The Phasor approach: Application to FRET analysis and Tissue autofluorescence

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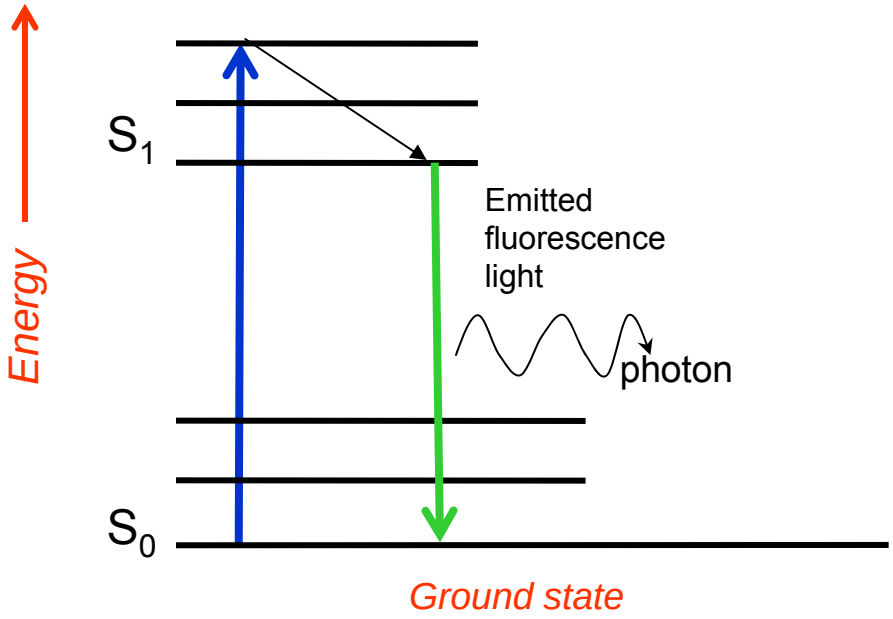
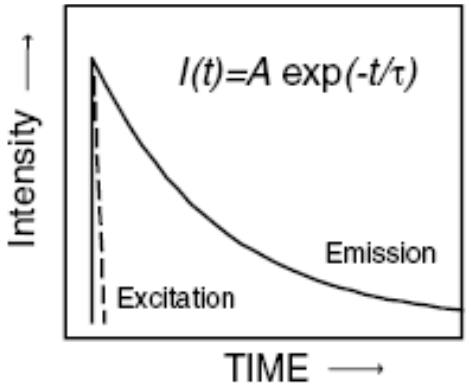
Outline

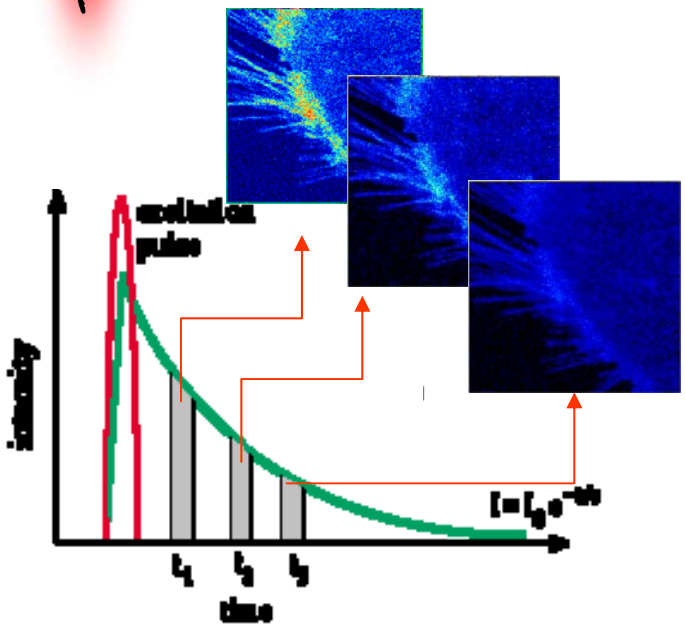
- Background: Lifetime
- Intro to Fluorescence Lifetime Imaging Microscopy
- Motivation for FLIM
- The Phasor approach
- Rac Activation using FLIM
- Conclusions

Lifetime: Background



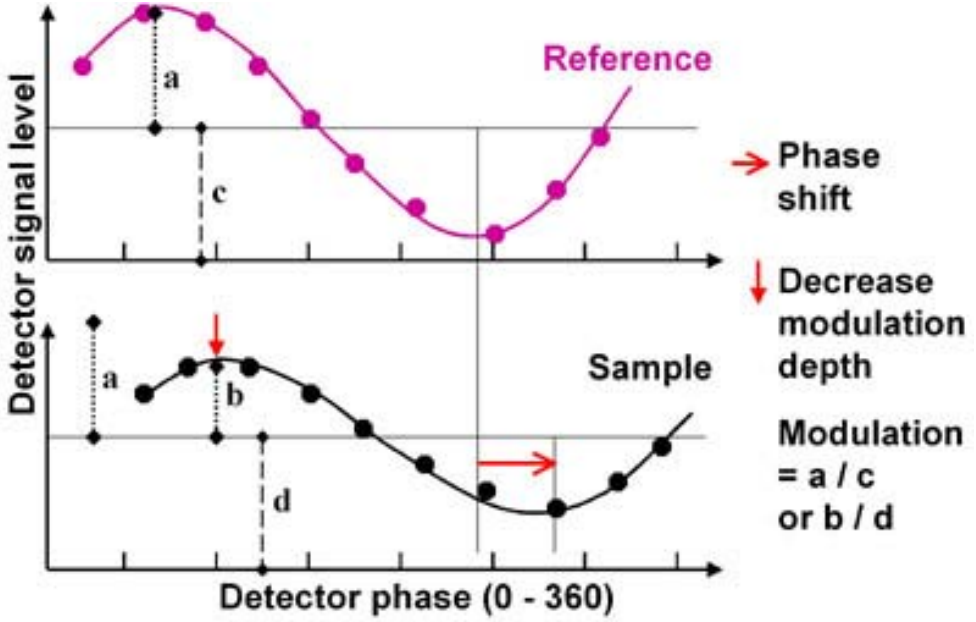


Time Domain and Frequency Domain FLIM



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 O_2 concentration in the cell or in tissues**

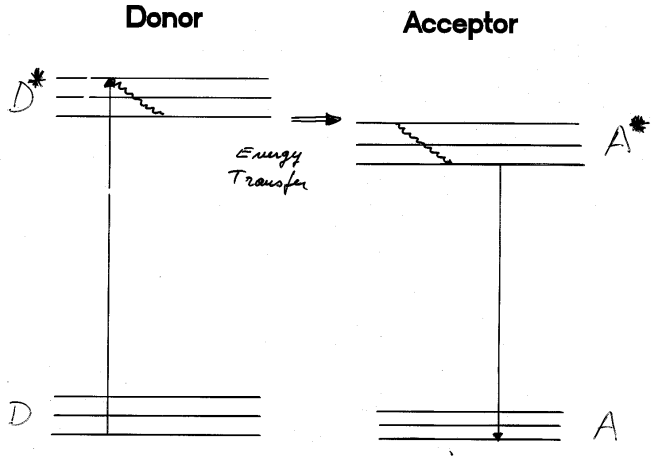
Förster Resonance Energy Transfer (FRET) (T. Förster,1949)

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.

$$D^* + A \underset{k_{-T}}{\overset{k_T}{\rightleftharpoons}} D + A^*$$

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



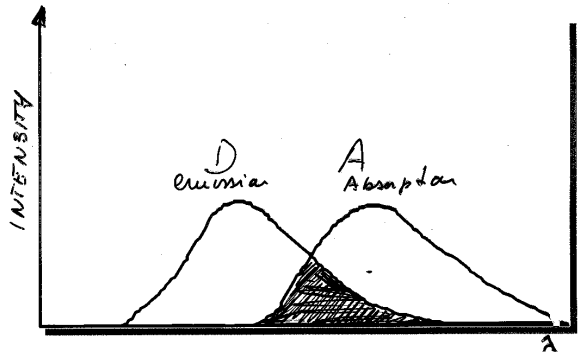
Because of rapid internal conversion the process A* to D* is very unlikely to occur unless the donor is of the same kind of the acceptor (Homotransfer).
FRET strongly depends on the distance between the two groups.
The theory was developed by Förster in 1949 who calculated the rate of transfer to be

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

τ_D is the lifetime of the donor in the absence of the acceptor.
R is the distance between the two groups
Ro is called the Föster characteristic distance

$$R_0 = 9.7x10^3\left(J\kappa^2 n^{-4}\Phi_D\right)^{1/6}$$

n is the refractive index of the medium
Φ_D is the quantum yield of the donor
k^2 is a complex geometrical factor which depends on the relative orientation of donor and acceptor.



J is a measure of the spectral overlap

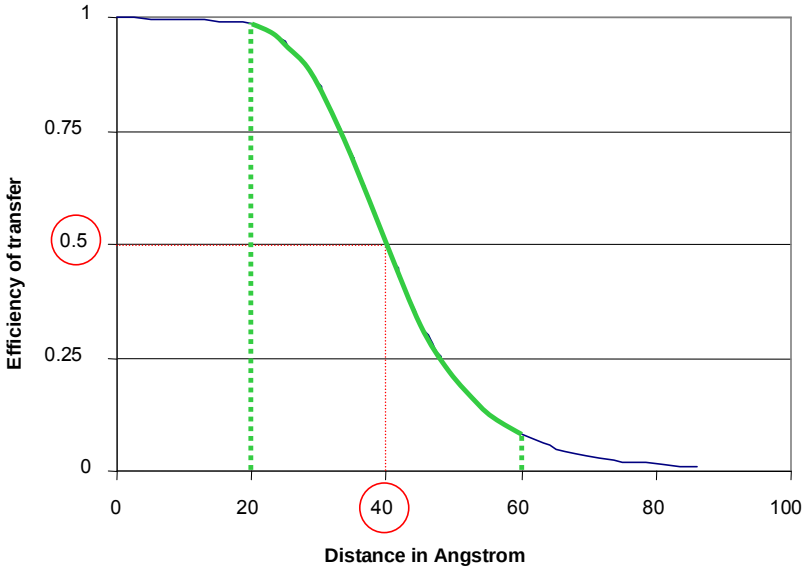
Energy transfer studies give information
•Distance between groups
•Orientation of two groups
Notice that D and A can be the same kind of molecule provide emission and absorption overlap.

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 ~0.5 R_0 and ~1.5R_0

Energy transfer efficiency (E)

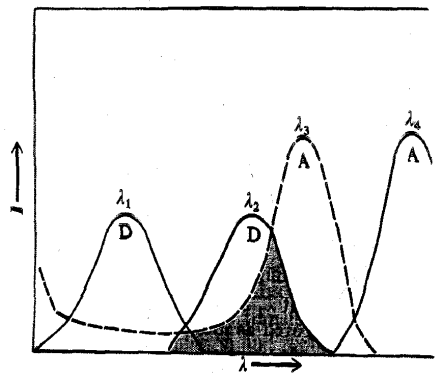
$$E = \frac{k_T}{k_T + \sum_{i \neq T} k_i}$$

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

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

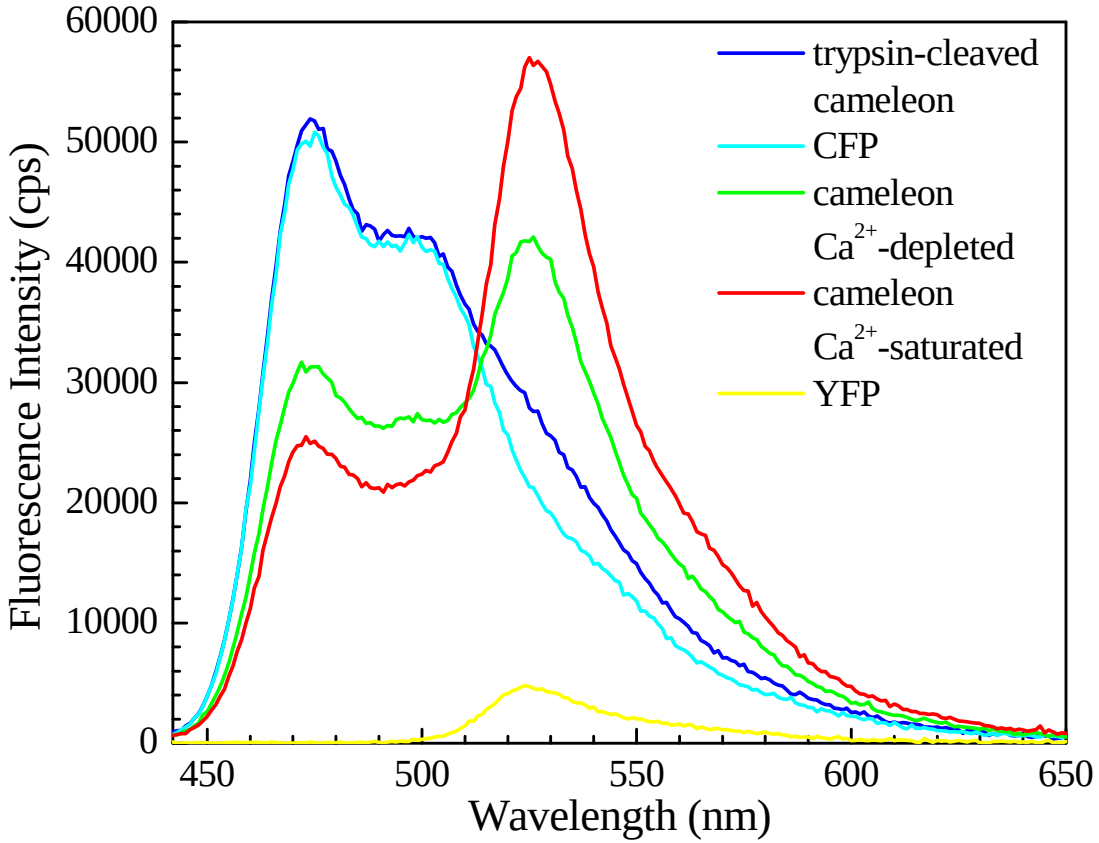
In presence of the donor the total fluorescence intensity at λ_4 is

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

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.

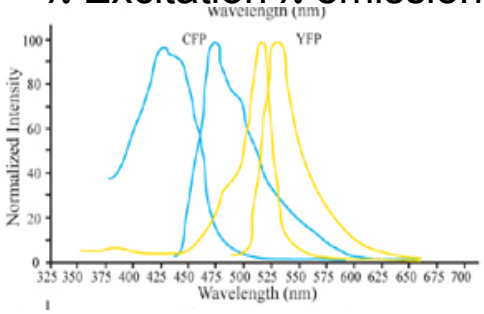
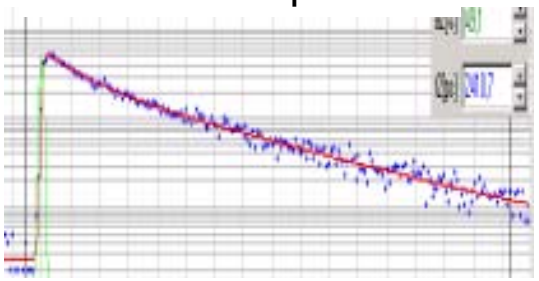
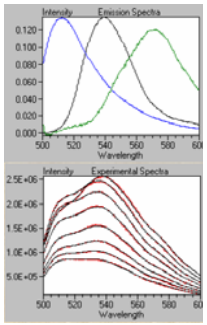
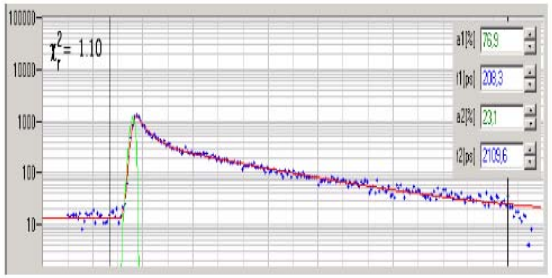
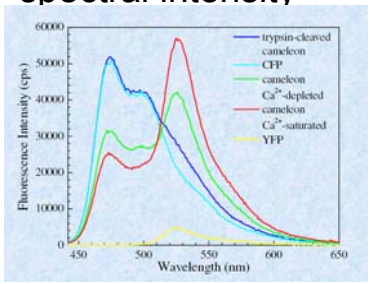
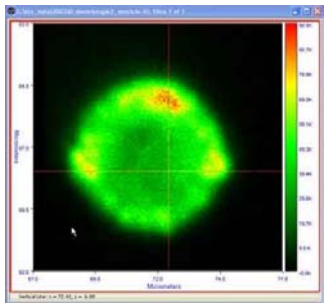
$$\frac{\tau_{D+A}}{\tau_D} = 1 - E$$



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	λ Excitation λ emission 	Lifetime Components 
2) Demixing of multiple species in a pixel	Spectral demixing 	Multiexponential analysis 
3) Identification of processes: FRET	Ratio of acceptor/donor spectral intensity 	Quenching of donor lifetime 

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!

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.

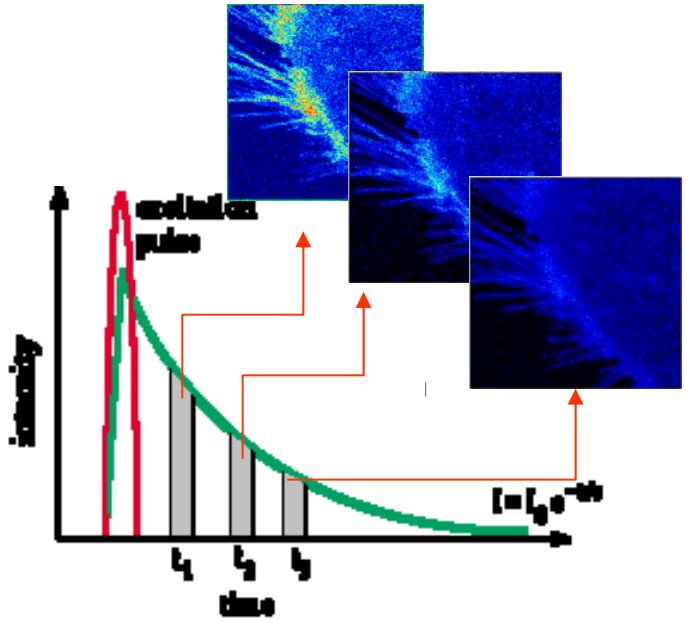
We need to go to a new “space”

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

This is what we need: the phaser!

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

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



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

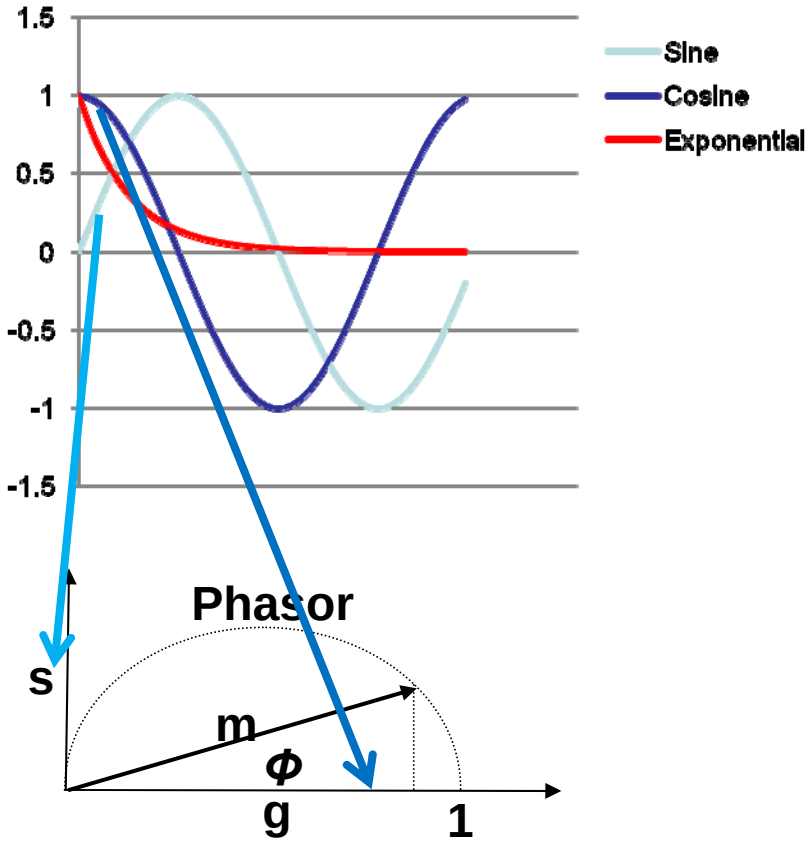
The interval between the excitation flashes, and 1st excited photon is measured

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

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

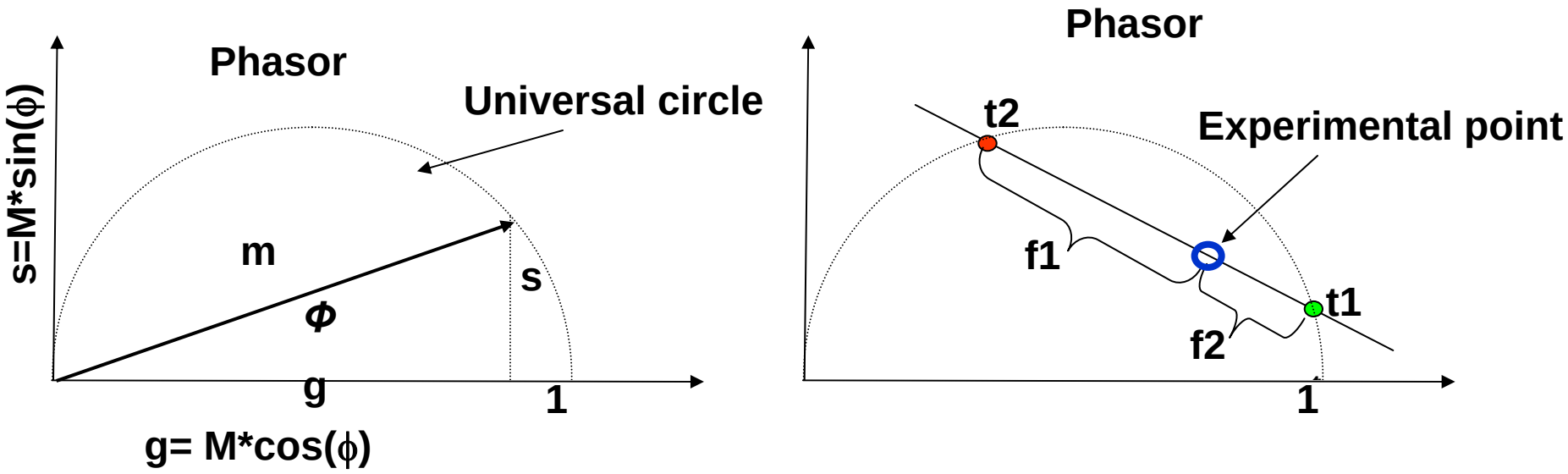
$$s_i(\omega) = \int_0^\infty I(t)\sin(\omega t)dt \,/\, \int_0^\infty I(t)dt$$

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



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

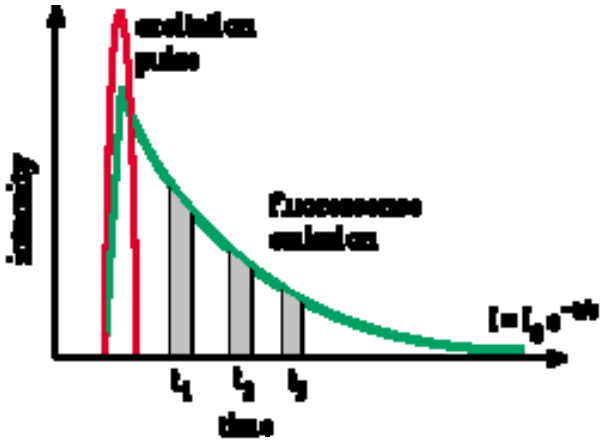
The algebra of phasors



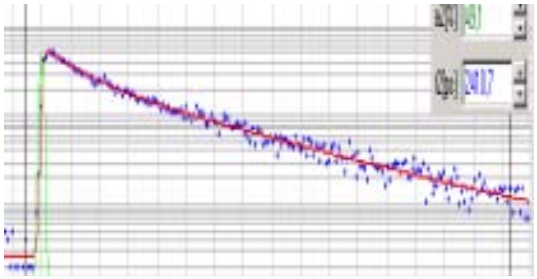
Simple rules to the Phasor plot:

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

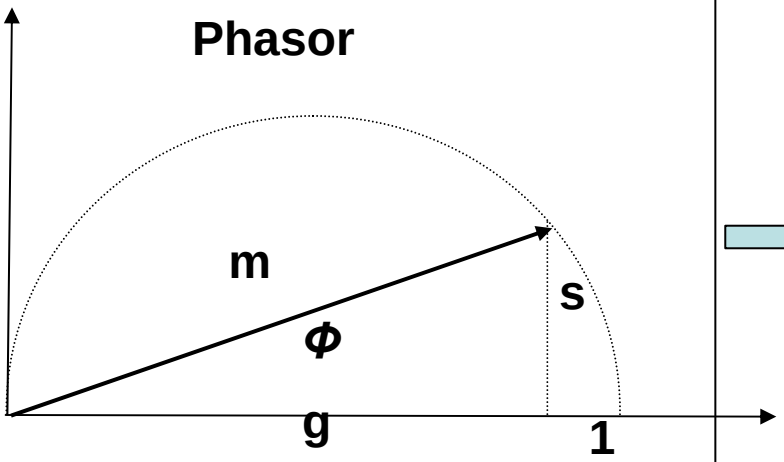


Fitting of exponentials

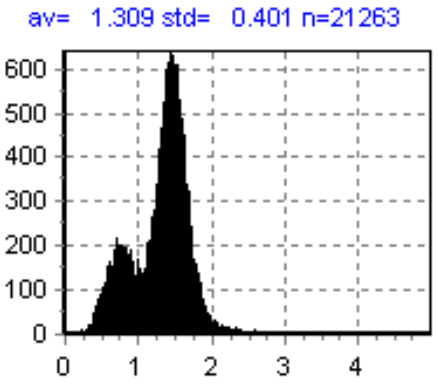


Multiexponential analysis

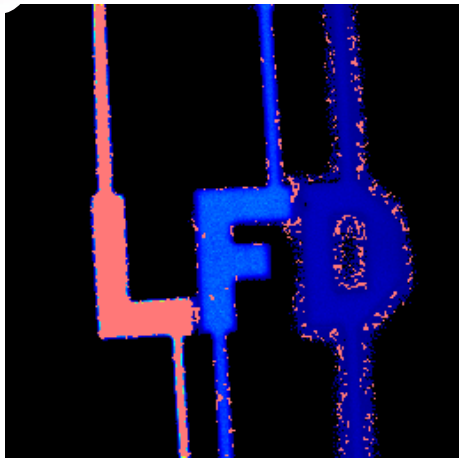
Phasor

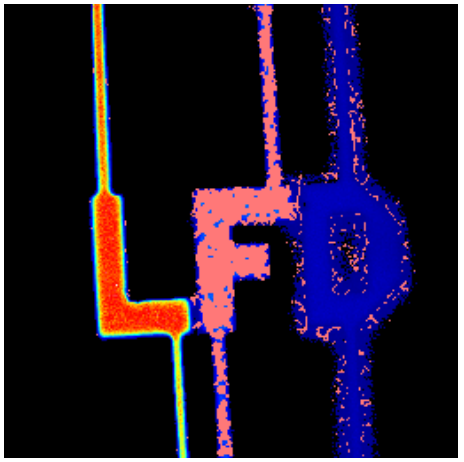


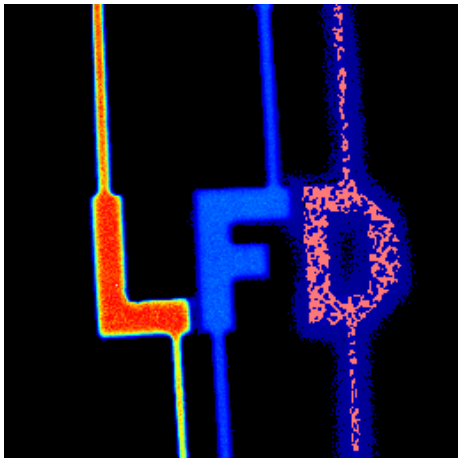
Lifetime distribution

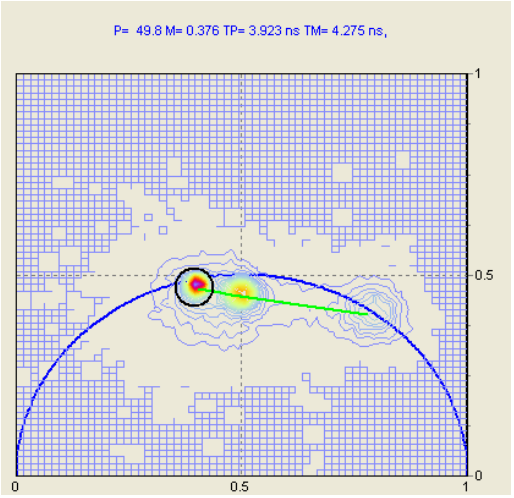


Separating Single exponential lifetimes using the phasor approach

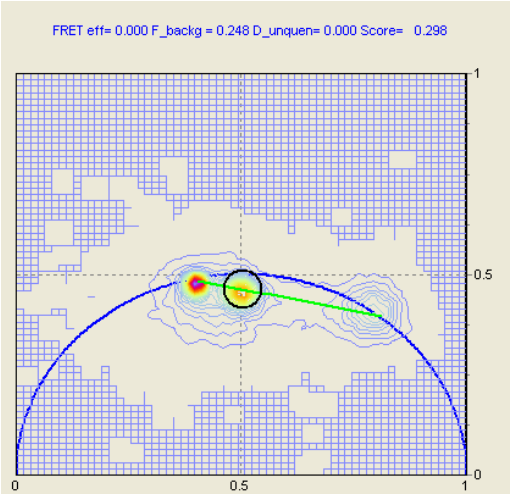




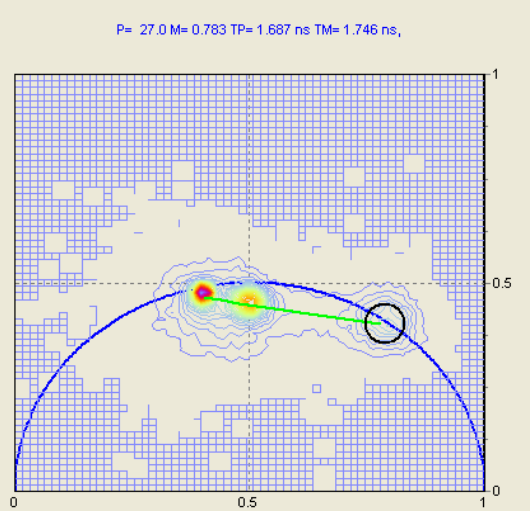




Fluorescein



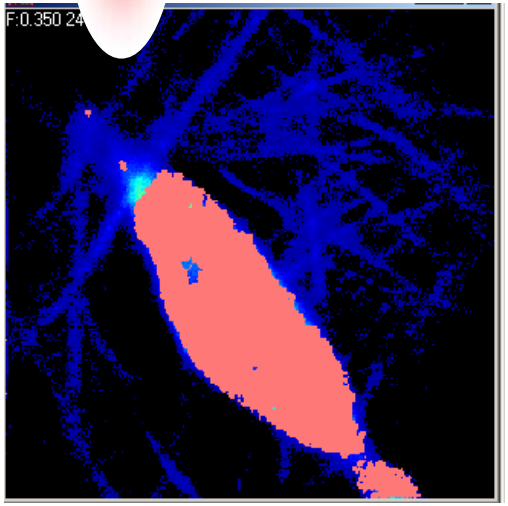
Mixture



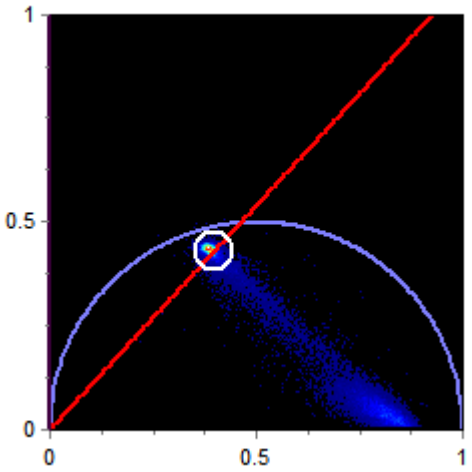
Rhodamine B1

Pax-eGFP CHO-k1 in collagen

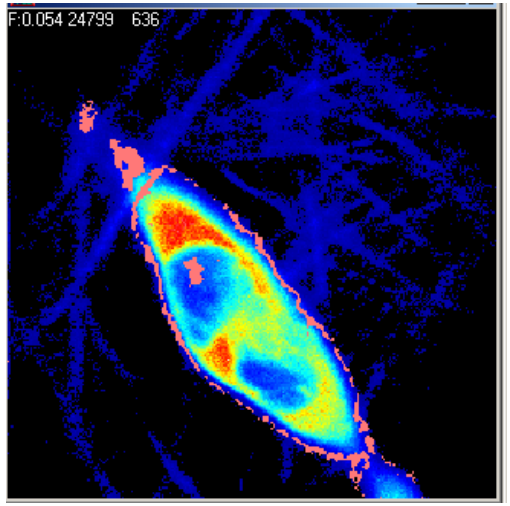
referenced with Fluorescein @ 905nm



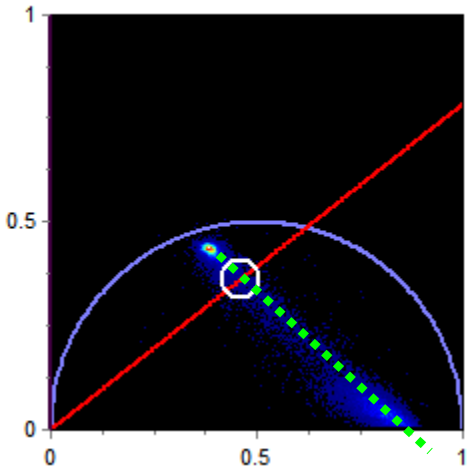
P= 47.2 M= 0.343 TP= 2.149 ns TM= 2.751 ns,



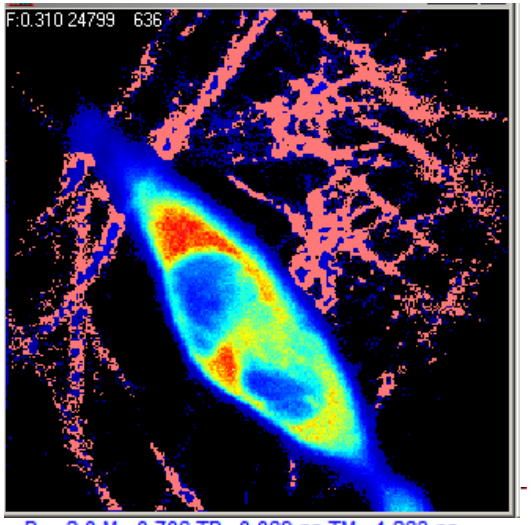
Lifetime of EGFP



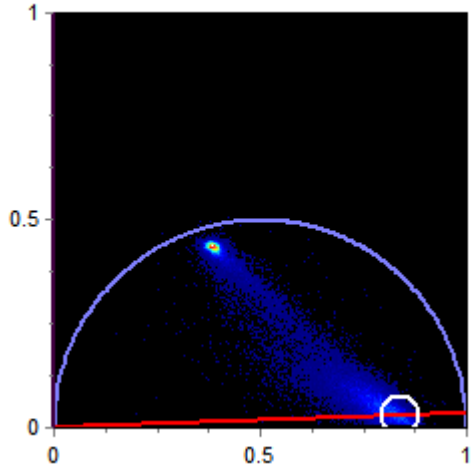
P= 38.2 M= 0.344 TP= 1.563 ns TM= 2.748 ns,



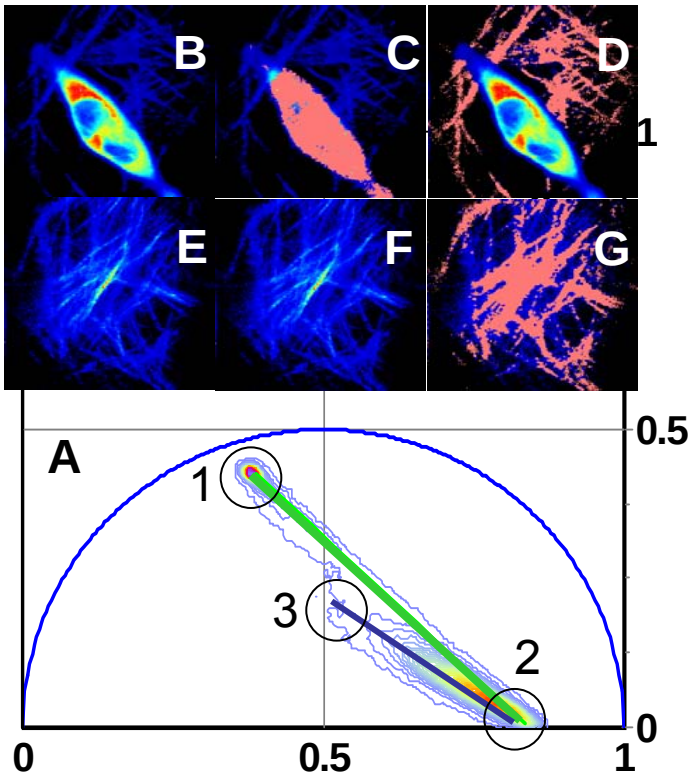
Combinations of Lifetimes



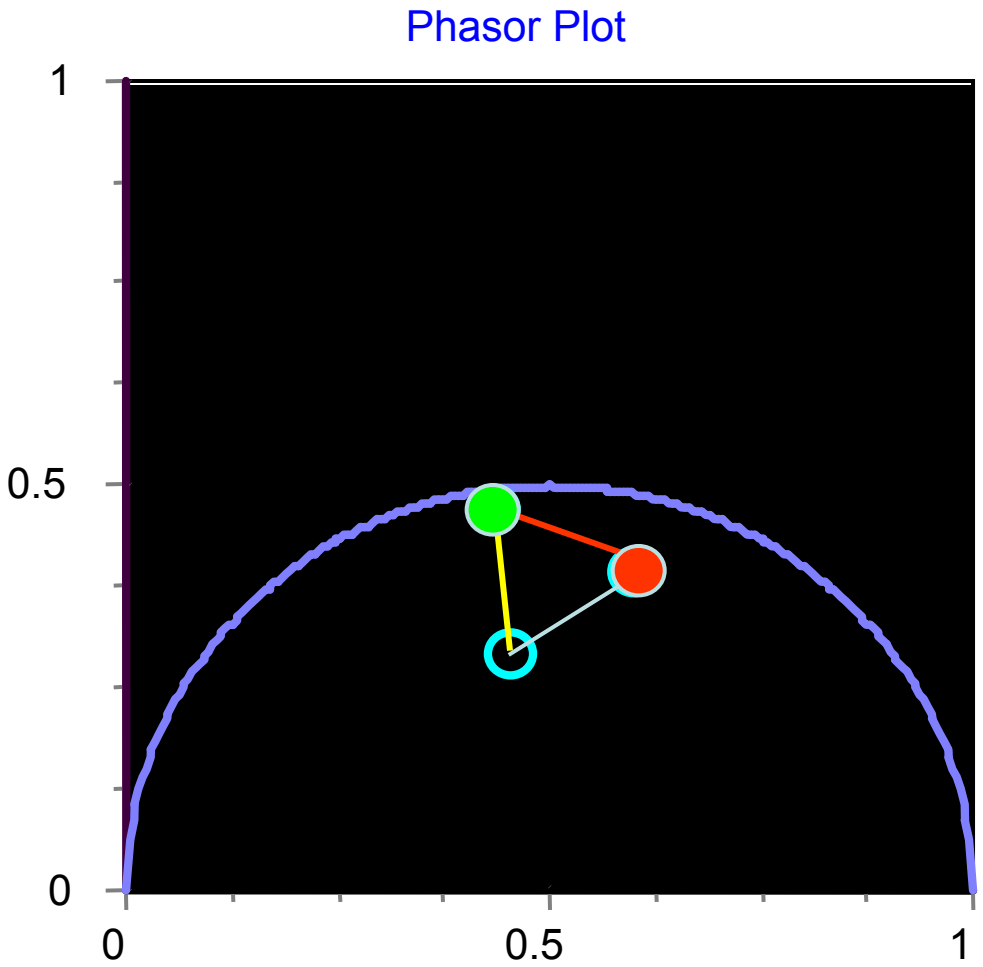
P= 2.0 M= 0.706 TP= 0.069 ns TM= 1.283 ns,



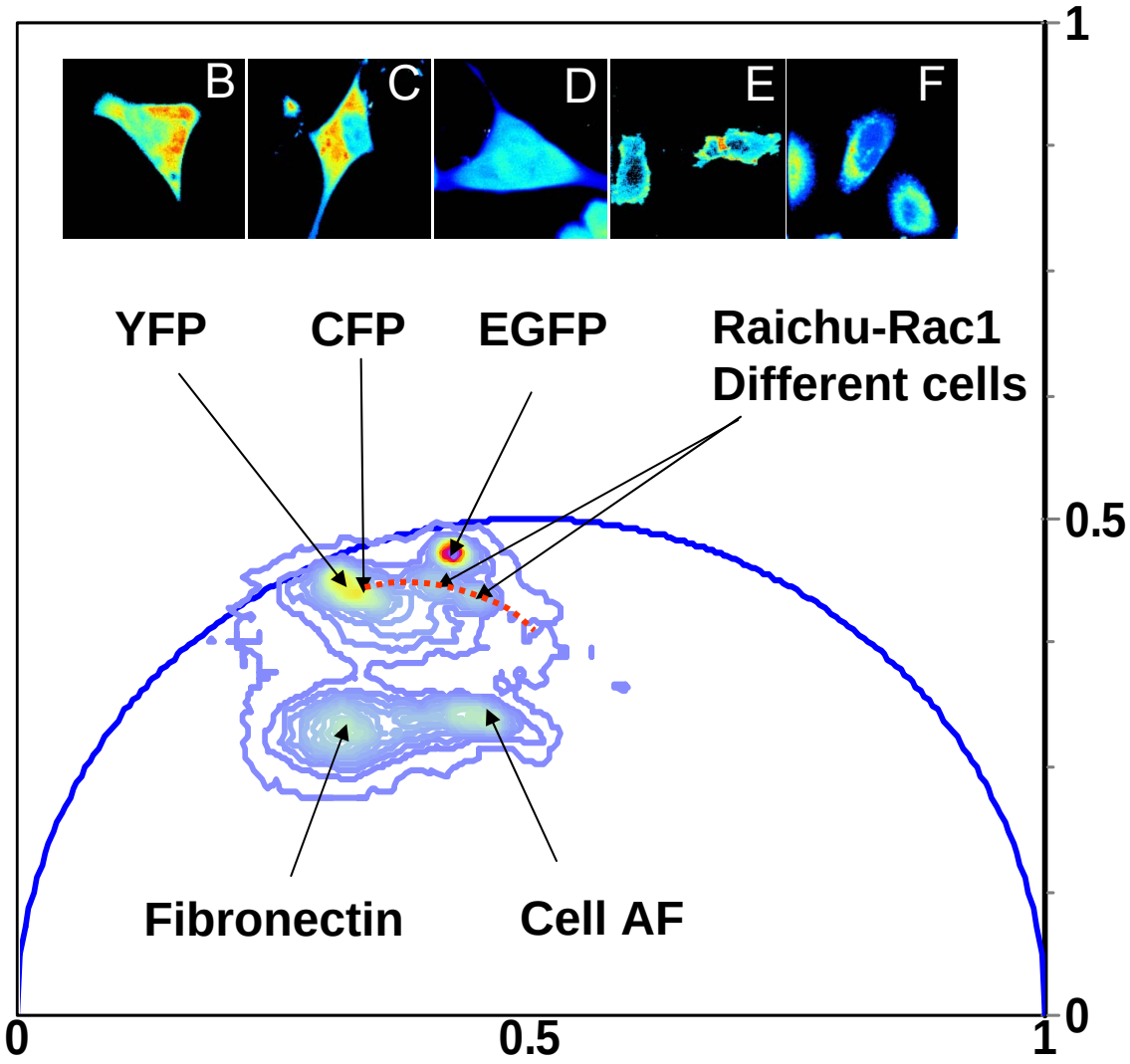
Lifetime of Collagen


B
C
D
E
F
G
A
1
2
3
0
0.5
1
0.5
1
0

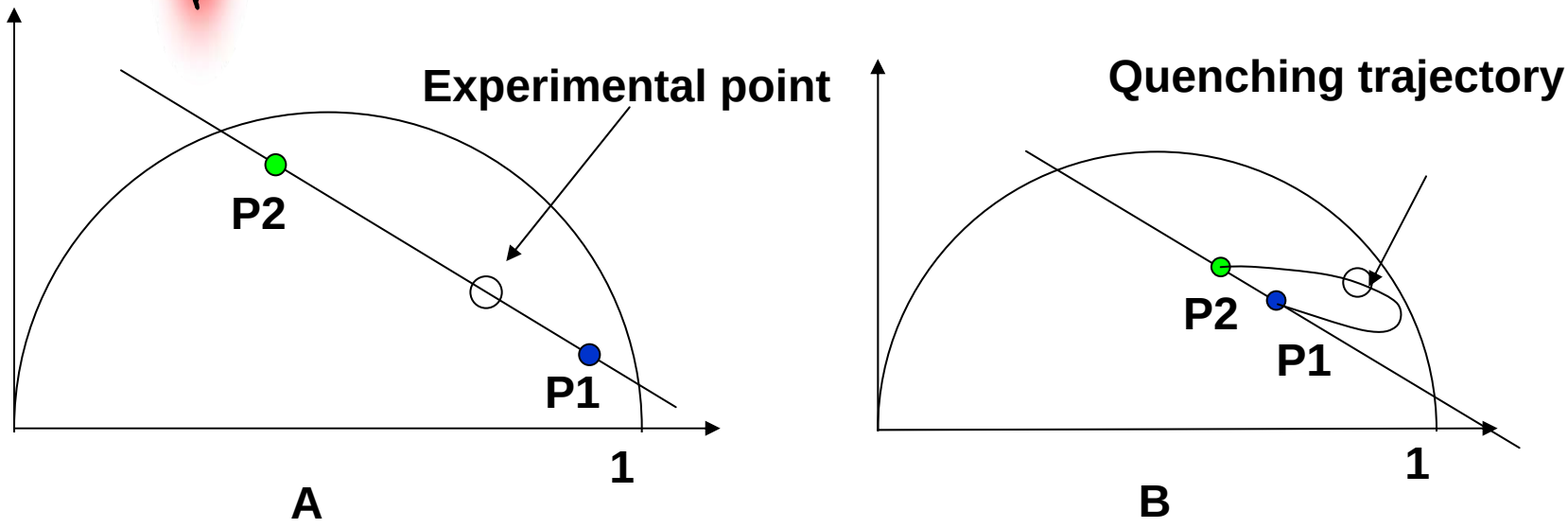
How to identify components?



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.


B
C
D
E
F
YFP
CFP
EGFP
Raichu-Rac1
Different cells
Fibronectin
Cell AF
1
0.5
0
0
0.5
1

How to distinguish two multi-exponential components from FRET?



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

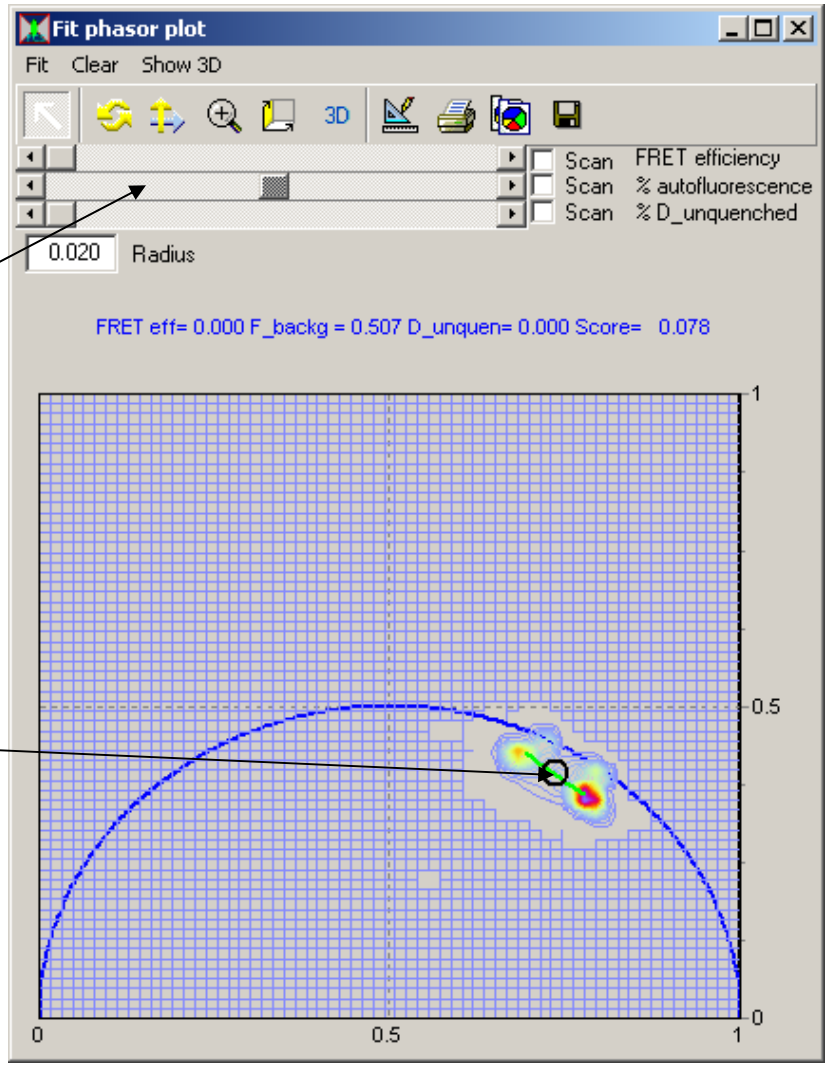
The fractional intensity calculator

1. Cl on the phasor plot (or enter the coordinates of the phasor manually) and assign the phasor to species 1.

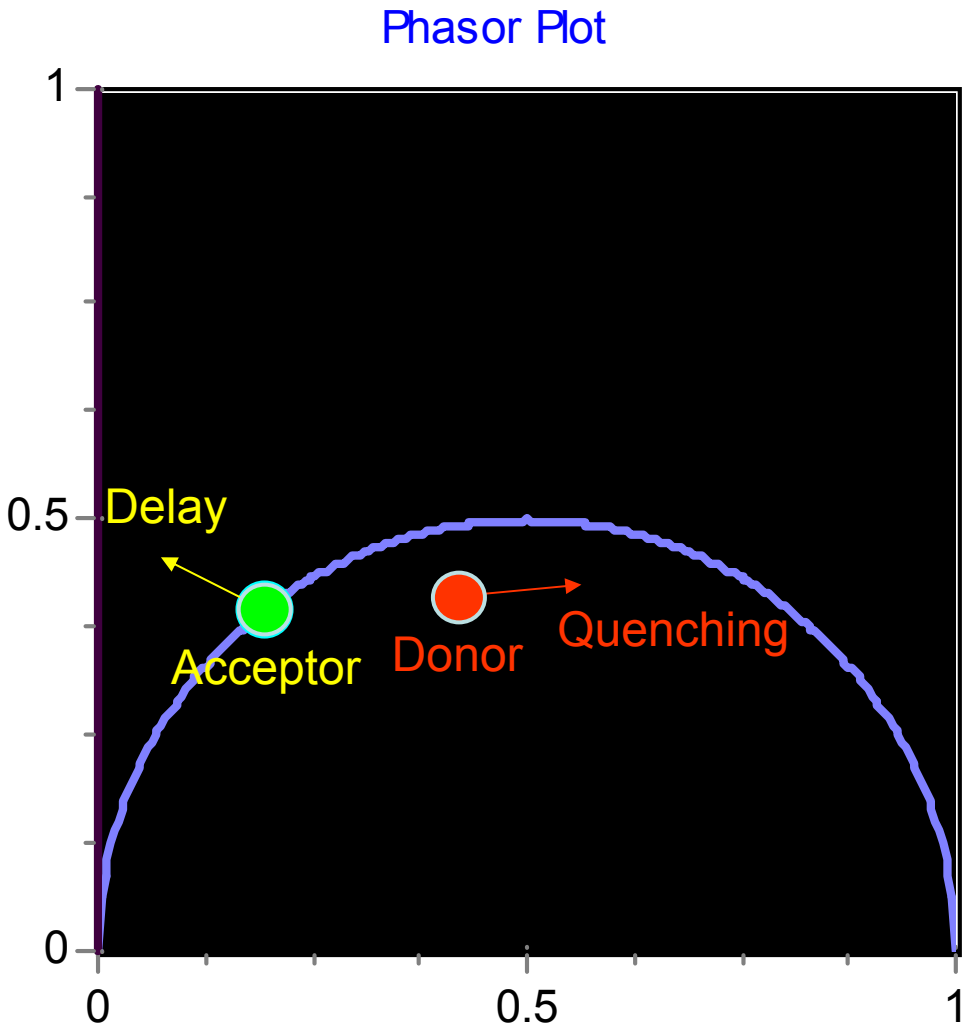
2. Click to assign the phasor to the phasor 2.

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?



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

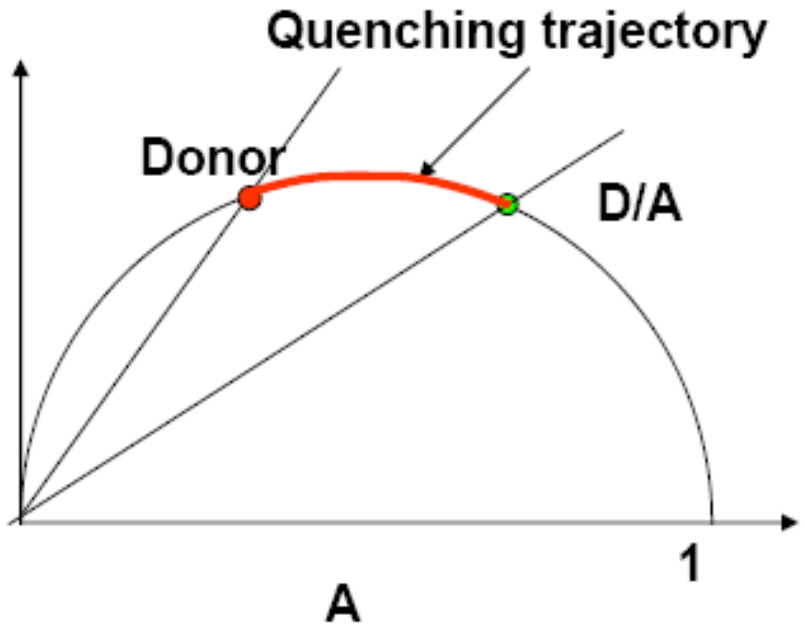
The calculator for FRET efficiencies from

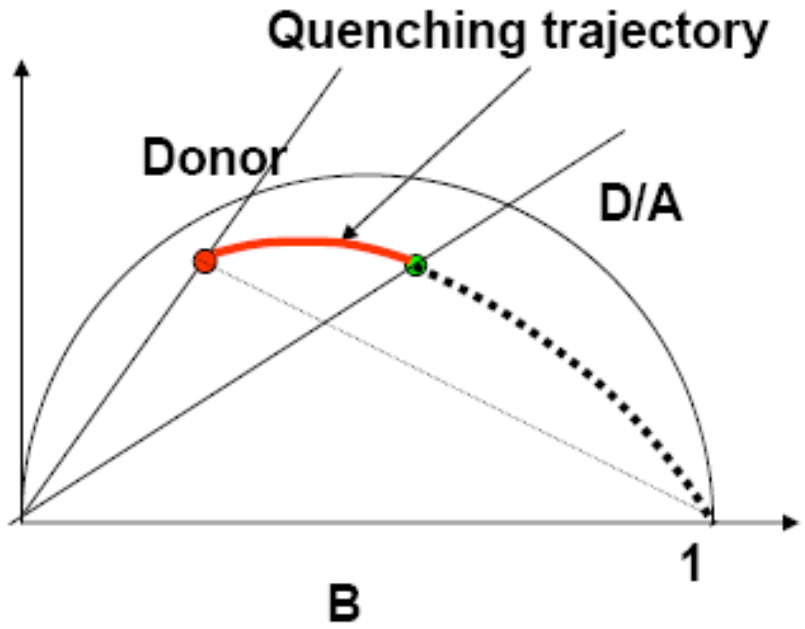
$$\text{FRET efficiency} = \frac{\tau_D - \tau_{FRET}}{\tau_D}$$

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

The FRET calculator

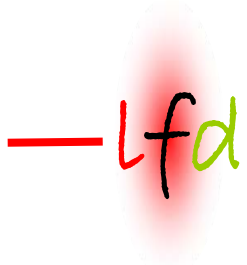
If we have a donor with a single exponential decay that is quenched by the presence of a acceptor. What should we expect?





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?

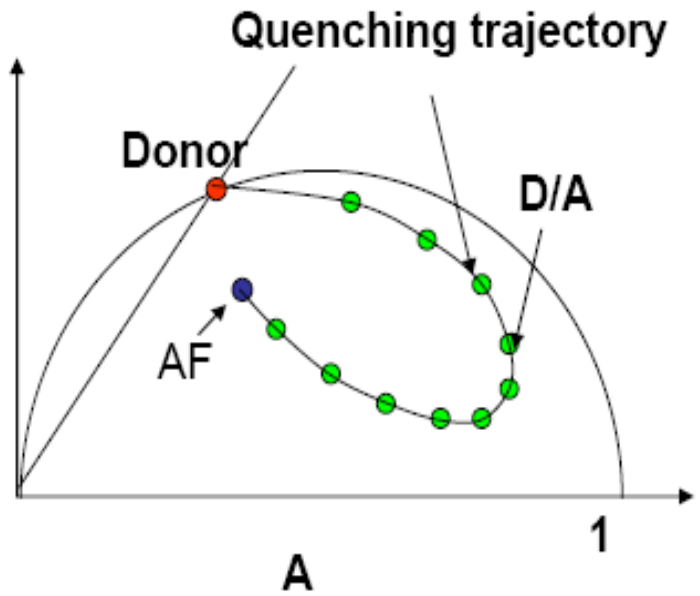


The FRET Calculator

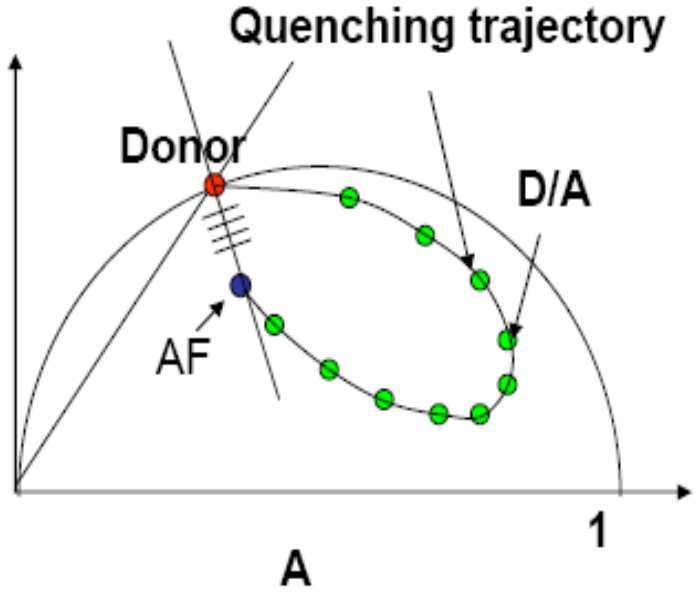
- Can we quench up to zero lifetime?
- Even if we quench all the DONOR, we still are left with the autofluorescence.
- The final point is not at zero but at the autofluorescent phasor!!!

The FRET Calculator

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

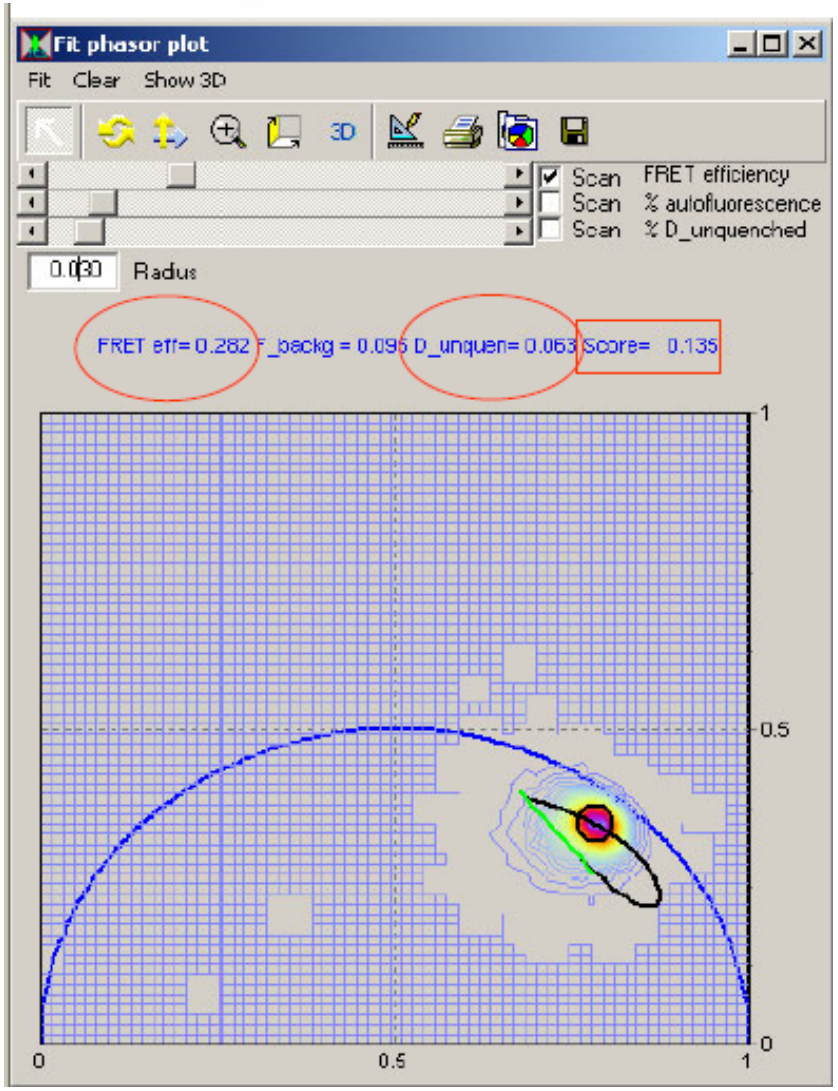


As the lifetime of the Donor is quenched, the phasor of the quenched Donor is added to the phasor of the autofluorescence



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

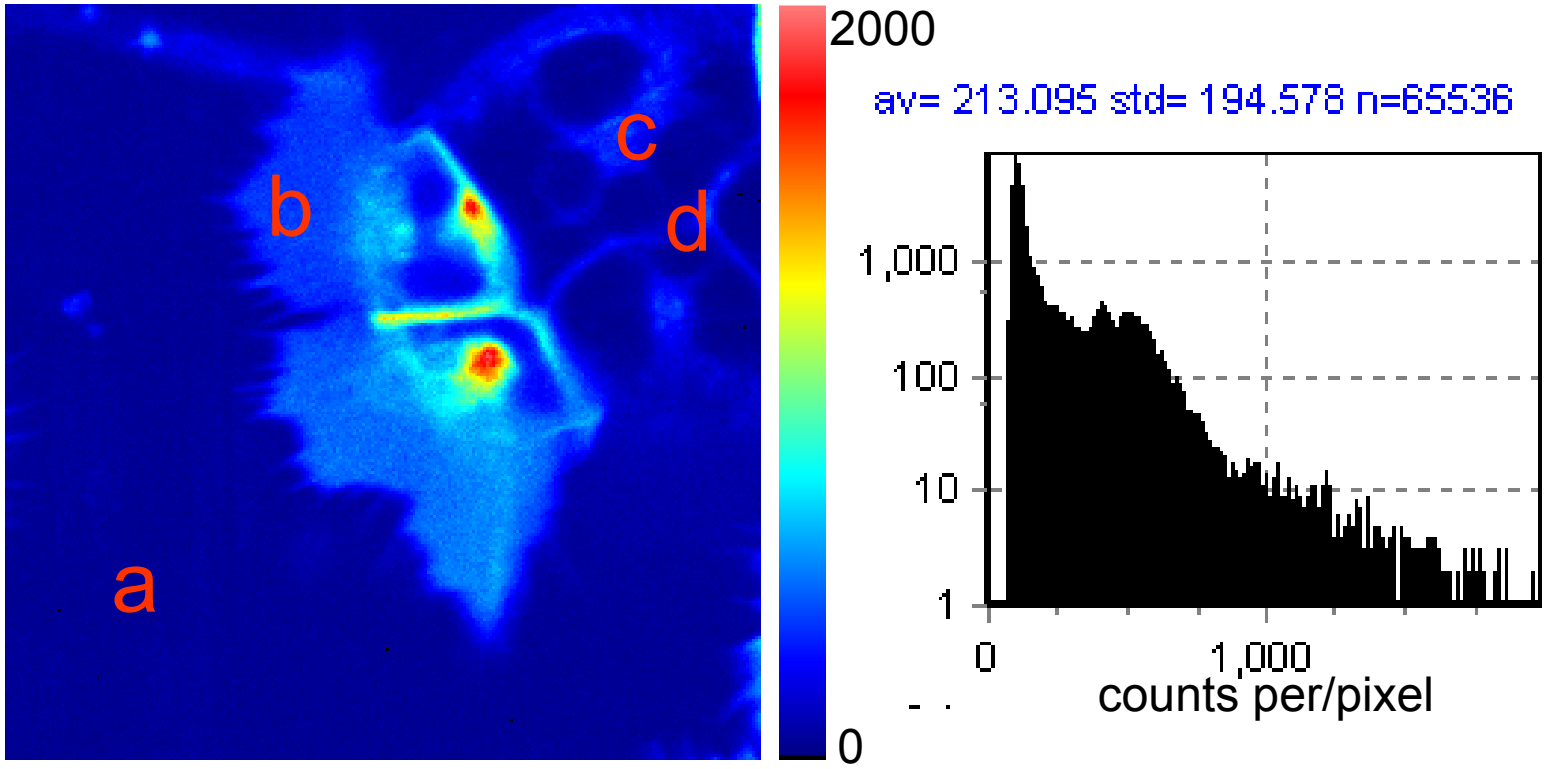
The FRET calculator



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

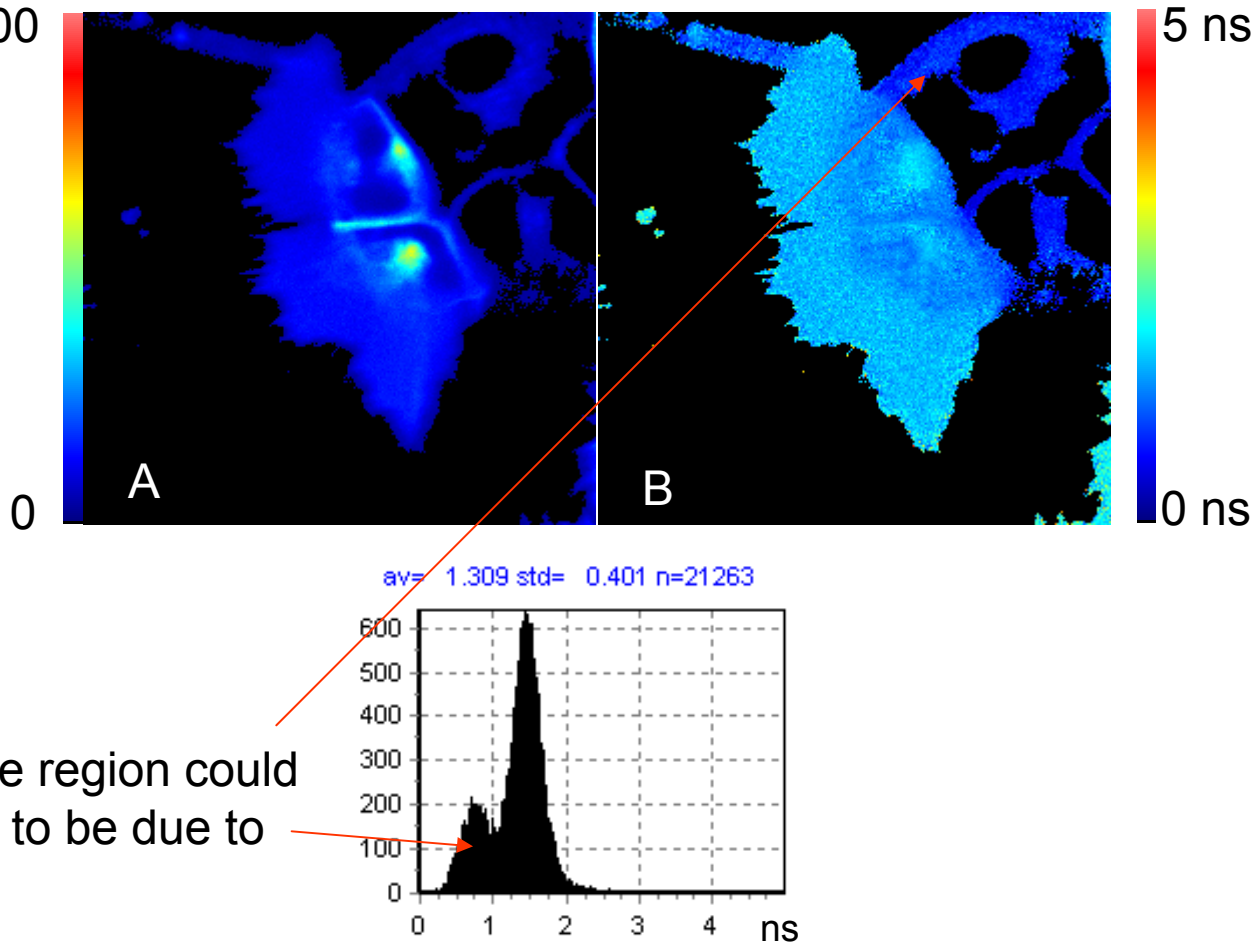


Several regions the image can be identified corresponding to **a)** background (2 exponentials) **b)** cell 1 bright (2 exponentials) **c)** cell 2 dim, **d**) cell junctions dim.

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

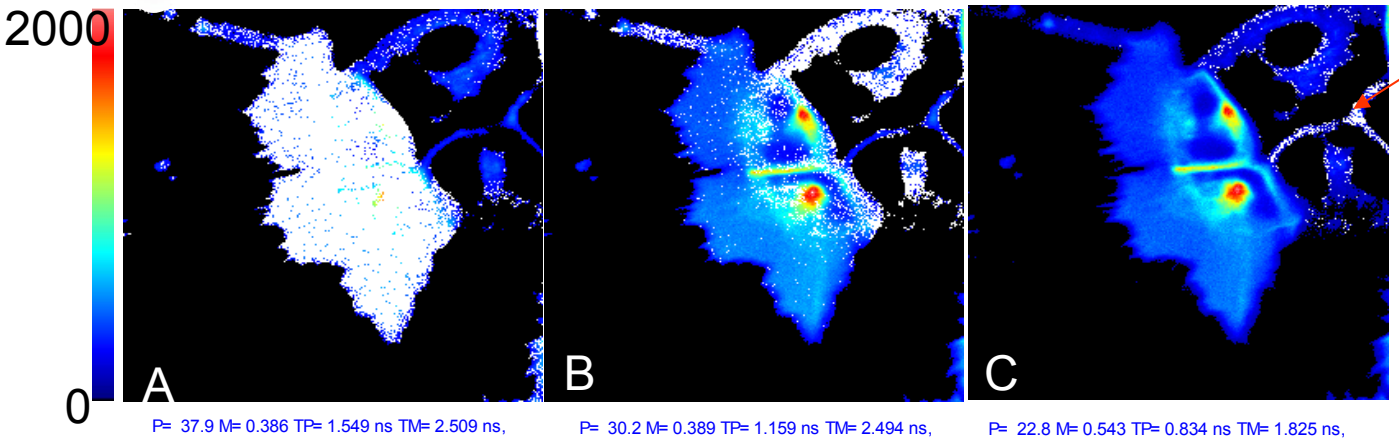
ımage obtained using B&H 830 in our 2-photon microscope



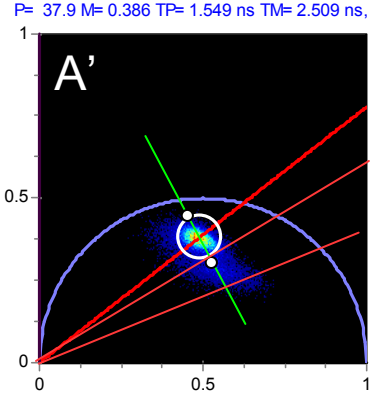
Shorter lifetime region could be interpreted to be due to FRET

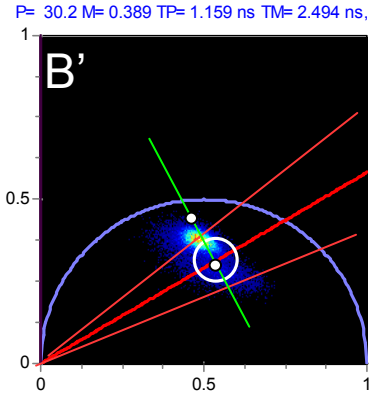
Donor+acceptor+ligand. A) intensity image after background subtraction, B) τ_p image

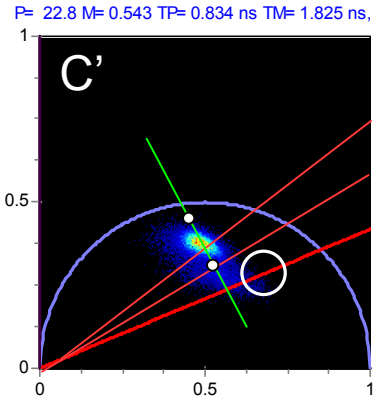
Identification of FRET using the phasor plot

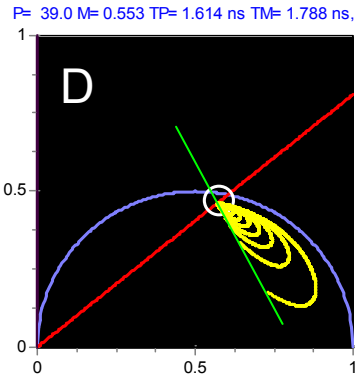


FRET only occurs at the cell junctions



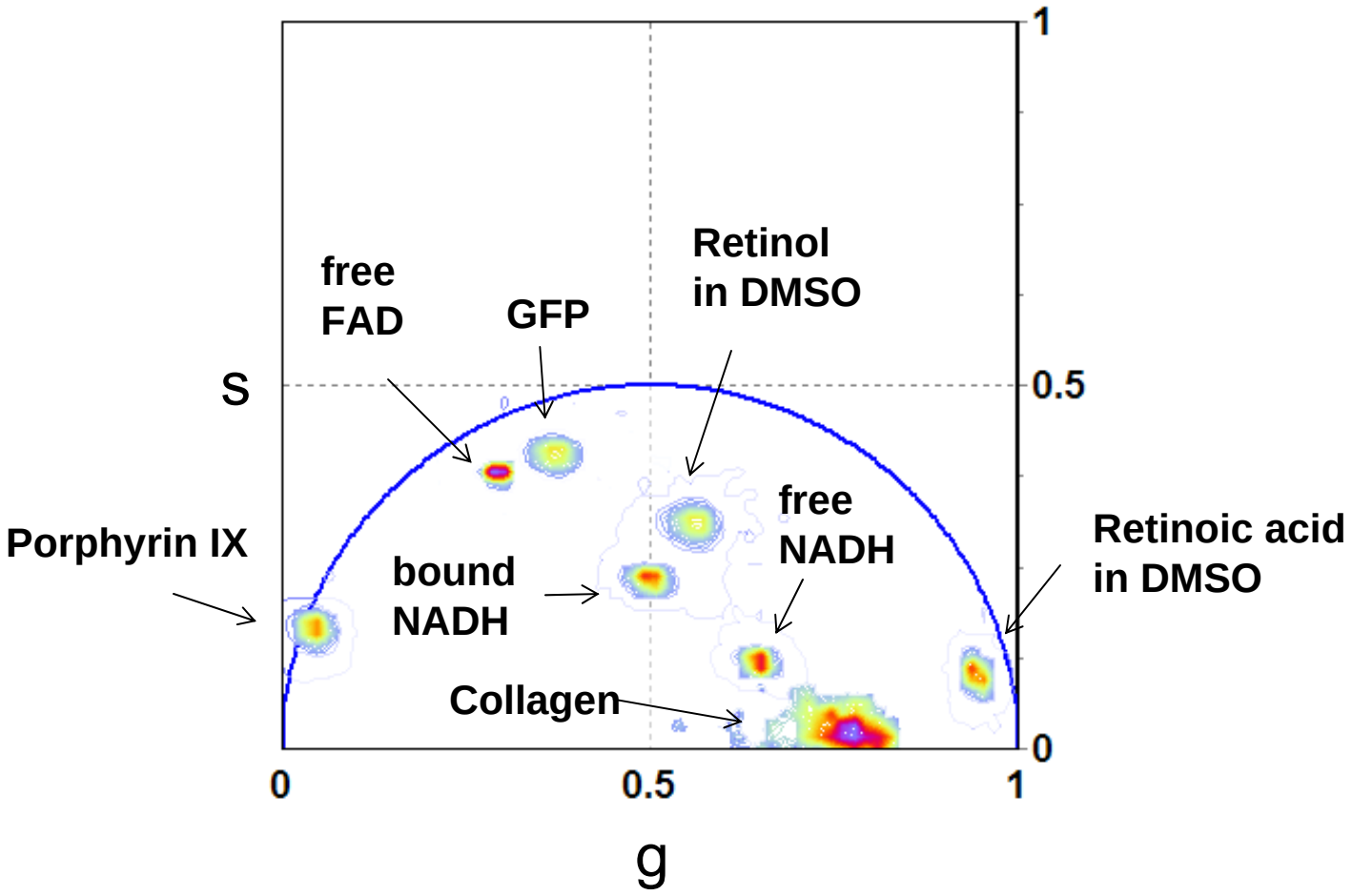






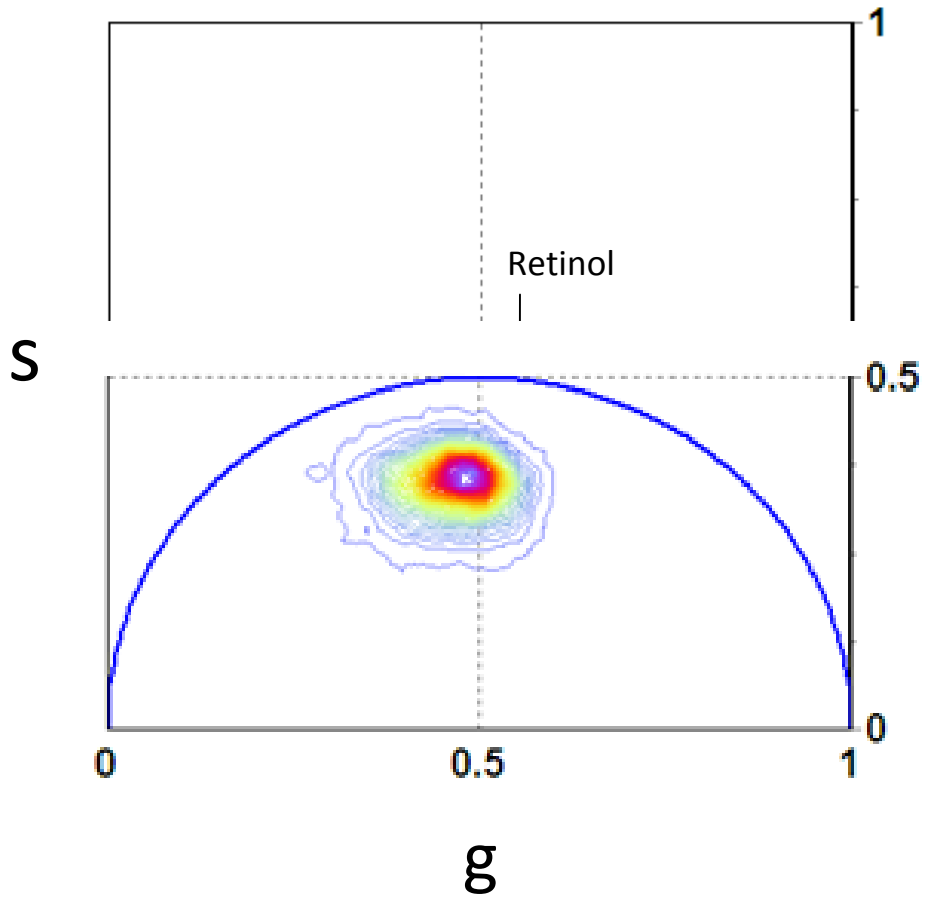
Selecting regions of the phasor diagram. Selecting the region in **A'** (donor +acceptor) the part in white lights up (**A**). Selecting the region in **B'** (autofluorescence) the part in white in lights up (**B**). The color scale in **B'** has been changed to better show the region of the autofluorescence. 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**

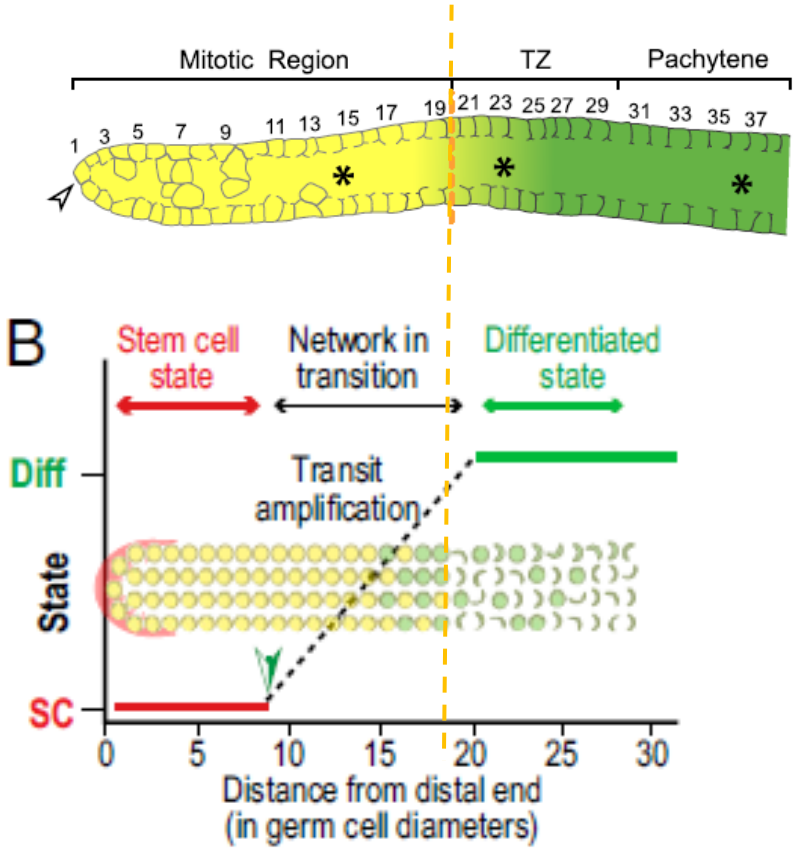
✓ **Cellular**

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

✓ Complex distribution of autofluorescence

✓ Redox ratio (NADH/NAD+ ~ 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

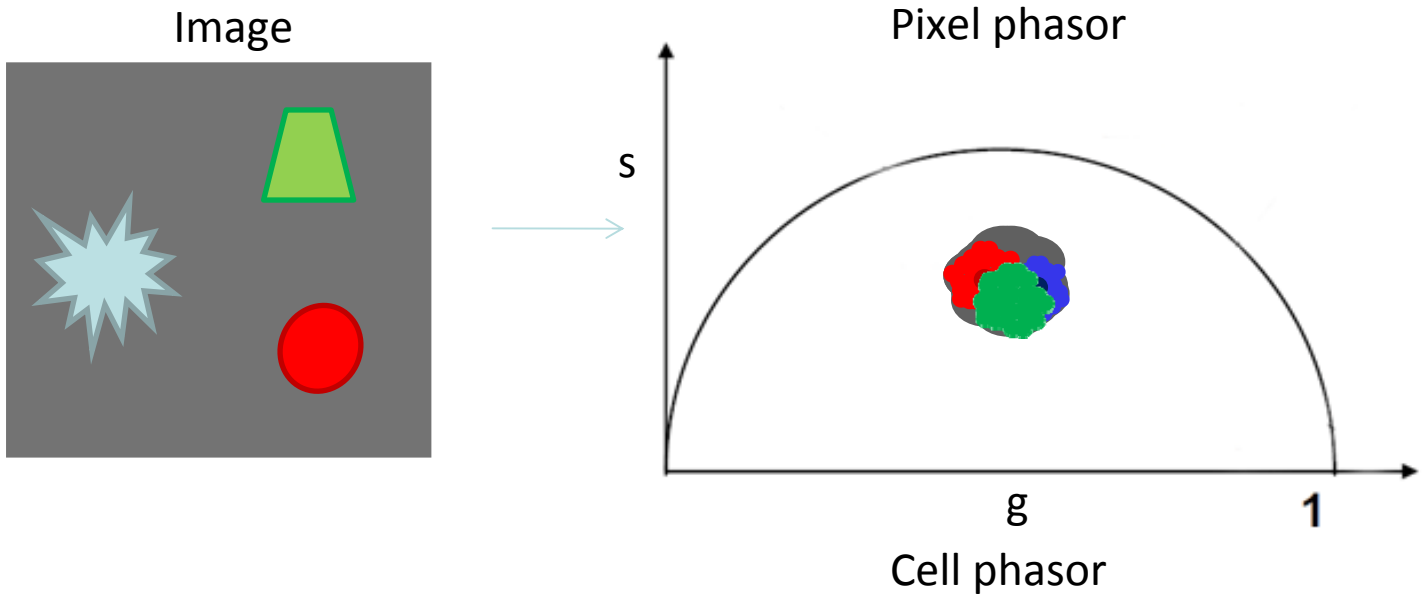
- ✓ The distal pool: undifferentiated cells maintained in a "stem cell-like state"
- ✓ proximal pool contains cells that are closer to differentiating

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

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

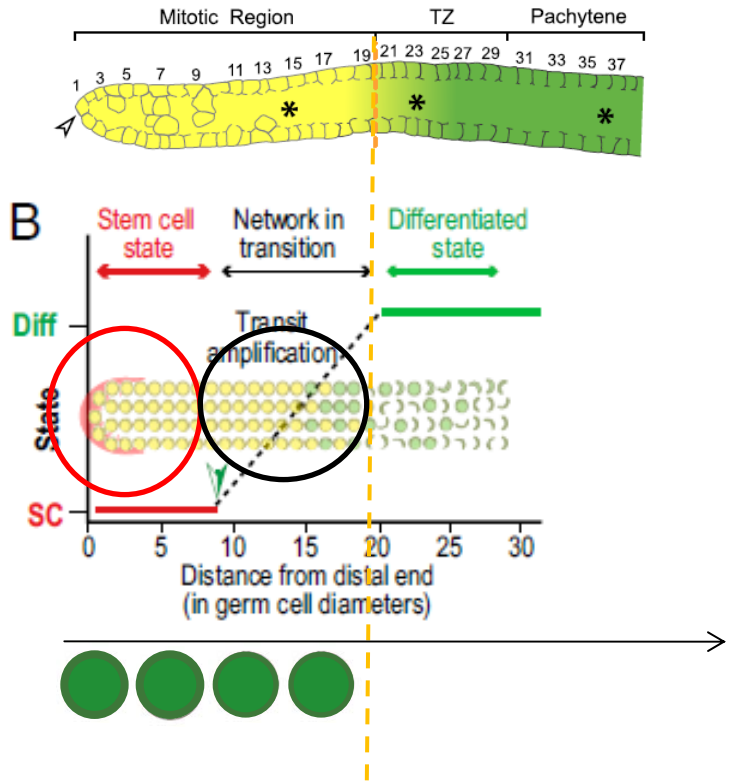
Crittenden et al. Mol Biol Cell, 2006; Cinquin et al. PNAS 2010

Image Segmentation:
from pixel phasor plot to cell phasor plot



- ✓ Phasor average value of cells
- ✓ Better resolution
- ✓ Metabolic state of cells
- ✓ Cell phasors can be statistically attributed to the 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 µs,
SimFCS software

Mitotic region : stem cells niche

- ✓ **Distal pool:** undifferentiated cells maintained in a "stem cell-like state"
- ✓ **Proximal pool:** contains cells that are closer to differentiating

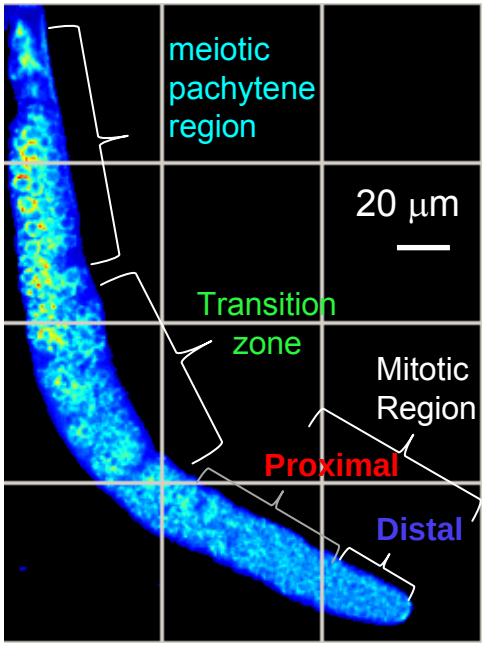
Transition zone : cells that have differentiated and entered meiotic prophase (crescent-shaped DNA)

Meiotic pachytene region: progression through meiosis.

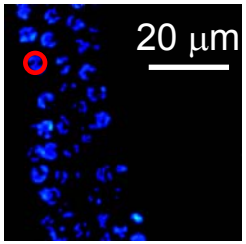
Cinquin et al. PNAS 2010

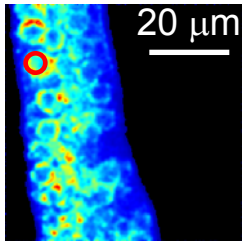
Stem cell metabolic “states” in C.elegans

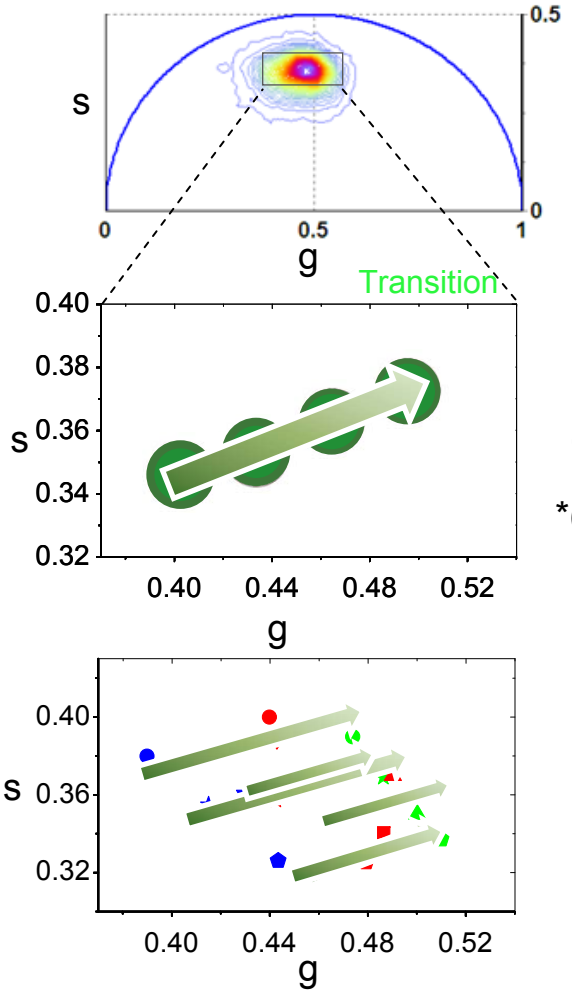
@740nm
@880nm
NADH, FAD
histone-GFP fusion
in germ line nuclei



Cell selection







Pixel phasor

Cell phasor

*(t-test, p<0.05)

N=6

In the large phasor cluster we distinguish statistically different subclusters

Stringari et al. PNAS 2011

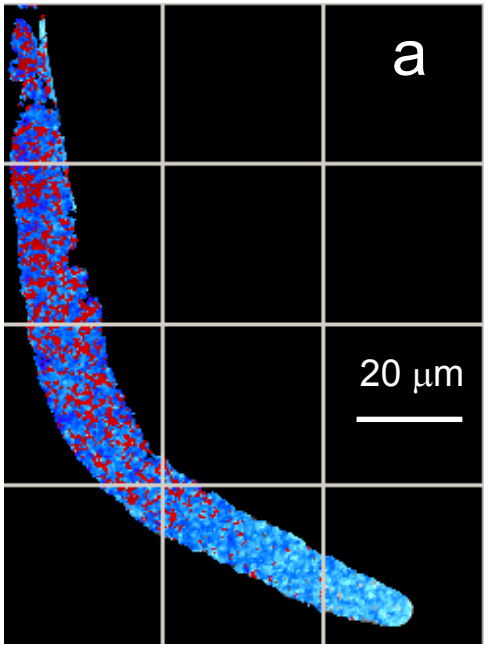
Mapping relative concentration of metabolites

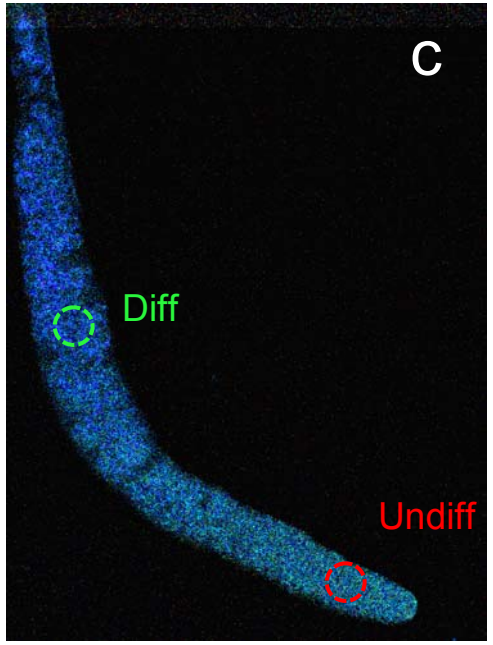
Redox balance and modulation of stem cell self-renewal and differentiation

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

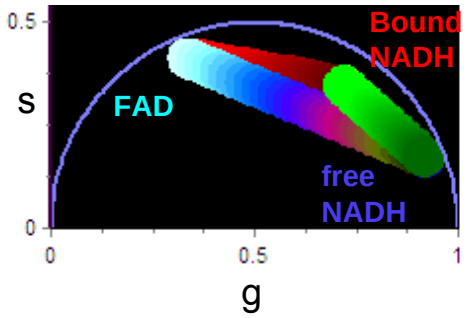
✓Signaling molecules that promote differentiation cause progenitor to become more oxidized .

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

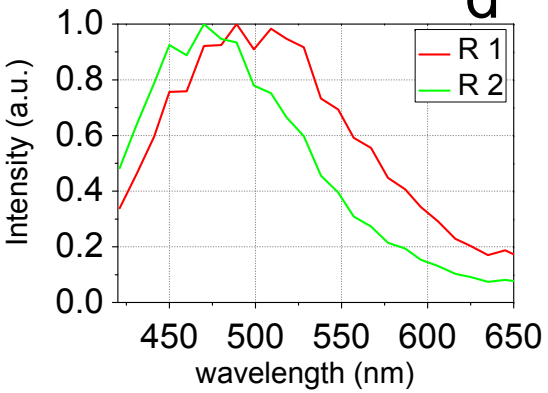




b

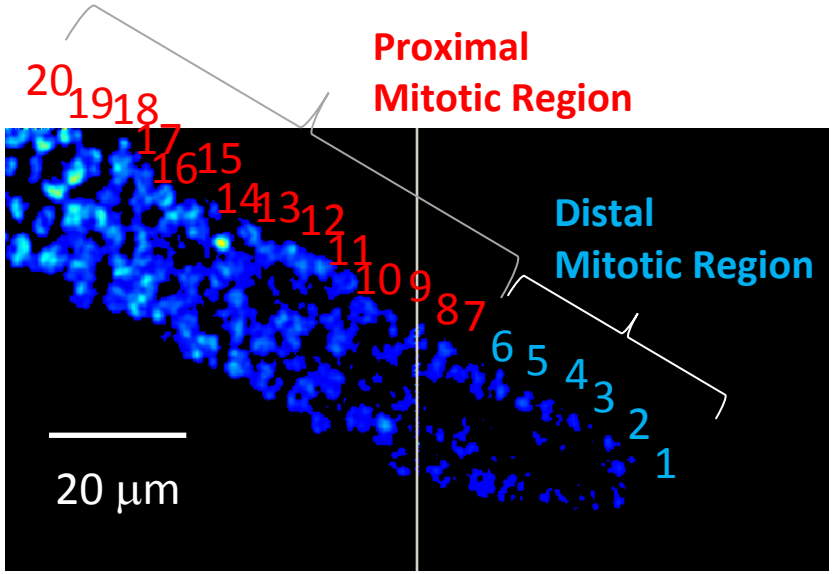


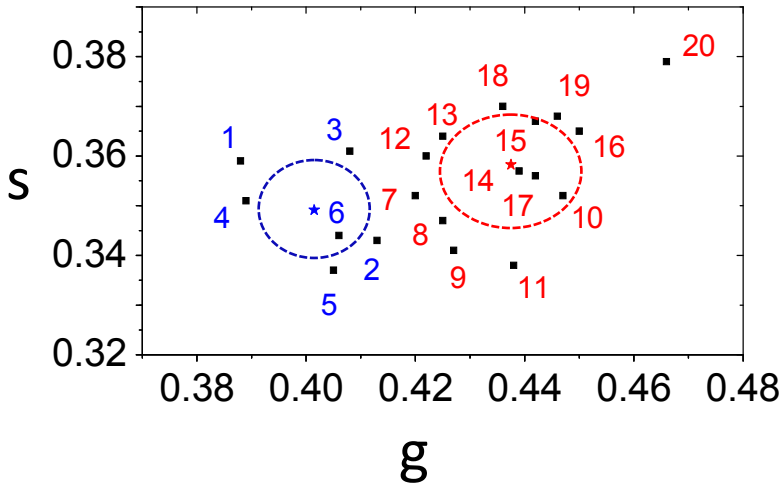
d



Stringari et al. PNAS 2011

Single cell phasor plot distinguishes metabolic states of cells





✓ Evolution of the cell phasor fingerprints during differentiation

✓Gradient of metabolic states of cells.

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

Conclusions

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

Current work and future directions

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

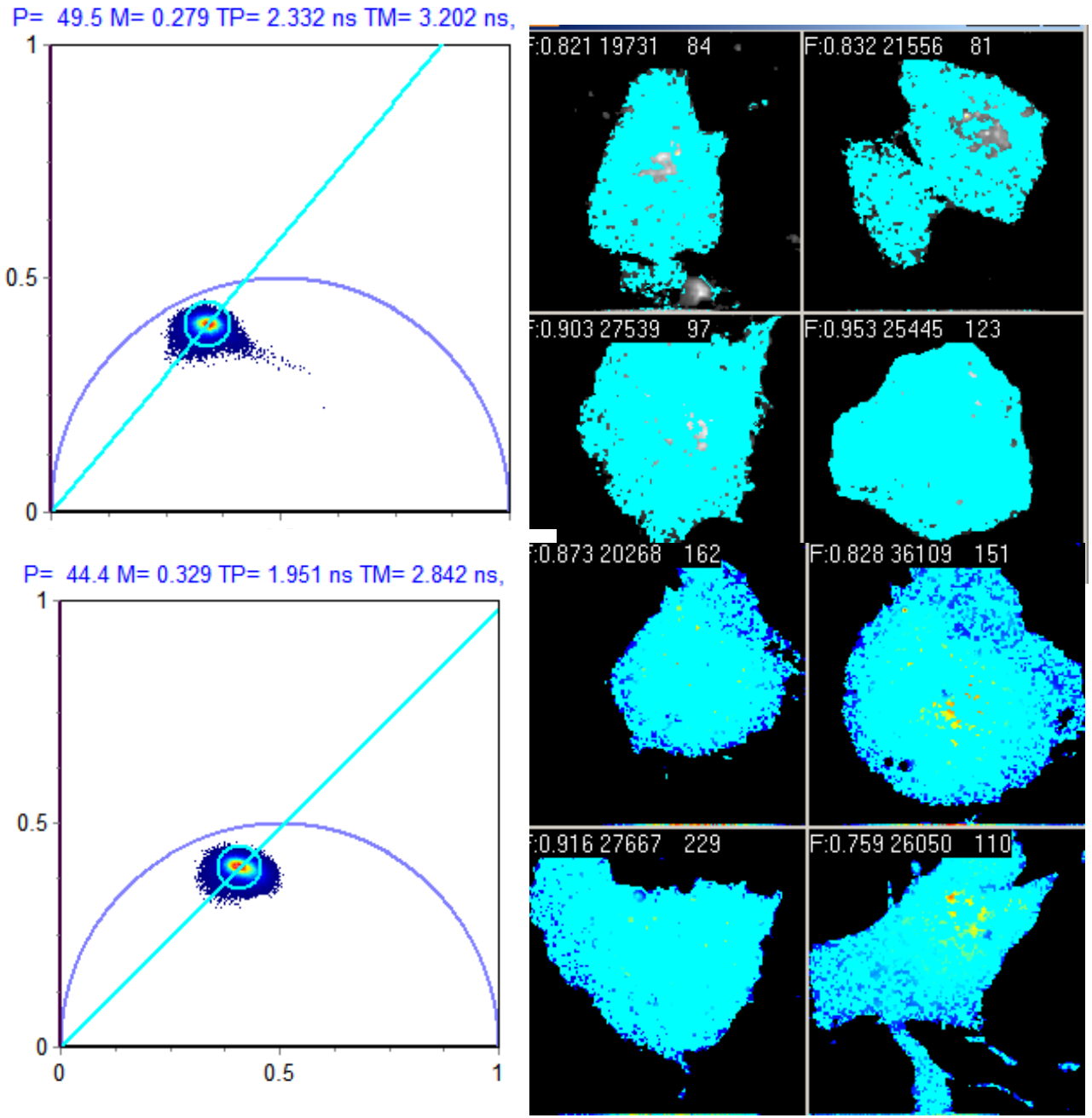
The Phasor approach: Application to Biosensor FRET analysis

Enrico Gratton, Michelle Digman, Liz Hinde, Santiago Aguilar and Magdee Hugais

University of California at Irvine

COS7 cells, CFP only and with KH 449-521 before and after addition of EGF

Images are the average of 10 frames at 2 s each
Images taken on M3, Excitation at 840 nm , 40mW (outside)
Emission filter 460/60nm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0.5
0
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1
0.5
0
0
0.5
1
F:0.821 19731 84
F:0.832 21556 81
F:0.903 27539 97
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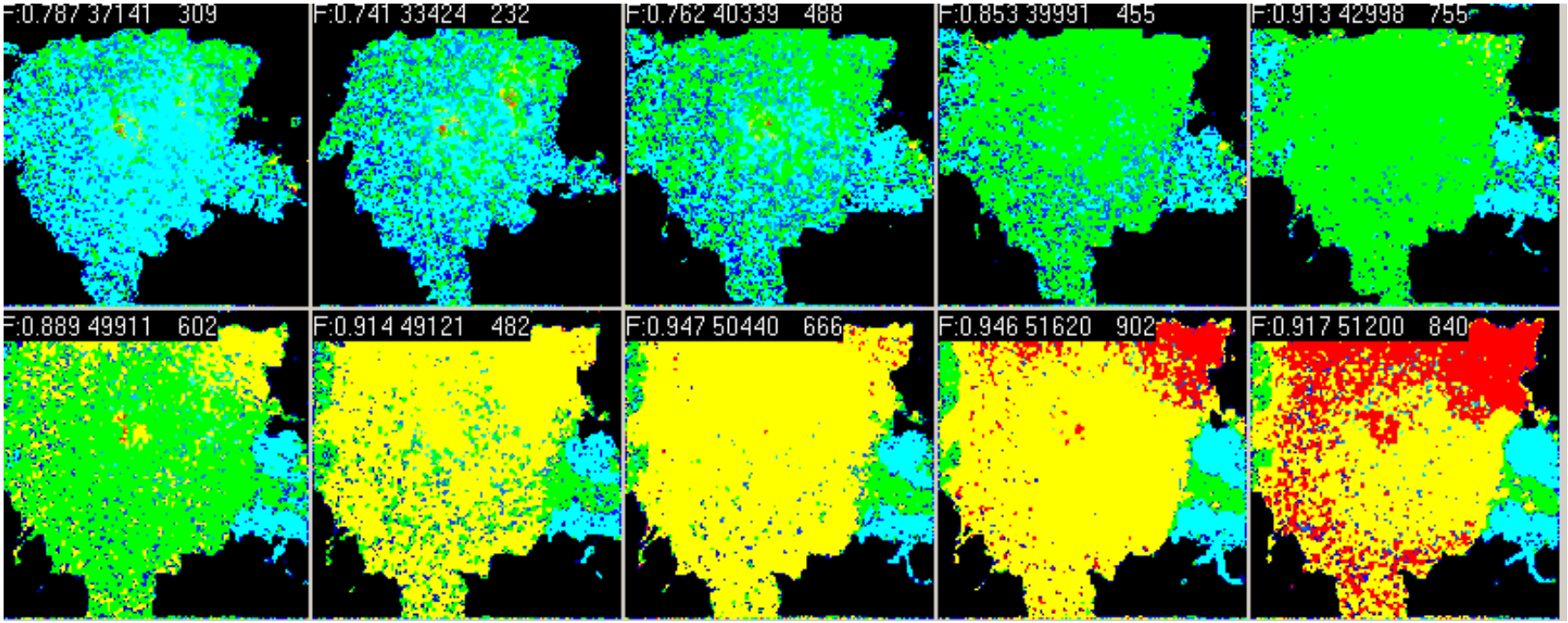
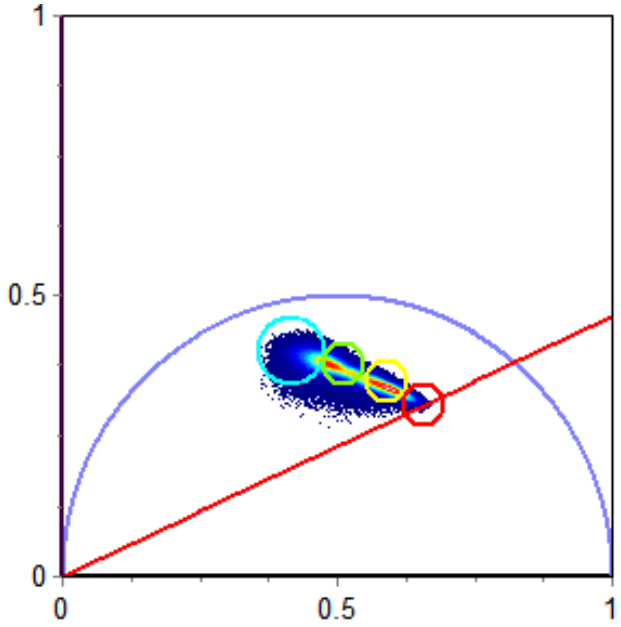
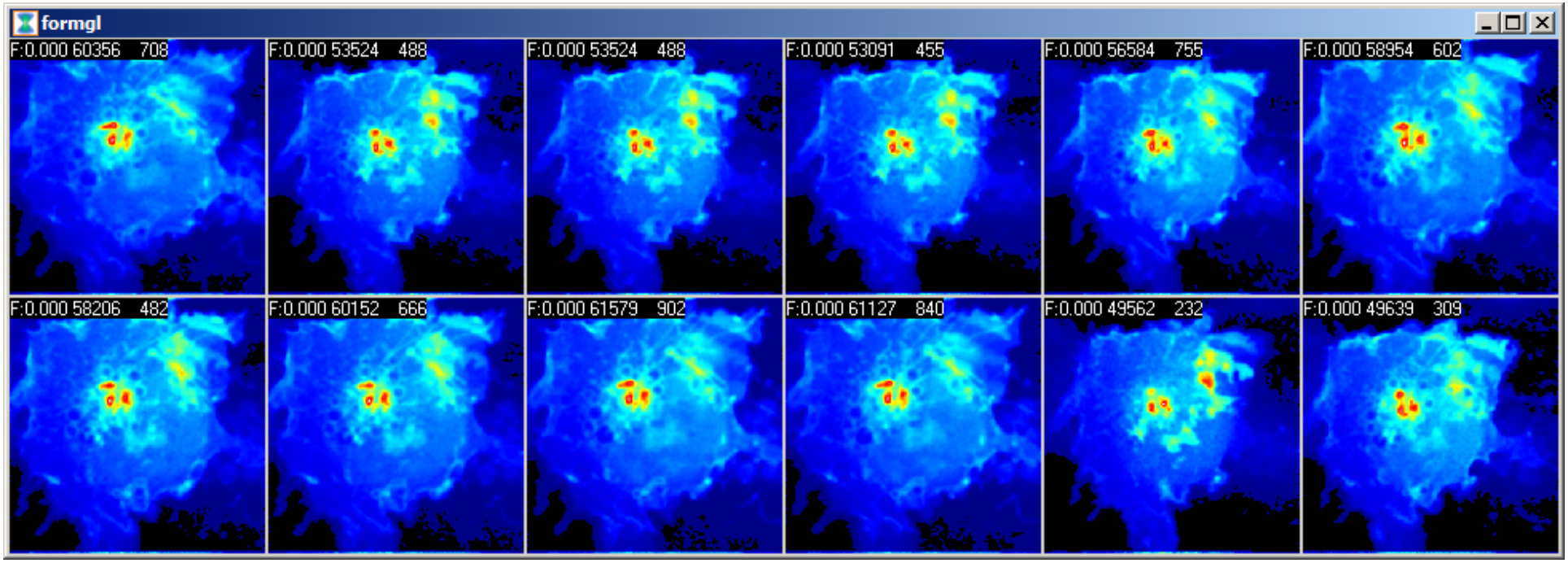
F:0.916 27667 229
F:0.759 26050 110

CFP



KH Rac 449-521 biosensor before addition of EGF (first image) and with increasing time after addition of EGF (about every 30s).




formgl
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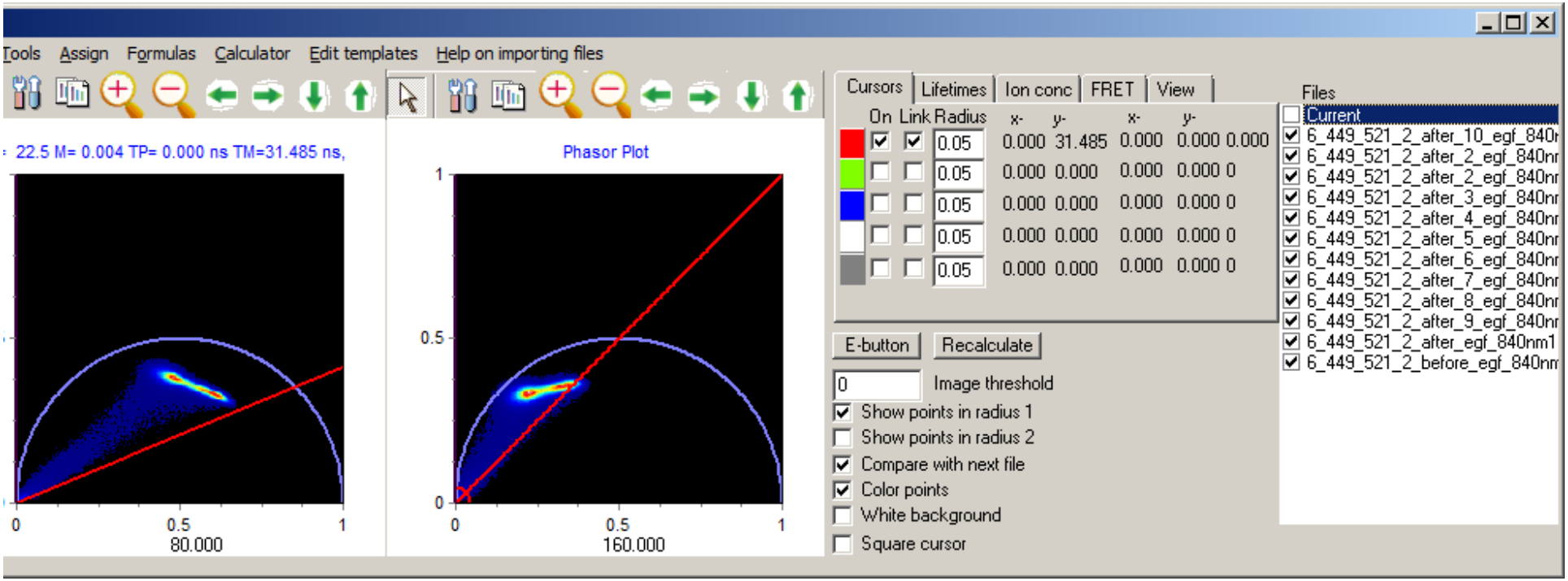
Tools Assign Formulas Calculator Edit templates Help on importing files
22.5 M= 0.004 TP= 0.000 ns TM=31.485 ns,
Phasor Plot
0
0.5
1
80.000
160.000
Cursors Lifetimes Ion conc FRET View
On Link Radius x- y- x- y-
0.05 0.000 31.485 0.000 0.000 0.000
0.05 0.000 0.000 0.000 0.000 0
0.05 0.000 0.000 0.000 0.000 0
0.05 0.000 0.000 0.000 0.000 0
0.05 0.000 0.000 0.000 0.000 0
E-button
Recalculate
0
Image threshold
Show points in radius 1
Show points in radius 2
Compare with next file
Color points
White background
Square cursor
Files
Current
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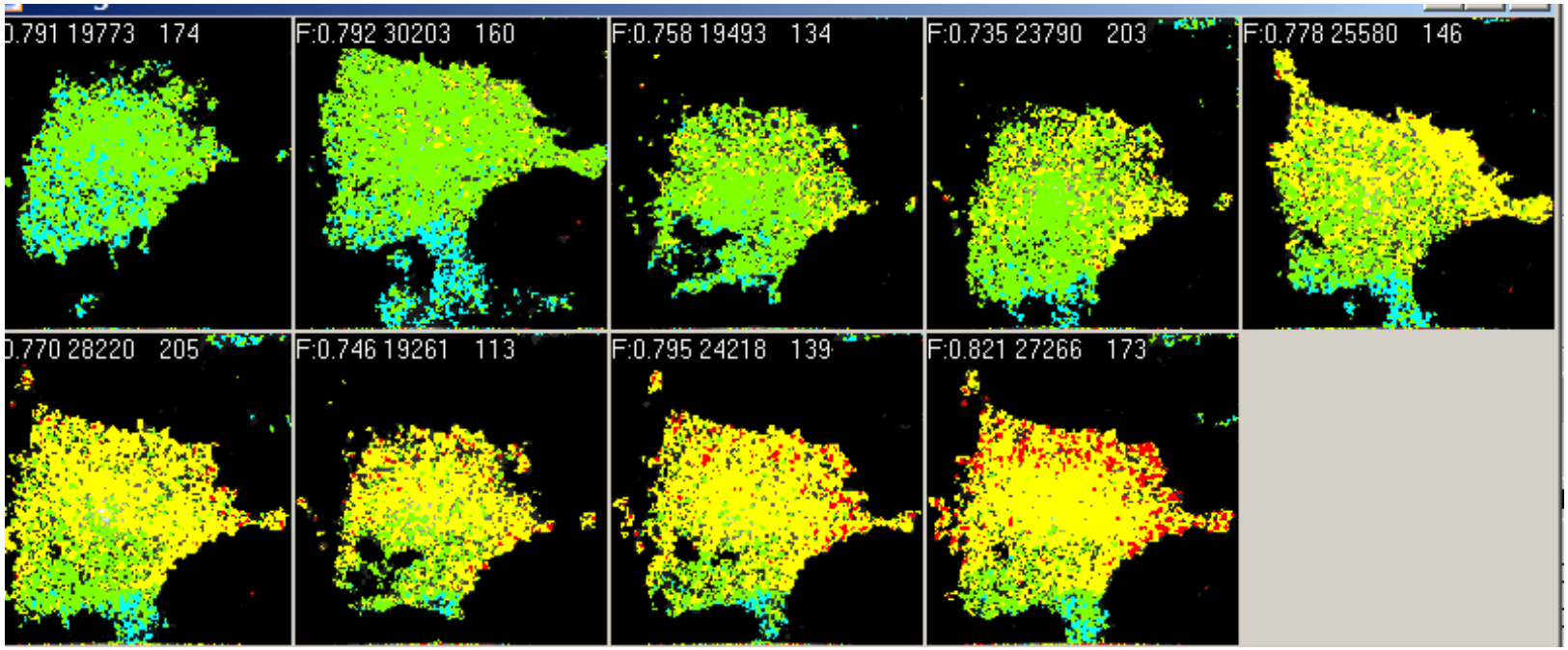
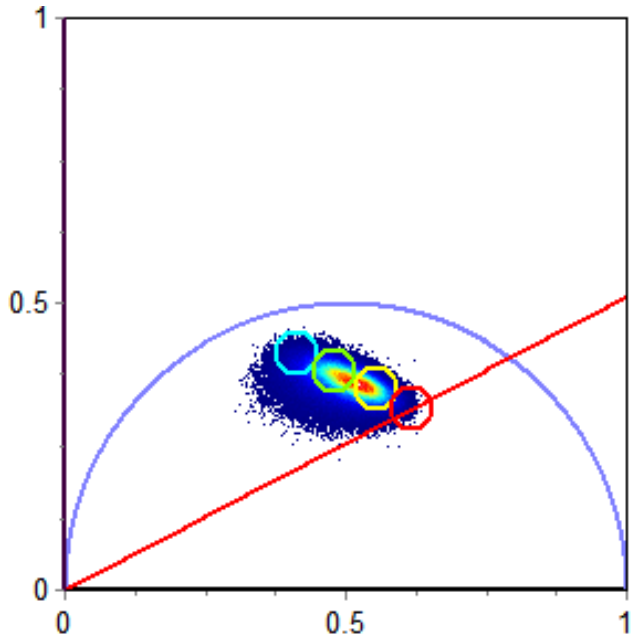
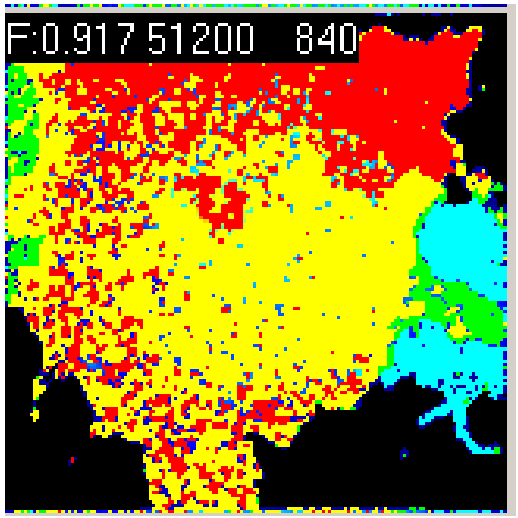


KH Rac 449-521 biosensor with increasing time after addition of EGF (about every 30s).

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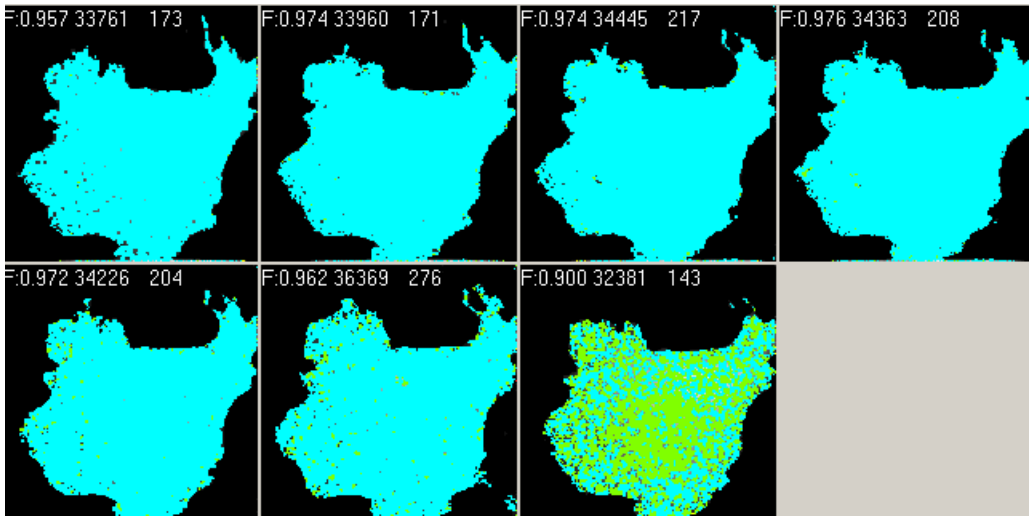
F:0.957 33761 173
F:0.974 33960 171
F:0.974 34445 217
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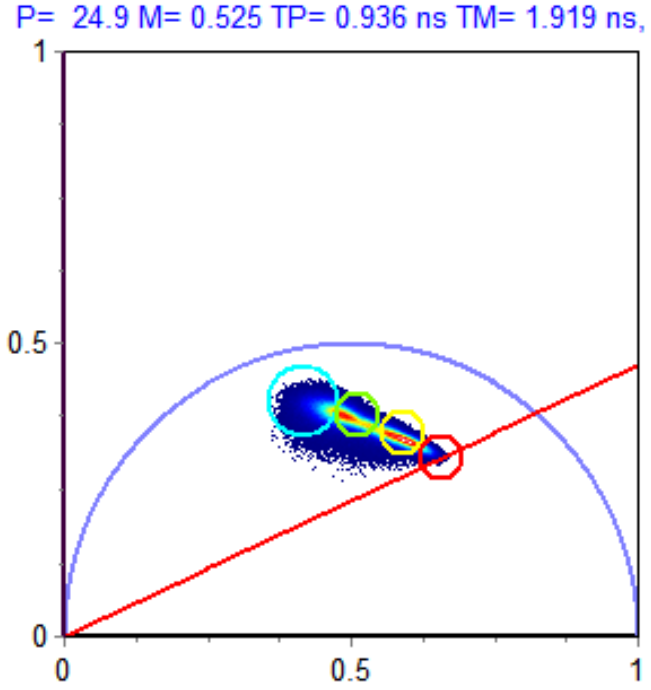
F:0.900 32381 143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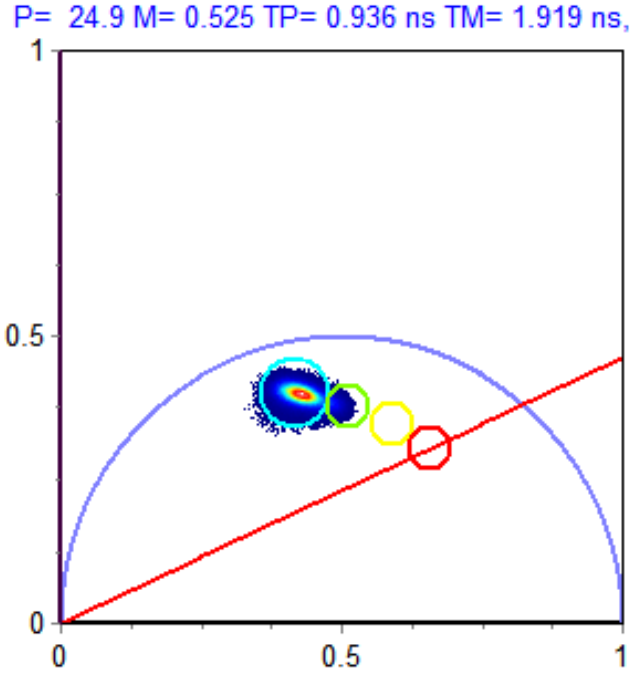
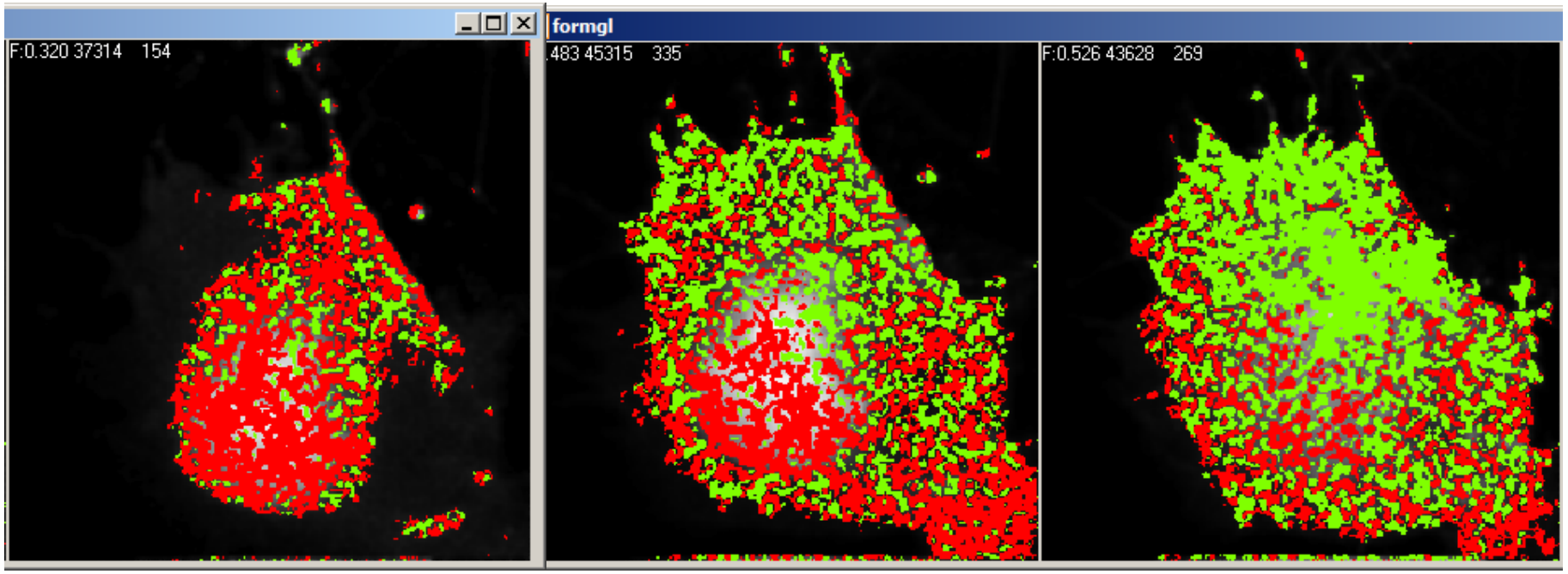
0.5
1

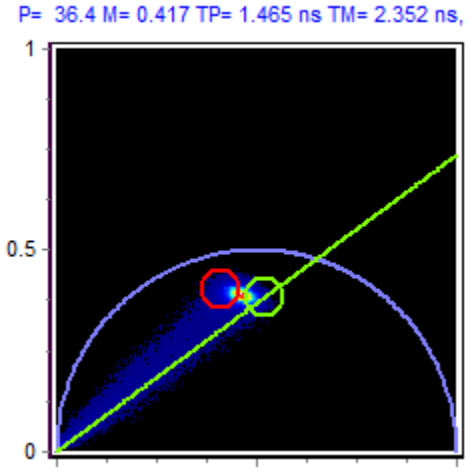

P= 24.9 M= 0.525 TP= 0.936 ns TM= 1.919 ns,
1
0.5
0
0
0.5
1

Before EGF

After EGF time 1

After EGF time 2





Features of the new approach

Many of the obstacles in FLIM data analysis can be removed.
The accuracy of lifetime determination is improved

The speed of data analysis is reduced to almost instantaneous for an entire image or several images

The analysis is “global” over the image and across images.

The interpretation of the FLIM experiment is straightforward.
Minimal prior spectroscopy knowledge is needed

The Phasor analysis method can be applied to all modes of data acquisition (frequency-domain and time-domain)

Ion concentrations can be calculated

Conclusions

By representing “molecular species” rather tan sum of exponential decays the phasor approach reduces the problem of fitting exponential components to exploration of regions of the phasor plot

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

You don't 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