# The Phasor approach: Application to FRET analysis

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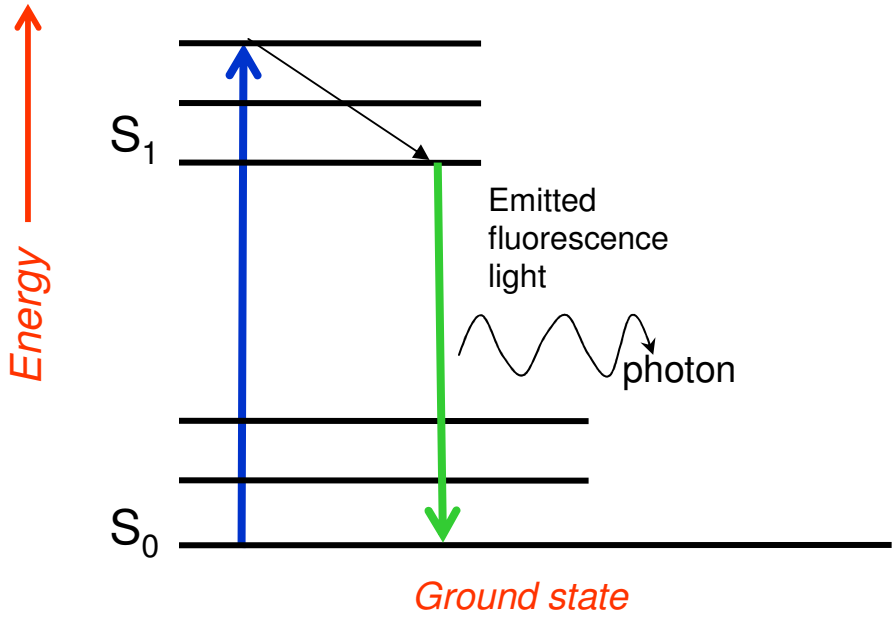
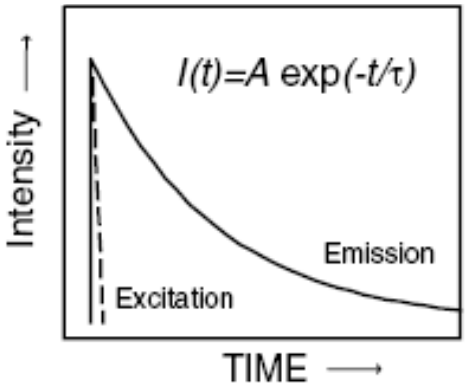
# Outline

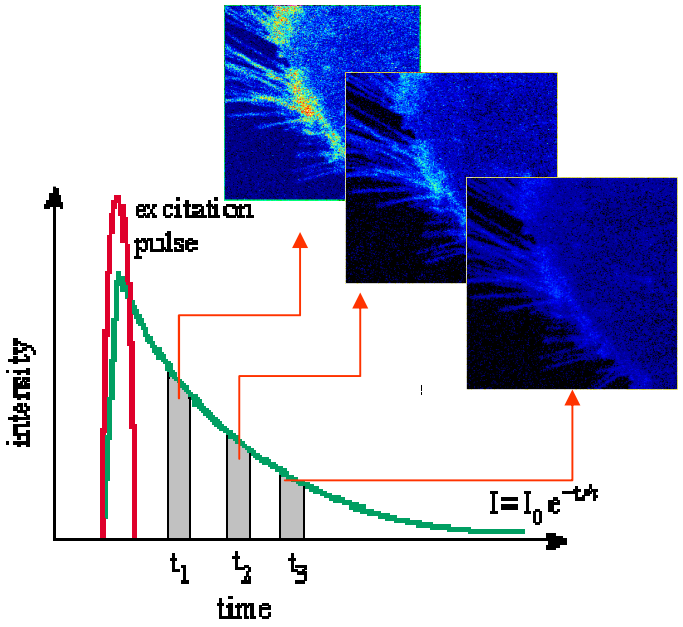
- Background: Lifetime
- Intro to Fluorescence Lifetime Imaging Microscopy
- Motivation for FLIM
- The Phasor approach
- Rac Activation using FLIM
- Future Prospects

# Lifetime: Background



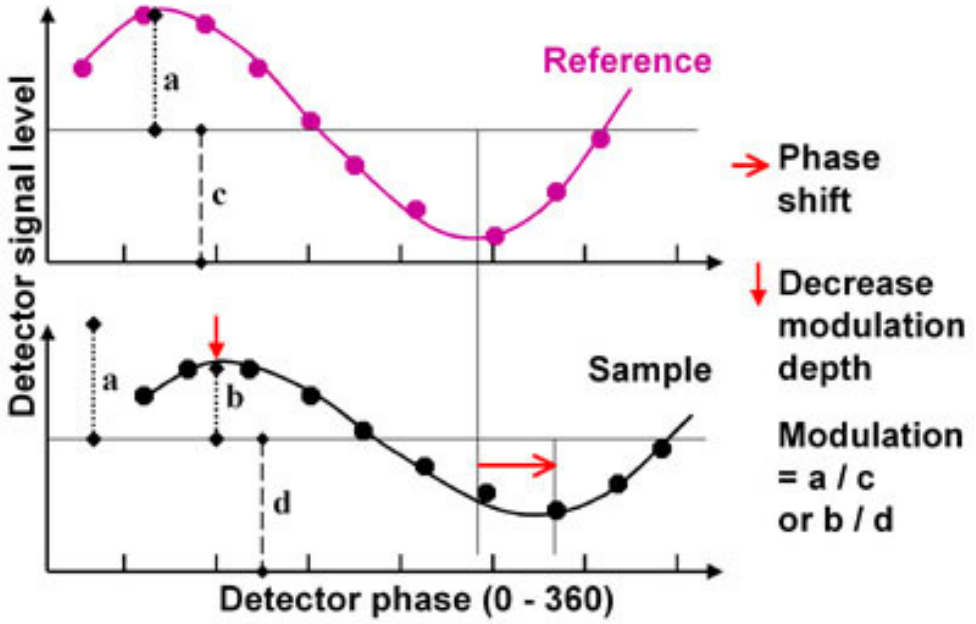



# Time Domain and Frequency Domain FLIM




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.

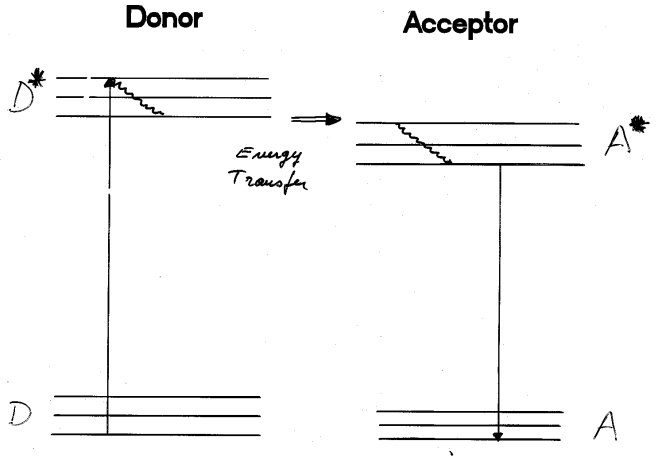
**Description of the process**

Consider a molecule called 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 called 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



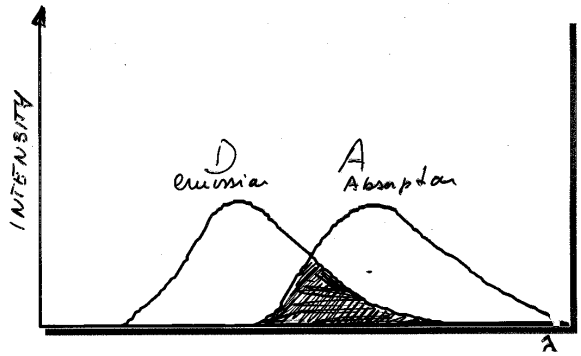
Because of rapid internal conversion the process A* to D* is very unlikely to occur unless the donor is of the same kind of the acceptor.
FRET strongly depends on the distance between the two groups.
The theory was developed by Förster in 1949 who calculated the rate of transfer to be

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

$\tau_D$ 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
$\Phi_D$ 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**

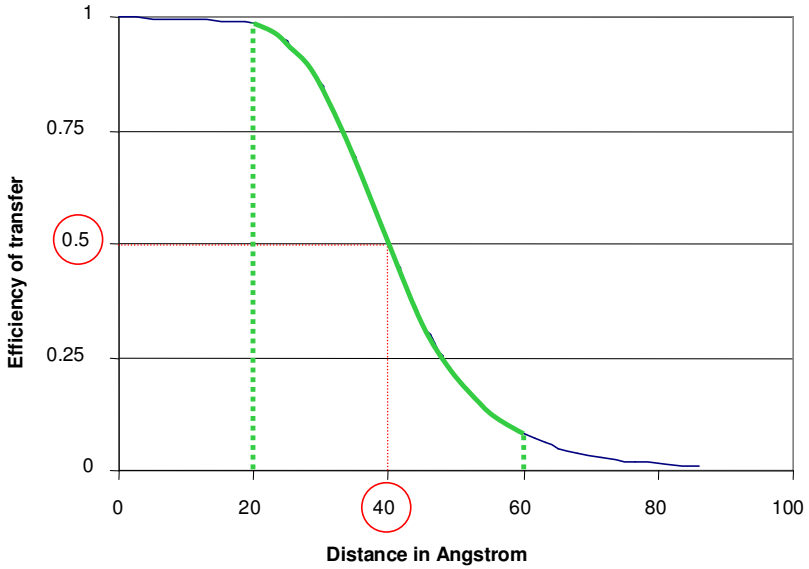
**Energy transfer studies give information**
•Distance between groups
•Orientation of two groups
Notice that D and A can be the same kind of molecule provide emission and absorption overlap.

# 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 ~1.5$R_0$

How to perform an energy transfer experiment?
Define

Energy transfer efficiency ( *E*)

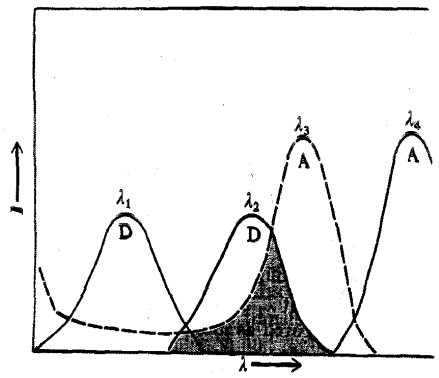
$$E = \frac{k_T}{k_T + \sum_{i \neq T} k_i}$$

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 intensity of the acceptor fluorescence in the absence and presence of the donor.




The sample is excited at $\lambda_1$. The fluorescence at $\lambda_4$ of the acceptor can be observed in the absence of the donor to check if there is any contribution of direct acceptor excitation

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

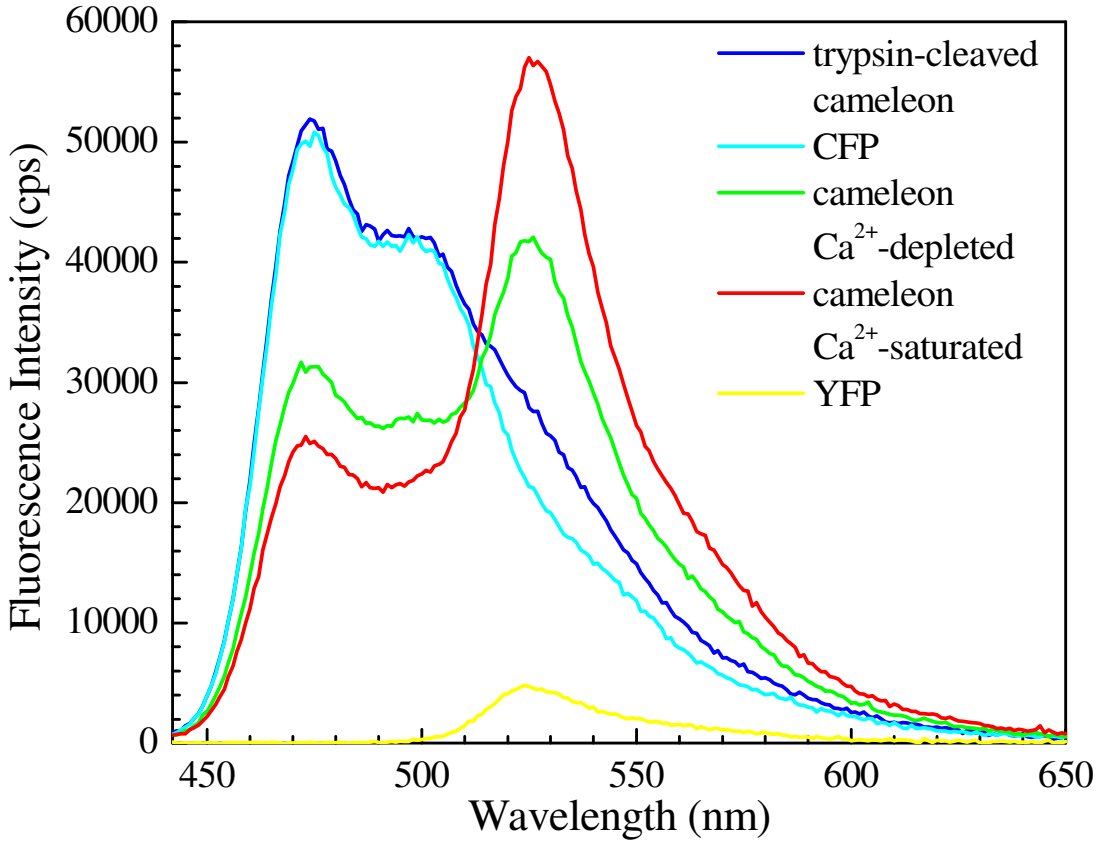
In presence of the donor the total fluorescence intensity at $\lambda_4$ is

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

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.

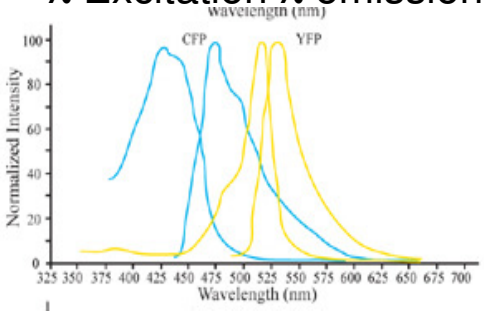
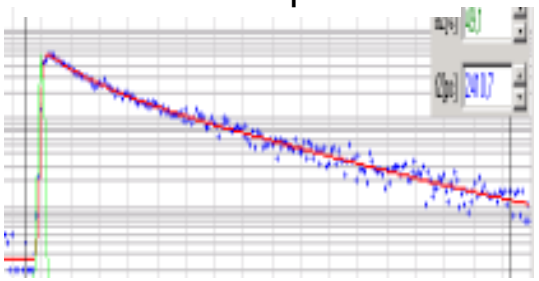
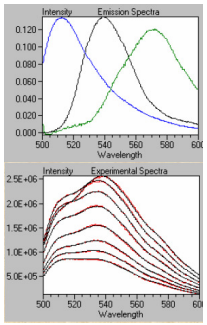
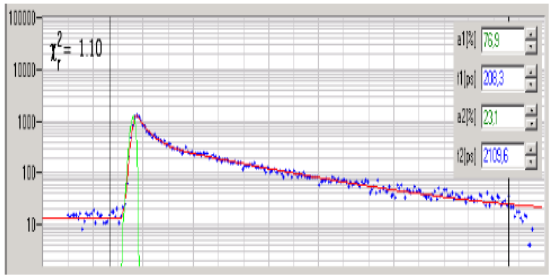
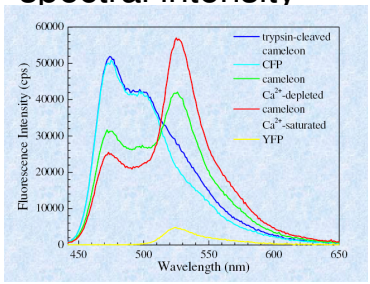
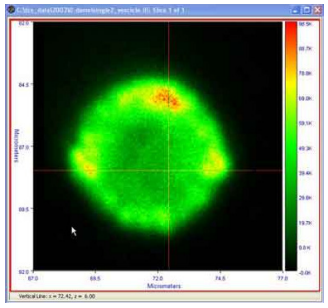
$$\frac{\tau_{D+A}}{\tau_D} = 1 - E$$




# 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.

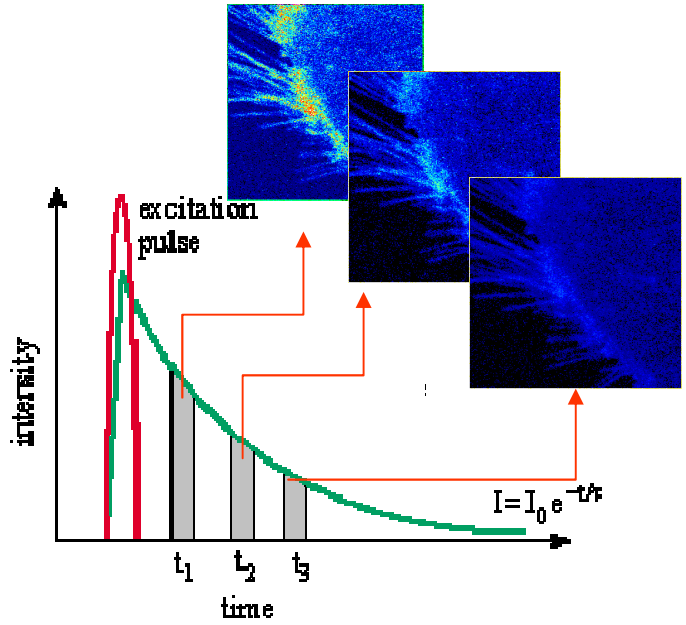
We need to go to a new “space”

# 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.

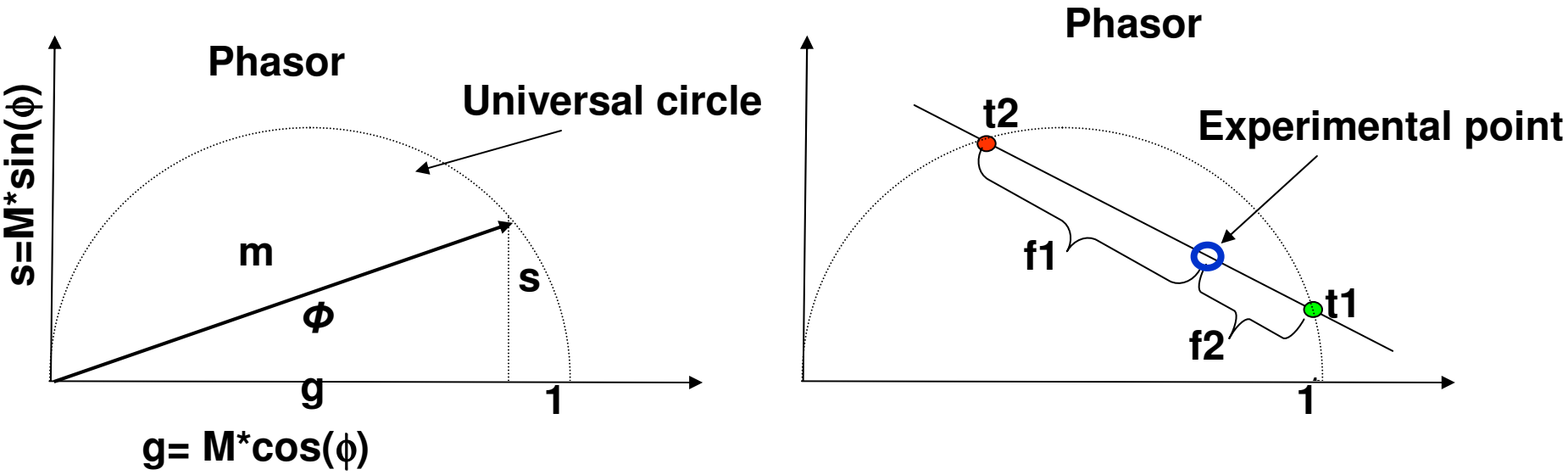
# How to calculate the components g and s of a phasor from the time decay?




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

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

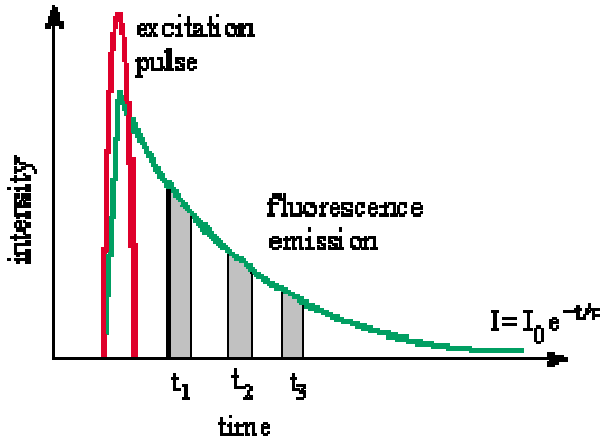
# The algebra of phasors



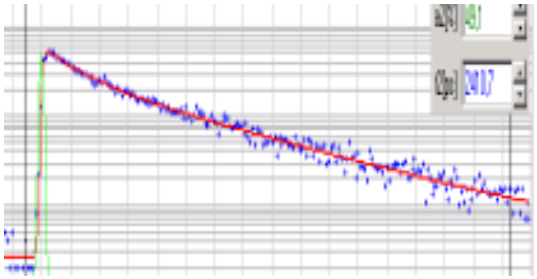

Simple rules to the Phasor plot:

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

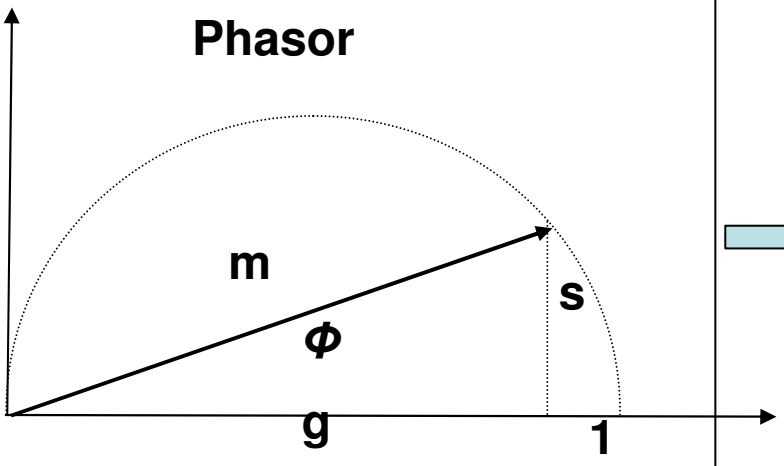



## Fitting of exponentials

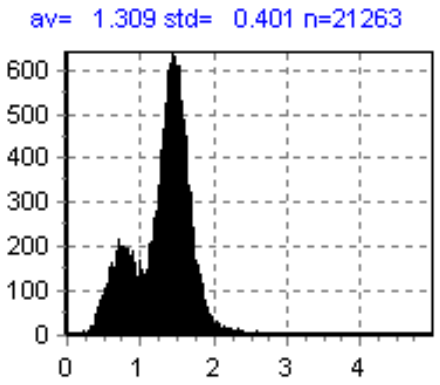


Multiexponential analysis

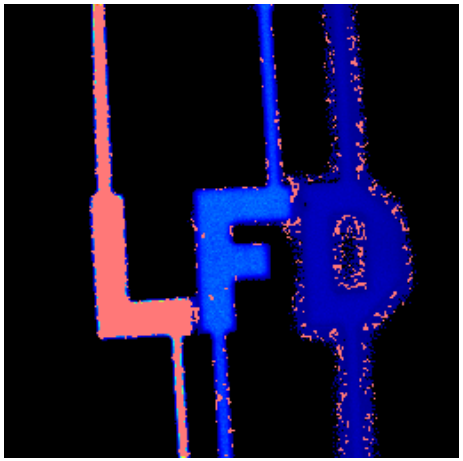
## Phasor

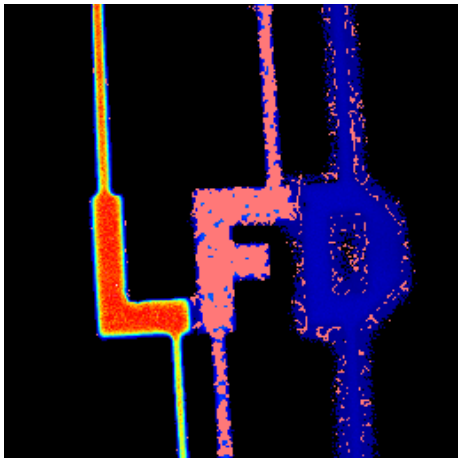


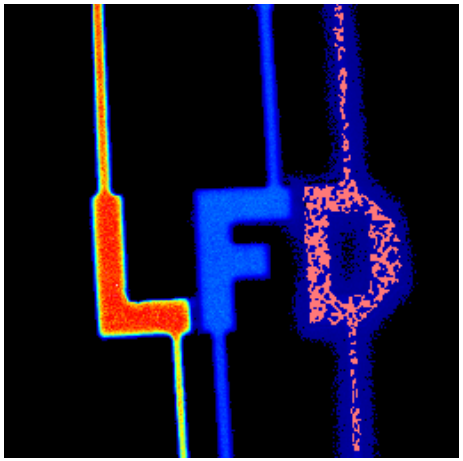

## Lifetime distribution

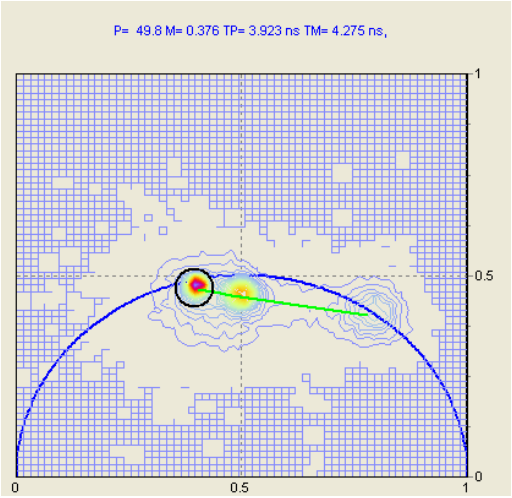


# Separating Different Single exponential lifetimes using the ISS Fast FLIM system

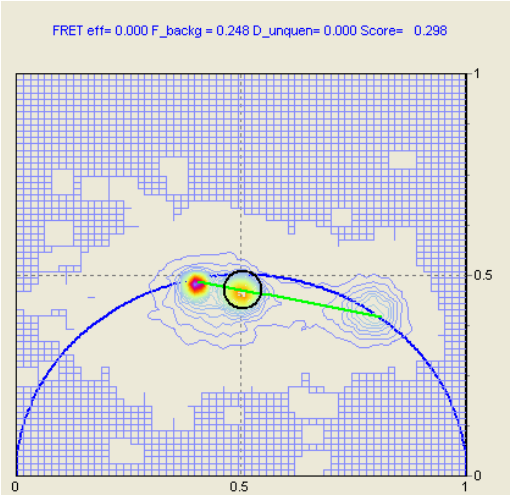




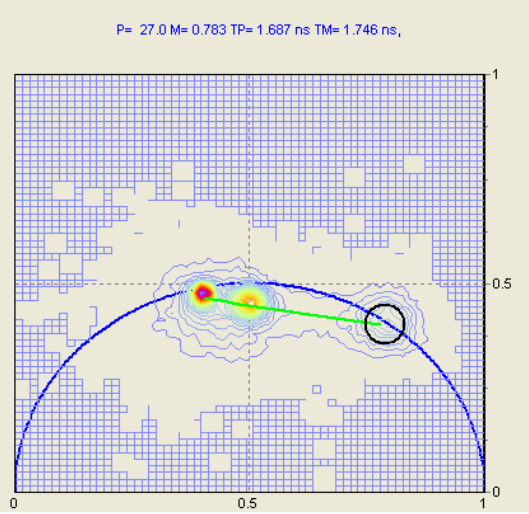





Fluorescein



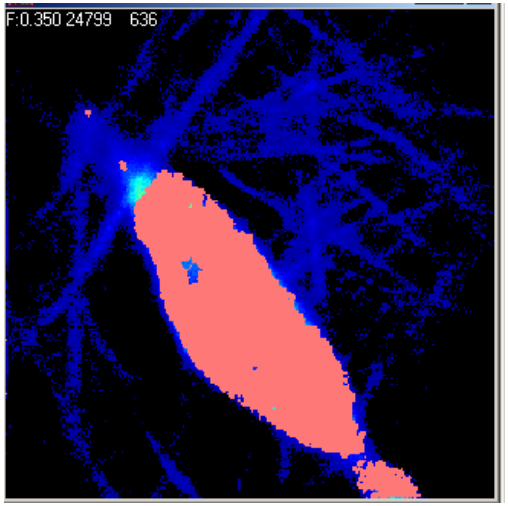

Mixture



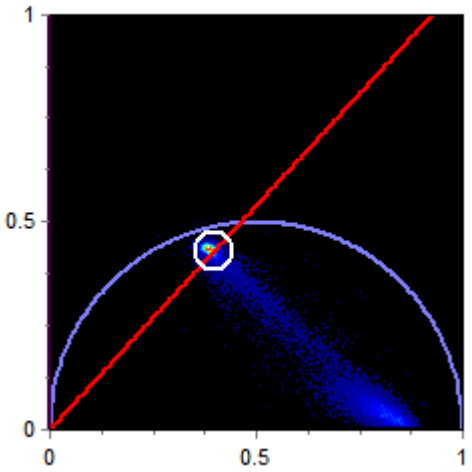

Rhodamine B1

# Pax-eGFP CHO-k1 in collagen

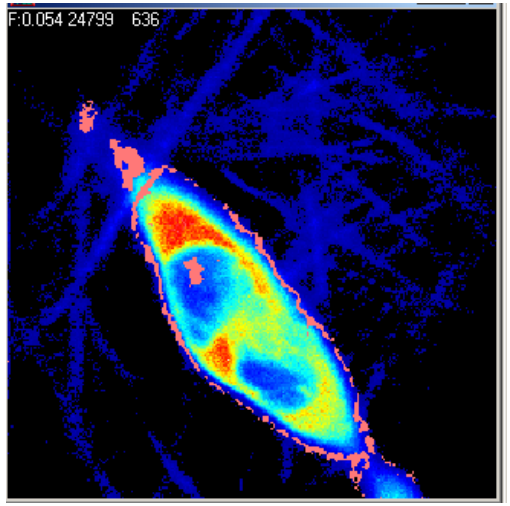
referenced with Fluorescein @ 905nm



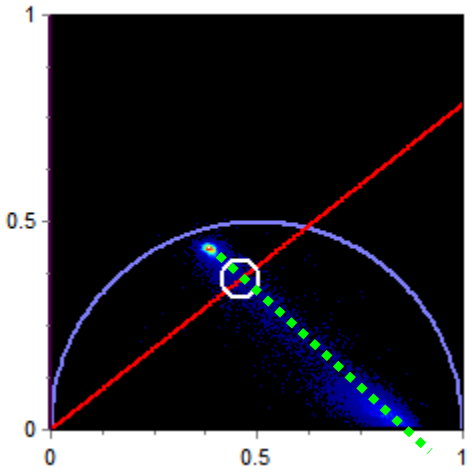

P= 47.2 M= 0.343 TP= 2.149 ns TM= 2.751 ns,



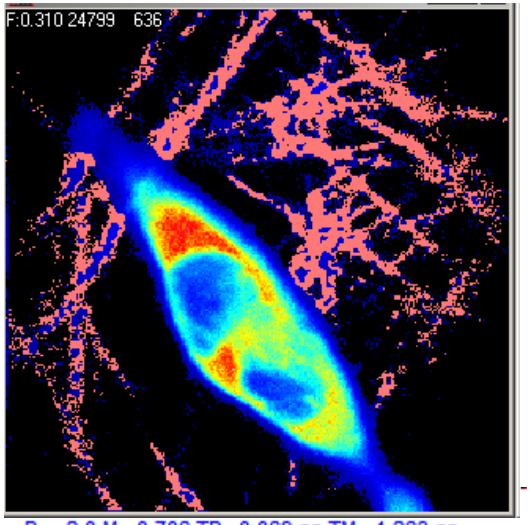

Lifetime of EGFP



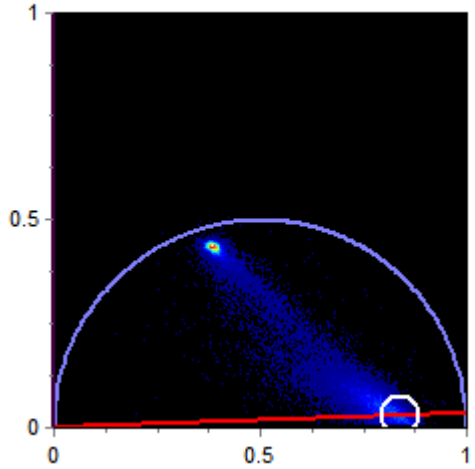

P= 38.2 M= 0.344 TP= 1.563 ns TM= 2.748 ns,



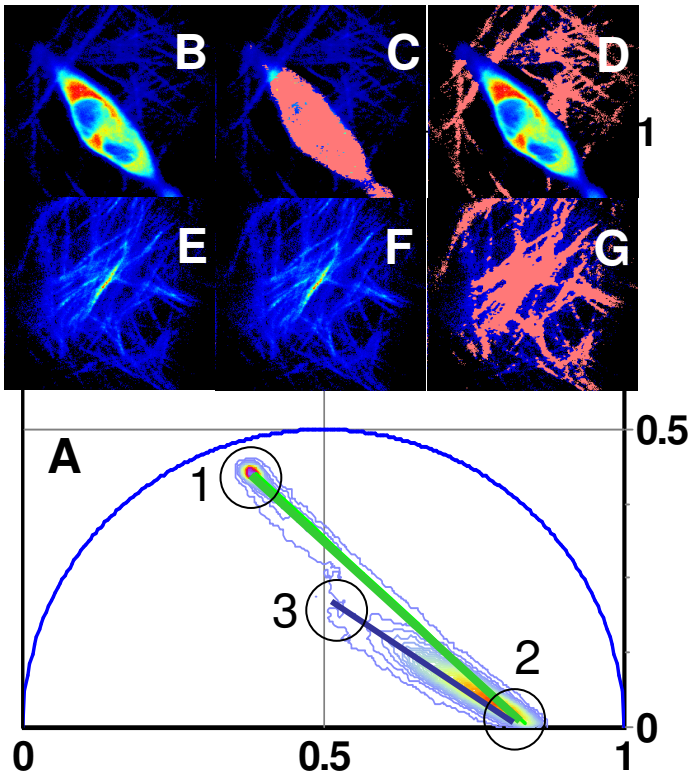

Combinations of Lifetimes



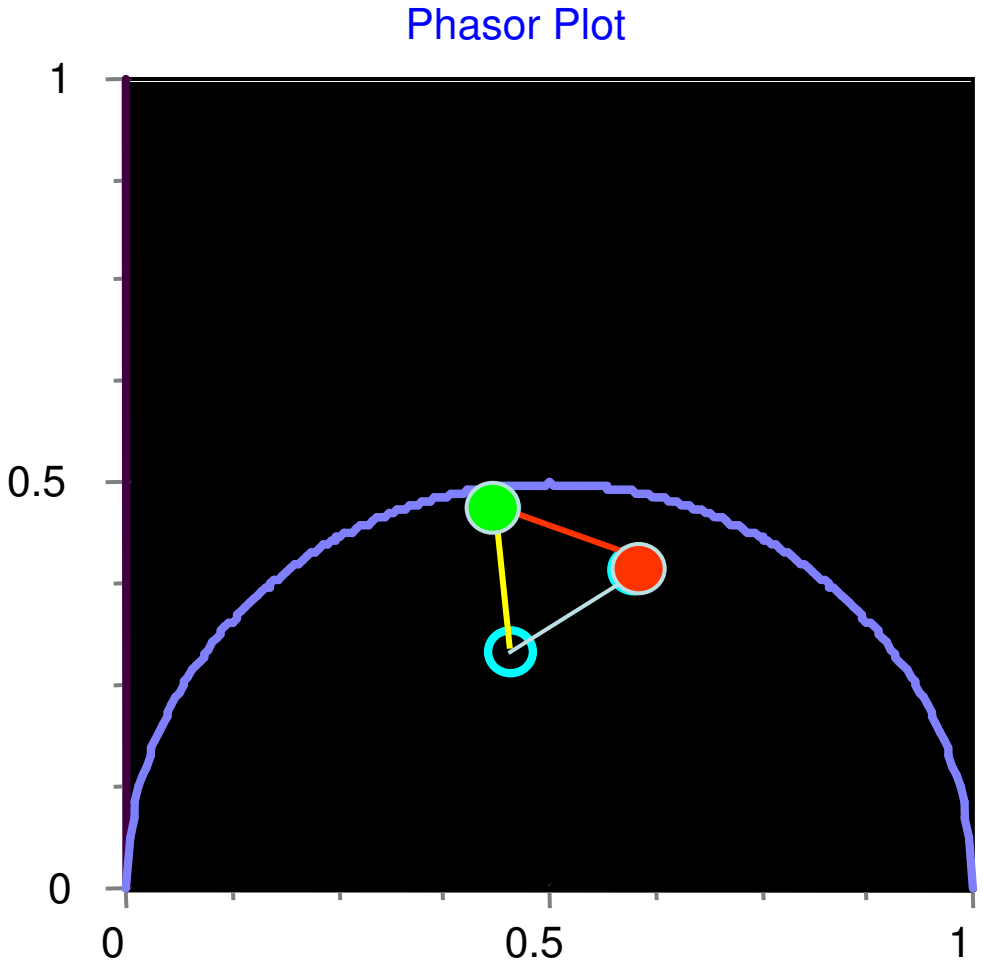

P= 2.0 M= 0.706 TP= 0.069 ns TM= 1.283 ns,



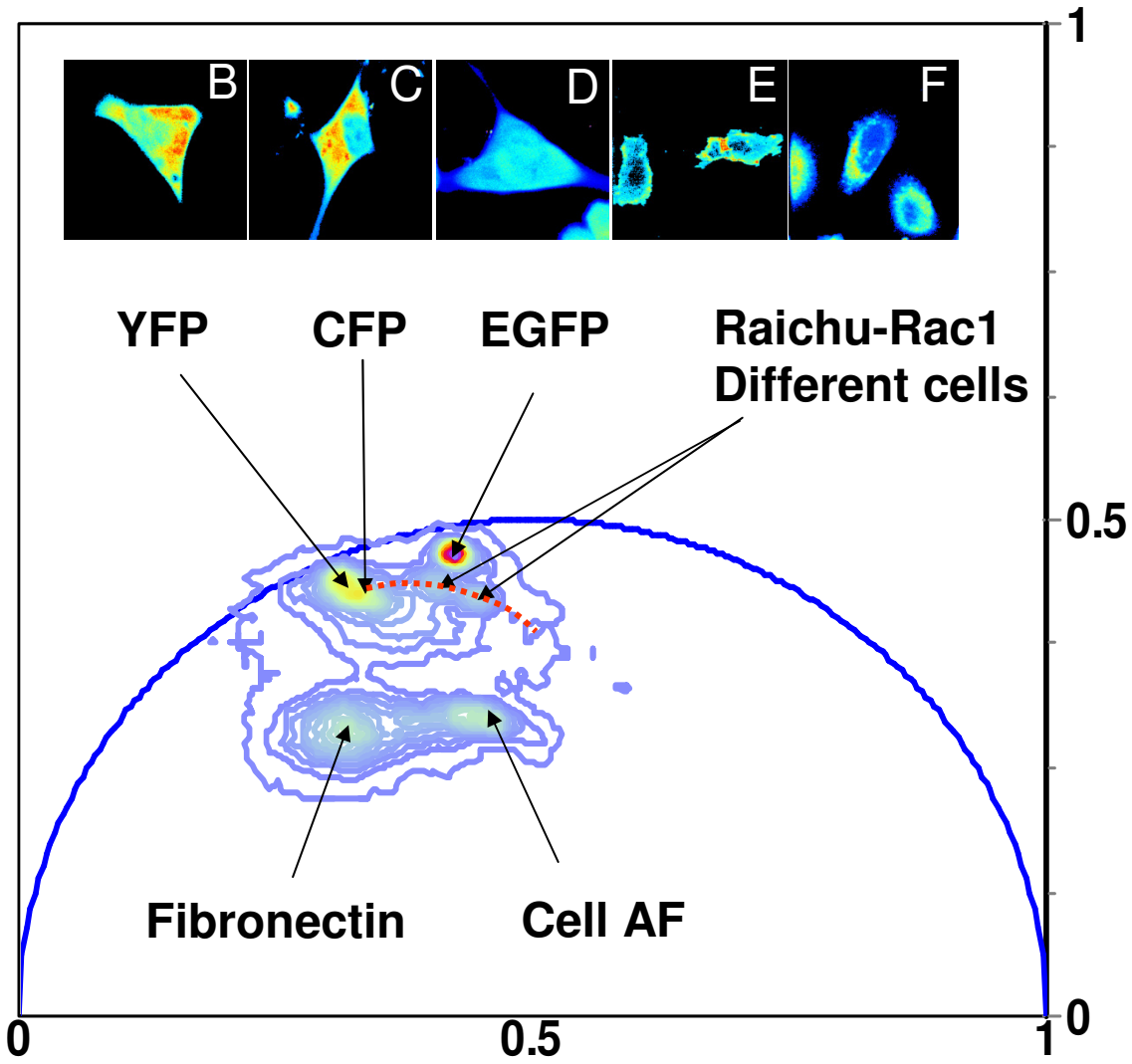

Lifetime of Collagen


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

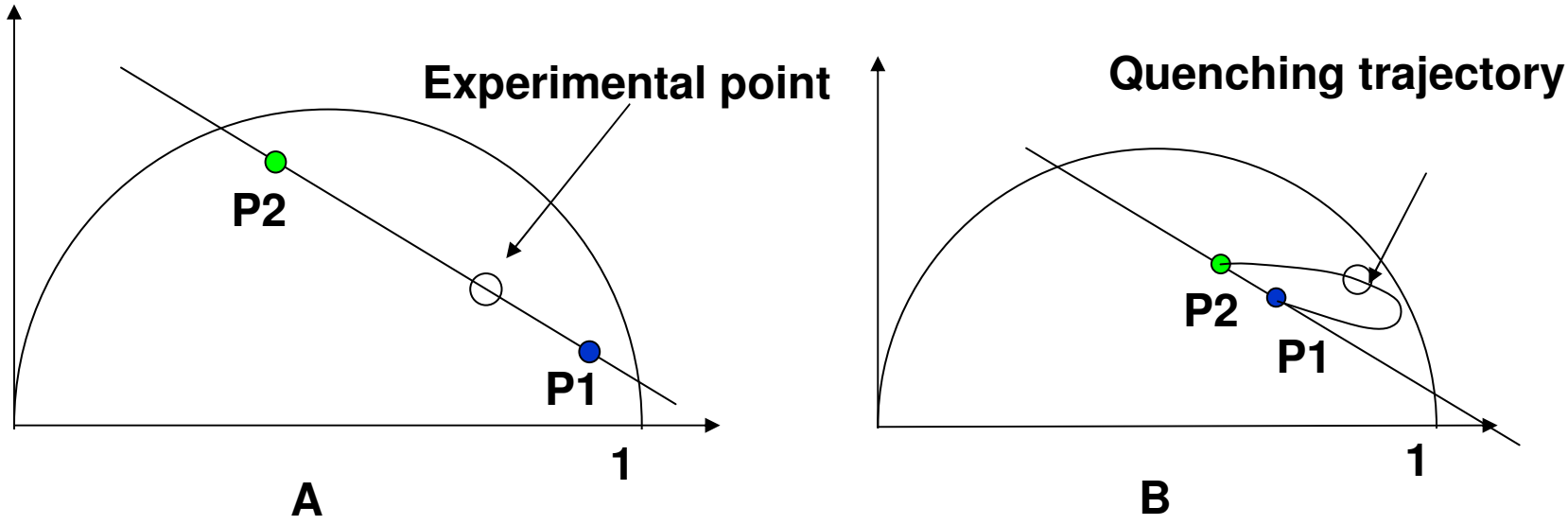
# How to identify components?




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

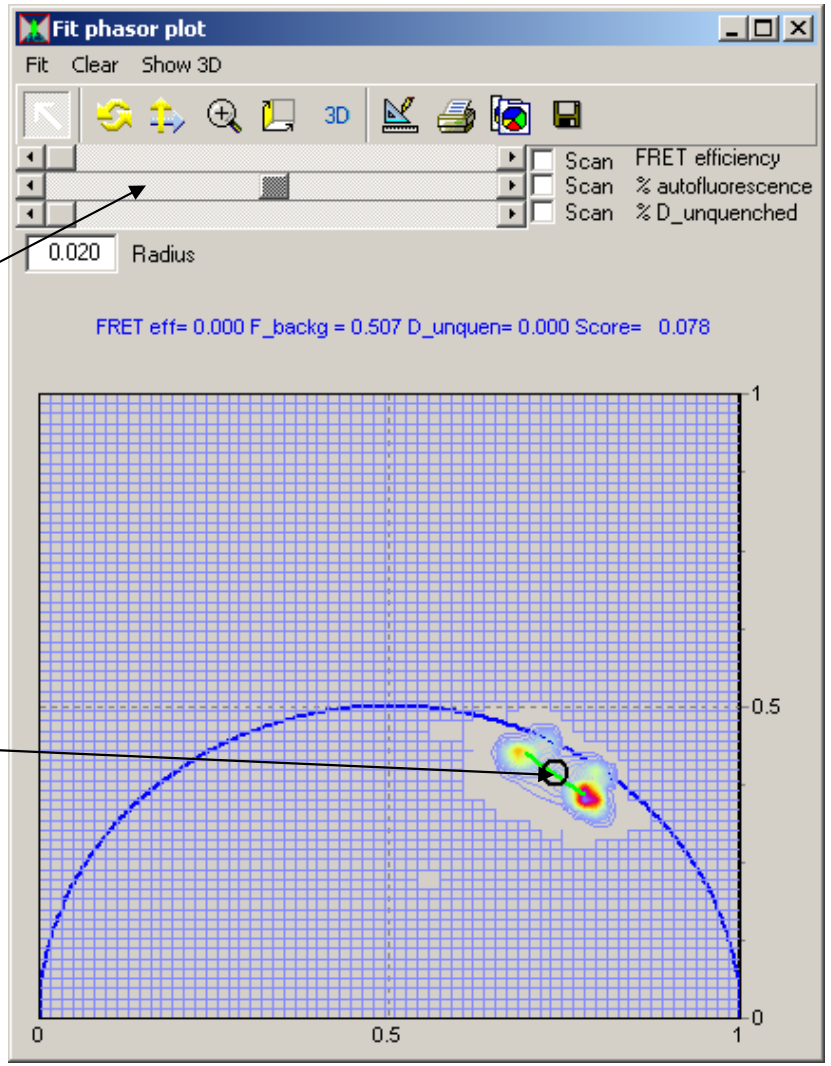
# The fractional intensity calculator

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

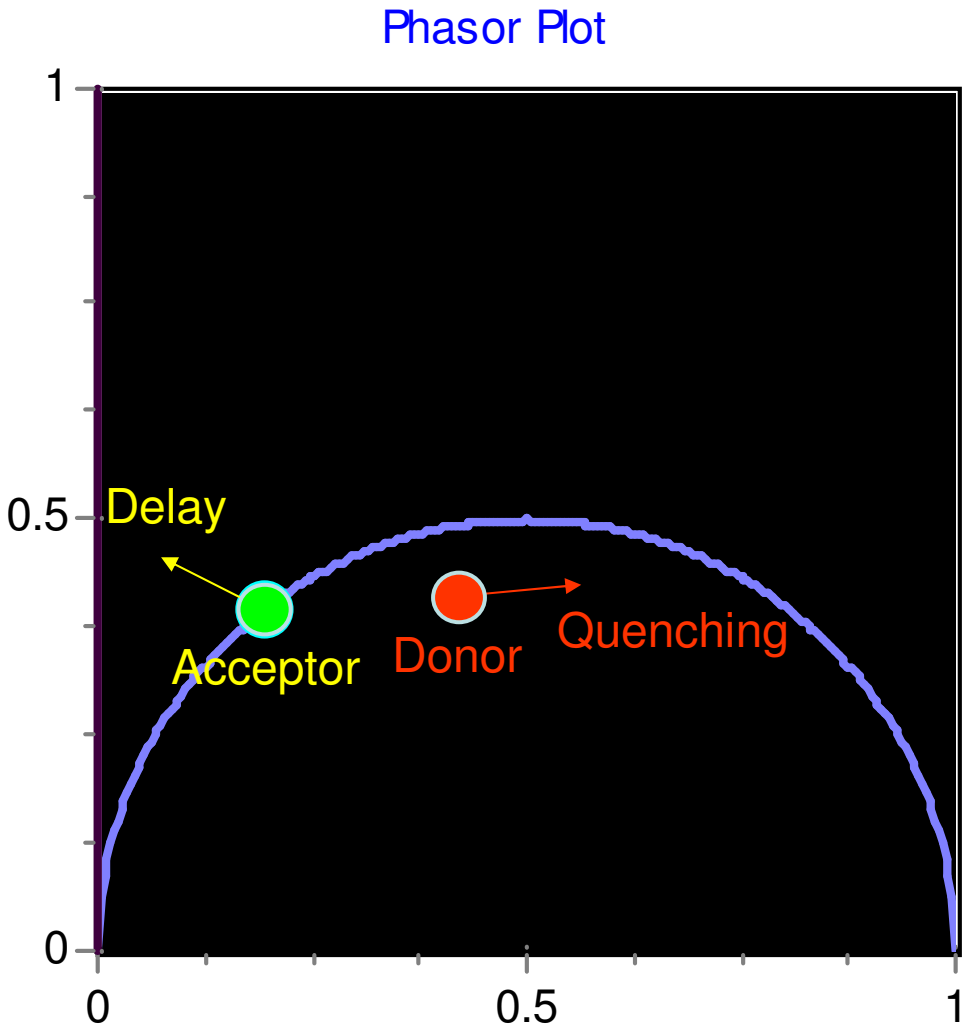
2. Click to assign the phasor to the phasor 2.

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?




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

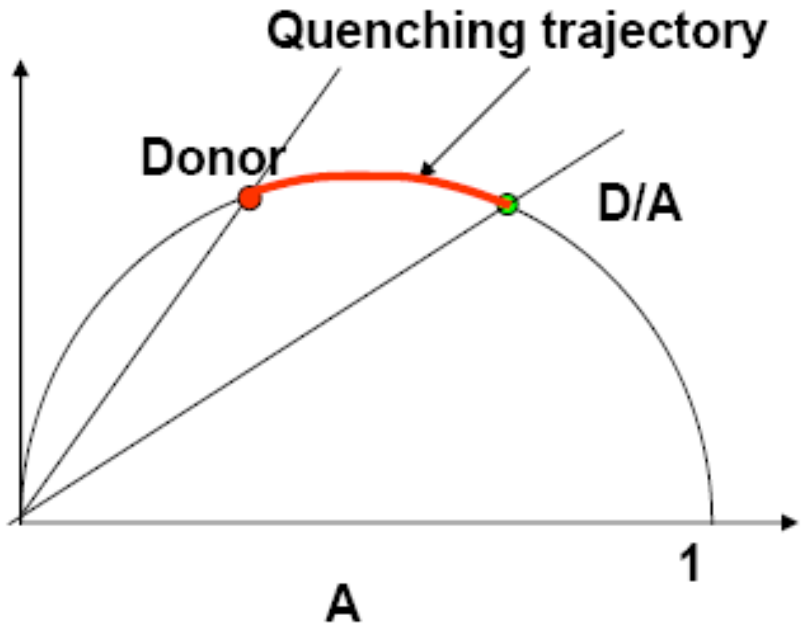
The calculator for FRET efficiencies from

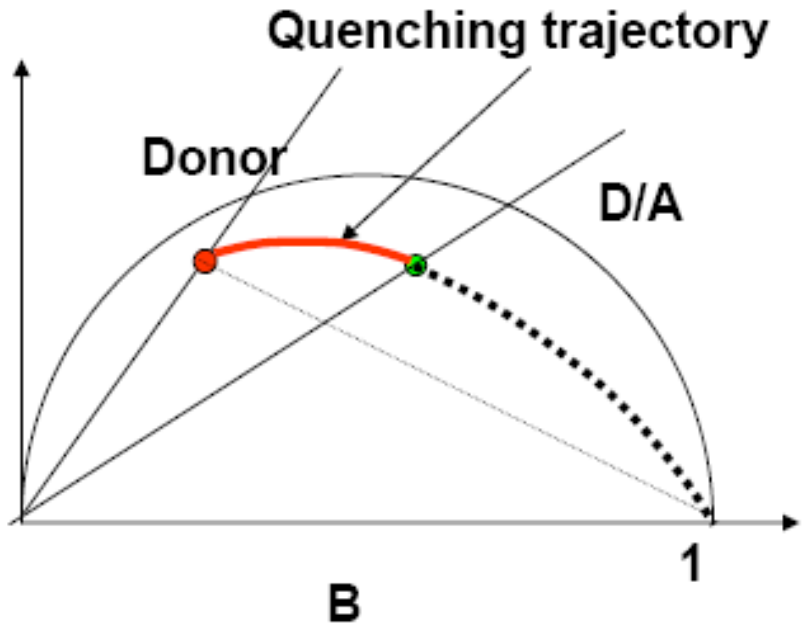
$$\text{FRET efficiency} = \frac{\tau_D - \tau_{FRET}}{\tau_D}$$

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**

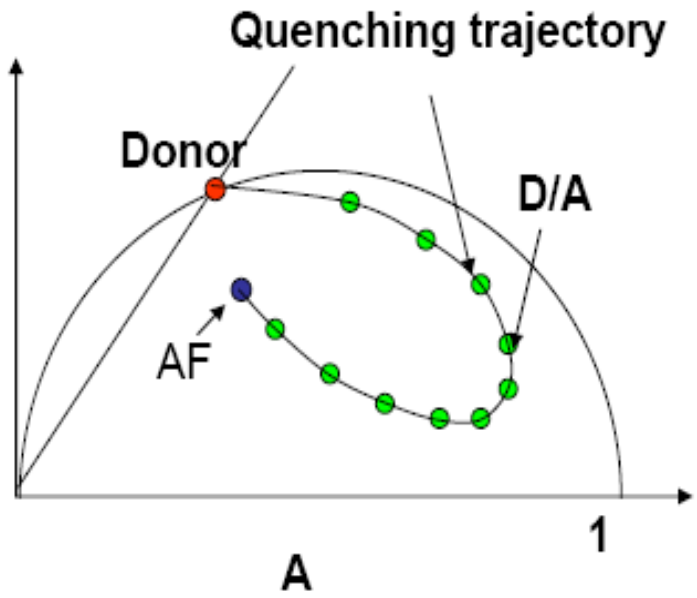
**The lifetime of the donor is along a different "trajectory", Why is the trajectory an arc rather than a line to the (1,0) point?**

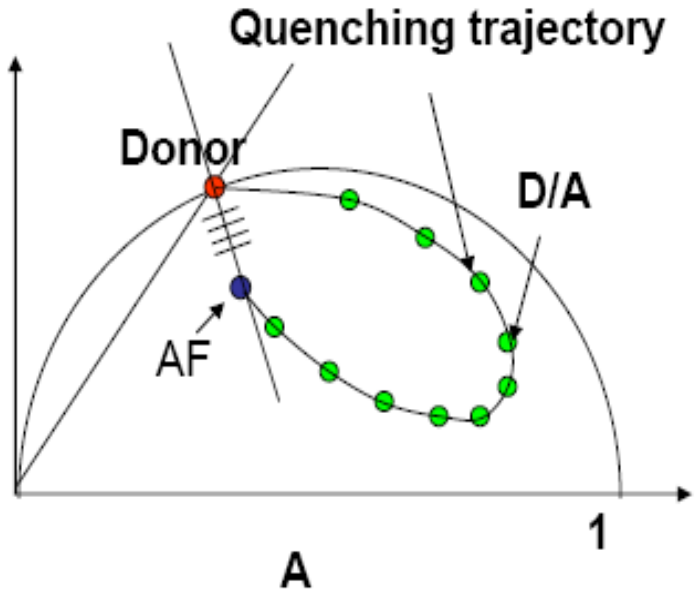
# The FRET Calculator

- 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!!

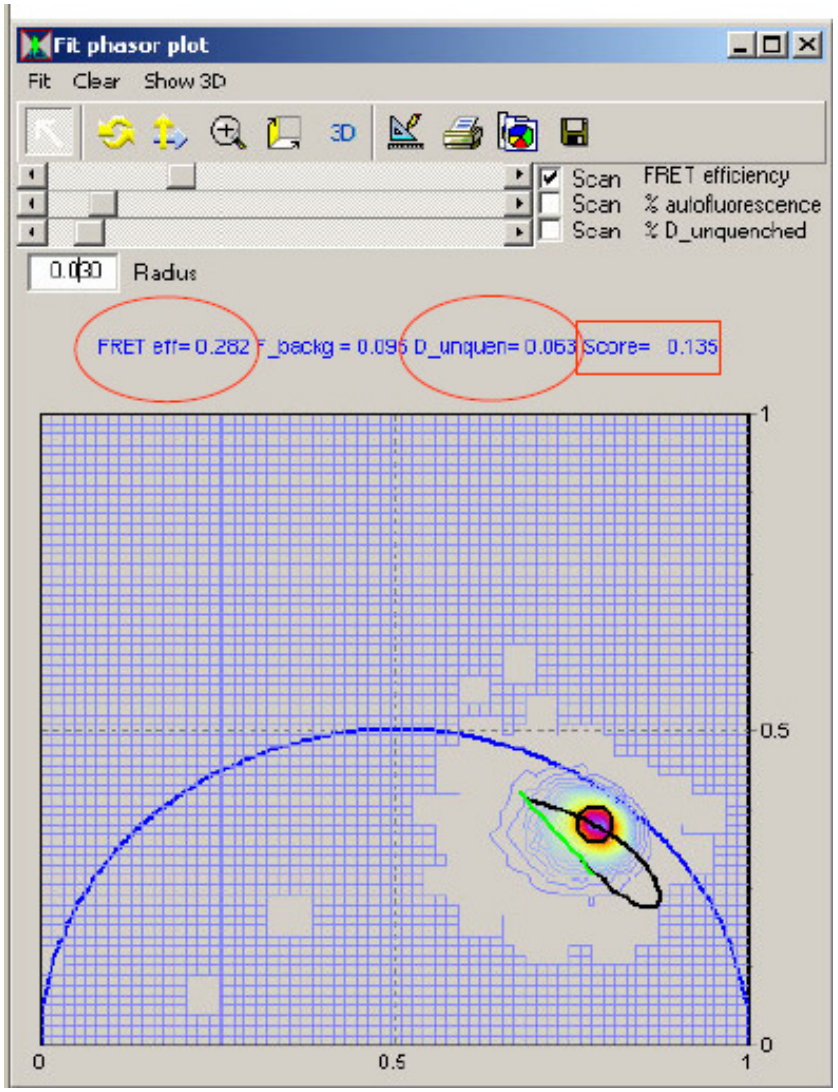



**As the lifetime of the Donor is quenched, the phasor of the quenched Donor is added to the phasor of the autofluorescence**

**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**

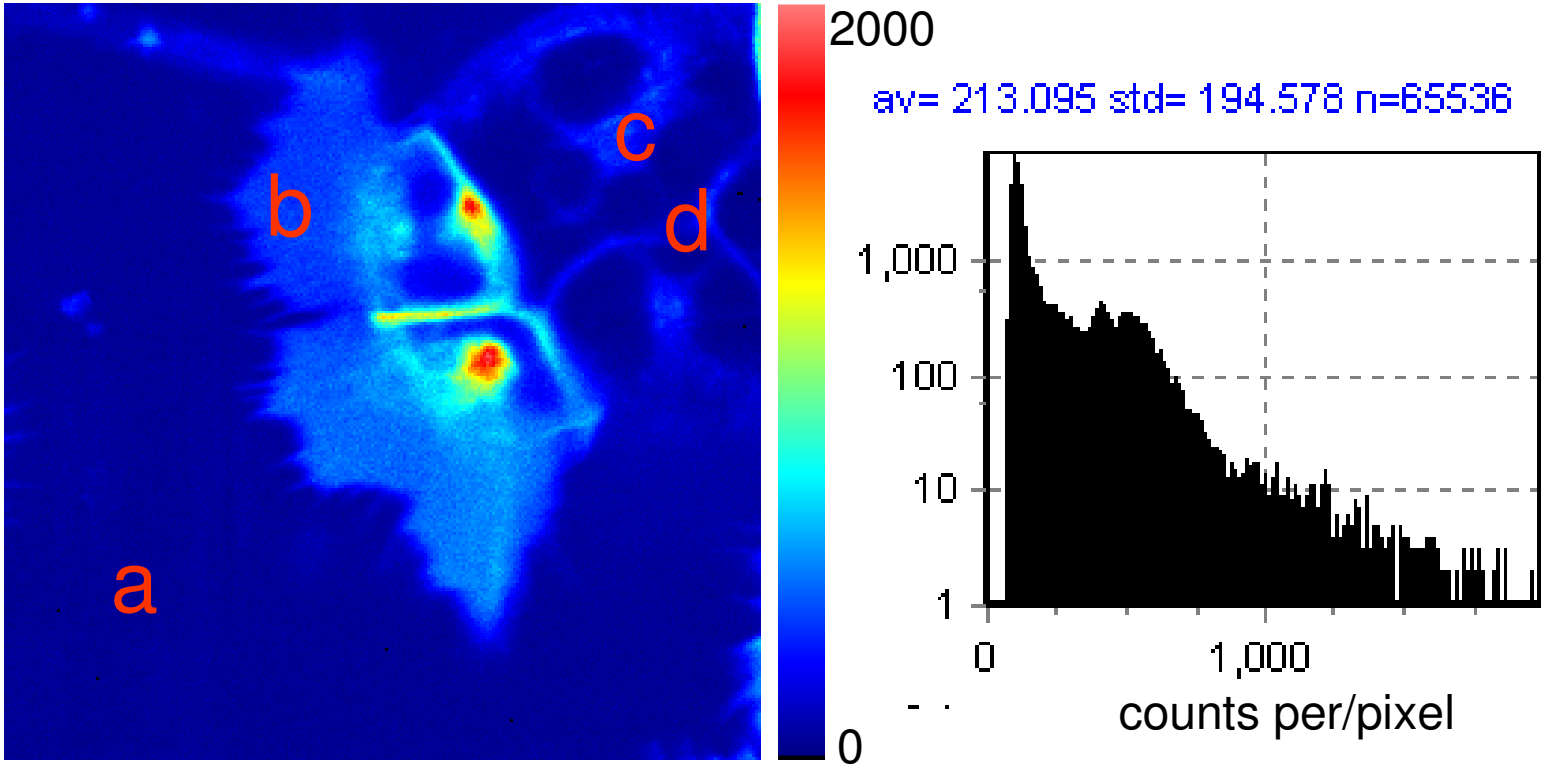
# The FRET calculator




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

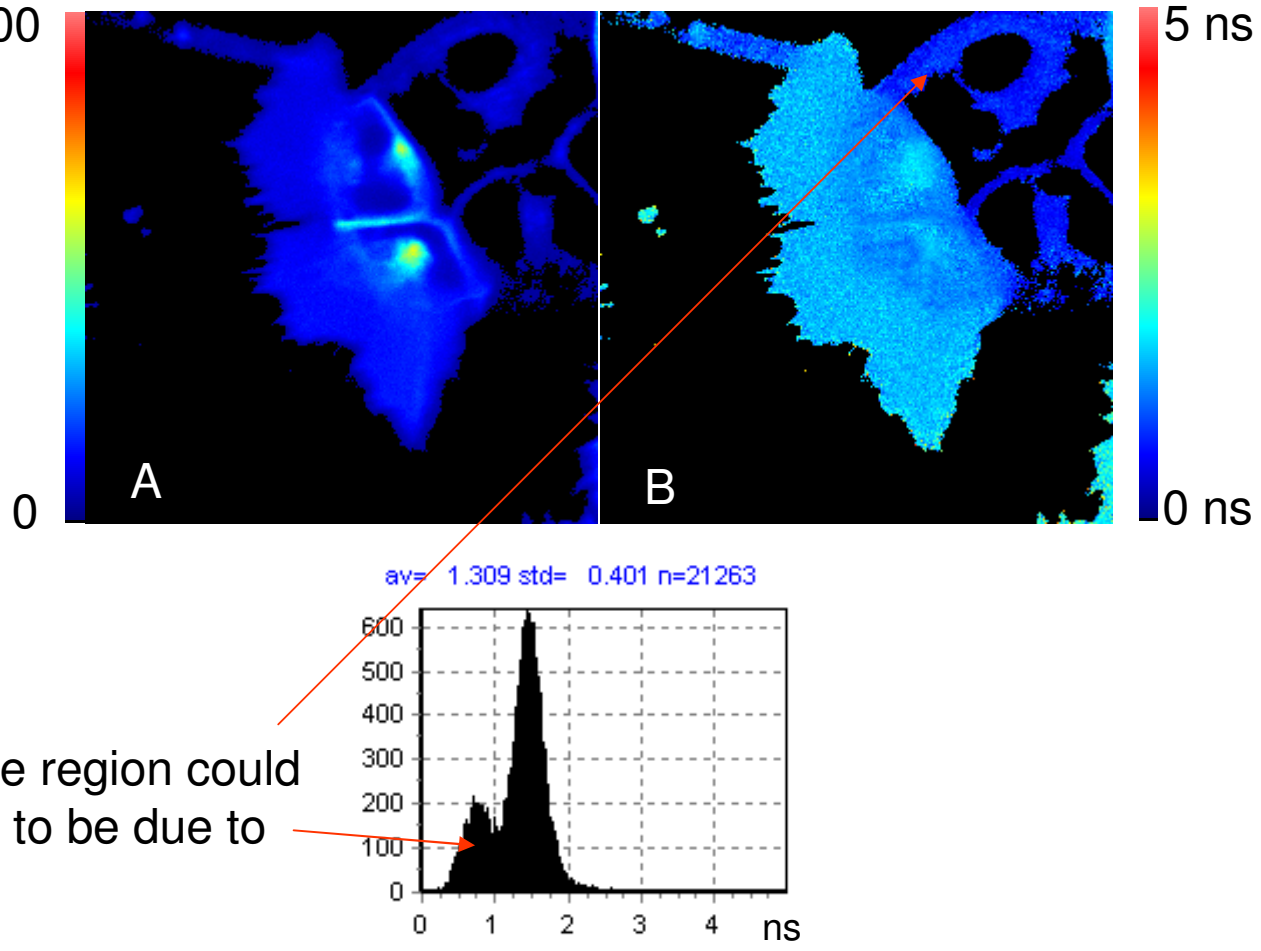



Several regions the image can be identified corresponding to **a)** background (2 exponentials) **b)** cell 1 bright (2 exponentials) **c)** cell 2 dim, **d**) cell junctions dim.

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

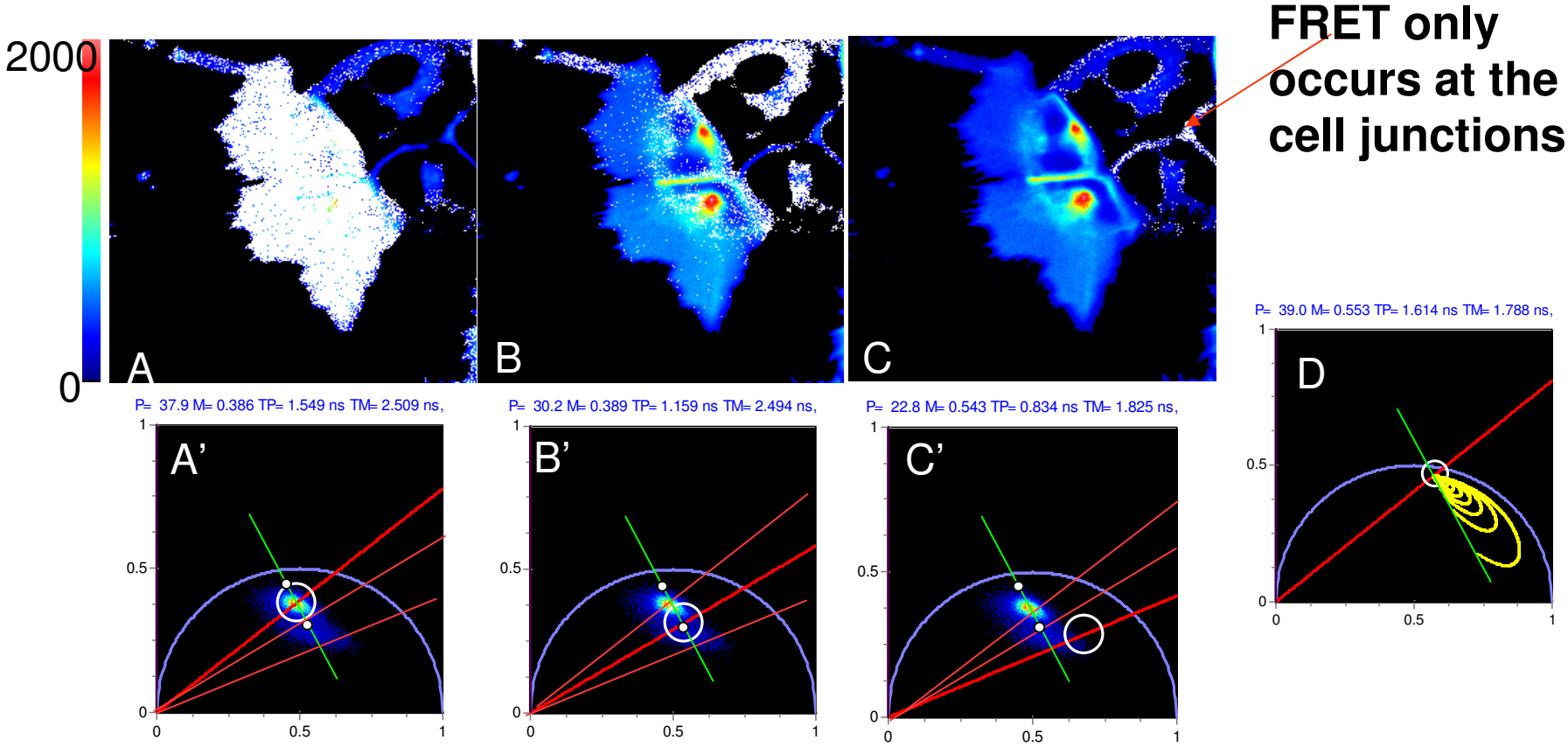
**Image obtained using B&H 830 in our 2-photon microscope**




Shorter lifetime region could be interpreted to be due to FRET

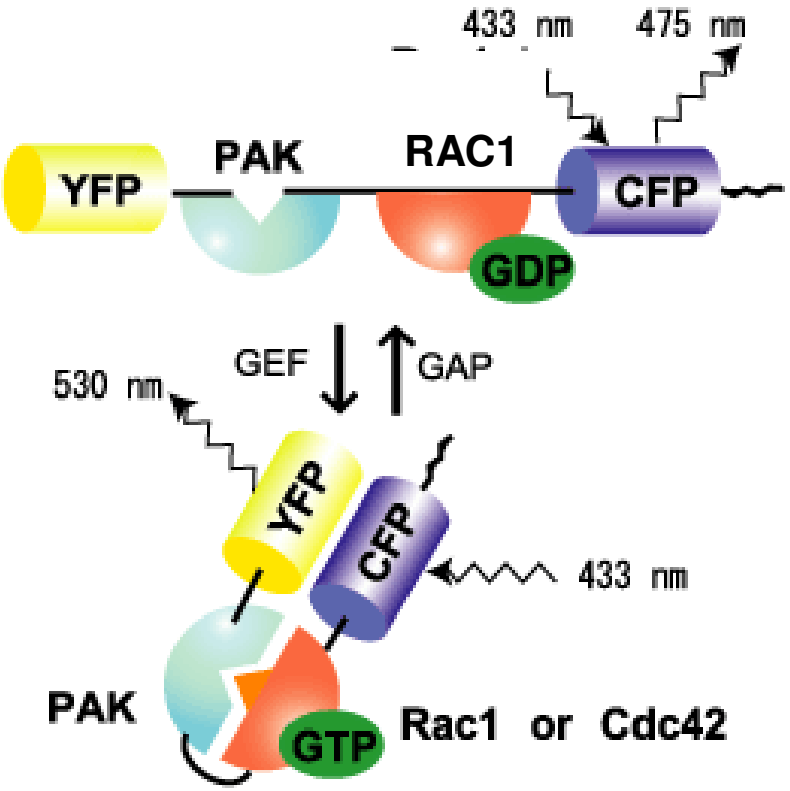
**Donor+acceptor+ligand. A) intensity image after background subtraction, B) $\tau_p$ image**

# Identification of FRET using the phasor plot




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**.

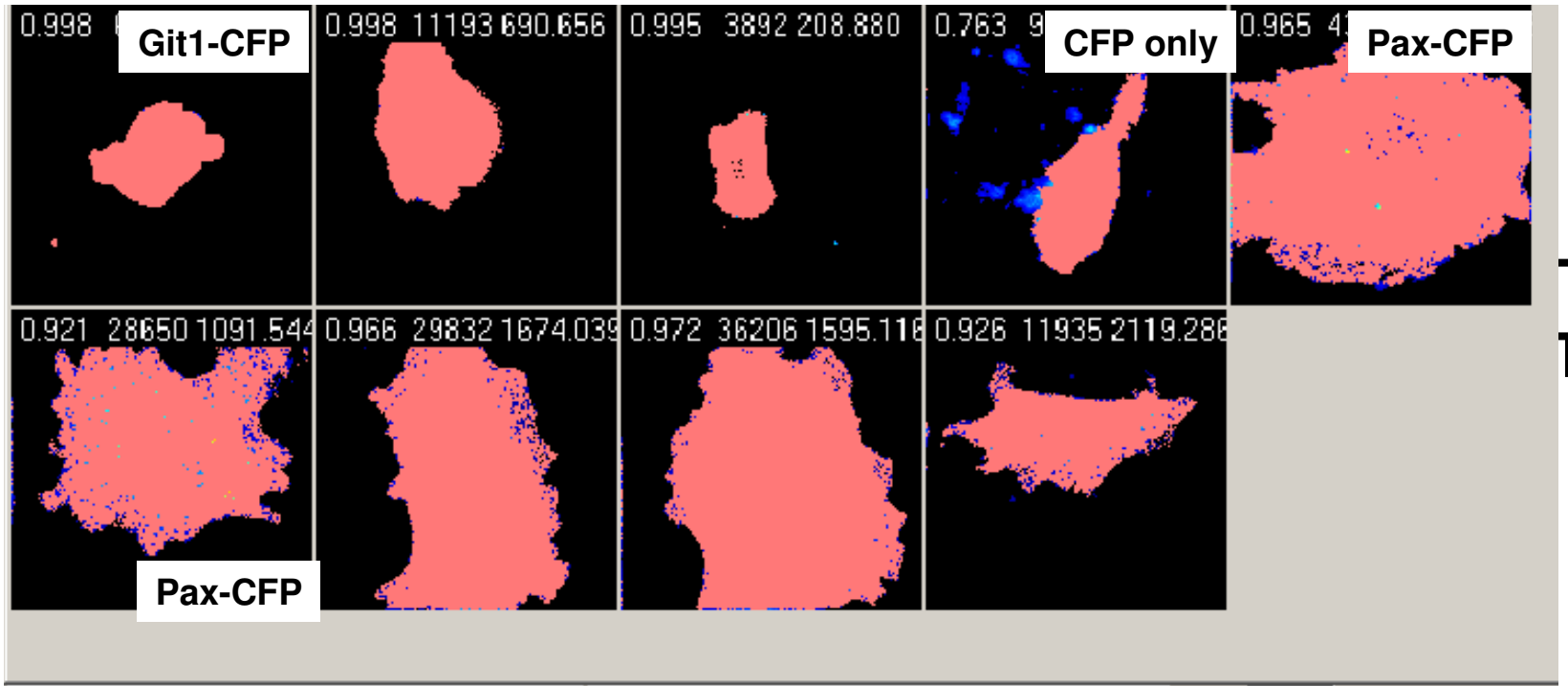
# Raichu-Rac1 FLIM/FRET analysis



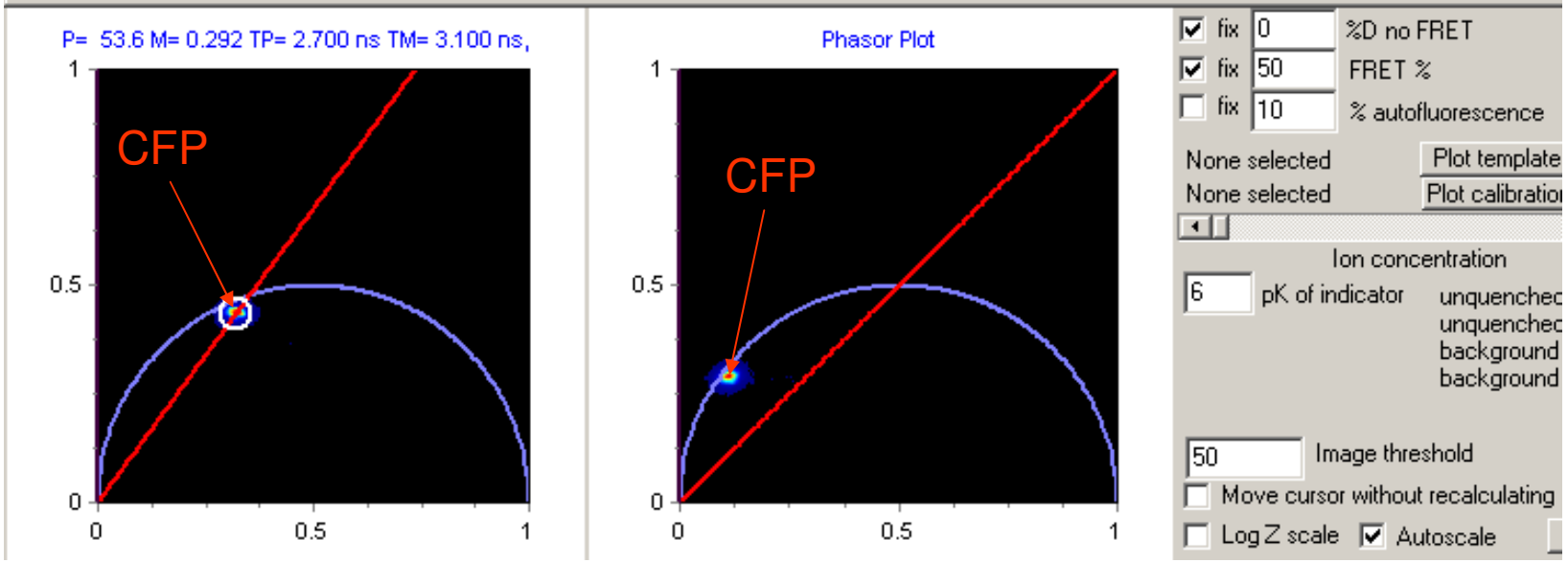

Fluorescence Resonance Energy Transfer
(FRET)

http://www.biken.osaka-u.ac.jp/biken/shuyouvirus/e-phogemon/raichu-Rac.htm

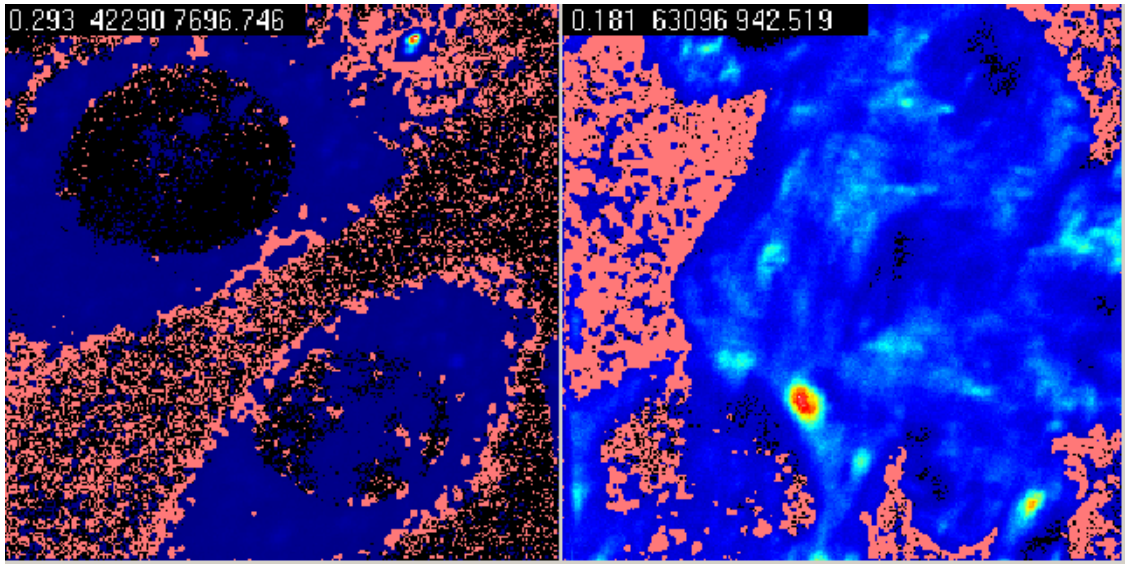
# Locating the CFP phasor



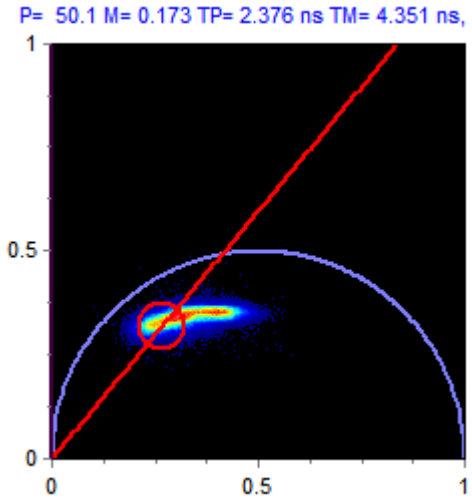

**DONOR:**
**CFP**
**TauP=2.7**
**TauM=3.1**



# Lifetime of the background

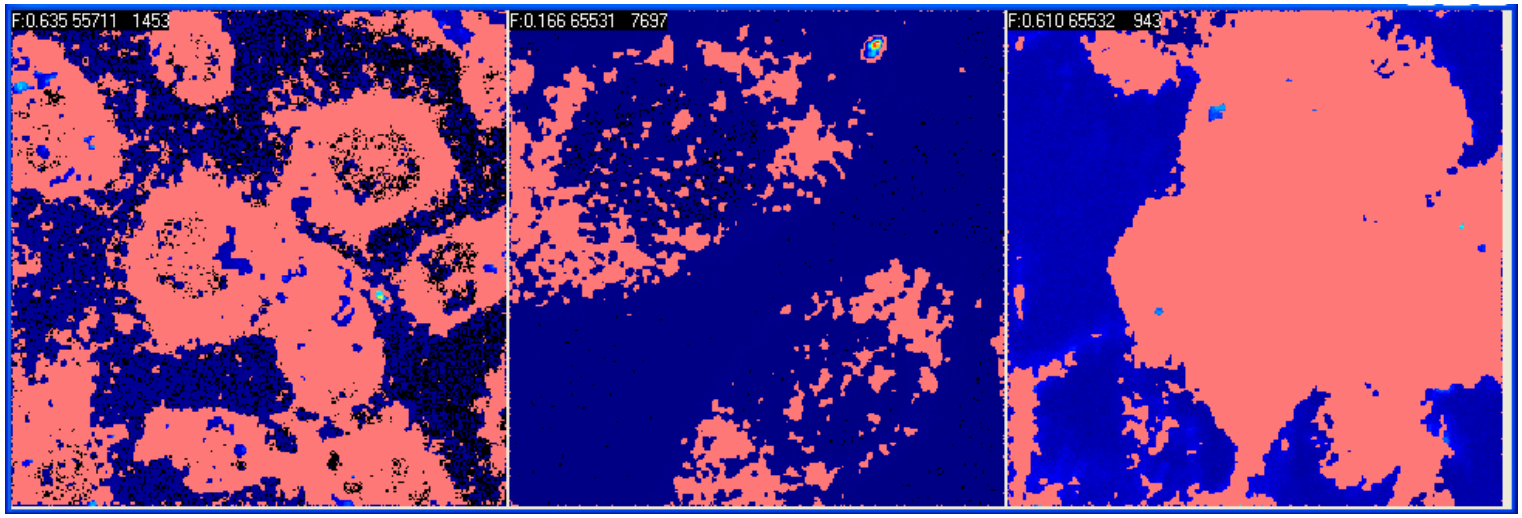



**TauP=2.4**
**TauM=4.4**

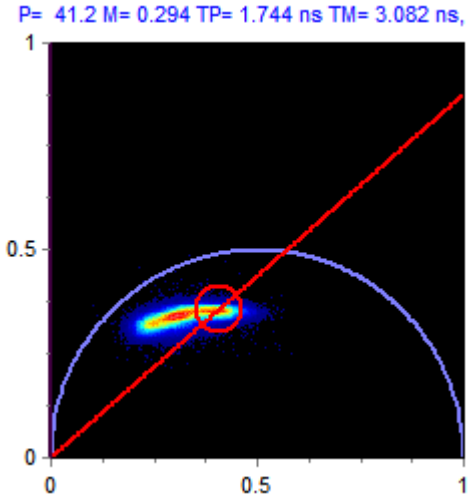


# Determining the cell autofluorescence phasor

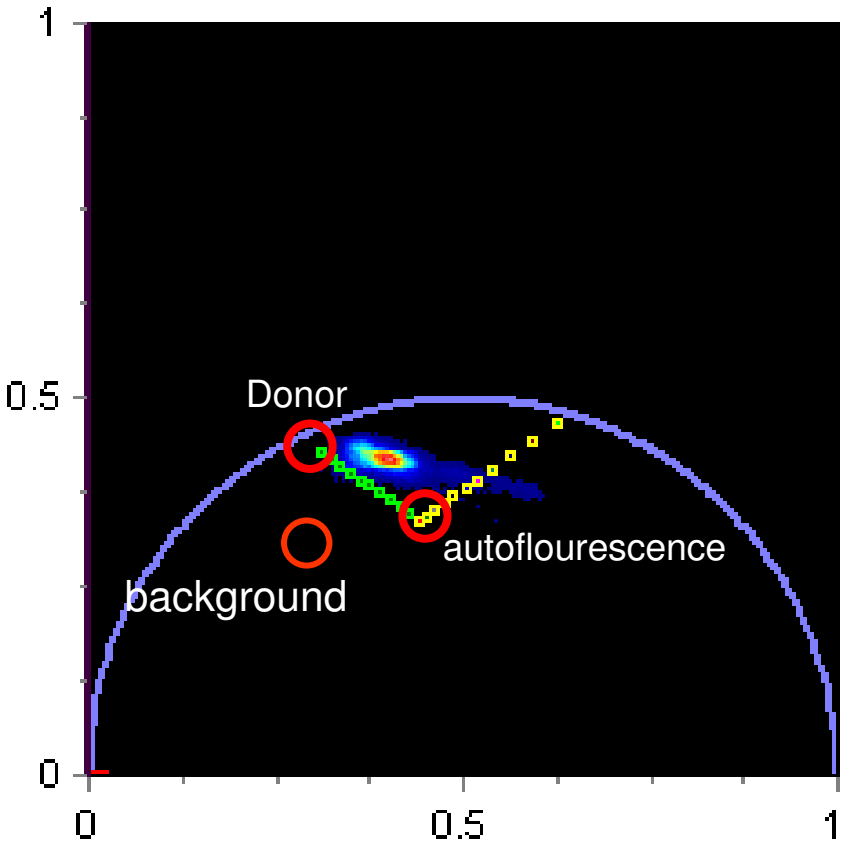
Cells only, no CFP

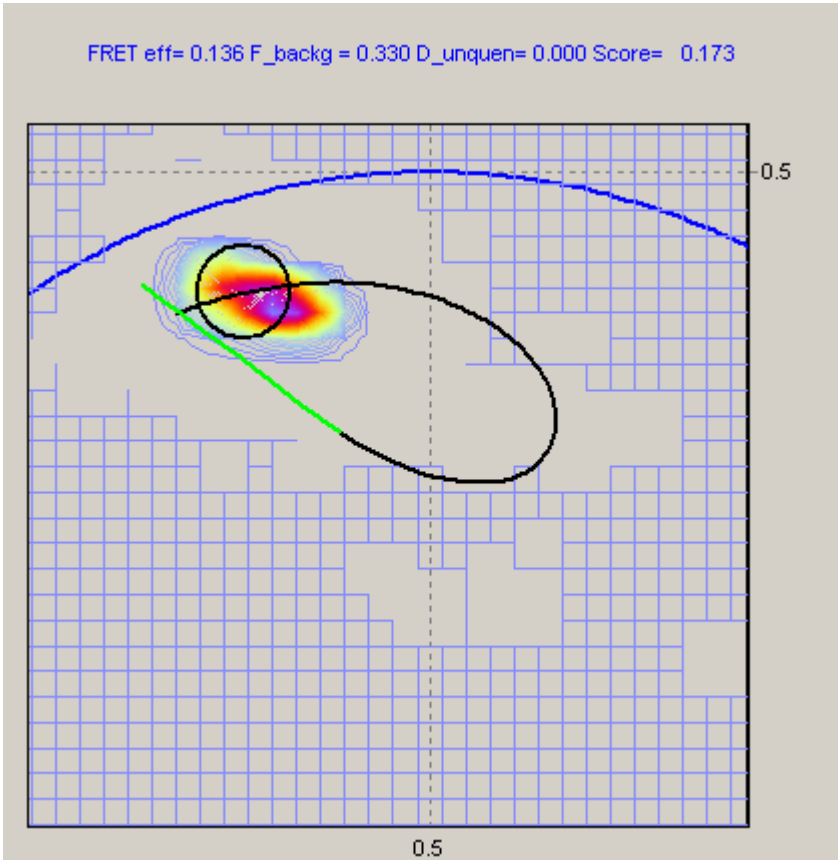



**TauP=1.7**
**TauM=3.0**



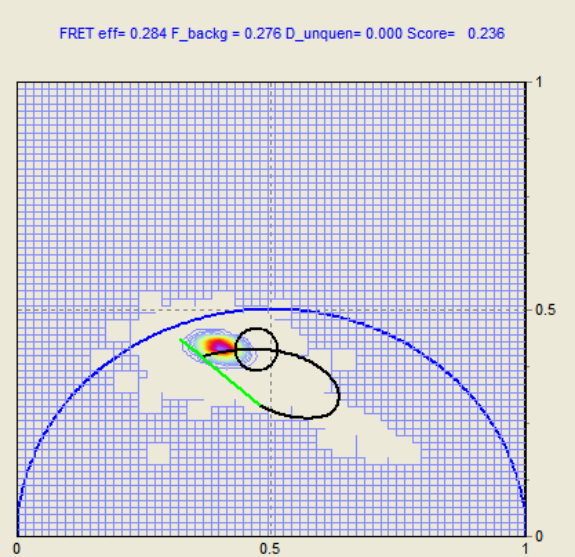
# Calculating FRET trajectories

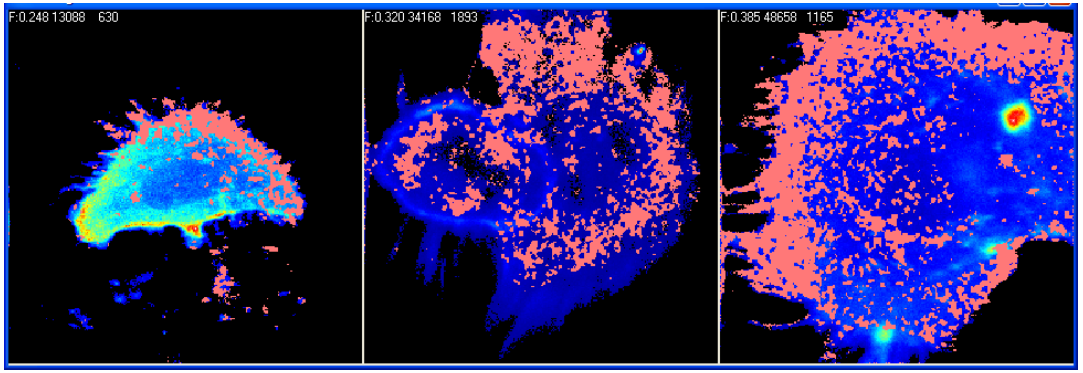


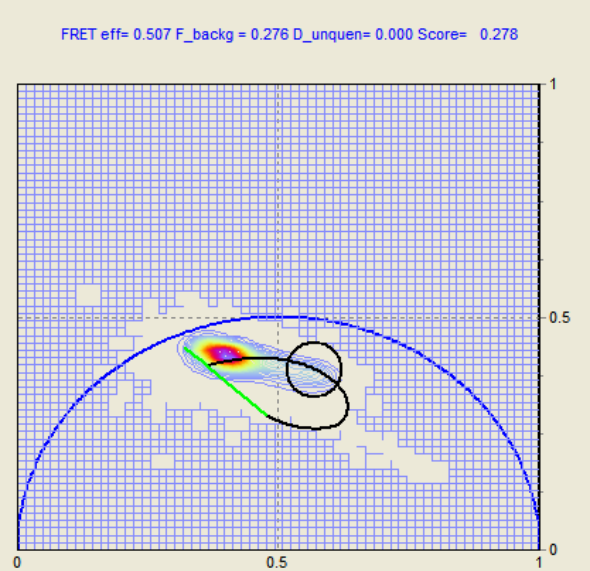
# Wt Raichu 1011 in MEF

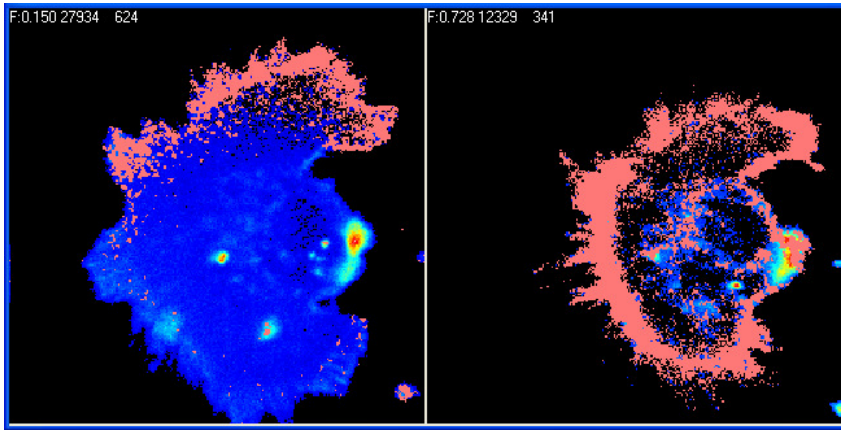
**Rac1 is activated diffusely with an increasing gradient toward the leading edge**



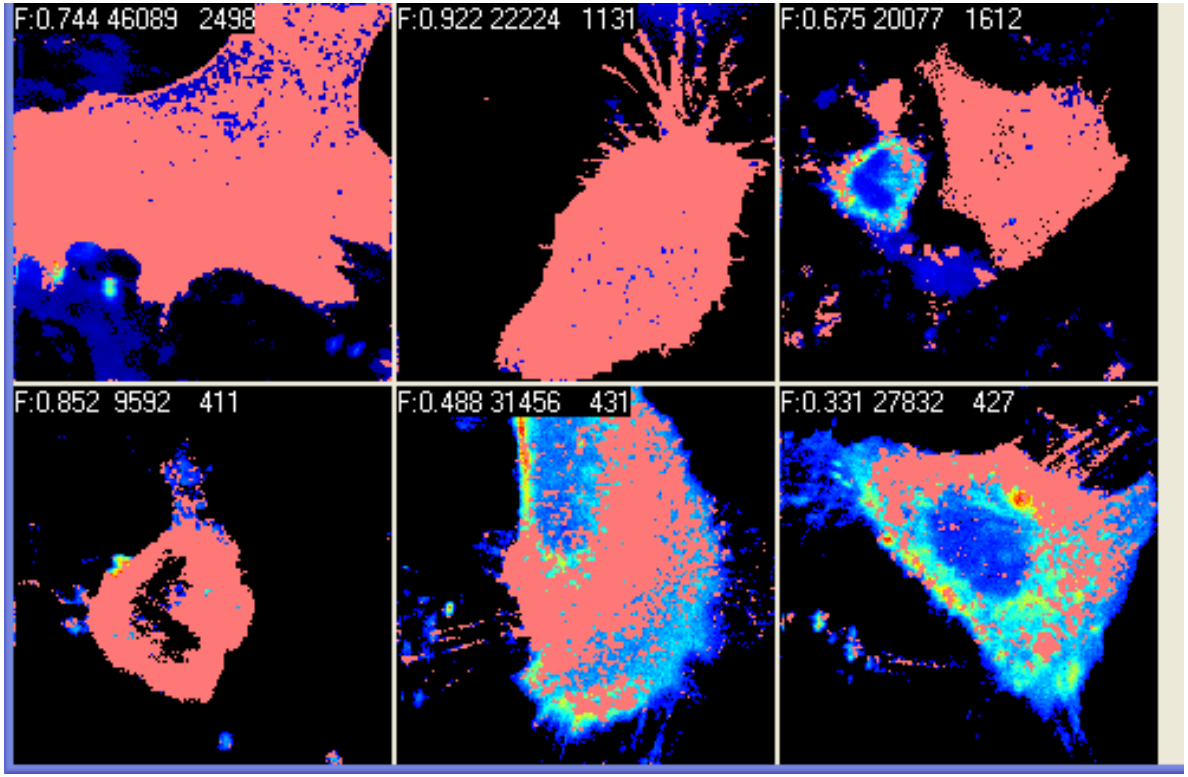

28% FRET efficiency

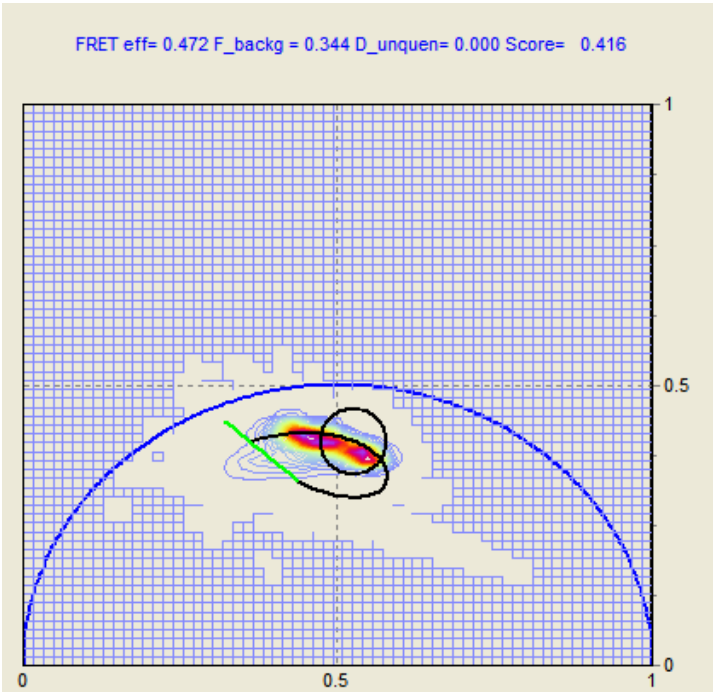



50% FRET efficiency

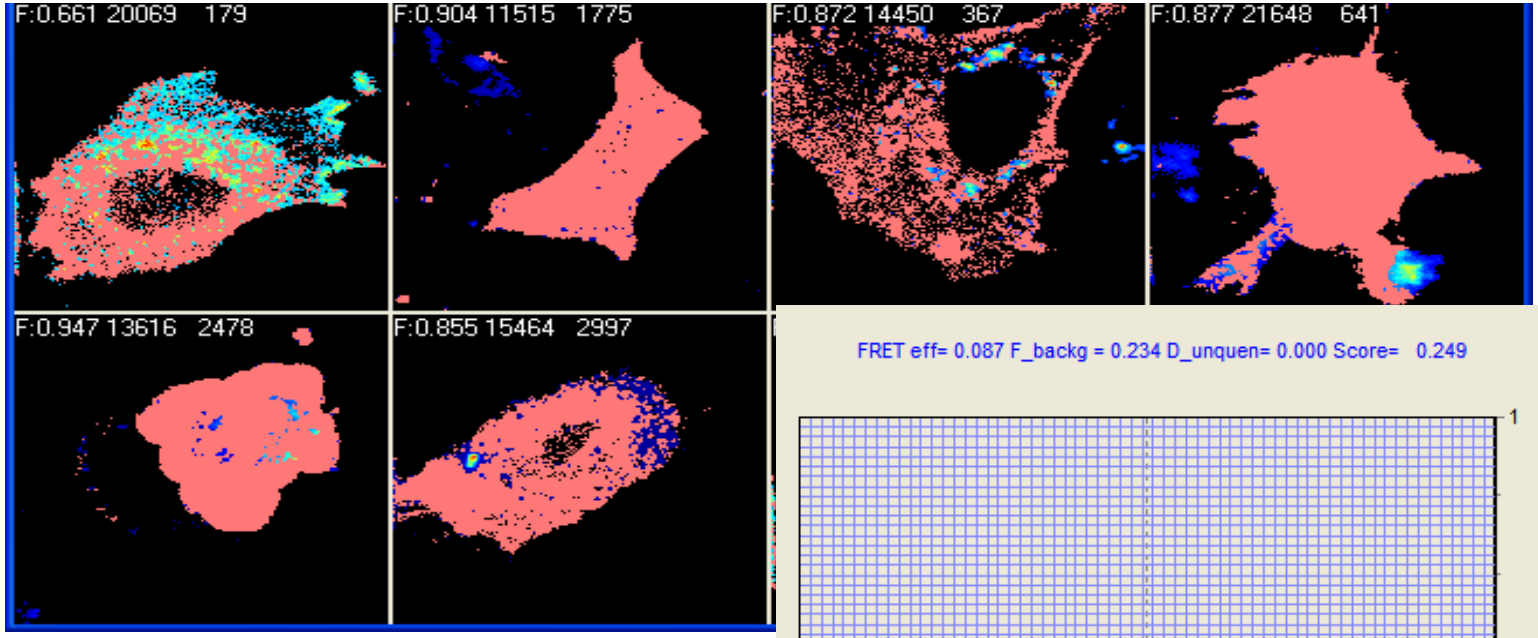
# Constitutively active (V12Rac): Raichu-Rac1 1012

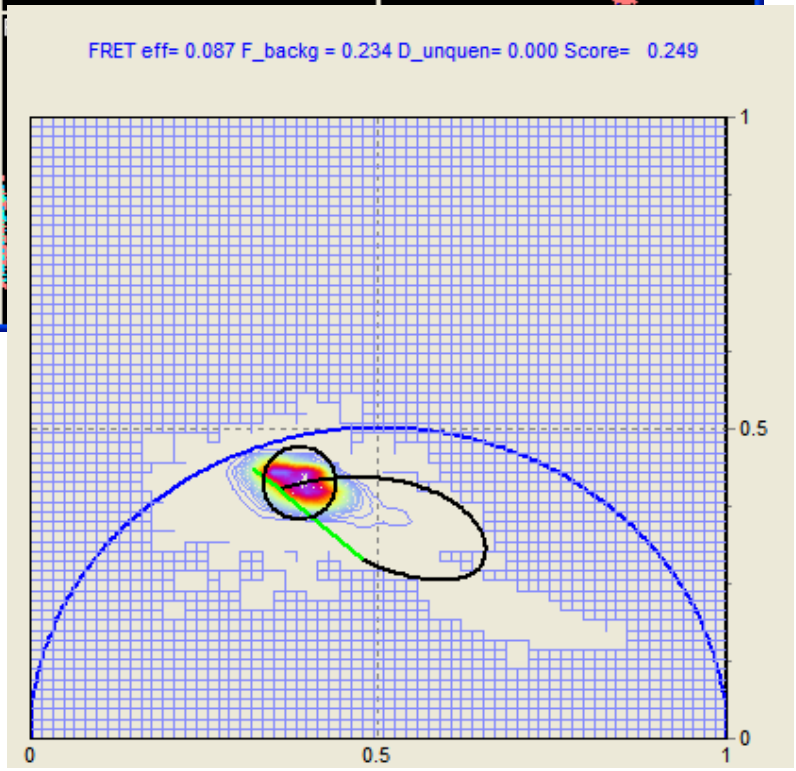



**47% FRET efficiency**

# Non-active (N17Rac): Raichu-Rac1 1013




**<8 % FRET efficiency**

# Raichu-RAC1 summary

- The wt Raichu-Rac1 1011 variant shows FRET efficiency of 28-50%
- The wt Raichu-Rac1 1012 variant shows FRET efficiency of 47-66%
- The wt Raichu-Rac1 10123 variant shows FRET efficiency of <8%

# 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