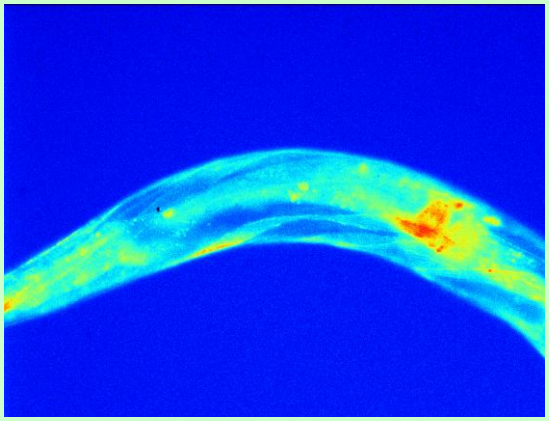
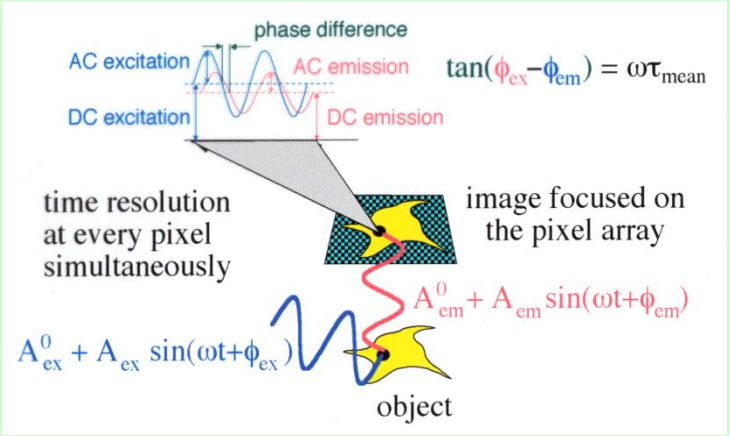
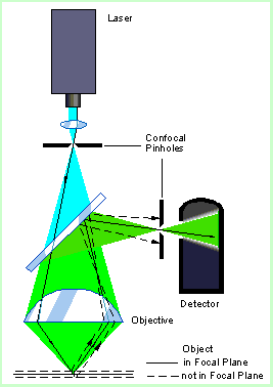
What is behind all those lifetimes anyway? Why are they useful?

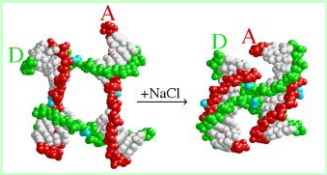


Organisms

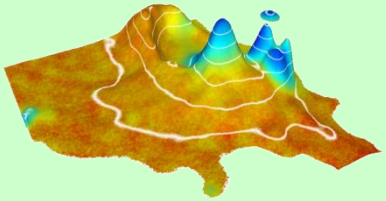




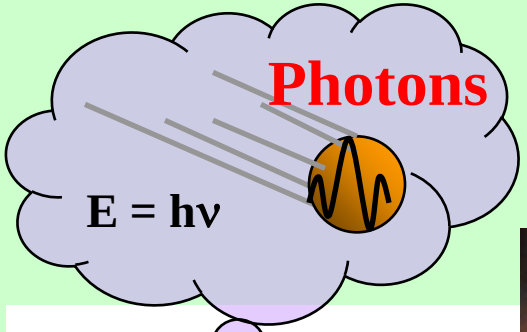
What is behind all those lifetimes anyway? Why are they useful?

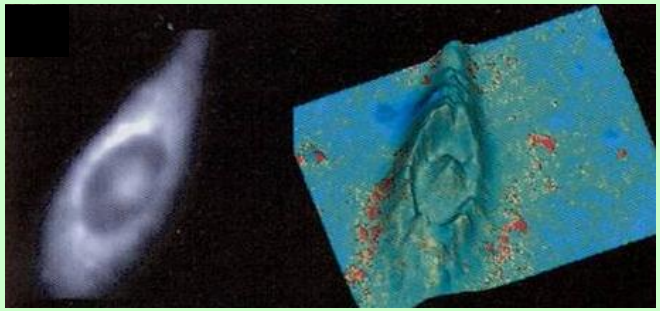


Molecules



Medical Imaging

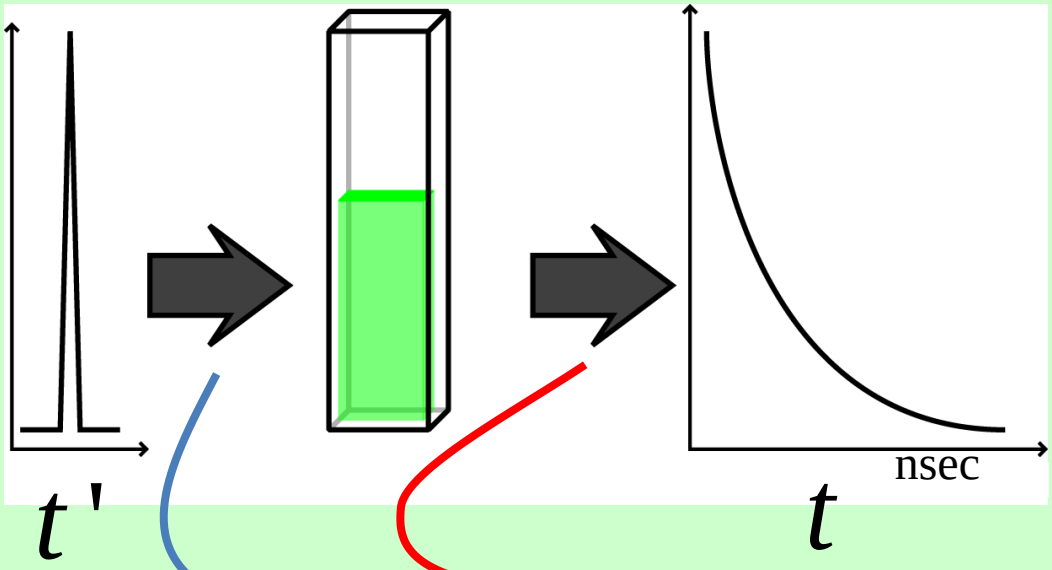


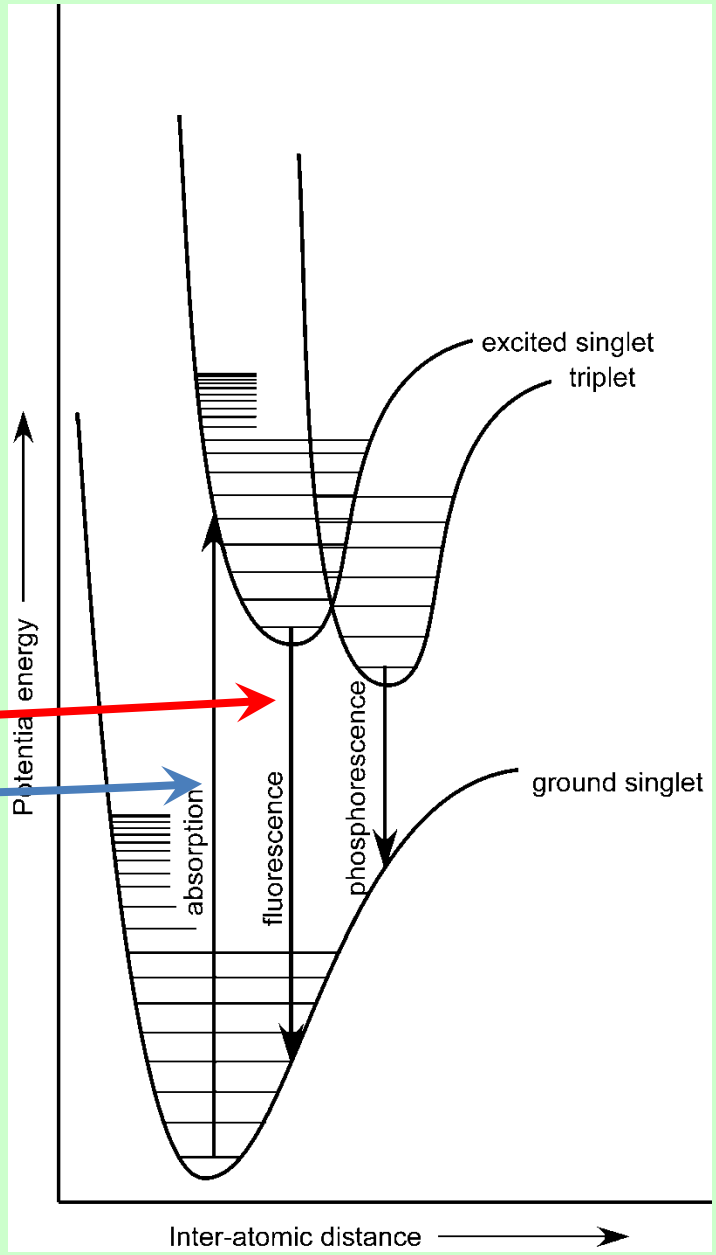


Cells

How do photons talk to molecules

"What is a lifetime?"



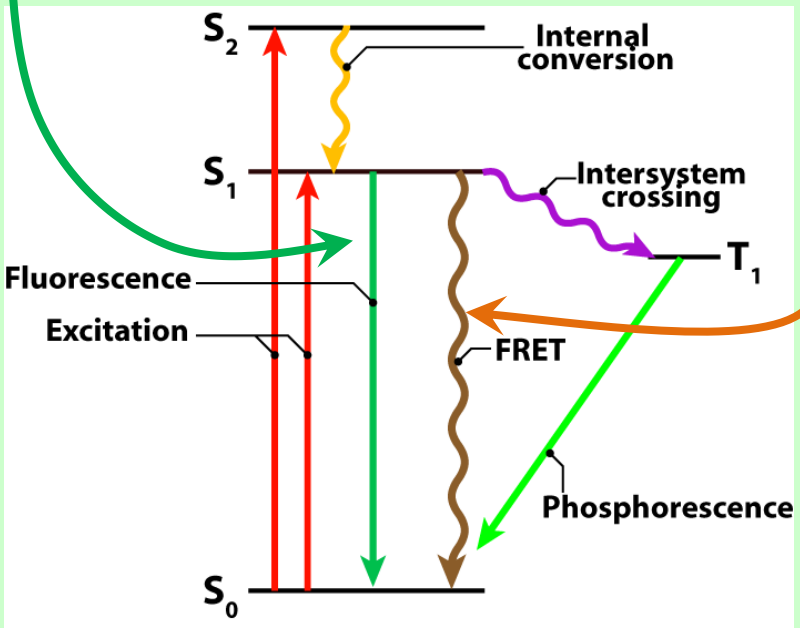


$$F_0 \exp(-(t - t')/\tau)$$

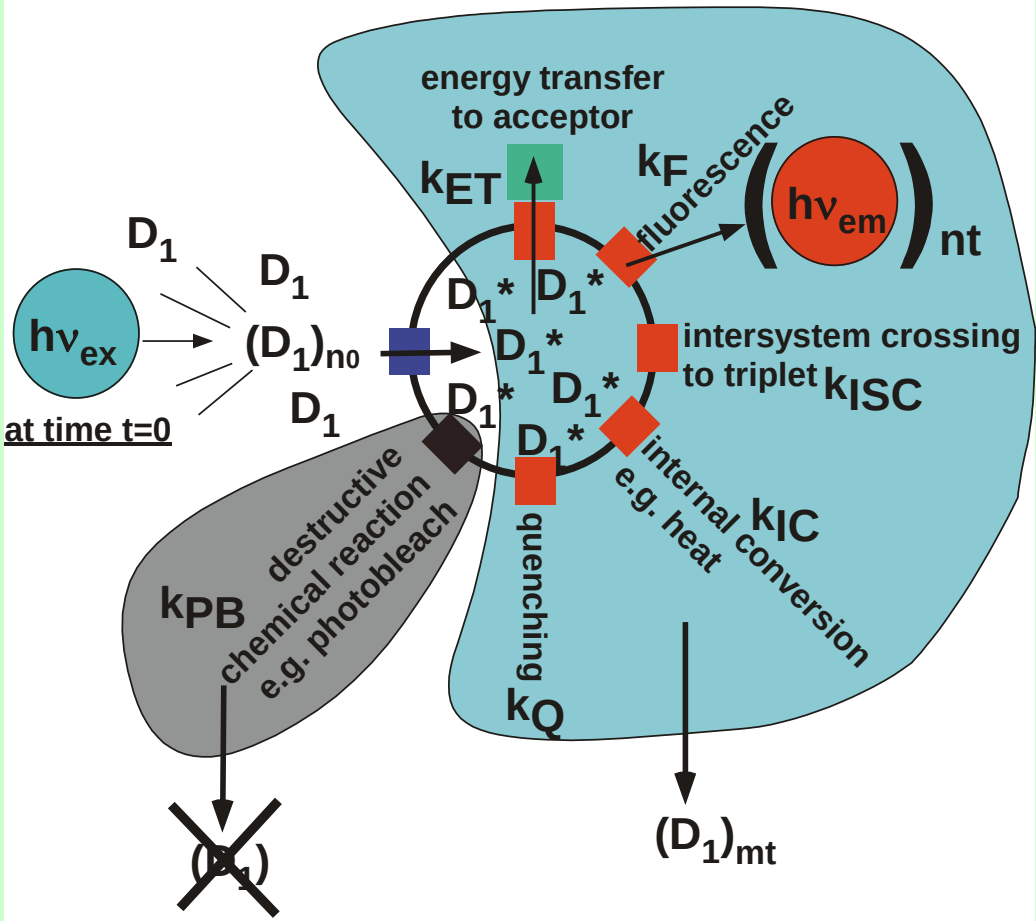
"What can lifetimes tell us?"

Fluorescence photons are the"message" from a spy (the fluorophore). We are actually interested in the other pathways out of the excited state.

e.g. FRET



MANY PHOTONS ($h\nu_{ex}$ & $h\nu_{em}$) AND MANY ESCAPE DOORS

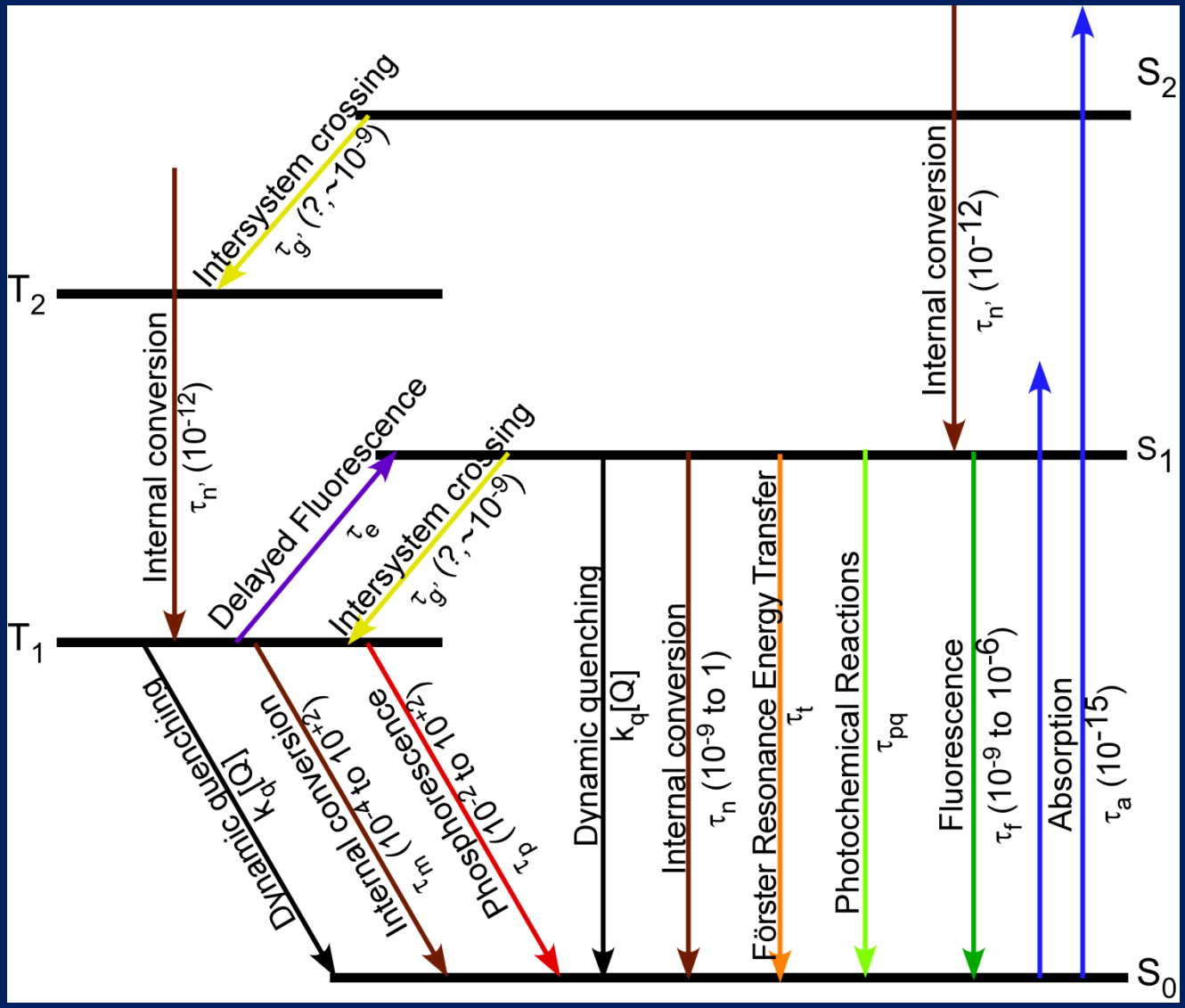


lifetime of the excited state:

$1/\tau = k_T + k_F + k_{ISC} + k_{IC} + k_Q + k_{PB} = \sum_j k_j$

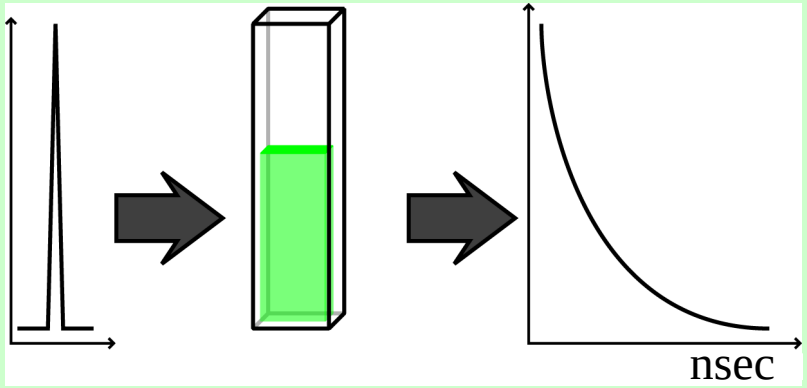
Quantum yield of the i^{th} process = $\dfrac{k_i}{\sum_j k_j}$

There are many pathways for an excited chromophore to return to the ground state

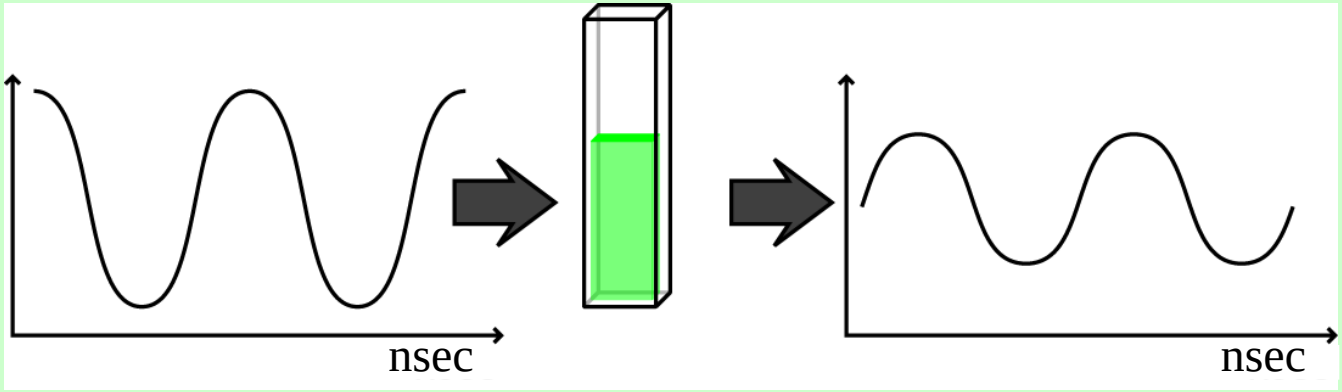


How do we measure lifetimes?

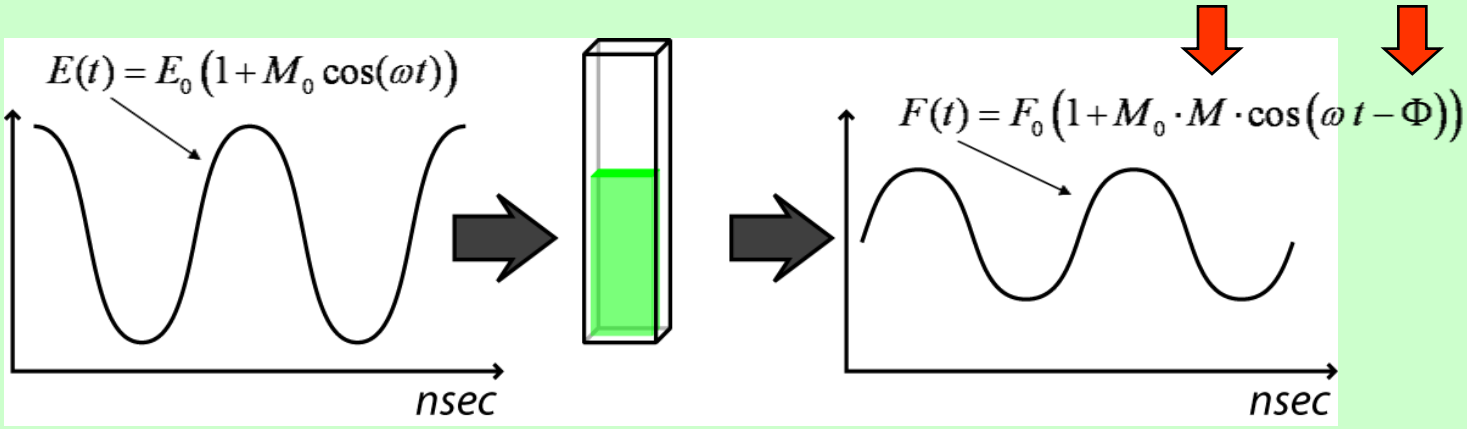
$$F_0 \exp(-(t - t')/\tau)$$

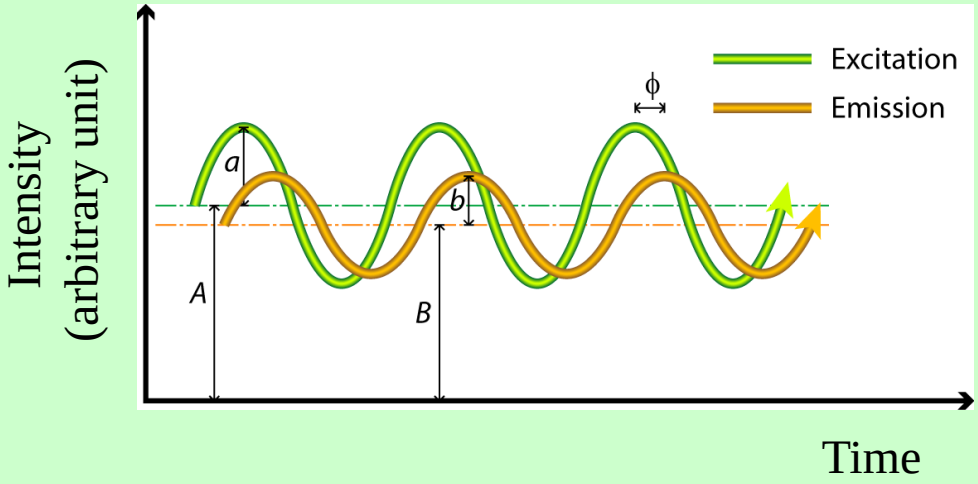


$$F(t) = M\cos(\omega t + \phi)$$



Frequency domain lifetime measurement





$$M = \frac{b/B}{a/A} = \frac{1}{\sqrt{1 + (\omega \tau_M)^2}}$$

$$\Phi = \tan^{-1}(\omega \tau_\Phi)$$

This is the way we will discuss today

Usually there are several lifetime components
- See later how to handle this -

$$F(t)_{meas} = \int_0^t E(t')F_\delta(t-t')dt'$$

Time-domain:

$$F_\delta(t-t')_{meas} = \sum_i F_{\delta,i}(t-t') = \sum_i F_{0,i}\exp(-(t-t')/\tau_i)$$

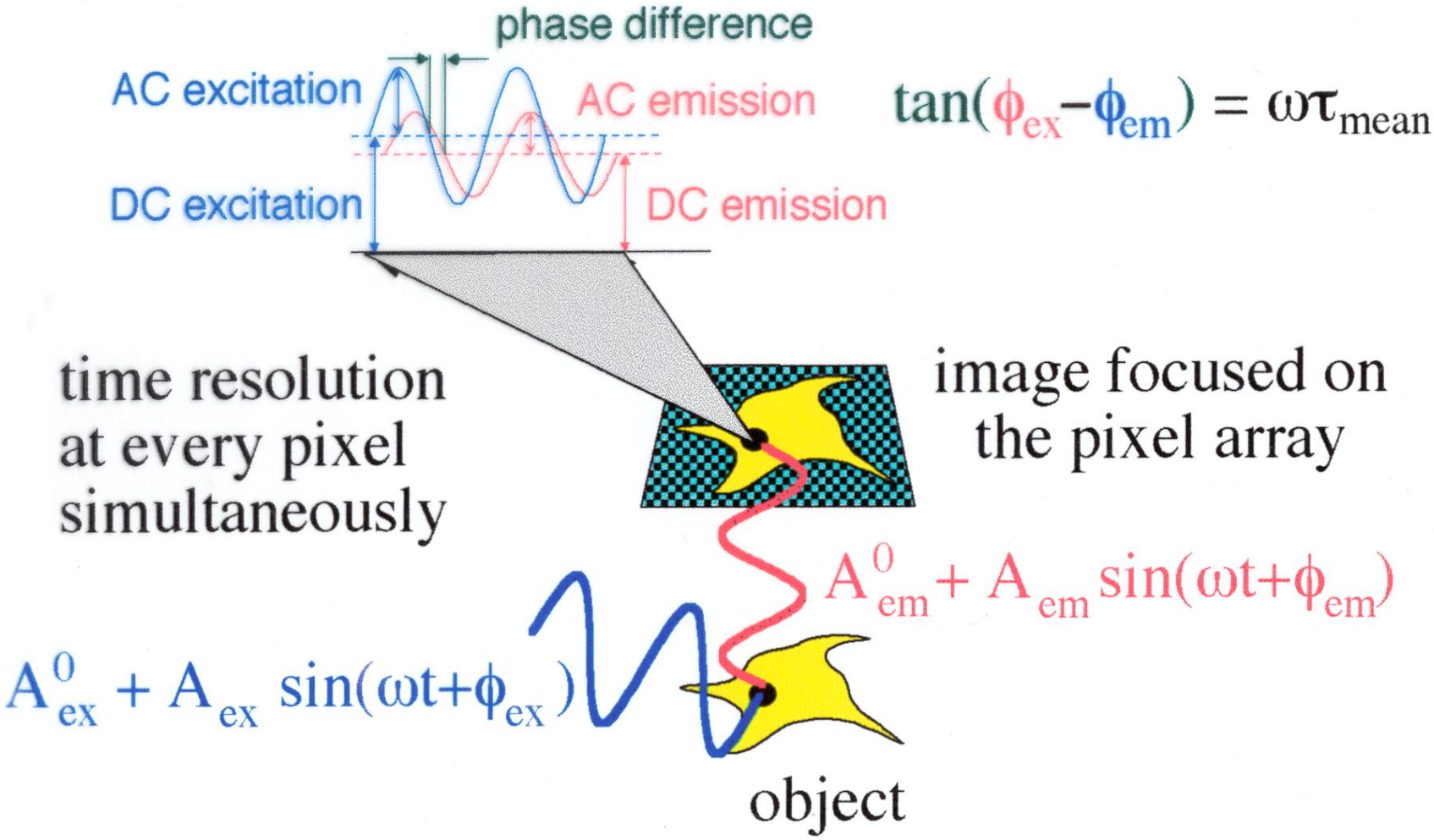
Frequency domain:

Excitation repetitive pulse; e.g. $\rightarrow \quad \propto \cos(\omega t)$

$$F(t)_{meas} = \left[\sum_i F_{0,i}\tau_i + \sum_i \frac{F_{0,i}\tau_i}{1+j\omega\tau_i}e^{j\omega t}\right] = \left[\sum_i F_{0,i}\tau_i + e^{j\omega t}\sum_i \frac{F_{0,i}\tau_i}{\sqrt{1+(\omega\tau_i)^2}}e^{-j\tan^{-1}\omega\tau_i}\right]$$

$$\frac{F(t)_{meas}}{F_{meas,ss}} = 1+\sum_i \frac{\alpha_i}{1+j\omega\tau_i}e^{j\omega t} = 1+e^{j\omega t}\sum_i \alpha_i M_i\left[\cos(\phi_{i,\omega})+j\sin(\phi_{i,\omega})\right]$$

We want to measure fluorescence lifetimes <u>in a fluorescence image</u> at <u>every location in the cell</u>.



Measuring Nanosecond fluorescence lifetimes at many pixels in an image used to be difficult

First we look at some early attempts

THE REVIEW OF SCIENTIFIC INSTRUMENTS VOLUME 30, NUMBER 6 JUNE, 1959

Microscope Phase Fluorometer for Determining the Fluorescence Lifetimes of Fluorochromes

BENJAMIN D. VENETTA
Department of Anatomy, Western Reserve University School of Medicine, Cleveland 6, Ohio

1959

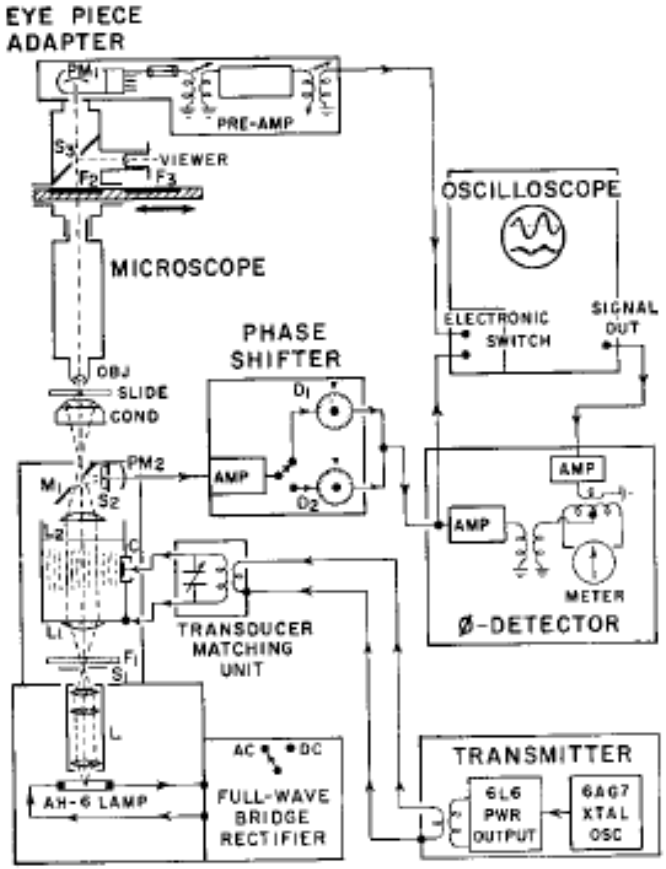


FIG. 1. Block diagram of the microscope phase fluorometer.

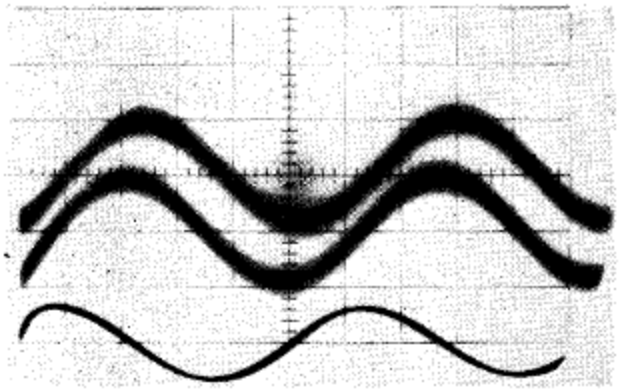


FIG. 5. The transmitted light signal, fluorescent light signal, and the tracer signal (sweep speed: 0.034 μsec/cm).

$$\tan\Delta\phi = \omega\tau.$$

The instrument was capable of dissecting the image into areas of interest, and can therefore be classified as an imaging fluorescence lifetime instrument. Lifetime measurements were carried out on "fluorphores bound to the **nuclei of tumor cells, as well as autofluorescence of biological tissue samples**."

MEASUREMENT OF FLUORESCENCE DECAY TIME IN LIVING CELLS

CH. N. LOESER, ELLEN CLARK, MARJORIE MAHER and H. TARKMEEL

University of Connecticut Health Center, Department of Anatomy, Farmington, Conn. 06032, USA

Experimental Cell Research 72 (1972) 480–484

1972

"Ascites tumor cells, liver cells, fibroblasts, bacteria, and cell fractions, after incubation with a fluorochrome and appropriate washing, can be suspended in a cuvette (or in the case of single cells, placed on a microscope slide) and the fluorescent decay time can be read out digitally in nanoseconds. The instrument is most accurate where actual decay values are > 2 ns! "

Table 1. *Intracellular fluorescence decay times of ANS, TNS, BP, and 2-AN*

Medium[a]	Cell type	Decay time (nsec)
0.3×10^{-4} M ANS	Ascites	7.8 ± 0.2[b]
0.3×10^{-4} M TNS[c]	Ascites	8.8 ± 0.1
16 % saturated BP	Ascites	15.2 ± 0.1
0.3×10^{-4} M 2-AN	Ascites	16.3 ± 0.1
0.3×10^{-4} M ANS	*Bacterium megaterium*	10.3 ± 0.3

[a] BP was made up as a saturated solution in propylene glycol and diluted with saline. The other compounds were made up in Krebs-Ringer, pH 7.3 ± 0.1.
[b] Standard error.
[c] Limited solubility in aqueous solution.

1976-
1979

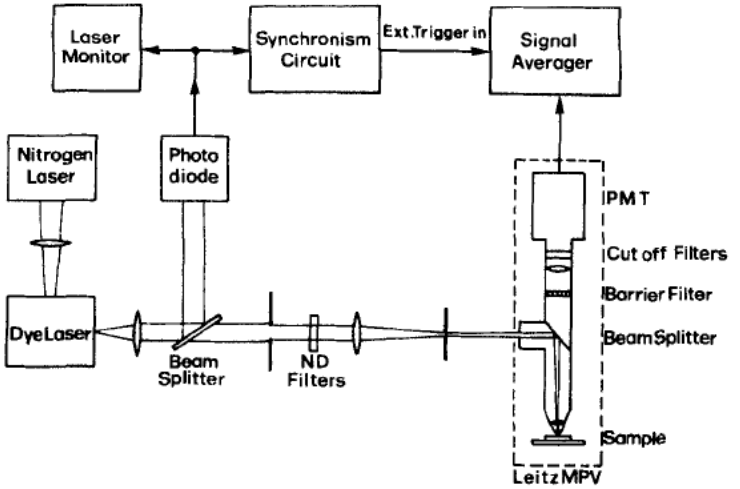
FLUORESCENCE OF COMPLEXES OF QUINACRINE MUSTARD WITH DNA. I. INFLUENCE OF THE DNA BASE COMPOSITION ON THE DECAY TIME IN BACTERIA

G. BOTTIROLI,* G. PRENNA,*† A. ANDREONI,‡ C. A. SACCHI‡ and O. SVELTO‡

*Centro di Studio per l'Istochimica del C.N.R., Istituto di Anatomia Comparata dell'Università, Pavia, Italy and ‡Centro de Studio per l'Elettronica Quantistica e la Strumentazione Elettronica del C.N.R., Istituto di Fisica del Politecnico, Milano, Italy

Photochemistry and Photobiology, Vol. 29, pp. 23 28. 1979.

$$\tau = \frac{\int_0^\infty t n(t)\,dt}{\int_0^\infty n(t)\,dt} = \tau_D\left(1 - \frac{\sqrt{\pi}}{2}\frac{[Y]}{[Y]_0}\right),$$



The fluorescence of several bacterial DNAs stained with quinacrine mustard have been investigated using a laser microfluorometer with **a spatial resolution of ~0.3 *micro*-m and a temporal resolution of ~0.3 ns** connected to a digital signal averager.

We explain this result on the basis of an energy transfer mechanism between dye molecules intercalating AT:AT sequences (donors) and dye molecules bound to either GC:GC or GC:AT sequences (acceptors).

Andreoni, **A.**, Sacchi, C.A., Svelto, O., Longoni, A., Bottiroli, G., and Prenna, G., in ***Proceedings of the Third European Elecrro-Optics Conference***, H.A. Elion, Editor, SPIE, Washington, 258-270, (1976).

Fluorescence Decay Analysis in Solution and in a Microscope of DNA and Chromosomes Stained with Quinacrine

DONNA J. ARNDT-JOVIN, SAMUEL A. LATT, GEORGE STRIKER AND THOMAS M. JOVIN

THE JOURNAL OF HISTOCHEMISTRY AND CYTOCHEMISTRY

Vol. 27, No. 1, pp. 87–95, 1979

Fluorescence Lifetimes of Quinacrine Bound to DNA and Poly[d(A-T)] (Three-Component Analysis)[a]

DNA or polymer[b]	(A-T)[c]	(A-T)[d]	rte[e]	⟨τ⟩ (nsec)	τ_1 (nsec)	fce %	τ_2 (nsec)	fce %	τ_3 (nsec)	fce %	$\langle r_w^2 \rangle$	⟨c⟩
Poly[d(A-T)][f]	1	1	1	18	1.1	6	7.9	33	26	60	2.71	1040
Clostridium acidurici	0.7	0.24	.2	14	3.0	29	11	36	27	34	1.20	566
Proteus mirabilis	0.6	0.14	.15	14	3.3	29	13	45	30	25	2.34	2326
Bacillus subtilis	0.55	0.098	.12	12	2.6	35	11	39	27	26	1.26	437

1979

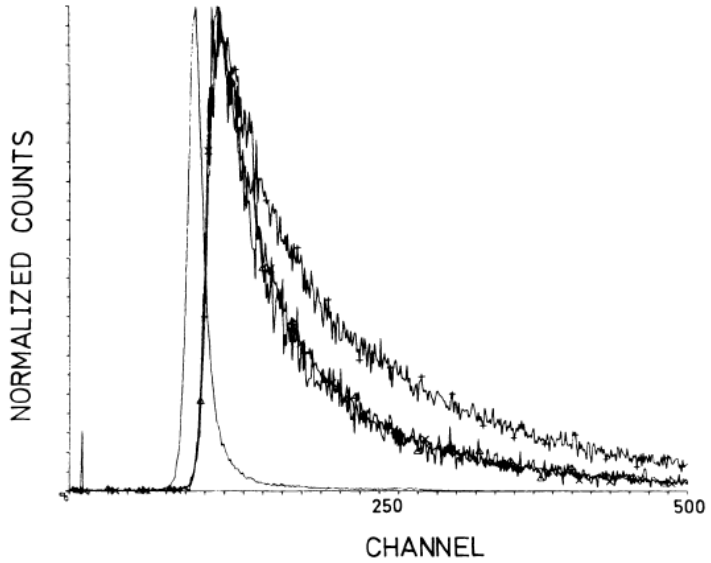


FIG. 4. Fluorescence decay curves for quinacrine bound to cytologic samples taken by microfluorometry. Decay curves were taken on the microscope as described in Materials and Methods and data were analyzed is in Table III, 0.127 nsec/channel. Solid line, flash lamp; open triangle, nuclei from a normal human XY male; ×, nuclei from *Drosophila virilis*; +, nuclei from *Samoae leonensis*.

The new laboratory based FLIM instruments were first reported about 1989

What changed later in the 1980s?

Light sources, detectors (Intensifiers, CCDs), computers, etc.

Parts became available commercially; major progress in microscopes

Commercial packages for image analysis and date handling and display

Interest grew in the biology community for quantitative imaging

By now the landscape has changed drastically

Now many firms delivering FLIM instruments

100s of publications

Books dedicated to FLIM:

FRET and FLIM Techniques
Volume 33 (Laboratory Techniques in Biochemistry and Molecular Biology)
Ed by Theodorus W. J. Gadella

FLIM Microscopy in Biology and Medicine
Ed by Ammasi Periasamy and Robert M. Clegg

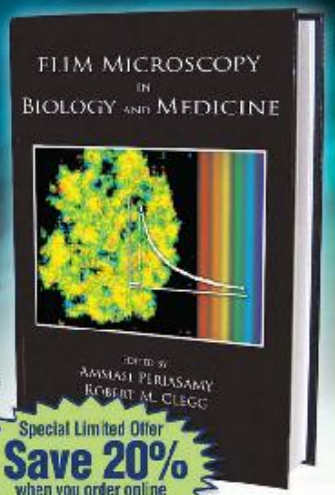
And
many general and specific reviews.

C7890 CFL

NEW

Offers Dramatic Opportunities in Biomedical Research

FLIM Microscopy in Biology and Medicine



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Edited by **Ammasi Periasamy**
University of Virginia, Charlottesville, USA

Robert M. Clegg
University of Illinois, Urbana-Champaign, USA

Detecting Signals at the Single Molecule Level: Pioneering Achievements in Microscopy

Fluorescence lifetime imaging microscopy (FLIM) is an established tool for a variety of applications in biology and biomedical research. However, recent advances have led to such remarkable improvements in its capacity for contrast and sensitivity that researchers can now employ it to detect signals at the single molecule level. FLIM also offers the additional benefit of independence from fluorophore concentration and excitation intensity. Moreover, its unique sensitivity makes it an excellent reporter of conformational changes and of variations in the molecular surroundings of biological molecules.

Most of this improvement and discovery has occurred during the past decade and to date, information that would benefit a broad range of researchers remains scattered in the literature. Edited by two of the top pioneers in the field, **FLIM Microscopy in Biology and Medicine** presents the fundamentals of FLIM along with a number of advanced considerations so that a wider audience can appreciate recent and potential improvements that make it such a valuable tool.

In addition to reviewing the latest developments, applications, and approaches to data analysis, the book also takes measure of the current state of the field, presenting the pros and cons of different methods and suggesting where improvements are required. The book also describes ancillary techniques related to the direct determination of lifetimes, including imaging fluorescence anisotropy for the study of molecular rotations.

New Opportunities for Biomedical Researchers...New Challenges for Microscopy Researchers

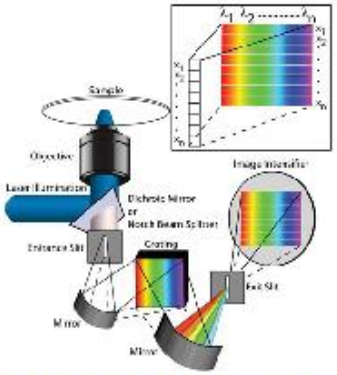
Discussion sections in all the chapters clearly show the challenges for implementing FLIM for various applications. Certain chapters discuss limits on the number of photons required for highly accurate lifetime determinations as well as the accuracy with which multiple, closely associated lifetime components can reliably be determined. Such considerations are important for users when selecting the most advantageous method of FLIM to use for a particular application.

While this book provides an introduction for those new to FLIM, it gathers a wealth of material to enhance the work of experts involved with pioneering technological improvements or research opportunities in this unique and promising area of microscopy.

See Reverse for Table of Contents and Ordering Information

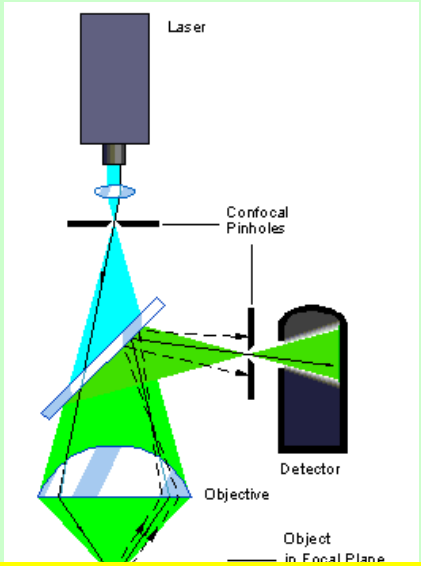
Features

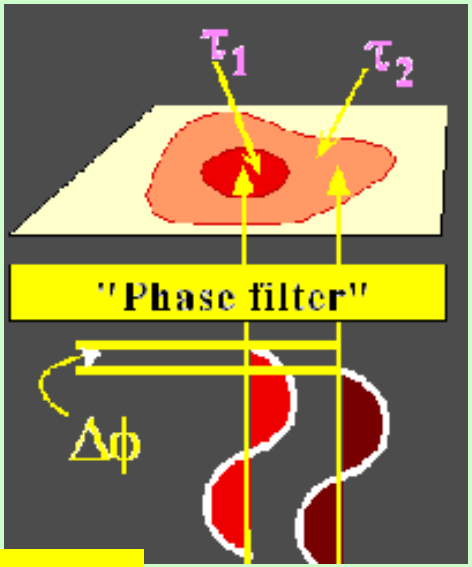
- Brings together the contributions of those pioneering the field
- Covers issues related to data acquisition and data analysis
- Addresses the advantages and disadvantages of FLIM in various biological and clinical research areas
- Compares FLIM measurements to other techniques
- Addresses the fundamentals of dynamic fluorescence measurements and the basic pathways of de-excitation available to electronically excited molecules



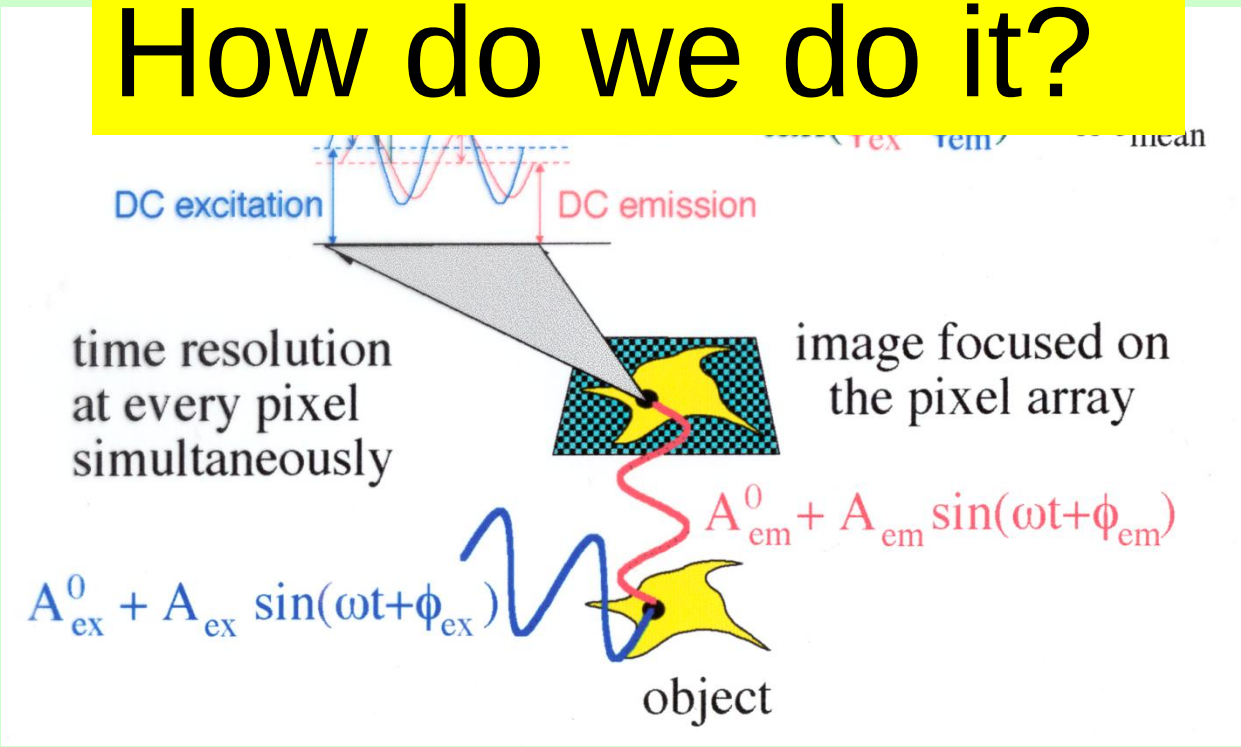
Catalog no. C7890, July 2009, c. 472 pp.
ISBN: 978-1-4200-7890-9, $99.95 / £63.99

2 WAYS
TO DO IT
2-hν scanning
&
full-field
FLI





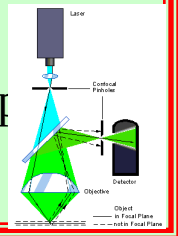
How do we do it?



Fluorescence lifetime-resolved imaging microscopy (FLI)

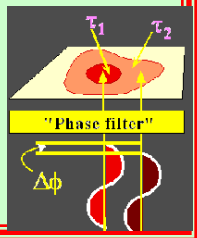
Scanning 2-hν FLI

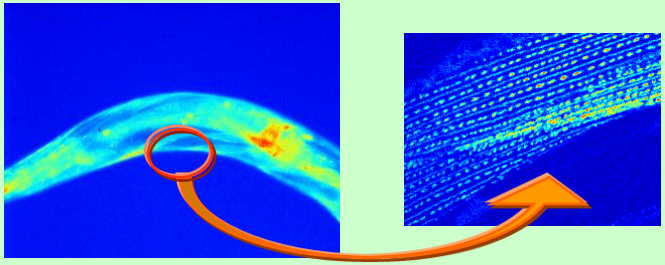
- Spatial confinement of excitation-diffraction limited focussing
  0.3 m x 1 m ($h\nu_{ex}$=700 nm, NA=1.3)
- confocal effect
- Little or no photodamage outside of 2-hν region
- Depth of penetration
- 3-D images possible
- UV-excitation (localized)
- PM detection - multifrequencies - Fourier spectrum
- Detection straight forward
- Photoactivation of caged comp

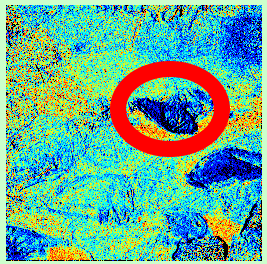


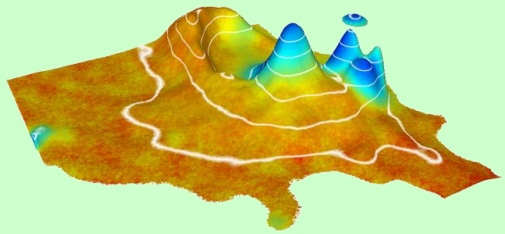
Full-field FLI

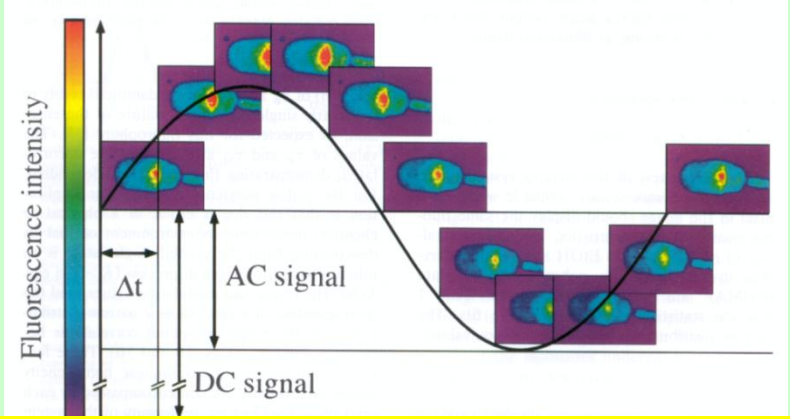
- Simultaneous pixel measurement
- Attach to any microscope
- Simplicity of optical construction & operation
- FLIE (endoscopy)
- Real-time applications
- CCD data acquisition (long integration times possible without unreasonable total measurement time)
- Phosphorescence (DLIM)
- 3-D possible with image deconvolution; spinning disk
- Rapid time resolution for kinetics in millisecond range.



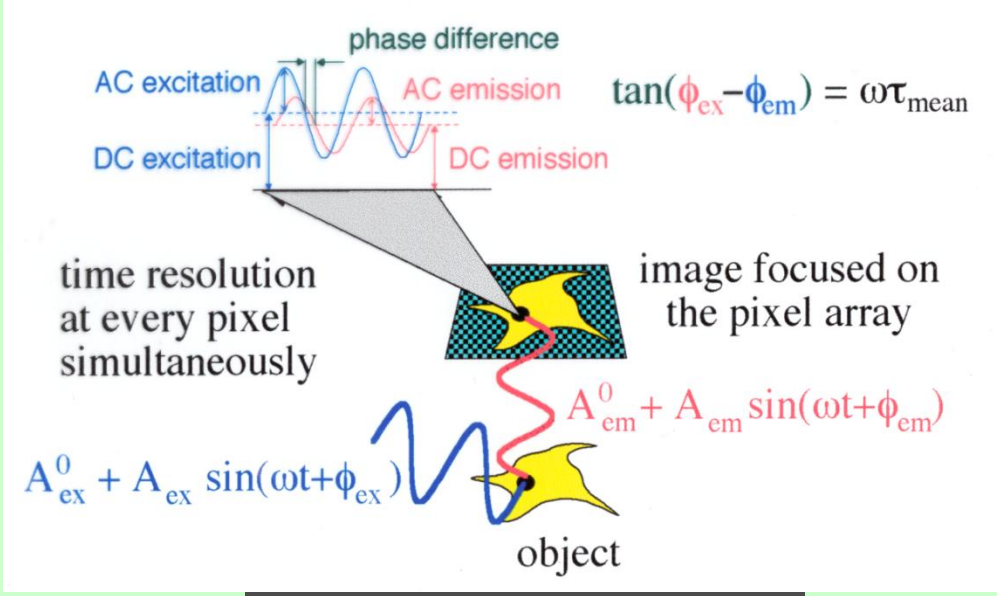


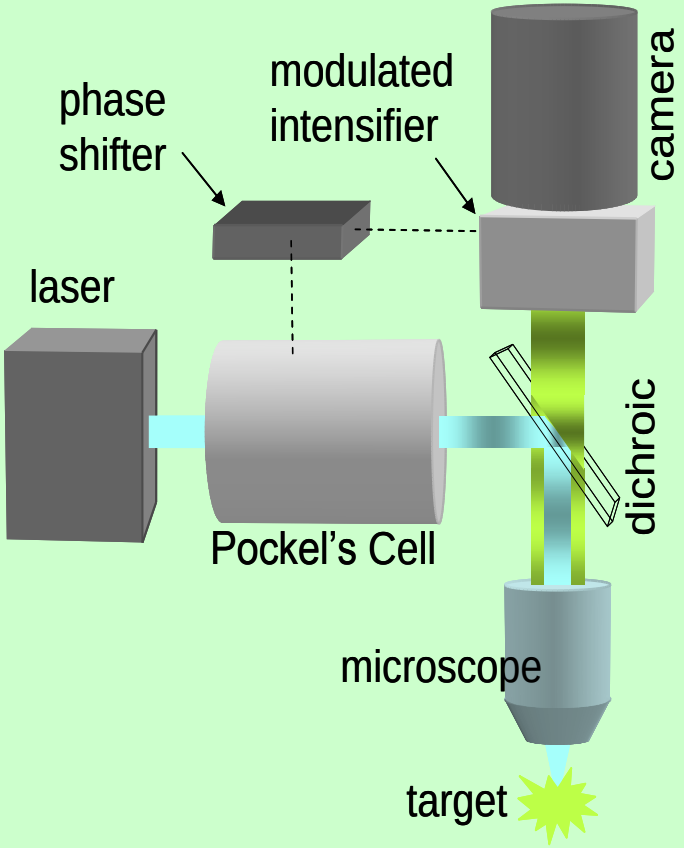


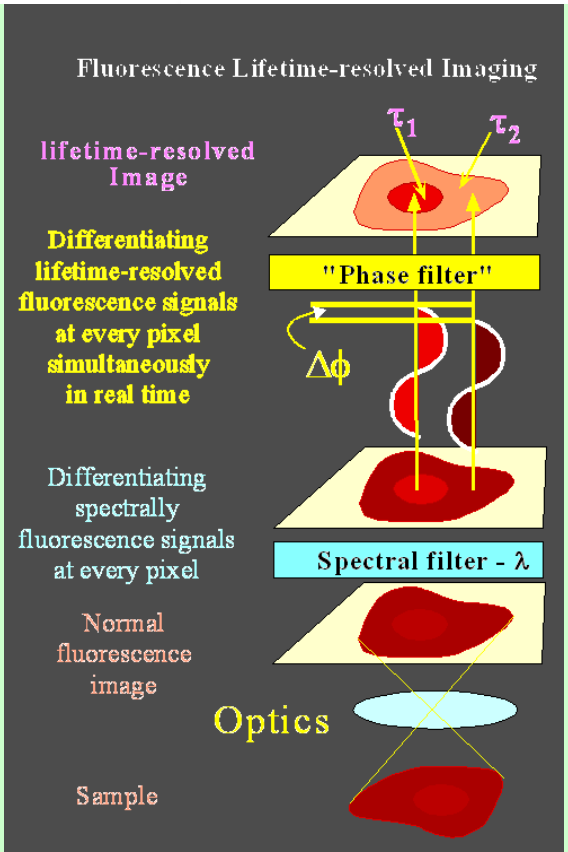




Boar sperm labeled with a lifetime dye molecule; Note the variation of the fluorescence intensity over the period of the excitation modulation.

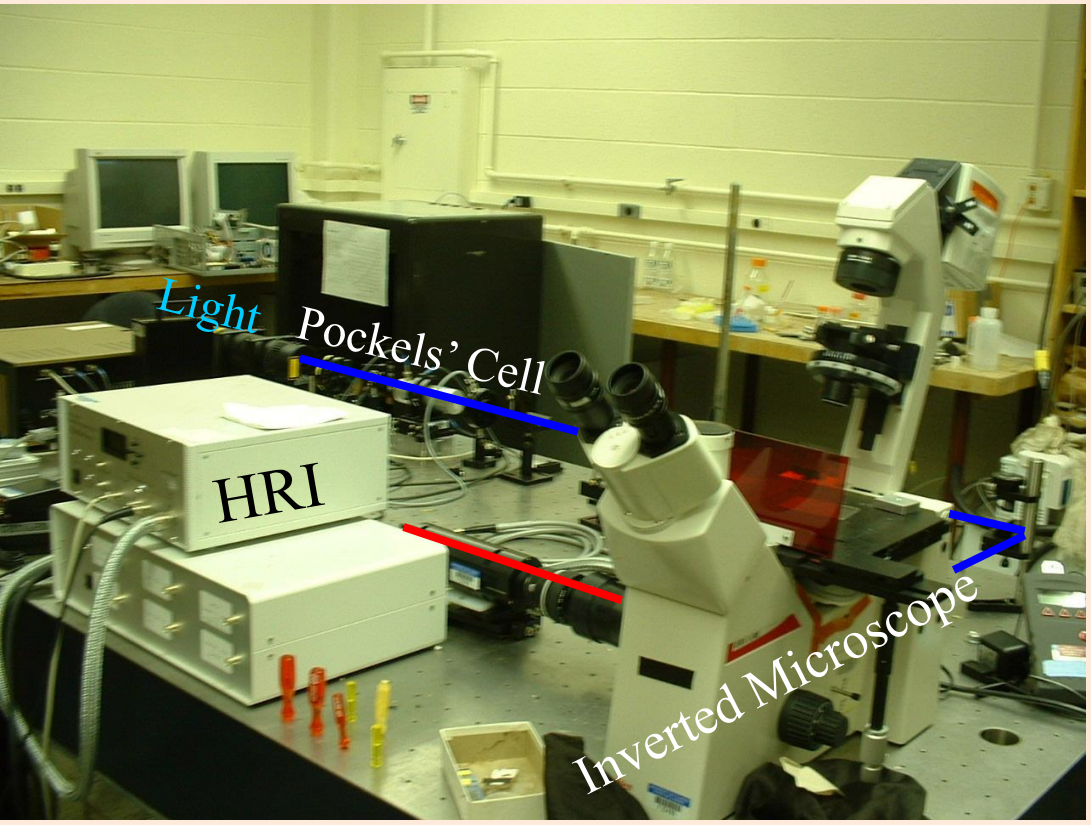


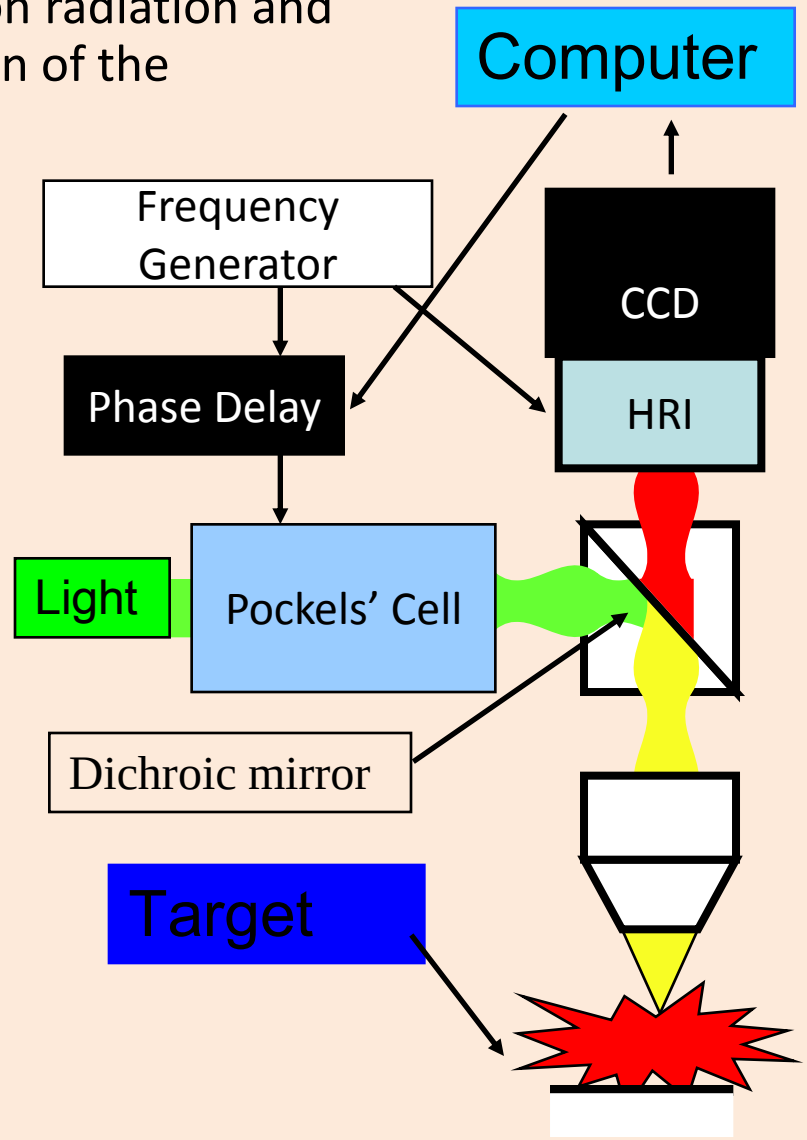




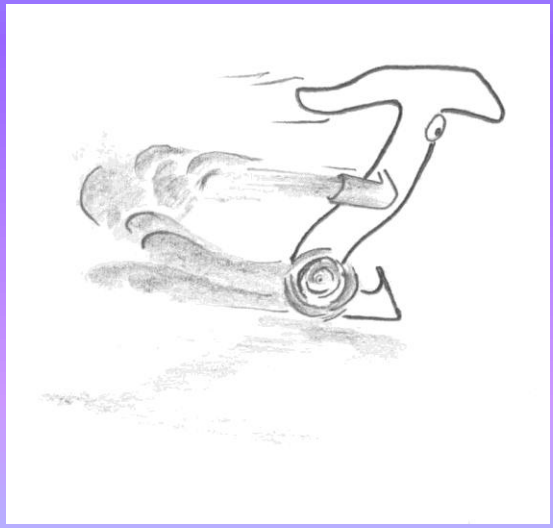
Experimental setup of FLIM

FLIM is operated by modulating the excitation radiation and observing the phase delay and demodulation of the fluorescence emission

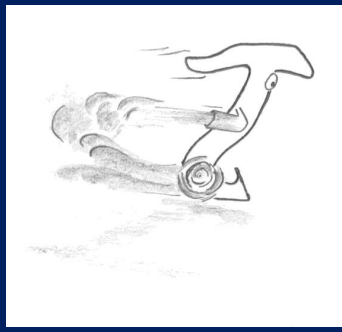




We want to make the measurements fast



Importance of rapid  display

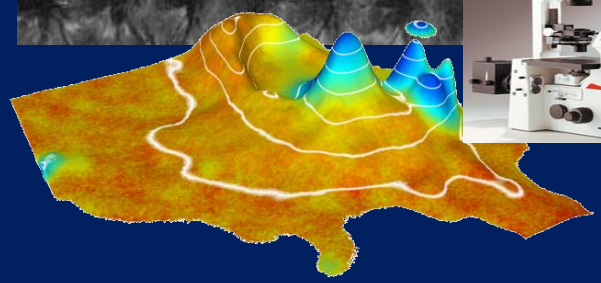


with large information content

Make the information in the image intelligible to the user

Real time communication to user

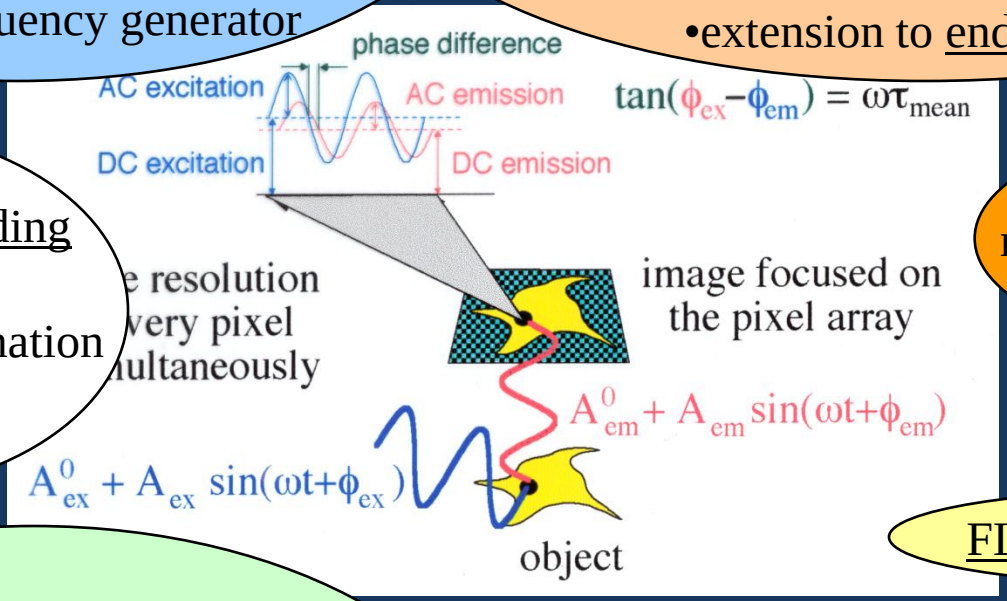
Medical Imaging



What can we do with it?

Detailed analysis of lifetimes
- accurate lifetimes
- component analysis
- multifrequencies
- global lifetime analysis
- HF chip frequency generator

Fast Lifetime-resolved imaging
- rapid data acquisition
- real-time updated display
- informative image display
- suppression & enhancement of components
- use of hardware to increase speed
- extension to endoscopes



Improving & extending
- image contrast
- component discrimination
- 3-D capability

FLI
reactive oxygen species determination

FLI ion concentrations

FLI pH determination

Combine & quantify features
- lifetime-resolution
- spatial parameters
- spectral (+FLI) component analysis
- correction and utilization of “artifacts”
- global analysis

[photolysis, scattering, absorption]

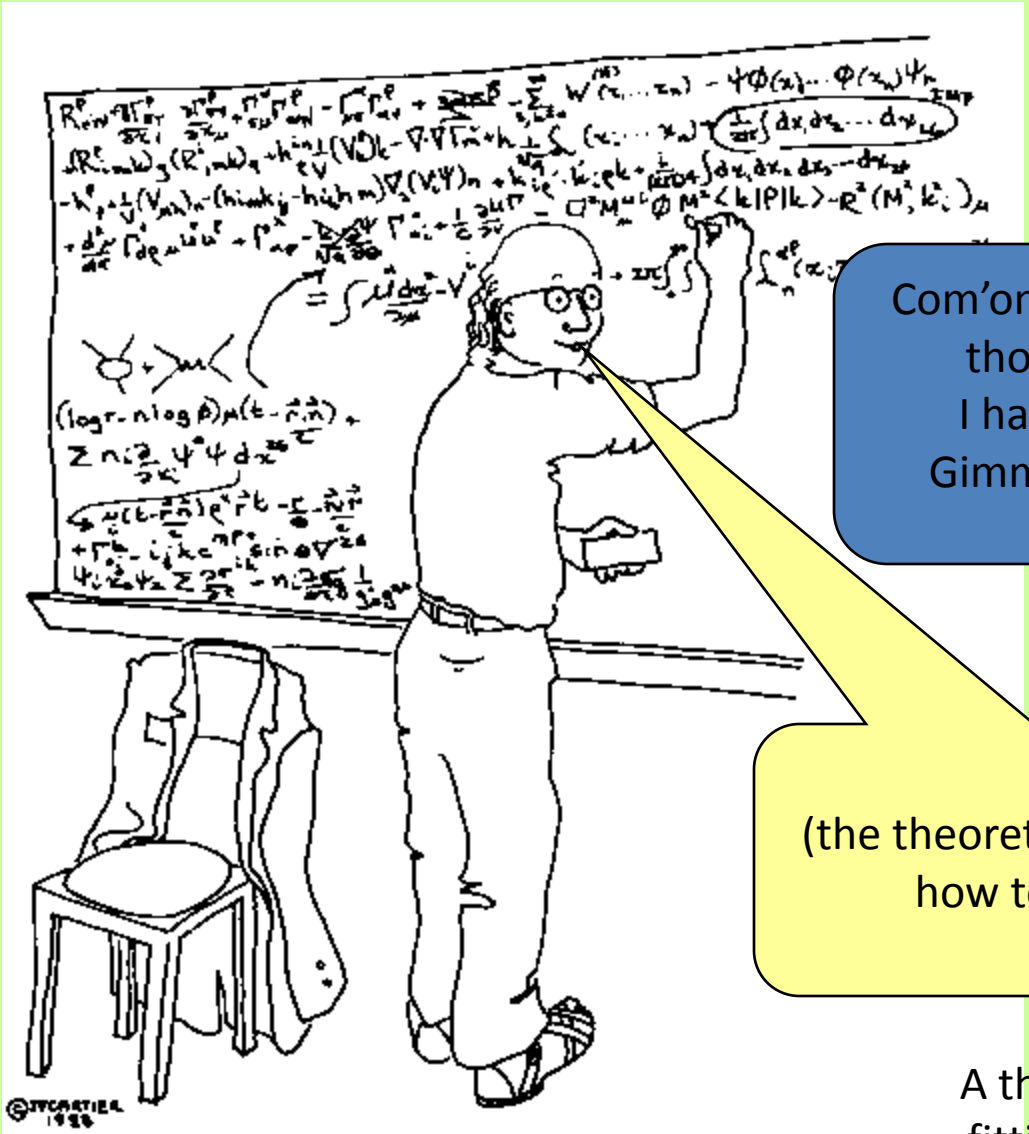
FRET applications
- Direct quantitative efficiencies
- combination of FLI with other methods

[ratio imaging, photobleach FRET]

SO, now we seem all set.

BUT…..

How do you interpret fluorescence decay?



"At this point we notice that this equation is beautifully simplified if we assume that space–time has 92 dimensions."

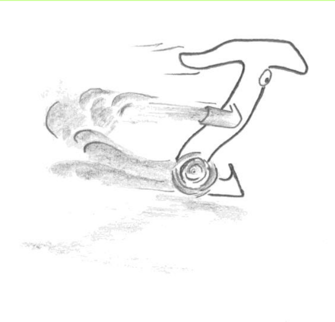
Com'on!, I don't want all those equations!
I have 10^6 pixels!!
Gimme an easy way!

Experimentalist

Here I
(the theoretician) can tell you how to do it right.

A theoretician solving the equations for fitting the fluorescence decay to multi-exponentials

We could fit the data analytically



I see three exponentials!

ou'd like it. Just like green eggs and ham!

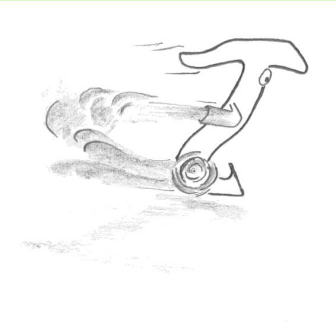
$$f_1 + f_2 \equiv 1$$

$$f_s \equiv a_s \tau_s \big/ \sum a_s \tau_s$$

$$\tau_2 = \frac{\beta + \omega\tau_1}{\beta\omega^2\tau_1 - \omega}$$

$$\beta = \frac{M\cos\Phi - (1+\omega^2\tau_1^2)^{-1}}{M\sin\Phi - \omega\tau_1(1+\omega^2\tau_1^2)^{-1}}$$

$$f_2 = \frac{M\cos\Phi - (1+\omega^2\tau_1^2)^{-1}}{(1+\omega^2\tau_2^2)^{-1} - (1+\omega^2\tau_1^2)^{-1}}$$



What now?

Model Independent Analysis
Some different ways to parameterize lifetime-resolved data

$$1/(1+j\omega t) = M_i\left[\cos(\phi_{i,\omega}) + j\sin(\phi_{i,\omega})\right]$$

$$x = M_i\cos(\phi_{i,\omega}) \text{ and } y = M_i\sin(\phi_{i,\omega})$$

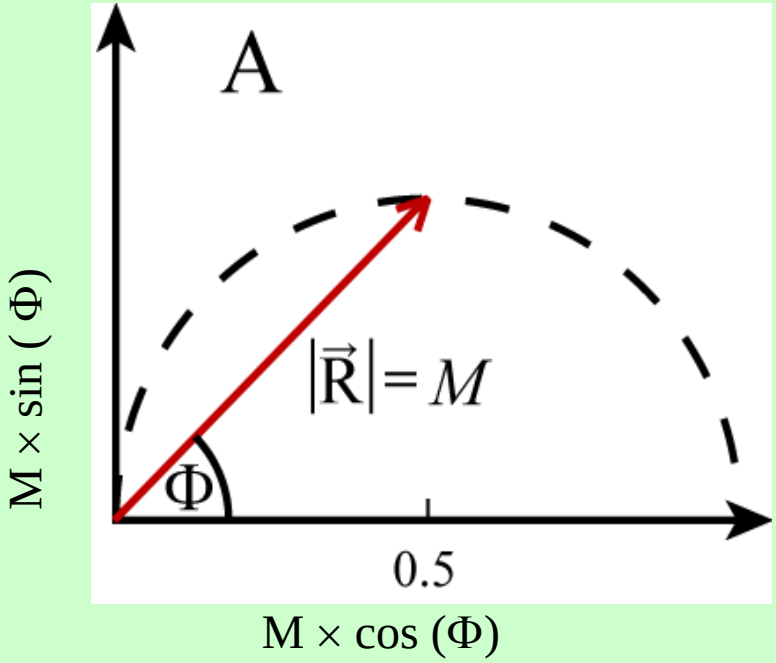
$$j = \sqrt{-1}$$

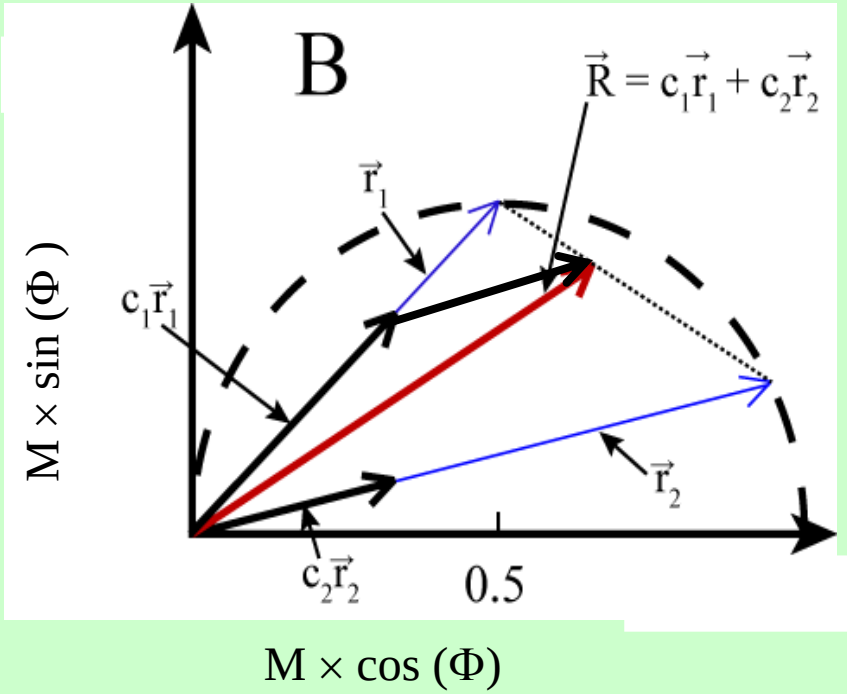
Frequency domain lifetime measurement

Data analysis with a polar plot representation

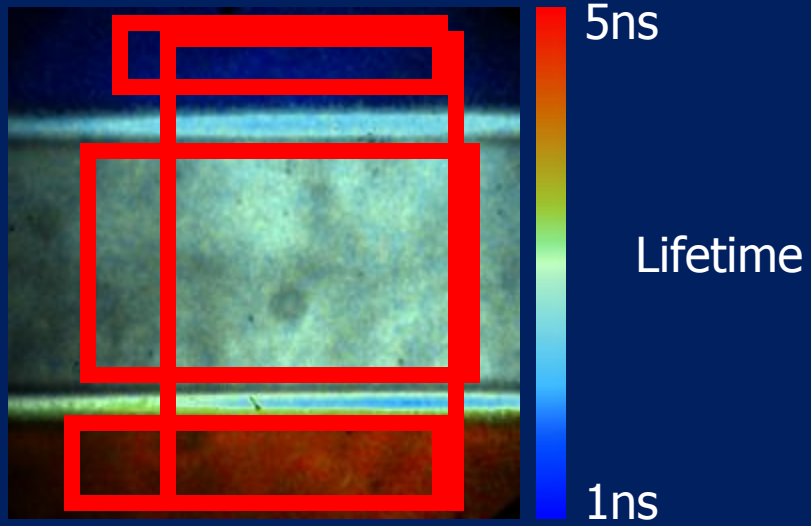
Demodulation = $M = \frac{b/B}{a/A} = \frac{1}{\sqrt{1+(\omega\tau_M)^2}}$

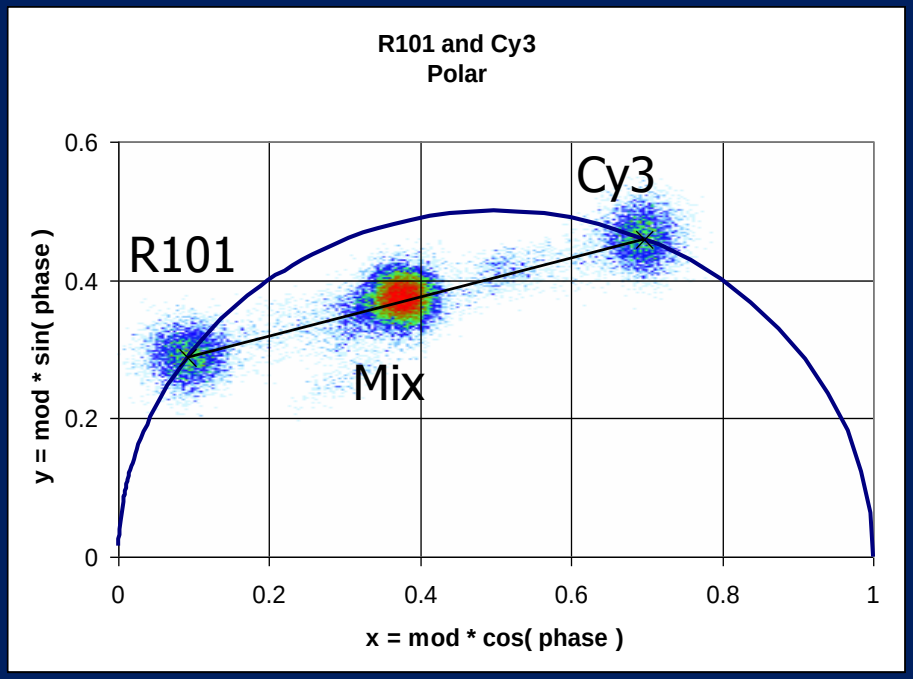
Phase shift = $\Phi = \tan^{-1}(\omega\tau_\Phi)$

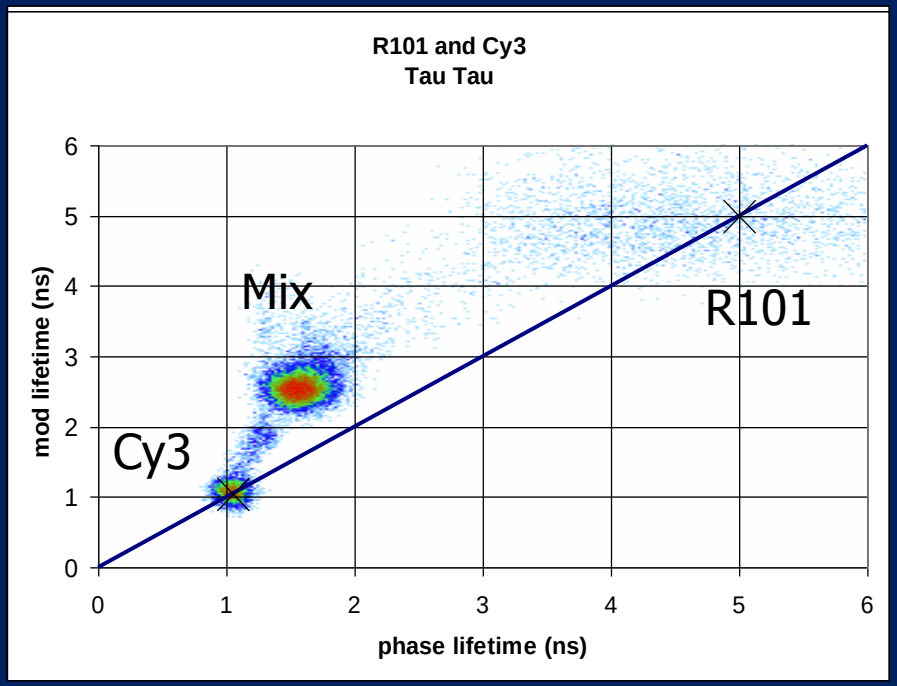




good for any signal $\propto \frac{1}{1+i\omega\tau}$ (for instance dielectric dispersion)


5ns
Lifetime
1ns


R101 and Cy3
Polar
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1
y = mod * sin(phase)
x = mod * cos(phase)
Cy3
R101
Mix

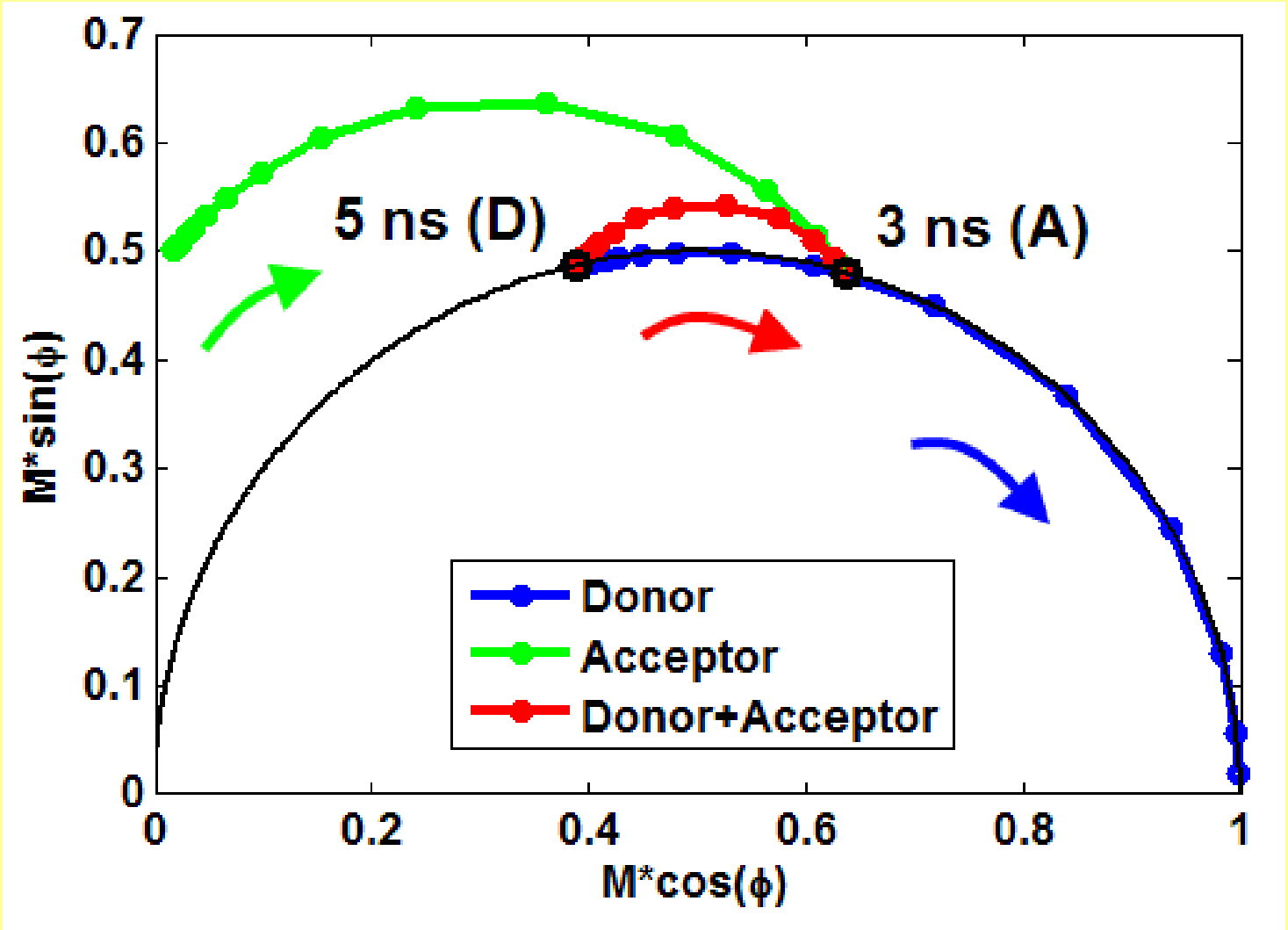

R101 and Cy3
Tau Tau
6
5
4
3
2
1
0
0
1
2
3
4
5
6
mod lifetime (ns)
phase lifetime (ns)
Mix
R101
Cy3

Observing the fluorescence of:

Product species of an excited state reaction

Product and directly excited species

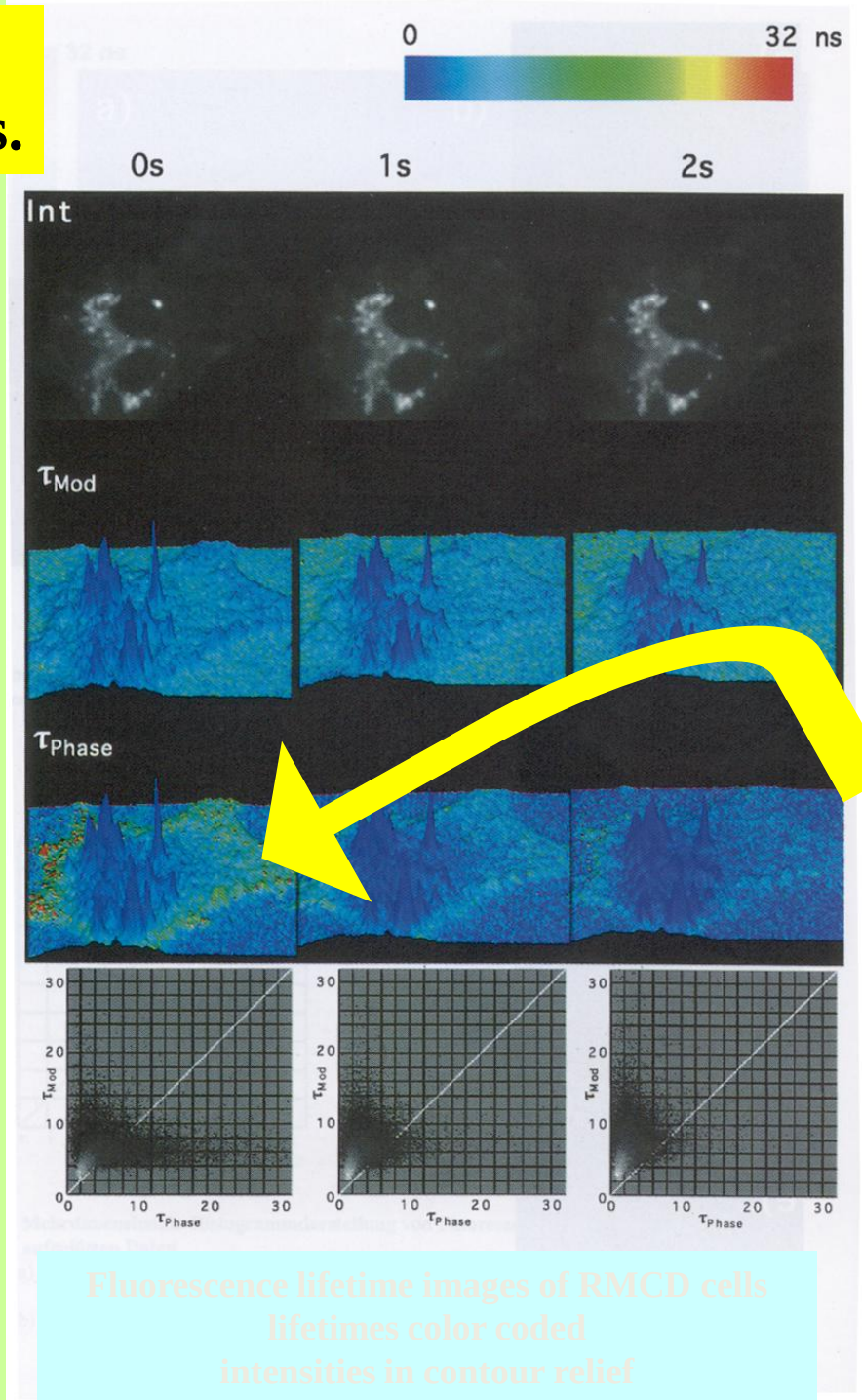
Directly excited species



Enough talking! Now let's see some real measurements!

Medical imaging tumor diagnostics.

Endoscopy & Microscope



Fluorescence lifetime images of RMCD cells
lifetimes color coded
intensities in contour relief

Time-resolved images showing the presence of the monomer form of porphyrin, and the photolysis of the monomer form of PPIX.

Monomeric PPIX 20 ns Is photolabile. Used for phototherapy.

Photodiagnostics and phototherapy

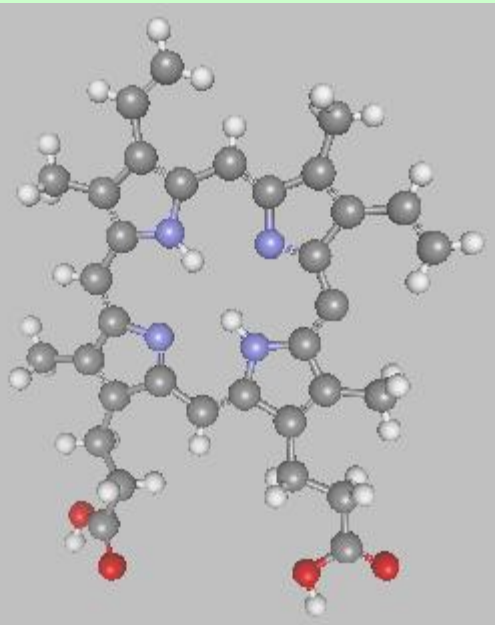
ALA

8 x 

δ-aminolevulinic-acid

Normal cellular

heme synthesis



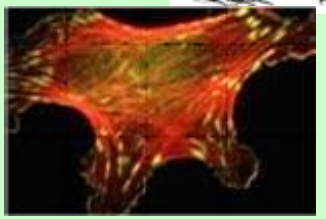
PpIX-protoporphyrin IX

Fluorescence

or

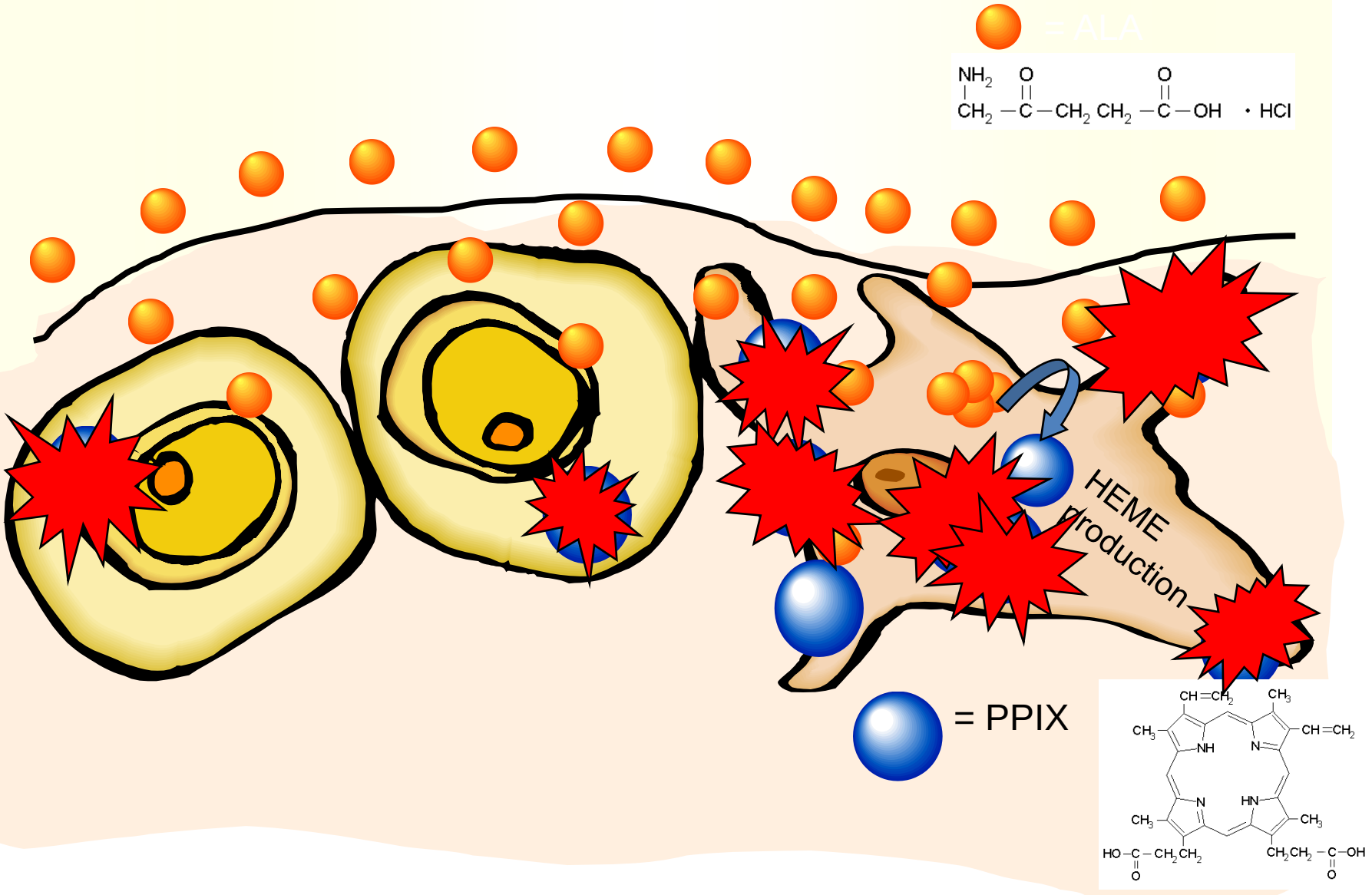
$^1O_2$

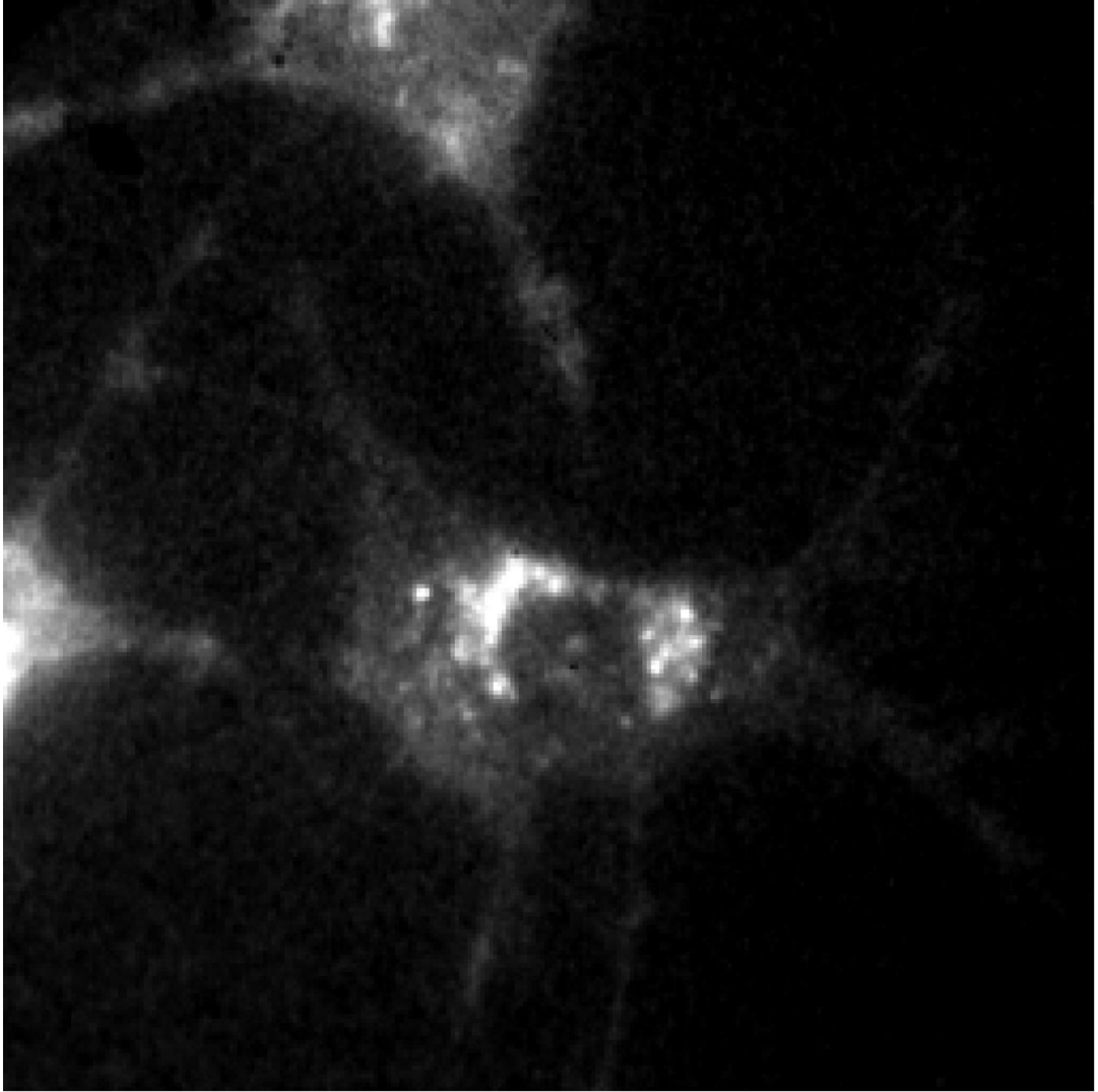
hν

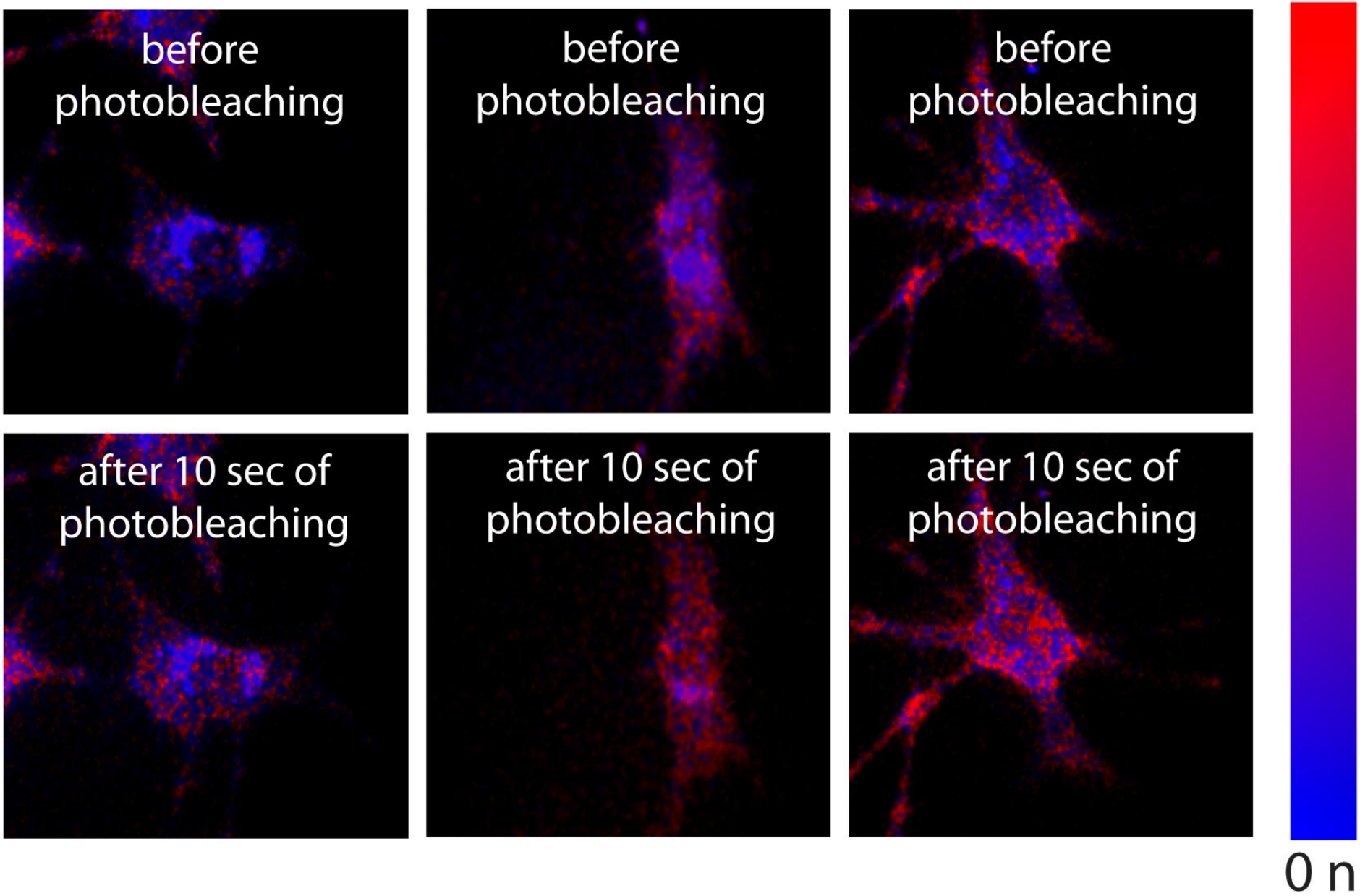


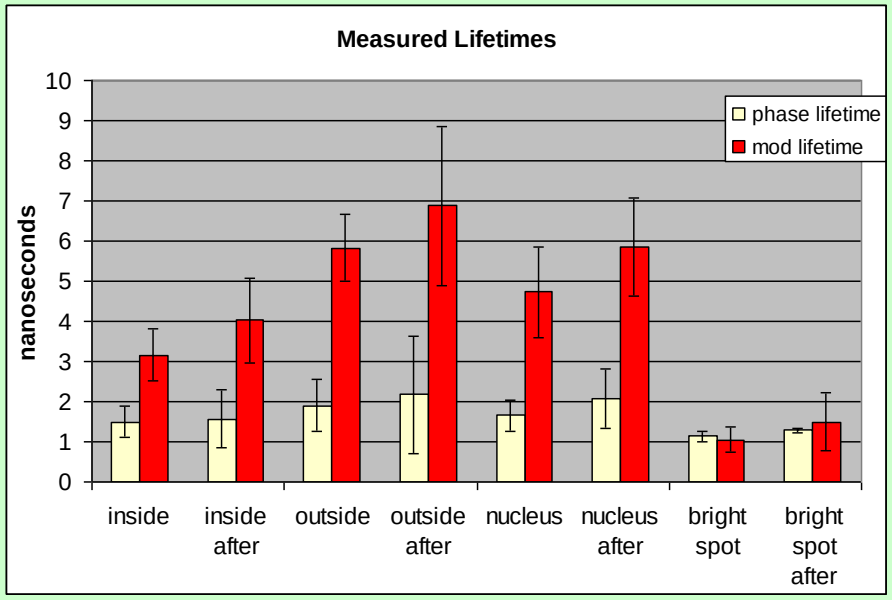
The monomer of PpIX forms ROS and is used for phototherapy

PDD and PDT using ALA

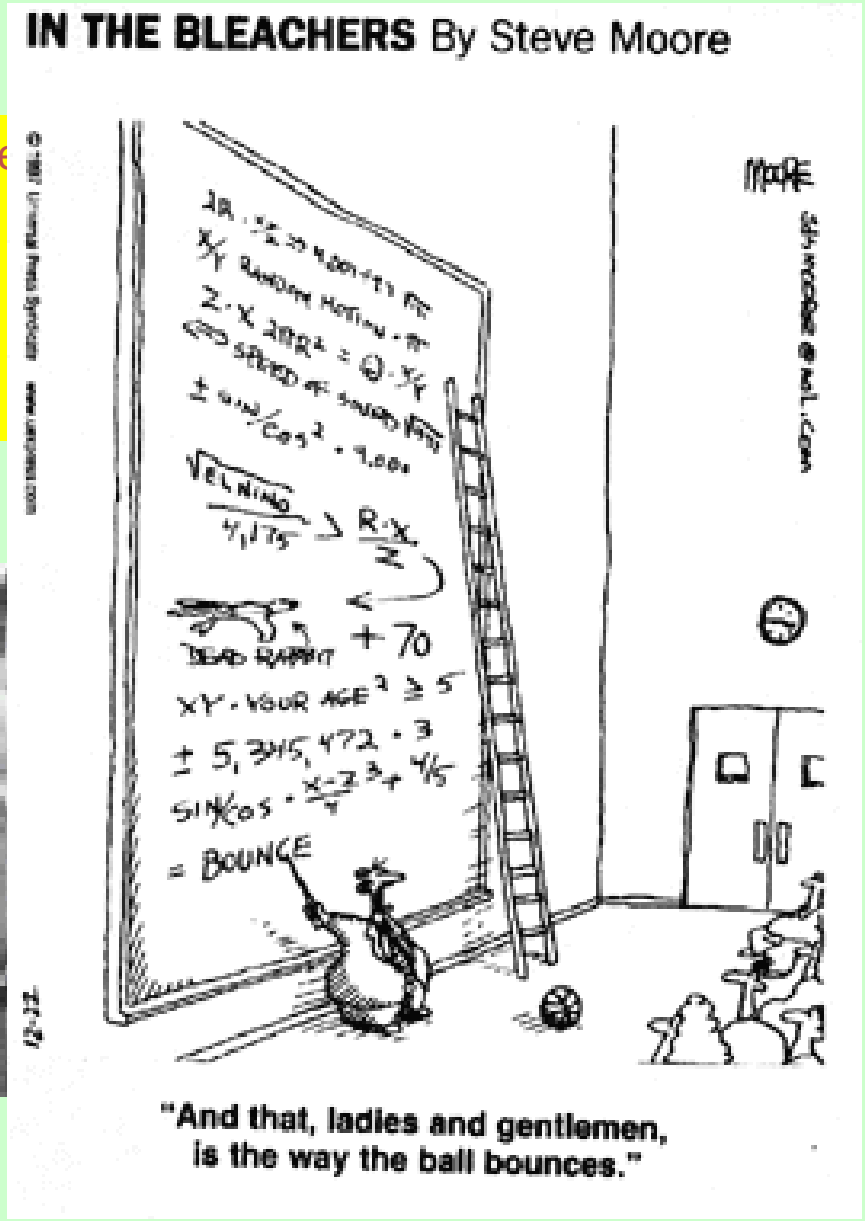


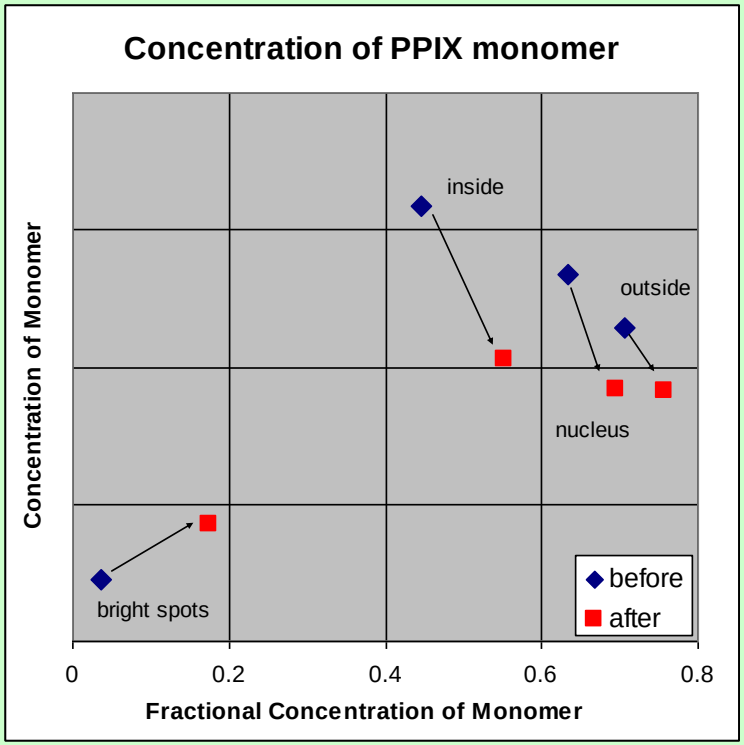



before photobleaching
before photobleaching
before photobleaching
after 10 sec of photobleaching
after 10 sec of photobleaching
after 10 sec of photobleaching
0 ns



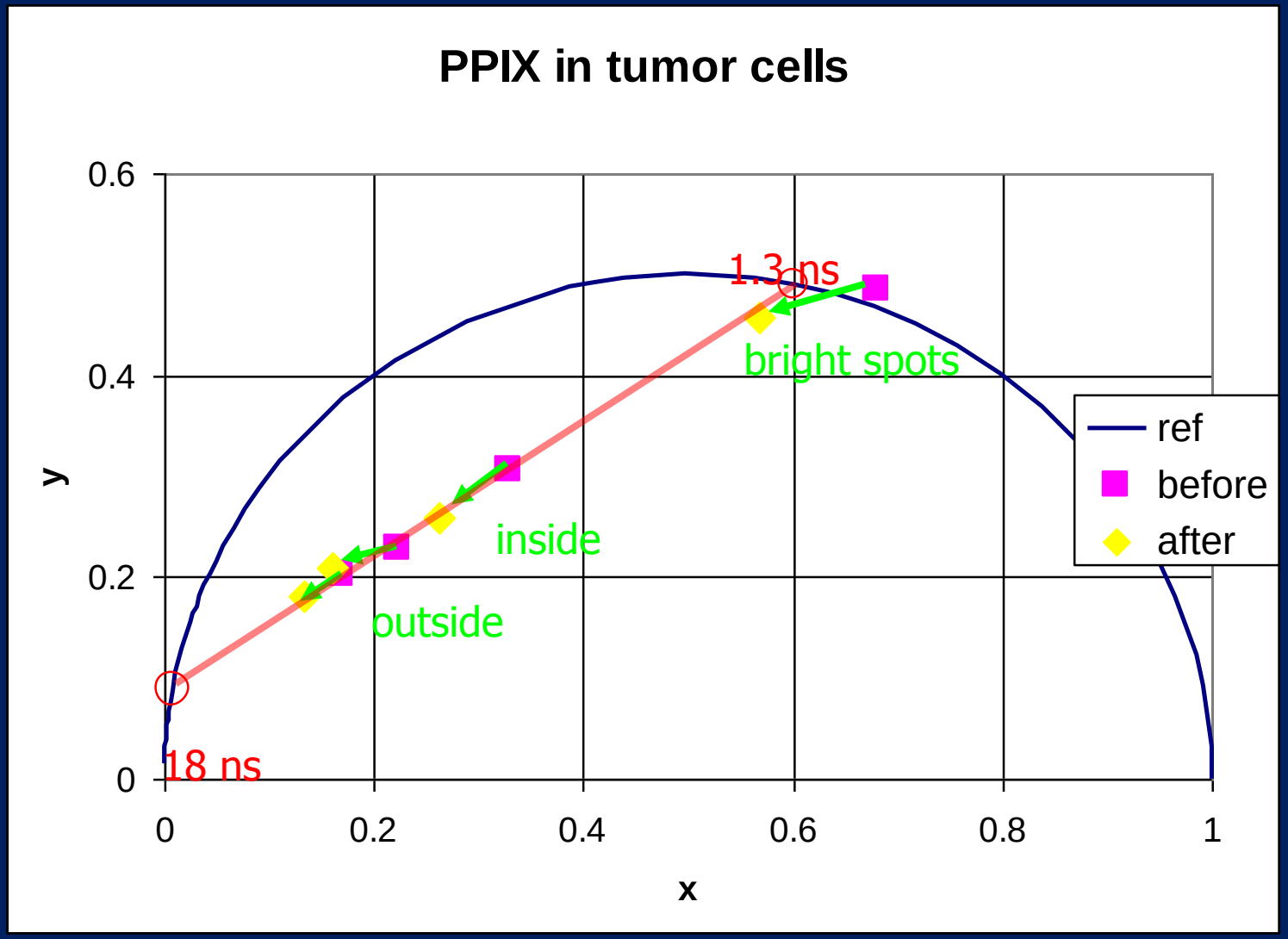
But sometimes that complicates things





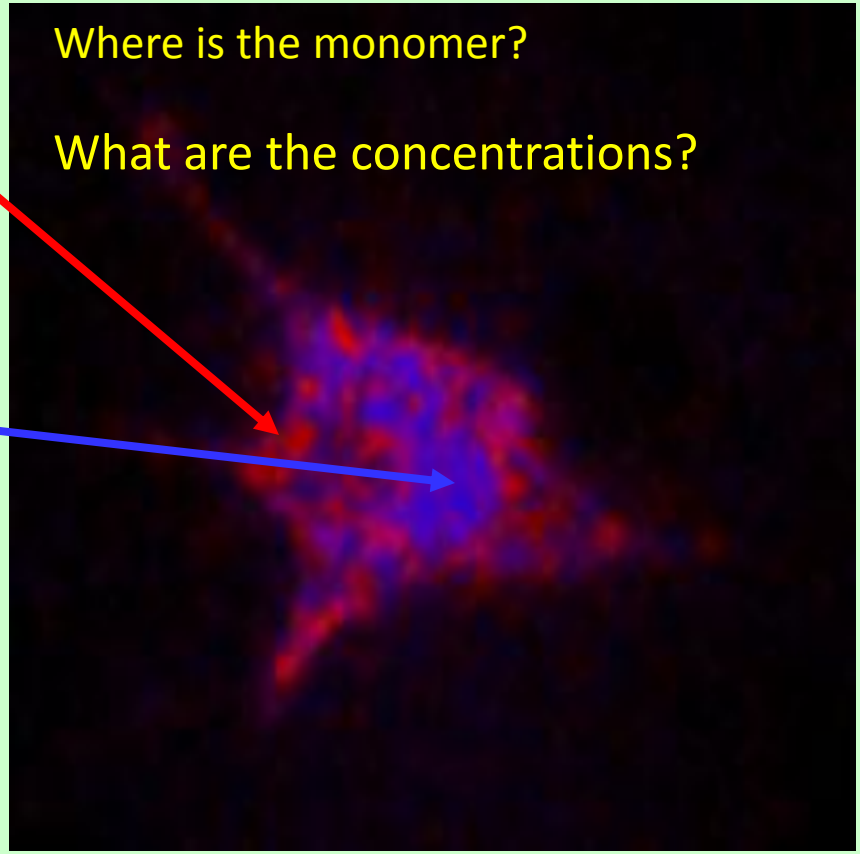
Polar Plot analysis

Fluorescence intensity and dynamic response change upon illumination



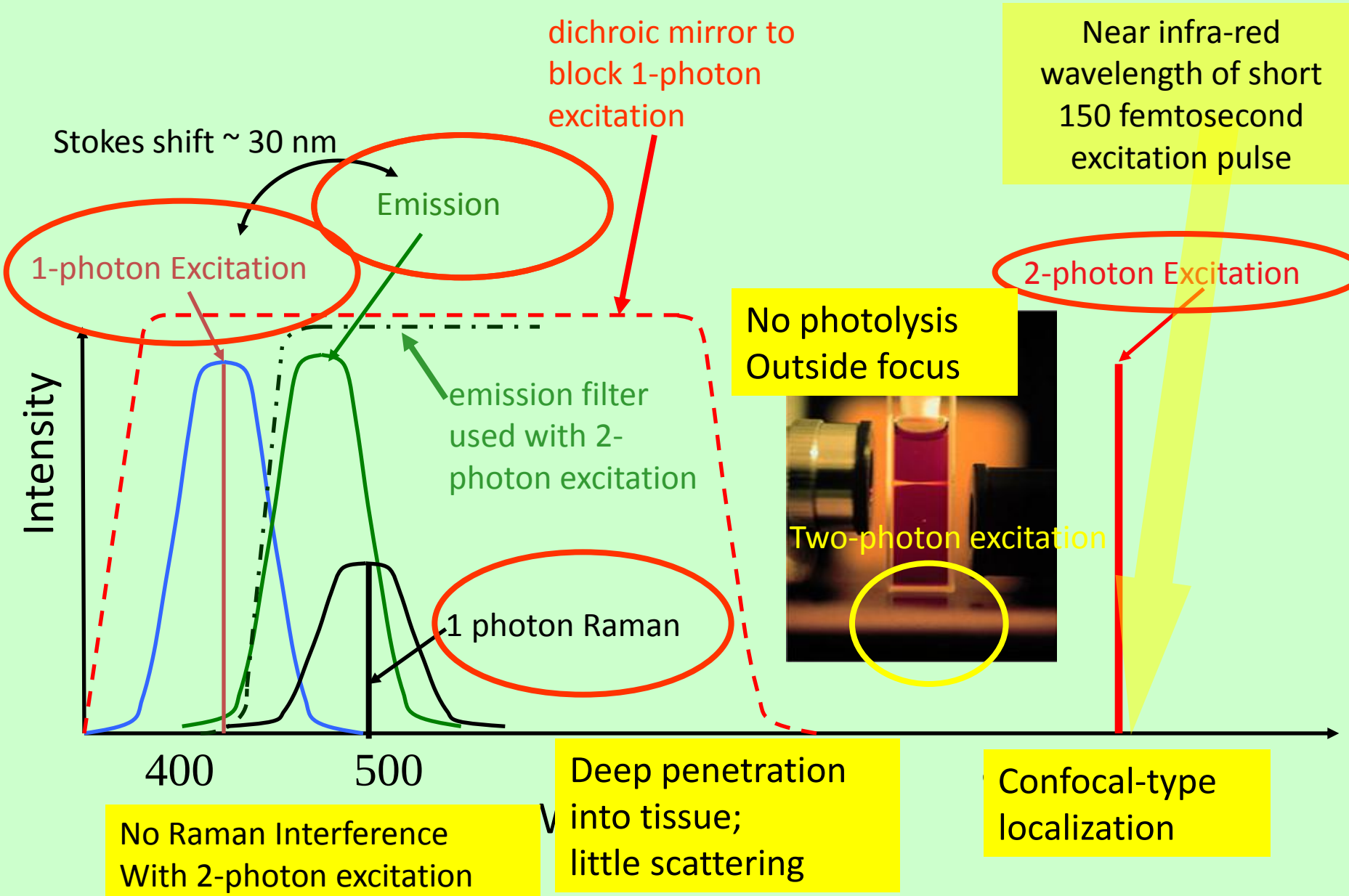
Protoporphyrin IX

	Lifetime	Location
Monomer	17-18 ns	
Dimer	~2 ns	
Higher Multimers	<2 ns	



Monomers and Multimers ≈ same spectra

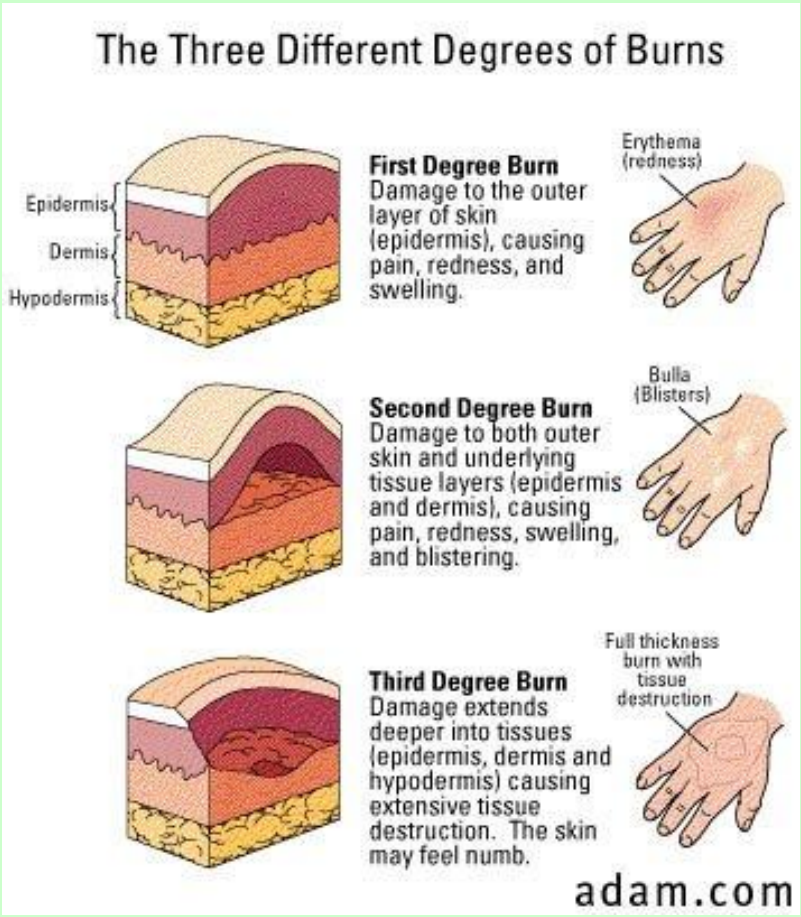
Two photon excitation: Separation of Excitation and Emission



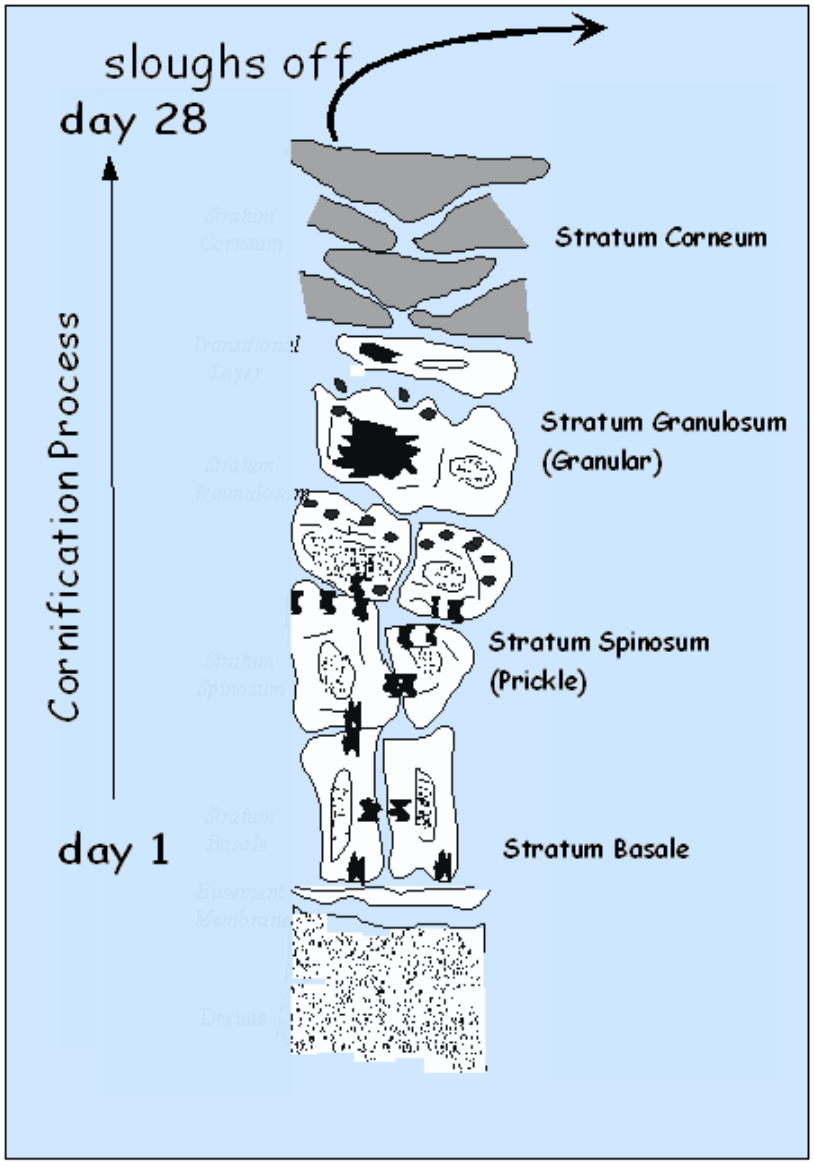
What do photons have to do with our everyday life?

A lot!

Sunbathing can be (is) dangerous



Fundamental Questions on Sunscreen Behavior in the Skin



Dr. Kerry M. Hanson

Do sunscreens sensitize ROS in the skin?

Where do sunscreens localize?

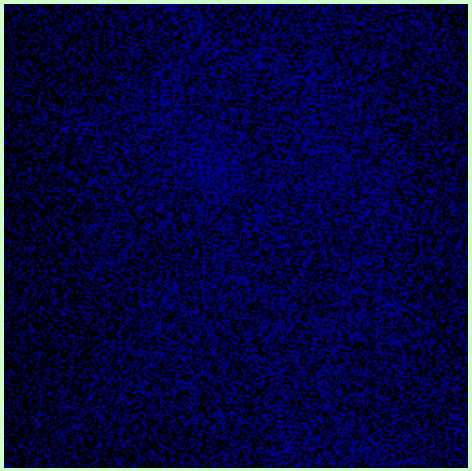
- Do they penetrate the cell or nuclear membrane?
- Do they penetrate the Stratum Corneum Barrier?

Does sunscreen *in vivo* photochemistry have photobiological influence?

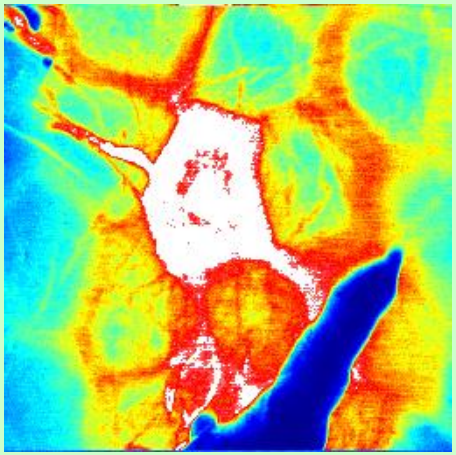
- Pyrimidine Dimerization
- Immunomodulation
- Photoaging

2-hν excitation FLI

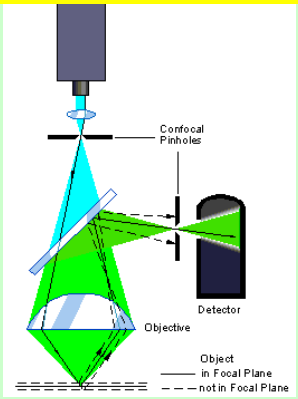
Before UV-B



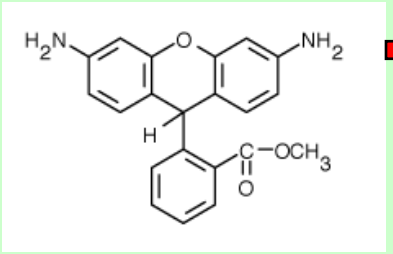
After UV-B



UV-B-induced Oxidation of the Lipid Matrix of the Stratum Corneum



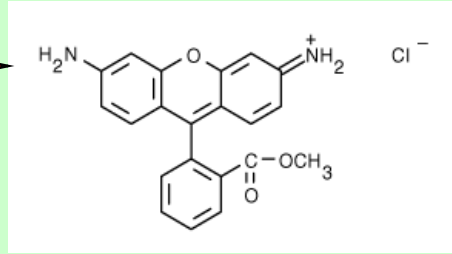
Dihydrorhodamine 123



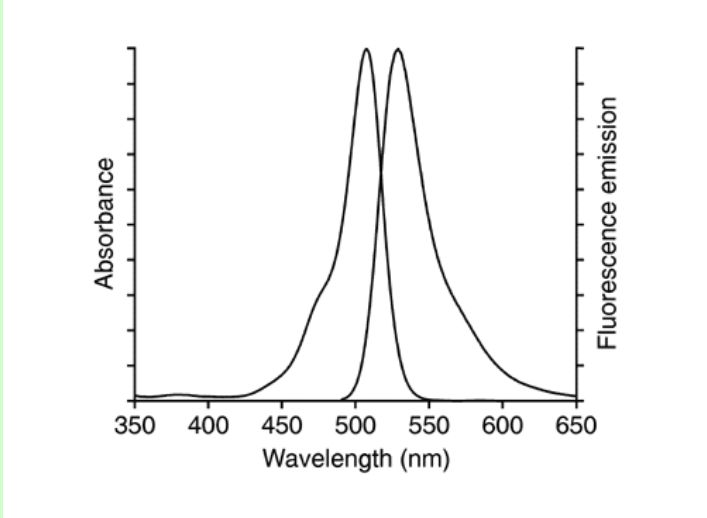
Before and After 1 Sub-Erythemal UV-B Dose

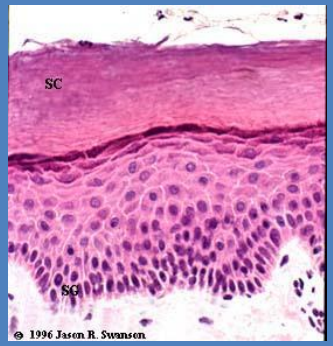
Reactive oxygen species

Rhodamine 123



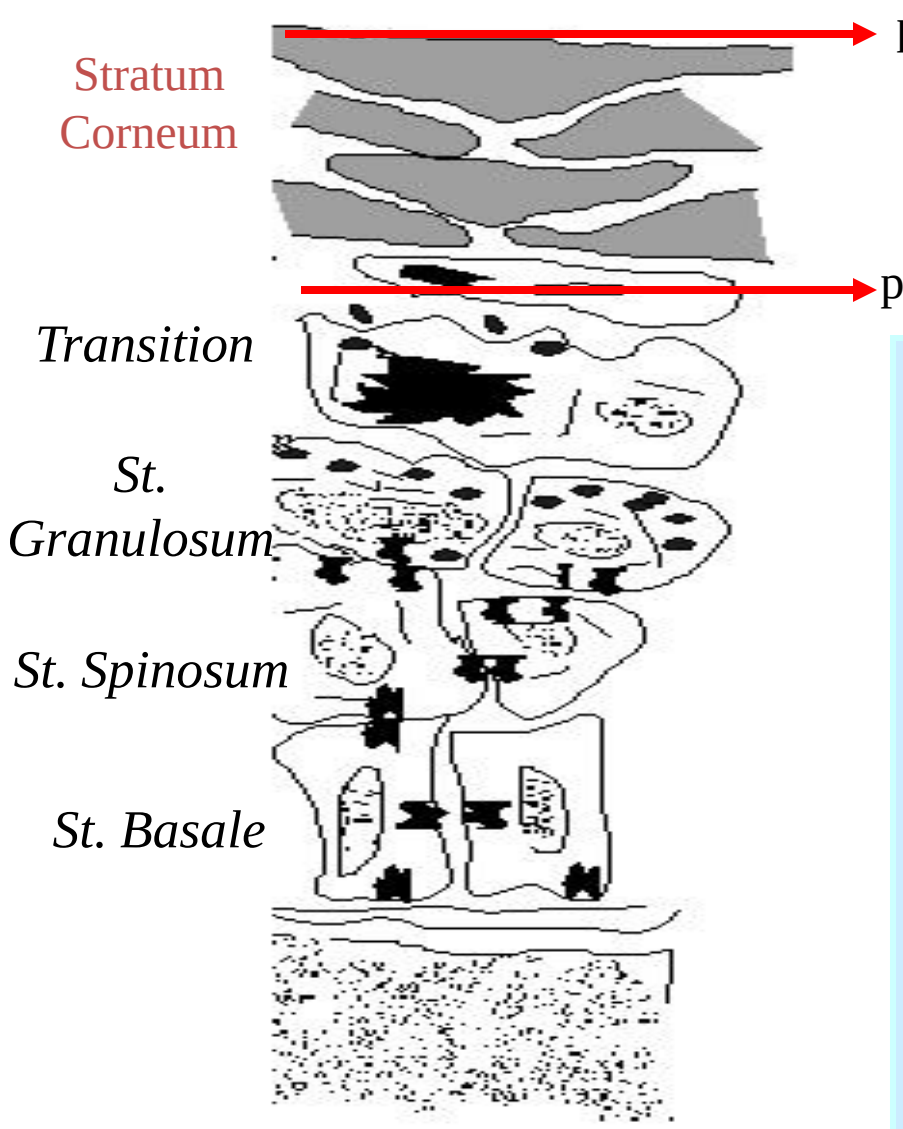
Use FLI to determine the concentration of the ROS through the indicator fluorescence. Lifetimes are concentration independent.





Human Epidermal *Stratum Corneum*

Stratum Corneum pH Gradient



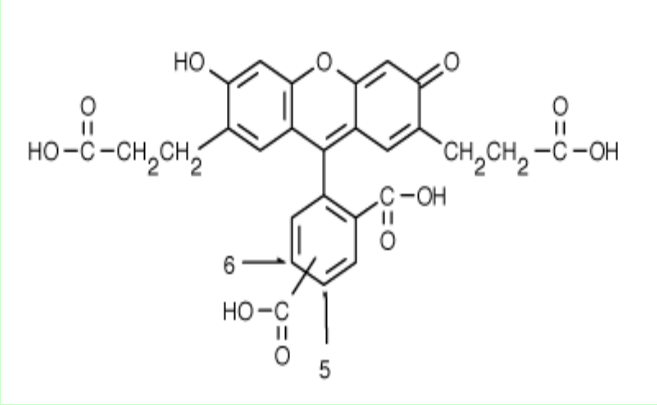
ΔH^+ 100-1000 times in 20μm!

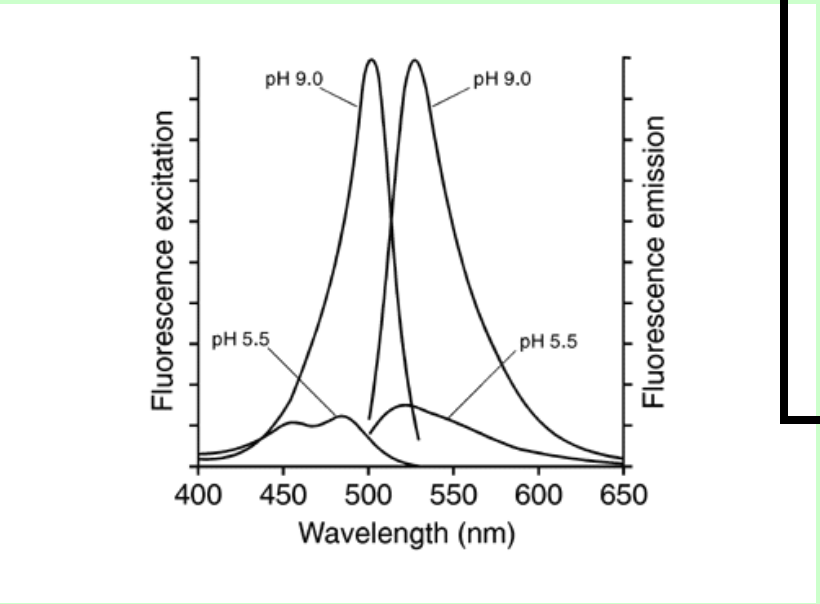
- Surface of skin is acidic
- pH Gradient affects
  - barrier function
  - skin disease (ichthyosis, dermatitis-$$$ health care)
- Only Tape-Stripping/Flat-Electrode experiments done to date = no resolution, cannot understand origin of gradient

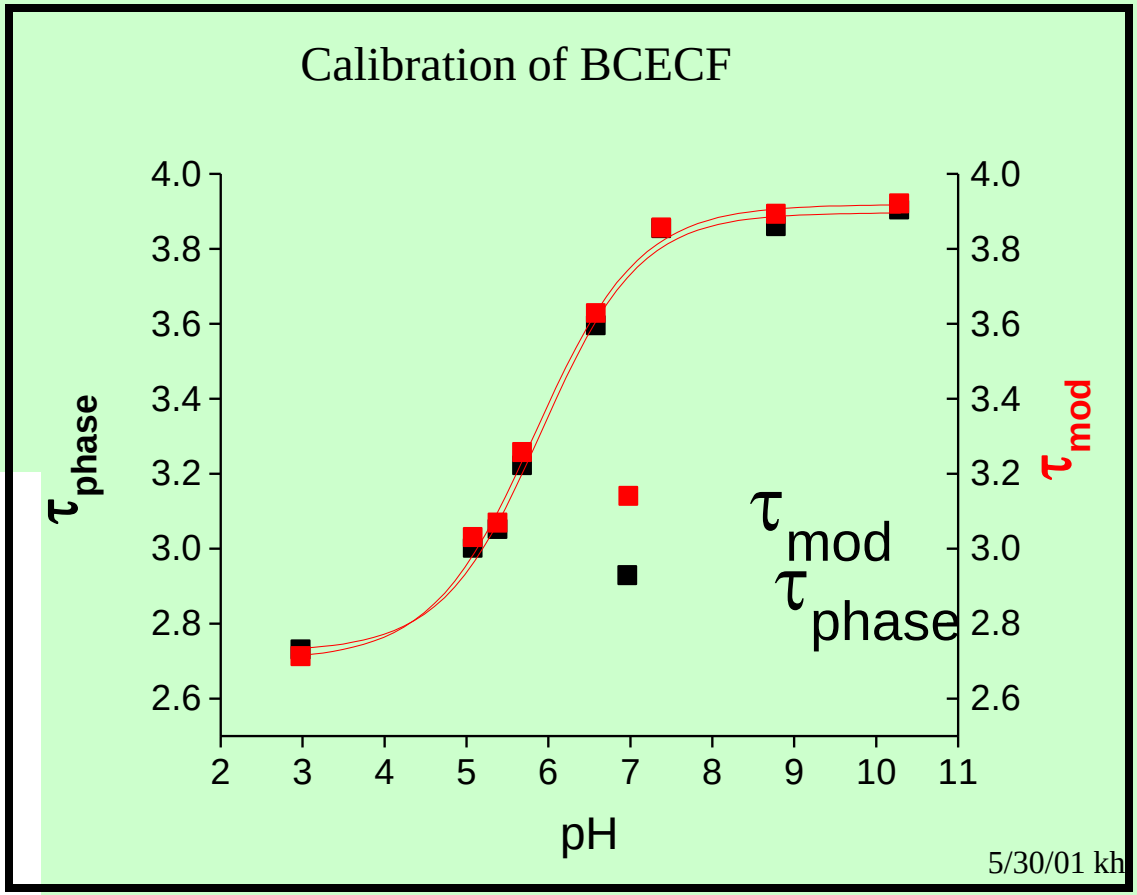
Hanson, Barry, Behne *et al.*

Two-Photon FLIM to Determine pH in the Stratum Corneum

BCECF: Lifetime-sensitive pH indicator







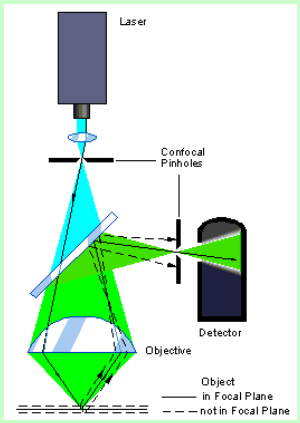
Hanson, Barry, Behne *et al.*

Lipid Matrix Has A Lower pH than Corneocytes & Viable Cells

Intensity

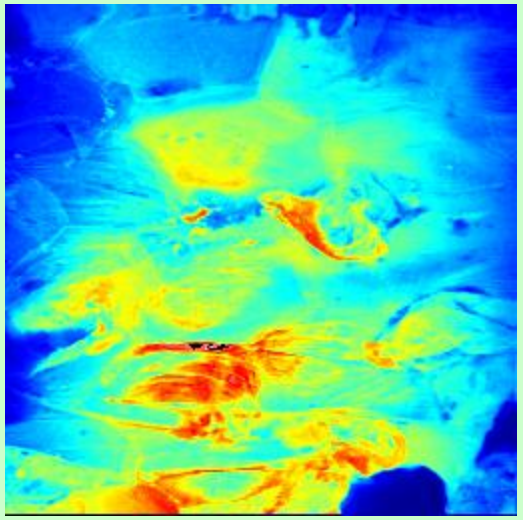
τ_{mod}

z=0 μm
Skin
Surface



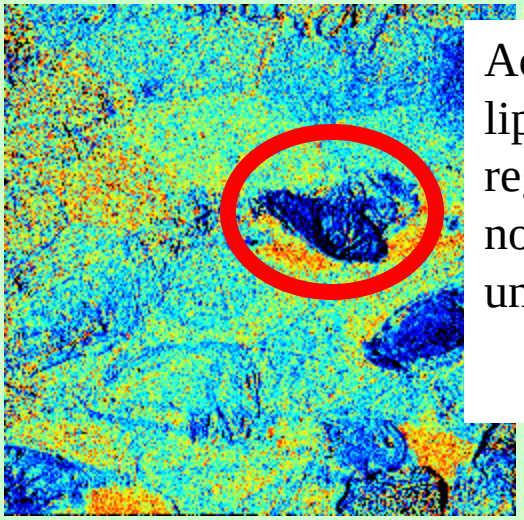
1500

0



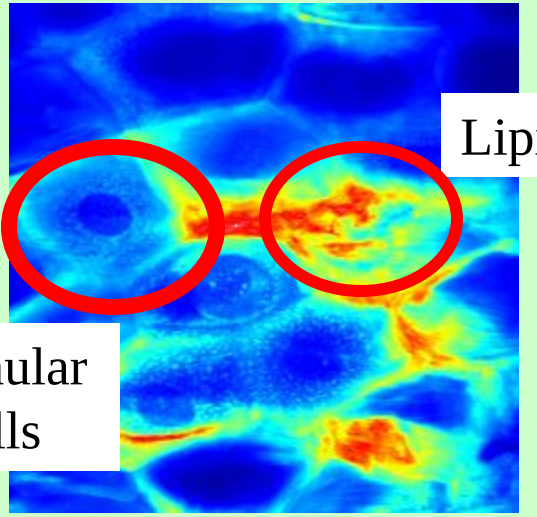
4 ns

2 ns



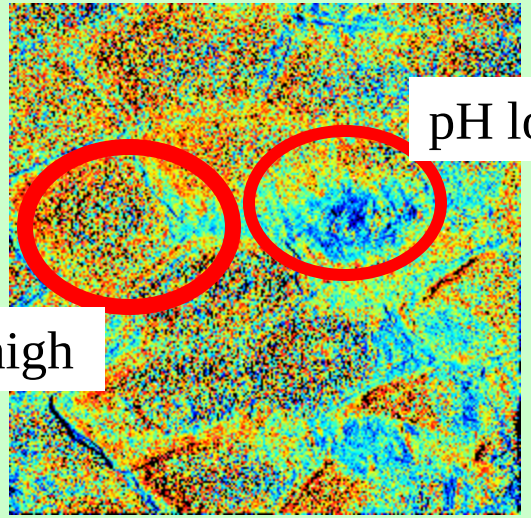
Acidic lipid regions, not uniform

z=15 μm
Corneum-
Granulosum
Junction



Lipid

Granular cells



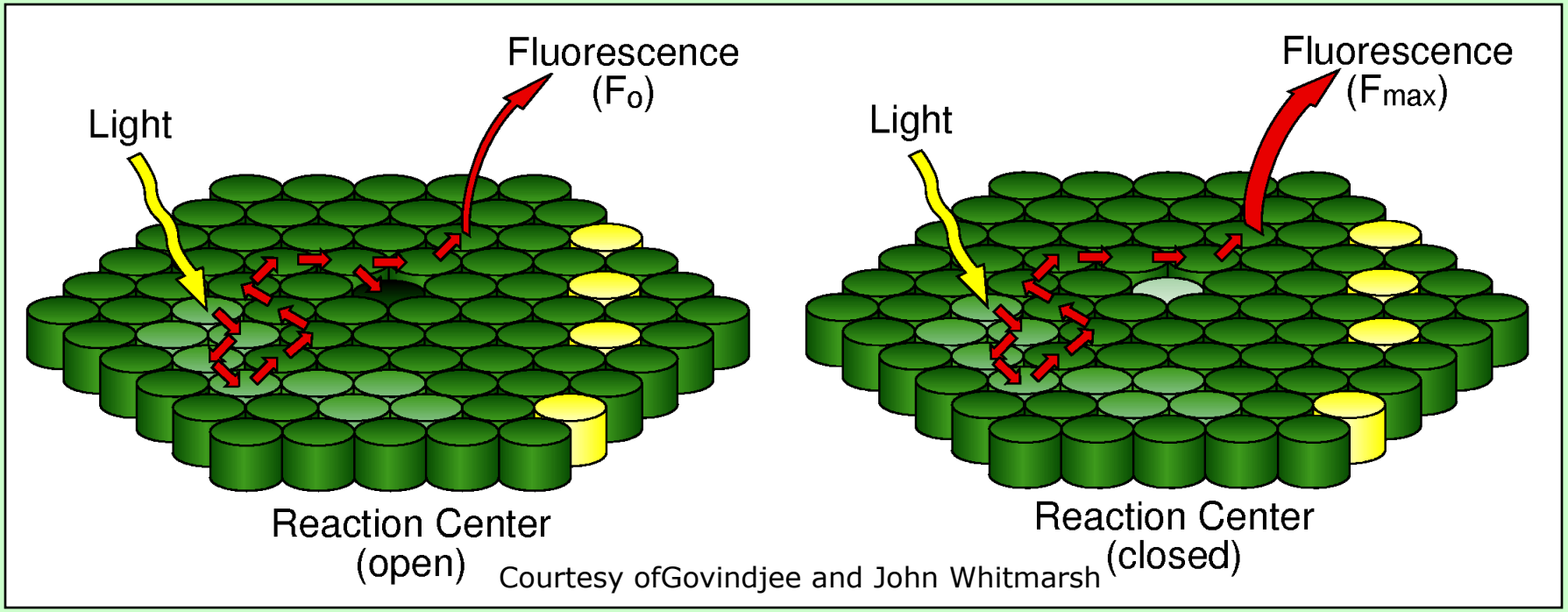
pH low

pH high

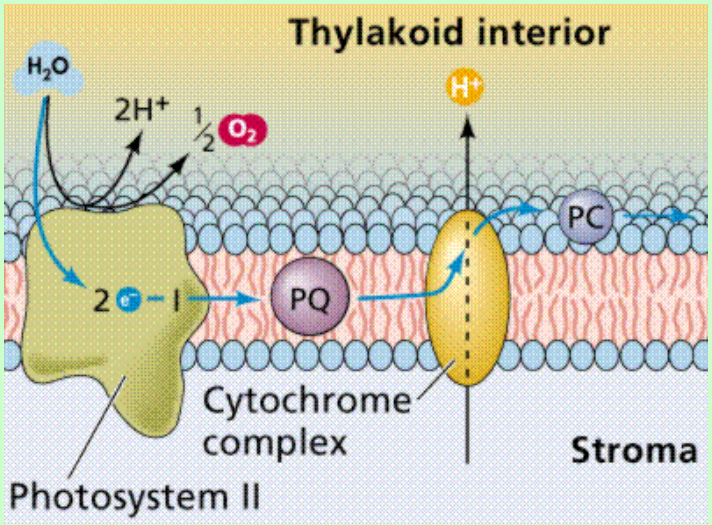
Hanson, Barry, Behne *et al.*

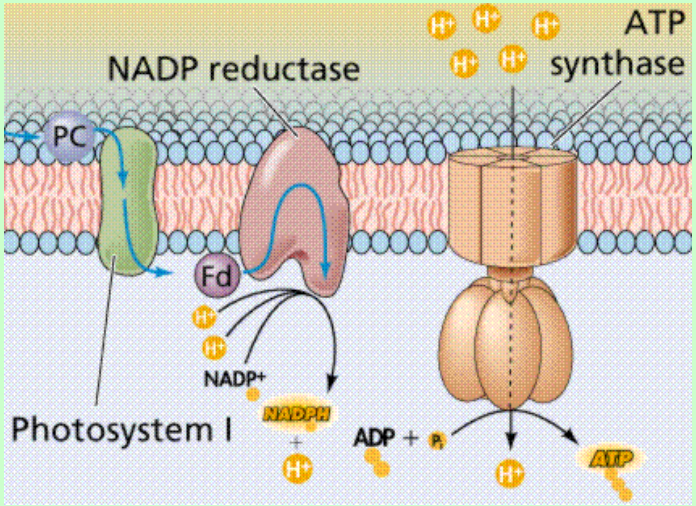
Using FLI for measuring Photosynthesis mechanisms and As a tool for plant health

Fluorescence monitors the photochemistry at the reaction center

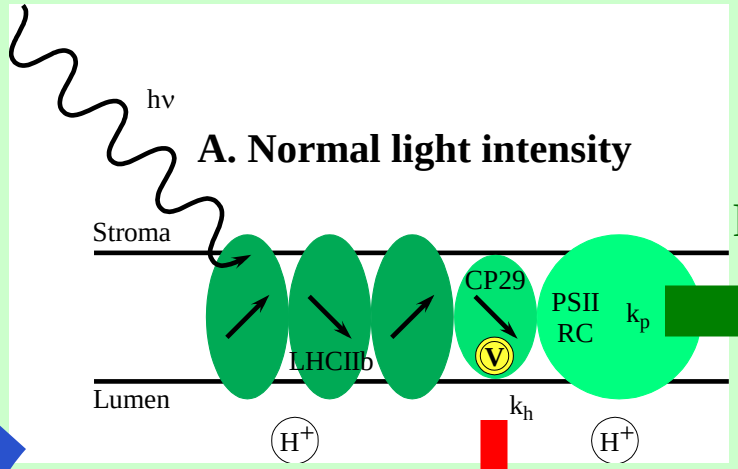


Courtesy ofGovindjee and John Whitmarsh





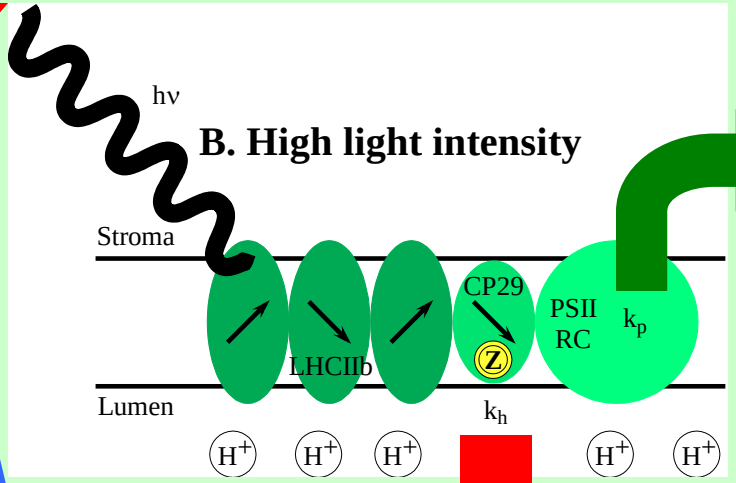
Real-Time Fluorescence Lifetime-Resolved Images of individual cells of Wild Type and NPQ mutants of *Chlamydomonas reinhardtii*



Photochemistry

violoxanthin

heat

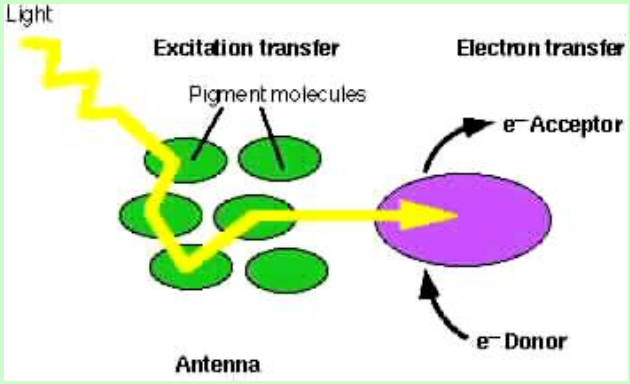


Photochemistry

zeaxanthin

Deepoxidase

heat

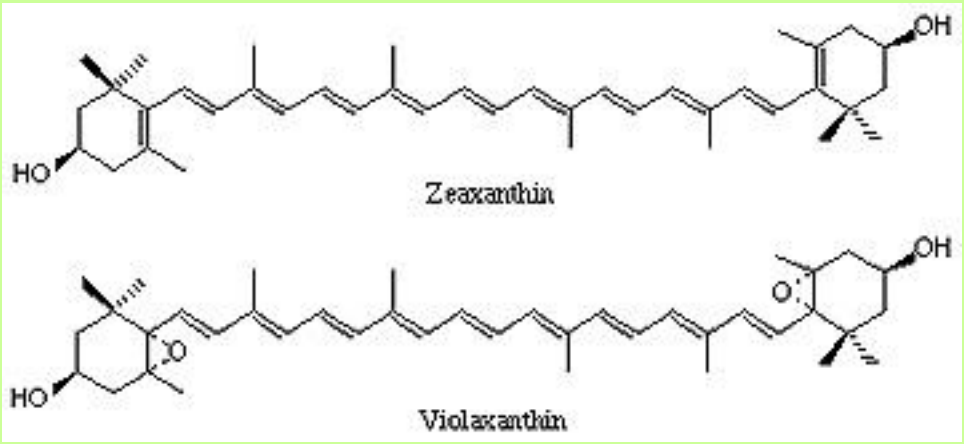


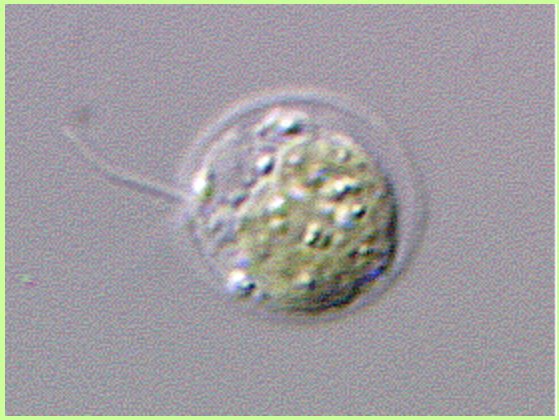
The mutants (NPQ1 and NPQ2) used in this work, accumulate violoxanthin and zeaxanthin, respectively. Fluorescence transient studies showed strong quenching for NPQ2 in comparison to the WT and NPQ1.

Fast-FLI for studying the role of the Xanthophyll cycle in the non-photochemical quenching process in single cells.

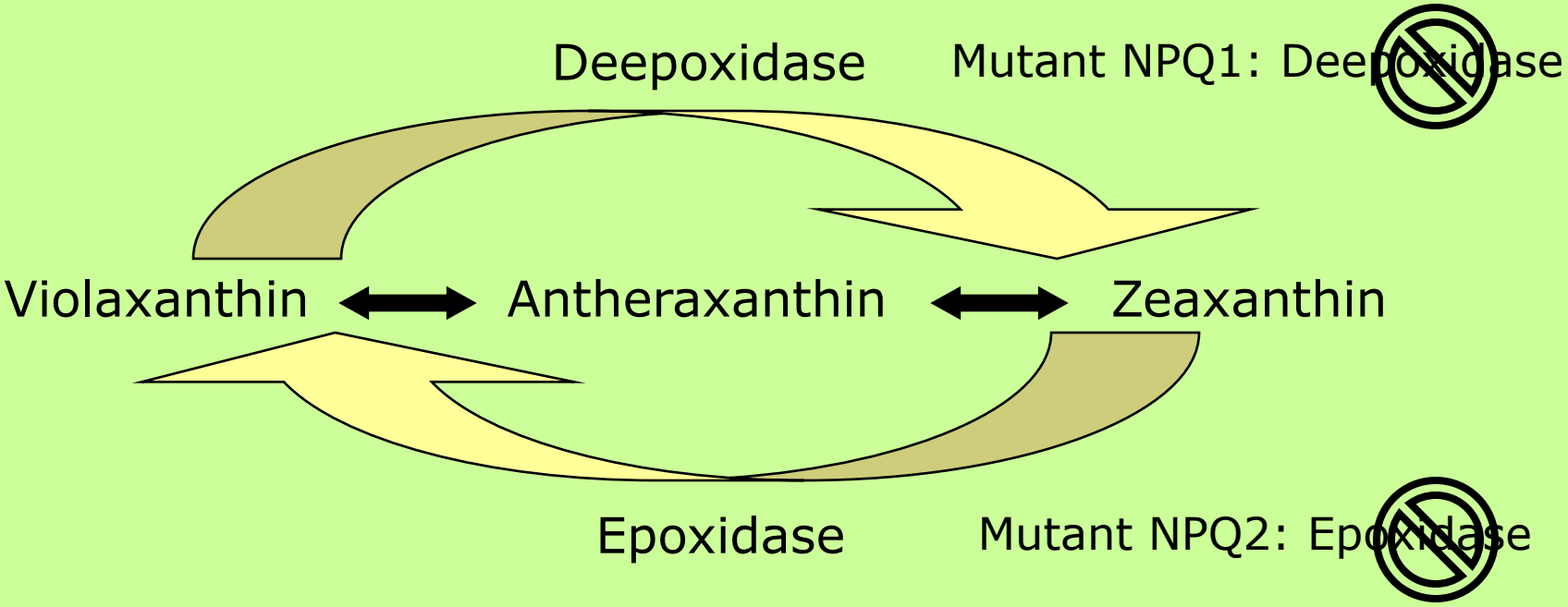
NPQ mutants of the green alga *Chlamydomonas reinhardtii*

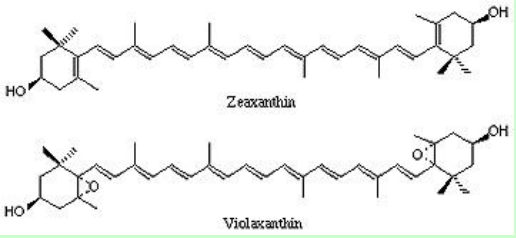
Mutants from Krishna K. Niyogi





The Xanthophyll cycle



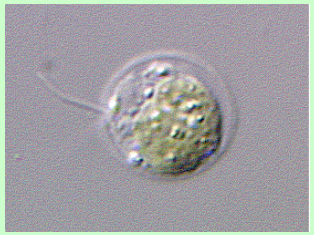


The Xanthophyll cycle

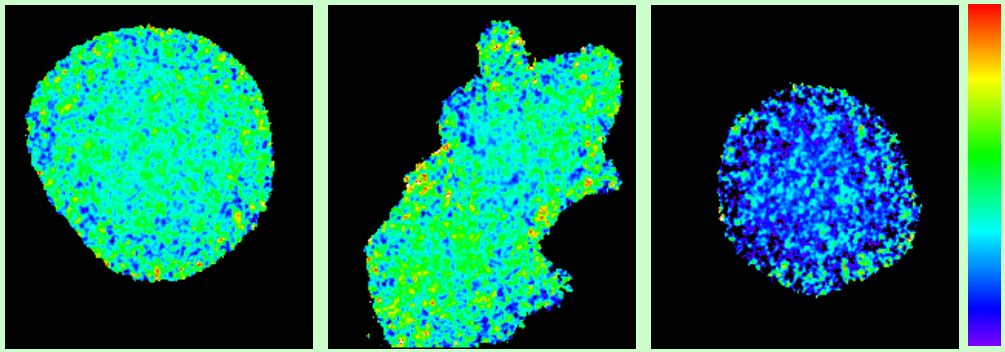
Deepoxidase

Violaxanthin ⟷ Antheraxanthin ⟷ Zeaxanthin

Epoxidase

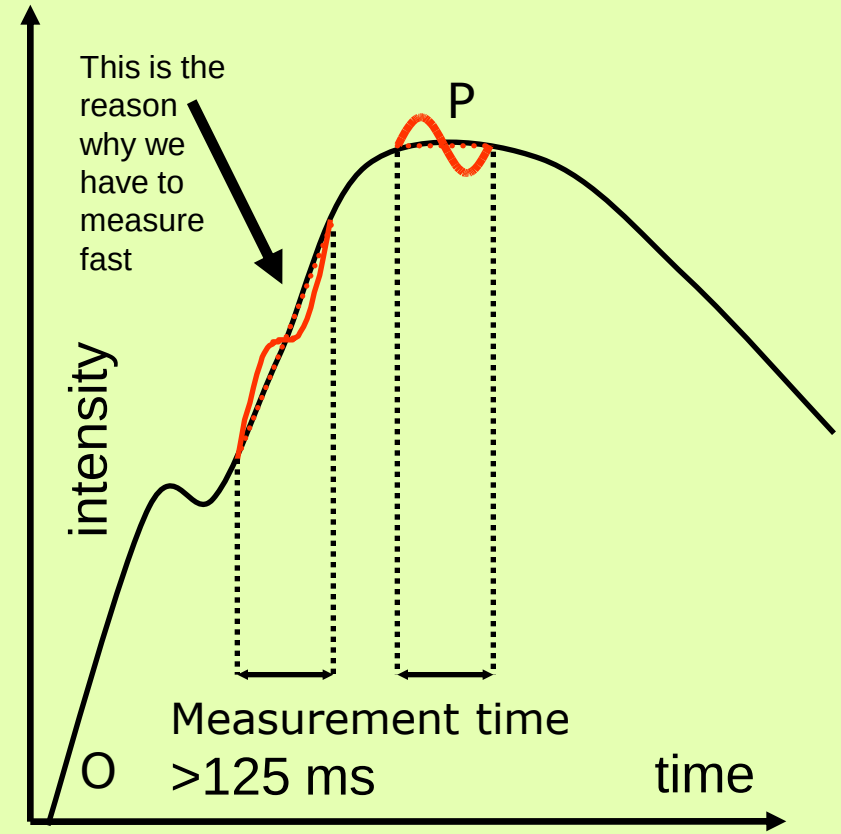


Single cells of *Chlamydomonas reinhardtii* wild-type (WT)
and non-photochemical quenching (NPQ) mutant

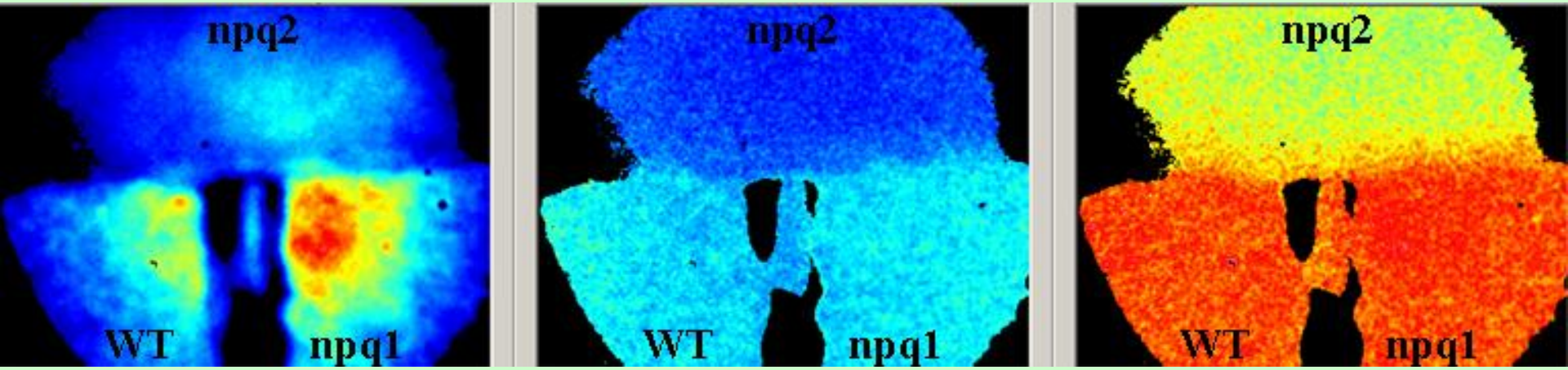


WT NPQ1 NPQ2

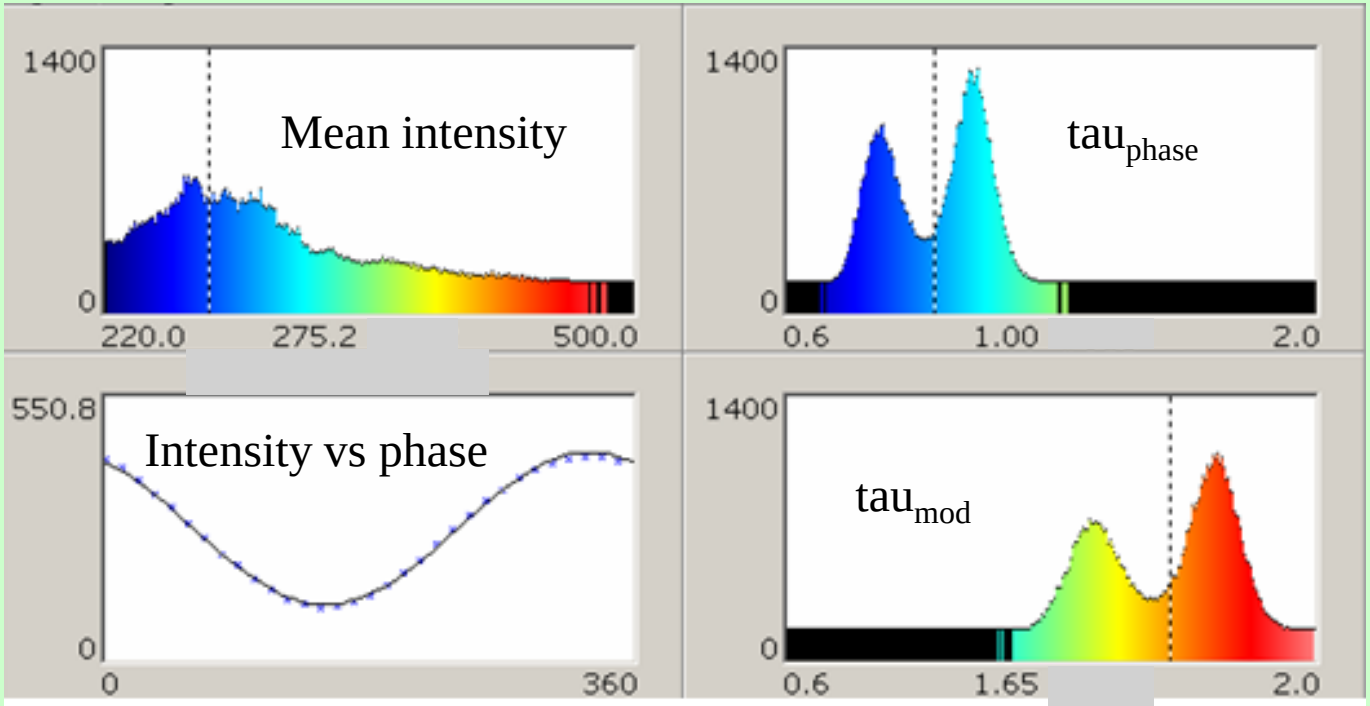
Deepoxidase Epoxidase



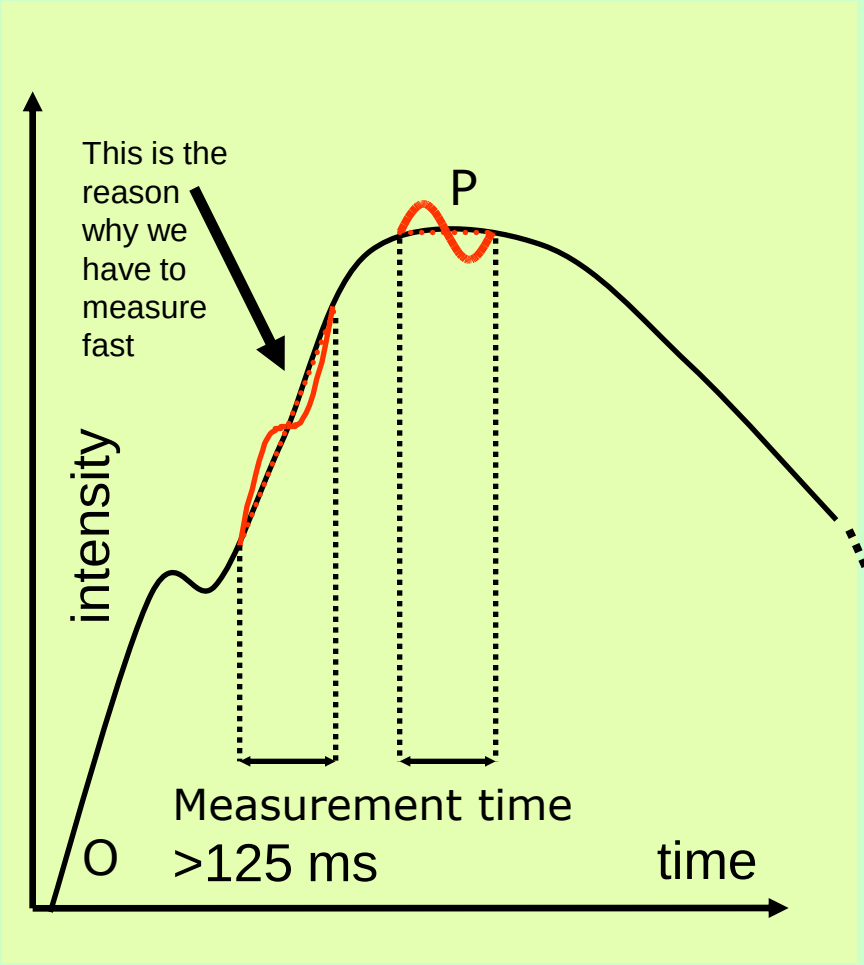
Mean Intensity Phase Lifetime Modulation Lifetime



0.6 ns 2.0 ns

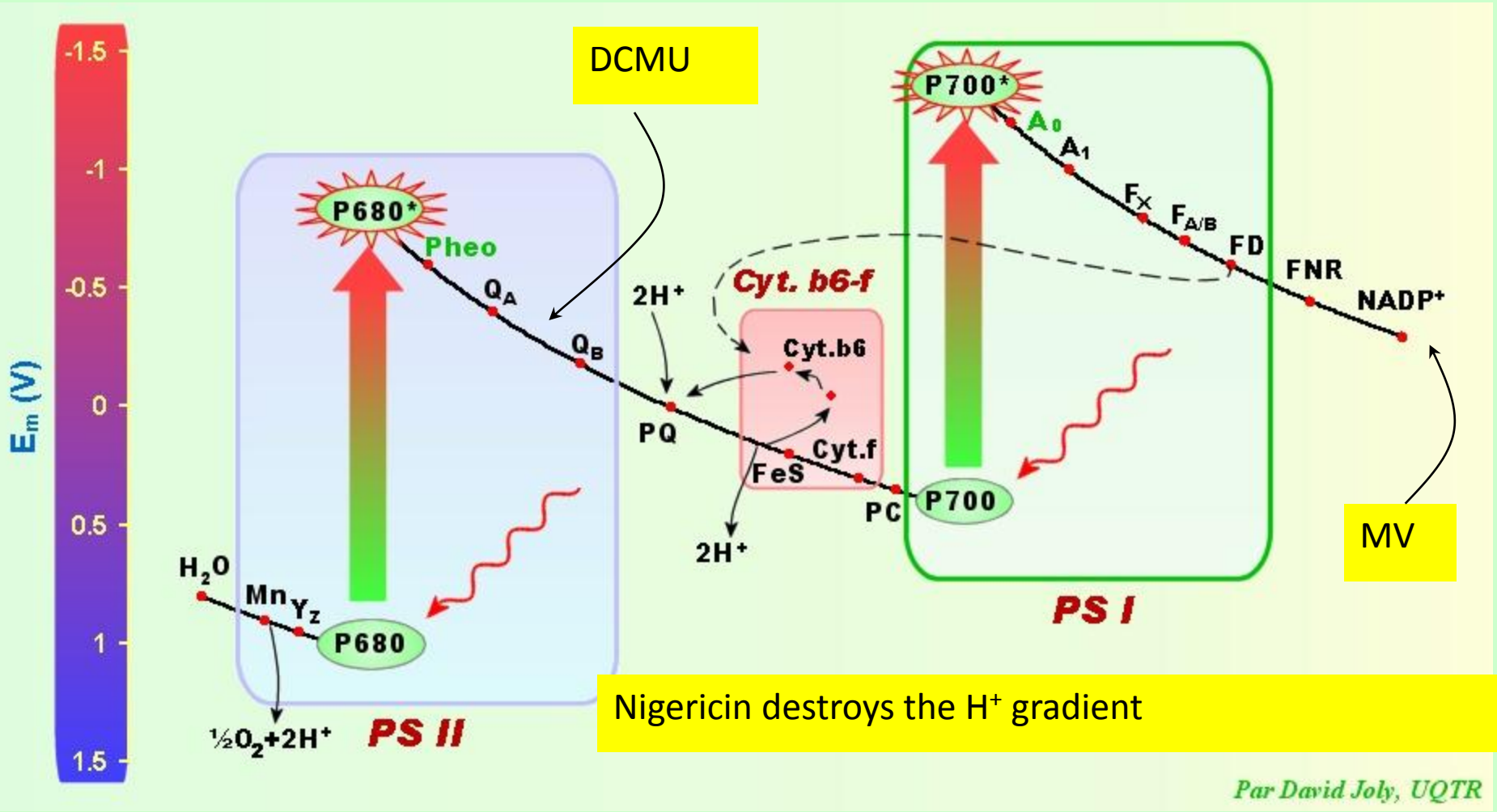


Kinetics



?

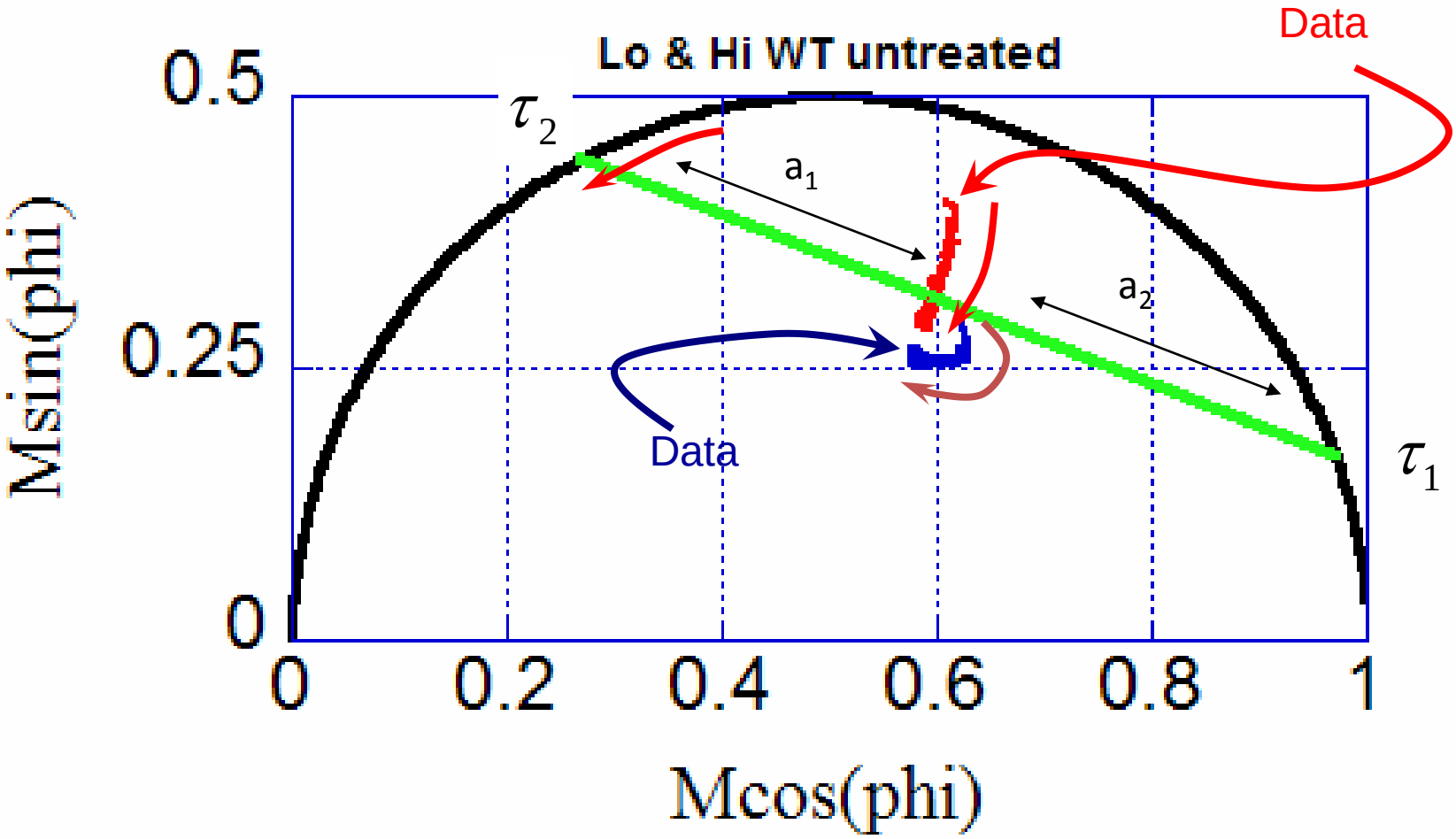
Long times ---250 seconds



Npq1 has ***no xanthophyll cycle*** and has no ***Zeaxanthin*** – cannot quench

Npq2 has ***no xanthophyll cycle*** but has ***Zeaxanthin*** – can quench

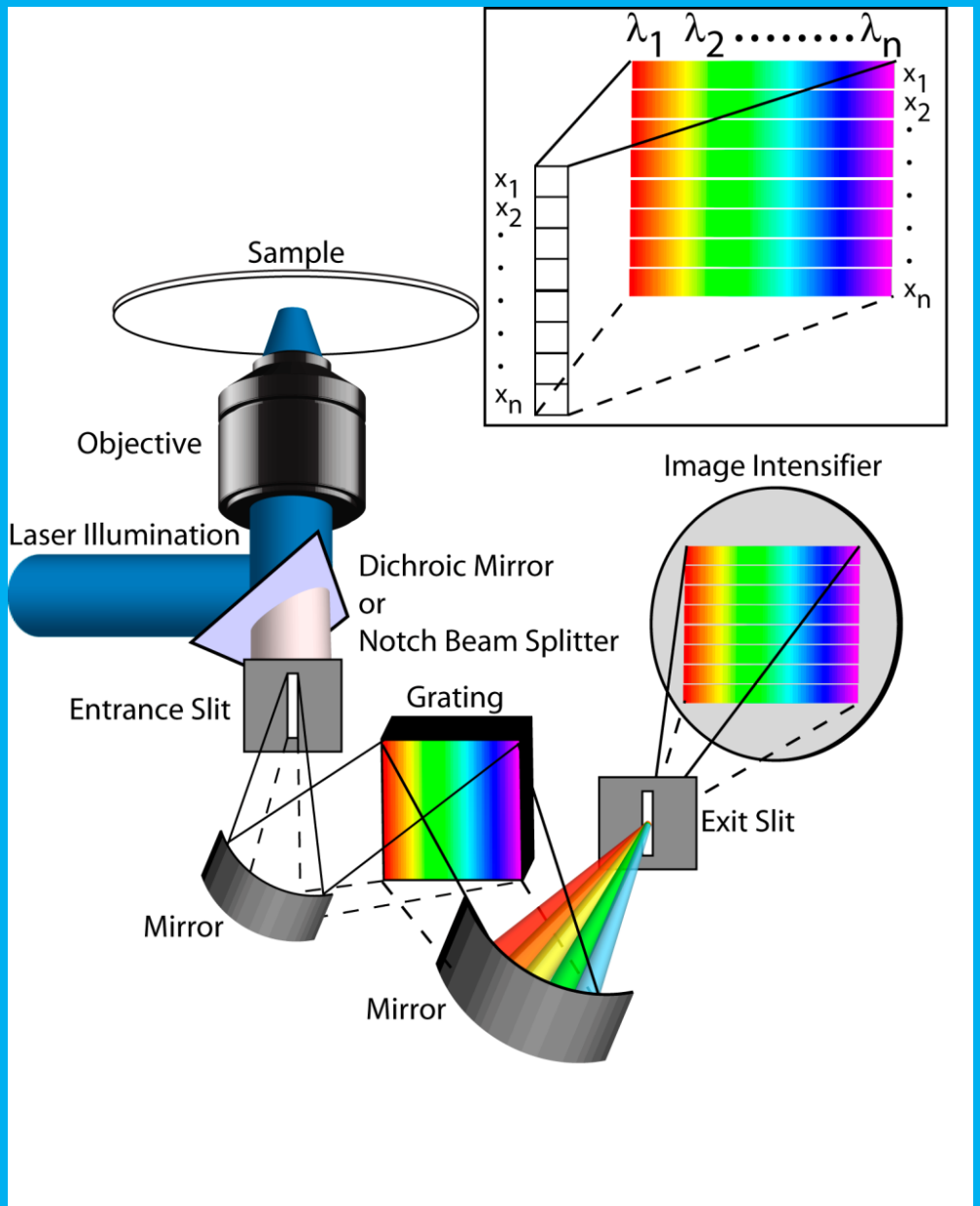
Lifetime lever



$$Signal = a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2}$$

Two lifetime pools of Chlorophyll Molecules

Spectral FLIM



2. two-lifetimes model, assuming spectrums are known:

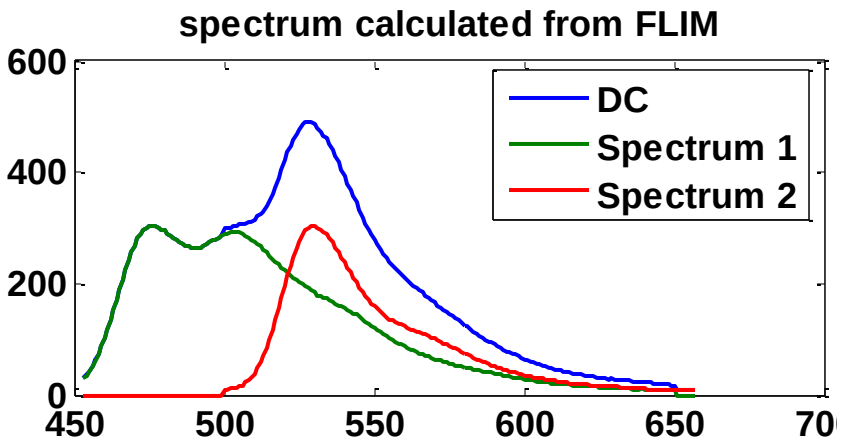
Should I weight the data??

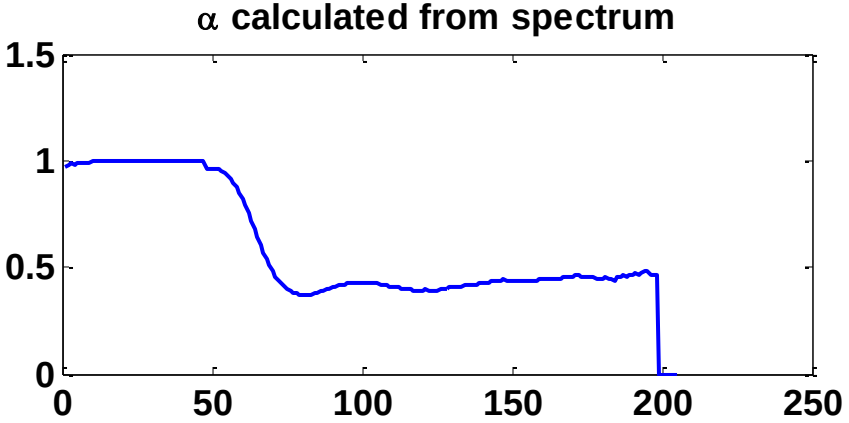
a) Fit the spectrum and fine α

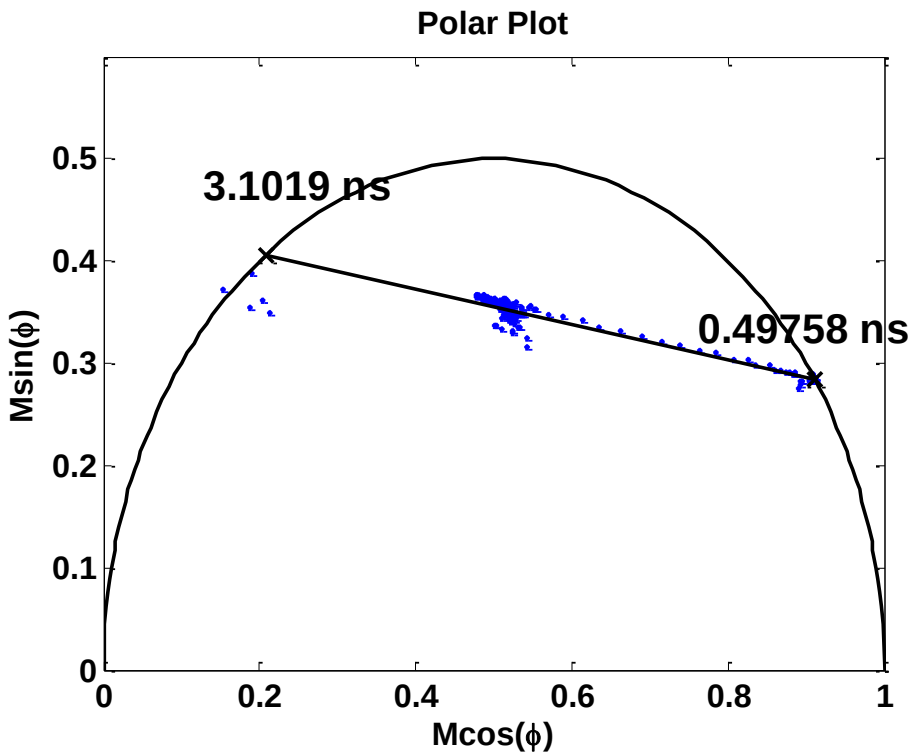
b) given α, use LSQF to find τ_1 and τ_2

$$x_{tot} = \alpha/(1+\omega^2\tau_1^2) + (1-\alpha)/(1+\omega^2\tau_2^2)$$

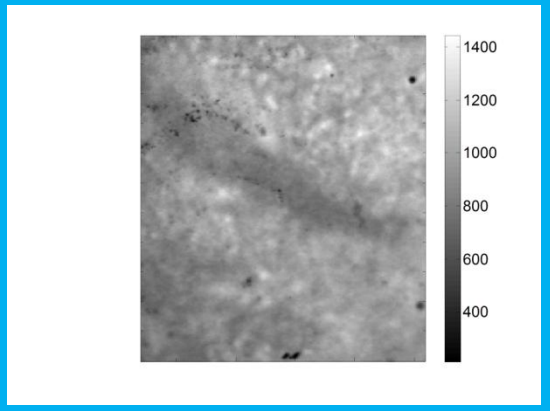
$$y_{tot} = \alpha\omega\tau_1/(1+\omega^2\tau_1^2) + (1-\alpha)\omega\tau_2/(1+\omega^2\tau_2^2)$$



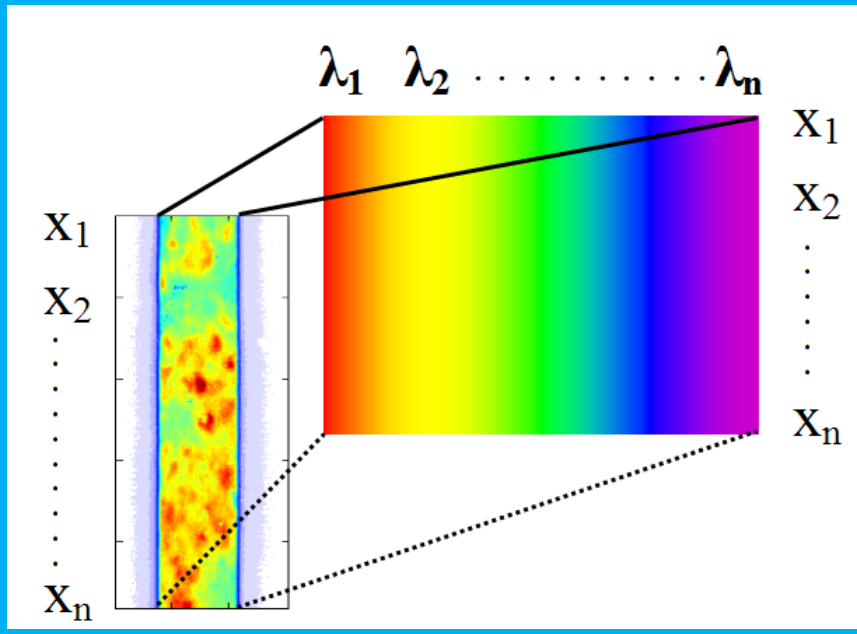




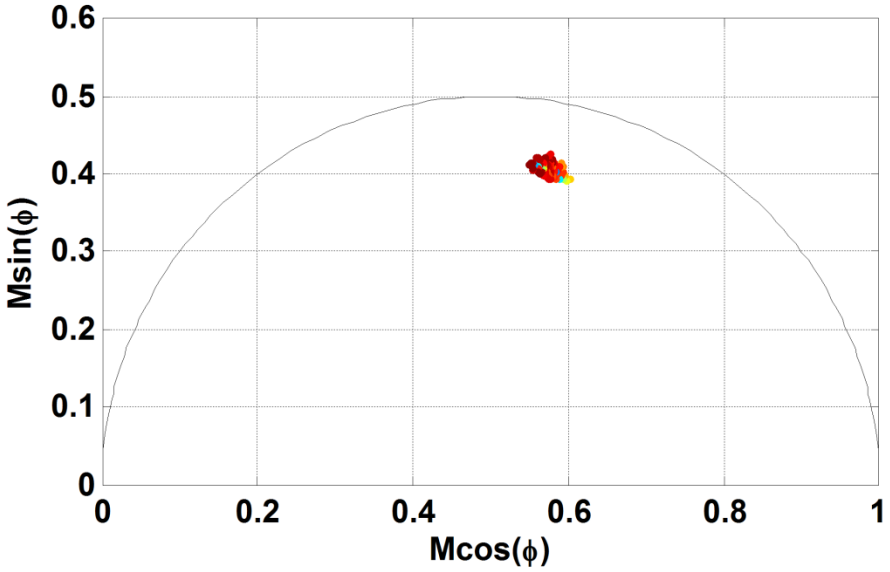
Original simulation→ 3ns & 0.5ns

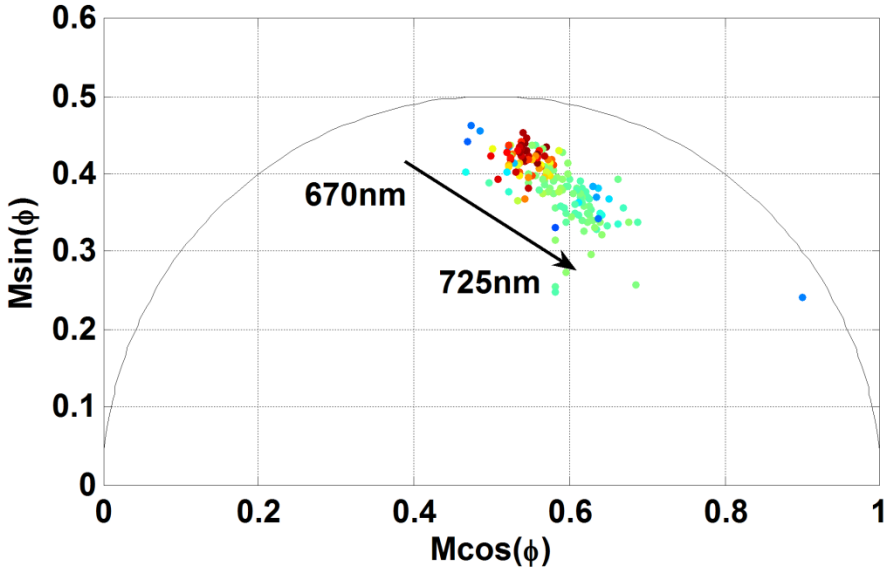


Avocado Leaves

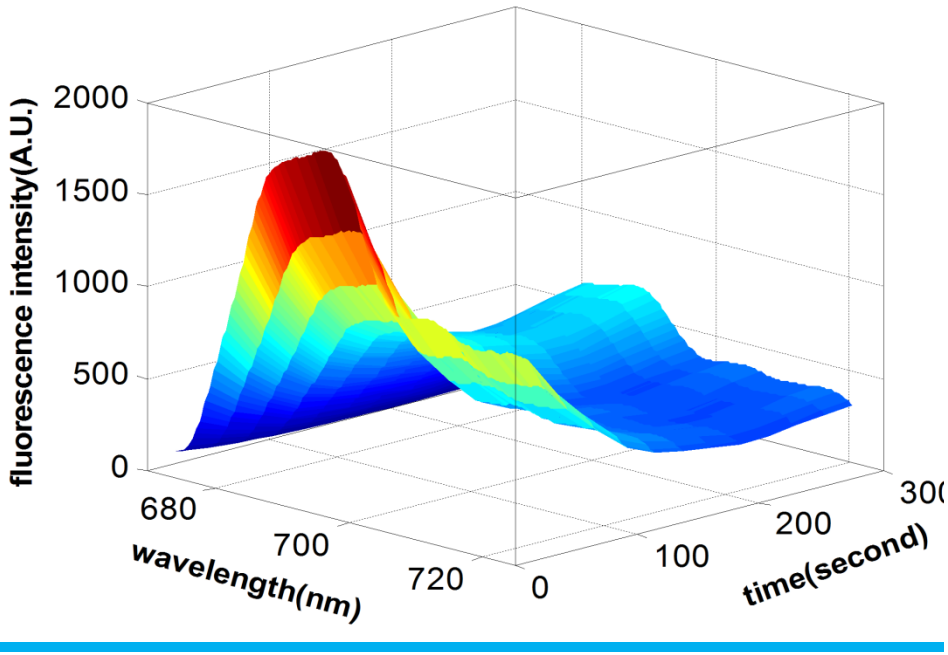


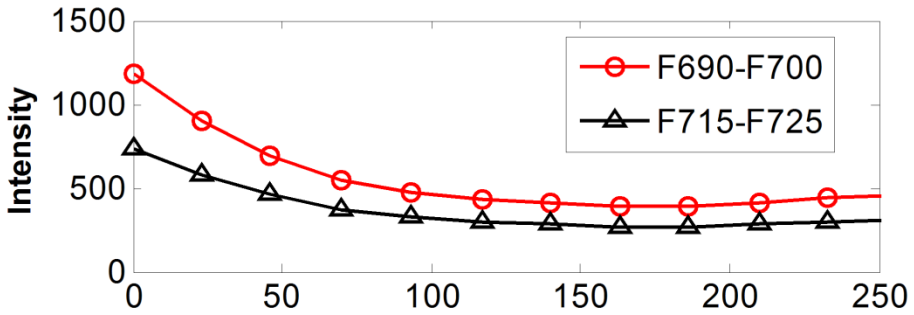
Different wavelengths – different lifetimes

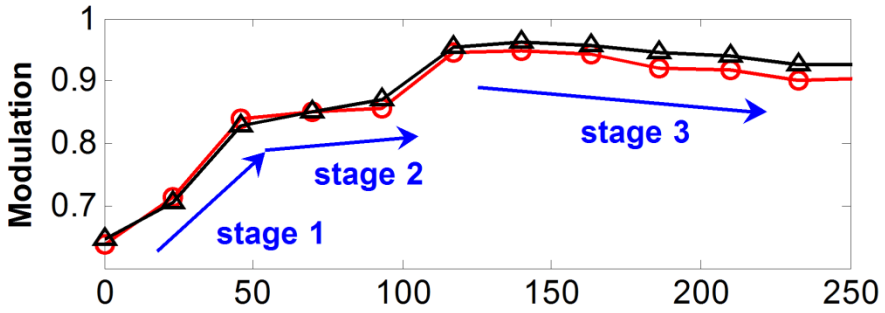


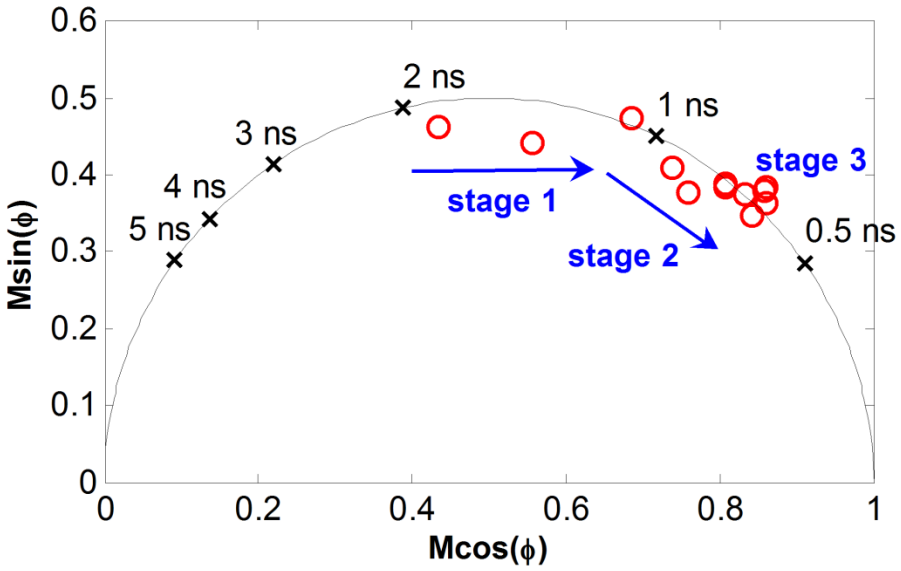


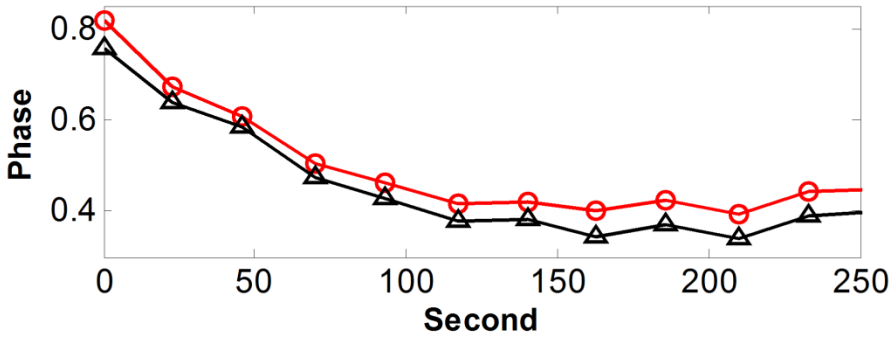
photoprotection



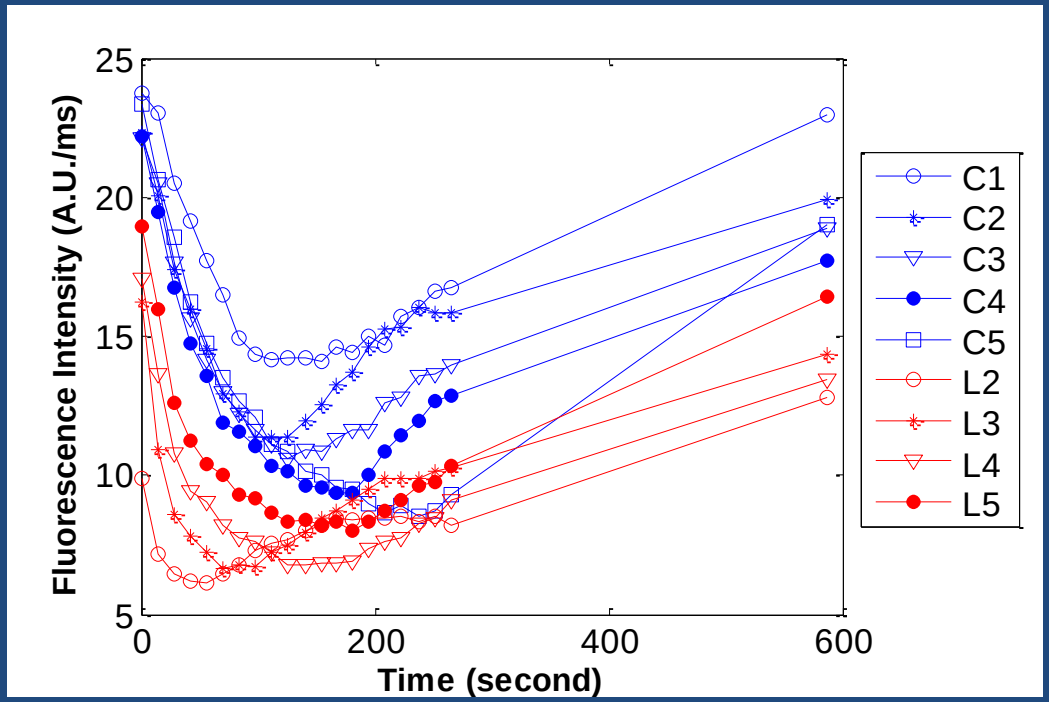


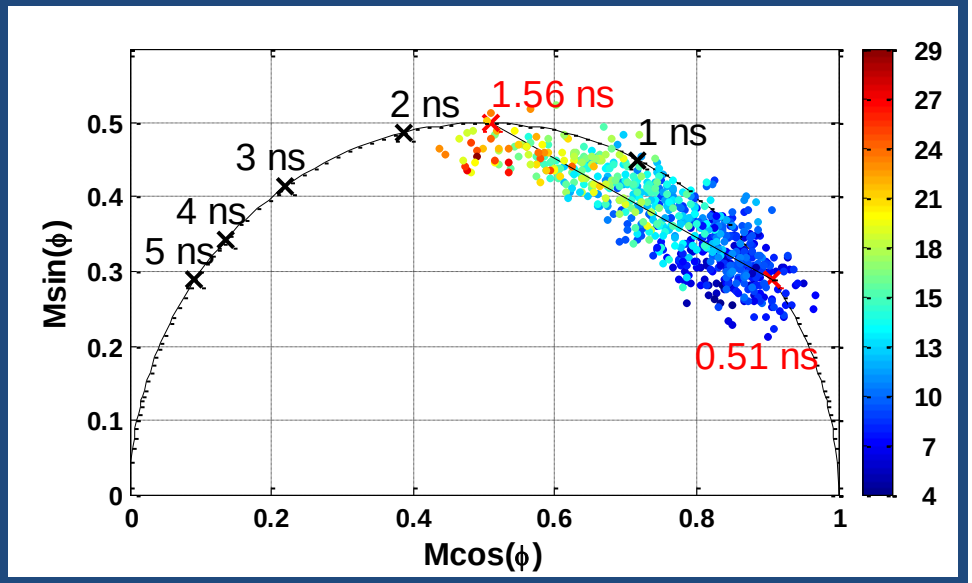


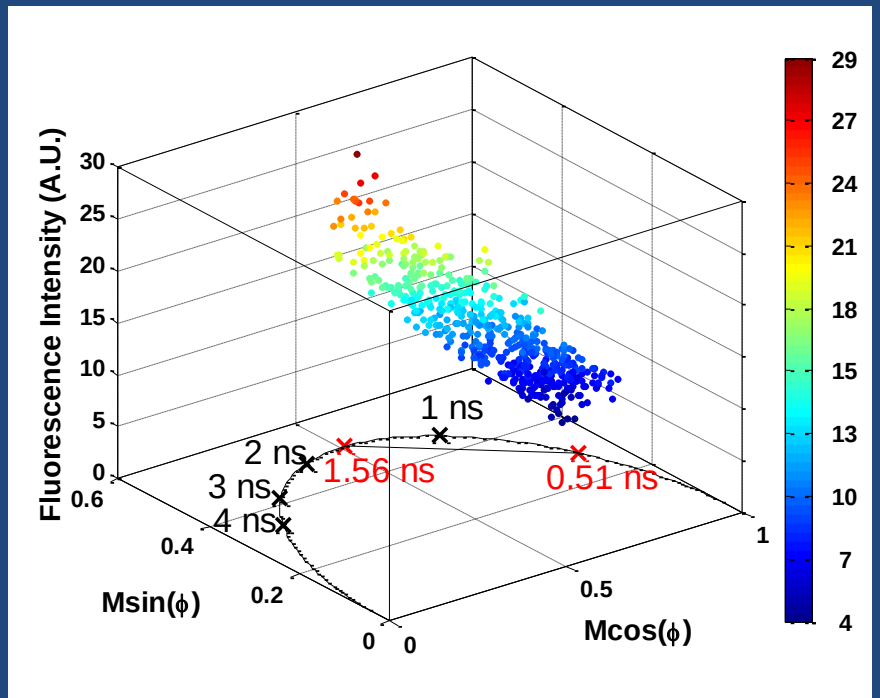




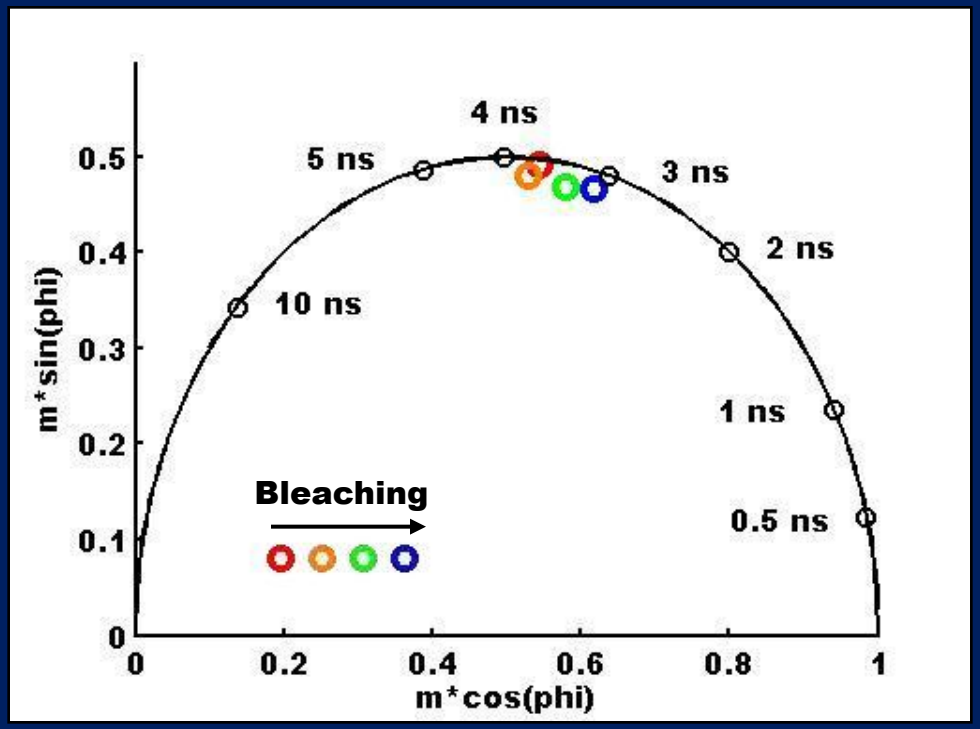
Avocado Leaves

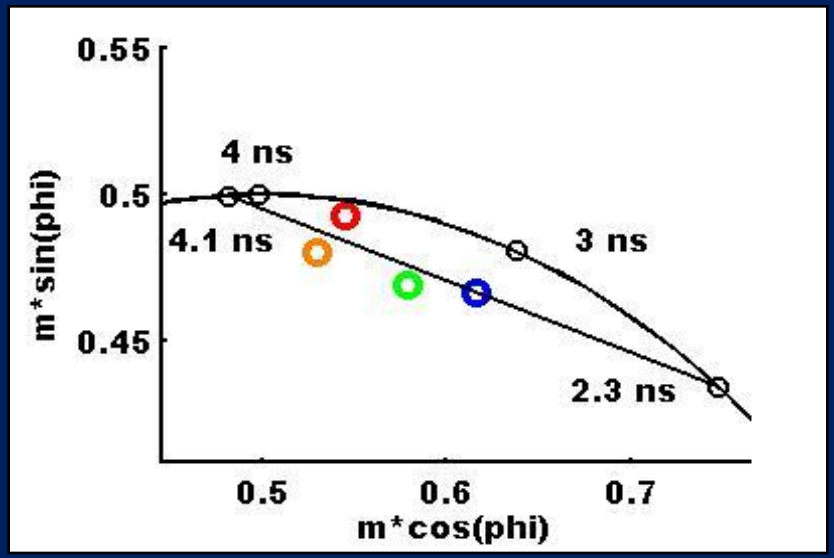

Fluorescence Intensity (A.U./ms)
Time (second)
C1
C2
C3
C4
C5
L2
L3
L4
L5


Msin(ϕ)
Mcos(ϕ)
2 ns
1.56 ns
1 ns
3 ns
4 ns
5 ns
0.51 ns


Fluorescence Intensity (A.U.)
Msin(ϕ)
Mcos(ϕ)
1 ns
2 ns
1.56 ns
3 ns
4 ns
0.51 ns

The Polar Plot describing the bleaching of ECFP





Two lifetimes for ECFP; and the two species of molecules do not interconvert.

The slower lifetime undergoes photolysis faster than the faster lifetime

A unique way to determine multiple lifetimes.

Combining
morphology + lifetime resolution
Localized spatial frequencies

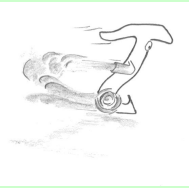
$$w(x,y,a)=\iint g\left(\frac{x-x'}{a},\frac{y-y'}{a}\right)f(x',y')\,dx'dy'$$

Wavelets

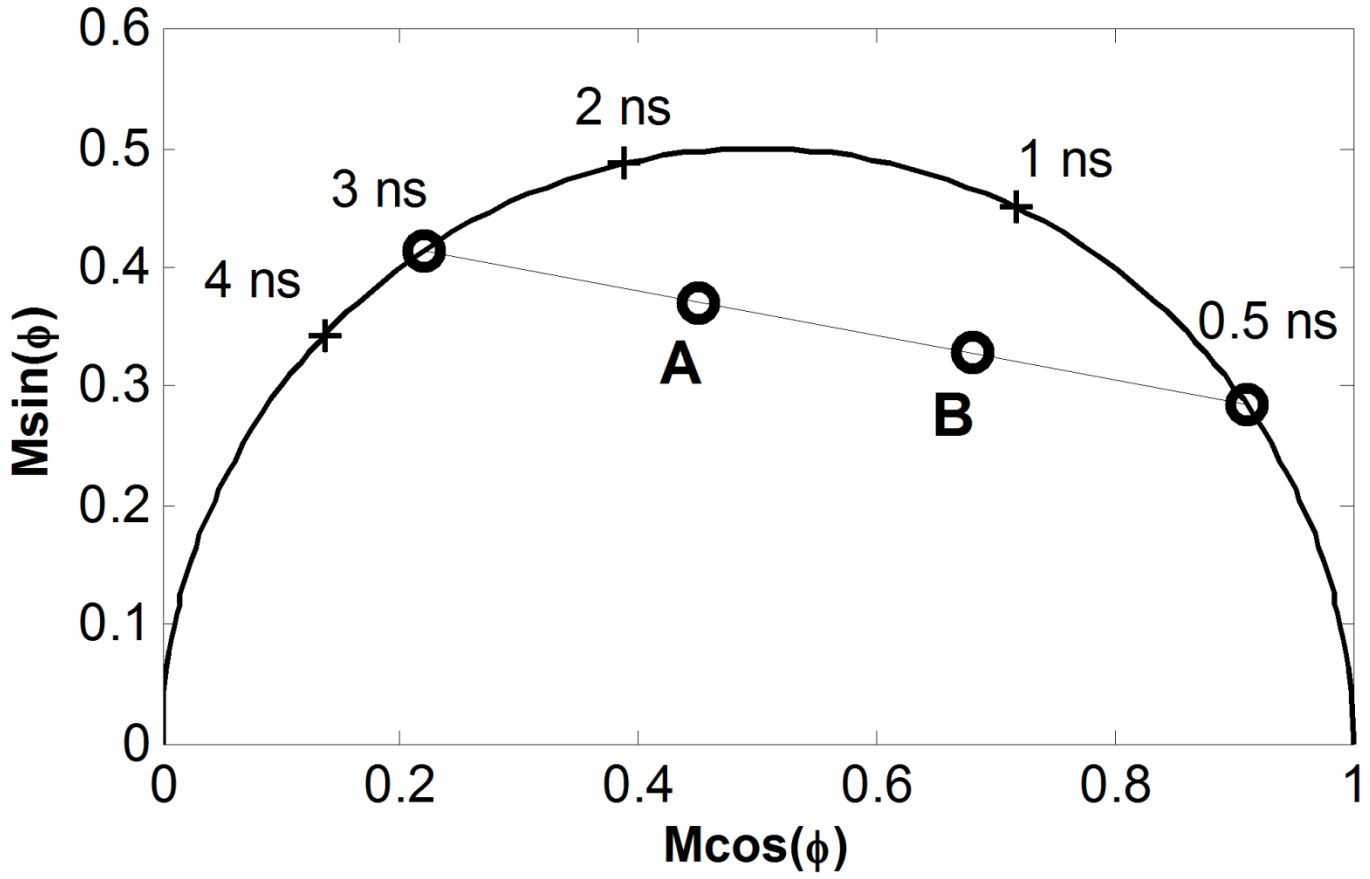
+

Denoising

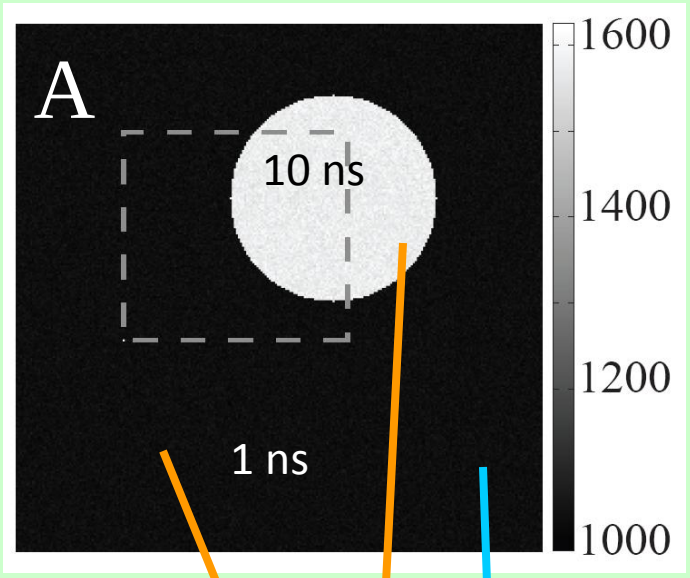
Chasing lifetimes in the noise

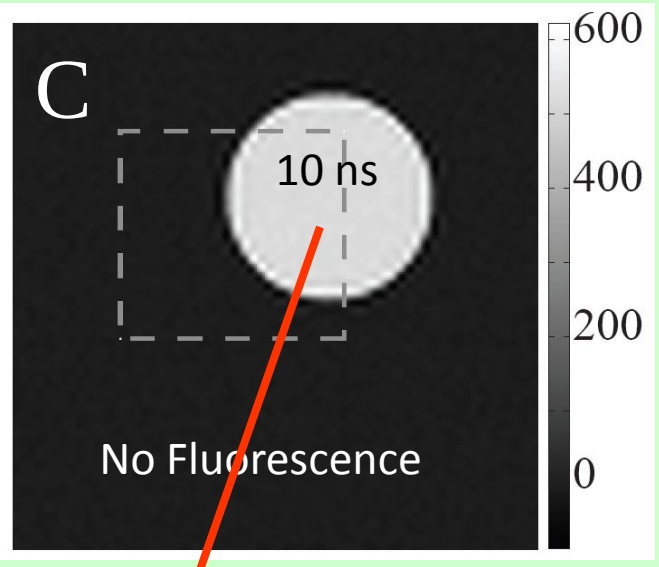


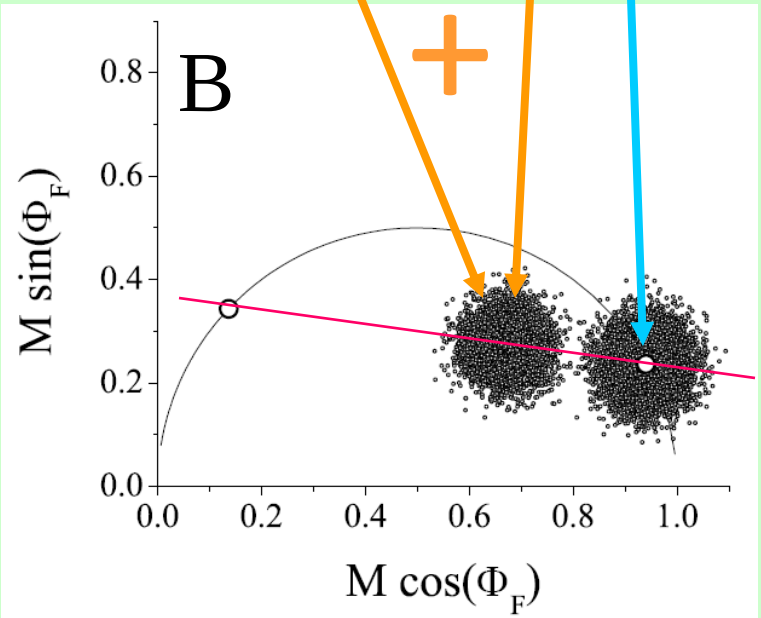
Remember the polar plot

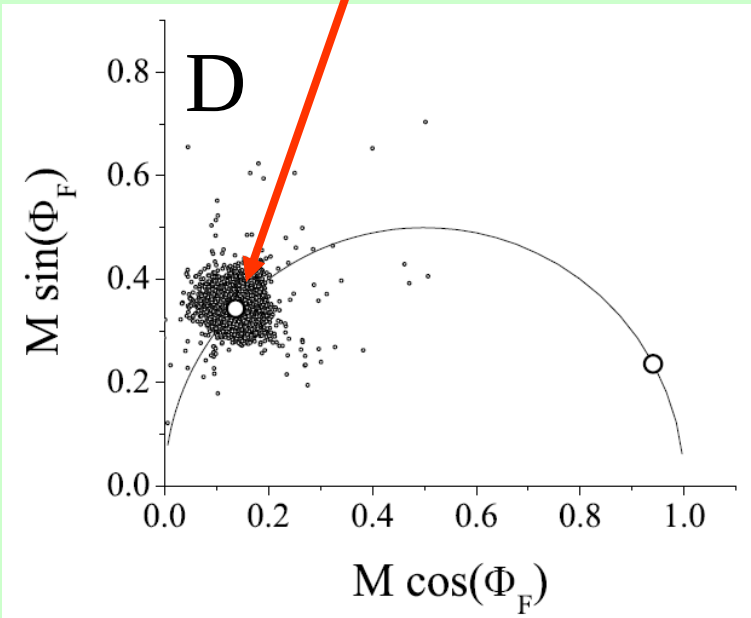


combination of wavelet analysis and FLIM with simulated data
Background constant
The wavelet analysis has completely removed the background contribution

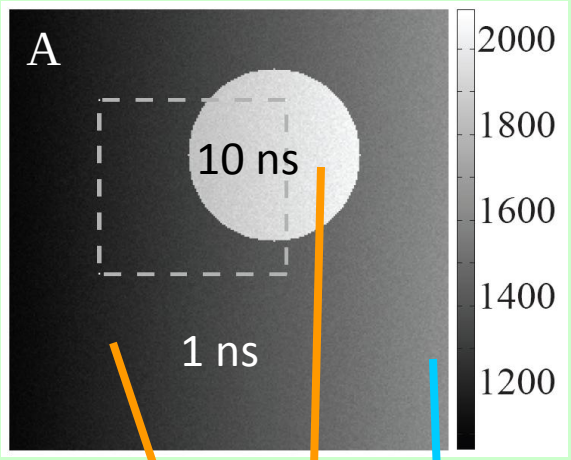


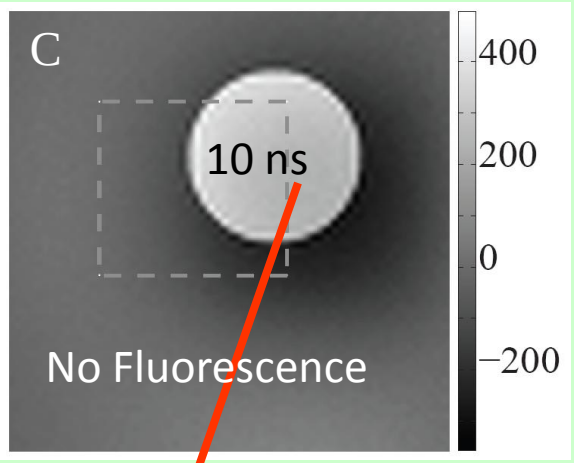


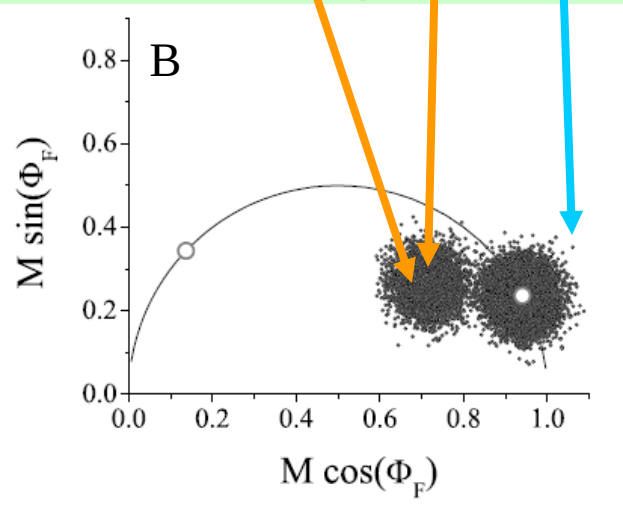


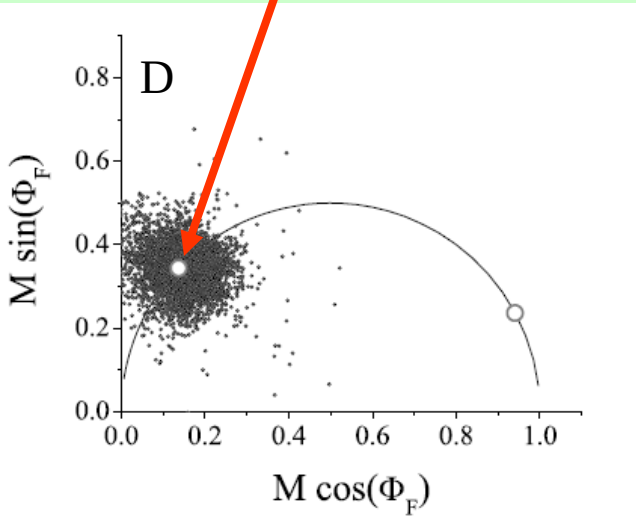


Combination of wavelet analysis and FLIM with simulated data
The background in this simulated data is increasing in amplitude with a constant gradient from left to right.





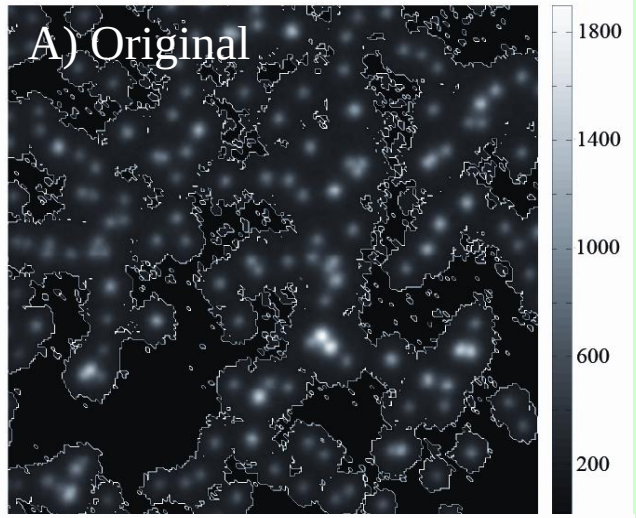


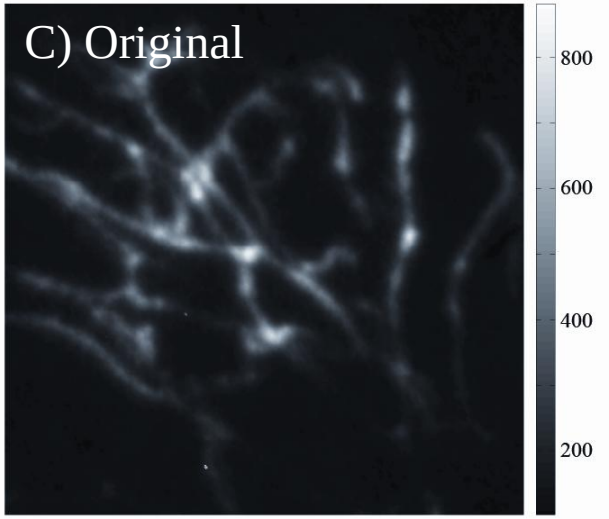


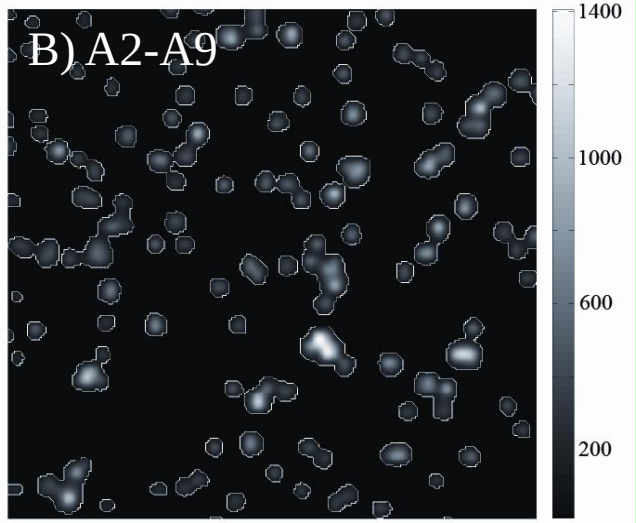
Finding morphology using wavelets

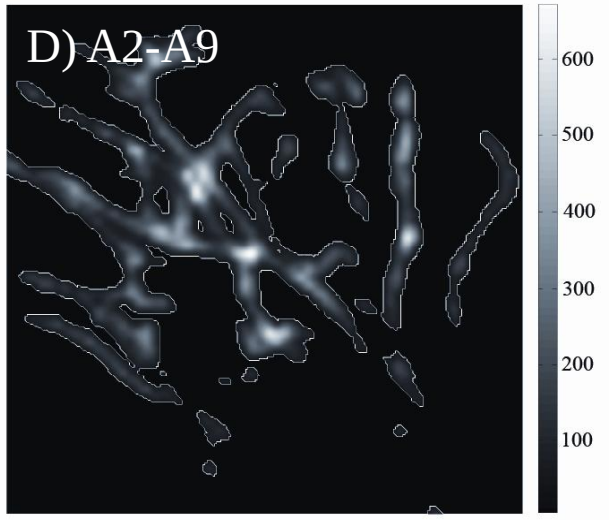
Background subtraction using wavelet on the **fluorescent beads** image (A and B) and on the **dendrites in a *Drosophila melanogastor* larva** expressing membrane-tagged GFP (C and D). The original images (A and C) and the edited image analyzed with wavelet (B and D) are compared.

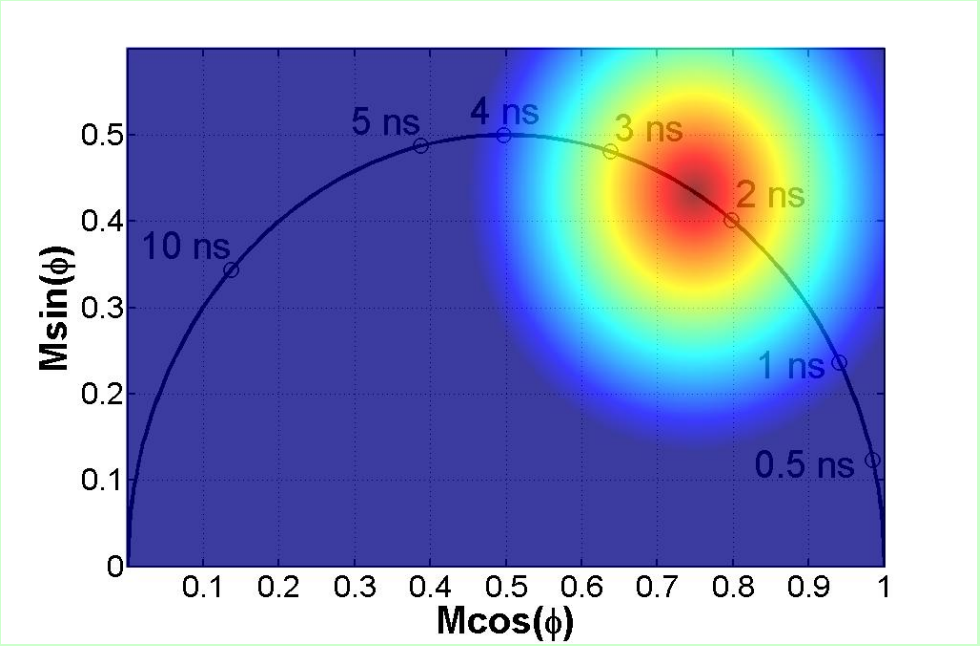
final images are reconstructed by the 'wrcoef2' function from the difference in the approximation data level 2 (containing both high and low spatial frequency components) and level 9 (containing mostly low spatial frequency component)





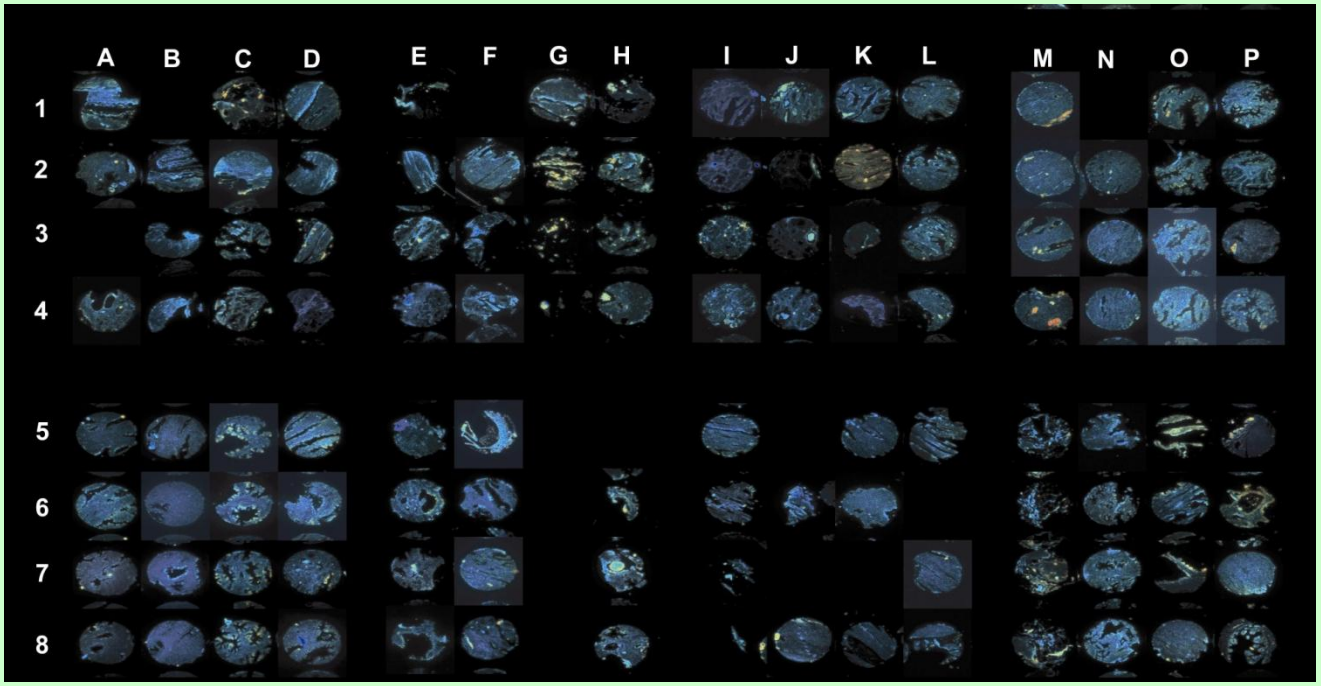


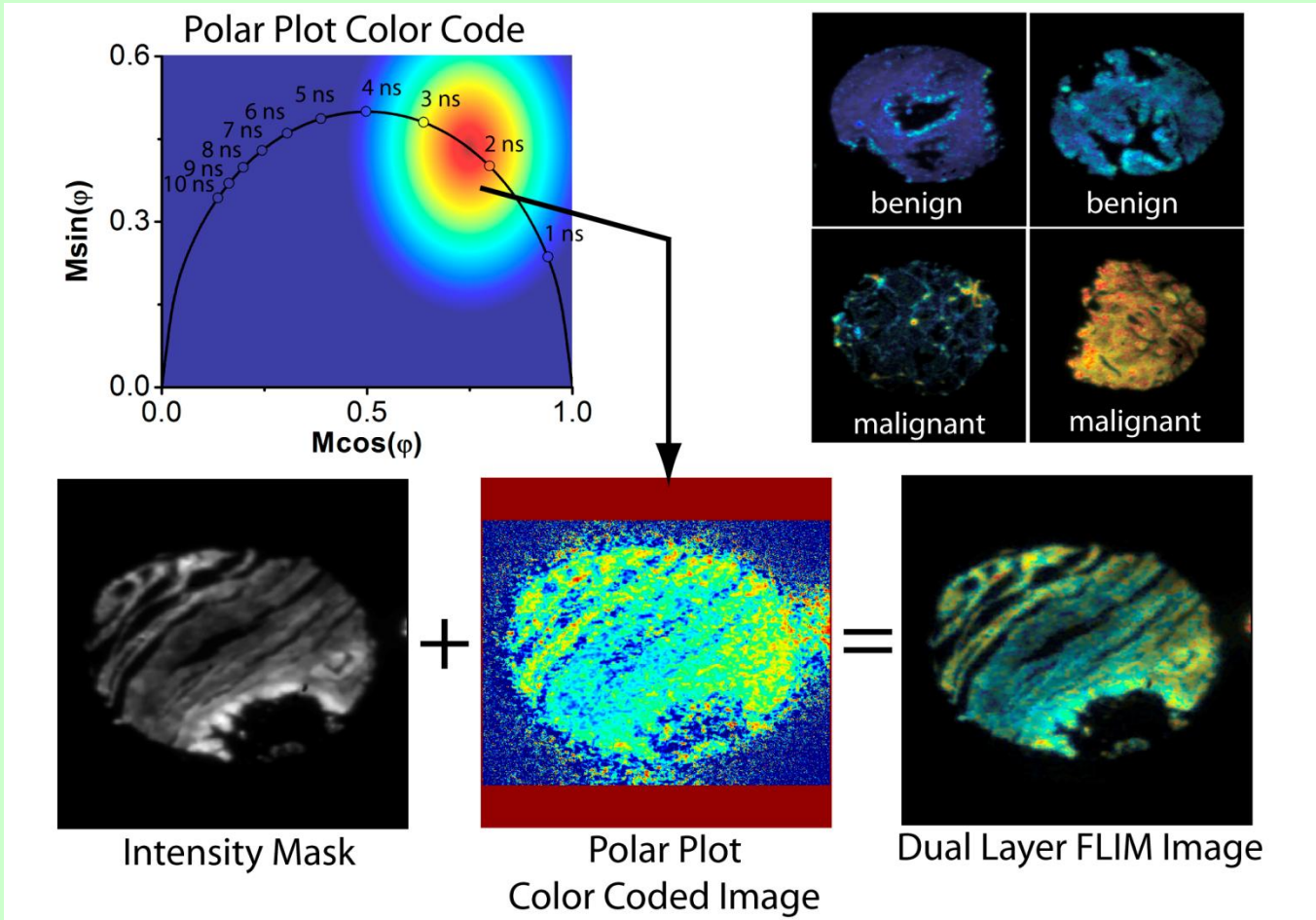




Polar plot color code for representing prostate tissue FLIM data

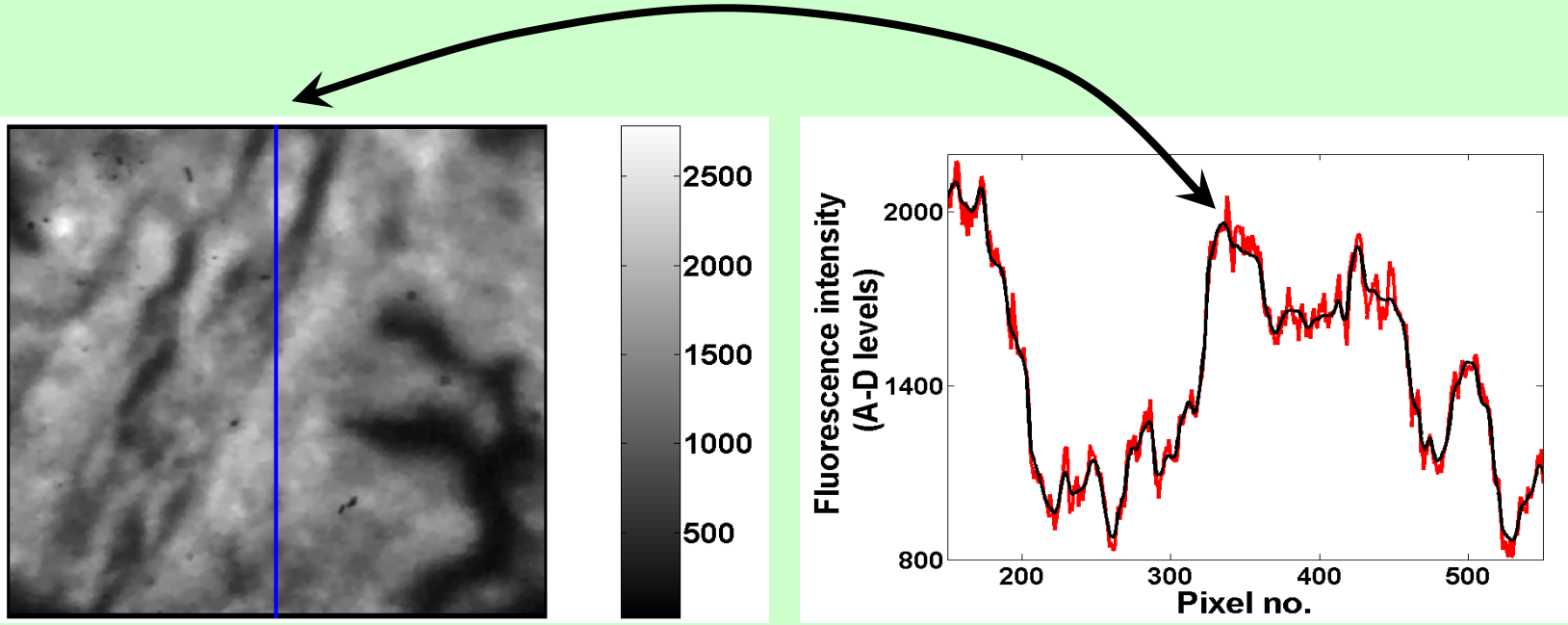
FLIM image of a prostate tissue biopsy microarray





Combining morphological features and FLIM signals (wavelets)
and
Using denoising to assist in the overall analysis

Variance stabilized Gaussian Denoising



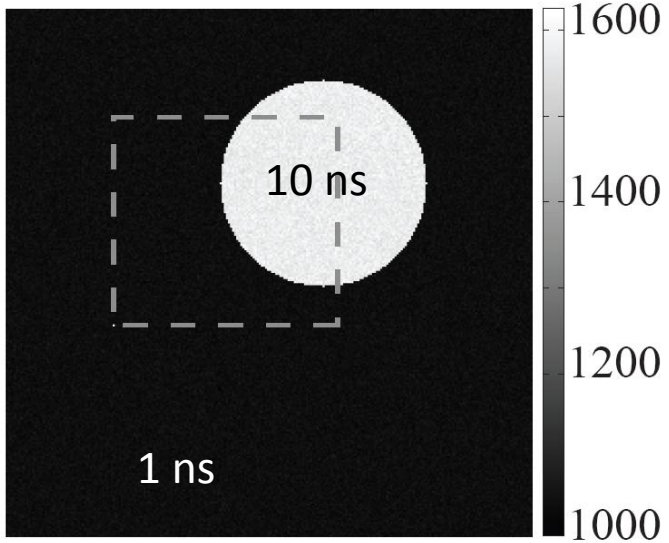
Red – raw data; Black – denoised

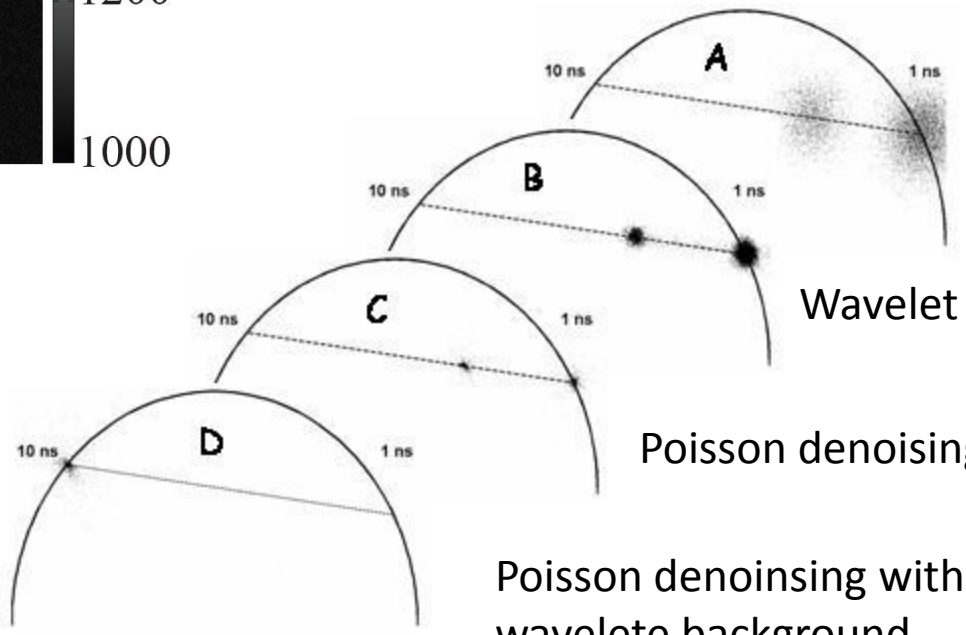
Line profile from an image of prostate tissue

Spatial frequency cuts (intervals) can be selected

Edges not smoothed

Polar Plots





Raw data

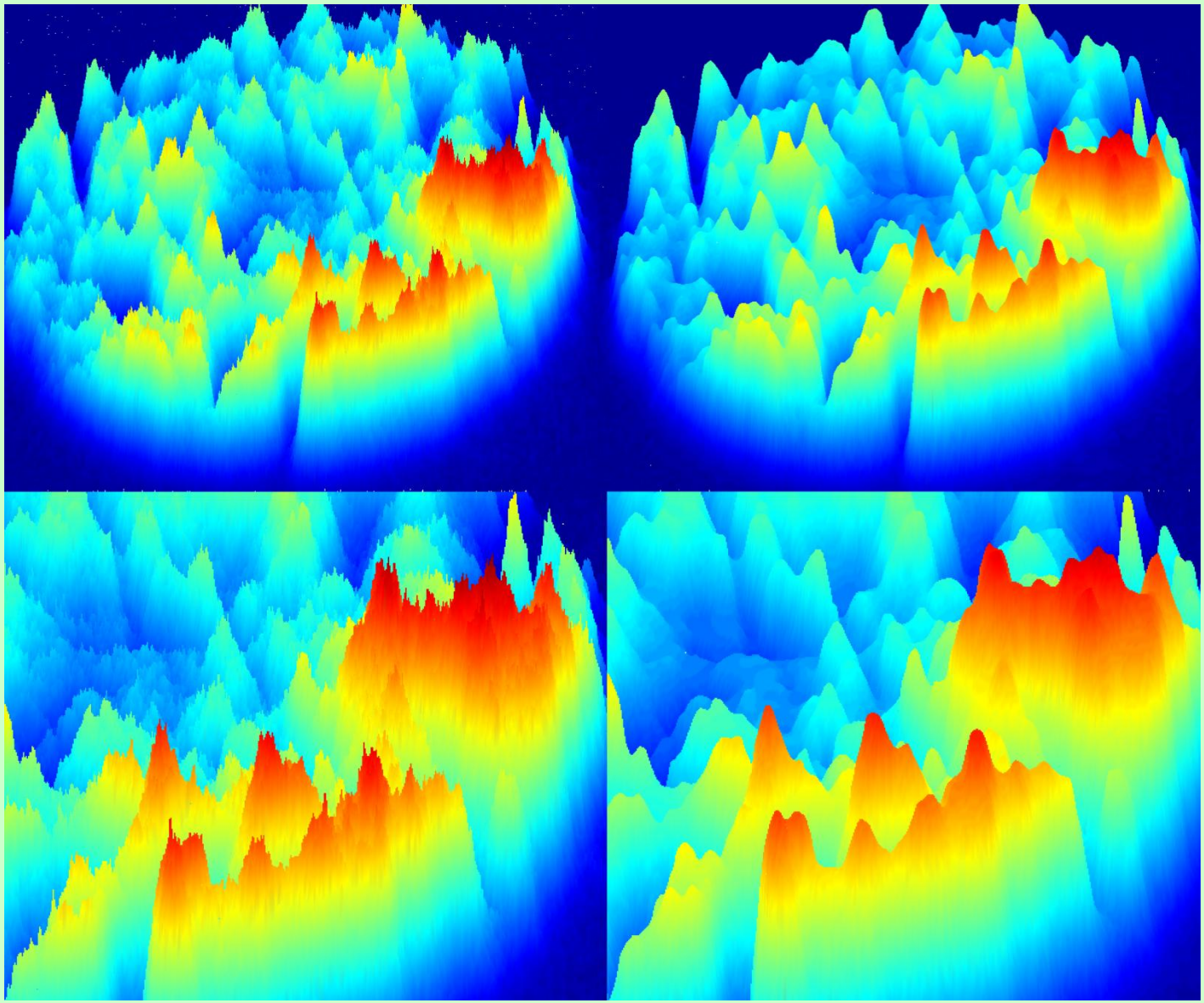
Wavelet smoothing

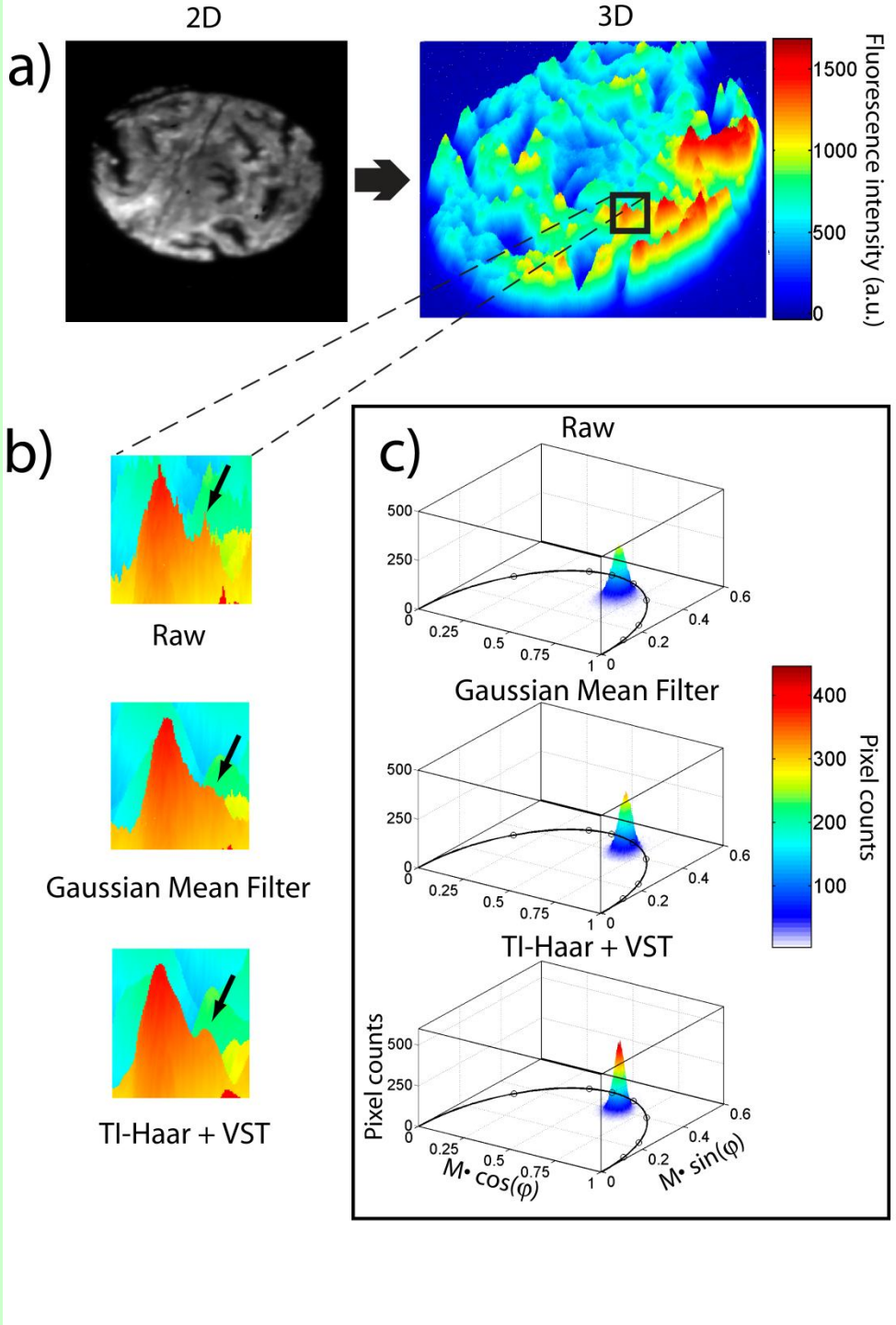
Poisson denoising

Poisson denoinsing with wavelete background subtraction

Left: raw fluorescence intensity images of a prostate tissue core

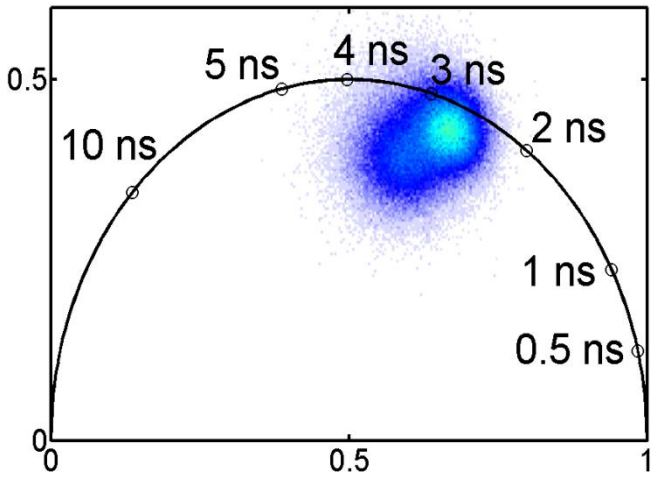
Right: denoised images

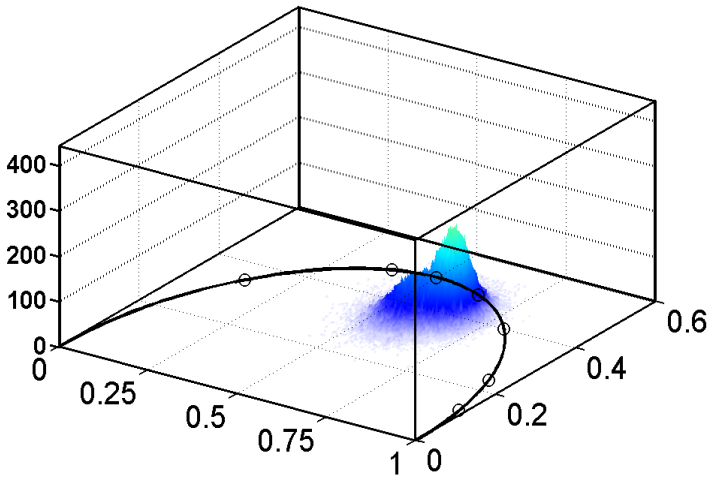


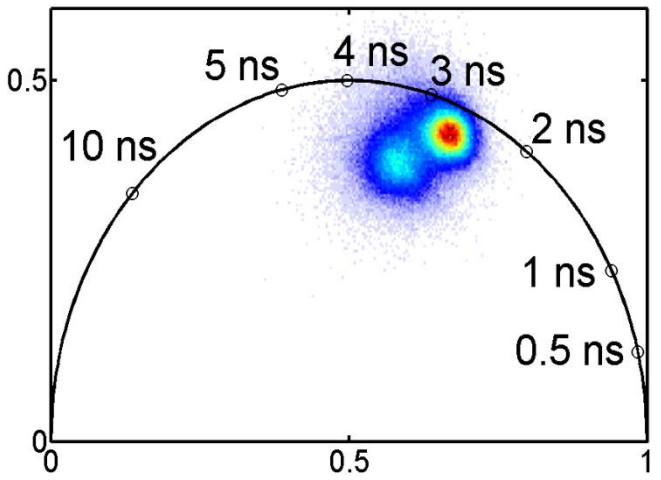

a)
2D
3D
1500
1000
500
0
Fluorescence intensity (a.u.)
b)
Raw
Gaussian Mean Filter
TI-Haar + VST
c)
Raw
500
250
0
0.25
0.5
0.75
1
0.6
0.4
0.2
Gaussian Mean Filter
TI-Haar + VST
400
300
200
100
Pixel counts
Pixel counts
M• cos(φ)
M• sin(φ)

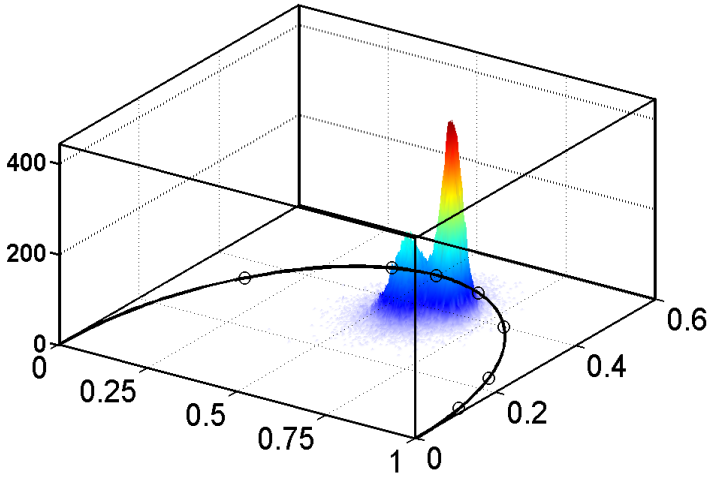
Polar plot histograms

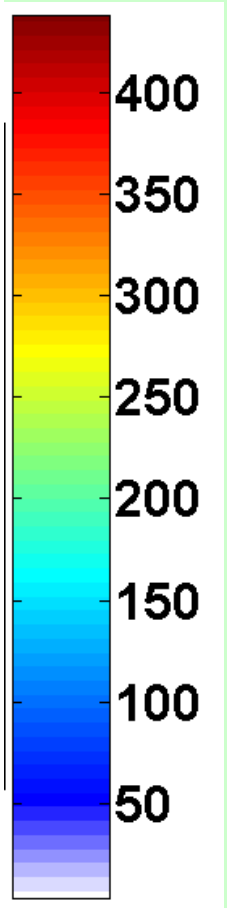
of entire FLIM images of a benign and a malignant prostate tissue core
Top: **before** denoising
Bottom: **after** denoising

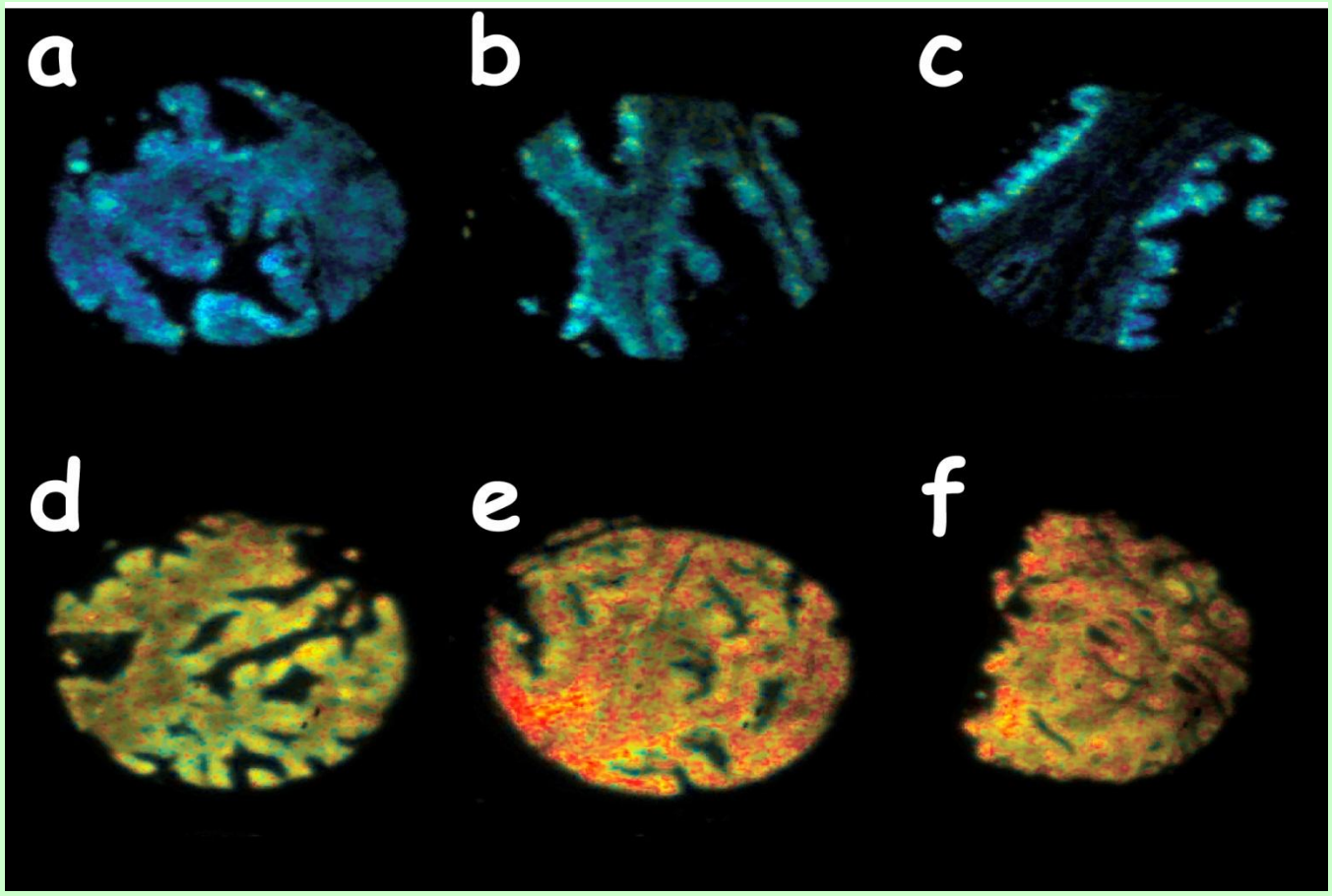




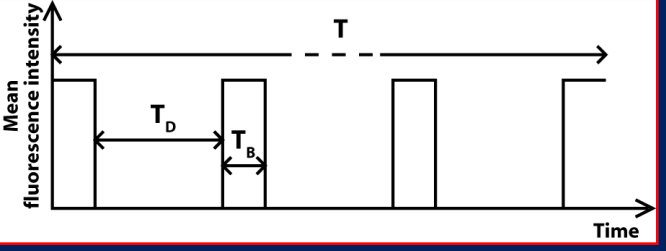




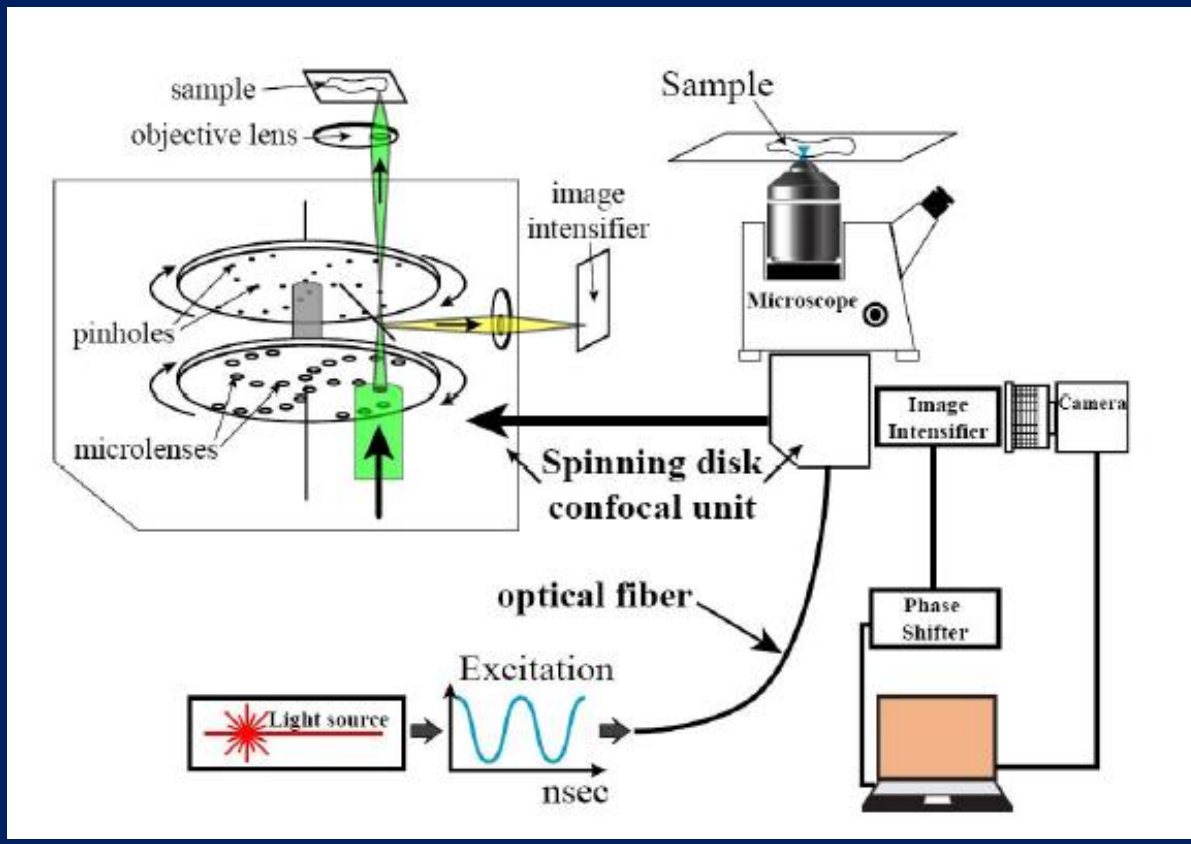




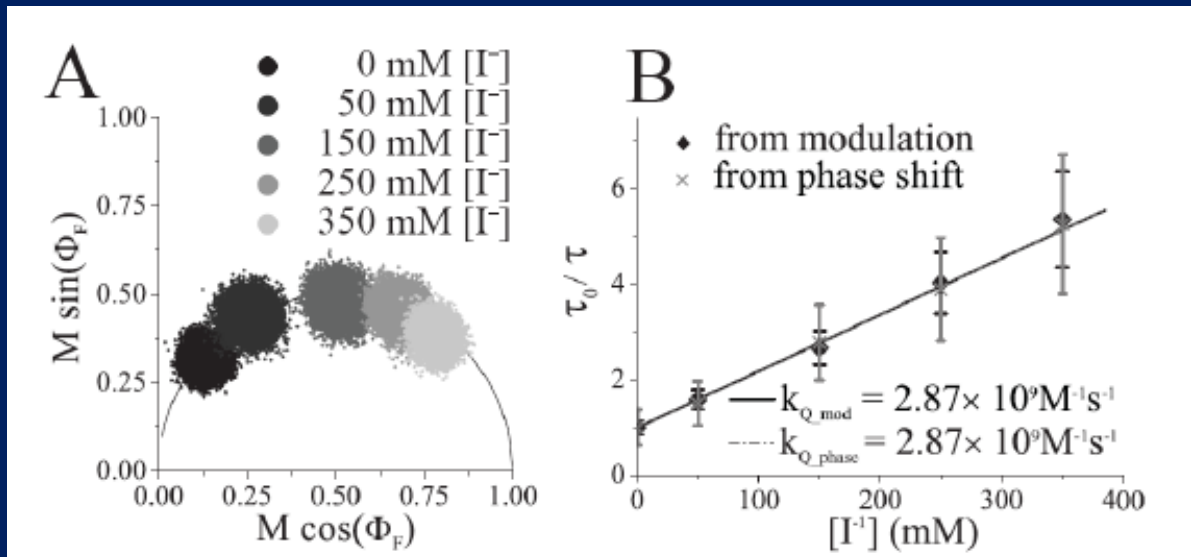
Dual layer FLIM images (intensity image used to mask color coded lifetime image) - Color indicates the fluorescence lifetime distribution of each pixel. Blue indicates normal fluorescence lifetimes while red indicates a significant shift in fluorescence lifetimes from benign tissue. **a-c)** Benign tissue cores. **d)** Low-grade cancer. **e & f)** High-grade cancer. Note that the lifetime distributions can be complex (the fluorescence lifetimes reflect multiple species – i.e., free and enzyme-bound species). The color coding represents an overall shift in the relative amounts of each species and therefore accomplishes representation of complex data in an easily visible fashion.



Spinning Disk Full-Field Flim 3rd dimension



The polar plot analysis of FLIM data from a set of fluorescein solutions having different concentrations of iodide, which quenches the fluorescence emission from fluorescein in diffusion controlled encounters



Full Field FLI

Peter Schneider

Oliver Holub

Christoph Gohlke

Glen Redford

(polar plot ALA–PPIX)

+

Spinning disk, wavelets and denoising

Chittaton Buranachi, Bryan Spring, Rohit Bhargava

(dendritesALA–PPIX, prostate FLIM, redox sensor)

Yi-Chun Chen (Polar Plot, spectral FLIM, photosynthesis)

John Eichorst & Peter (Yingxiao) Wang

Photosynthesis:

Govindjee

Oliver Holub

Christoph Gohlke

Gregor Heiss

Shizue Matsubara