

The Phasor approach: Application to FRET analysis and Tissue autofluorescence

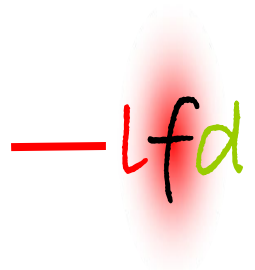
Laboratory for Fluorescence Dynamics
University of California at Irvine

—lfd

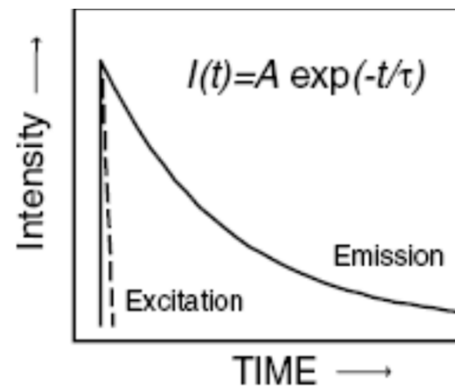
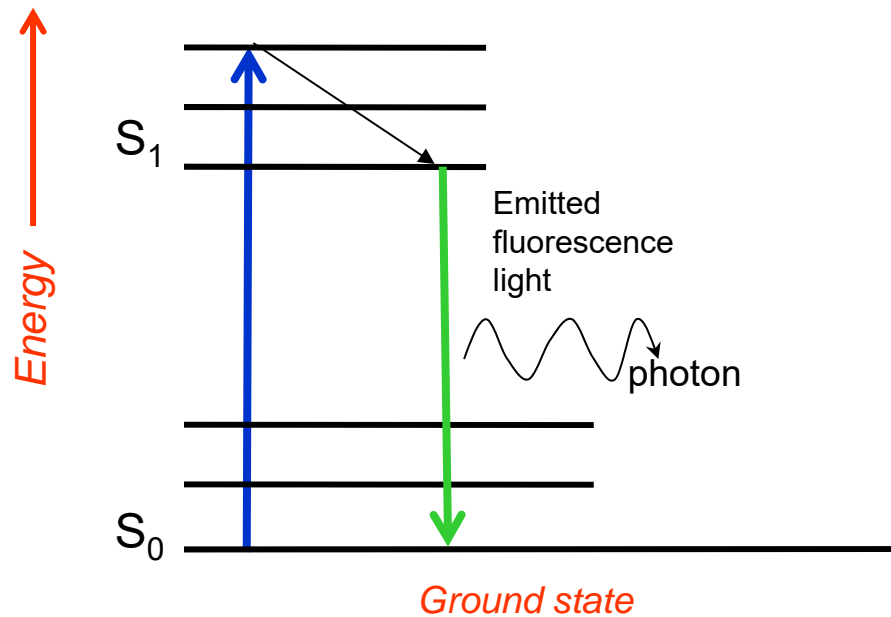


Outline

- Background: Lifetime
- Intro to Fluorescence Lifetime Imaging Microscopy
- Motivation for FLIM
- The Phasor approach
- Rac Activation using FLIM
- Conclusions

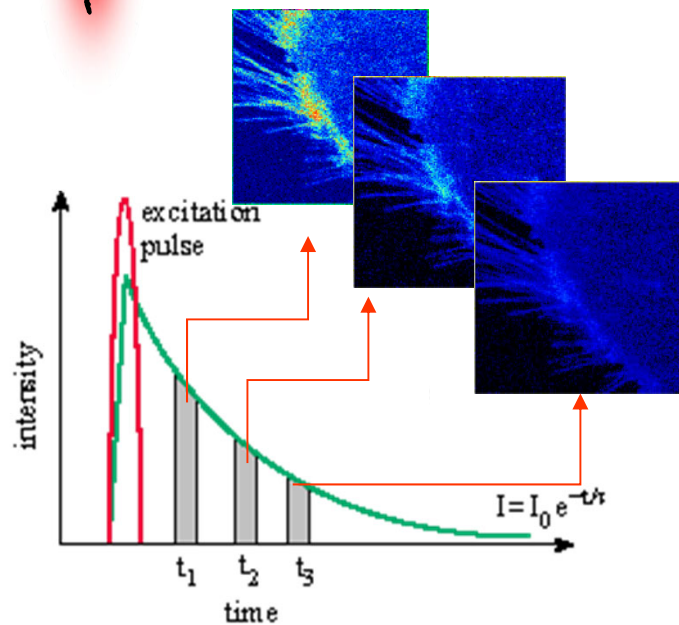


Lifetime: Background



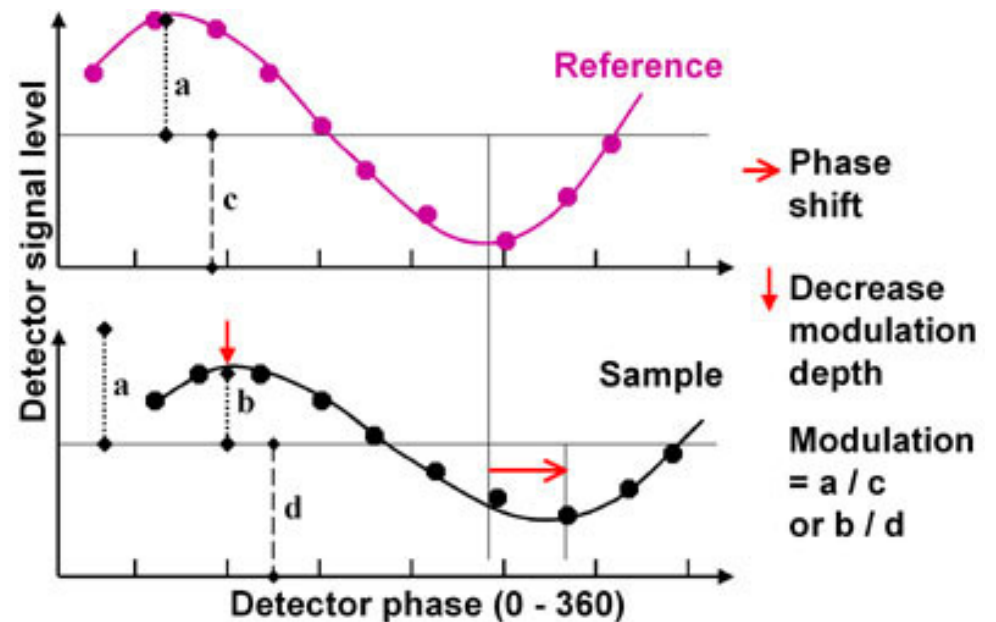
Time Domain and Frequency Domain FLIM

— lfd



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

The histogram of the time intervals between the excitation flash, and 1st emitted photon is measured



A sample is excited by a modulated light source

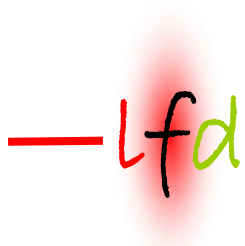
The fluorescence emission has the same frequency but is modulated and phase-shifted from the excitation source



Why do FLIM?

FLIM is used for :

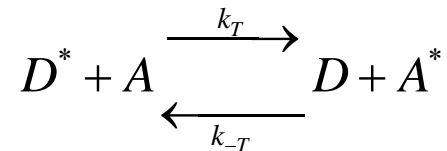
- **FRET**
- **Intracellular mapping of Ion concentration and pH imaging**
- **Biochemical reactions (oxidation/reduction) processes**
 - **NAD and NADH**
- **Long lifetime imaging (phosphorescence).**
 - **For example O₂ concentration in the cell or in tissues**



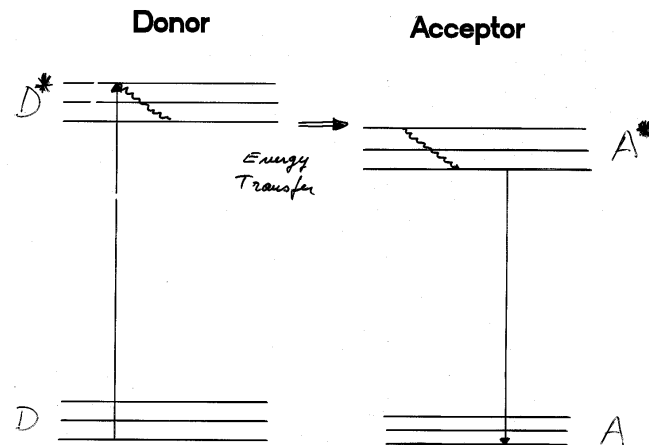
Förster Resonance Energy Transfer (FRET) (T. Förster, 1949)

An excited molecule can transfer energy to another molecule even if the other molecule is far away.

Description of the process: Consider a molecule (donor D) which absorbs light. After absorption, because of the fast internal conversion, the molecule will be at the bottom (lower vibrational state) of the excited state. If the donor emission energy coincides with the absorption energy of a different molecule (acceptor A), a resonance process can take place. The energy transfer occurs at a rate k_T .



The acceptor rapidly decays to the bottom of the excited state. From this level the acceptor molecule can decay by fluorescence emission or by non-radiative processes



Because of rapid internal conversion the process A^* to D^* is very unlikely to occur unless the donor is of the **same kind of the acceptor (Homotransfer)**.

FRET strongly depends on the distance between the two groups.

The theory was developed by Förster in 1949 who calculated the rate of transfer to be

$$k_T = \frac{1}{\tau_D} \left(\frac{R_0}{R} \right)^6$$

τ_D is the lifetime of the donor in the absence of the acceptor.

R is the distance between the two groups

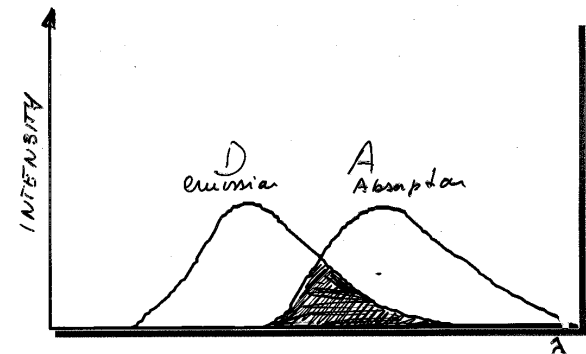
R_0 is called the Förster characteristic distance

$$R_0 = 9.7 \times 10^3 \left(J \kappa^2 n^{-4} \Phi_D \right)^{1/6}$$

n is the refractive index of the medium

Φ_D is the quantum yield of the donor

κ^2 is a complex geometrical factor which depends on the relative orientation of donor and acceptor.



J is a measure of the spectral overlap

Energy transfer studies give information

- Distance between groups
- Orientation of two groups

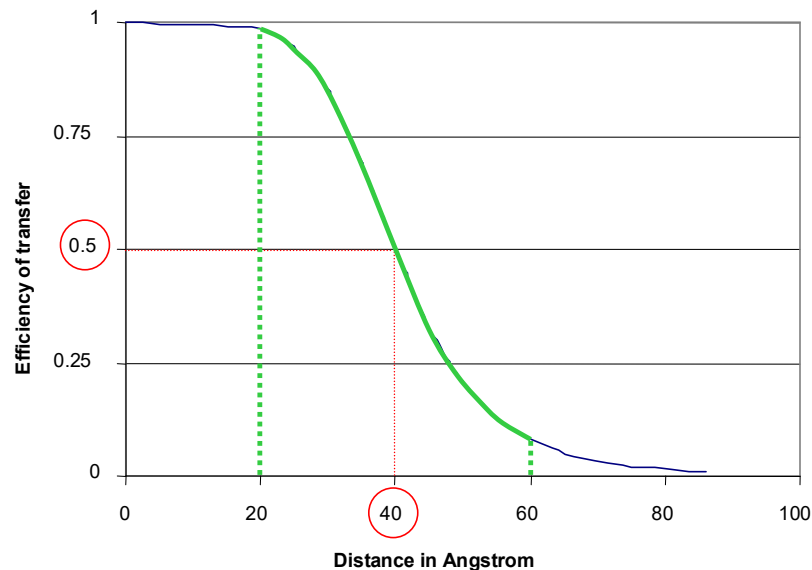
Notice that D and A can be the same kind of molecule provide emission and absorption overlap.

Distance dependence of the energy transfer efficiency (E)

$$R = \left(\frac{1}{E} - 1 \right)^{1/6} R_0$$

Where R is the distance separating the centers of the donor and acceptor fluorophores, R_0 is the Förster distance.

The efficiency of transfer varies with the inverse sixth power of the distance.



R_0 in this example was set to 40 Å.

When the E is 50%,
 $R=R_0$

Distances can generally be measured between $\sim 0.5 R_0$ and $\sim 1.5 R_0$

How to perform an energy transfer experiment?

— lfd

Energy transfer efficiency (E)

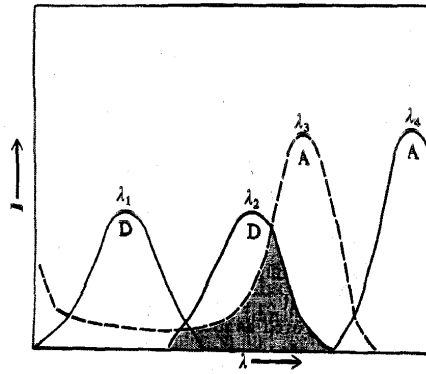
$$E = \frac{k_T}{k_T + \sum_{i \neq T} k_i}$$

Where k_T is the rate of transfer and k_i are all other deactivation processes.

Experimentally, E can be calculated from the fluorescence lifetimes or intensities of the donor determined in absence and presence of the acceptor.

$$E = 1 - \frac{\tau_{da}}{\tau_d} \quad \text{or} \quad E = 1 - \frac{F_{da}}{F_d}$$

If the acceptor is **fluorescent**, then it is possible to measure the fluorescence of the acceptor in the absence and presence of the donor.



The sample is excited at λ_1 . The fluorescence at λ_4 of the acceptor can be observed in the absence of the donor to check if there is any contribution of direct acceptor excitation

$$F_{A\lambda_1, \lambda_4} \cong \varepsilon_{A\lambda_1} C_A \Phi_{A\lambda_4}$$

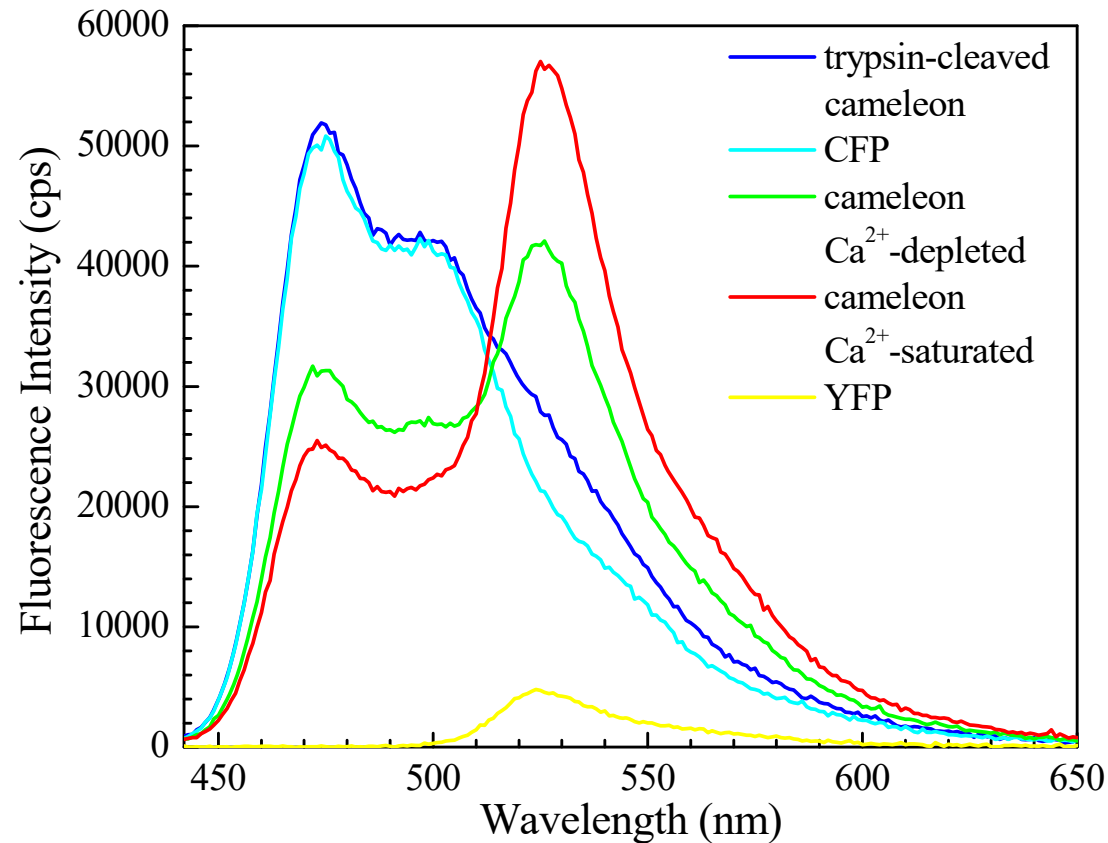
In presence of the donor the total fluorescence intensity at λ_4 is

$$F_{A+D\lambda_1, \lambda_4} \cong \varepsilon_{A\lambda_1} C_A \Phi_{A\lambda_4} + \varepsilon_{D\lambda_1} C_D E \Phi_{A\lambda_4}$$

measuring F_A and F_{A+D} , the energy transfer efficiency E can be obtained

An independent measurement of E can be obtained by comparing the decay time of the donor in the presence and absence of the acceptor.

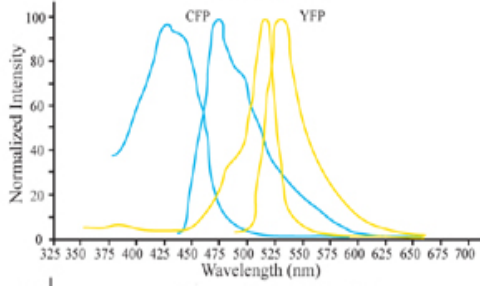
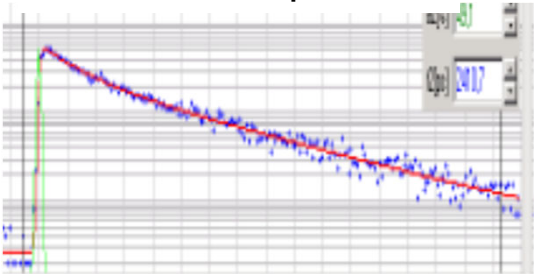
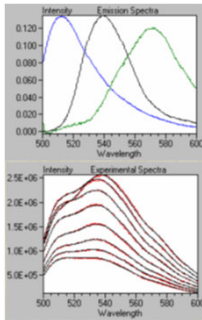
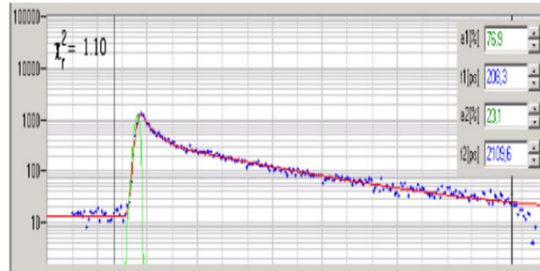
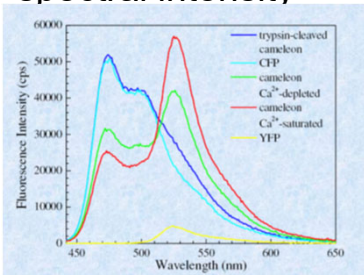
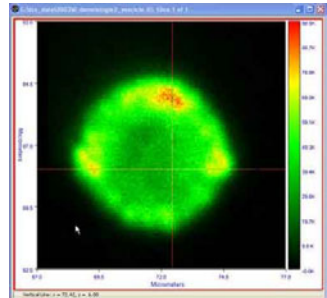
$$\frac{\tau_{D+A}}{\tau_D} = 1 - E$$



Summary of the FRET detection

- Quenching of the donor (intensity and lifetime)
- Increase of the acceptor fluorescence
- Decrease of the steady-state polarization
- Change of the lifetime of the acceptor (?)

Conceptual approaches to Spectroscopy

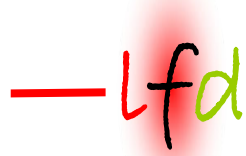
	Using the Spectra	Using the fluorescence decays
1) Identification Molecular Species	<p>λ Excitation λ emission</p> 	<p>Lifetime Components</p> 
2) Demixing of multiple species in a pixel	<p>Spectral demixing</p> 	<p>Multiexponential analysis</p> 
3) Identification of processes: FRET	<p>Ratio of acceptor/donor spectral intensity</p> 	<p>Quenching of donor lifetime</p> 



The challenges of FLIM

- At every pixel there are contributions of several fluorescent species, each one could be multi-exponential.
- To make things worse, we can only collect light for a limited amount of time (100-200 microseconds per pixel) which result in about **500-1000** photons per pixel.
- This is barely enough to distinguish a double exponential from a single exponential decay.
- Resolving the decay at each pixel in multiple components involves fitting to a function, and is traditionally a complex computational task “for experts only”.

A major problem is **data analysis and interpretation**



Major issues with FLIM

- Rather difficult technique
- Fitting is slow
- Results depend on initial conditions
- Interpretation requires expertise

Can we avoid all these problems?

- No expertise necessary
- Instantaneous results
- Independent of initial choices
- Quantitative results
- Intuitive simple interface

A new approach: no more fits!

—lfd

We propose a change in paradigm: Use a different representation of the decay where each molecular species has its own unique representation and where each process (FRET, ion concentration changes) is easily identified.

We need to go to a new “space”

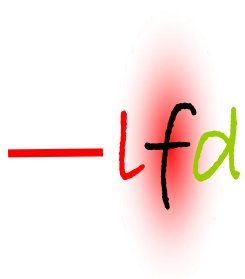
The phasor space and the universal circle (From Star-Trek)

—lfd

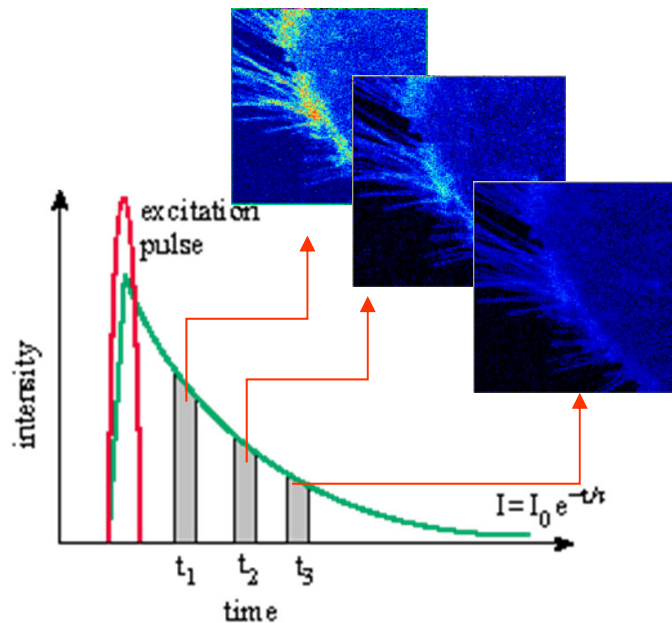


This is what we need: the phaser!

- Where does this concept come from??
- We need some math.



How to calculate the components τ_g and τ_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

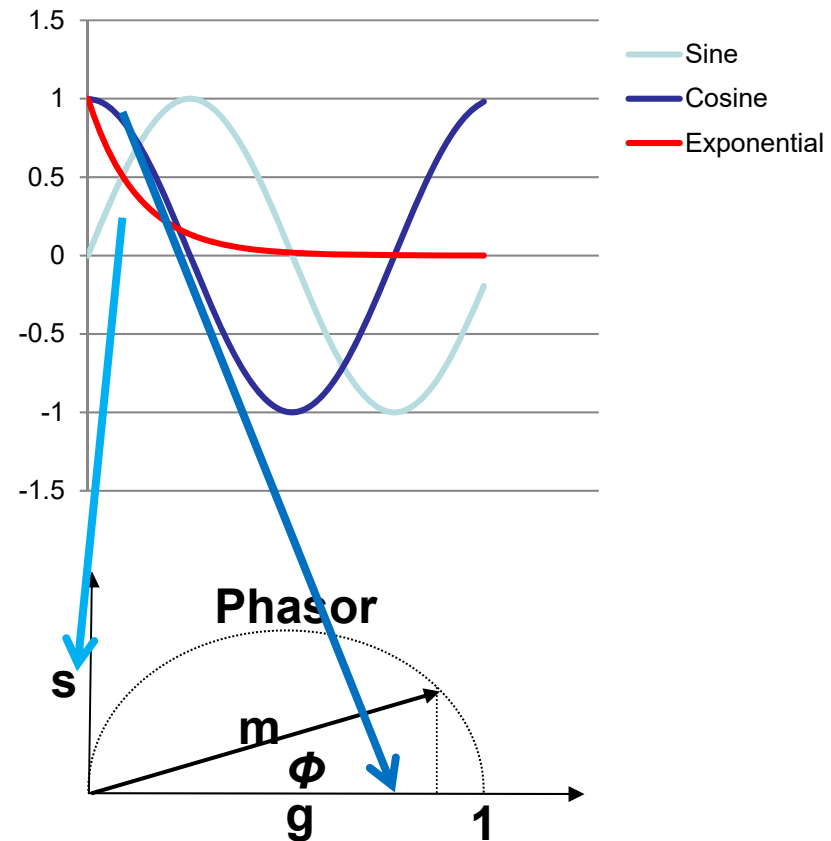
How to calculate the components g and s of a phasor from the time decay?

— lfd

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

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

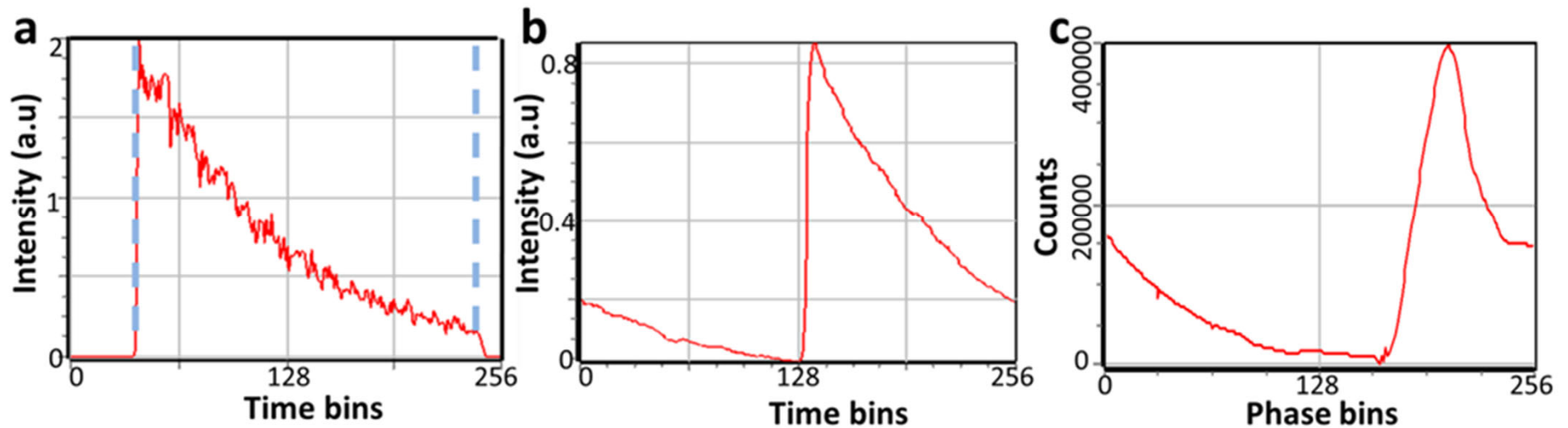
Time-domain
components of a phasor. $I(t)$ is
measured



Note that $I(t)$ can contain raw multiexponential data!!

What happens if you don't have the decay over a period?

80 MHz repetition laser frequency: period is 12.5 ns



The TAC range of 50 ns. Using a gain of 4 results in measurement over 12.5 ns.

But the useful range is only from channel 50 to 250 out of 256 bins (78%), and the entire period not being used makes the time record **incomplete**.

Using a gain of 2 makes the total range 25 ns, but an entire period can be extracted!

Example using the FLIMbox. The laser repetition period is 12.5 ns and it is divided into 256 bins, resulting in a 100% duty cycle

From The B&H publication

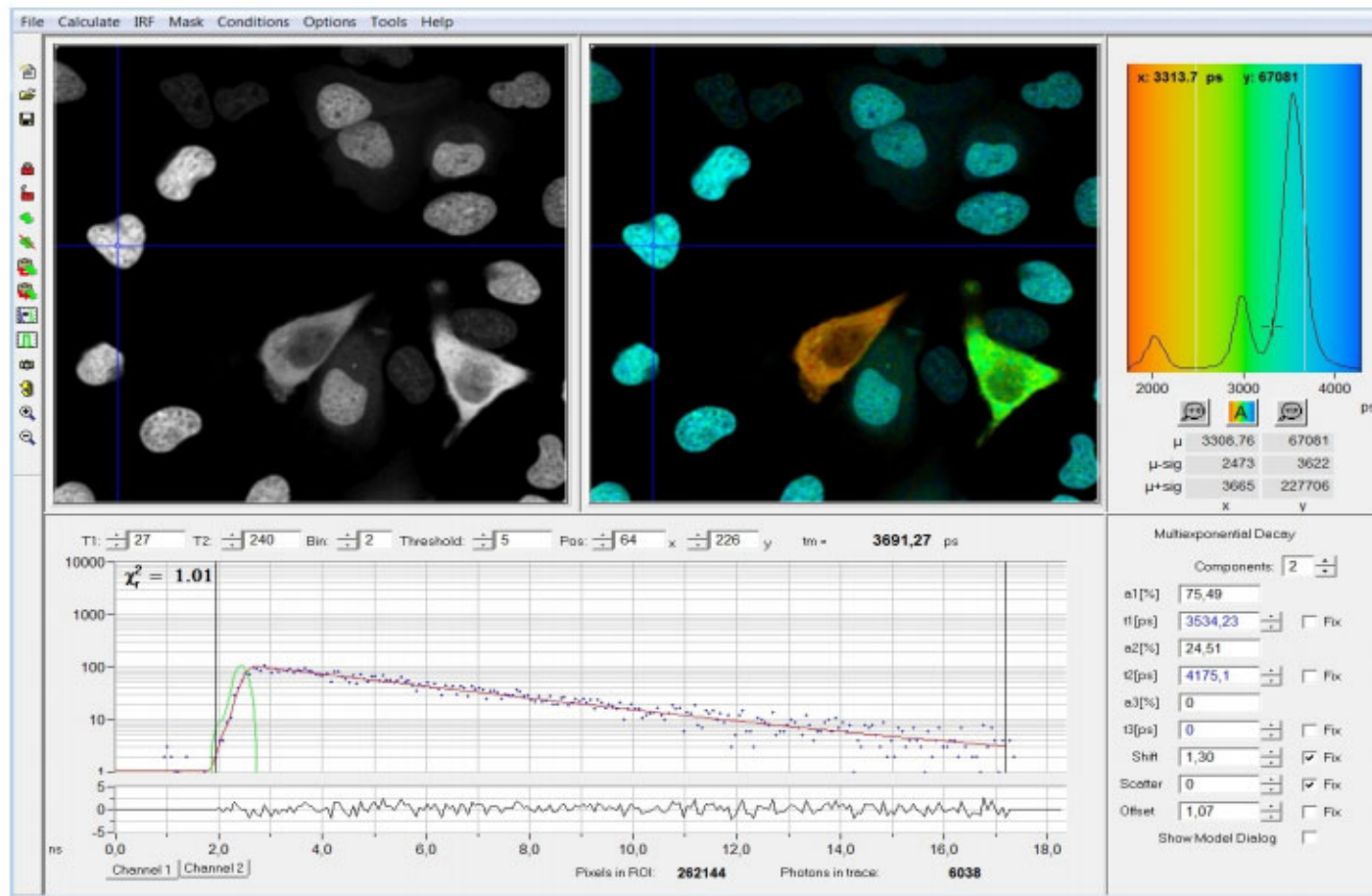


Fig. 2: SPCImage main panel. Intensity image, lifetime image, lifetime histogram, decay curve at selected position, and decay parameters at selected position. Recorded by bh Simple-Tau 152 FLIM system with Zeiss LSM 880.

Are other vendors of FLIM cards any better?

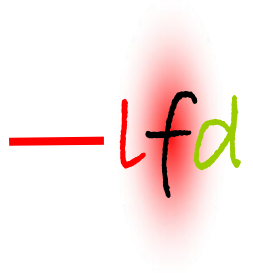
No, as far as I know. All the TCSPC cards have the same problem.

B&H show the problem, the other manufacturers hide it.

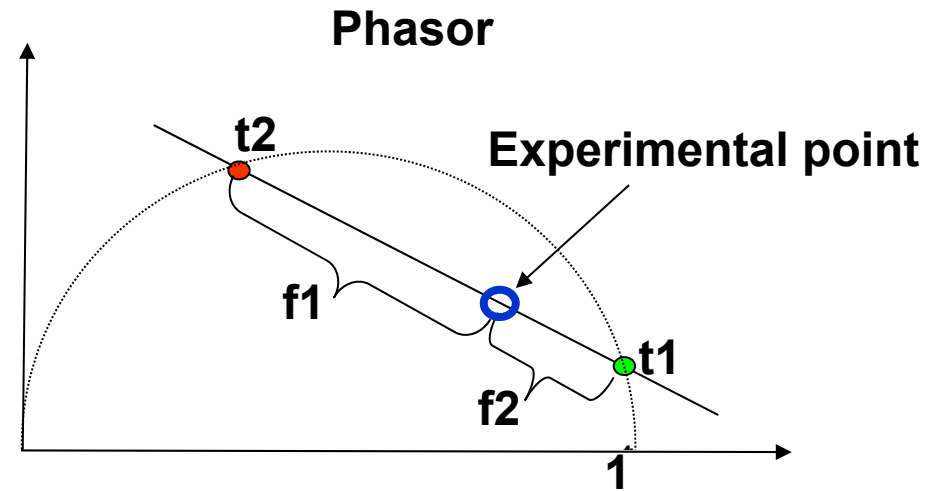
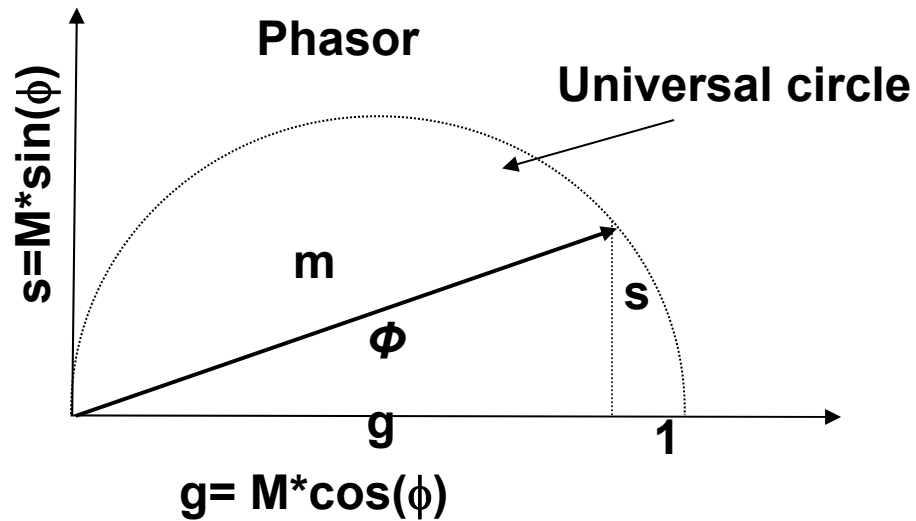
What is the consequence? You lose the fast and the slow decay.

So, long decay components do not appear in their instruments.

The results obtained with the Frequency domain FLIMbox cannot be replicated.



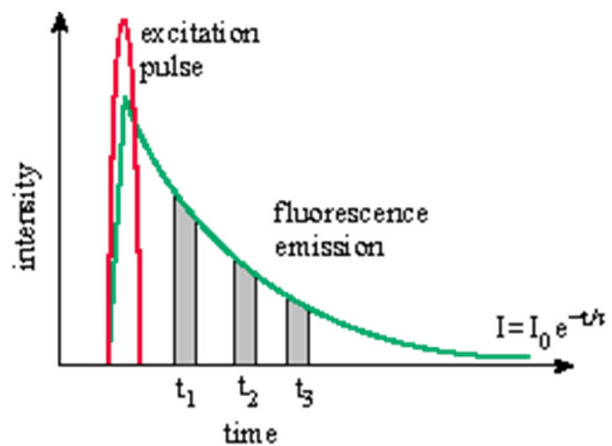
The algebra of phasors



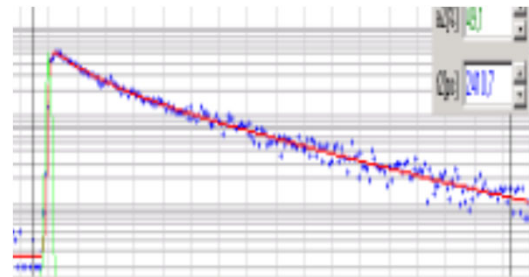
Simple rules to the Phasor plot:

- 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

From decay data

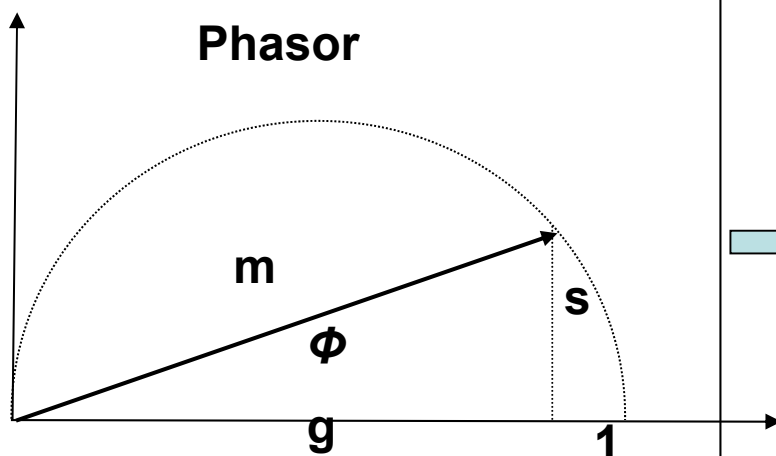


Fitting of exponentials

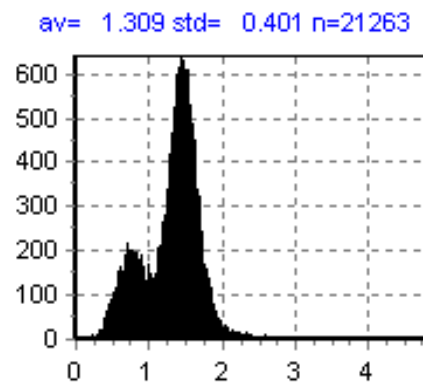


Multiexponential analysis

Phasor

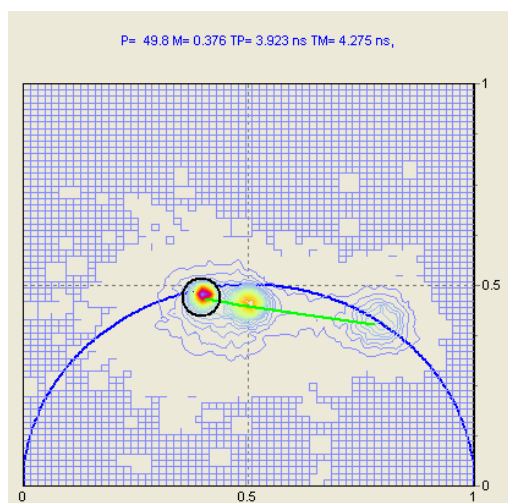
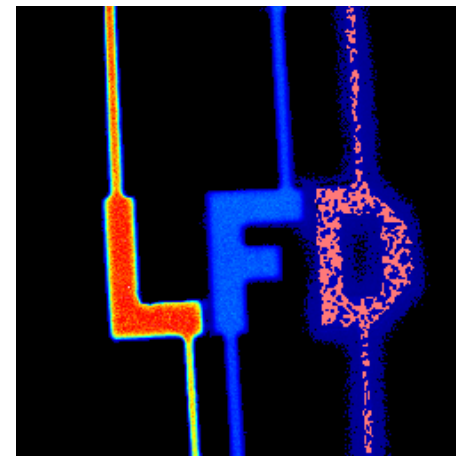
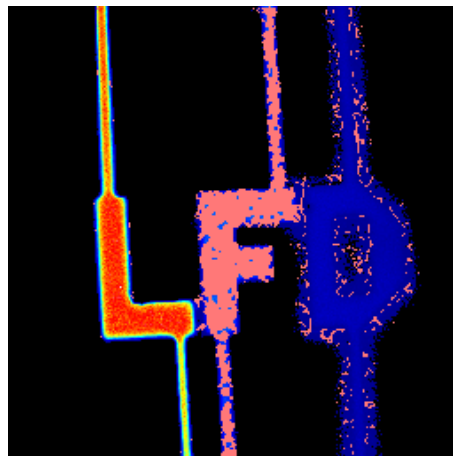
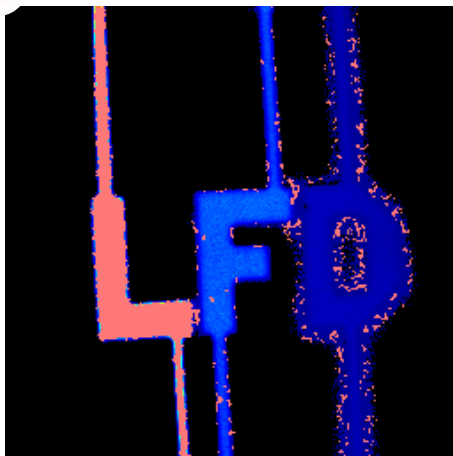


Lifetime distribution

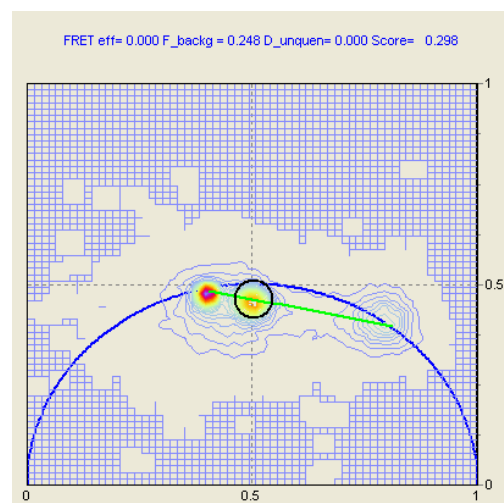


Separating Single exponential lifetimes using the phasor approach

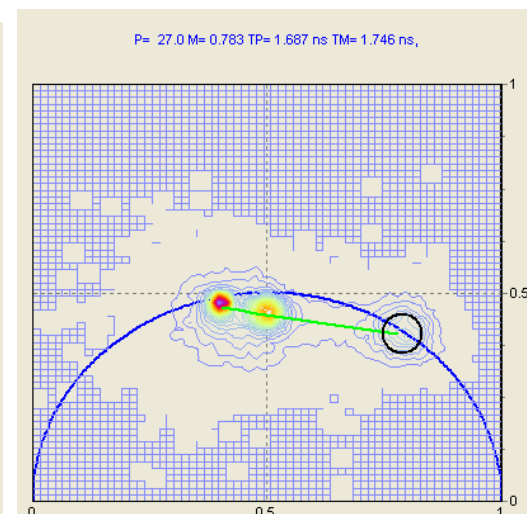
— *lfd*



Fluorescein



Mixture

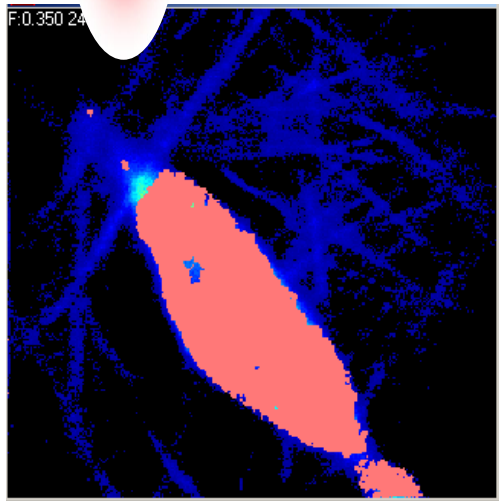


Rhodamine B1

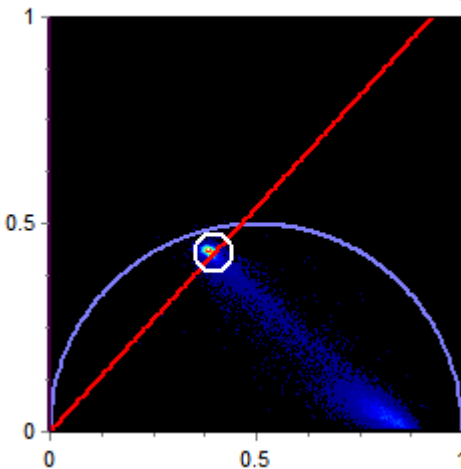


Pax-eGFP CHO-k1 in collagen

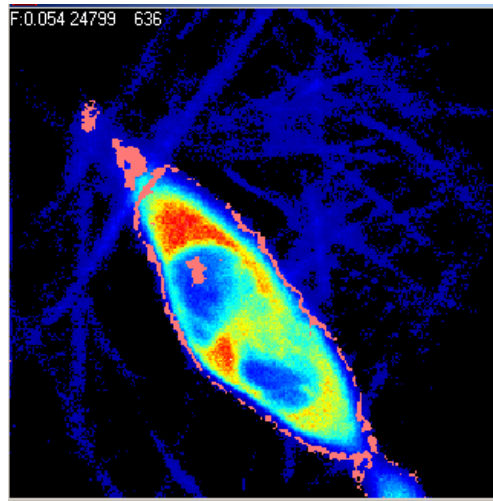
referenced with Fluorescein @ 905nm



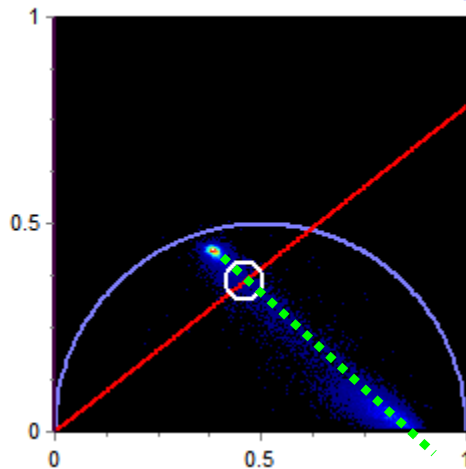
P= 47.2 M= 0.343 TP= 2.149 ns TM= 2.751 ns,



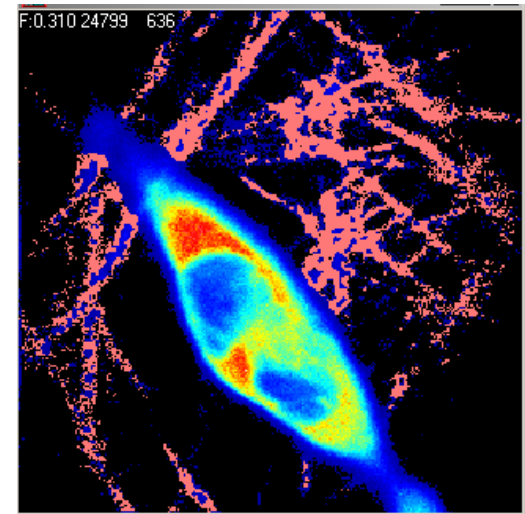
Lifetime of EGFP



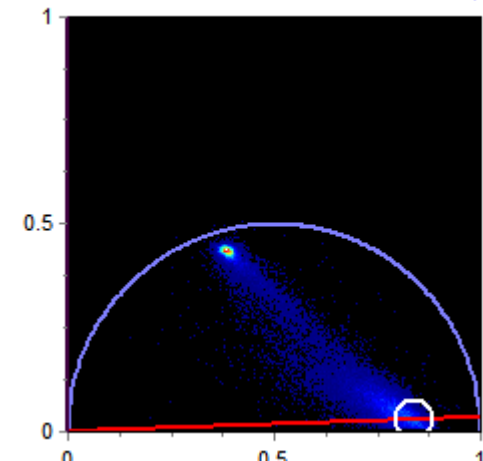
P= 38.2 M= 0.344 TP= 1.563 ns TM= 2.748 ns,



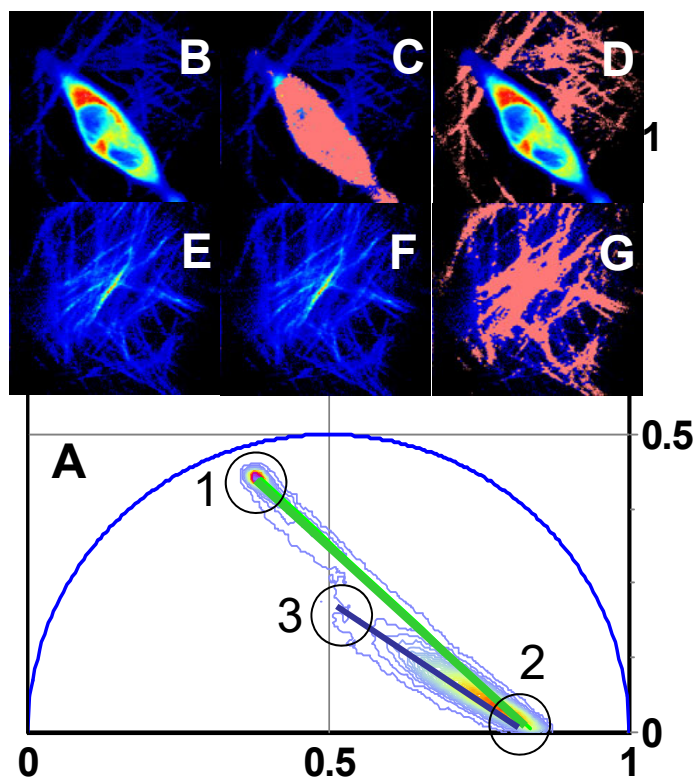
Combinations of Lifetimes

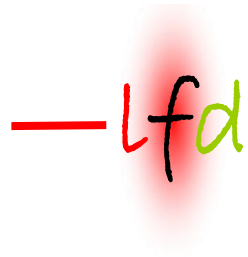


P= 2.0 M= 0.706 TP= 0.069 ns TM= 1.283 ns,



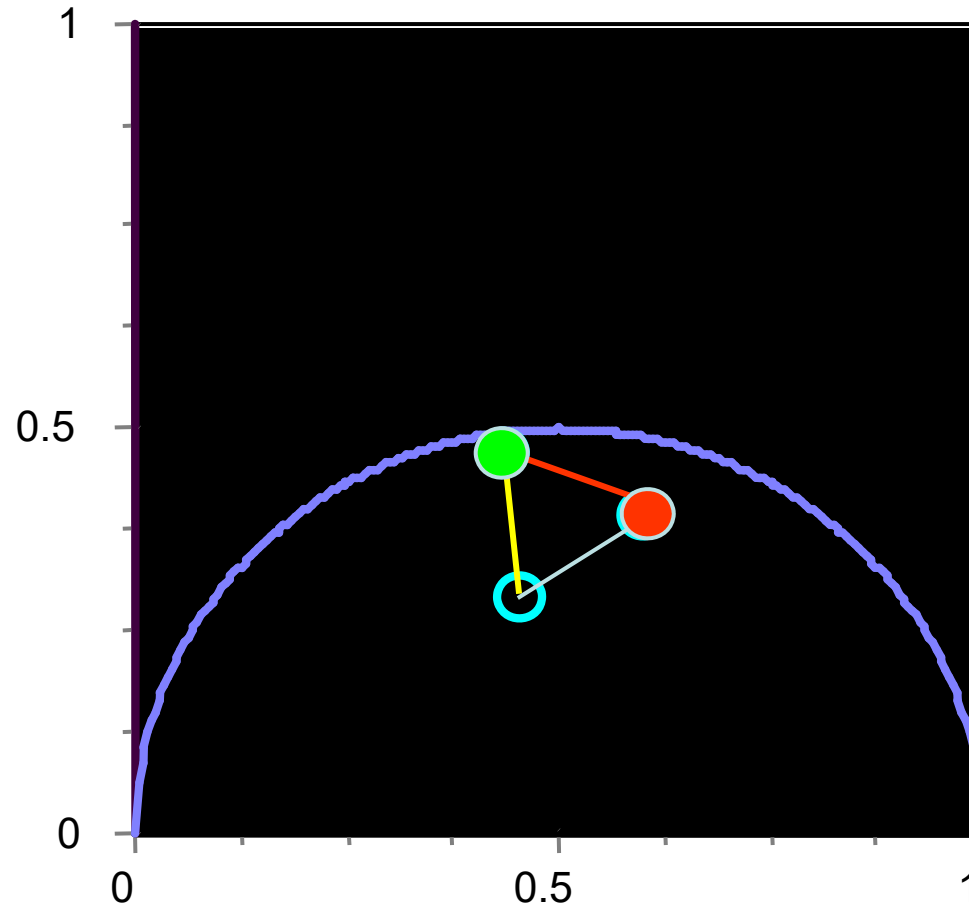
Lifetime of Collagen



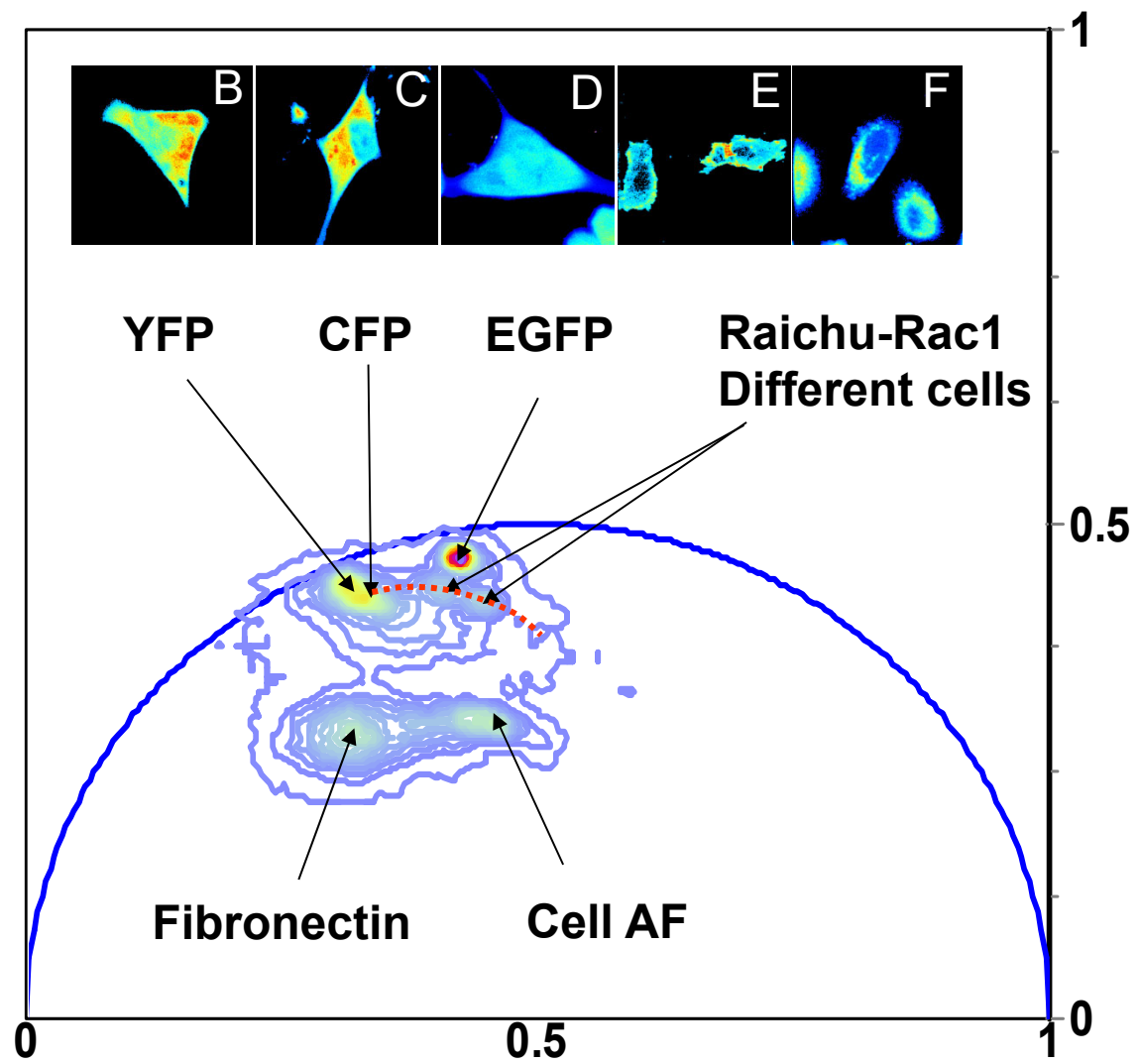


How to identify components?

Phasor Plot

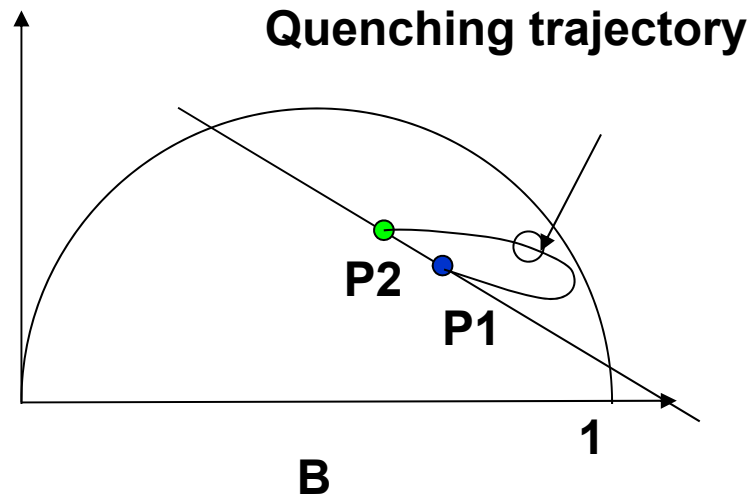
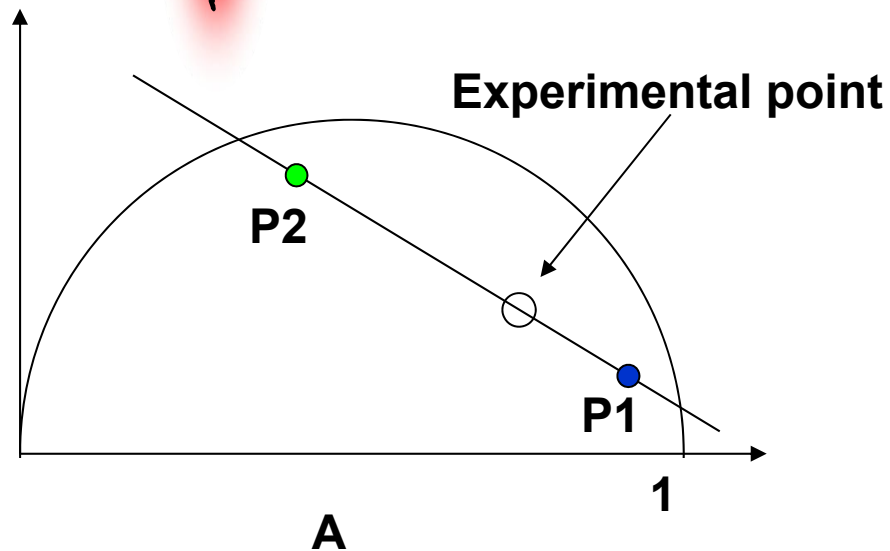


Phasors for common fluorophores. EGFP (green), autofluorescence (blue) and mRFP1 (red) (at 880 nm -2photon excitation). In any given pixel, mixture of EGFP and autofluorescence must be on the yellow line, mixtures of EGFP and mRFP1 must be on the red line. Mixtures of three of them must be inside the triangle with the corner in the 3 phasors.



How to distinguish two multi-exponential components from FRET?

— I_{fd}



Simple Rules for FRET:

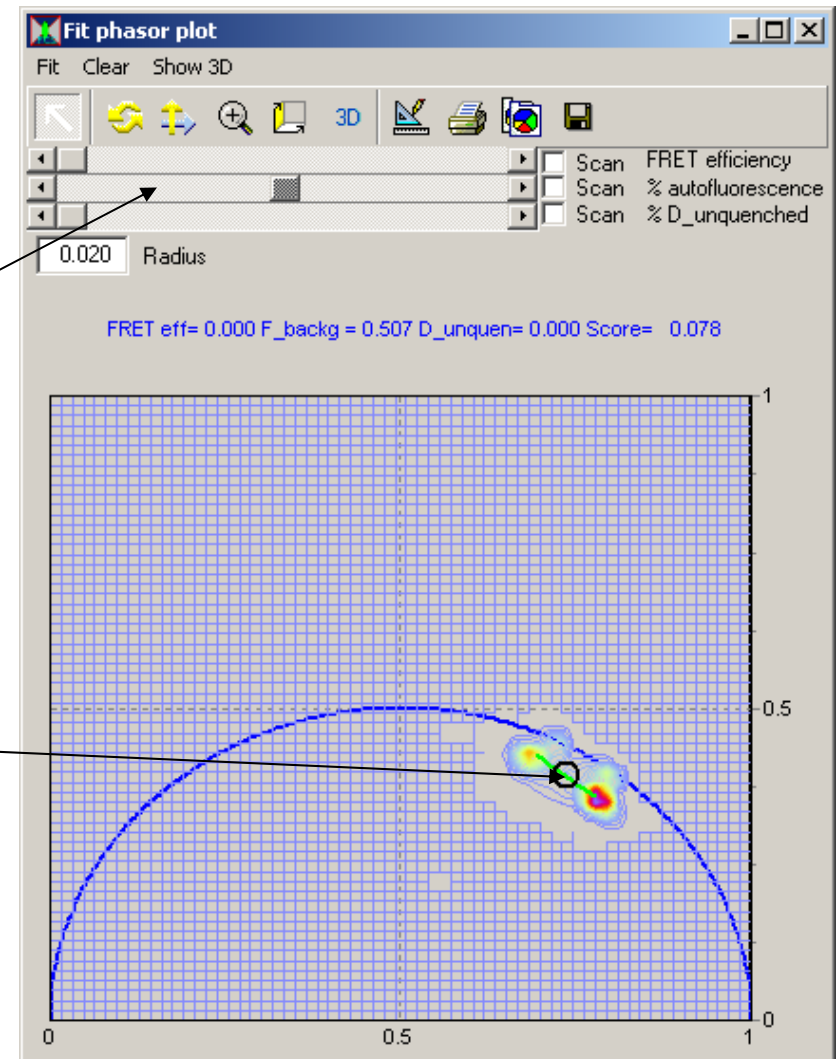
- 1) If the experimental point lies on a straight line then it is **NOT** FRET
- 2) FRET efficiencies follow a “quenching trajectory”
- 3) Quantitative FRET efficiencies can be obtained from the position on the quenching trajectory



The fractional intensity calculator

1. Click on the phasor plot (or enter the coordinates of the phasor manually) and assign the phasor to species 1.
2. Click to assign the phasor to the phasor 2.
3. The fractional (intensity weighted) contribution of the two phasors is calculated according to the sum rule of the phasor.

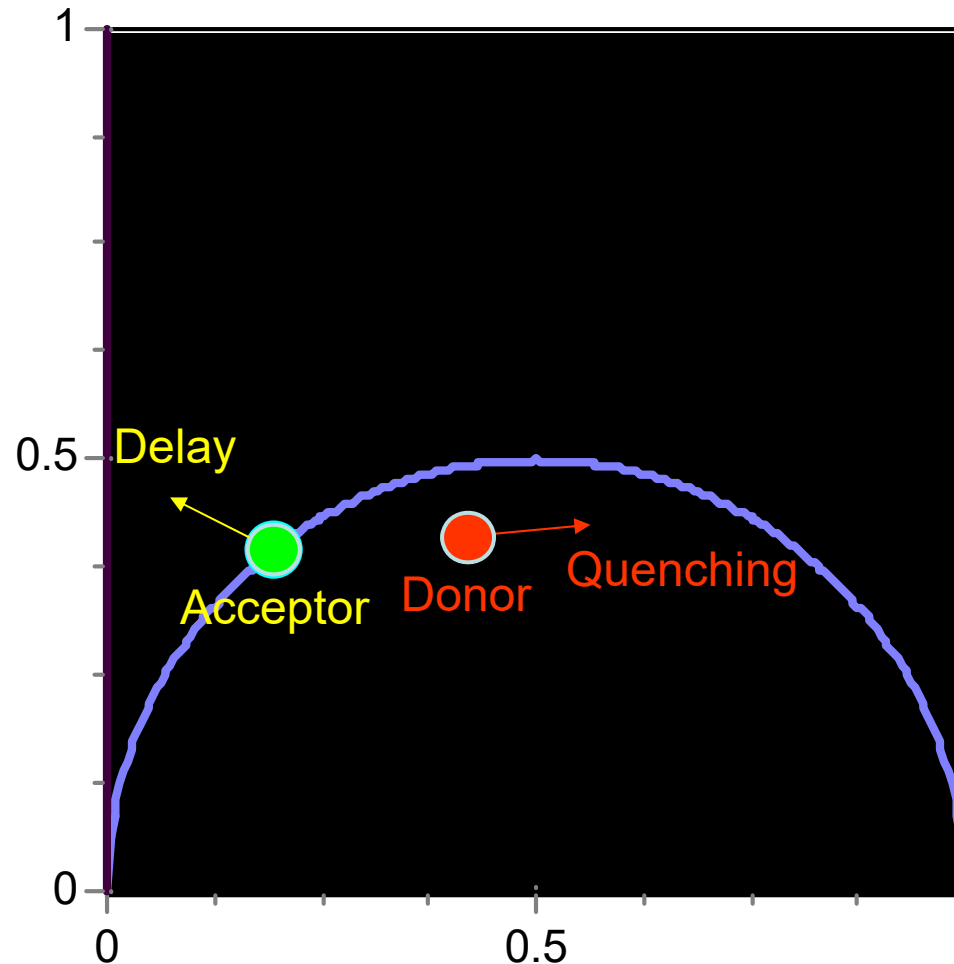
Moving this cursor, the circle will move in the phasor plot and automatically display the relative fraction of the two species (of the two phasors), independently on the number of exponential components necessary to describe the decay





How to identify processes?

Phasor Plot



Delay of the excitation of the acceptor due to FRET moves the acceptor phasor to the left (yellow arrow). If the delay is sufficiently long, the phasor could fall outside the semicircle. The donor phasor moves to the right (red arrow) due to quenching (shorter lifetime).

The principle of the calculator

Purpose: to generate trajectories in the phasor's plot corresponding to different processes

At present there are two functions programmed

The calculator for FRET efficiencies from

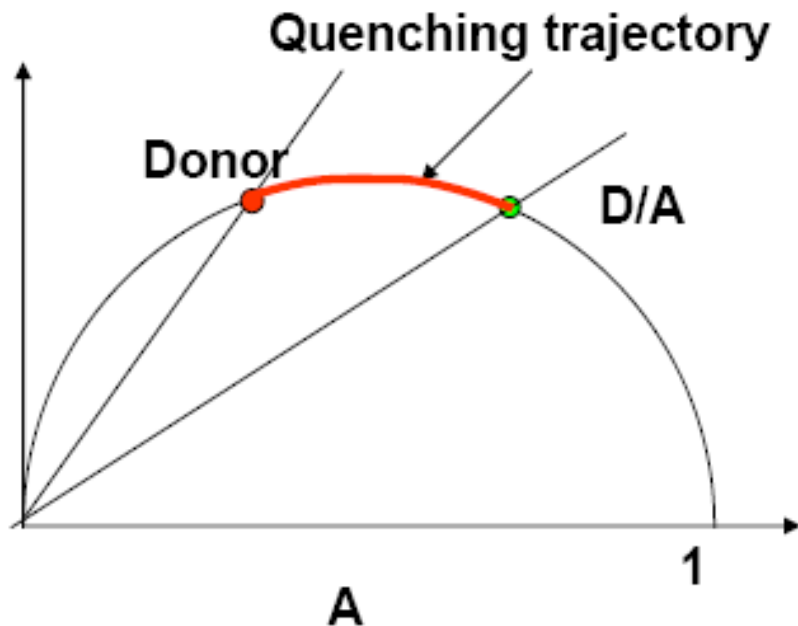
$$\text{FRET efficiency} = \frac{\tau_D - \tau_{\text{FRET}}}{\tau_D}$$

The calculator for ion concentrations from the relative contribution of the free and bound phasor.

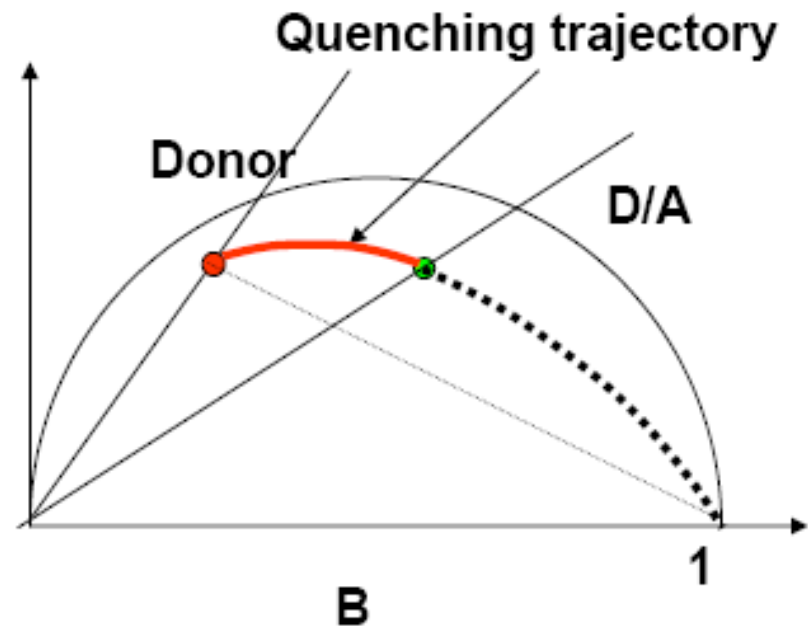
— τ_d

The FRET calculator

If we have a donor with a single exponential decay that is quenched by the presence of an acceptor. What should we expect?



The lifetime of the donor is quenched
The FRET efficiency can be calculated
by the ratio of the two lifetimes



The lifetime of the donor is along a
different “trajectory”, Why is the
trajectory an arc rather than a line to the
(1,0) point?



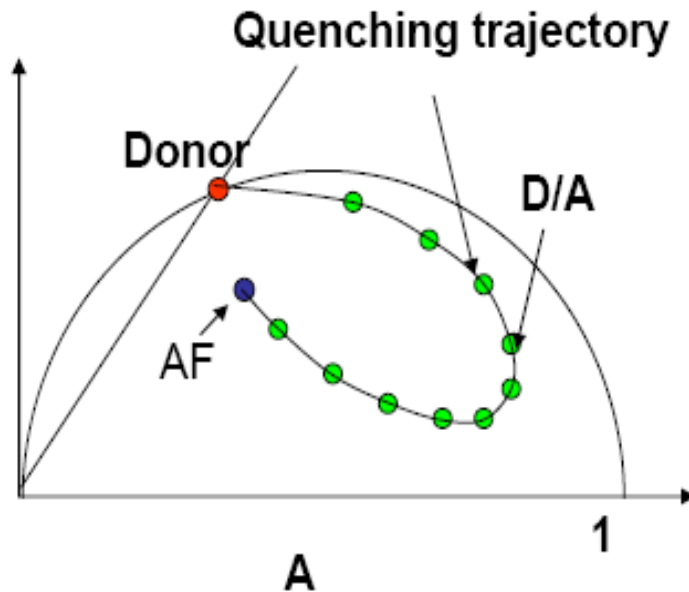
The FRET Calculator

- Can we quench up to zero lifetime?
- Even if we quench all the DONOR, we still are left with the autofluorescence.
- The final point is not at zero but at the autofluorescent phasor!!!

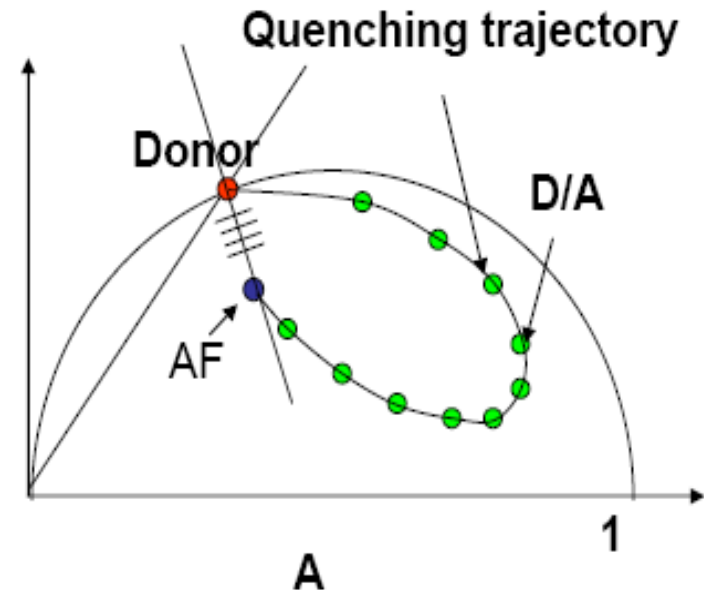


The FRET Calculator

After all the Donor is quenched, what is left? The cell autofluorescence!!



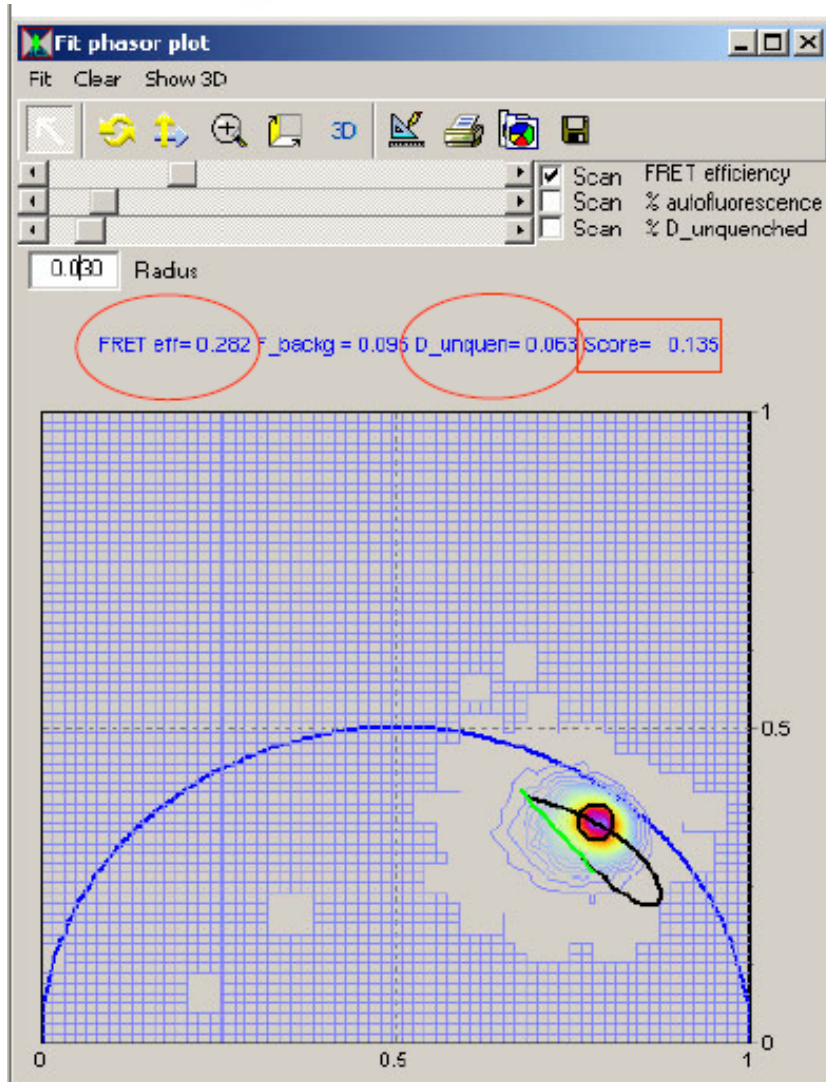
As the lifetime of the Donor is quenched, the phasor of the quenched Donor is added to the phasor of the autofluorescence



If there is a fraction of Donor that cannot be quenched, the final point will be along the line joining the Donor with the autofluorescence phasor



The FRET calculator

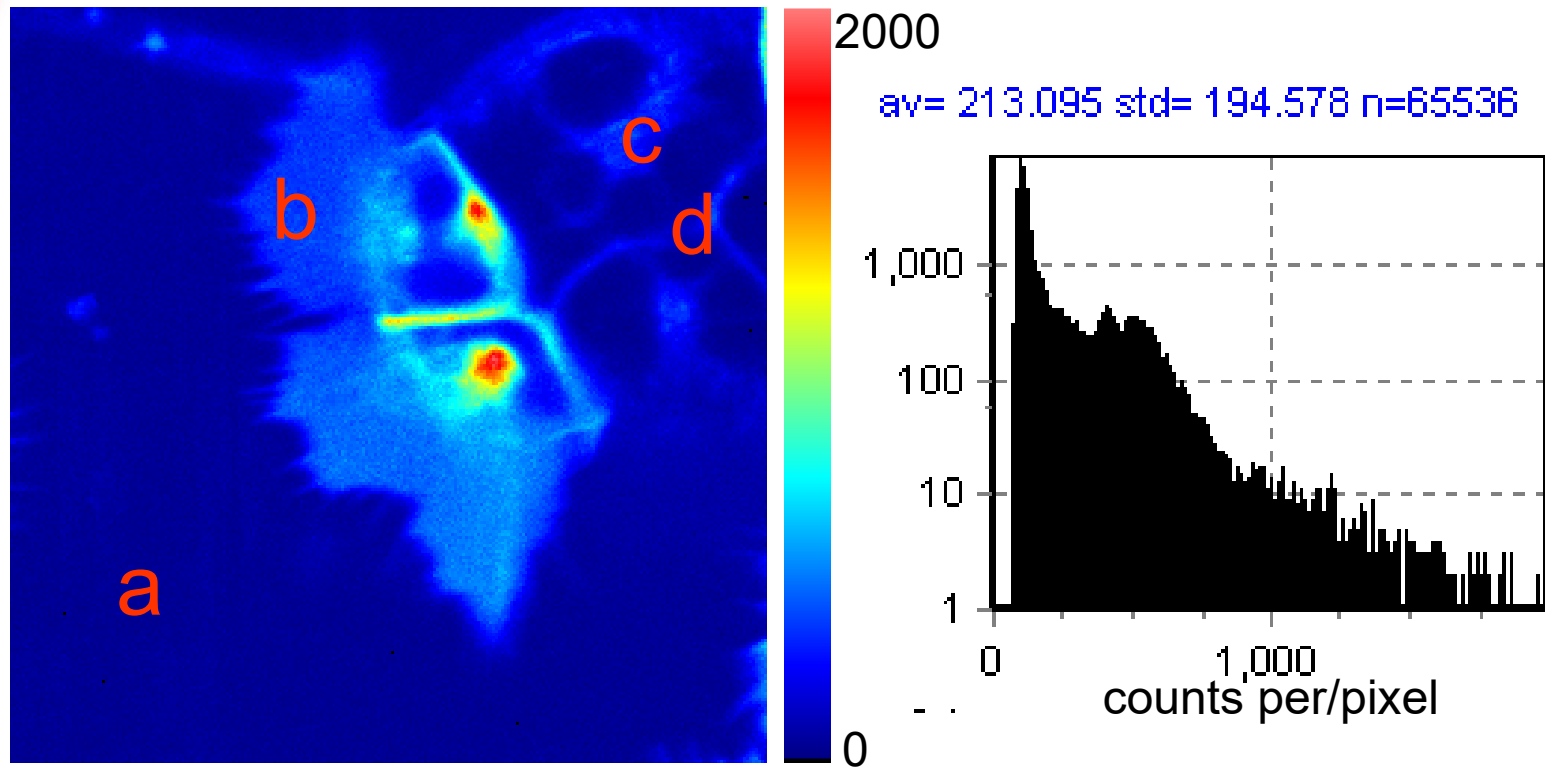


Information needed to calculate FRET:

- Donor phasor
- Autofluorescence phasor
- Amount of Donor that can't be quenched
- fractional contribution of autofluorescence and donor lifetimes



Example of FLIM analysis using phasors



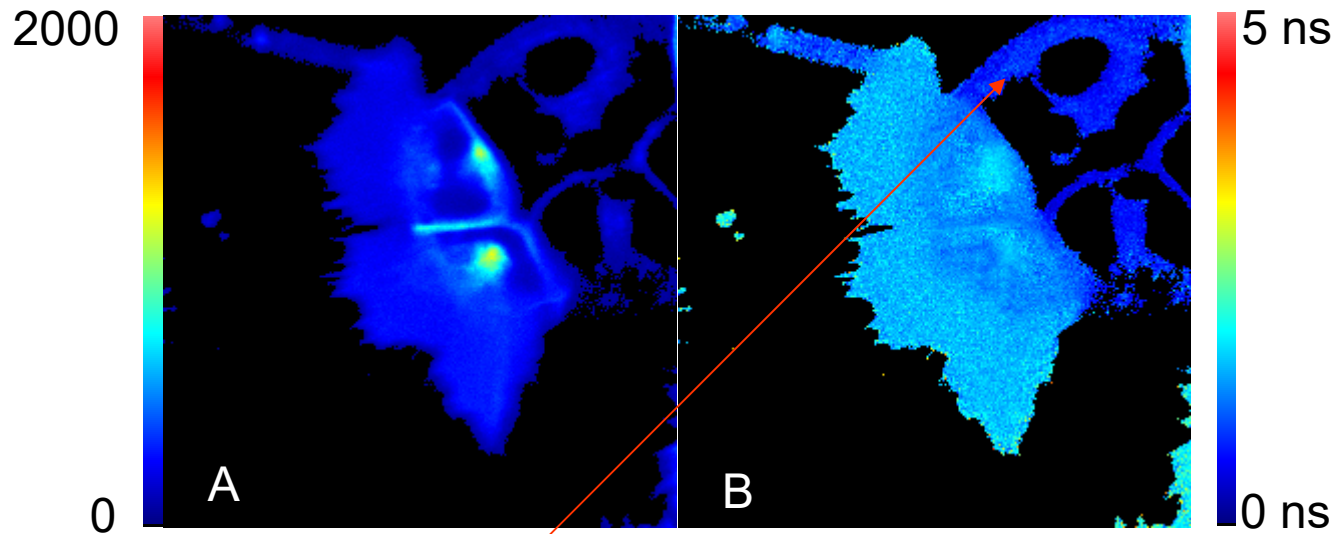
Several regions the image can be identified corresponding to **a**) background (2 exponentials) **b**) cell 1 bright (2 exponentials) **c**) cell 2 dim, **d**) cell junctions dim.

Image of cell expressing uPAR-EGFP and uPAR-MRFP receptor. Upon addition of a ligand, the receptor aggregates. FRET should occur at the cell junctions

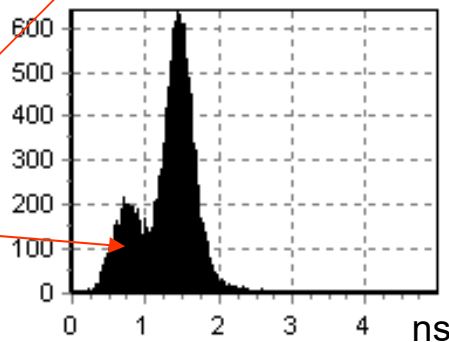


The pitfall of “conventional” FLIM analysis

image obtained using B&H 830 in our 2-photon microscope



av= 1.309 std= 0.401 n=21263



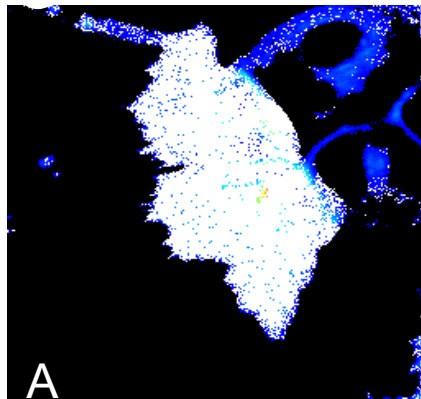
Shorter lifetime region could be interpreted to be due to FRET

Donor+acceptor+ligand. A) intensity image after background subtraction, B) τ_p image

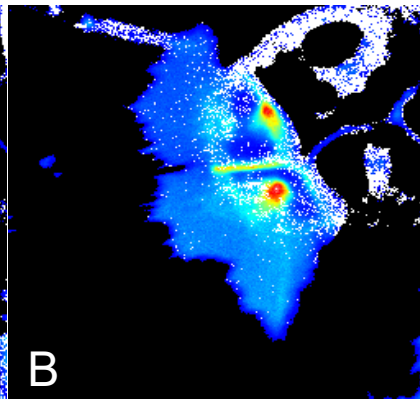
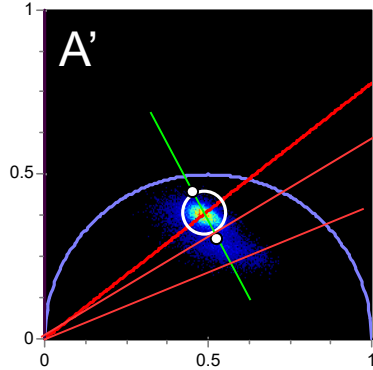


Identification of FRET using the phasor plot

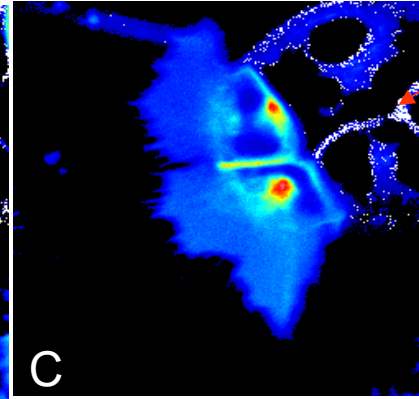
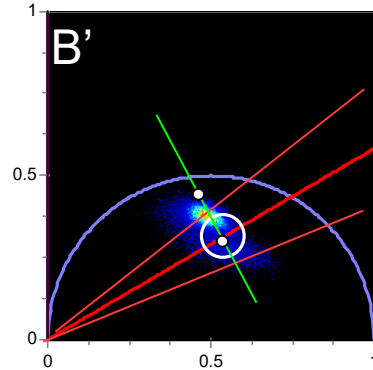
2000
0



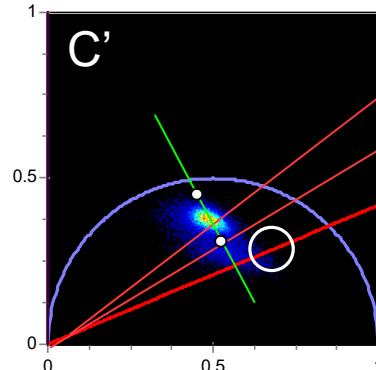
$P= 37.9$ $M= 0.386$ $TP= 1.549$ ns $TM= 2.509$ ns,



$P= 30.2$ $M= 0.389$ $TP= 1.159$ ns $TM= 2.494$ ns,

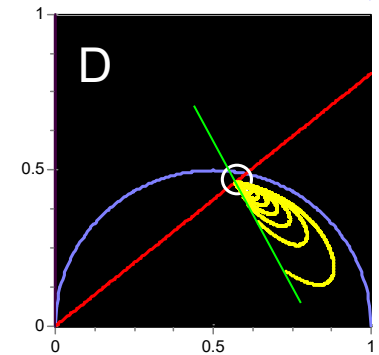


$P= 22.8$ $M= 0.543$ $TP= 0.834$ ns $TM= 1.825$ ns,



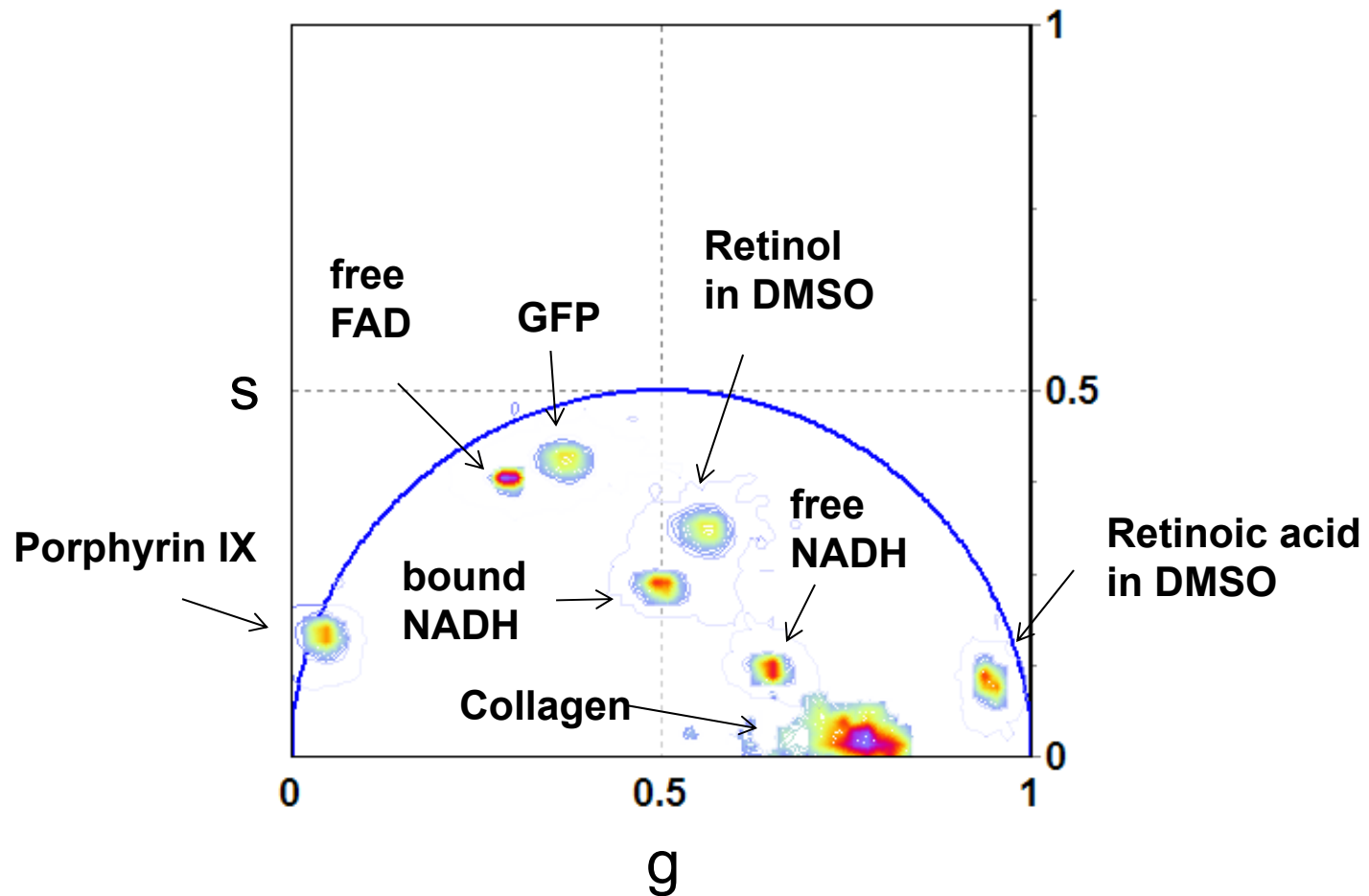
FRET only
occurs at the
cell junctions

$P= 39.0$ $M= 0.553$ $TP= 1.614$ ns $TM= 1.788$ ns,



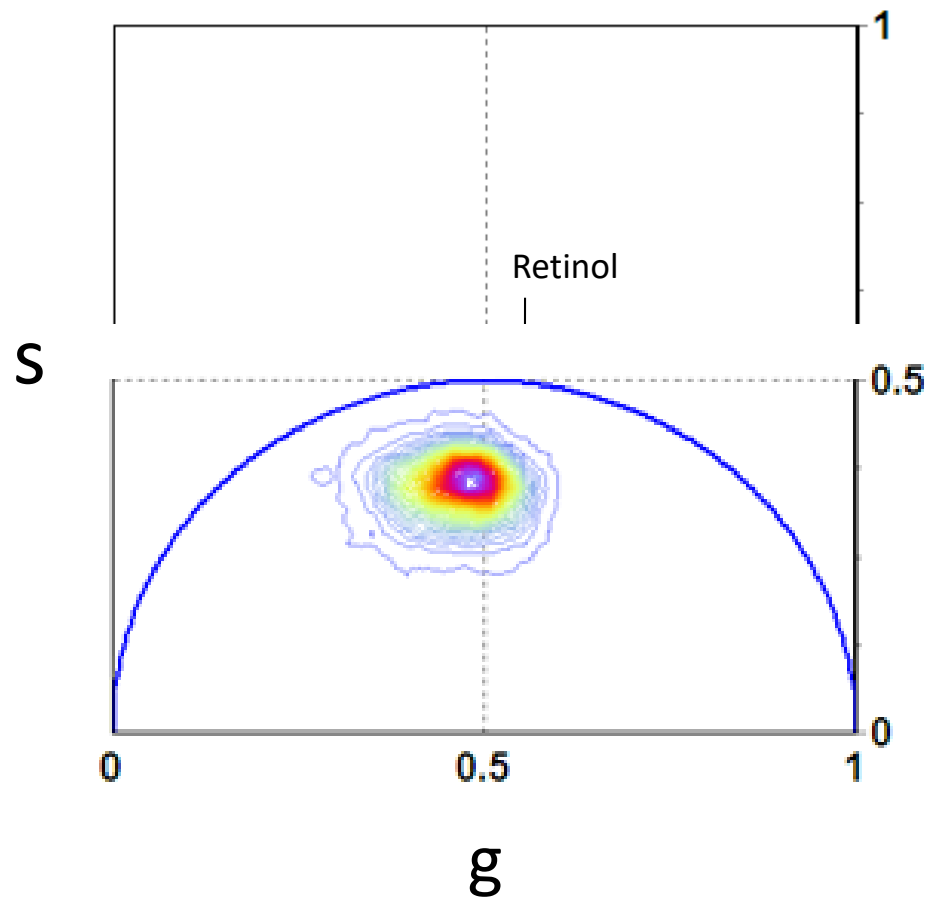
Selecting regions of the phasor diagram. Selecting the region in **A'** (donor +acceptor) the part in white lights up (**A**). Selecting the region in **B'** (autofluorescence) the part in white in lights up (**B**). The color scale in **B'** has been changed to better show the region of the autofluorescence. Selecting the region in **C'** (along the donor quenching line as shown in **D**) the part in white in at the cell junction lights up in **C**.

Phasor Fingerprint of pure chemical species....



Label free FLIM in Living tissues

Intrinsic Fluorophores



✓ Extracellular

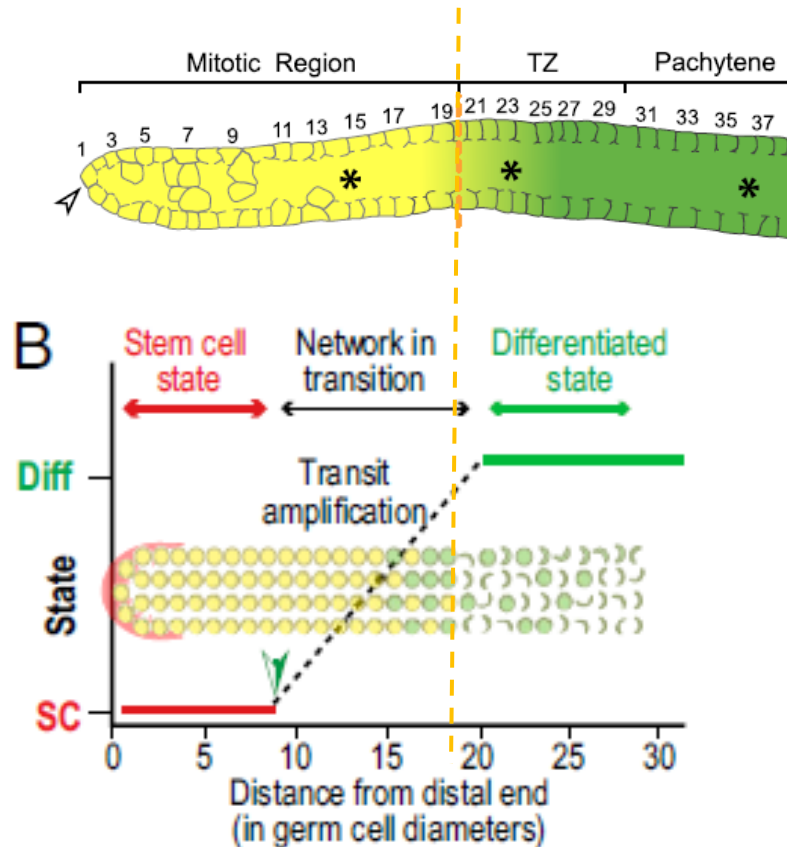
✓ Cellular

✓ NADH and FAD: metabolic coenzymes involved in oxidative phosphorylation and glycolysis.

✓ Complex distribution of autofluorescence

✓ Redox ratio ($\text{NADH}/\text{NAD}^+ \sim \text{free/bound NADH}$) reports on metabolic changes associated with cell carcinogenesis and differentiation

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



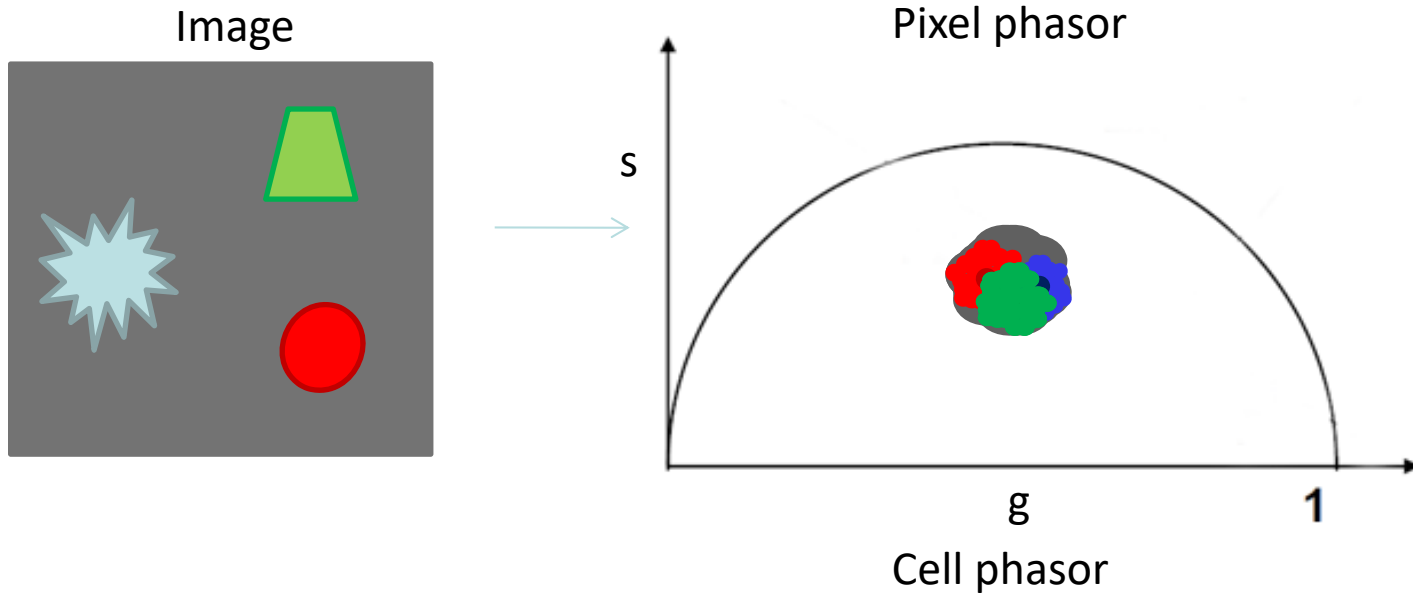
Mitotic region : stem cells niche

- ✓ 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)

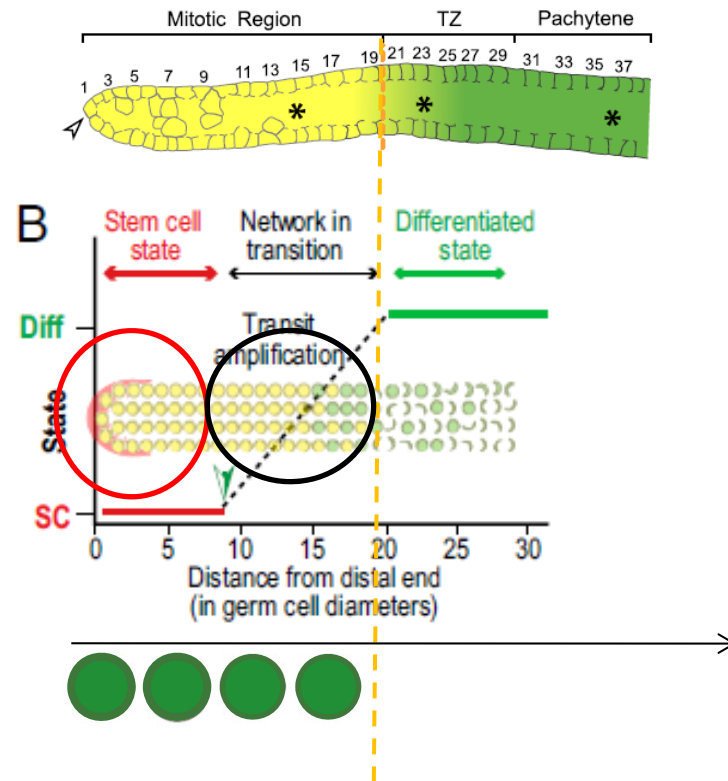
Meiotic pachytene region: cells that have further progressed through meiosis.

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 same 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 μ s,

SimFCS software

Mitotic region : stem cells niche

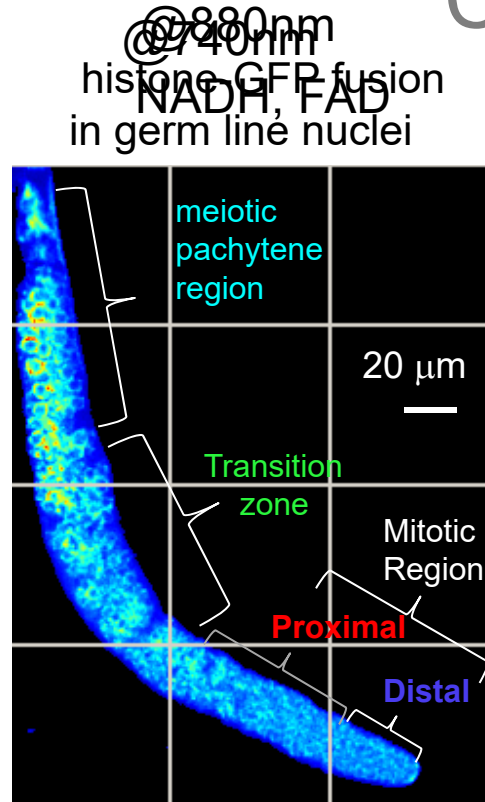
✓ **Distal pool**: undifferentiated cells maintained in a “stem cell-like state”

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

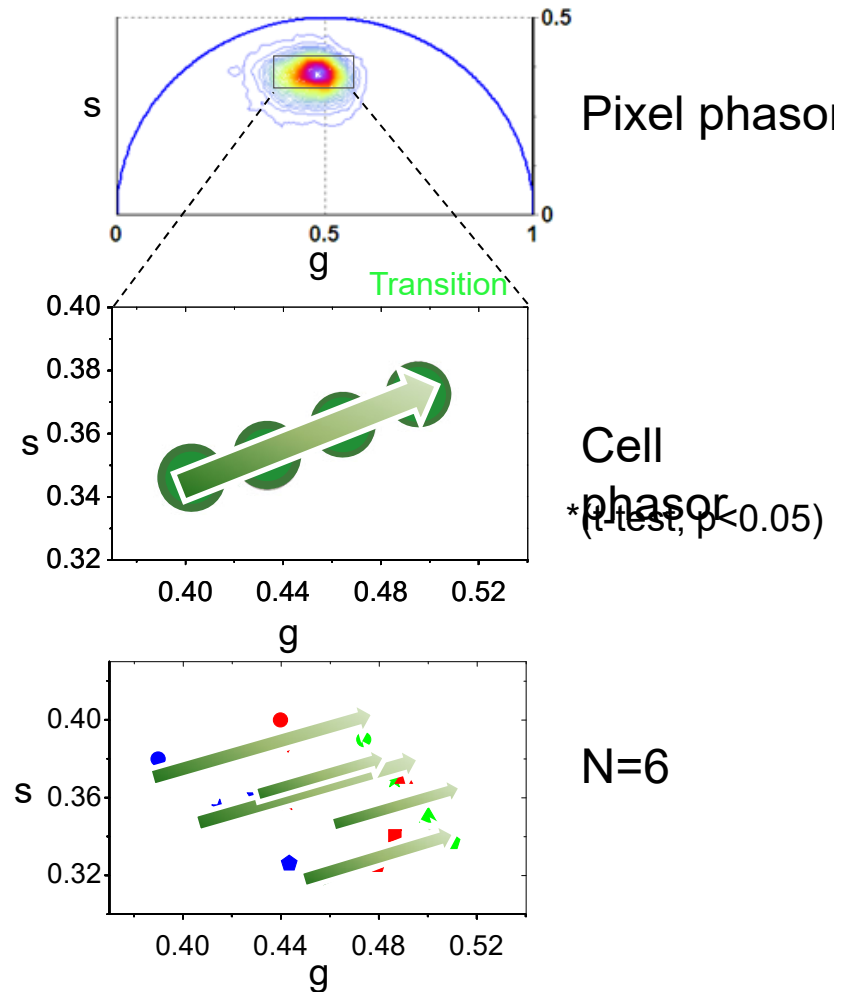
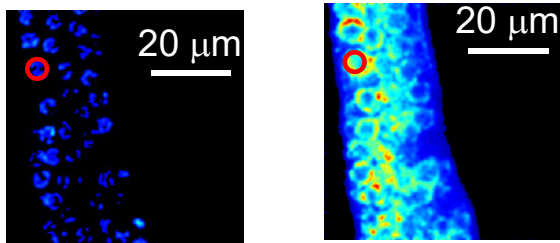
Transition zone : cells that have **differentiated** and entered meiotic prophase (crescent-shaped DNA)

Meiotic pachytene region: progression through meiosis.

Stem cell metabolic “states” in *C.elegans*



Cell selection



In the large phasor cluster we distinguish statistically different subclusters

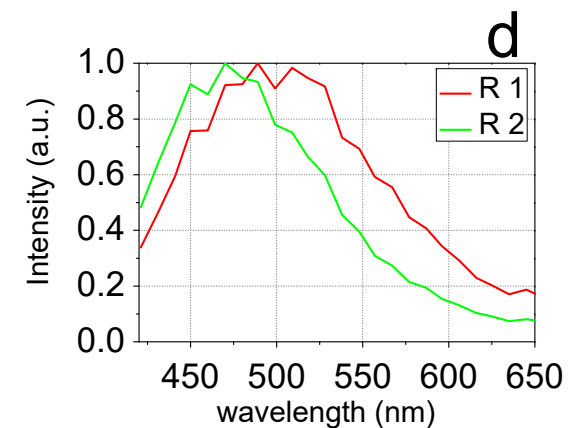
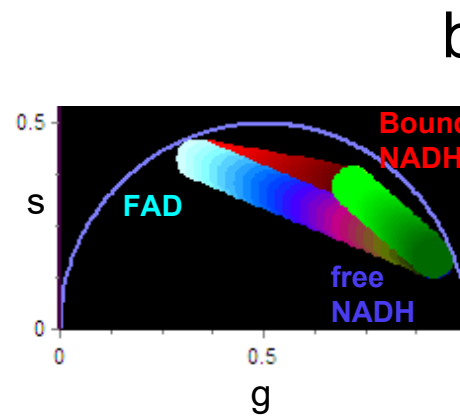
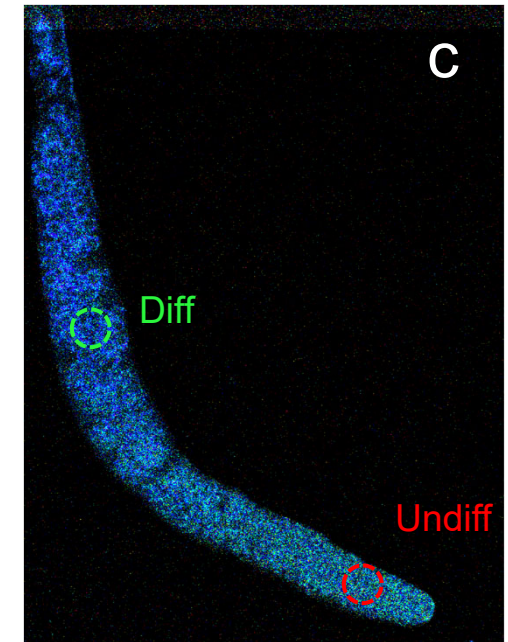
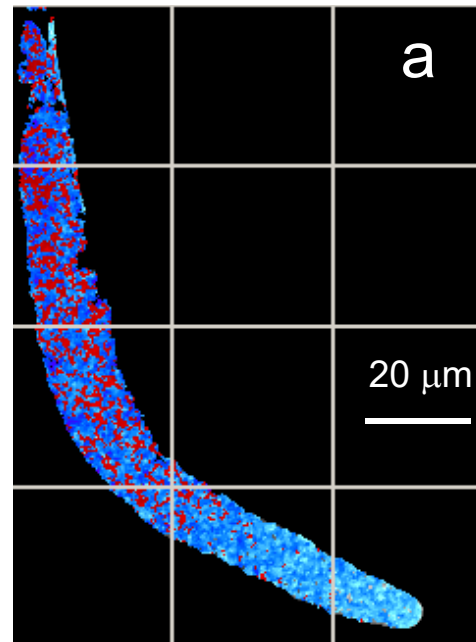
Mapping relative concentration of metabolites

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

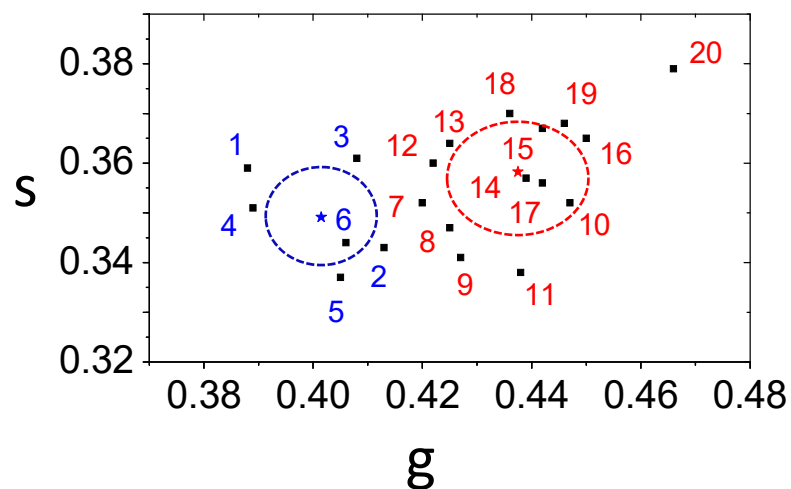
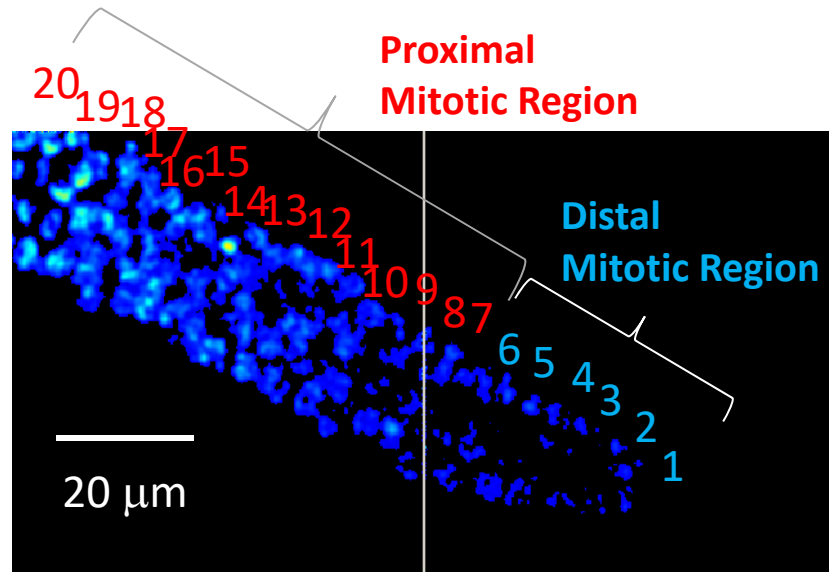
✓ Growth factors that promote self-renewal cause stem cell to become more reduced.

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

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



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)



Conclusions

By representing “molecular species” rather than summing exponential decays the phasor approach reduces the problem of fitting exponential components and exploration of regions of the phasor plot

The analysis of the trajectory in the phasor plot provides a quantitative resolution of “processes” such as linear combination of two (more species) and the calculation of FRET efficiencies by simple arithmetic.

You do not need to be an expert spectroscopist to resolve the molecular species present and to calculate ion concentration or FRET efficiencies.

It globally analyze many cells (different experiments) simultaneously

IT IS A RADICALLY DIFFERENT APPROACH