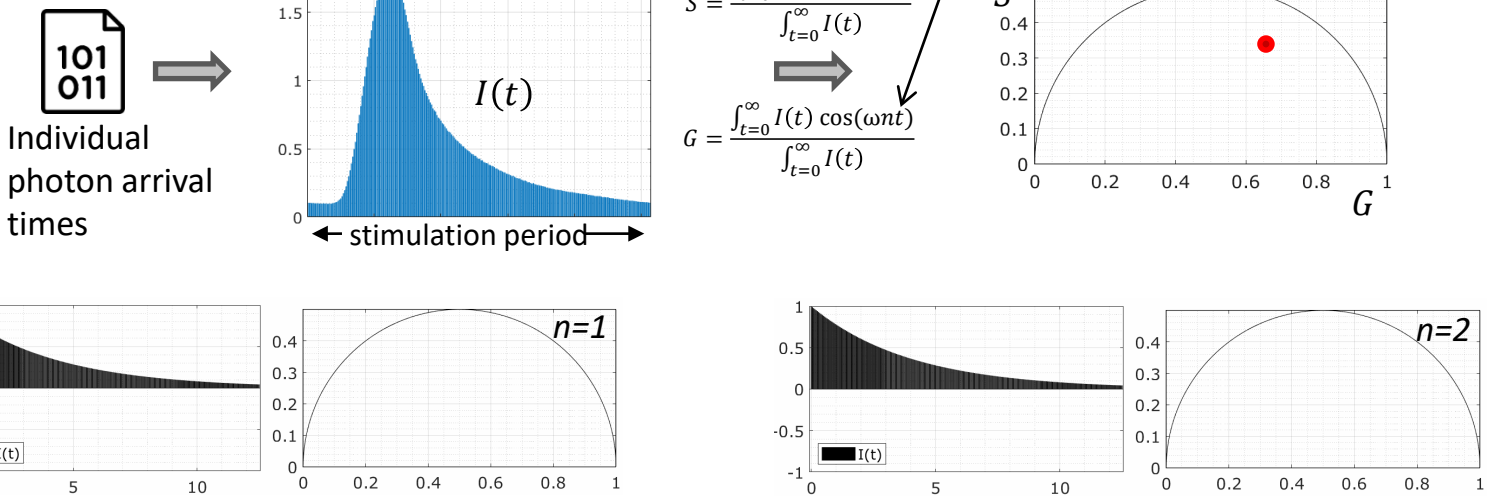
$\omega = 2\pi f$ where f is the linear modulation frequency

The Phasor Transform in Lifetime Microscopy



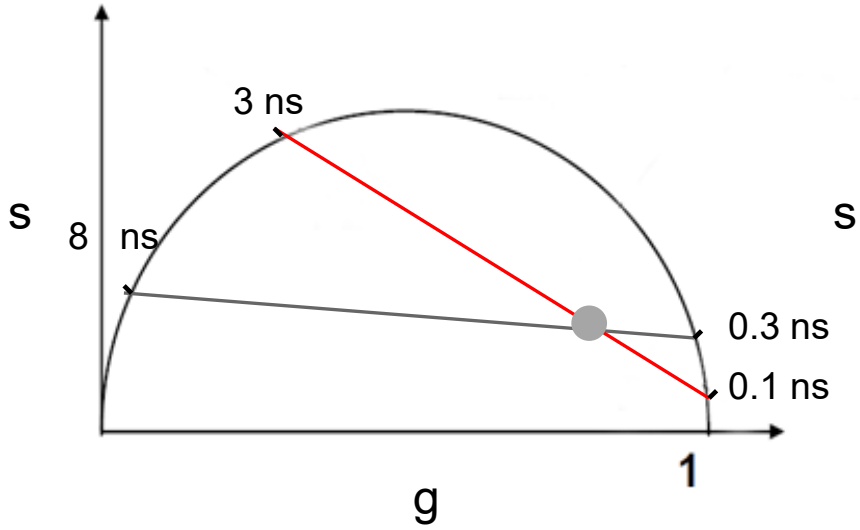
Slide credit: Alex Vallmitjana

Multi-harmonics Phasor analysis

Multi-harmonics analysis separates different molecular components that have the same location in the phasor plot at one harmonic but arise from different lifetime distributions.

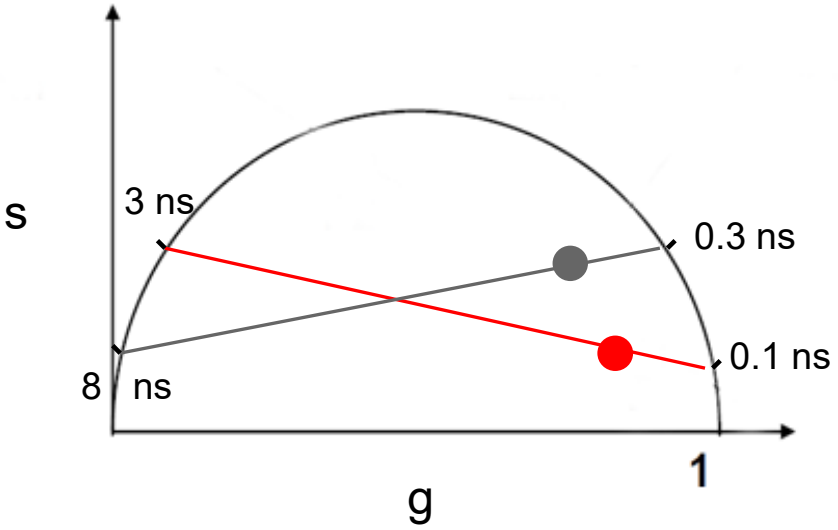
1st harmonic

Phasor tranformation
at $\omega = \omega_0$



2nd harmonic

Phasor tranformation
at $\omega = 2\,\omega_0$



$$g_{i,j}(\omega) = \sum_k \frac{f_k}{1+(\omega\tau_k)^2}$$

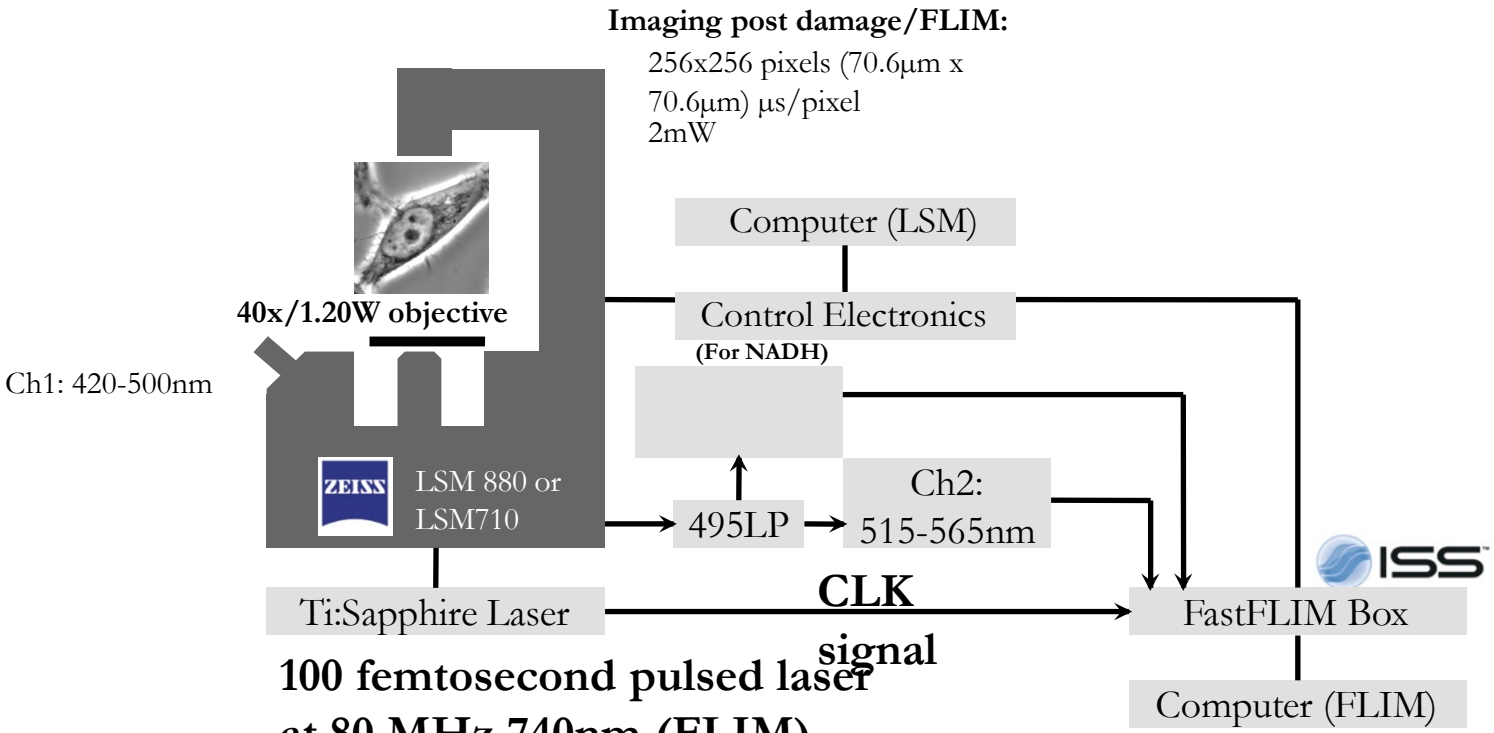
$$s_{i,j}(\omega) = \sum_k \frac{f_k \omega\tau_k}{1+(\omega\tau_k)^2}$$

$$\tan^{-1}\varphi = \omega\tau$$

f = 80 MHz

Slide credit: Chiara Stringari

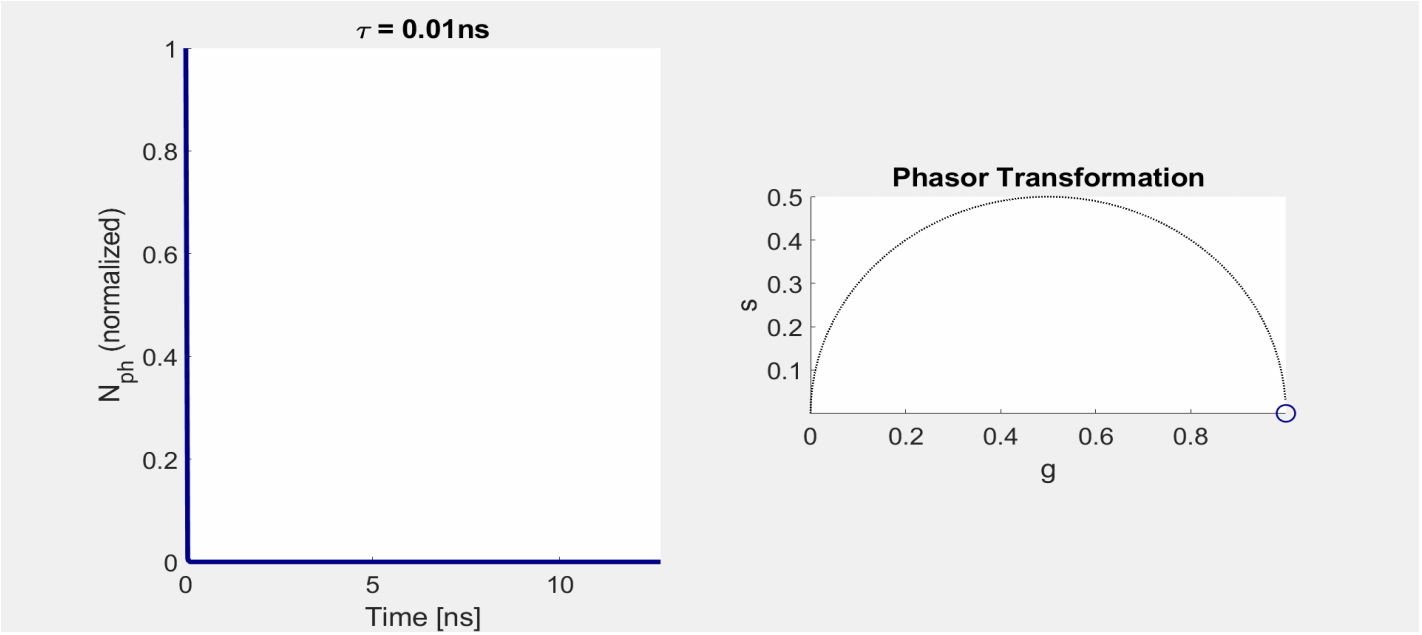
Microscope Schematic for FLIM



100 femtosecond pulsed laser at 80 MHz 740nm (FLIM), 780nm (laser damage)

Normal imaging: 488nm, 128x128, 4000 frames, 0.2us/pixel

Phasor FLIM



Phasor FLIM simulations by Dr. Lorenzo Scipioni

Digman MA, Caiolfa VR, Zamai M, Gratton E. **The phasor approach to fluorescence lifetime imaging analysis**. Biophys J. 2008;94(2):L14–L16. doi:10.1529/biophysj.107.120154

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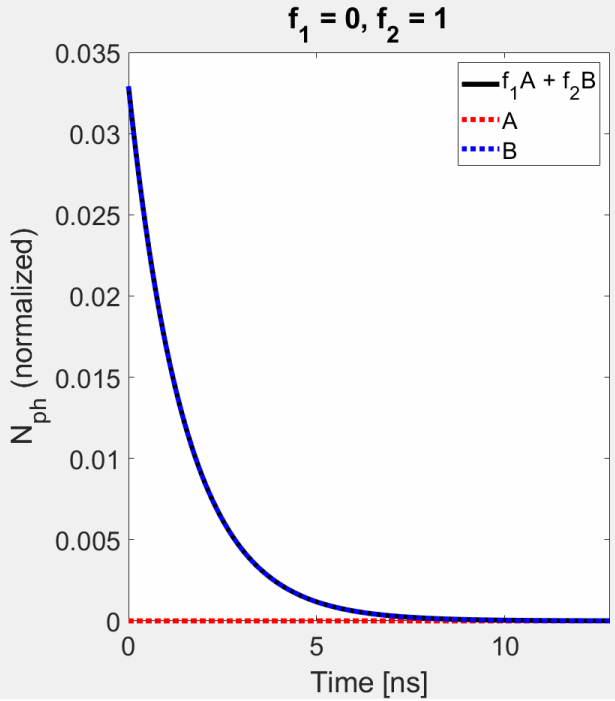
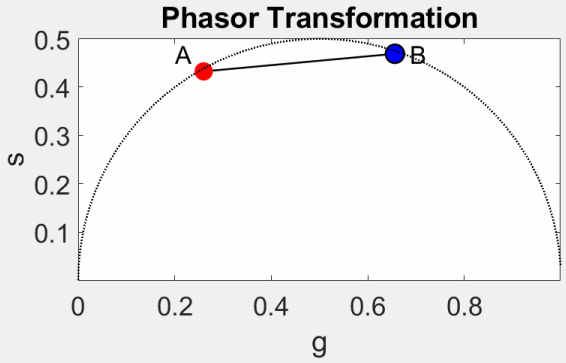
Lifetime Phasors – Linear Combination





Phasor FLIM simulations by Dr. Lorenzo Scipioni

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1. A sample contains two kinds of molecules. The first molecule has a fluorescence lifetime (single exponential) of 10 ns and its molar fraction is 10%. The second molecule has a lifetime (single exponential) of 2 ns and contributes for the rest 90% of the molar fraction. Show in a phasor plot (at 80 MHz) the position of the phasor of the individual species and of the mixture of the two species.

$$f = 80 \times 10^6 \text{ Hz}$$

$$\omega = 2\pi f$$

$$g = \frac{1}{1+\omega^2\tau^2}$$

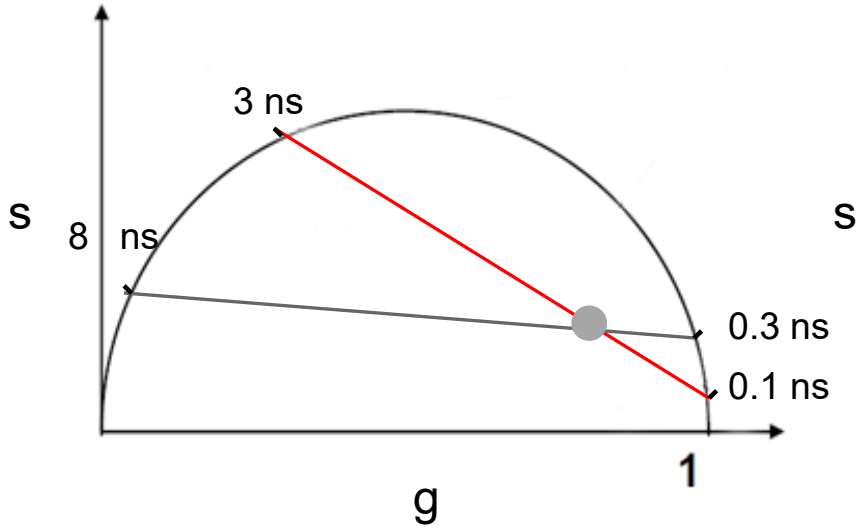
$$S = \frac{\omega\tau}{1+\omega^2\tau^2}$$

Multi-harmonics Phasor analysis

Multi-harmonics analysis separates different molecular components that have the same location in the phasor plot at one harmonic but arise from different lifetime distributions.

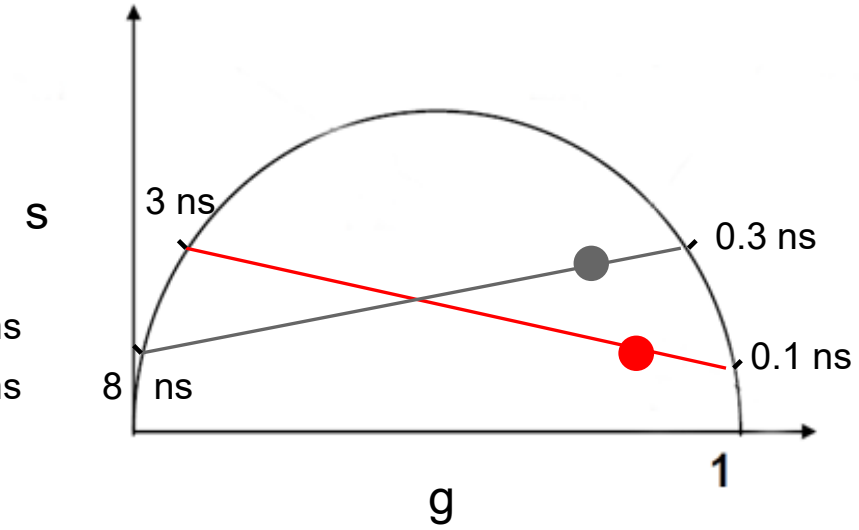
1st harmonic

Phasor tranformation
at $\omega = \omega_0$



2nd harmonic

Phasor tranformation
at $\omega = 2\,\omega_0$



$$g_{i,j}(\omega) = \sum_k \frac{f_k}{1+(\omega\tau_k)^2}$$

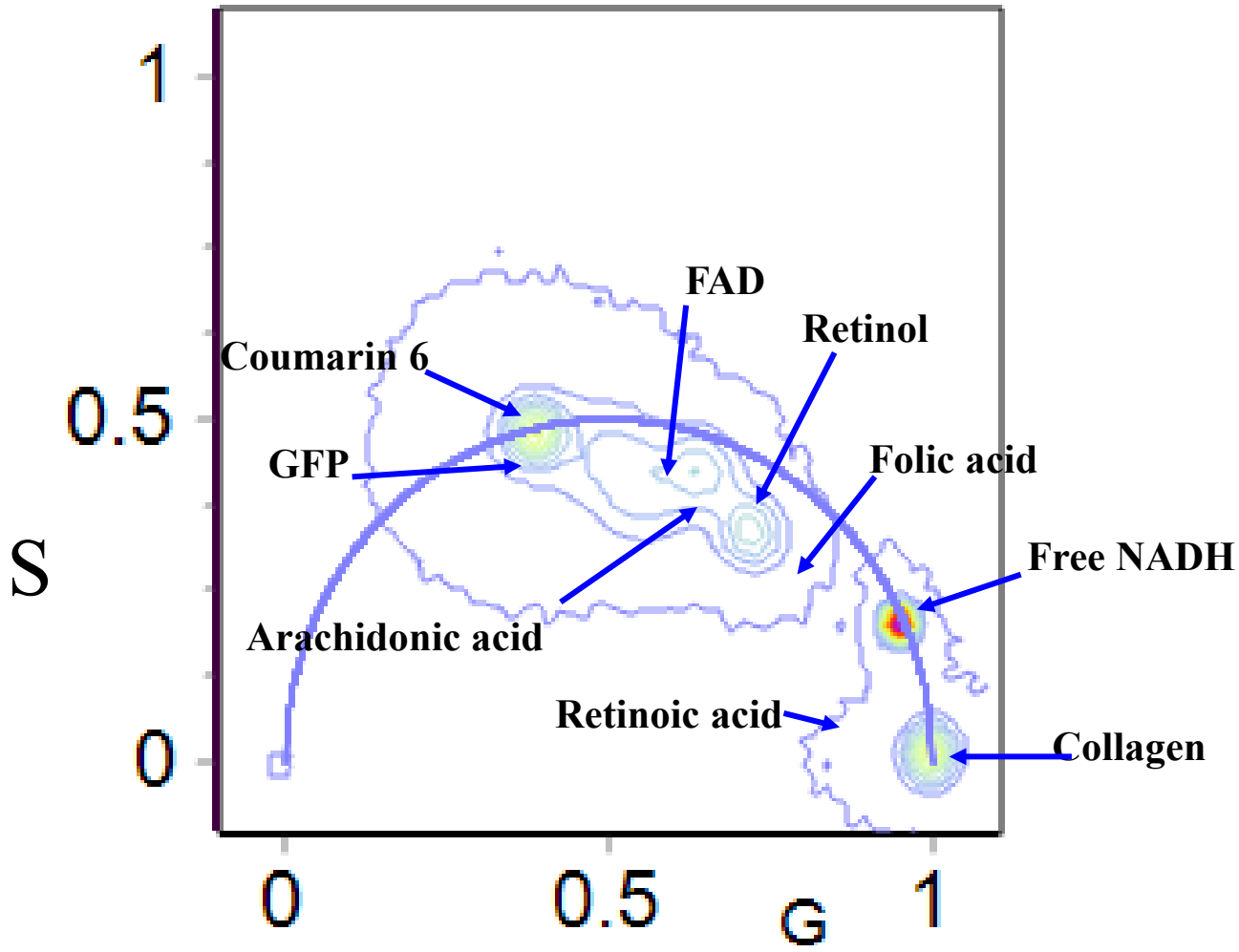
$$s_{i,j}(\omega) = \sum_k \frac{f_k \omega\tau_k}{1+(\omega\tau_k)^2}$$

$$\tan^{-1}\varphi = \omega\tau$$

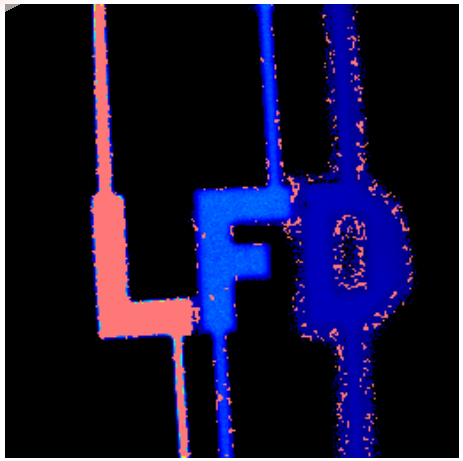
f = 80 MHz

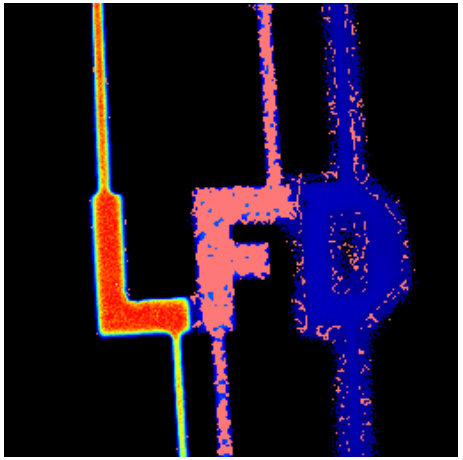
Slide credit: Chiara Stringari

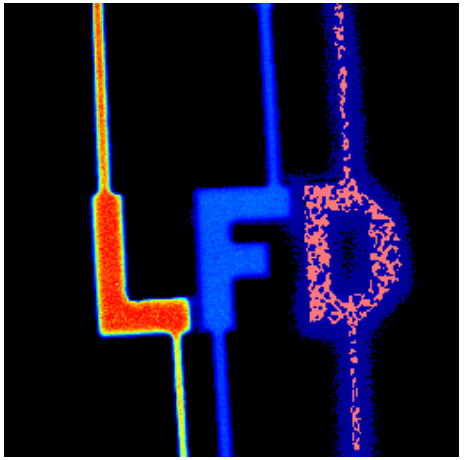
Phasor Fingerprint of pure chemical species….

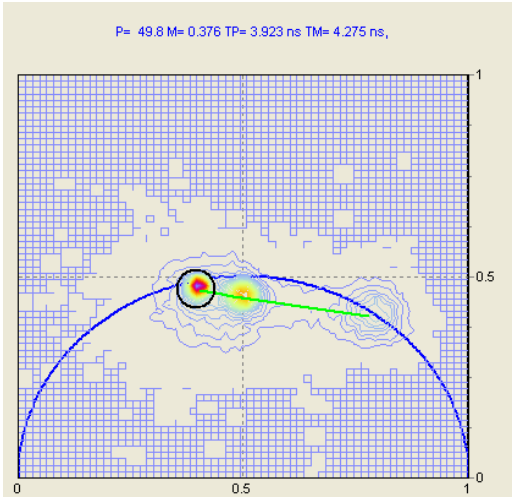


Separating Single exponential lifetimes using the phasor approach

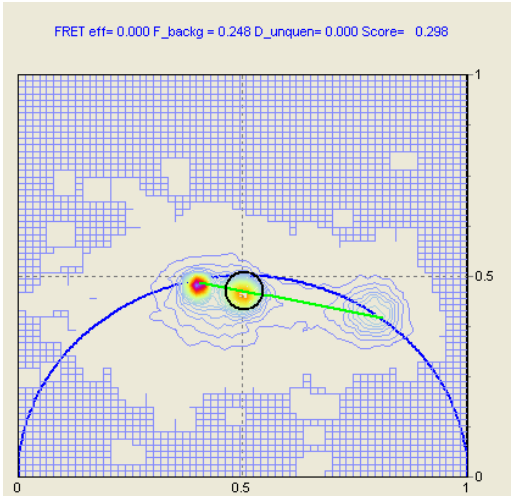




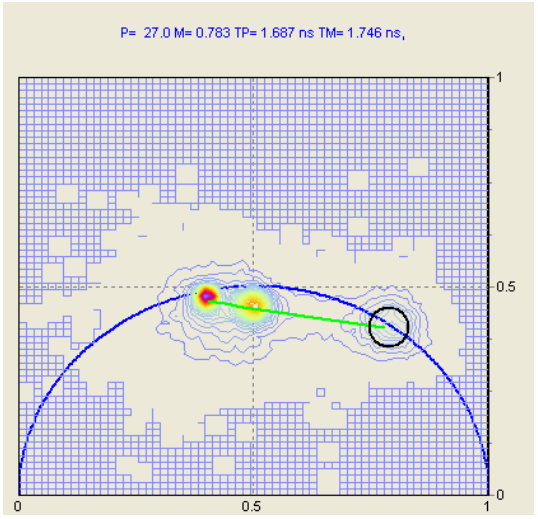




Fluorescein



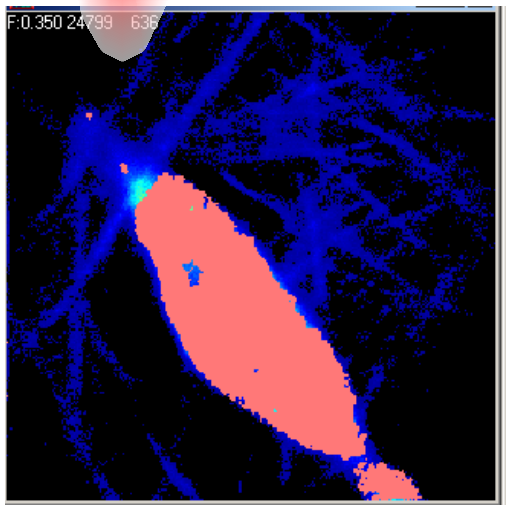
Mixture



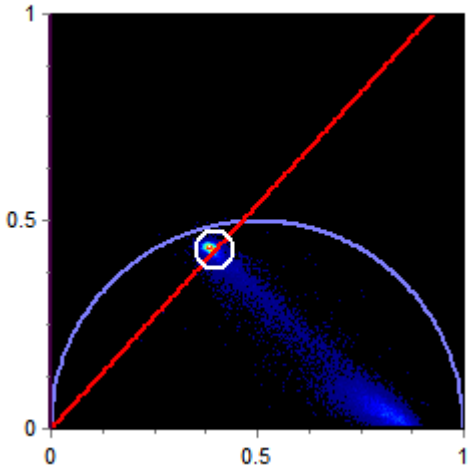
Rhodamine B1

Pax-eGFP CHO-k1 in collagen

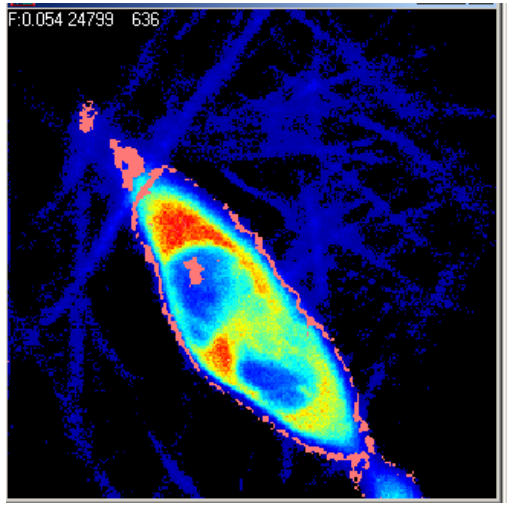
referenced with Fluorescein @ 905nm



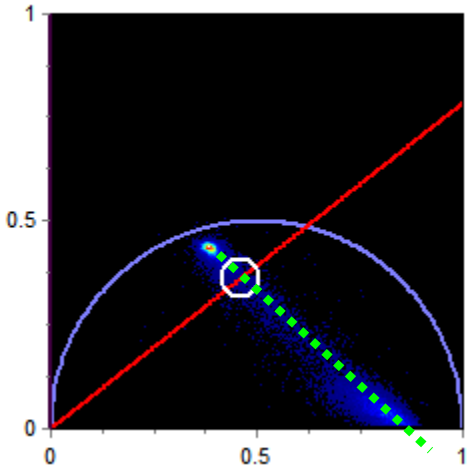
P= 47.2 M= 0.343 TP= 2.149 ns TM= 2.751 ns,



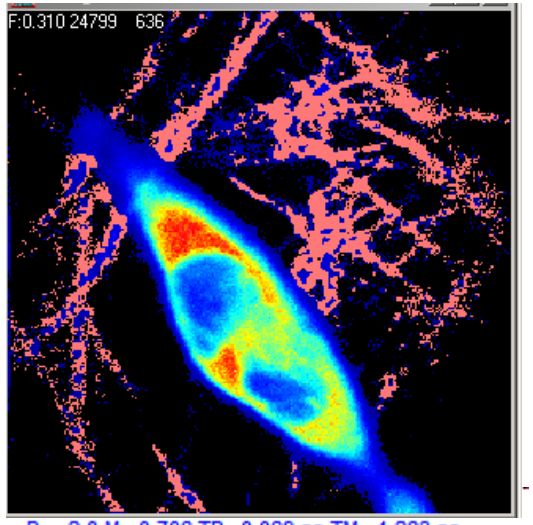
Lifetime of EGFP



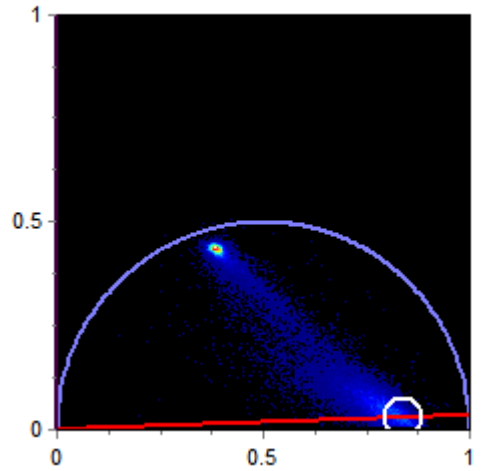
P= 38.2 M= 0.344 TP= 1.563 ns TM= 2.748 ns,



Combinations of Lifetimes

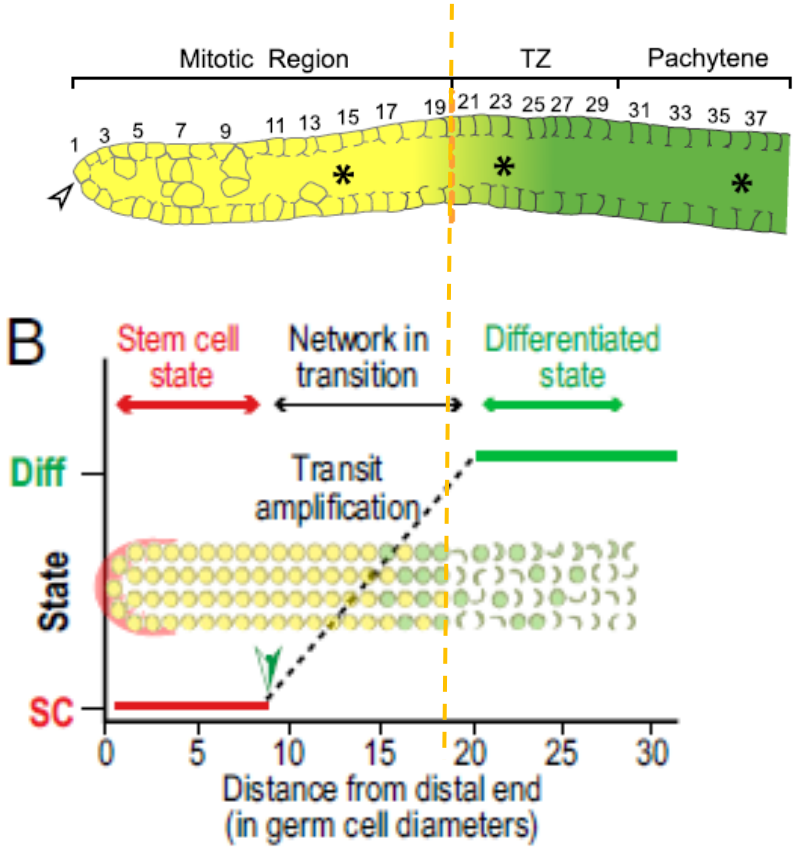


P= 2.0 M= 0.706 TP= 0.069 ns TM= 1.283 ns,



Lifetime of Collagen

C.Elegans germ line: a model for stem cell biology



Mitotic region : stem cells niche

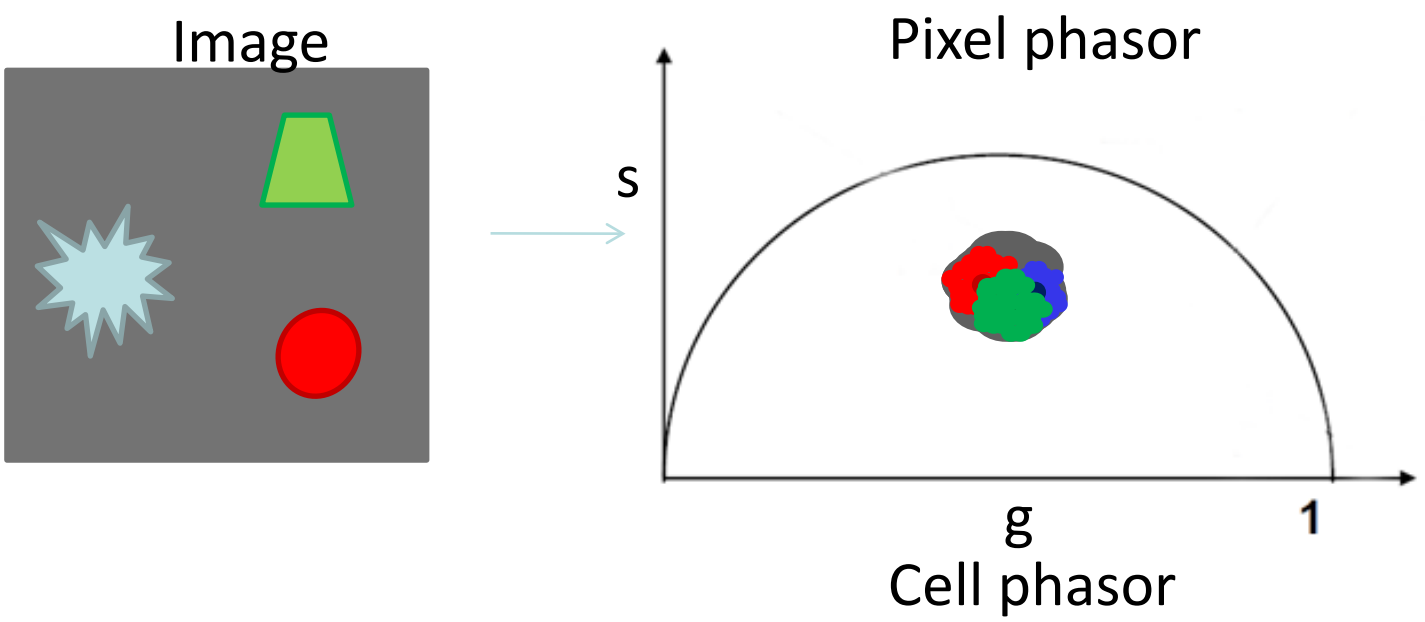
- ✓ The distal pool: undifferentiated cells maintained in a "stem cell-like state"
- ✓ proximal pool contains cells that are closer to differentiating

Transition zone : cells that have differentiated and entered meiotic prophase (crescent-shaped DNA)

Meiotic pachytene region: cells that have further progressed through meiosis.

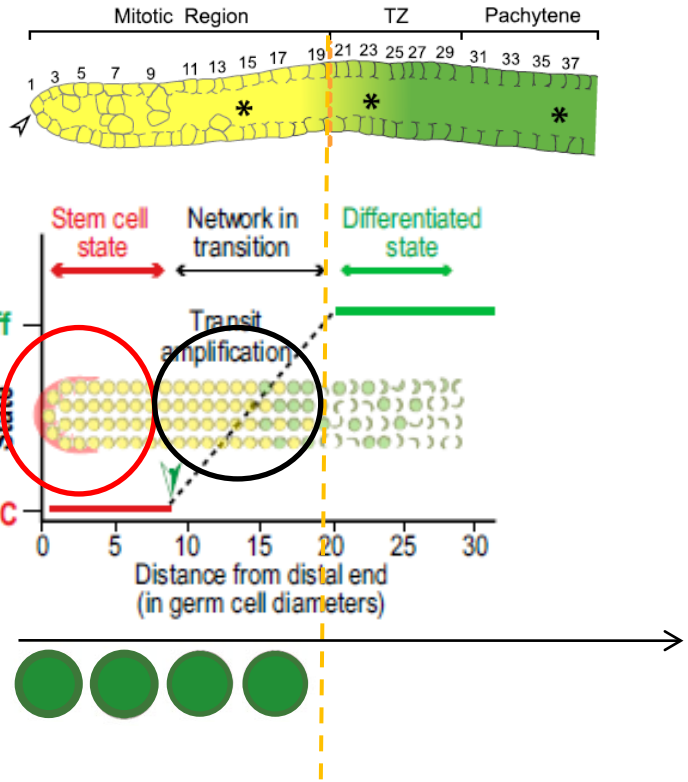
Crittenden et al. Mol Biol Cell, 2006; Cinquin et al. PNAS 2010

Image Segmentation:
from pixel phasor plot to cell phasor plot



- ✓ Phasor average value of cells
- ✓ Better resolution
- ✓ Metabolic state of cells
- ✓ Cell phasors can be statistically attributed to the s
  or different average phasor value

C.Elegans germ line: a model for stem cell biology



Experimental set-up

C.Elegans histone-GFP fusion in germ line nuclei
λ@ 880 nm and 740 nm

Ti: sapphire laser, 80 MHz, Zeiss 710,
ISS A320 FastFLIM, GaAs PMT,
40 x 1.2 NA,
Power ~ 5 mW, Pixel dwell time=25 μs
SimFCS software

Mitotic region : stem cells niche

✓ **Distal pool:** undifferentiated cells maintained in a "stem cell-like state"

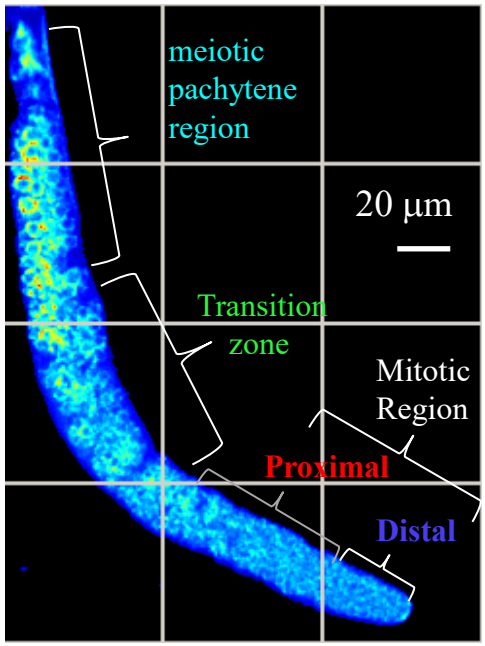
✓ **Proximal pool:** contains cells that are closer to differentiating

Transition zone : cells that have differentiated and entered meiotic prophase (crescent-shaped DNA)
Meiotic pachytene region: progression through meiosis.

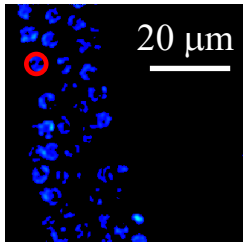
Cinquin et al. PNAS 2010

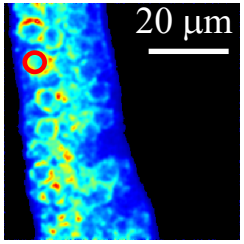
Stem cell metabolic “states” in C.elegans

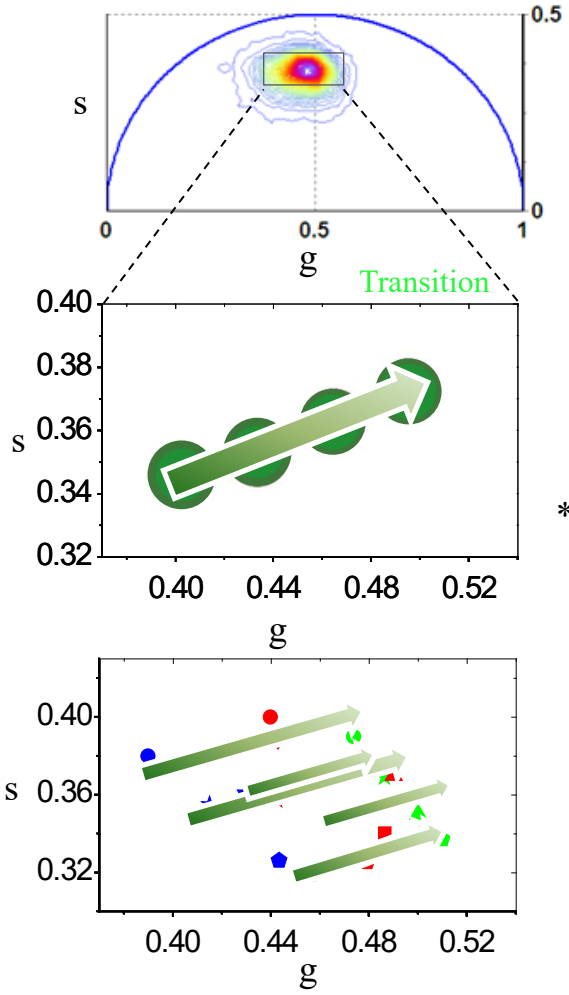
@740nm
@880nm
histone-GFP fusion
NADH, FAD
in germ line nuclei



Cell selection







Pixel phas

Cell phasor

*(t-test, p<0.05)

N=6

In the large phasor cluster we distinguish statistically different subclusters

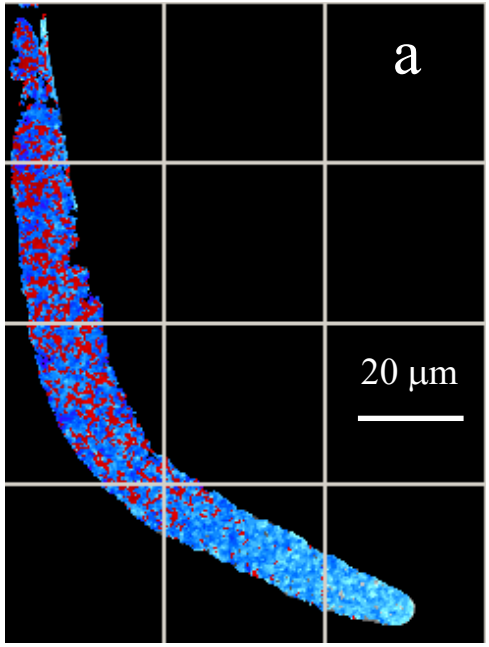
Stringari et al. PNAS 2011

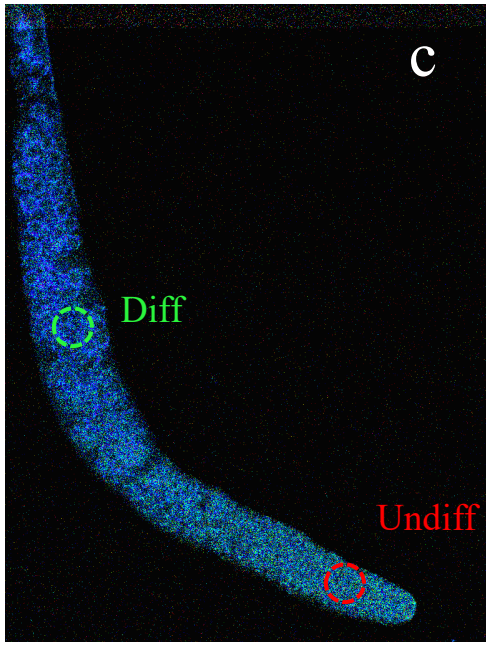
Mapping relative concentration of metabolites

Redox balance and modulation of stem cell self-renewal and differentiation

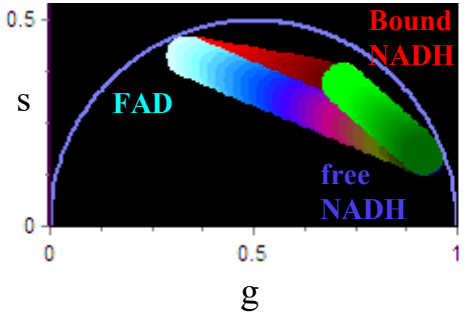
✓Growth factors that promote self-renewal cause stem cell to become more reduced.

✓Signaling molecules that promote differentiation cause progenitor to become more oxidized .

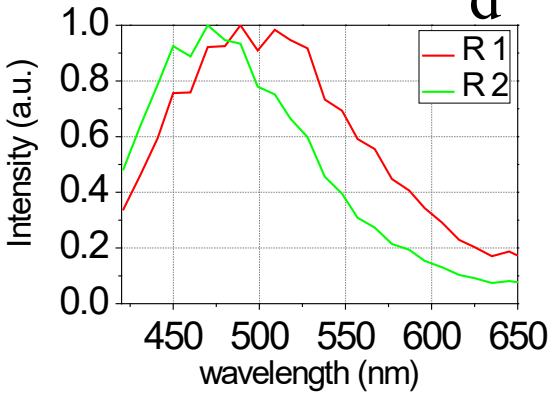




b



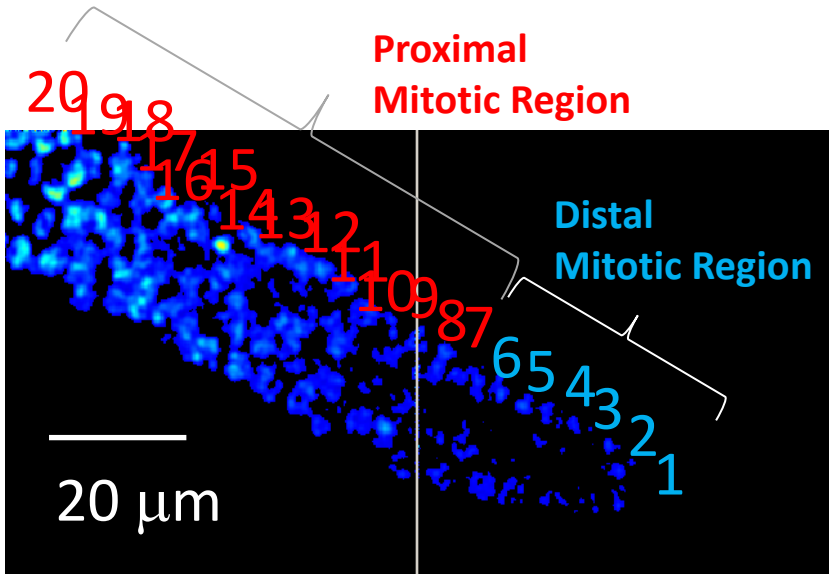
d

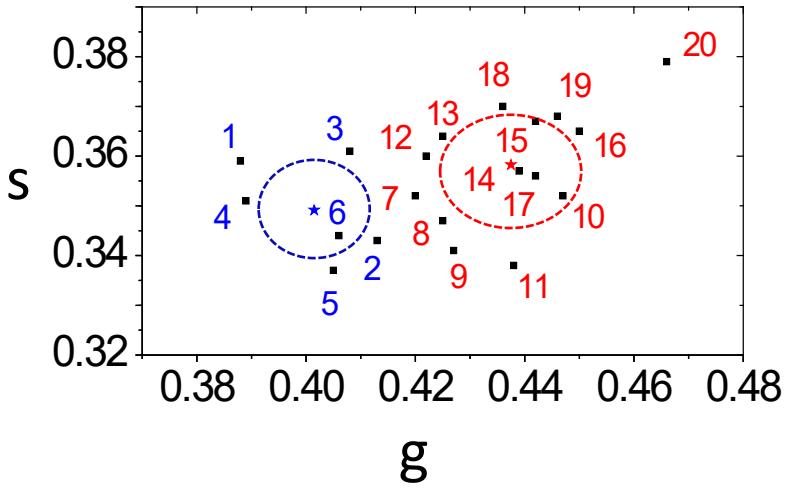


In agreement with in vitro study:
Uchugonova et al. J Biomed Opt 2008
Guo et al 2008 JBO

Stringari et al. PNAS 2011

Single cell phasor plot distinguishes metabolic states of cells





✓ Evolution of the cell phasor fingerprints during differentiation

✓ Gradient of metabolic states of cells.

✓ Phasor fingerprint heterogeneity among mitotic cells could reveal symmetric and asymmetric divisions occurring at the level of the niche.

Conclusions

- ✓ Image segmentation: Cell phasors
- ✓ Better resolution
- ✓ Discrimination of different metabolic states of cells, small differences in redox ratio
- ✓ We identify and map relative concentration of intrinsic fluorophores

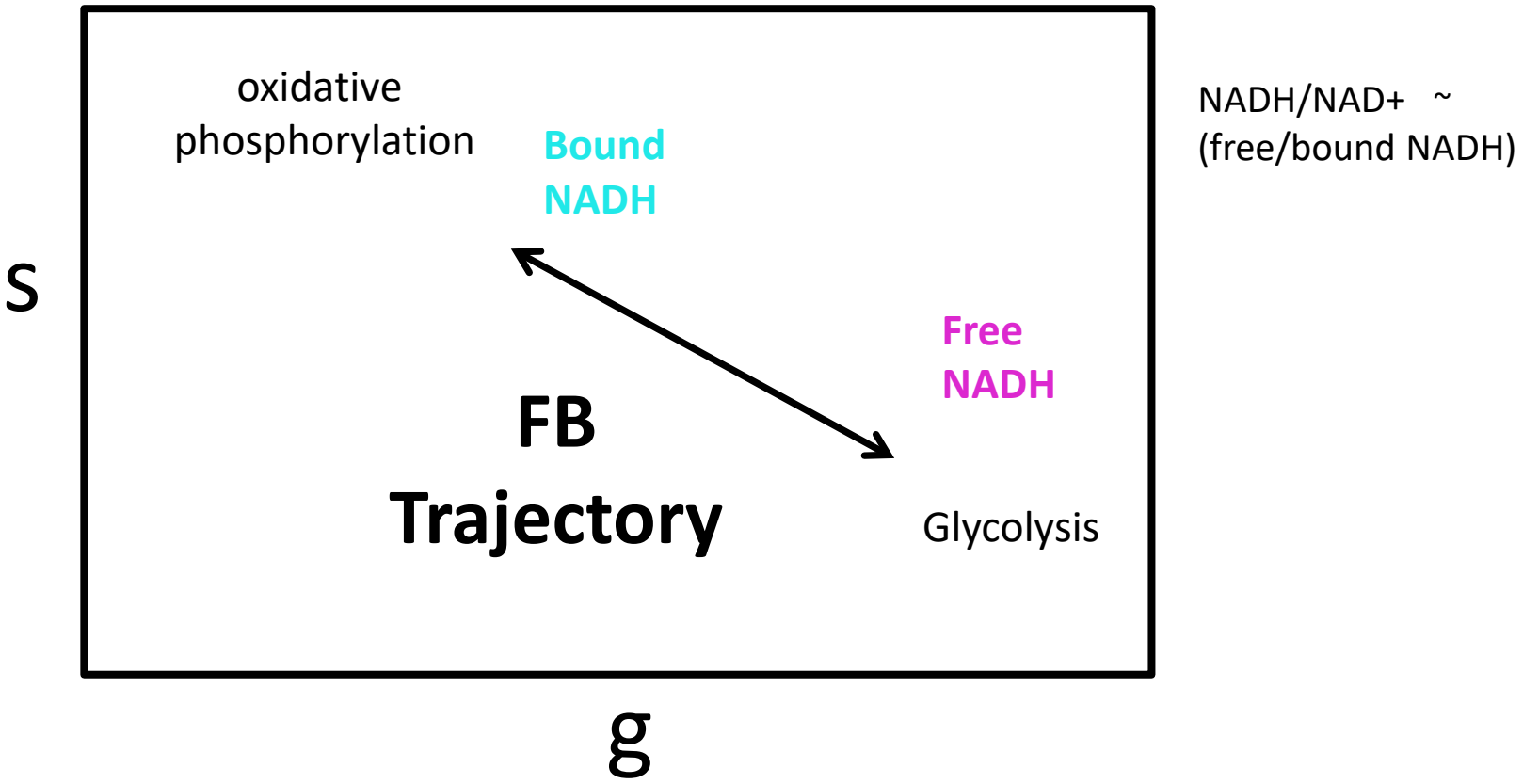
Current work and future directions

- ✓ Identify asymmetric and symmetric divisions and predict stem cell fate
- ✓ Metabolic evolution of differentiation pathways to different cell lineages.
- ✓ Metabolic pathways in colon cancer (Wnt signaling)

Metabolic Trajectory in the Phasor Plot

Free/bound NADH gradients associated with:

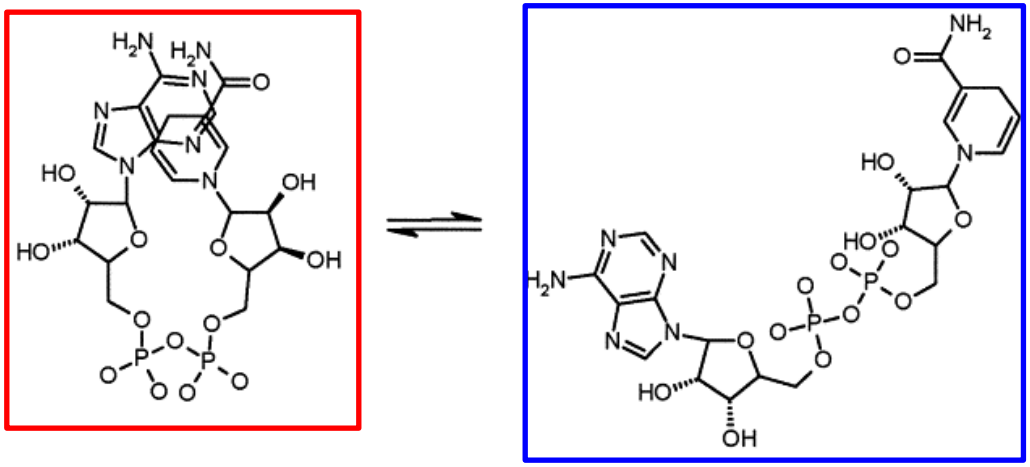
- Glycolysis/oxidative phosphorylation
- Oxidative stress
- cell proliferation
- differentiation
- Cancer



Non-invasive detection of metabolism using intrinsic autofluorescence

- Reduced form of the Nicotinamide adenine dinucleotide autofluorescent (NADH)
- This coenzyme that plays a role in production of energy in cells.
- In our study we excited NADH with a 2-photon laser to obtain the fluorescence lifetime.

Free NADH **Bound NADH**

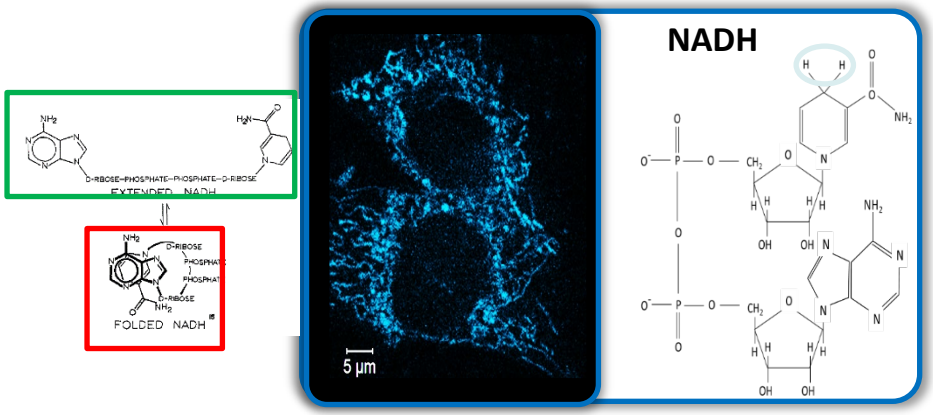


τ=0.4ns τ=3.4ns

Fig. 1. β-NADH equilibrium exist between the folded and unfolded conformations. Efficient energy transfer only occurs between the two chromophores in the folded conformation.

What is NADH & what is it's function?

- A the reduced form of Nicotinamide adenine dinucleotide (NADH) is found in all living cells.



- NADH binding is impacted by biochemical changes:
  - pH
  - Temperature
  - Other molecules eg. Chemical inhibitors

} Conformational and ionic changes at binding sites

- In metabolism NADH has several key roles:
  - It acts as a coenzyme in redox reactions where:
    - NAD^+ is an oxidising agent – accepts electrons and becomes reduced.
    - NADH is a reducing agent – donates electrons and becomes oxidised.
  - A precursor molecule in ADP-ribosylation:
    - Associated with cyclic ADP-ribose.
  - A substrate for:

Lakowicz, J. R., *et al., Analytical Biochemistry* (1992), Wright, B. K., *et al., Biophysical Journal* (2012), Wright, B. K., *et al., Microscopy Research and Technique* (2012)

FLIM Free and bound NADH as a Metabolic marker

"In vivo Multiphoton Fluorescence Lifetime Imaging of Protein-bound and Free NADH in Normal and Pre-cancerous Epithelia"

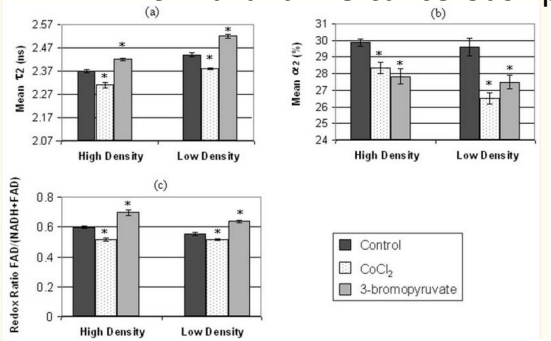
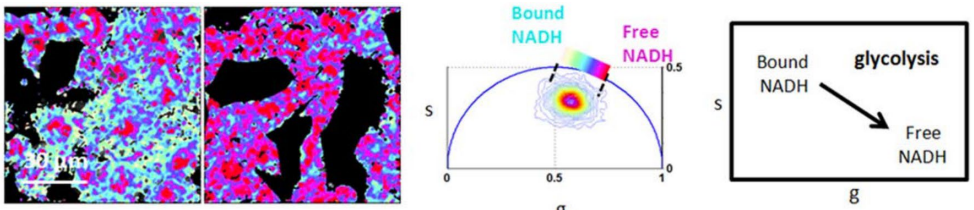


Figure. **Skala, M. C. et al. PNAS, 104, 19494–9 (2007).**

"Phasor –FLIM of Free and Protein Bound NADH Reveals Neural Stem Cell Differentiation Potential"

Low Glucose **High Glucose**



Stringari, C. et al. PLoS One, 7(11), 1-11 (**2012**).

Glycolysis

Free NADH Short Lifetime

Bound NADH Long Lifetime

Oxidative Phosphorylation

Lakowicz JR, et al. Proc Natl Acad Sci U S A. 1992;89(4):1271–1275
Bird, D. K. et al. Cancer Res. 65, 8766–8773 (**2005**).

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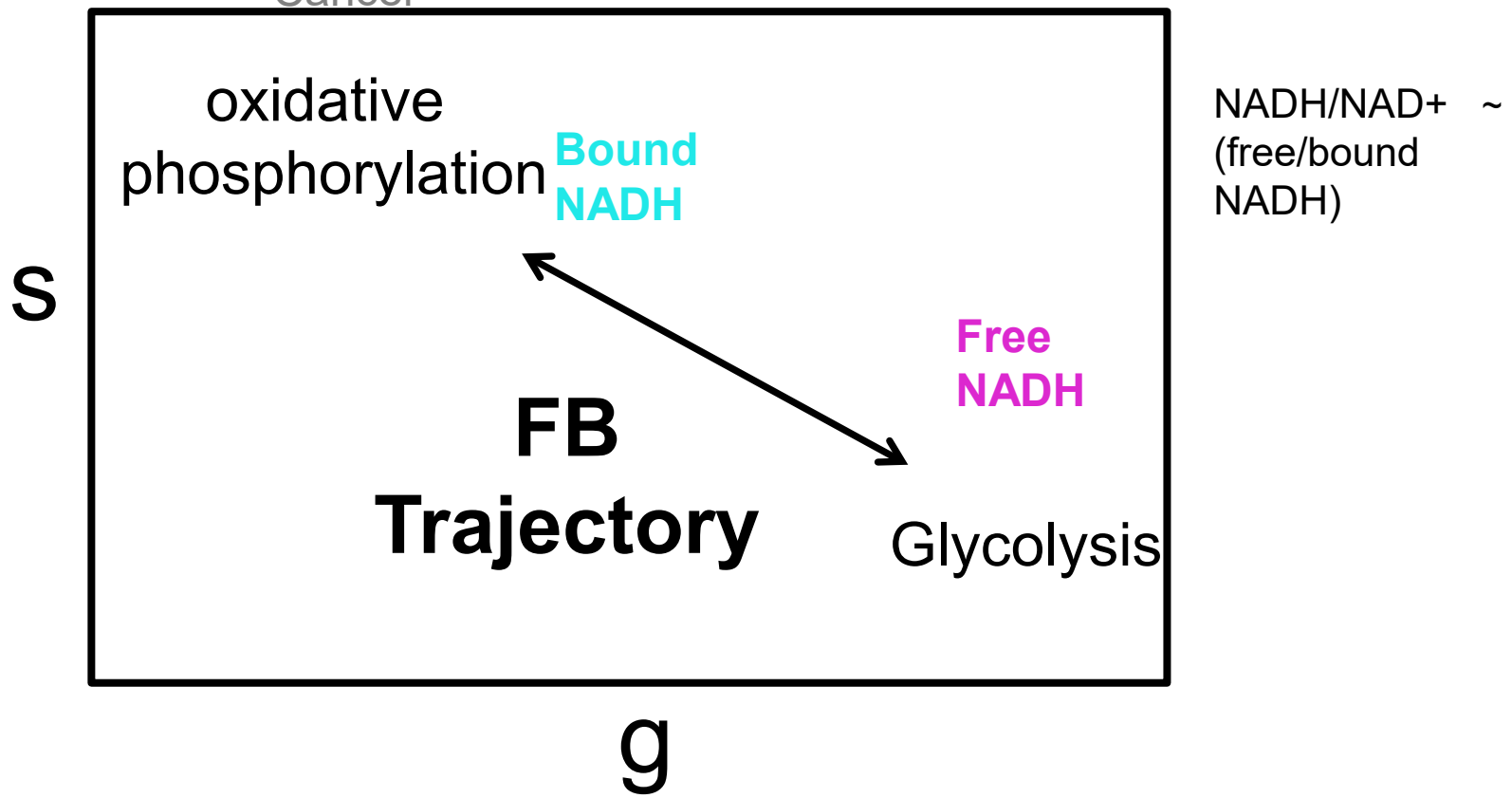
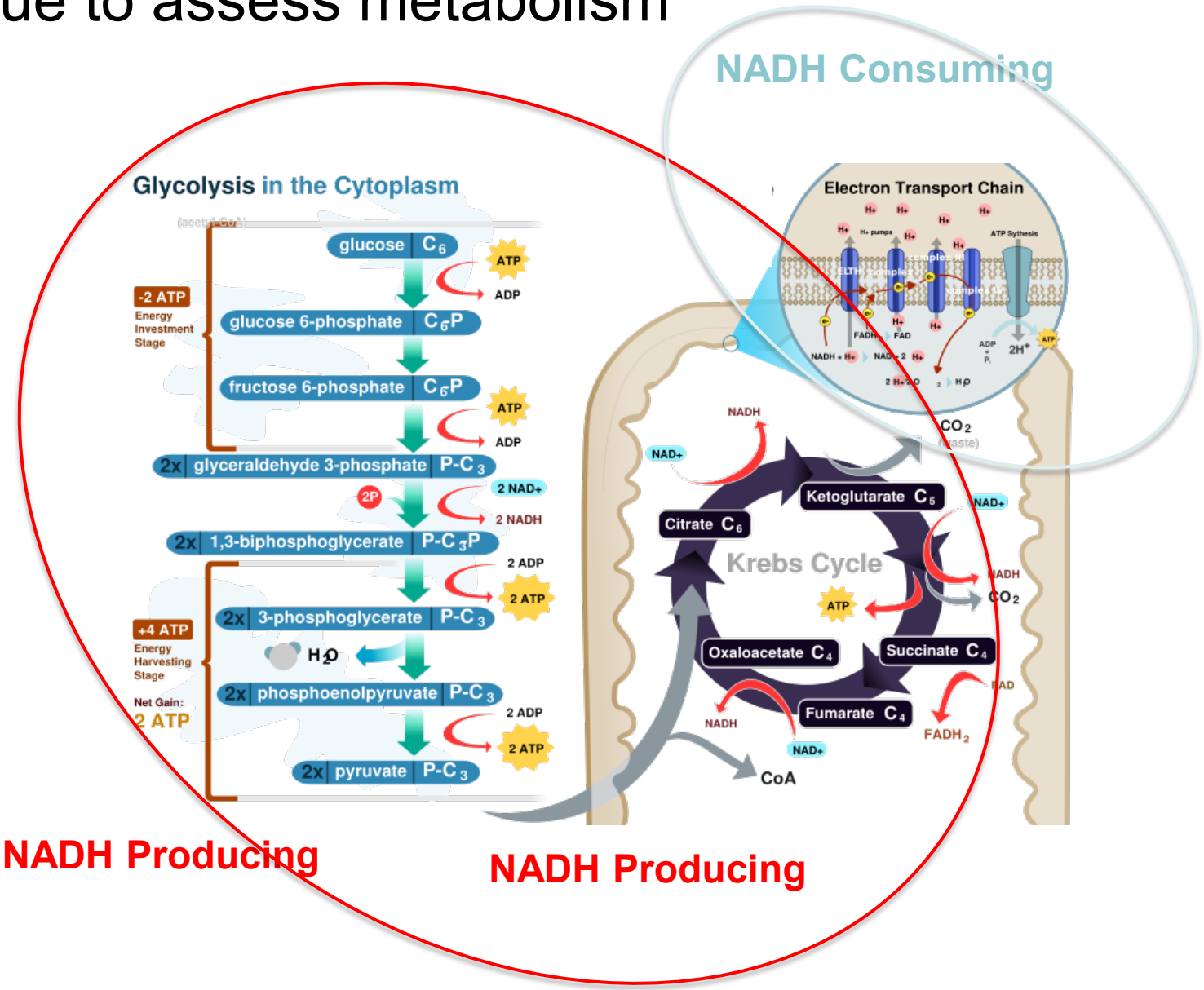
Metabolic Trajectory in the Phasor Plot

Free/bound NADH gradients associated with:

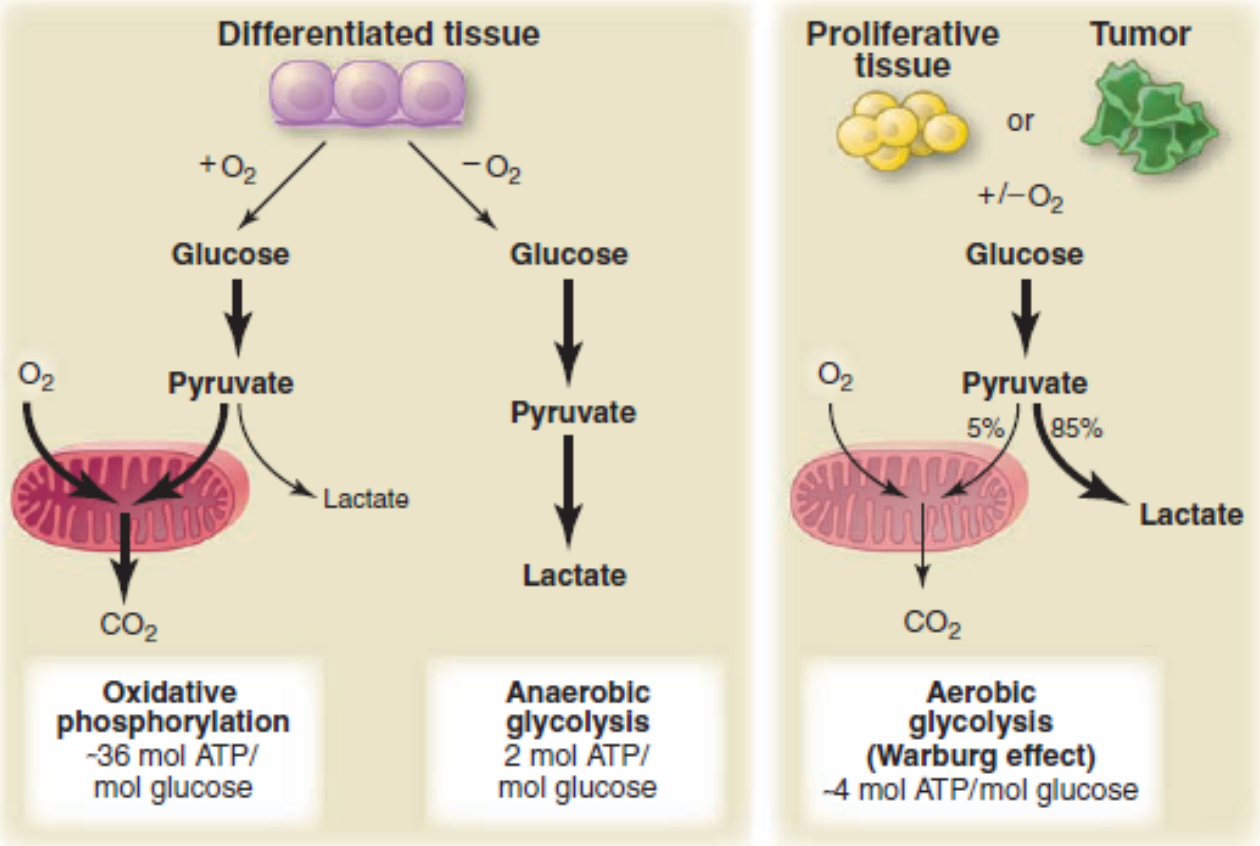
- Glycolysis/oxidative phosphorylation
- Oxidative stress
- cell proliferation
- differentiation
- Cancer



Phasor FLIM measurement of Free/Bound NADH in tissue to assess metabolism



Metabolism in tissues



LOW NADH/NAD+ (LOW free/bound NADH)

HIGH NADH/NAD+ (**HIGH** free/bound NADH)

Cairns et al. Nature reviews Cancer 2011Heiden, *et al. Science* 2009

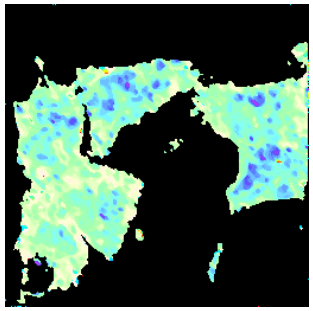
Metabolic Trajectory

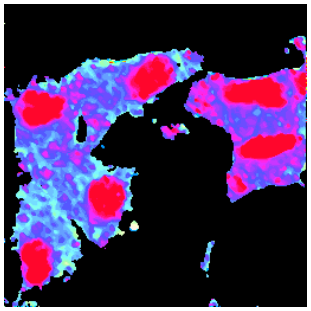
Oxidized NAD+/ Reduced NADH --> Free/bound NADH

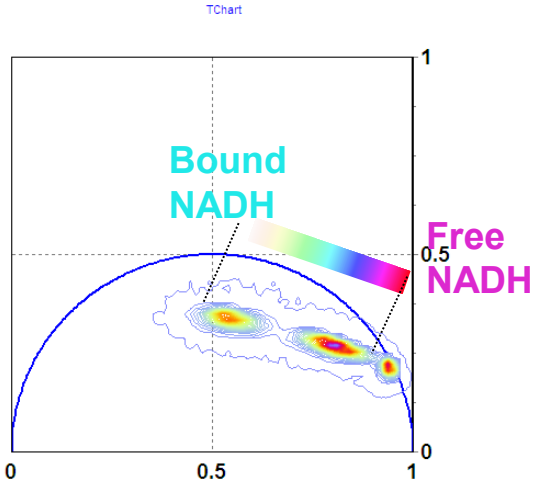
Electron chain inhibition

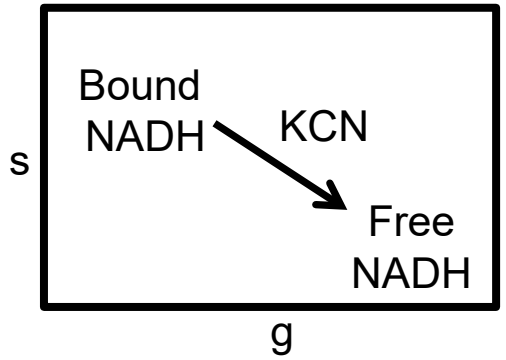
Before

KCN





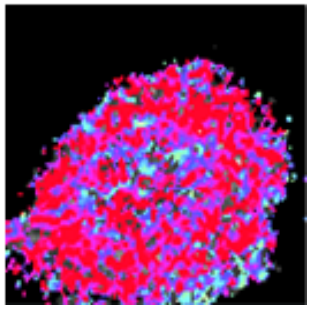


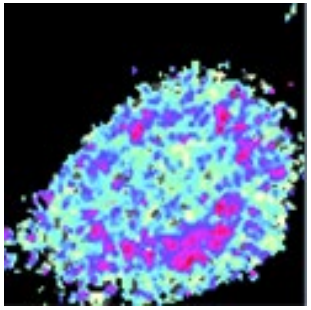


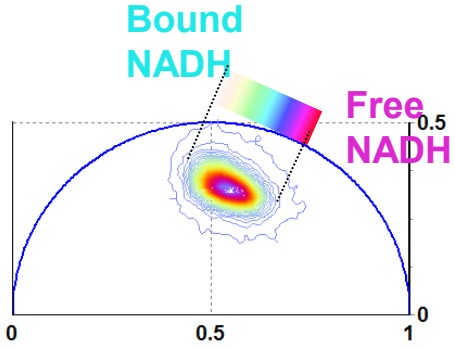
Oxidative stress

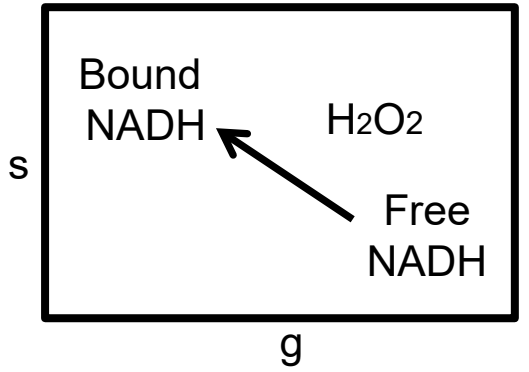
Before

H_2O_2

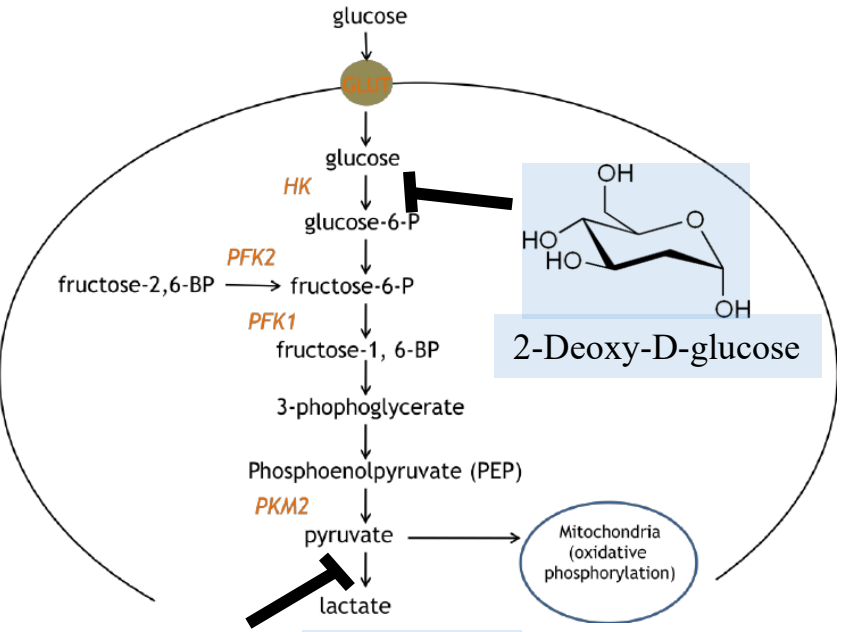




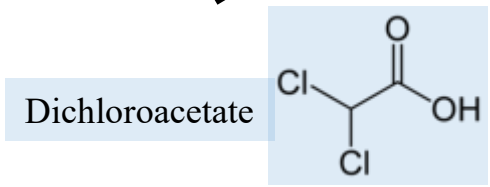




Glucose metabolism

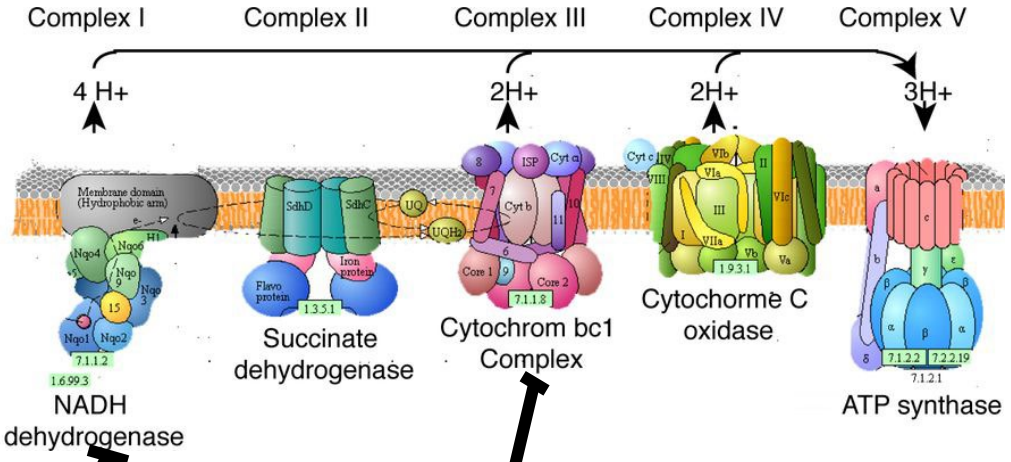


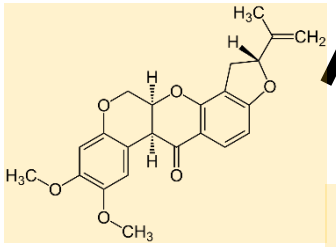
2-Deoxy-D-glucose



Dichloroacetate

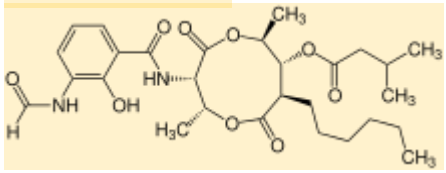
Weinstein, BioRxiv (2019)





Rotenone

Antimycin A

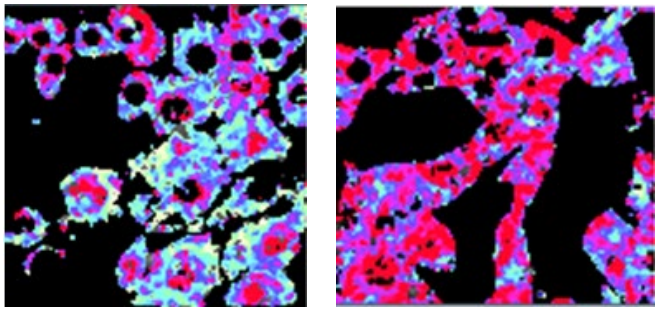


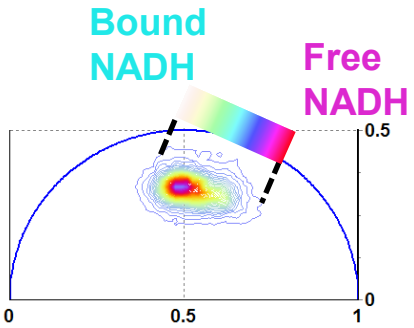
Metabolic Trajectory
glycolysis/ oxidative phosphorylation --> Free/bound NADH

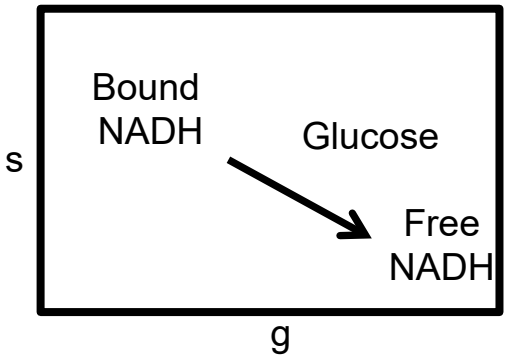
Glucose uptake

4.5 mM glucose

22 mM glucose



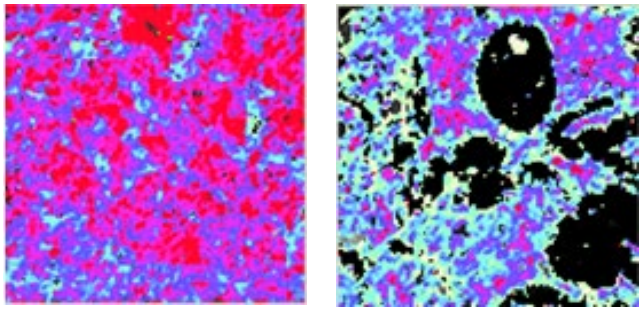


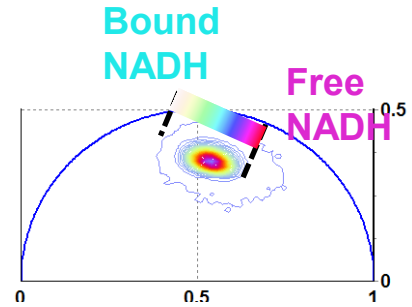


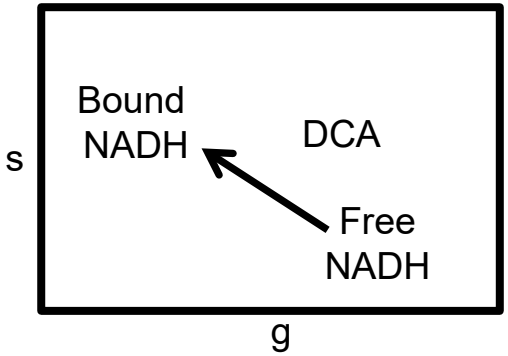
Glycolysis inhibition Dichloroacetate (DCA) metabolic-targeting cancer drug

control

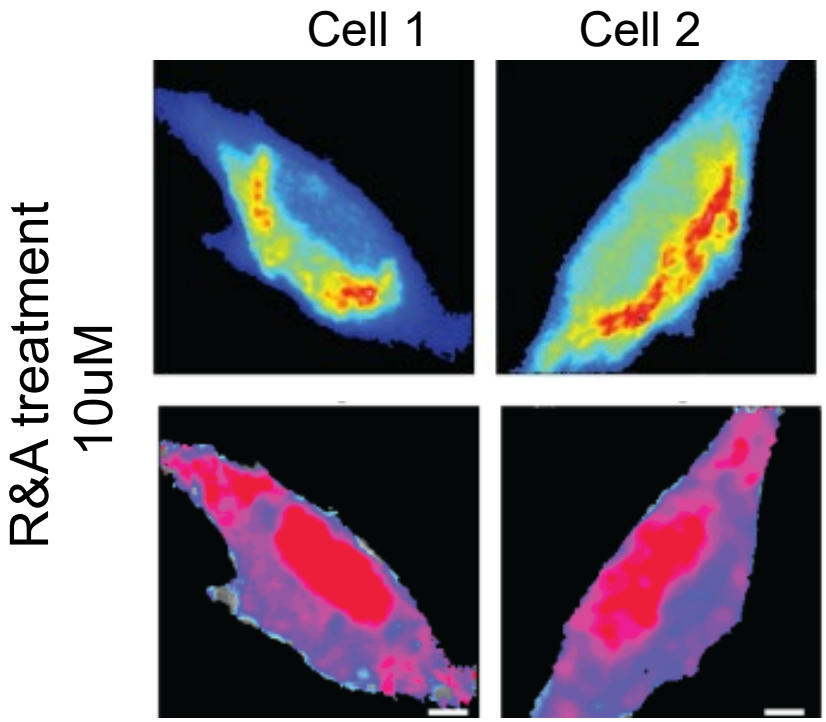
DCA

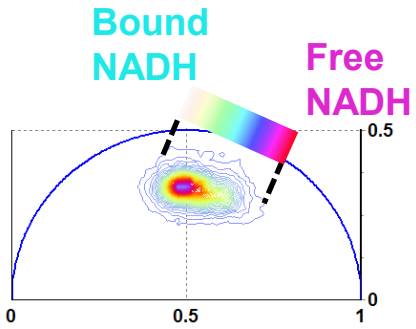


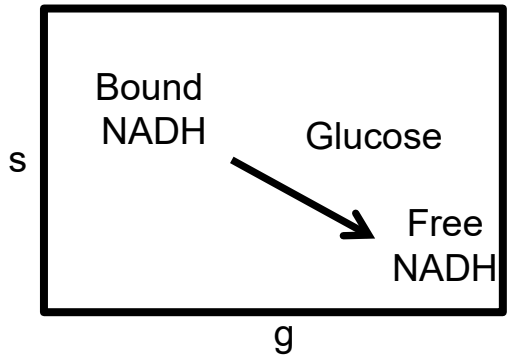




Shutting down Oxidative Phosphorylation: Rotenone + Antimycin A



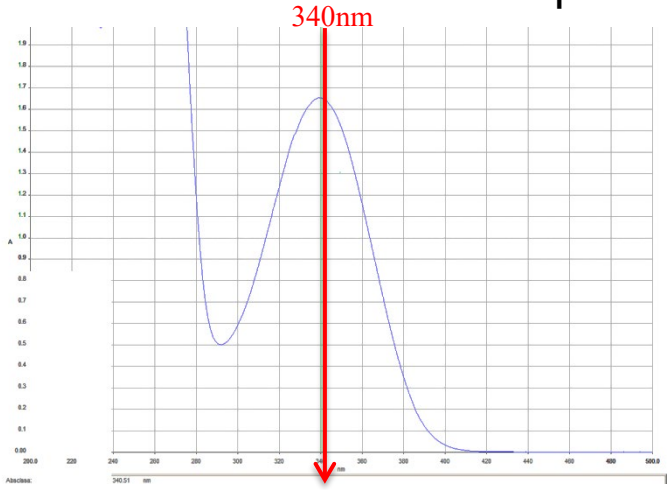




Procedures for FLIM experiments

FLIM:

1. We took an absorbance measurement of free Nicotinamide adenine dinucleotide (NADH) between 200-500nm. The peak of the absorbance band was 340 nm:



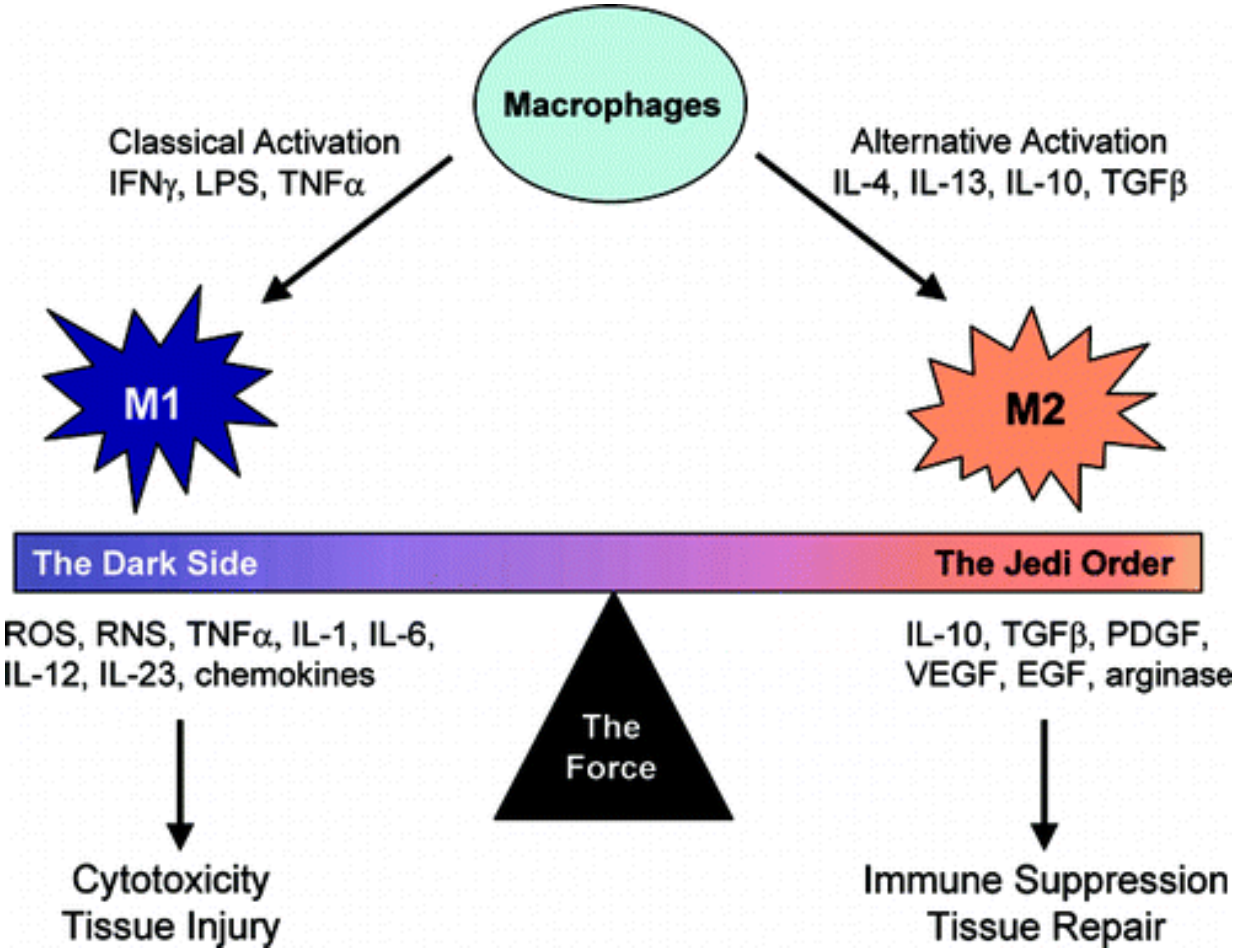
2. For the control, we took free NADH and bound NADH as a reference to mark the phasor lifetime on the polar plot

3. We took M0, M1, M2 cells, plated on glass coverslips coated with fibronectin

4. Microscope setup: use 2-Photon laser set to 740 nm to excite free and bound NADH. The fluorescence emission was collected using a PMT with a BP filter: 420-500nm.

5. Used SimFCS software to analyze FLIM data using the phasor plot

M1 vs. M2 Macrophages



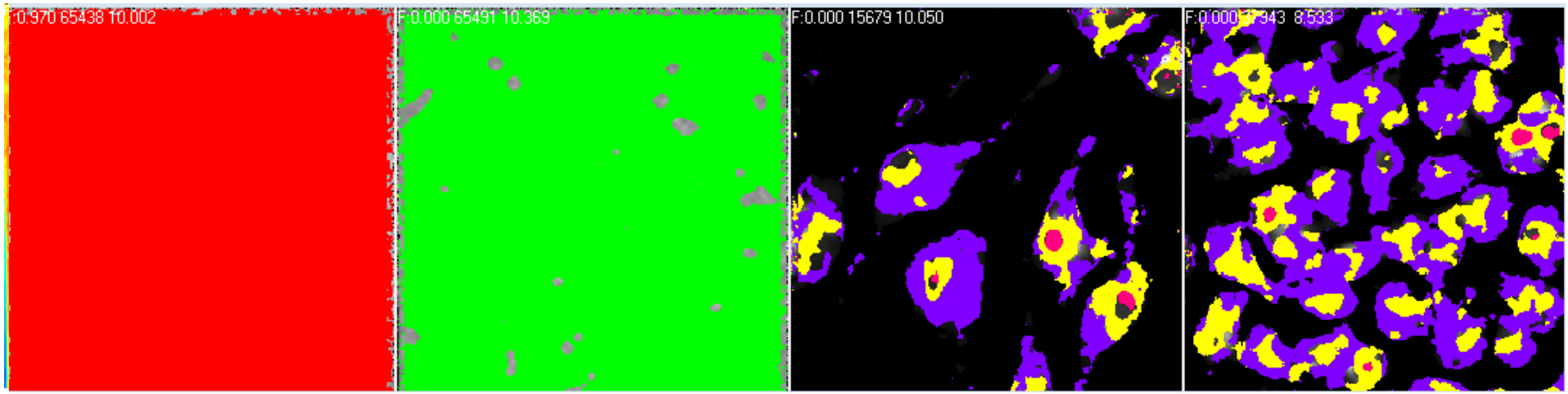
http://www.drthrasher.org/page167.html

M1 stimulated cells have an overall **decreased** bound NADH population which indicates a shift towards **glycolysis**

M2 stimulated cells have an overall **increased** bound NADH population which indicates a shift towards **OXPHOS**

Results of FLIM Study

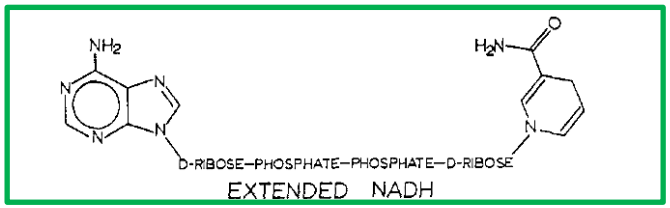
NADH

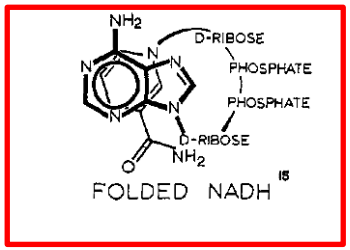


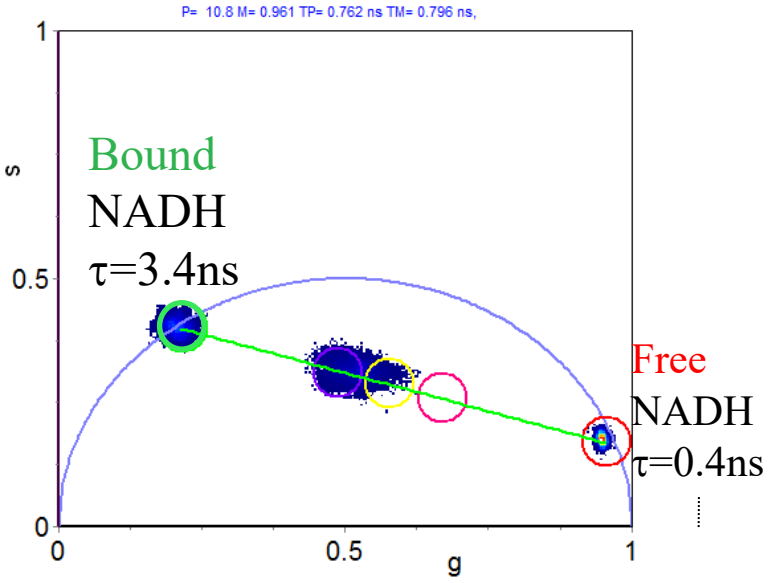
Free NADH

Bound NADH to LDH

M0 macrophages on glass







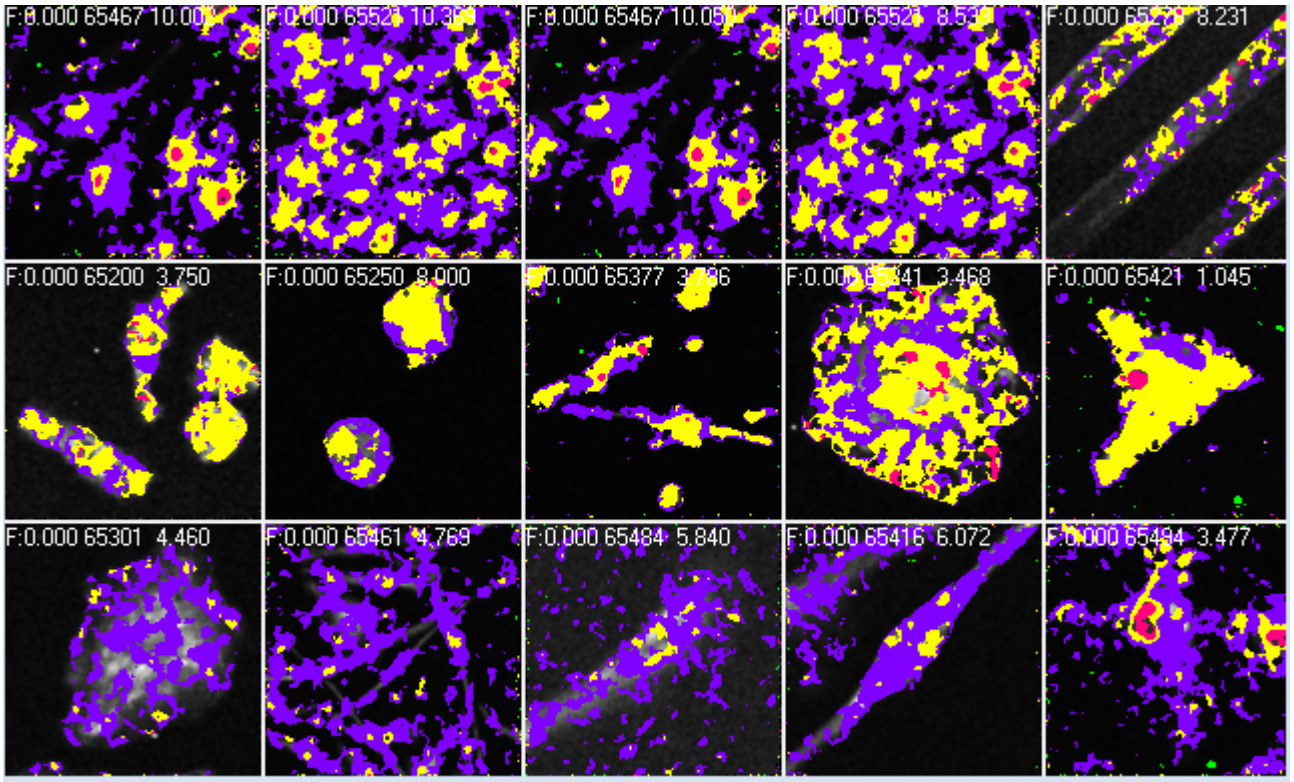
Results of FLIM Study

M0 vs M1 vs M2

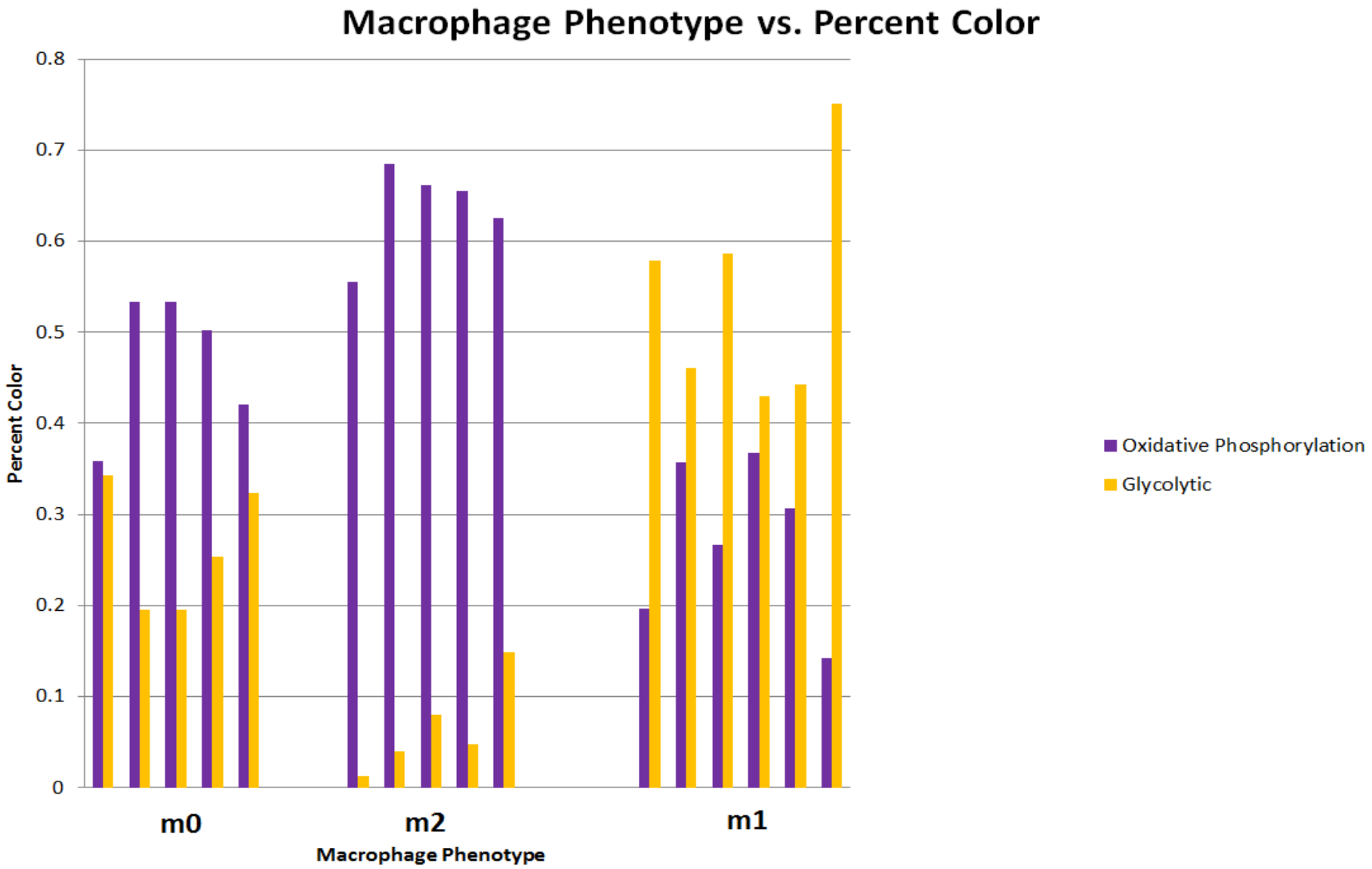
M0
Macrophages

M1 Macrophages
LPS, corresponding to proinflammatory macrophages.

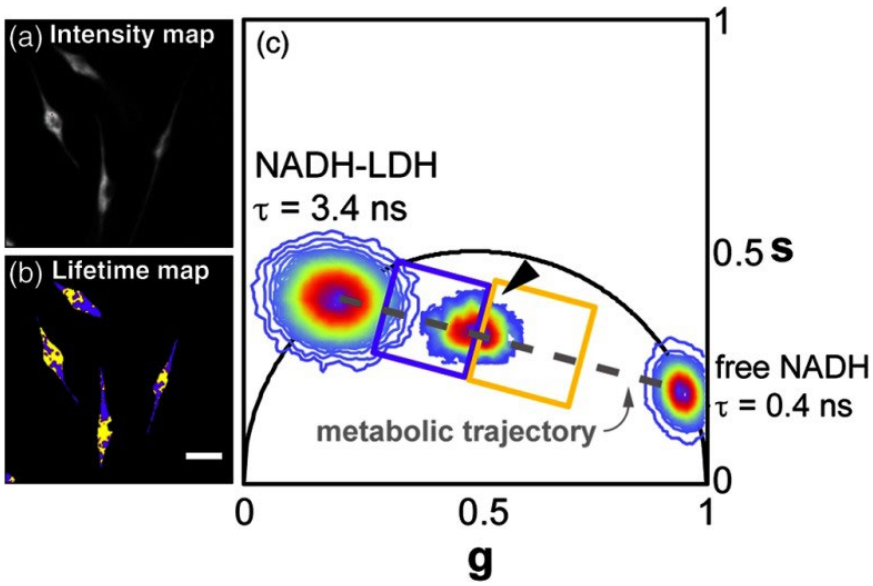
M2
Macrophages

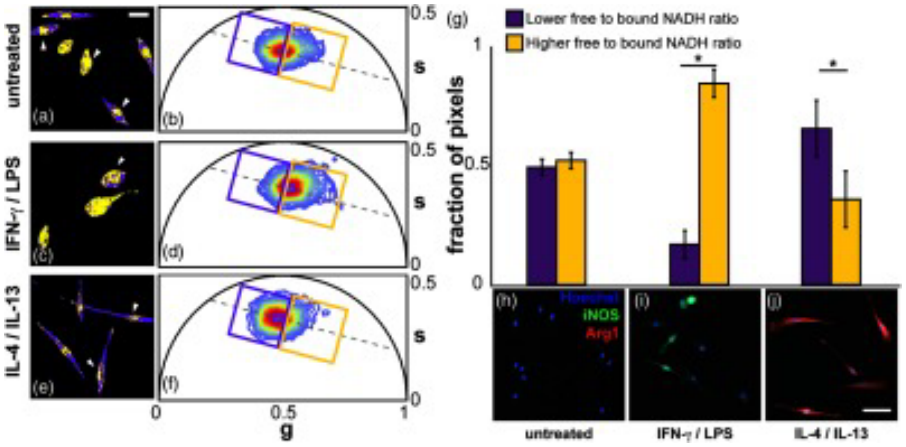


Results of FLIM Study



Polarized macrophages have different lifetime distributions





Alfonso-García A, Smith TD, Datta R, Luu TU, Gratton E, Potma EO, Liu WF. Label-free identification of macrophage phenotype by fluorescence lifetime imaging microscopy. J Biomed Opt. 2016 Apr 30;21(4):46005. doi: 10.1117/1.JBO.21.4.046005. PMID: 27086689; PMCID: PMC4833856.

Small Intestine (SI)

- ✓ Ti : sapphire laser, 80 MHz
- ✓@ 880 nm and @ 740 nm
- ✓ Power ~ 4 mW
- ✓ 40 x 0.8 NA water immersion WD=2mm
- ✓ ISS A320 FastFLIM
- ✓ Pixel dwell time: 25 µs/pixel
- ✓ PMT H7422P-40 of Hamamatsu

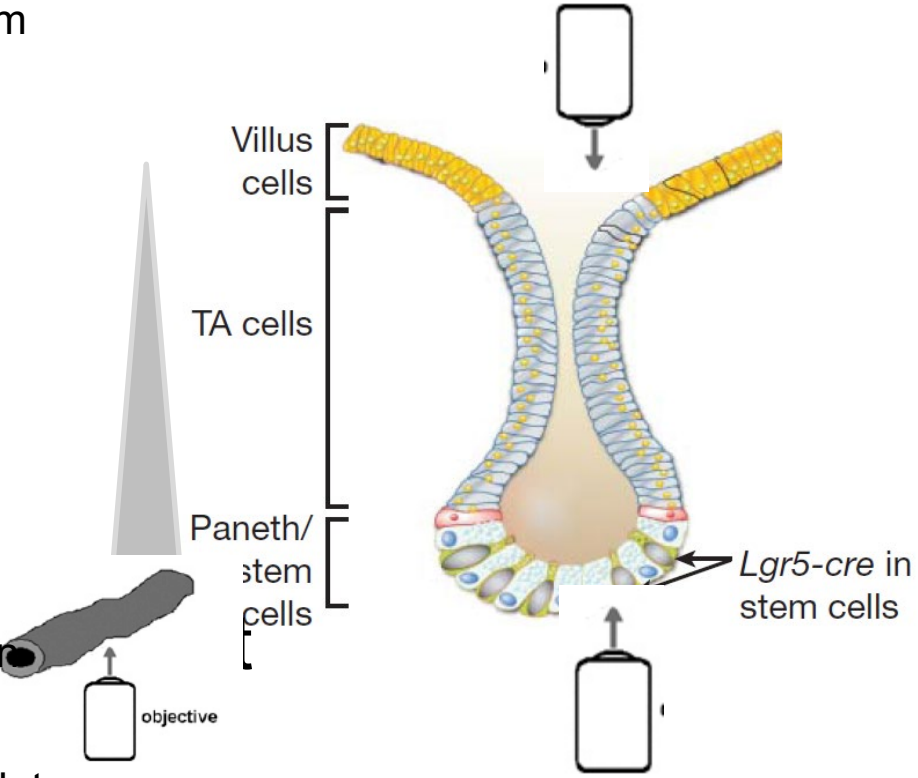
✓**Lgr5+GFP stem cells** at the base of the crypt

✓ alternated pattern with Paneth cells (“niche” cells)

✓The **Wnt gradient** controls the cell fate and proliferation along the crypt villus axis

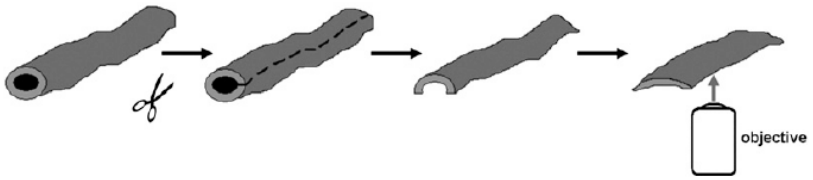
✓**tissue imaging**:

✓Lgr5+ stem cells located at the crypts base are responsible of generating the **intestinal cancer** (**aberrant Wnt**)



- from the outside of the gastric lumen

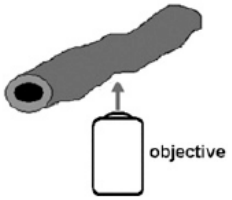
- the *ex-vivo* SI tube was cut and open flat

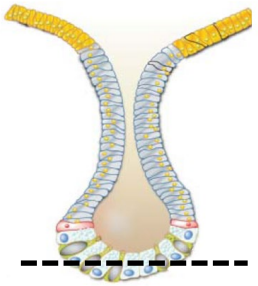


(Barker et al. Nature 2007 ; Barker et al. Nature 2009; van de Wetering et al. Cell 2002; Reya et. al Nature 2005)

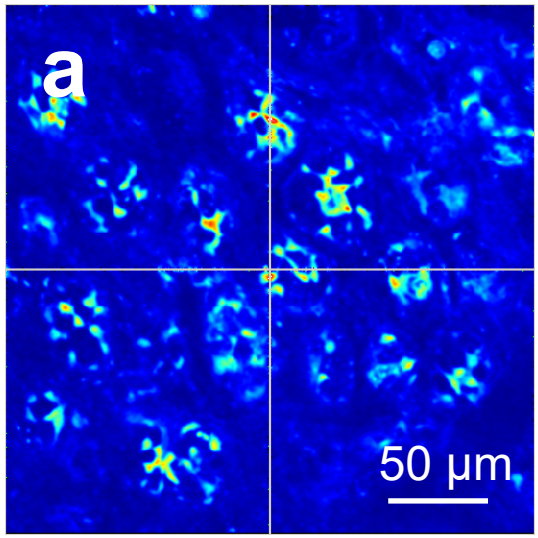
Origin of intrinsic contrast in the small intes

@ 880nm

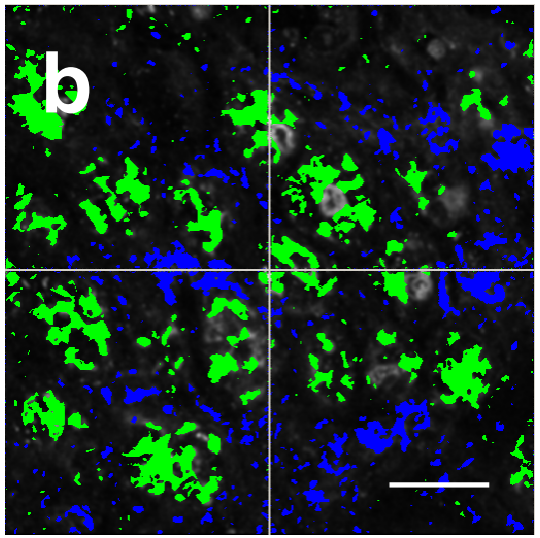


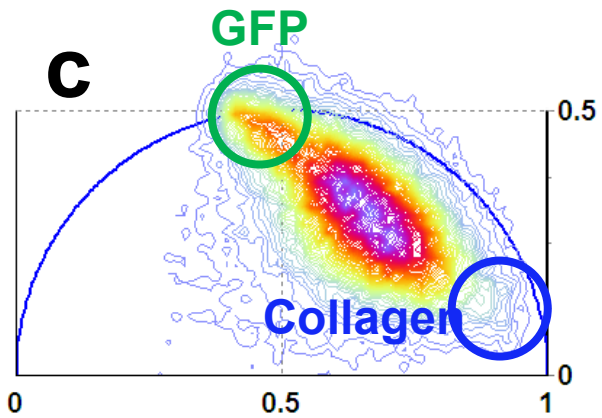


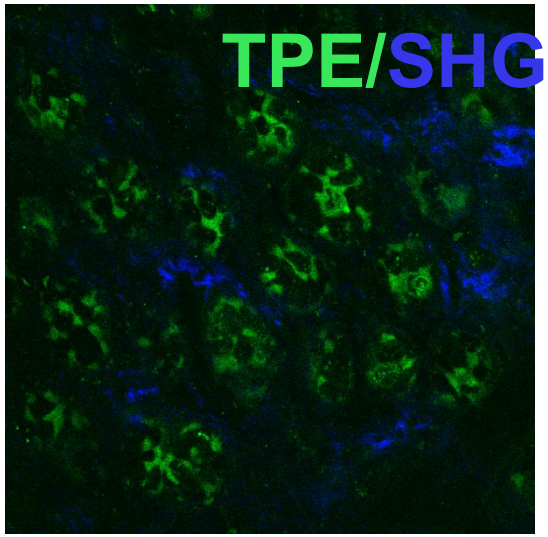
Intensity



FLIM map



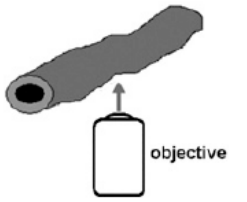


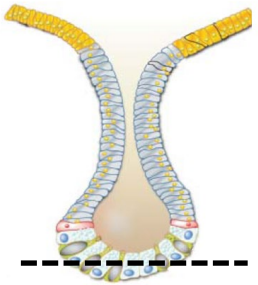


Stringari et al. “Identification of stem cells and metabolic gradients of small intestine epithelia by Fluorescence Lifetime Microscopy of NADH” submitted

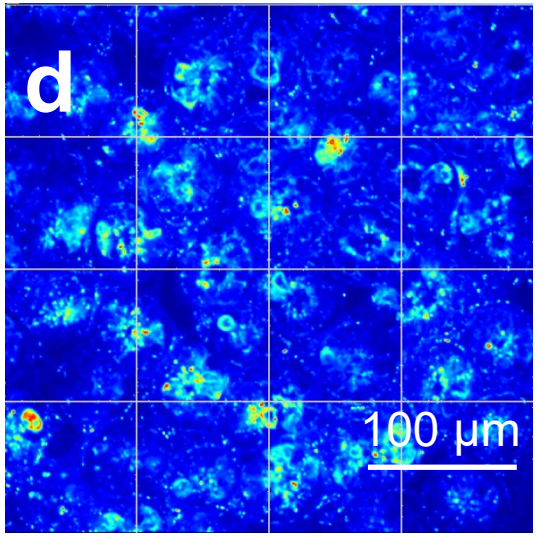
Origin of intrinsic contrast in the small intestine

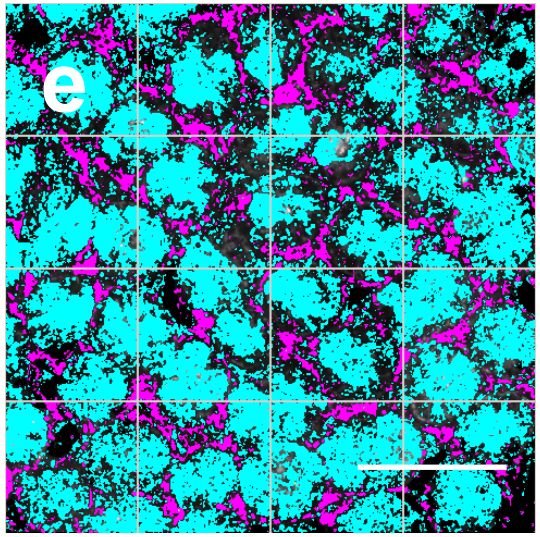
@ 740nm

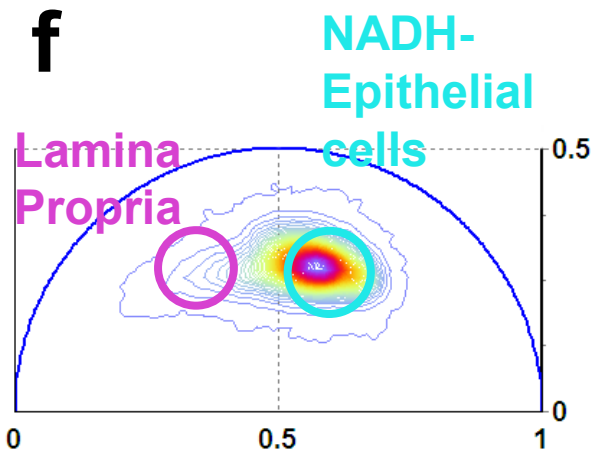




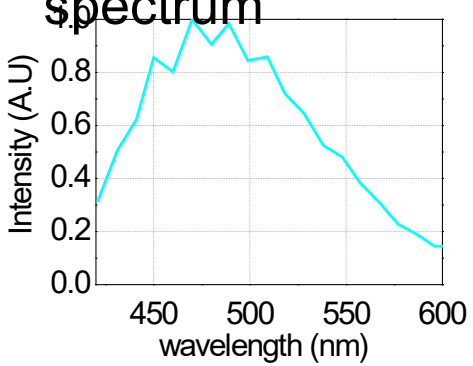
CRYPTs @ 740nm





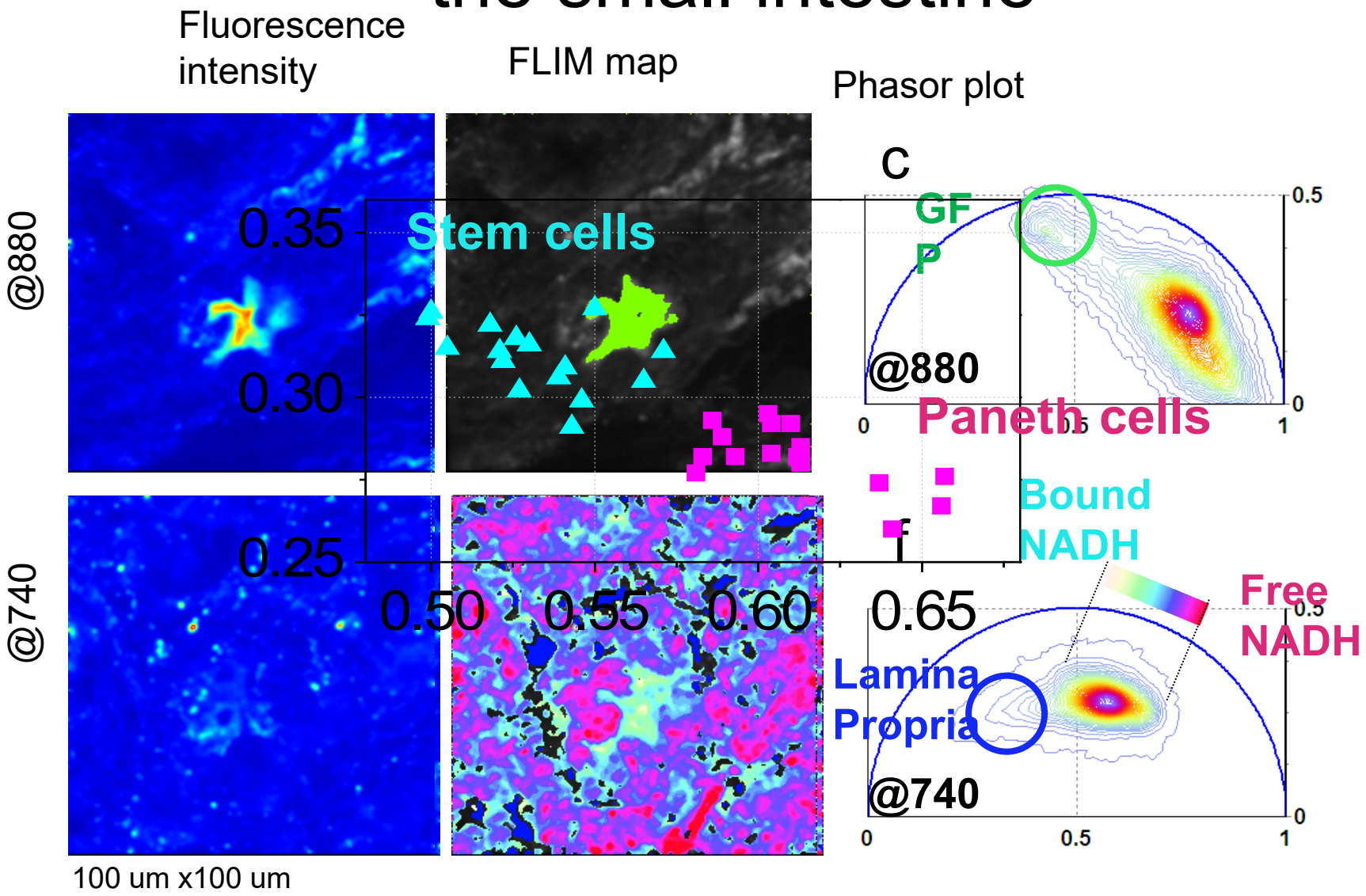


Emission spectrum



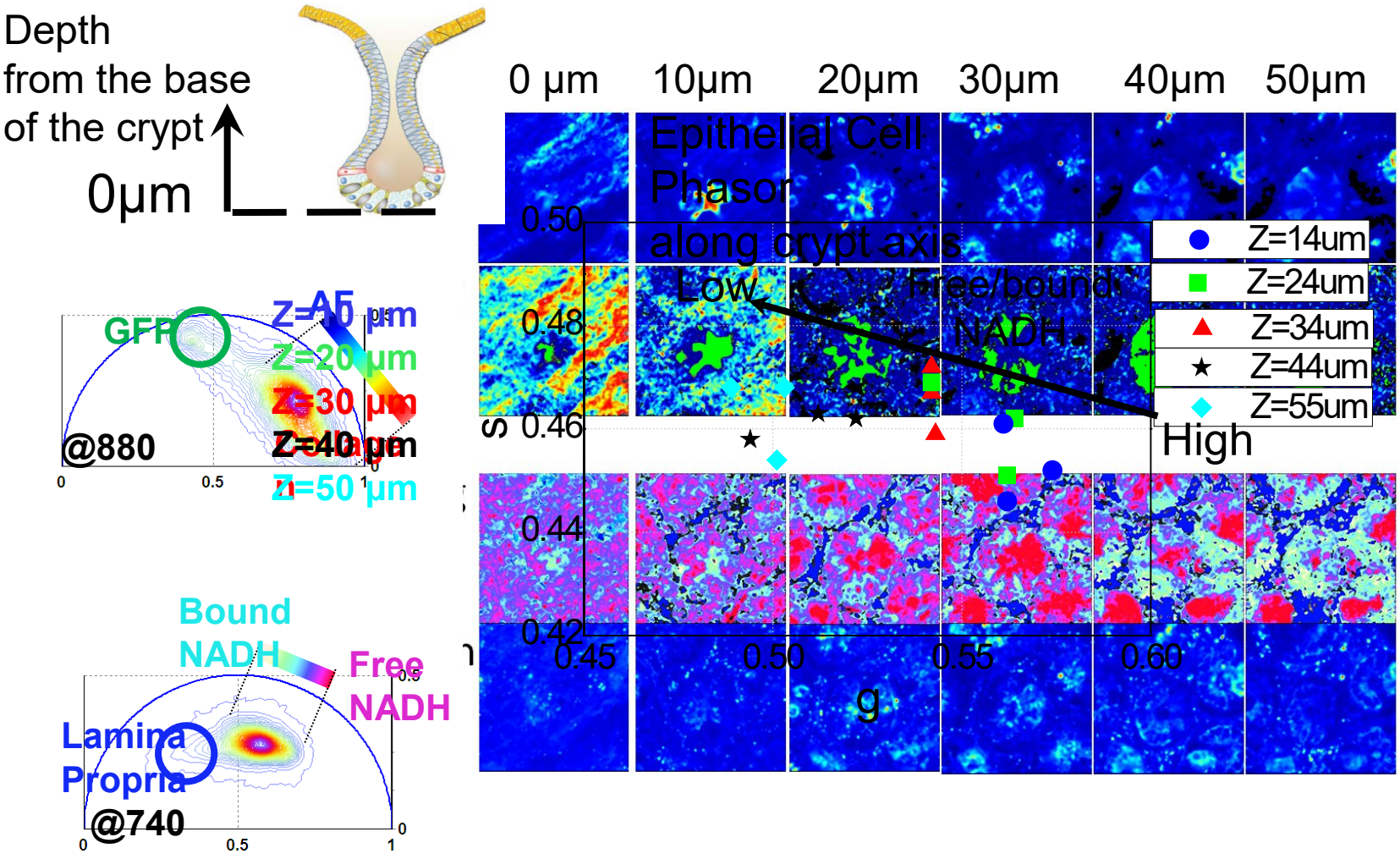
Stringari et al. “Identification of stem cells and metabolic gradients of small intestine epithelia by Fluorescence Lifetime Microscopy of NADH” submitted

Label free identification of stem cells in the small intestine



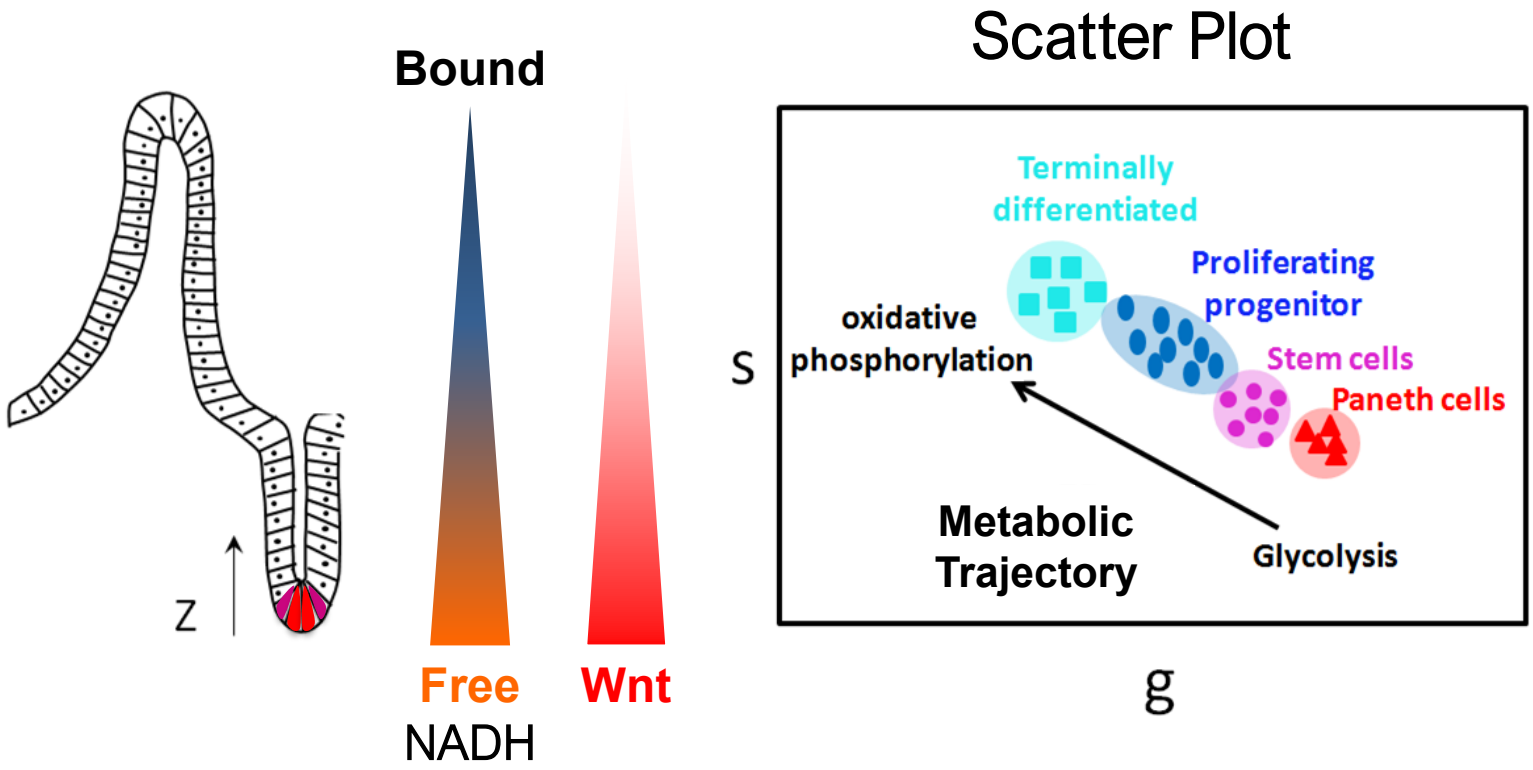
Stringari et al. “Identification of stem cells and metabolic gradients of small intestine epithelia by Fluorescence Lifetime Microscopy of NADH” submitted

Free/bound NADH gradient in the SI crypt



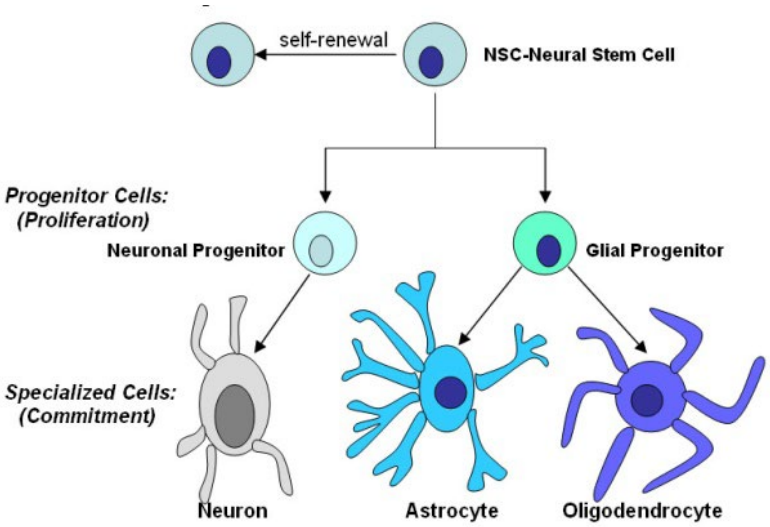
Stringari et al. “Identification of stem cells and metabolic gradients of small intestine epithelia by Fluorescence Lifetime Microscopy of NADH” submitted

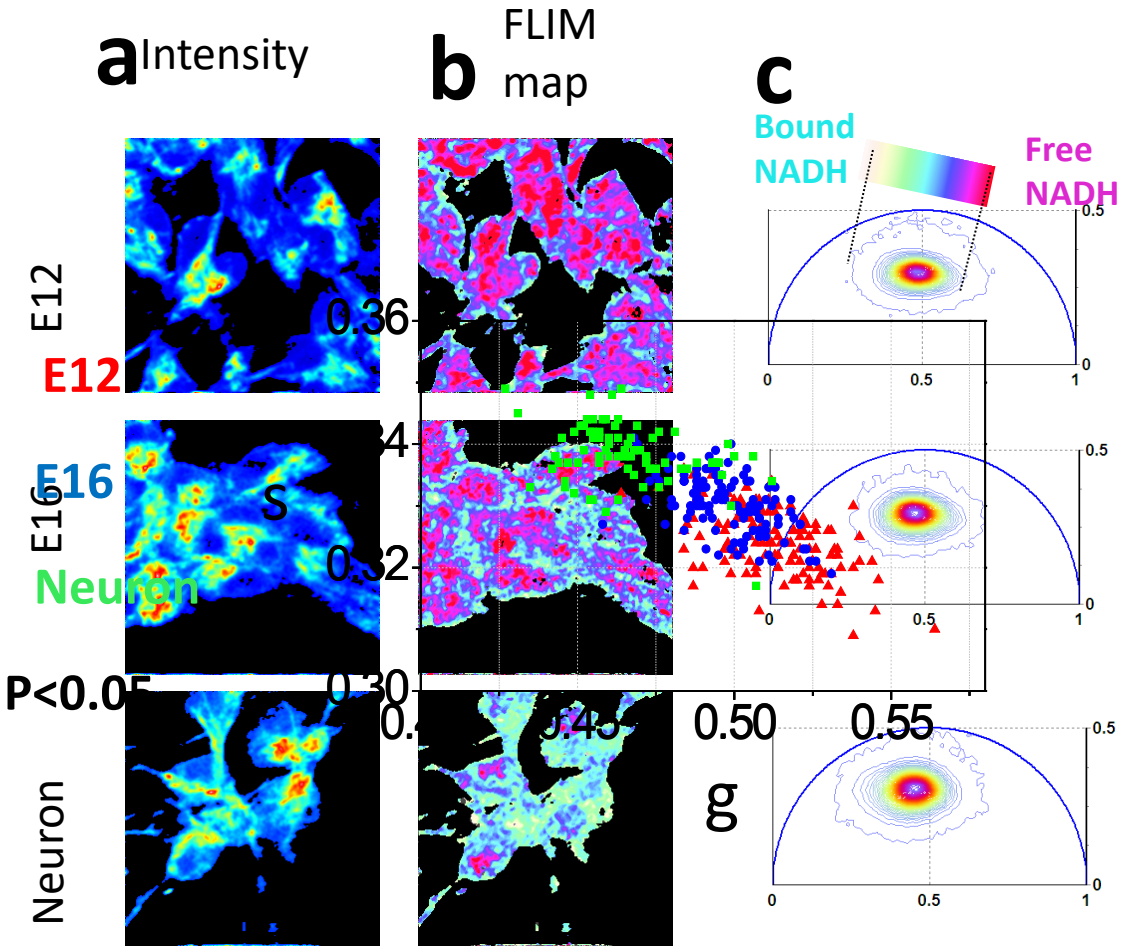
A link between Wnt and glycolysis and stem cells in the intestinal crypt



Stringari, C.,et al. 2012. Metabolic trajectory of cellular differentiation in small intestine by Phasor Fluorescence Lifetime Microscopy of NADH *Sci. Rep.* 2:568

NSPCs and neurons have a unique NADH Phasor FLIM metabolic signature





Free and protein-bound NADH Fluorescence Lifetime Microscopy distinguishes Neuronal Stem cell with different fate

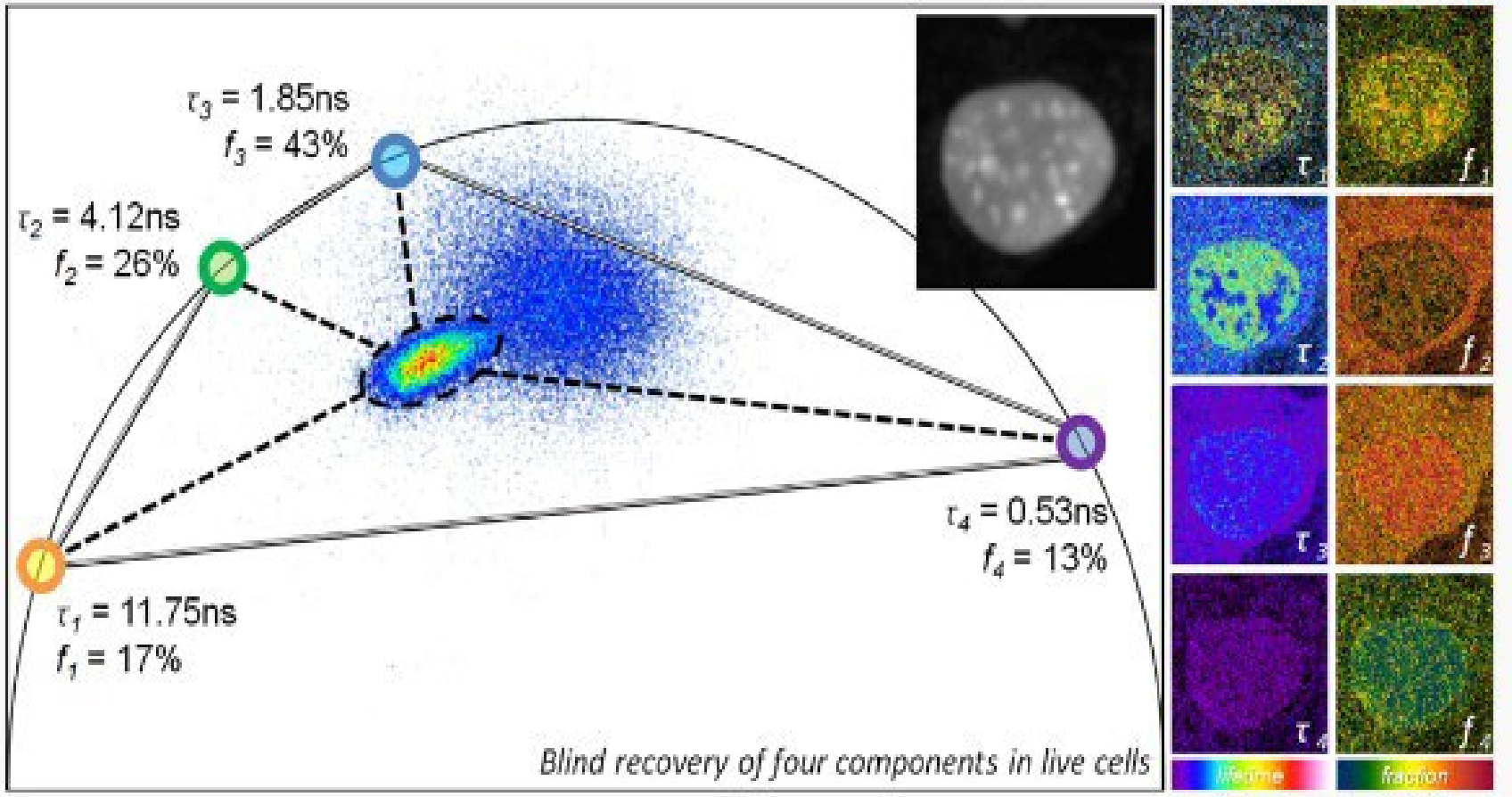
Conclusions

✓**Metabolic Trajectory** of free/bound NADH in the Phasor plot is related to NADH reduction/oxidation and reports on glycolysis, oxidative phosphorylation and oxidative stress

✓Phasor FLIM identifies different tissue components in the small intestine: epithelial cells, collagen fibers at the crypt base, lamina propria

✓Label-free identification of stem cells at the base of the small intestine by NADH fingerprint

✓Free/bound NADH 3D gradients associated to cell differentiation and proliferation in different biological systems



Research Article Vol. 12, No. 6 / 1 June 2021 / Biomedical Optics Express 3410
Biomedical Optics EXPRESS

Phasor-based image segmentation: machine learning clustering techniques

ALEX VALLMITJANA, BELÉN TORRADO, AND ENRICO GRATTON*

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*egratton22@gmail.com

pubs.acs.org/JPCB Article

Blind Resolution of Lifetime Components in Individual Pixels of Fluorescence Lifetime Images Using the Phasor Approach

Alexander Vallmitjana,§ Belén Torrado,§ Alexander Dvornikov, Suman Ranjit,* and Enrico Gratton*

Cite This: *J. Phys. Chem. B* 2020, 124, 10126–10137 Read Online

Methods and Applications in Fluorescence

PAPER

Resolution of 4 components in the same pixel in FLIM images using the phasor approach

Alexander Vallmitjana[1,4], Alexander Dvornikov[1,4], Belen Torrado[1,4], David M Jameson[2], Suman Ranjit[1,3,5] and Enrico Gratton[1,5]

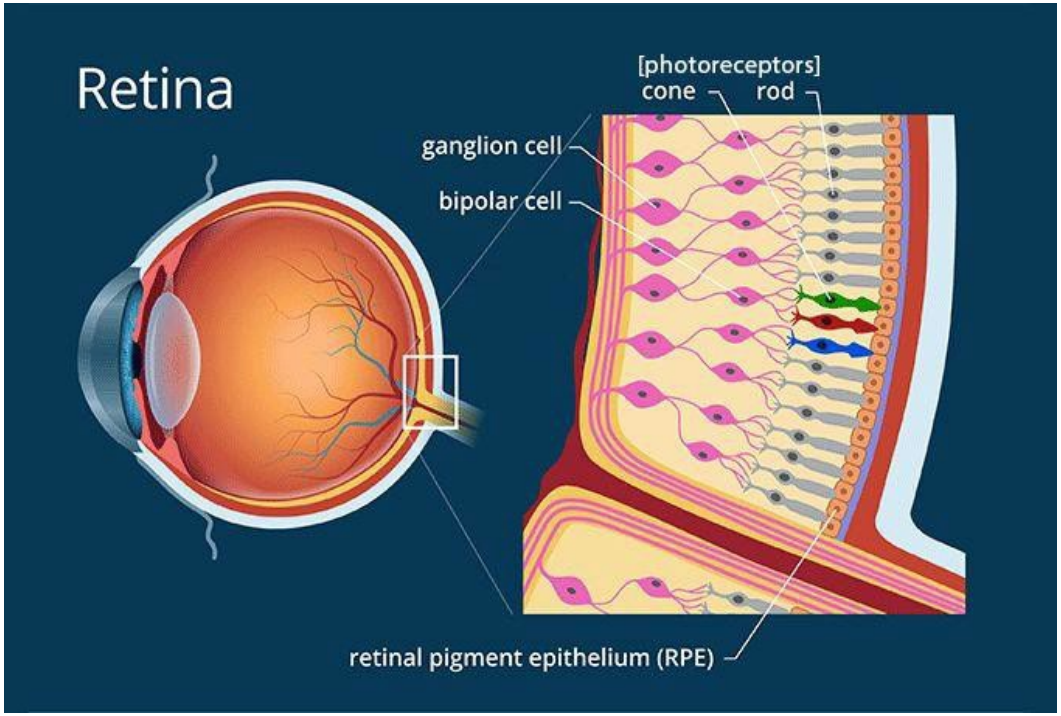
Laboratory for Fluorescence Dynamics

A national research center for biomedical fluorescence spectroscopy at the University of California, Irvine

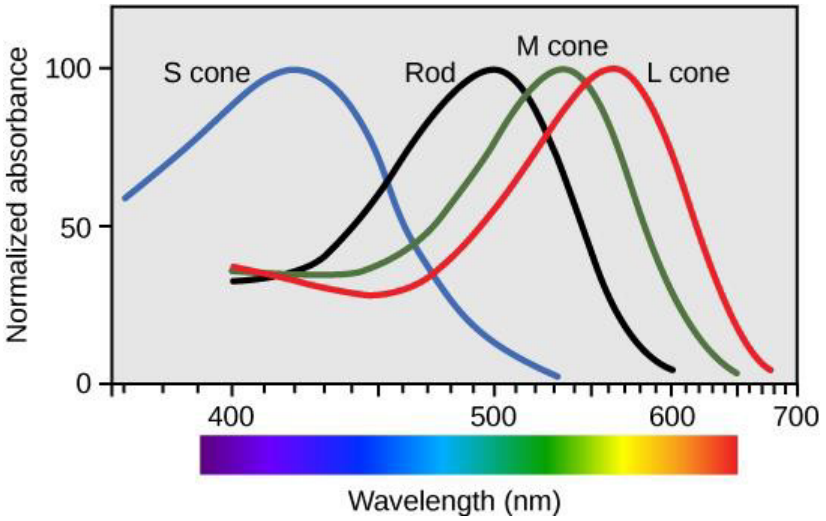
UCIrvine

Hyperspectral Imaging

The human eye as a spectral camera



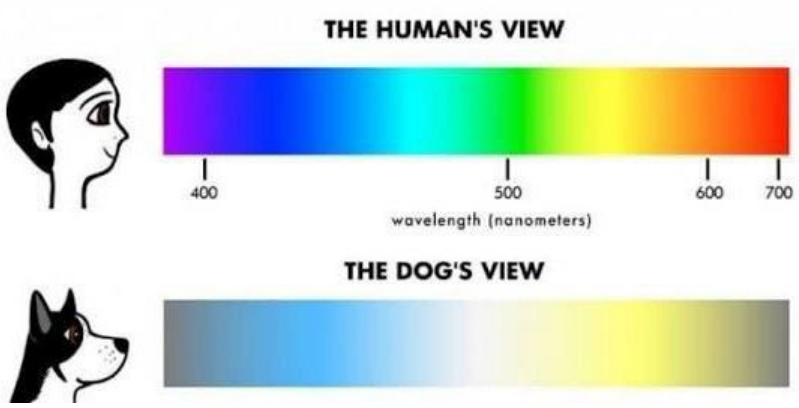
https://www.allaboutvision.com/eye-care/eye-anatomy/photoreceptors/



https://courses.lumenlearning.com/wm-biology2/chapter/transduction-of-light/

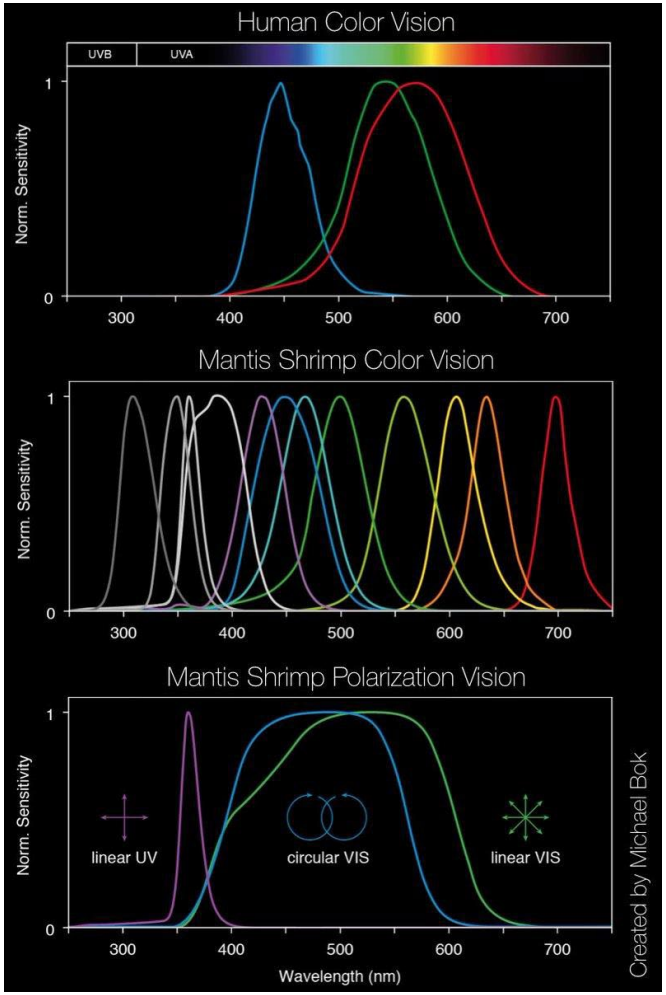
Not all spectral cameras are the same

https://www.quora.com/How-do-dogs-see-the-world-compared-to-humans



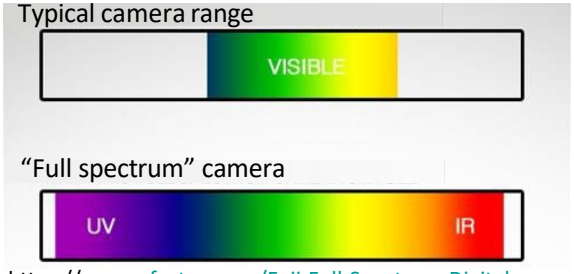
Someone is overdoing it…

https://www.wired.com



Twitter: @mikebok

Summarizing the perks of seeing more

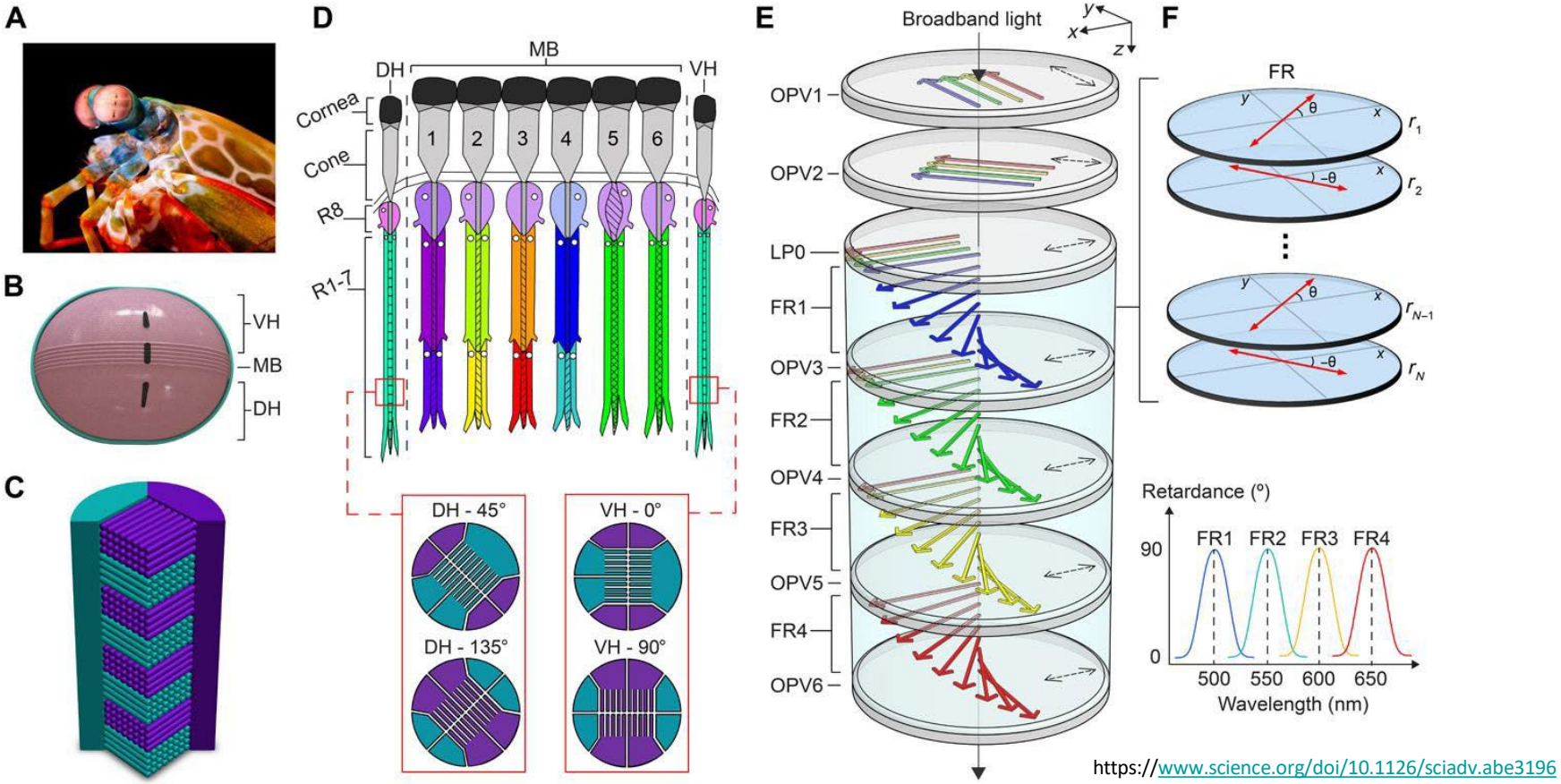


https://www.ufostop.com/Fuji-Full-Spectrum-Digital-Camera-IR-and-UV-p/camera-fsfuji-fullspectrum.htm

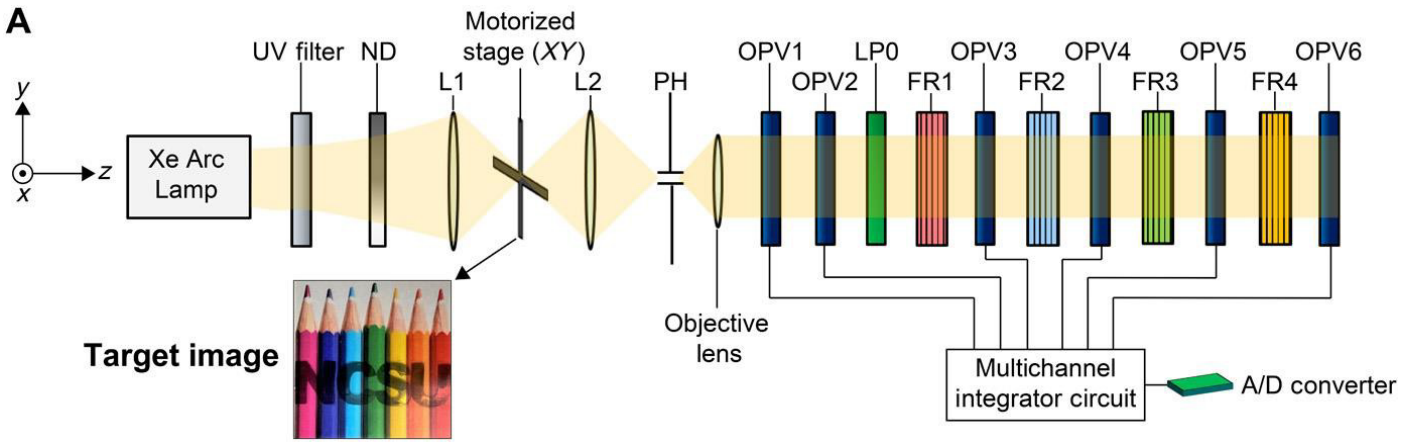
Why all this?
Nature inspires technology!

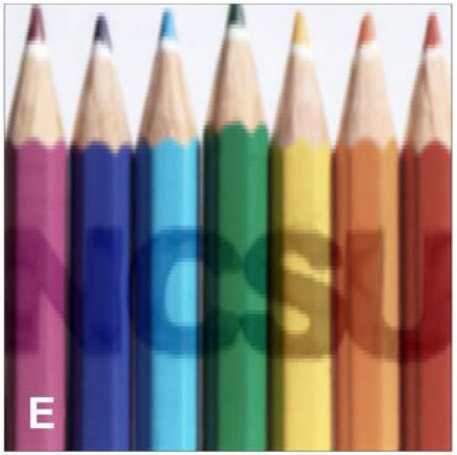
https://www.spencerscamera.com/full-spectrum-cameras.cfm

Inspired by nature Seeing like a mantis shrimp

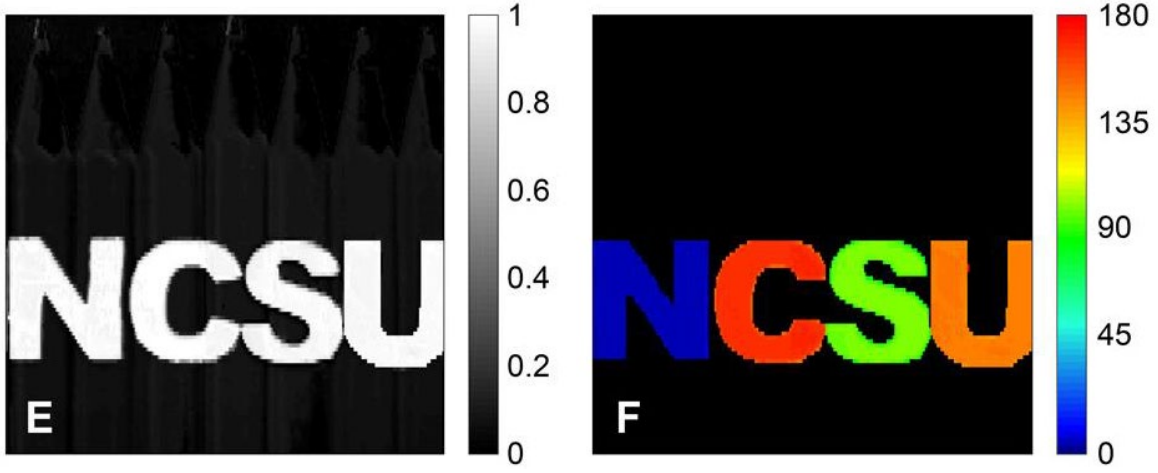


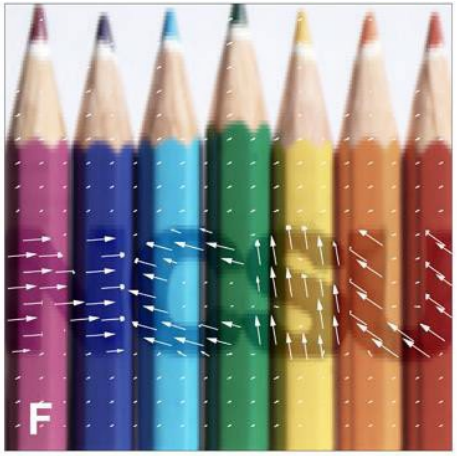
https://www.science.org/doi/10.1126/sciadv.abe3196





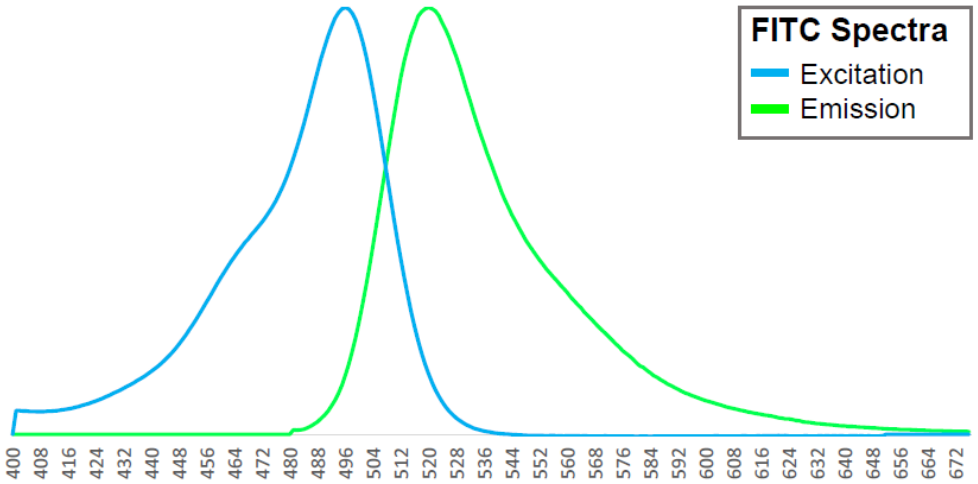
Mantis shrimp-inspired multispectral and polarization-sensitive (SIMPOL)





fluorescence

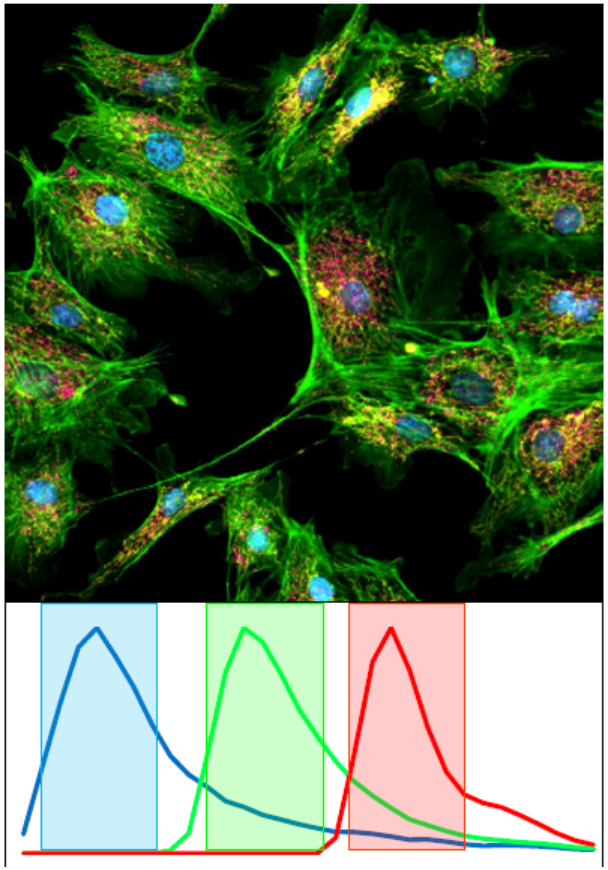
Fluorescence spectra



Why spectral imaging?

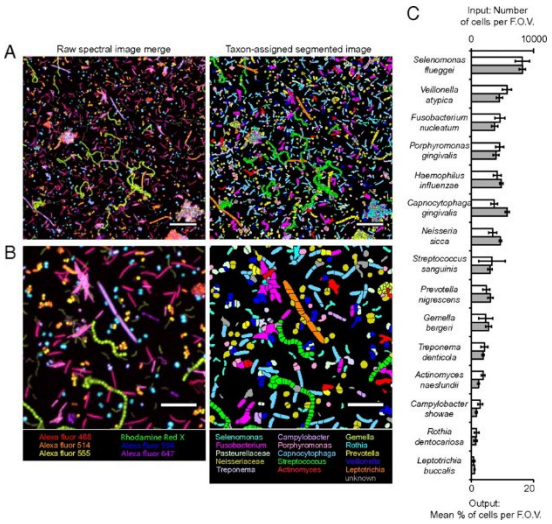
Conventional

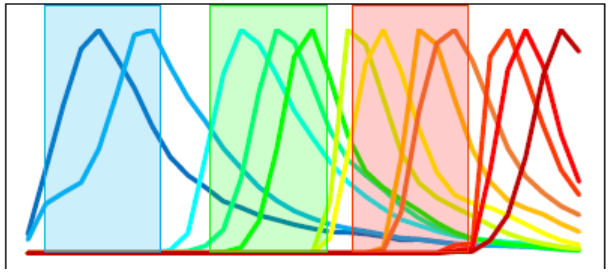
BPAE Cells - 3 Colors



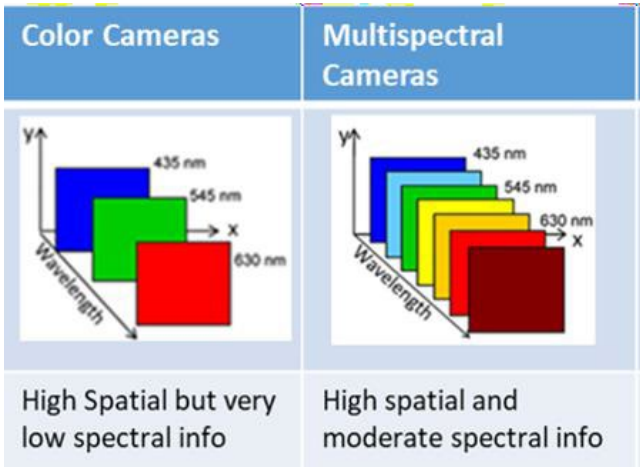
Spectral

Oral Plaque Biofilm - 12 Colors

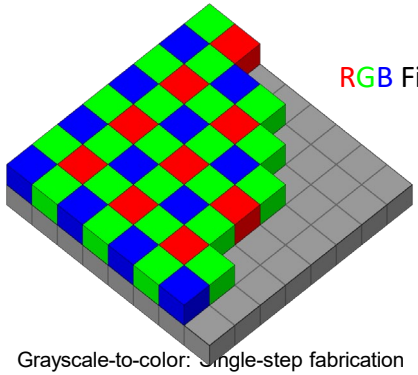




Valm et al. y
Proceedings of the National Academy of Sciences Mar 2011, 108 (10) 4152-4157;
DOI: 10.1073/pnas.1101134108

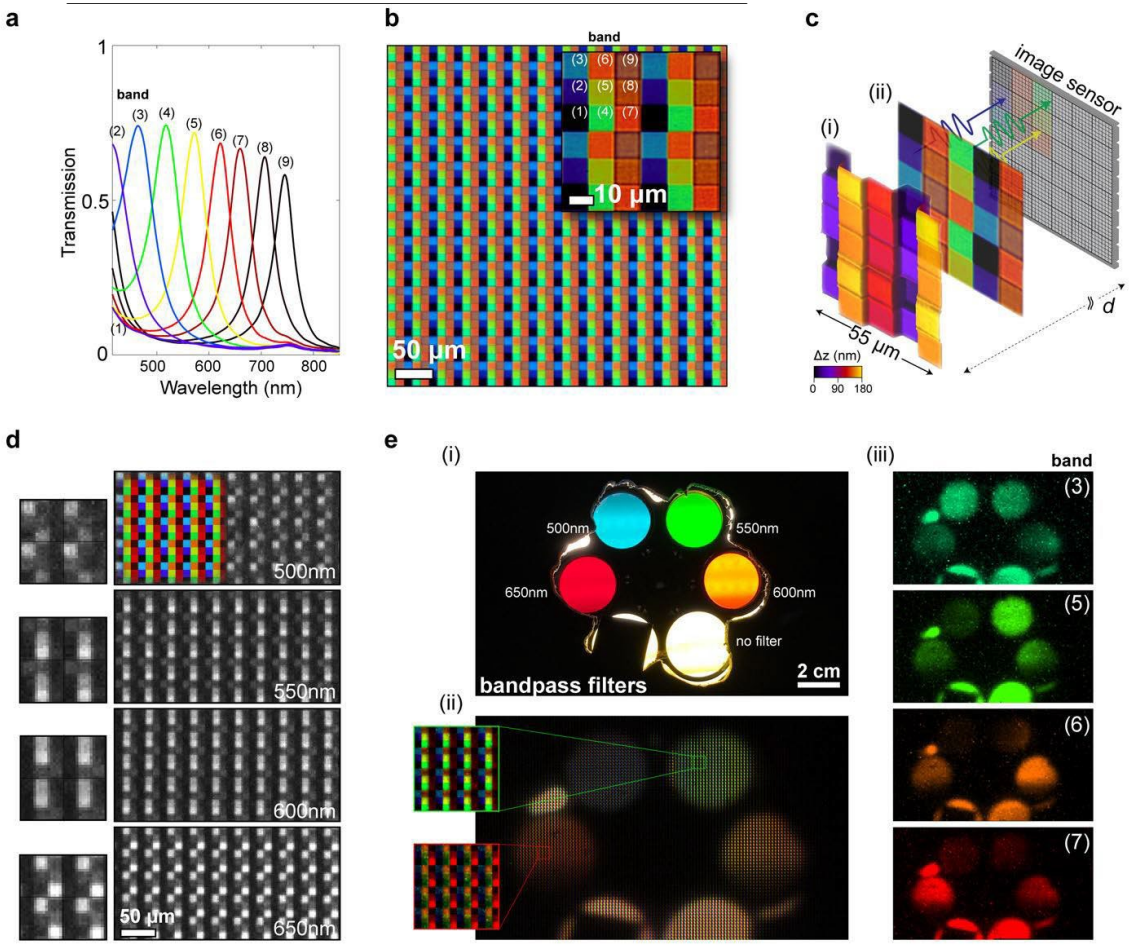


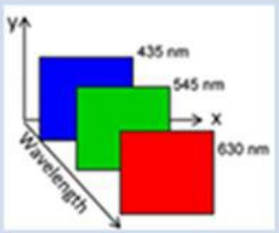
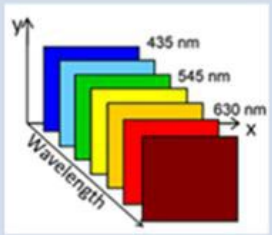
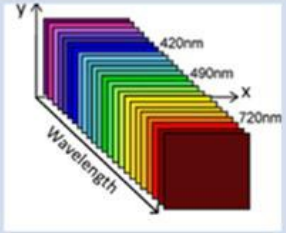
https://www.spectricon.com/spectral-imaging/



RGB Filters

Grayscale-to-color: Single-step fabrication of bespoke multispectral filter arrays Calum Williams1,2†, George S.D. Gor Sophia Gruber1,2, Timothy D. Wilkins Sarah E. Bohndiek2,3*

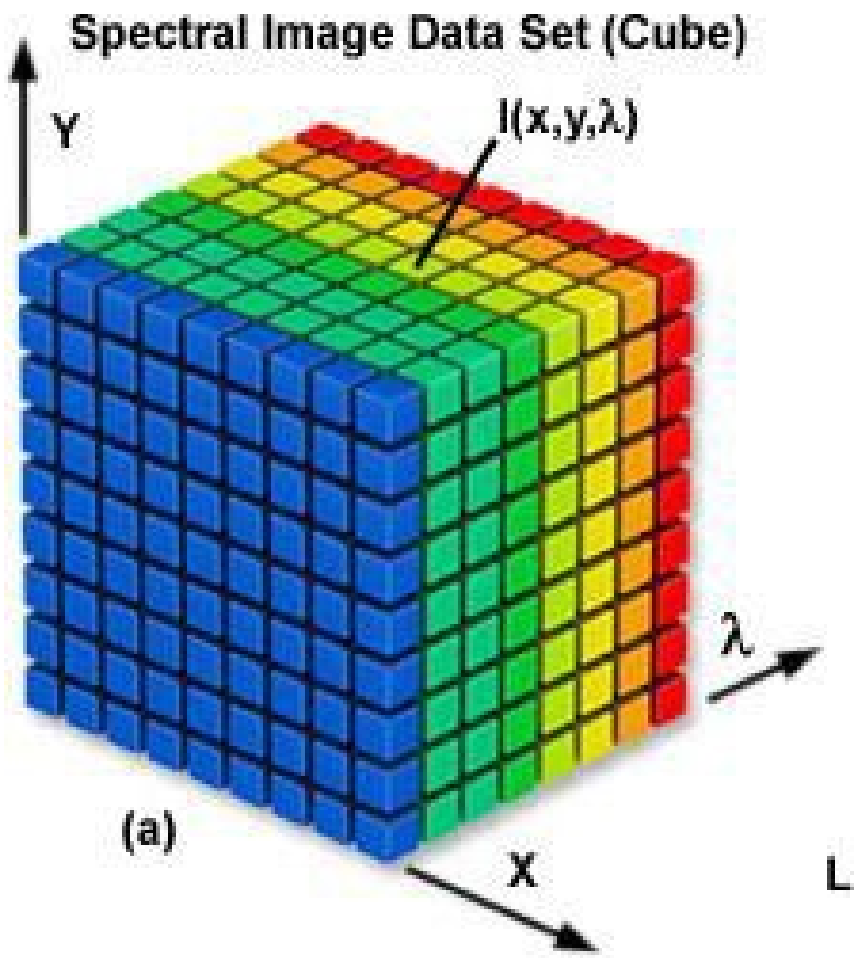


Color Cameras	Multispectral Cameras	Hyperspectral cameras
		
High Spatial but very low spectral info	High spatial and moderate spectral info	High spatial AND spectral info

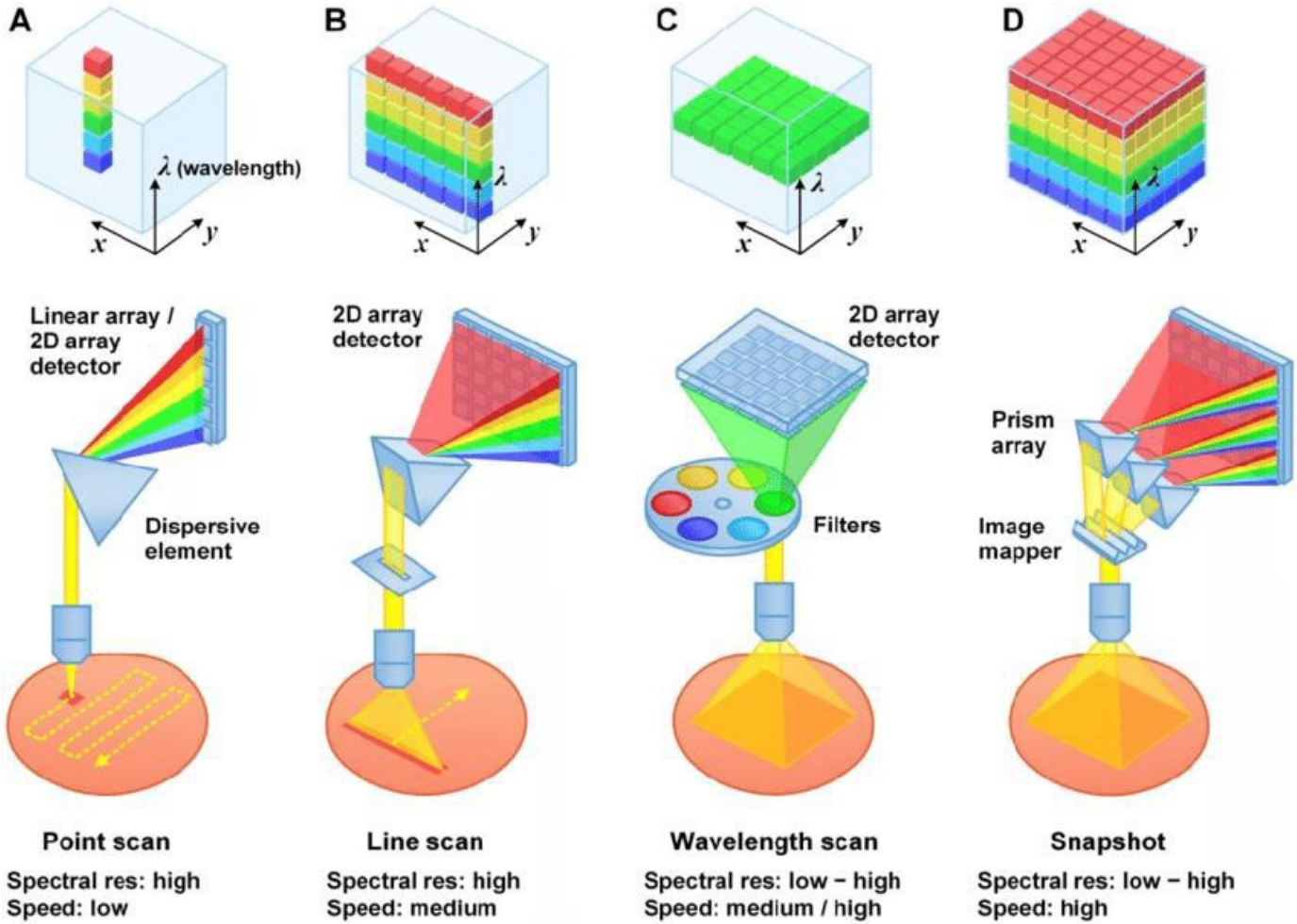
https://www.spectricon.com/spectral-imaging/

RGB : 3 colors
Multispectral : 4 to 15 colors
Hyperspectral : 16+ colors

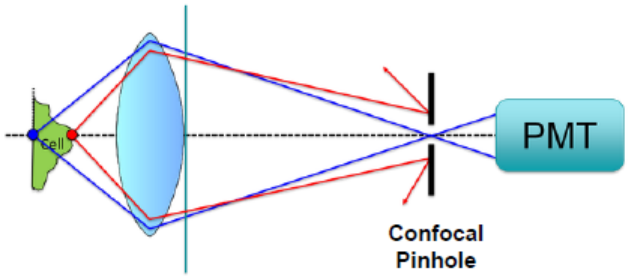
https://zeiss-campus.magnet.fsu.edu/articles/spectralimaging/introduction.html

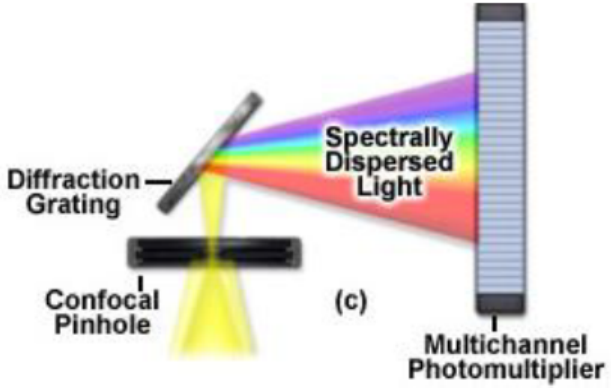


How do we collect spectral datasets?


A
λ (wavelength)
x
y
Linear array / 2D array detector
Dispersive element
Point scan
Spectral res: high
Speed: low
B
λ
x
y
2D array detector
Line scan
Spectral res: high
Speed: medium
C
λ
x
y
2D array detector
Filters
Wavelength scan
Spectral res: low – high
Speed: medium / high
D
λ
x
y
Prism array
Image mapper
Snapshot
Spectral res: low – high
Speed: high

Conventional vs spectral detection

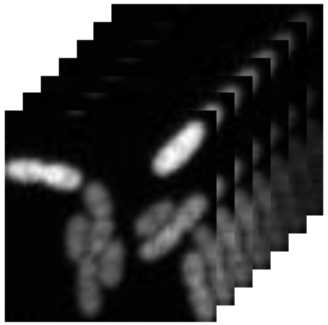




1 Channel
Sum of gated wavelengths

32 Possible Channels
Each a portion of gated wavelengths

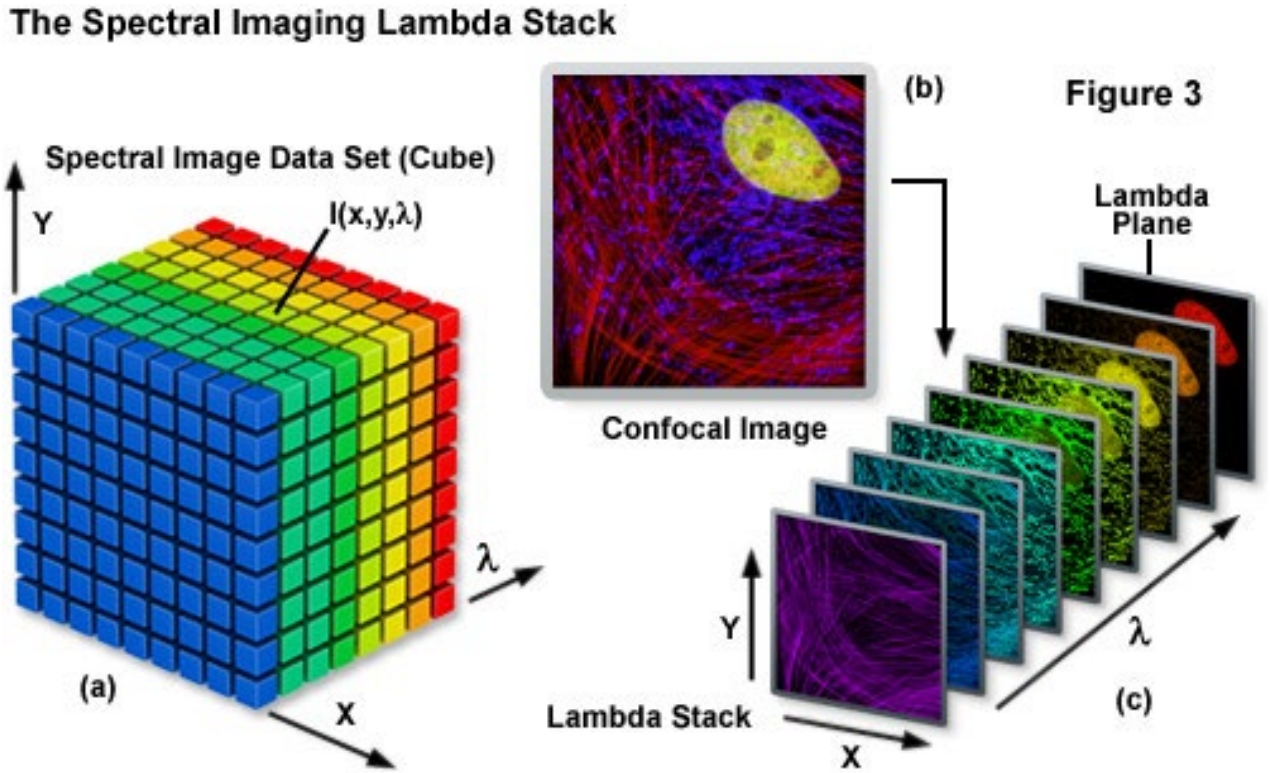
480:540nm =



λ stack

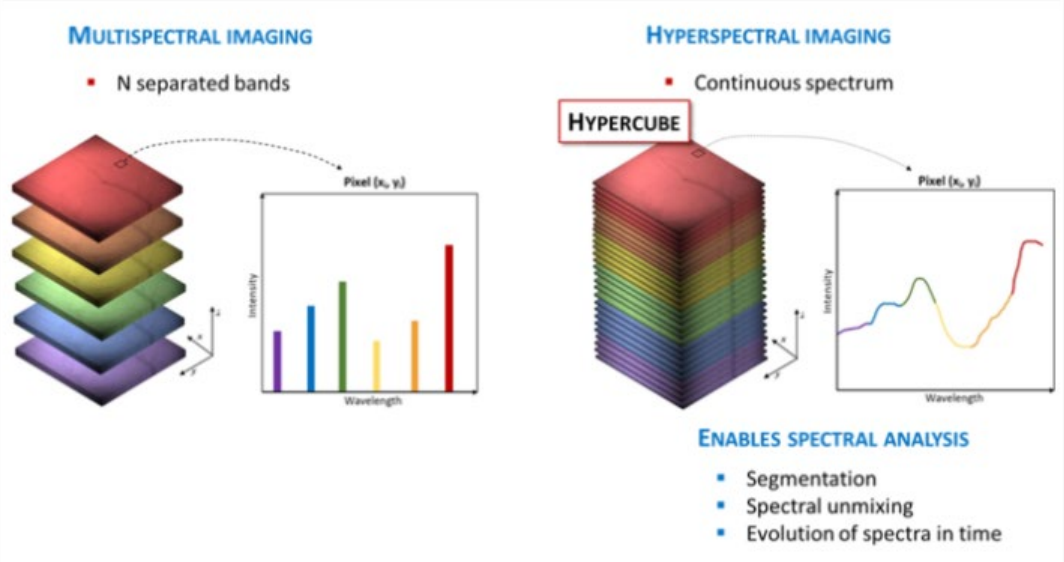
= 480nm
= 490nm
= 500nm
= 510nm
= 520nm
= 530nm
= 540nm

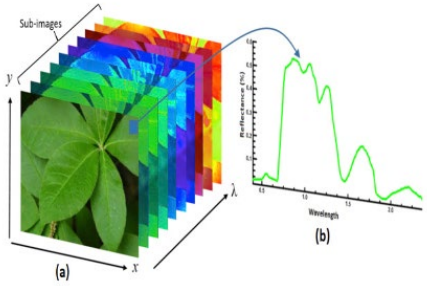
Using Hyperspectral emission detection



http://zeiss-campus.magnet.fsu.edu/articles/spectralimaging/introduction.html

What is Hyperspectral Imaging?

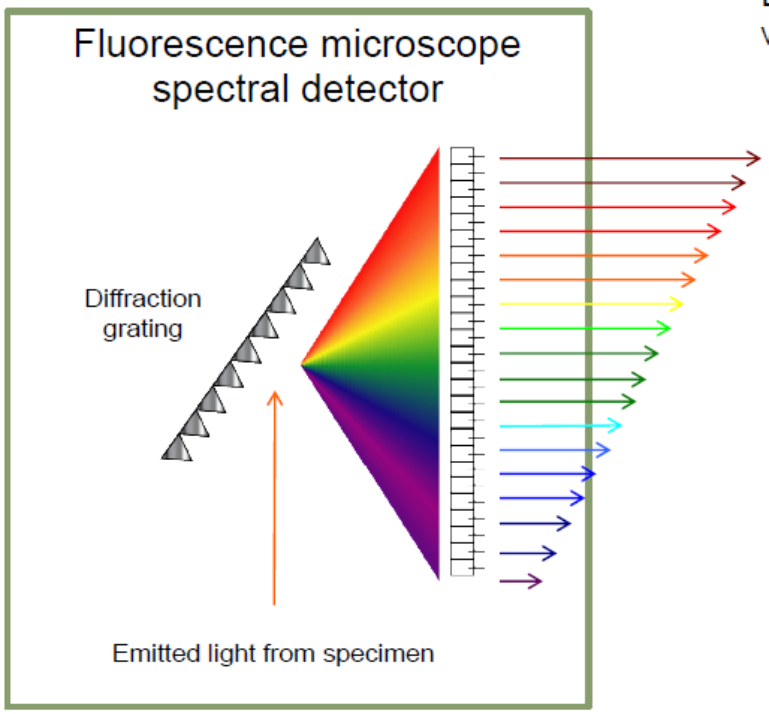




- hyperspectral imaging measures **the continuous spectrum of the light for each pixel of the scene** with fine wavelength resolution, not only in the visible but also in the near-infrared.

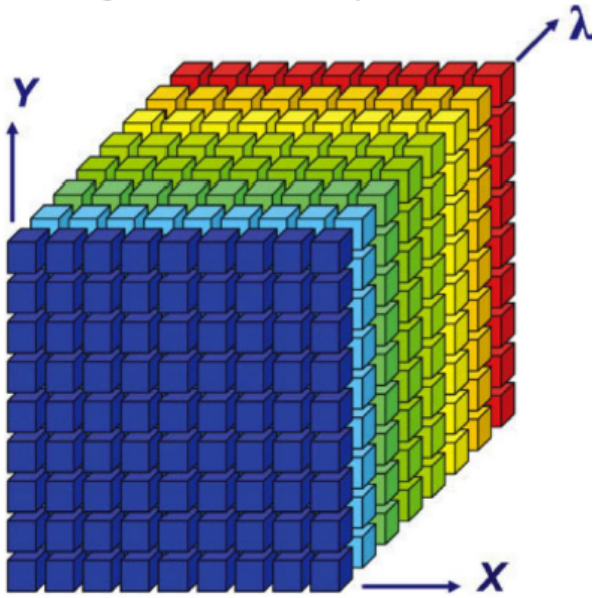
- The collected data form a so-called hyperspectral cube, in which two dimensions represent the spatial extent of the scene and the third its spectral content.

Spectral detection



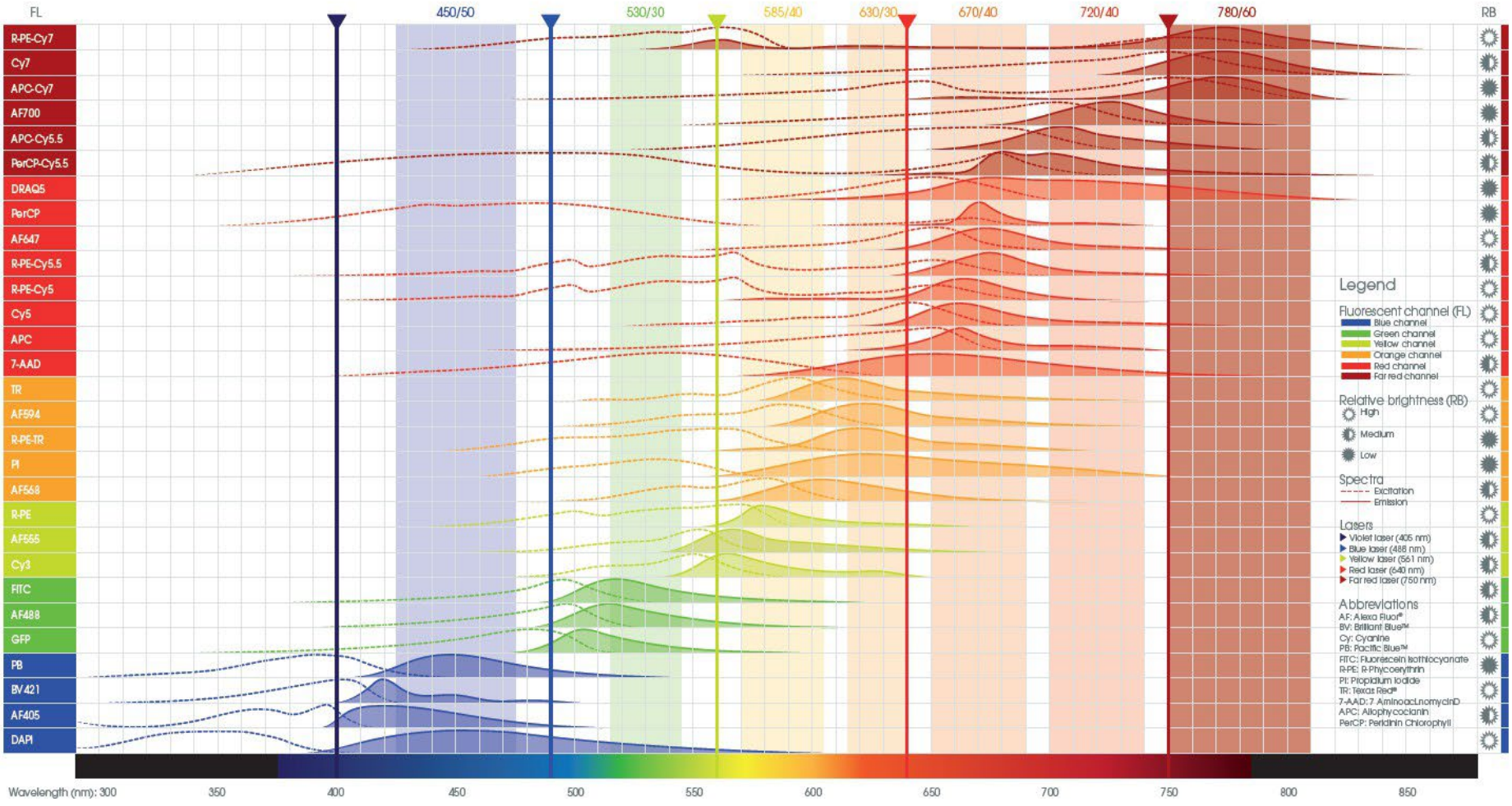
Dataset: λ stack

Like a Z-stack, but each slice represents wavelength rather than depth

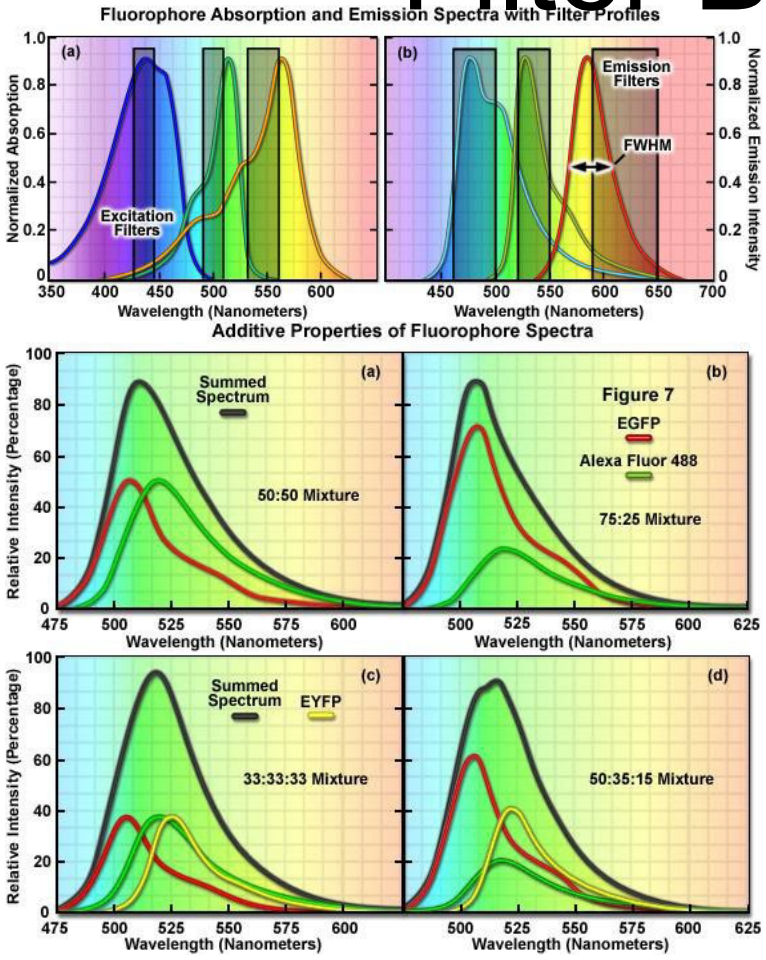


Spectral Image Data Cube

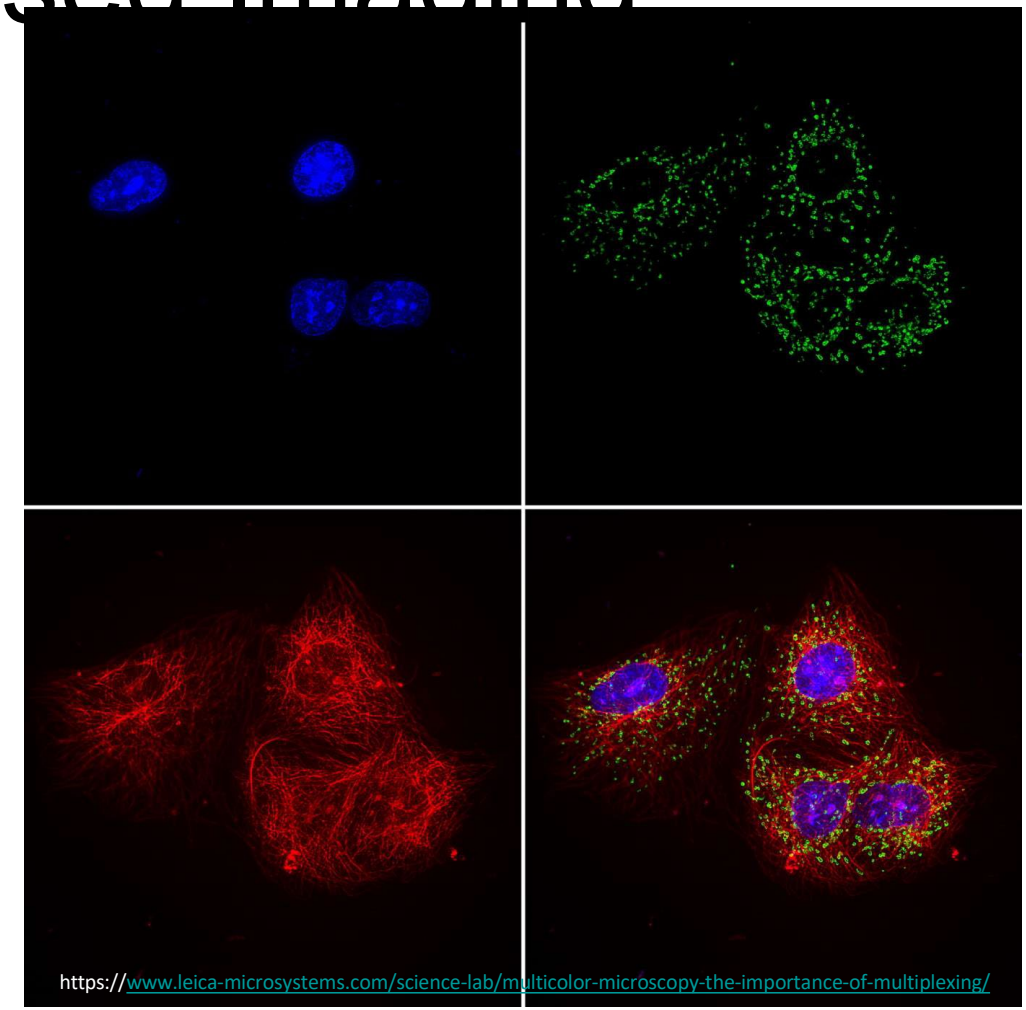
Garini Y, Young IT, McNamara G. 2006. *Cytometry*. 69A:735-747.


FL
450/50
530/30
585/40
630/30
670/40
720/40
780/60
RB
R-PE-Cy7
Cy7
APC-Cy7
AF700
APC-Cy5.5
PerCP-Cy5.5
DRAQ5
PerCP
AF647
R-PE-Cy5.5
R-PE-Cy5
Cy5
APC
7-AAD
TR
AF594
R-PE-TR
PI
AF568
R-PE
AF555
Cy3
FITC
AF488
GFP
PB
BV 421
AF405
DAPI
Legend
Fluorescent channel (FL)
Blue channel
Green channel
Yellow channel
Orange channel
Red channel
Far red channel
Relative brightness (RB)
High
Medium
Low
Spectra
Excitation
Emission
Lasers
Violet laser (405 nm)
Blue laser (488 nm)
Yellow laser (561 nm)
Red laser (640 nm)
Far red laser (750 nm)
Abbreviations
AF: Alexa Fluor®
BV: Brilliant Blue™
Cy: Cyanine
PB: Pacific Blue™
FITC: Fluorescein Isothiocyanate
R-PE: R-Phycoerythrin
PI: Propidium Iodide
TR: Texas Red®
7-AAD: 7-AminoactinomycinD
APC: Allophycocianin
PerCP: Peridinin Chlorophyll
Wavelength (nm): 300
350
400
450
500
550
600
650
700
750
800
850

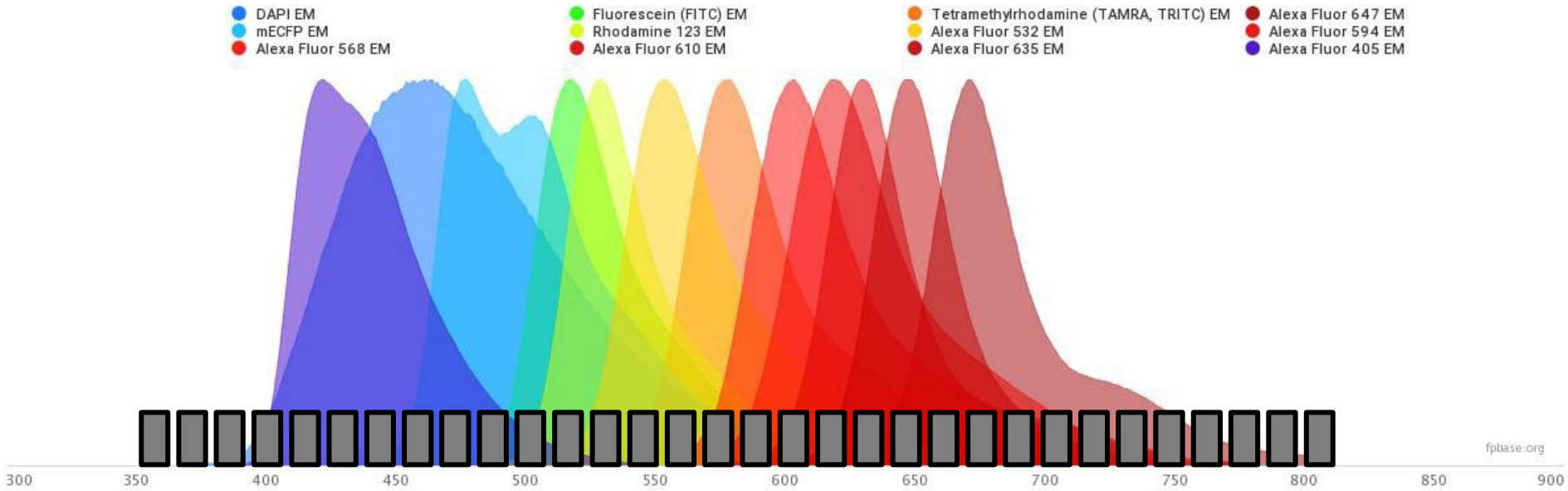
Filter Based Imaging

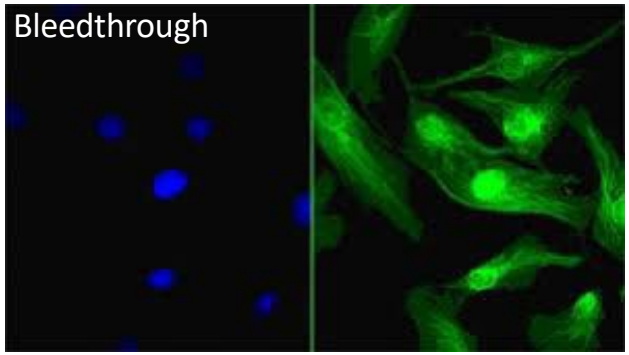


https://zeiss-campus.magnet.fsu.edu/articles/spectralimaging/introduction.html



https://www.leica-microsystems.com/science-lab/multicolor-microscopy-the-importance-of-multiplexing/


DAPI EM
mECFP EM
Alexa Fluor 568 EM
Fluorescein (FITC) EM
Rhodamine 123 EM
Alexa Fluor 610 EM
Tetramethylrhodamine (TAMRA, TRITC) EM
Alexa Fluor 532 EM
Alexa Fluor 635 EM
Alexa Fluor 647 EM
Alexa Fluor 594 EM
Alexa Fluor 405 EM
fpbase.org
300
350
400
450
500
550
600
650
700
750
800
850
900


Bleedthrough

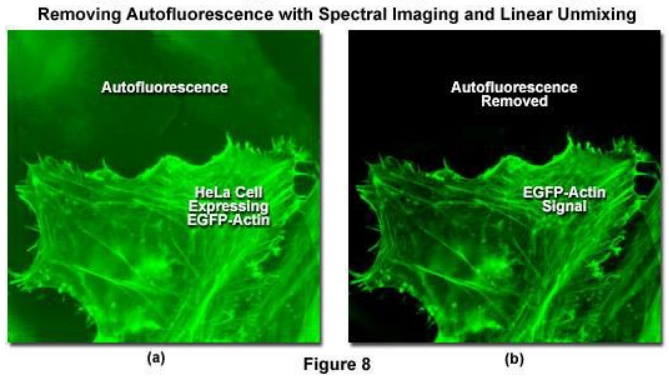
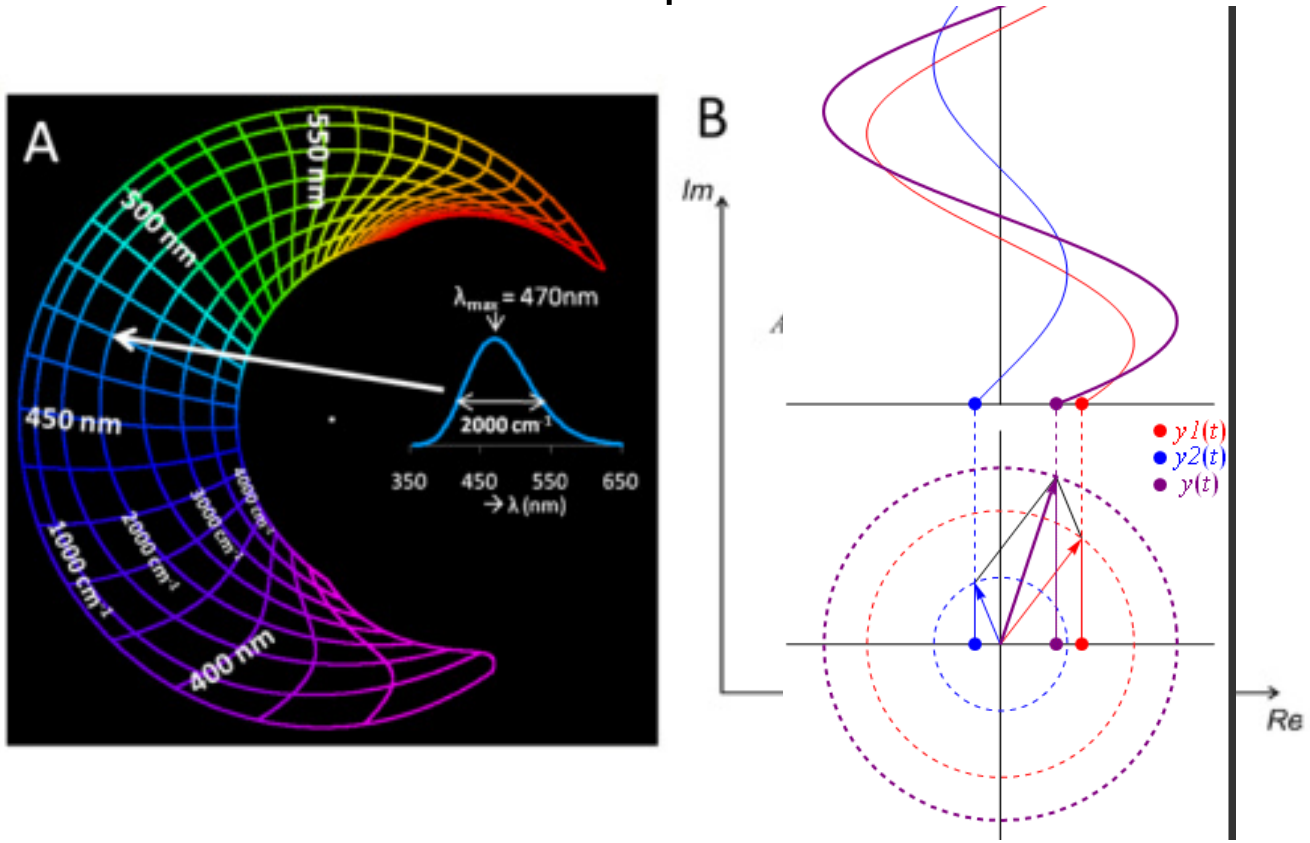

Removing Autofluorescence with Spectral Imaging and Linear Unmixing
Autofluorescence
HeLa Cell Expressing EGFP-Actin
Autofluorescence Removed
EGFP-Actin Signal
(a)
(b)

Figure 8

The Phasor approach in Hyperspectral imaging

Polar Plot representation



The sum of phasors as addition of rotating vectors

https://en.wikipedia.org/wiki/File:Sumafasores.gif

Fereidouni et al, Opt Express. 2012;20:12729–41

Phasor transformation

- Traditional spectral demixing
  - Requires prior knowledge of spectral profiles.
  - Problematic in identifying multiple similar species in a sample.
- Spectral Phasor analysis
  - Requires no prior knowledge of the species.
  - Combines traditional spectral analysis with Phasor analysis.
  - Enables the identification of the molecular environment.

- The phasor transformation calculates the sine and cosine of the Fourier of the spectrum.

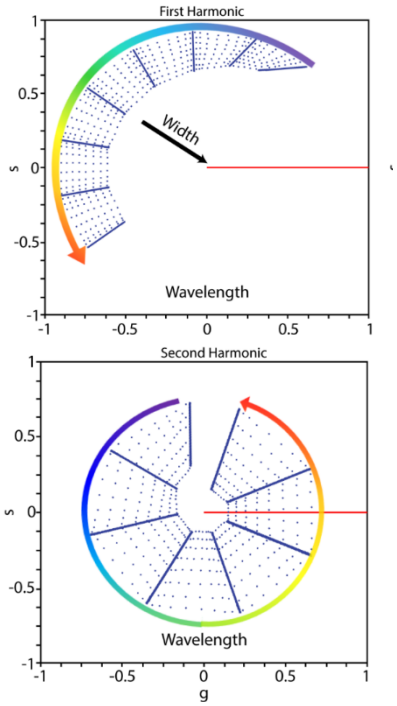
$$g = \frac{\sum_{\lambda} I(\lambda)\cos(2\pi n\lambda / L)}{\sum_{\lambda} I(\lambda)} \qquad s = \frac{\sum_{\lambda} I(\lambda)\sin(2\pi n\lambda / L)}{\sum_{\lambda} I(\lambda)}$$

- Individual fluorescent components have different spectra influenced by:
  - Biochemical environment
  - Binding
  - Energy Transfer

Garini, Y., *et al.. Cytometry Part A* (2006), Andrews, L. M. *et al., Biomedical optics express* (2013).
Digman, M. A., *et al., Biophysical Journal* (2008)., Fereidouni, F., *et al., Optics Express* (2012).
Cutrale, F., *et al., Methods and Applications in Fluorescence* (2013), Golfetto, O., *et al., Methods in Membrane Lipids* (2015).

Spectral Phasor Analysis

- For each harmonic, 2 coordinates are obtained → ‘g’ and ‘s’ Leads to the production of a polar phasor plot.

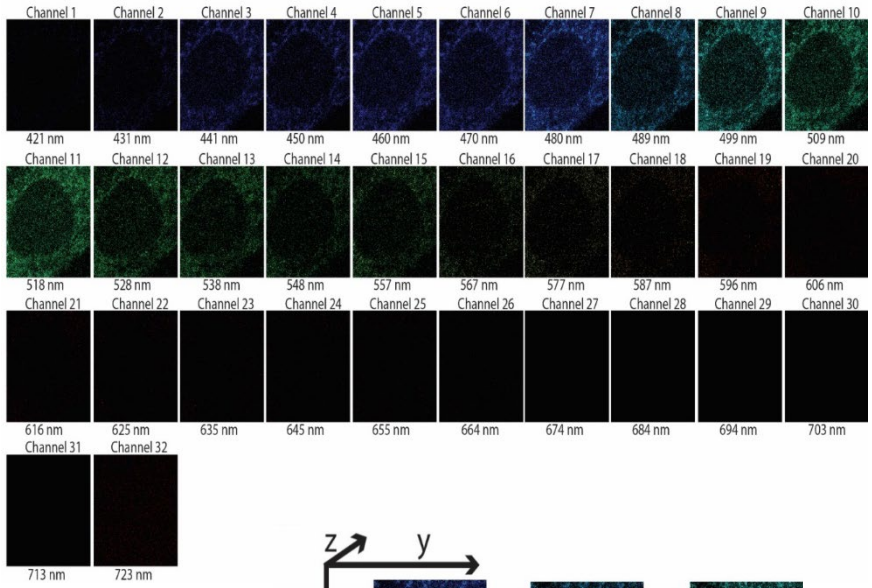


Golfetto, O., *et al., Methods in Membrane Lipids* (2015).

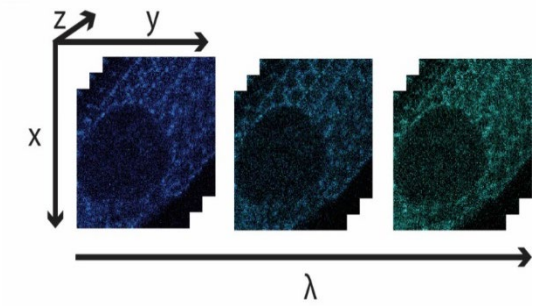
- The phasor transformation calculates the sine and cosine Fourier of the spectrum.
- For each Fourier harmonics, 2 coordinates are obtained, indicated by g and s.
- A point at coordinates (g,s) is called a phasor and it is represented in a polar plot.
- The angular position is proportional to the position of the average of the spectrum while the distance from the origin depends on the spectral width.

Microscopy Setup for Spectral data collection

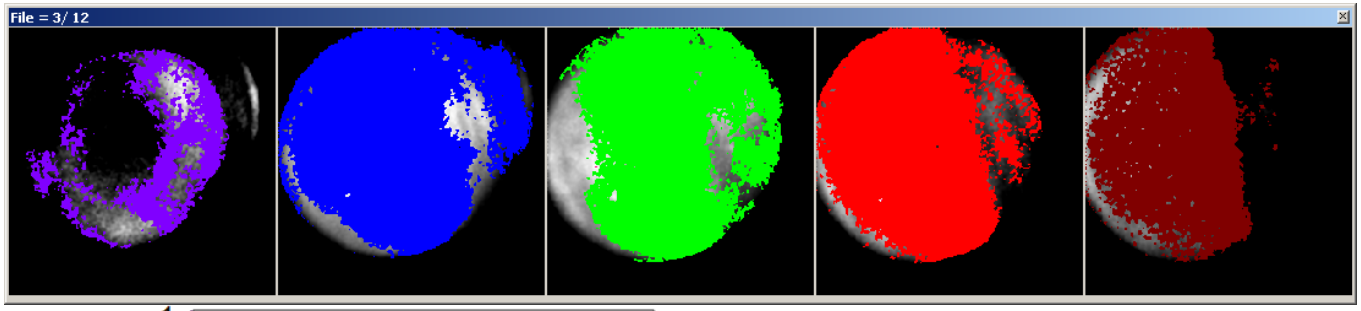
Zeiss LSM 710 coupled to a titanium: sapphire Mai Tai

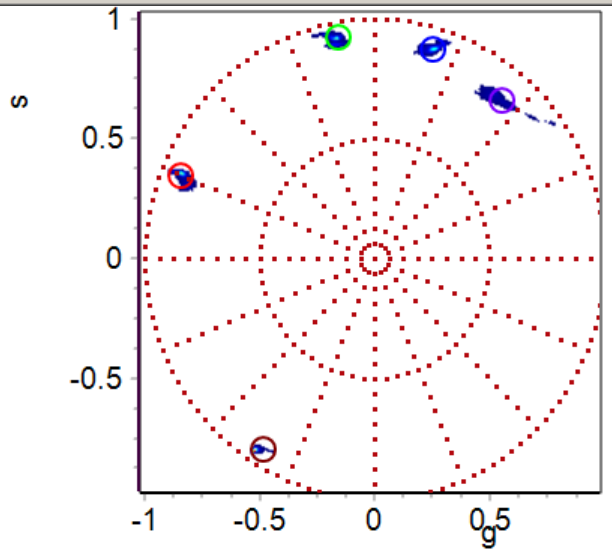


- Lambda scans collecting 32 channels at 9.7nm intervals
  - collected in 4 dimensions, ie λ as a function of x,y,z,t.
- Scan speed of 177µs
- Images can be collected from 256 x 256 up to 1024 x 1024 pixels



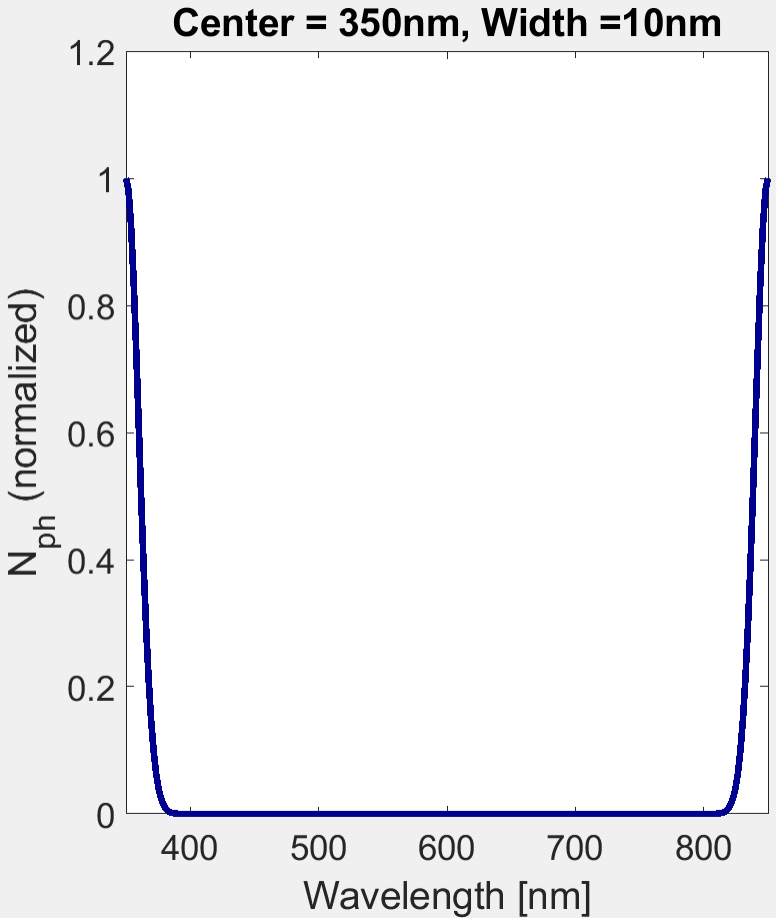
Spectral Emission using Phasors:

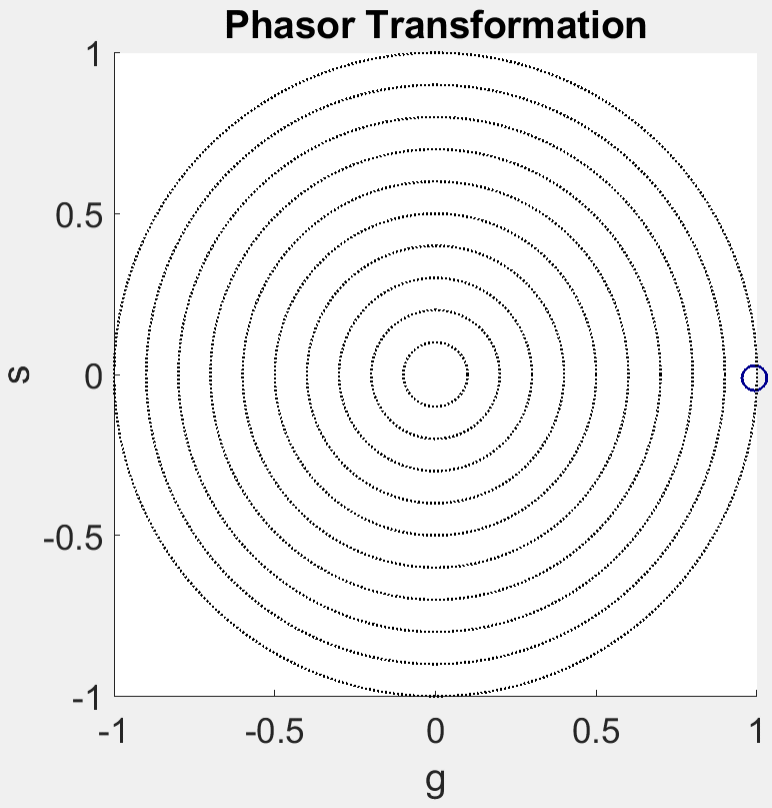




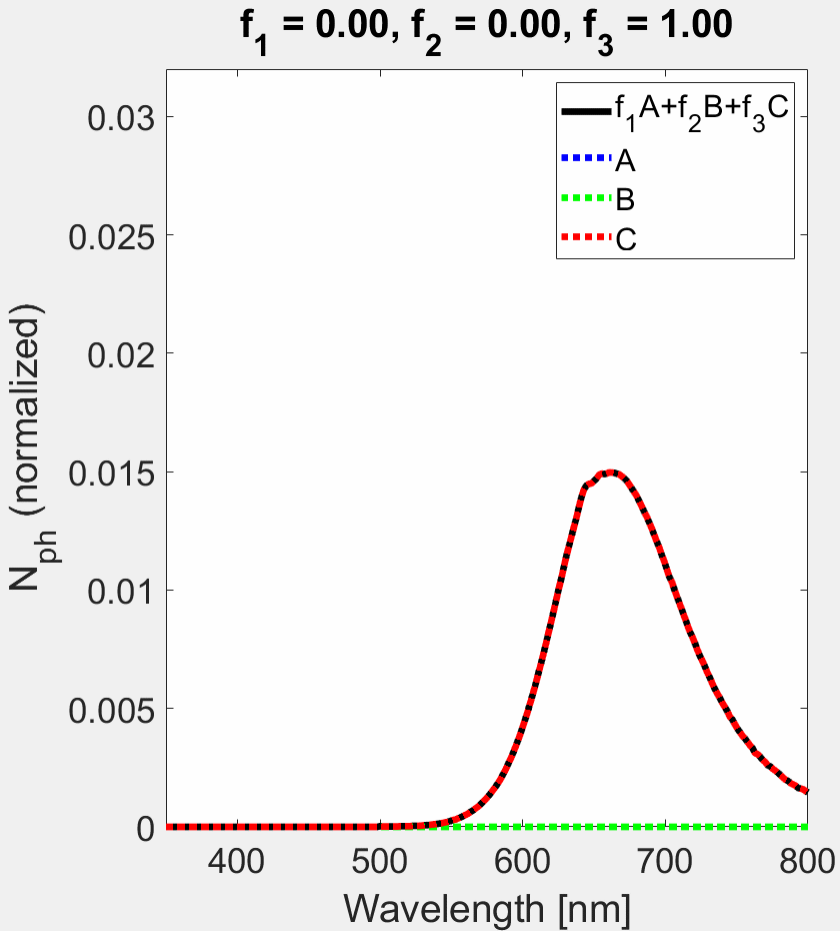
violet:458
blue: 479nm
green: 502nm
red:552nm
indigo: 621nm

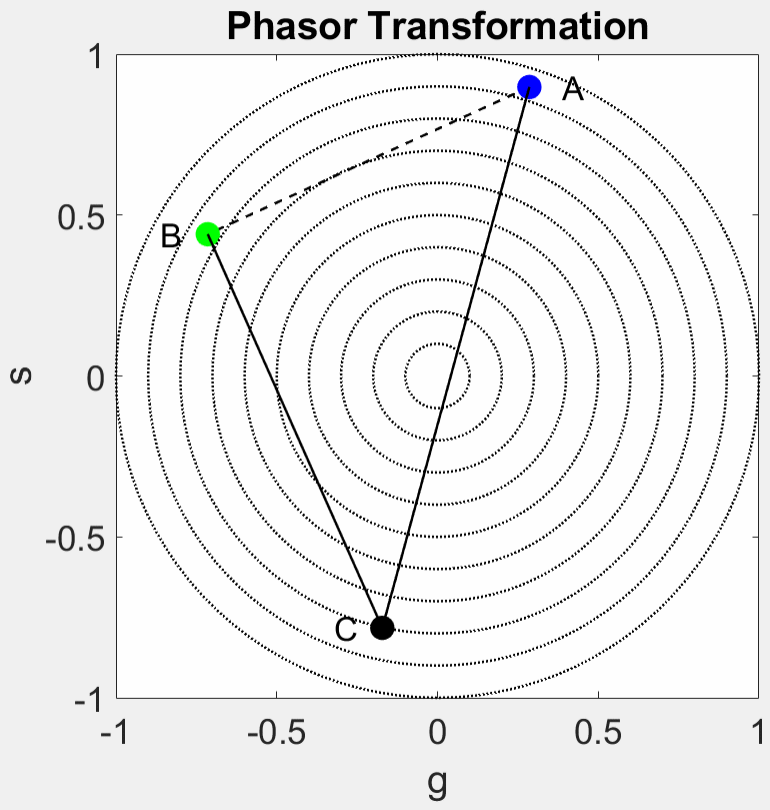
Spectral Phasors – Single spectrum



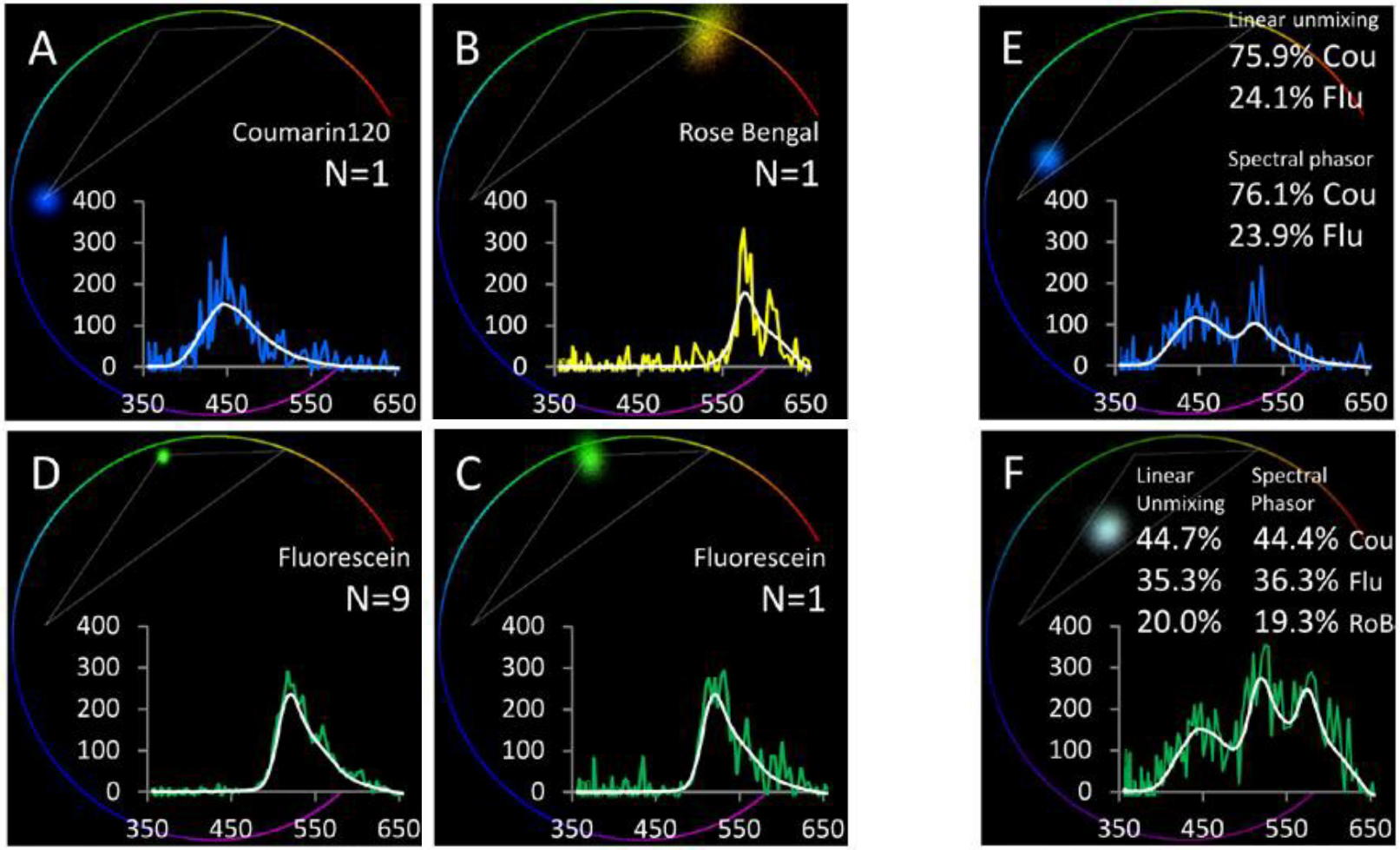


Spectral Phasors – Three spectra

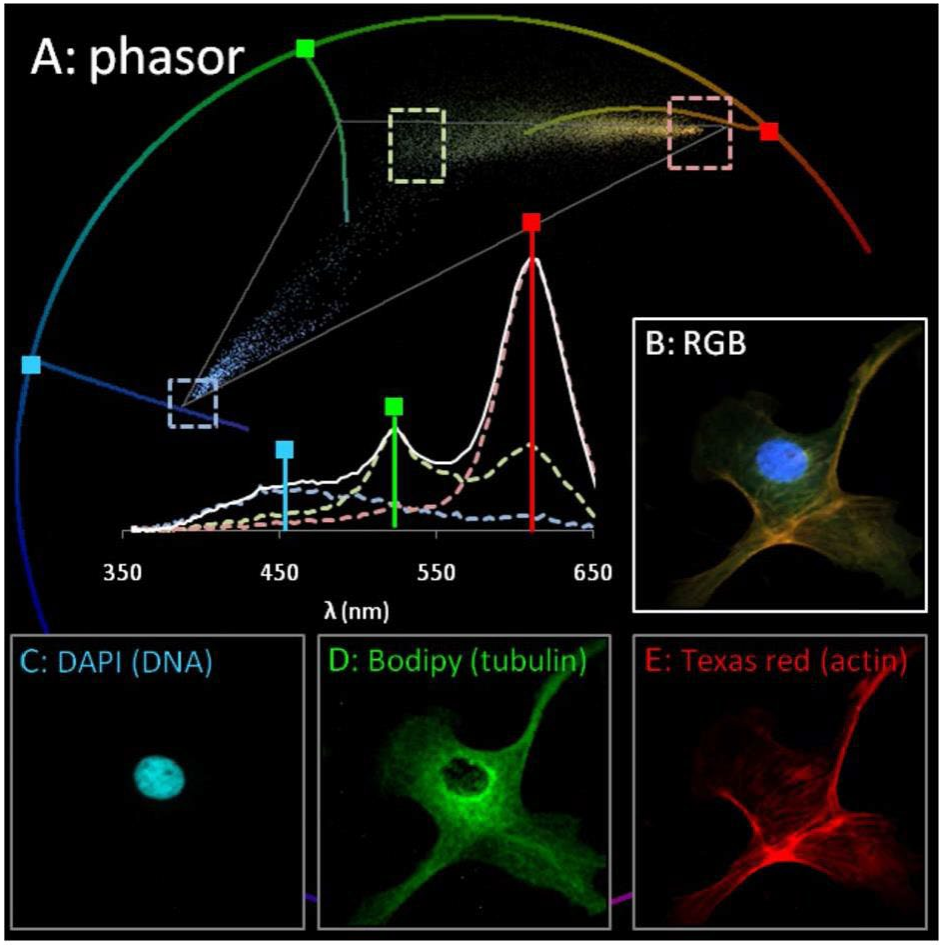


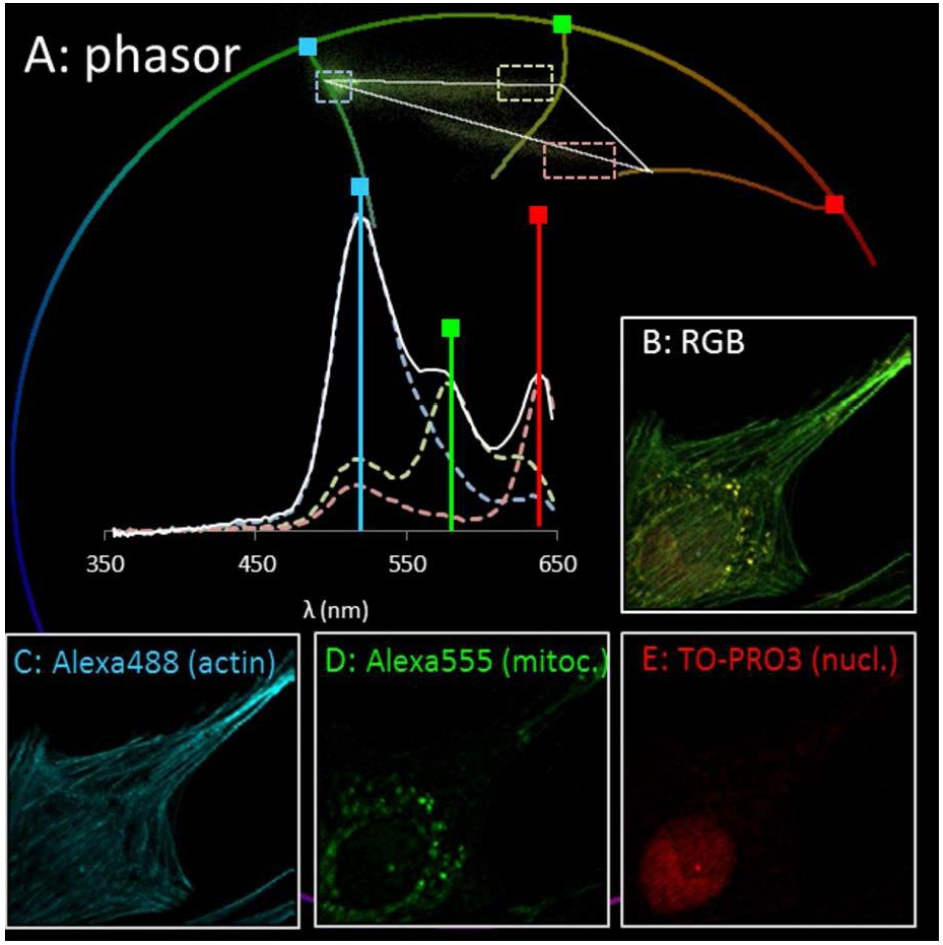


Spectral signature

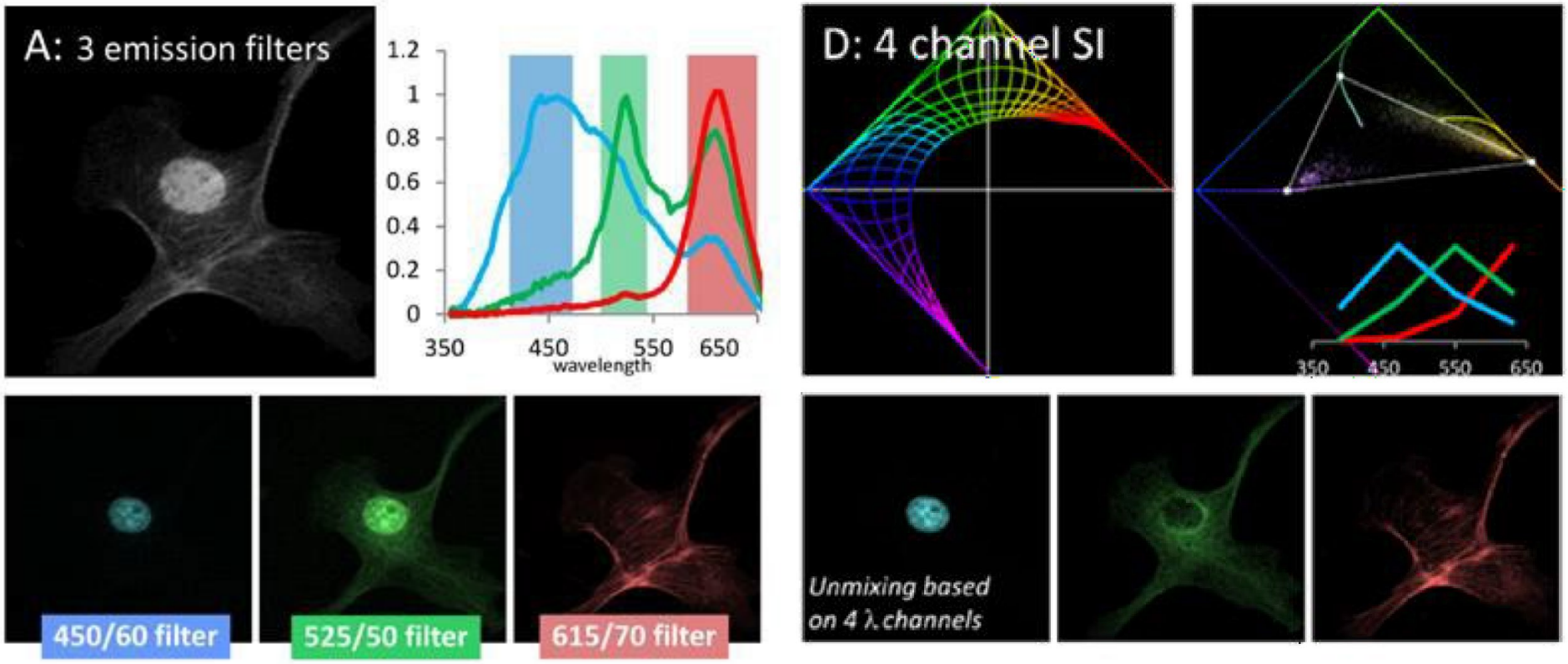


3-color unmixing

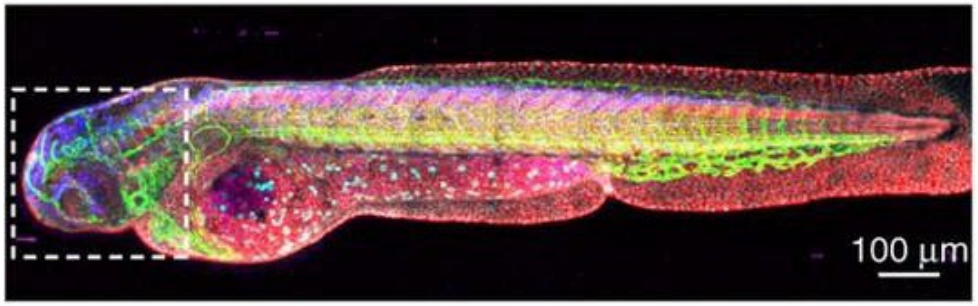


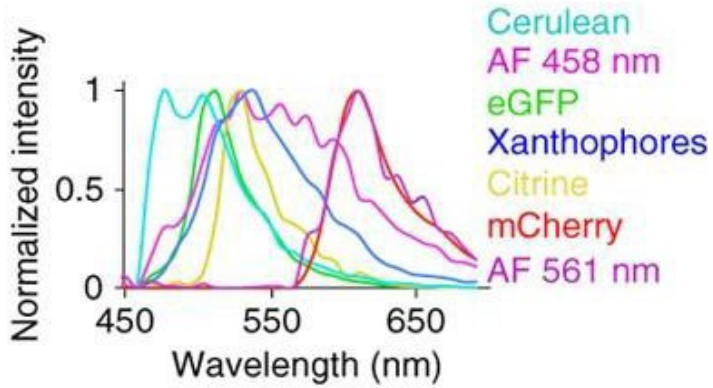


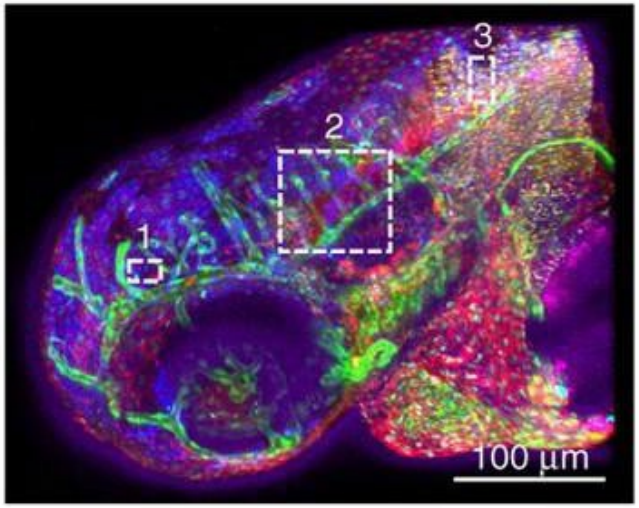
Number of spectral channels

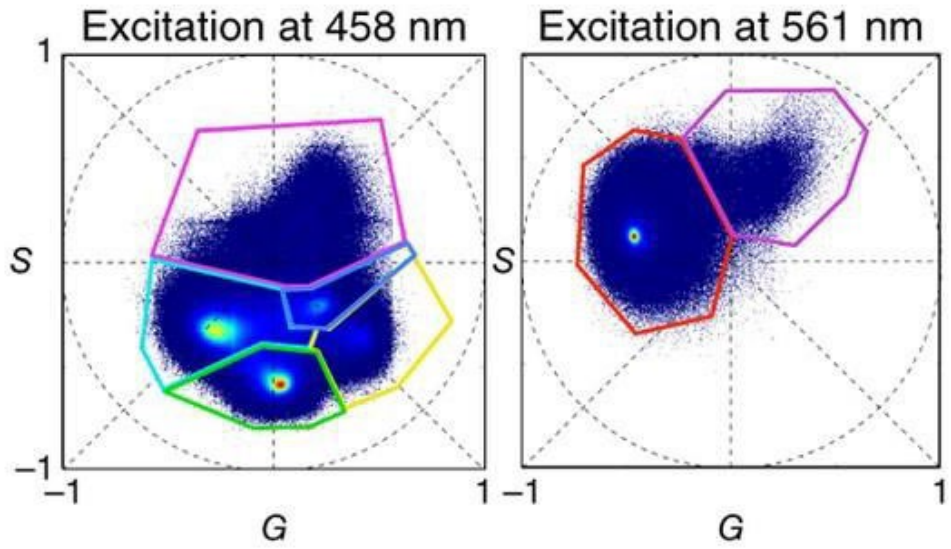


https://opg.optica.org/oe/fulltext.cfm?uri=oe-20-12-12729&id=233521

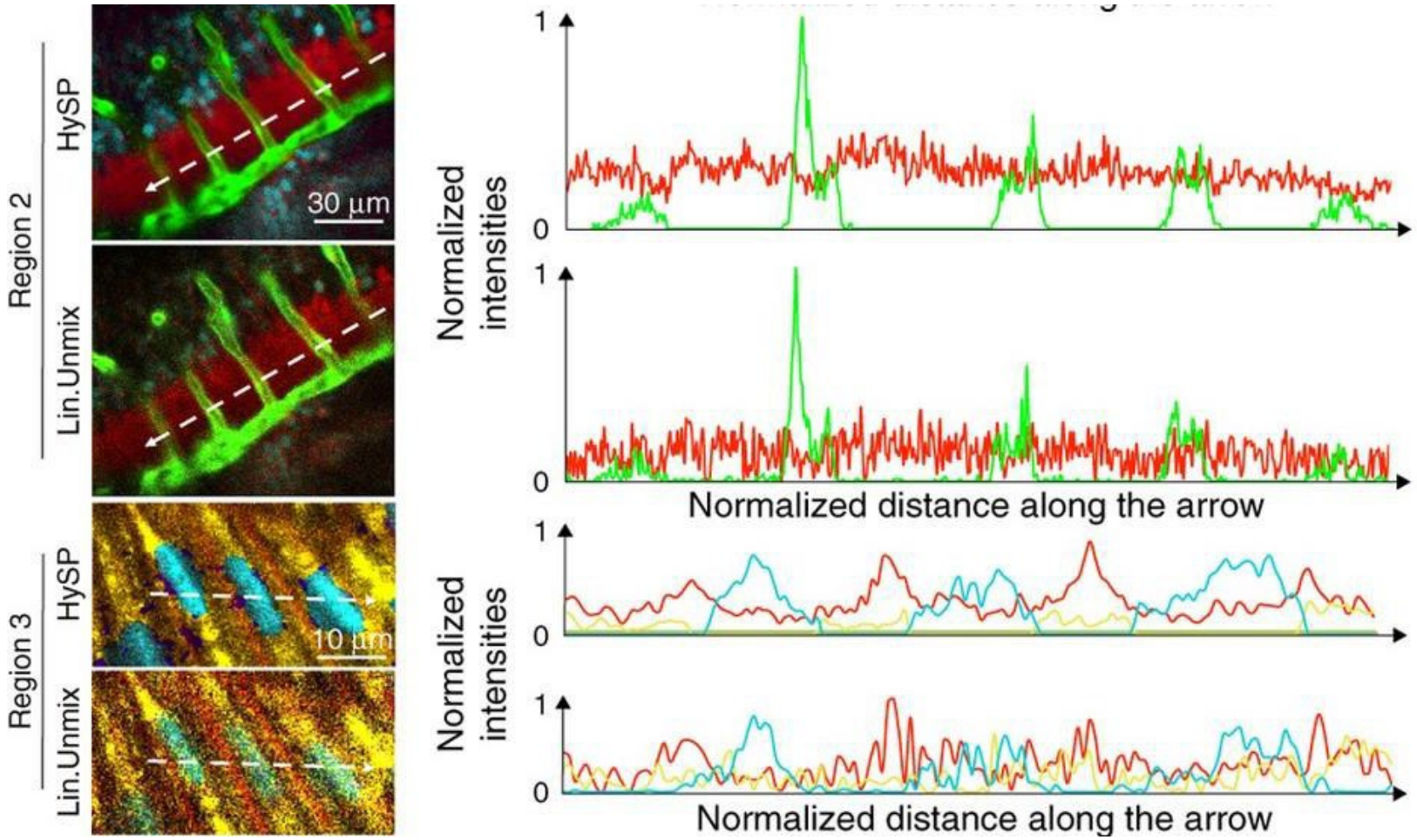




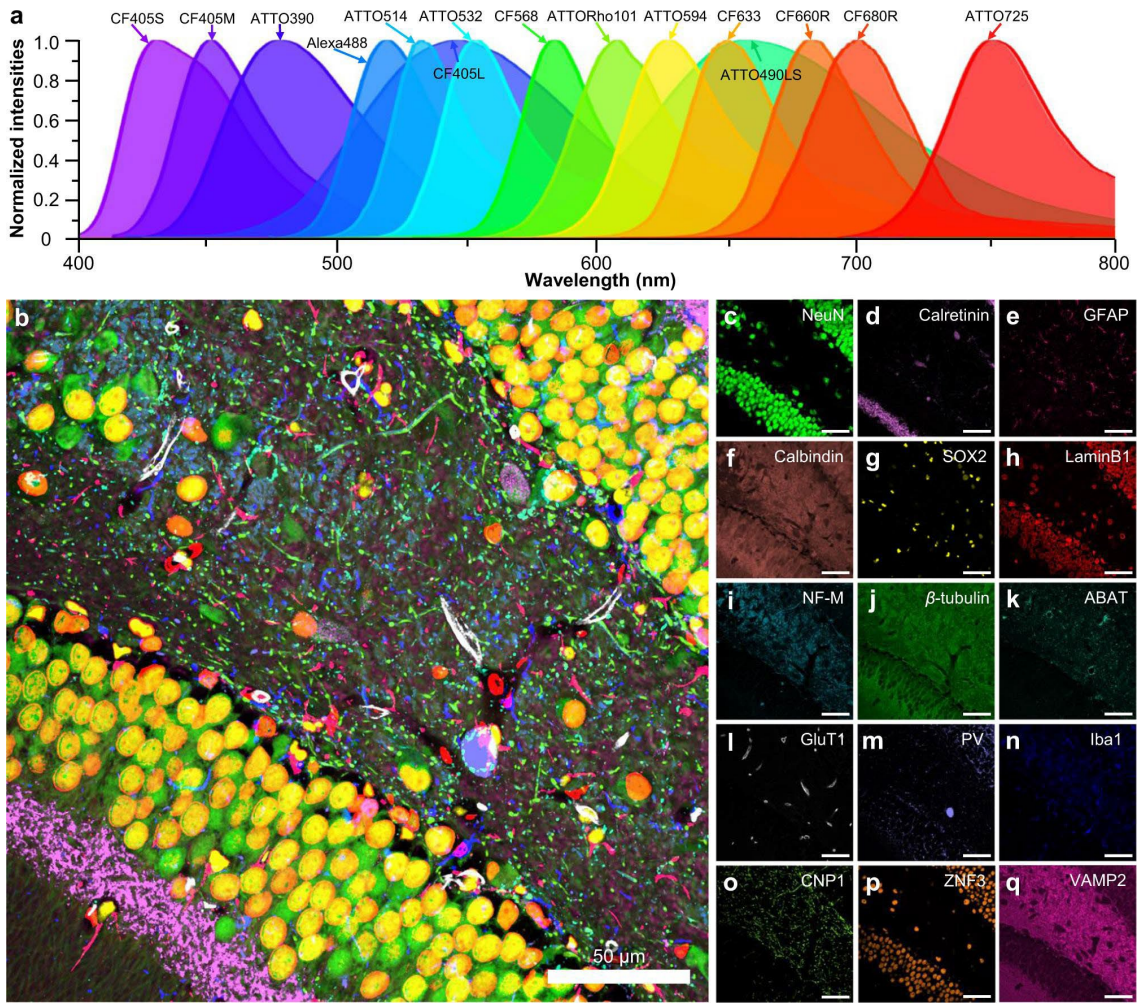




https://www.nature.com/articles/nmeth.4134/figures/2



https://www.nature.com/articles/nmeth.4134/figures/2

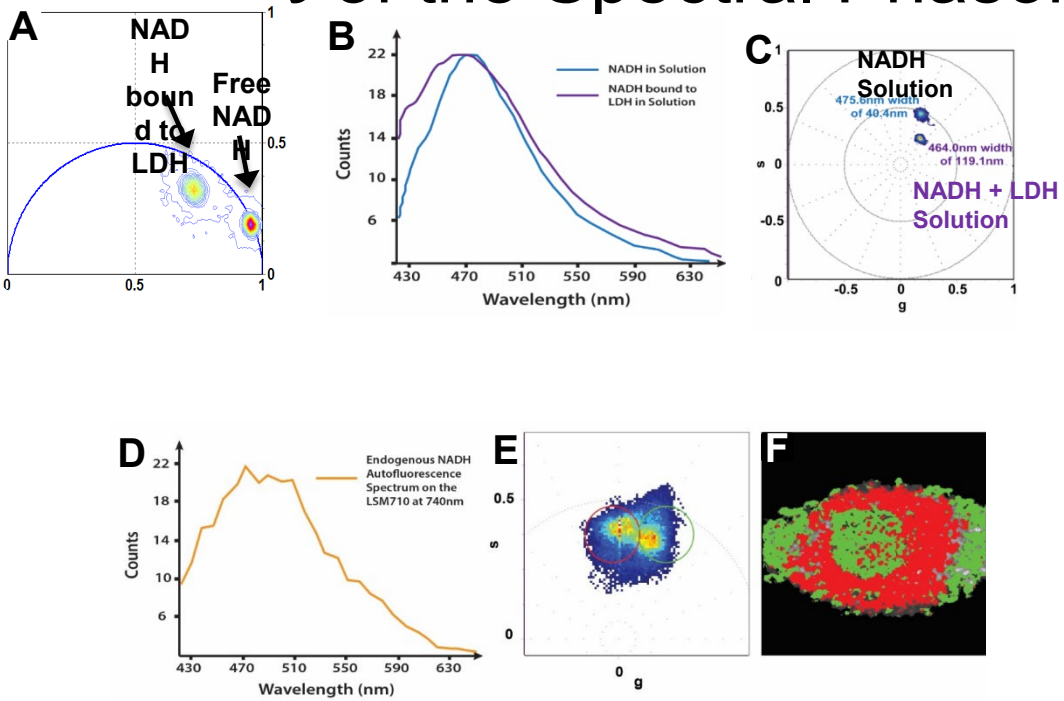


Fifteen-color multiplexed imaging of the mouse brain via PICASSO.

https://www.nature.com/articles/s41467-022-30168-z/figures/4

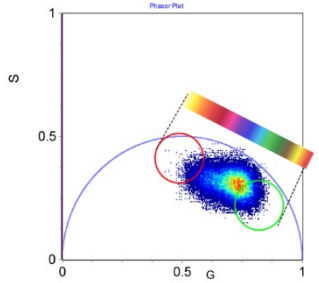
Spectral Phasor Analysis of NADH

- So where is NADH located on first harmonic of the Spectral Phasor?

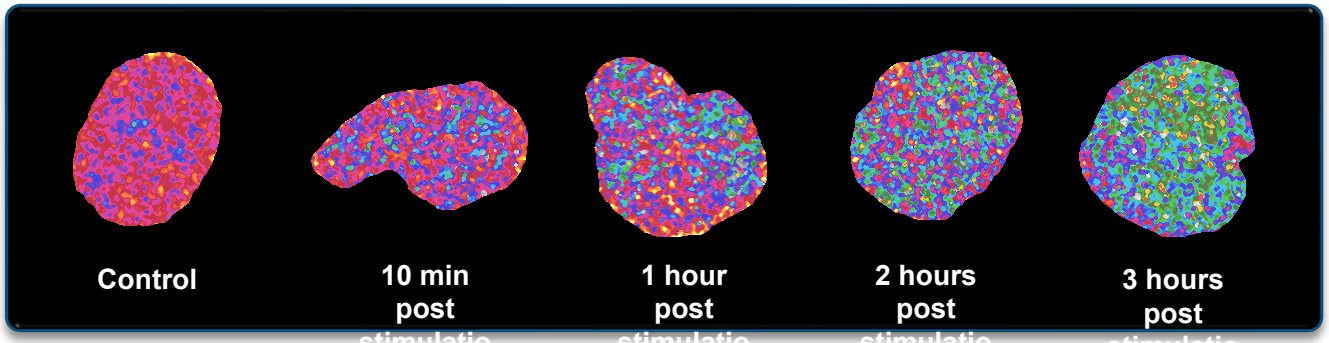


Individual Differentiating Cell FLIM and Spectral Phasor Analysis

- NADH Phasor FLIM

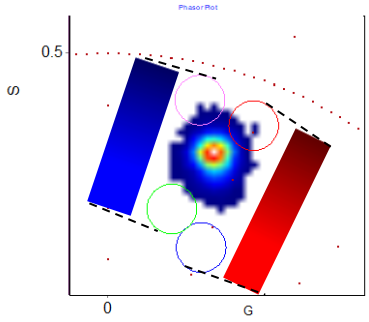


Linkage – 1.645ns to 0.526nm

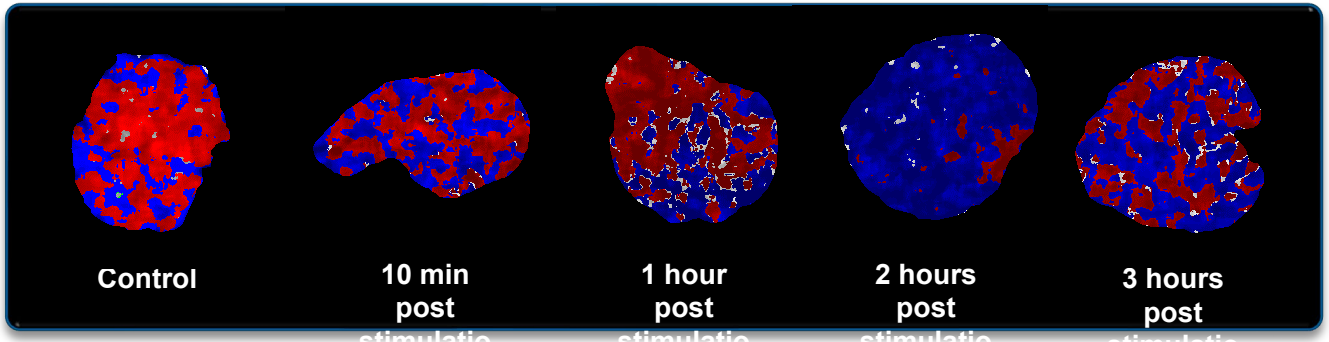


Wright, B. K., *et al., Biophysical Journal* (2012),Wright, B. K., *et al., Microscopy Research and Technique* (2012)

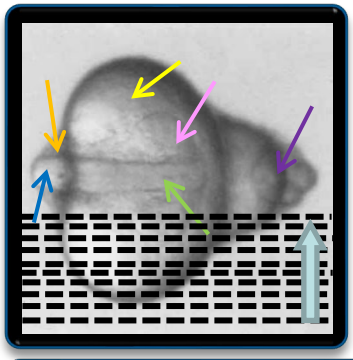
- NADH Spectral

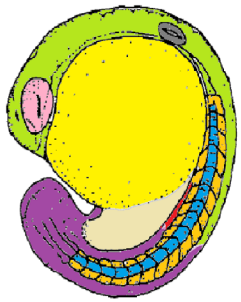


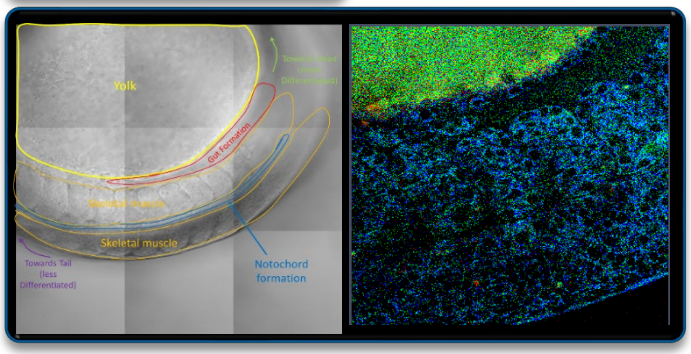
Blue Linkage – 480.1nm
Red Linkage – 473.4nm

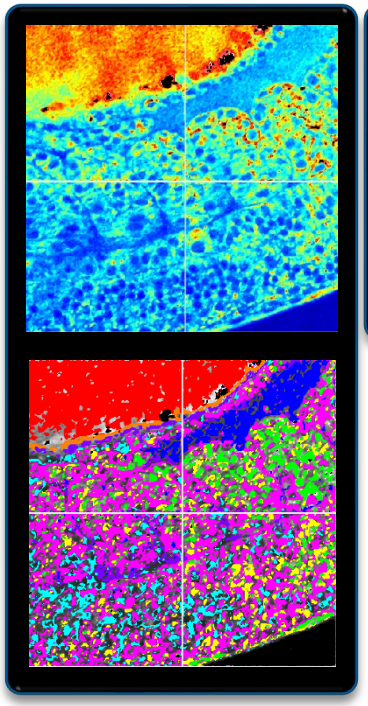


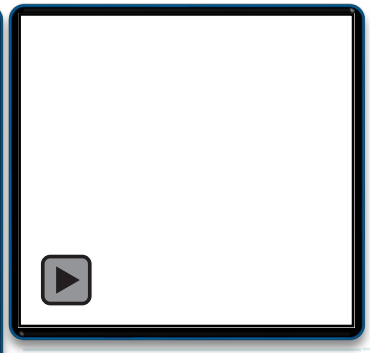
Identification of Tissues during Somitogenesis

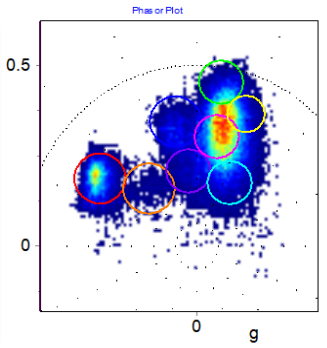








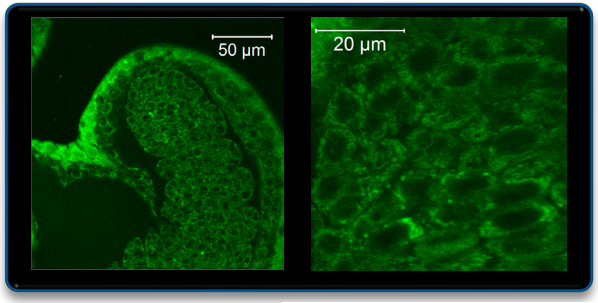


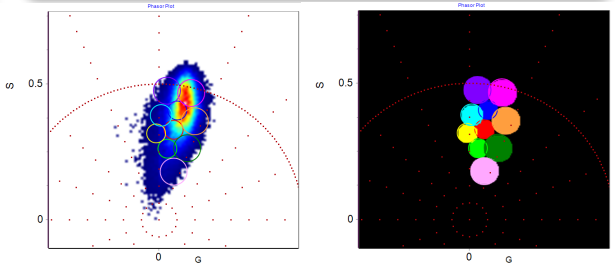


Cursor	Wavelength	Cursor	Wavelength
1	543.0nm	6	485.4nm
2	504.5nm	7	483.4nm
3	529.2nm	8	473.5nm
4	497.5nm		
5	467.3nm		

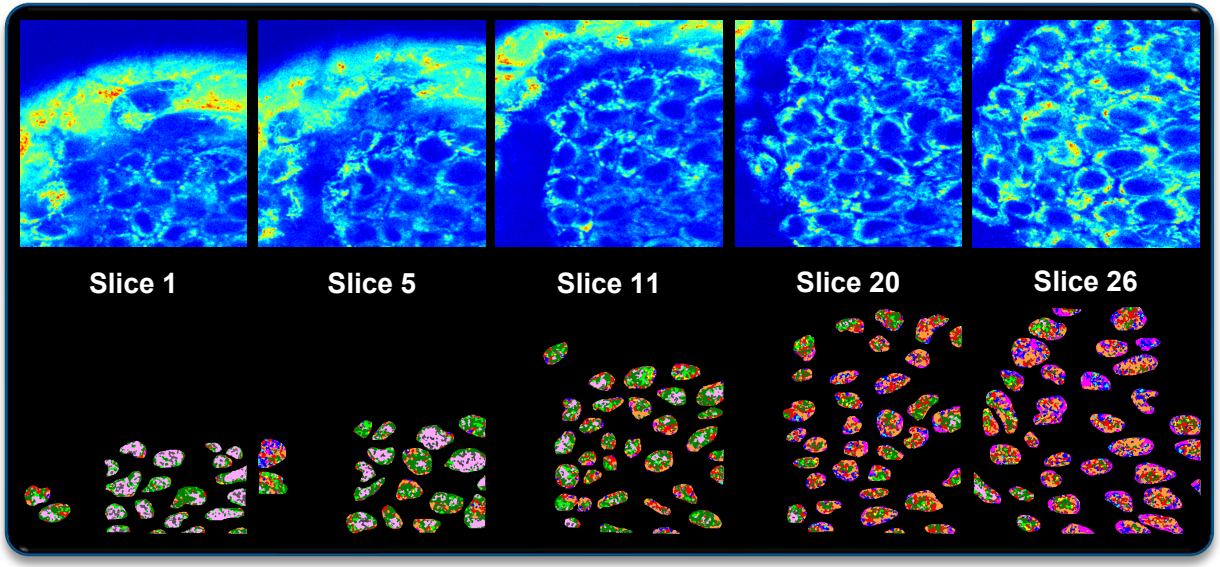
- What can Spectral Phasor tell us?
- Wavelength Range = 467.3nm – 543.0nm

Spectral Phasor Analysis of Tail Somite Nuclei





Cursor	Wavelength	Cursor	Wavelength
1	486.5nm	6	491.3nm
2	488.4nm	7	482.3nm
3	495.5nm	8	477.8nm
4	486.3nm	9	476.3nm
5	493.1nm	10	480.1nm



- Change from more **Light Pink** (480.1nm) and **Dark Green** (476.3nm) selections at outer surface of embryo to more **Dark Pink** (482.3nm), **Purple** (491.3nm), **Orange** (477.8nm) and **Dark Blue** selections (486.3nm) closer to the centre.

Acknowledgments

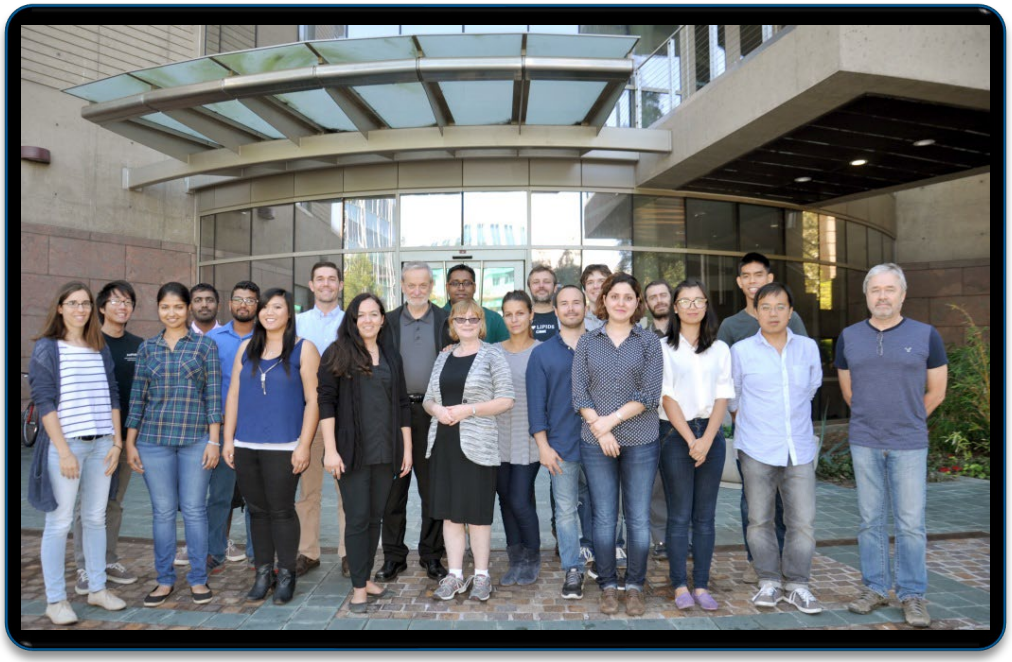
Michelle Digman
Enrico Gratton
Dave Jameson
Lorenzo Scipioni

Laboratory for Fluorescence Dynamics
& Digman Lab, UCI
Zeiss LSM 710 & SimFCS Software

Schilling Lab, UCI
Zebrafish Embryos

Arul Subramanian
Zebrafish Embryo prep

Mark Jones
Western Sydney University



https://www.phasorpy.org/stable/index.html

Section Navigation
- Introduction to PhasorPy
- Phasor coordinates from lifetimes
- Förster Resonance Energy Transfer
- Phasor plot
- Benchmarks
- Miscellaneous

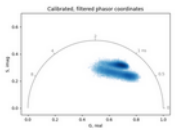
Tutorials

On this page
Benchmarks
Miscellaneous

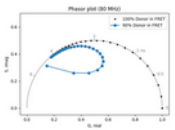
Tutorials

A gallery of examples that showcase how the PhasorPy library can be used to analyze time-resolved and hyperspectral fluorescence images using the Phasor approach.

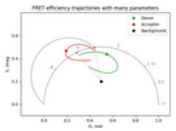
Some examples demonstrate the use of the programming interface in general, while others provide problem-oriented how-to guides for advanced applications.



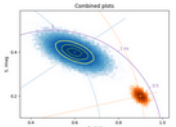
Introduction to PhasorPy



Phasor coordinates from lifetimes



Förster Resonance Energy Transfer



Phasor plot

Benchmarks

Benchmark phasor_from_signal

Miscellaneous

https://www.phasorpy.org/stable/index.html

PhasorPy

Phasor approach | Tutorials | API reference | Contributing | More

Search Ctrl + K

Section Navigation

- Introduction to PhasorPy
- Phasor coordinates from lifetimes
- Förster Resonance Energy Transfer
- Phasor plot
- Benchmarks
- Miscellaneous

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On this page
Benchmarks
Miscellaneous

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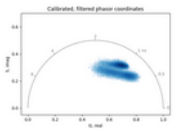
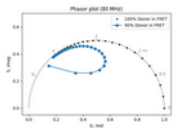
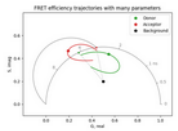


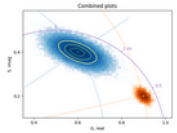
Introduction to PhasorPy



Phasor coordinates from lifetimes



Förster Resonance Energy Transfer



Phasor plot

Benchmarks

Benchmark phasor_from_signal

Miscellaneous

Acknowledgments

- ✓ **Dr. Chiara Stringari,** École Polytechnique, Paris, France
- ✓ **Enrico Gratton**, Laboratory for Fluorescence Dynamics, UIC
- ✓ **Marian Watermen and Kira Pate,** Department of Microbiology and Molecular Genetics, UCI
- ✓ **Robert Edward,** Department of Pathology, UCI
- ✓ **Peter Donovan ,** Sue and Bill Gross Stem Cell Research Center, UCI