Introduction to Number and Brightness analysis: the N&B approach

Recent developments in Image Correlation Spectroscopy

1. From single point fluctuation measurement to the analysis of entire images

2. ICS techniques in the confocal microscope and in the TIRF configuration using cameras
3. Could also be used with the spinning disk approach

RICS analysis of diffusion
tICS and kICS binding map
STICS and STICCS Velocity maps using
Number and Brightness map (N&B)

Why we need N&B? What kind of biological questions can it answer that

Intensity
RICS
FRAP

cannot answer?

How to measure the state of aggregation of proteins? For example the formation of dimers or oligomers?
Why is this question important?

Monomer-dimer equilibria in a membrane

Protein aggregation of misfolded protein in tissues

Protein oligomerization and complexes formation

Interactions

Signalling

Plaque formation

N&B in transfected COS 7 cells using human Htt exon 1 with varying lengths of polyglutamine fused to GFP (Httex1 97QP-GFP, Httex1 46Qp-GFP and Httex1 25QP-GFP)

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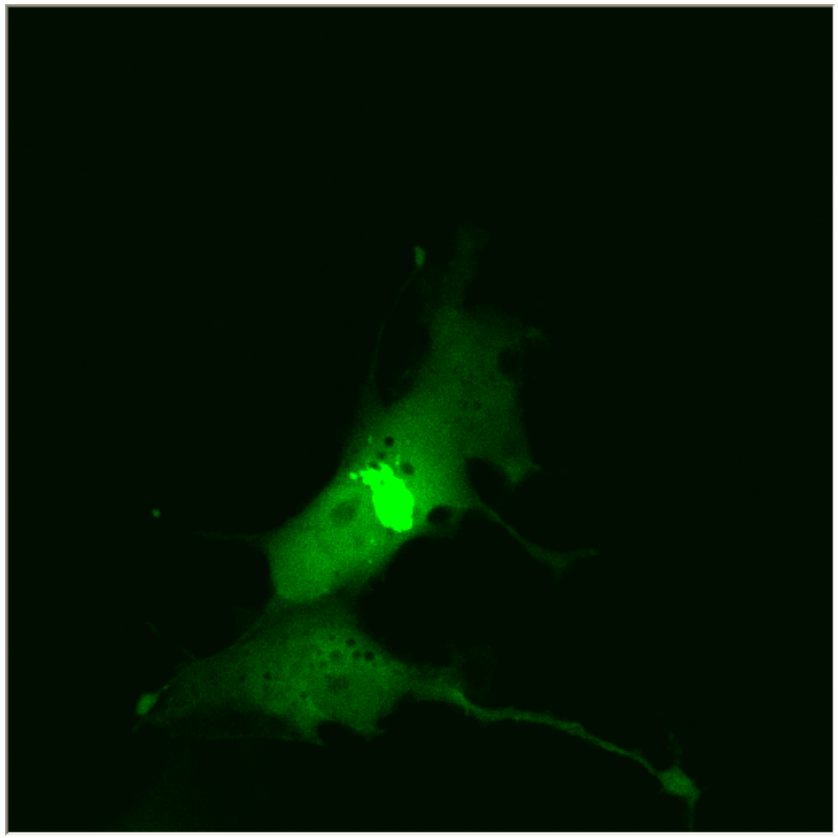
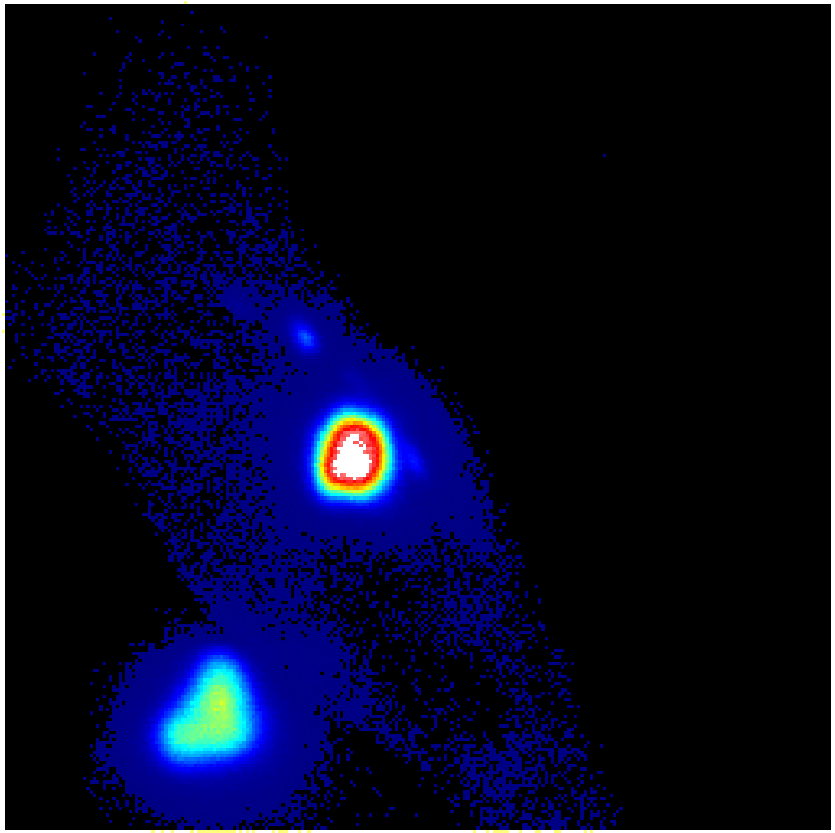
97QP after 24 hours

COS7: Zeiss 510 META 1-photon and 2-photon system



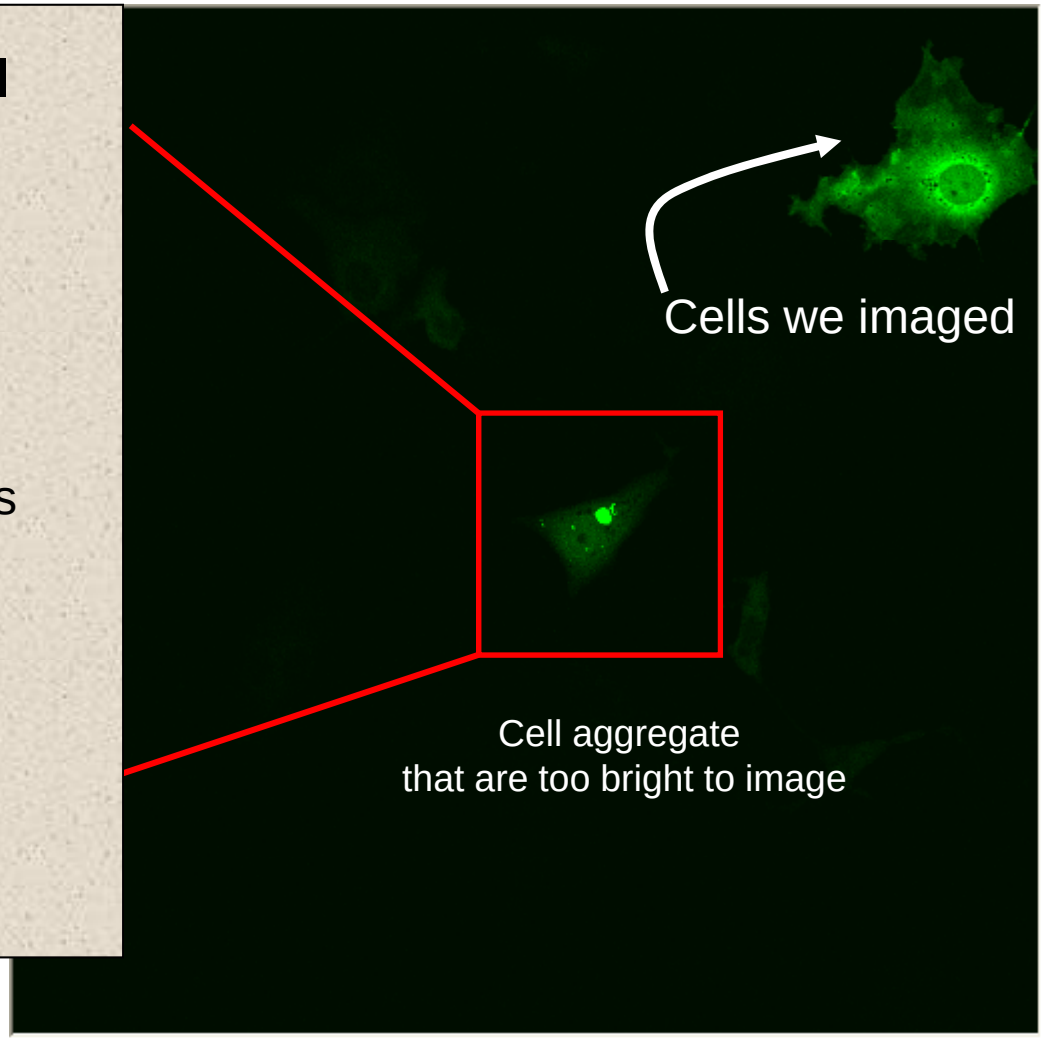
1-photon



2-photon

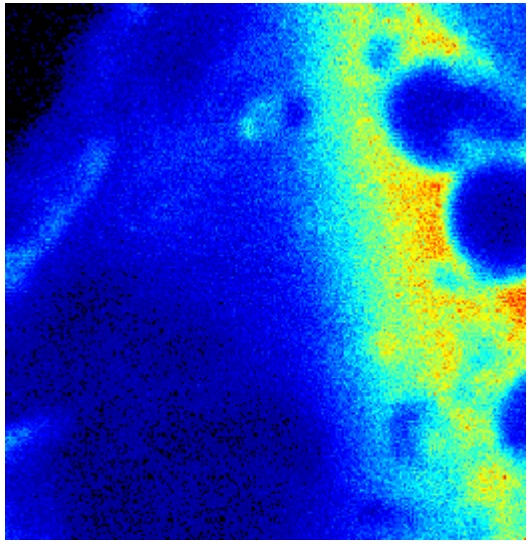
What are the Biological questions?

- Are there precursor aggregates before the plaque stage?
- Are there smaller aggregates (precursors to the large aggregate?
- Can we follow aggregation?
- Can we detect aggregates in the nucleus?



97QP in COS7 cells

QP971010.bin



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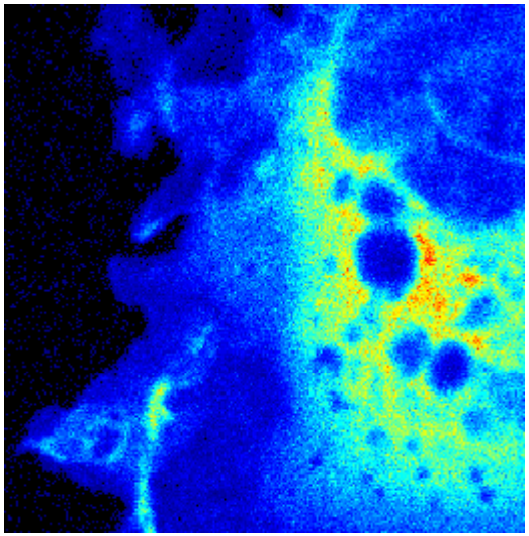

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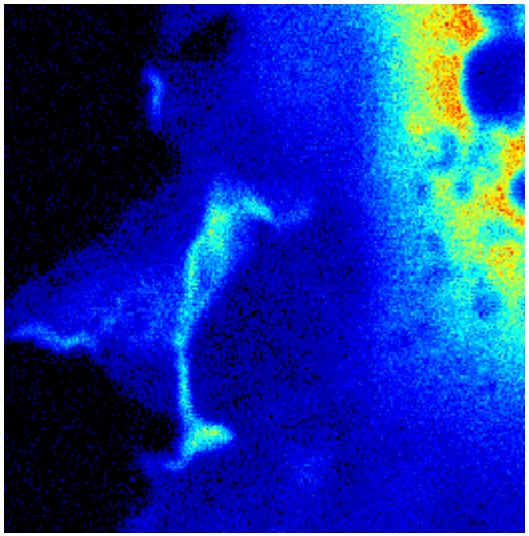
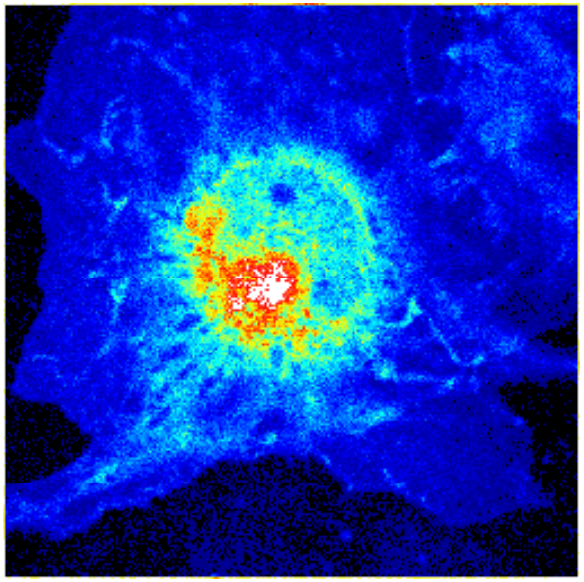
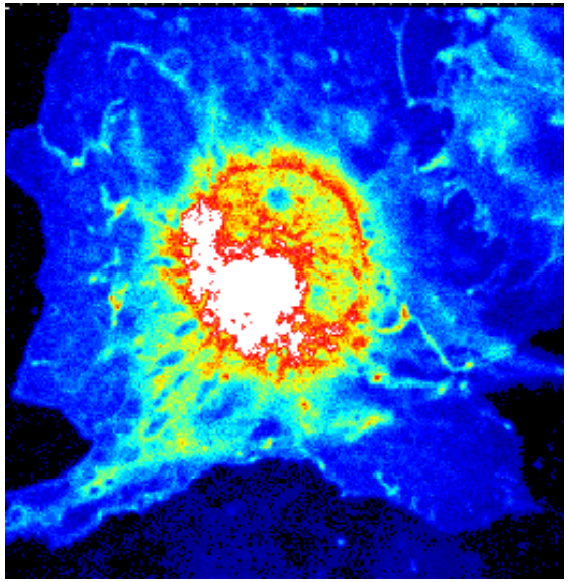


Averaged 4 frames for movie Total frames=100

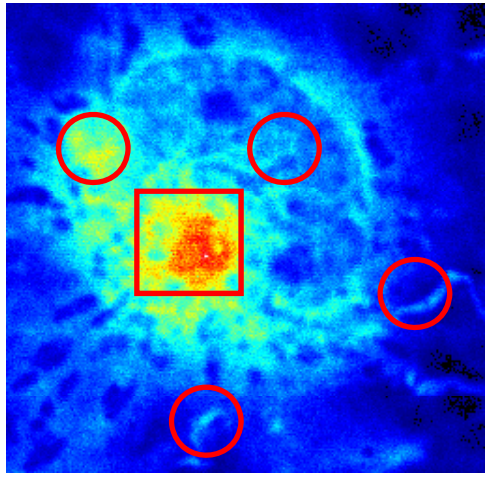
97QP97 at a very early stage of aggregation

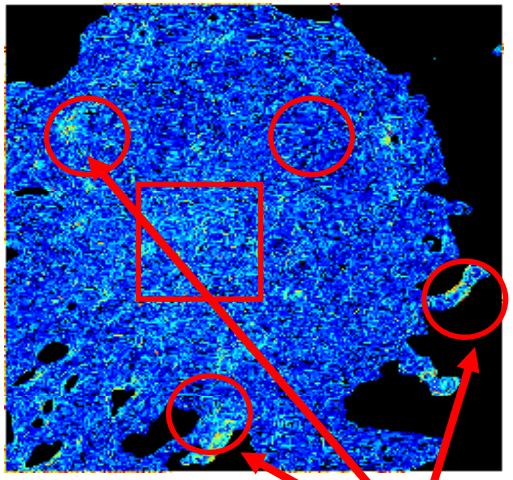


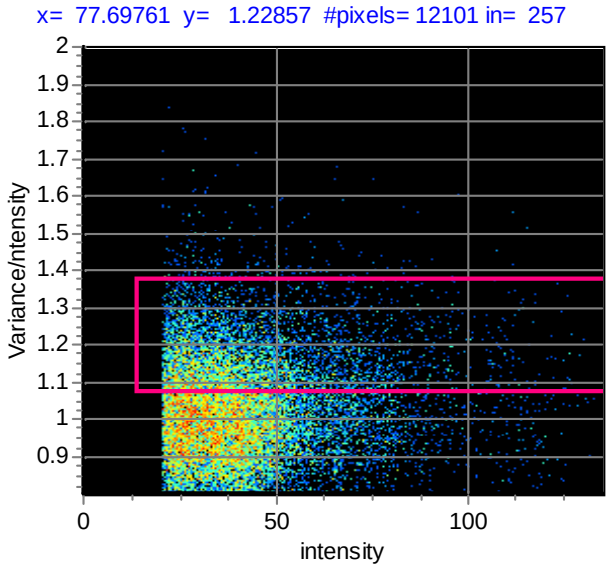


Are the protein aggregates of similar size in these regions?

B map







Larger aggregates only in the appendices

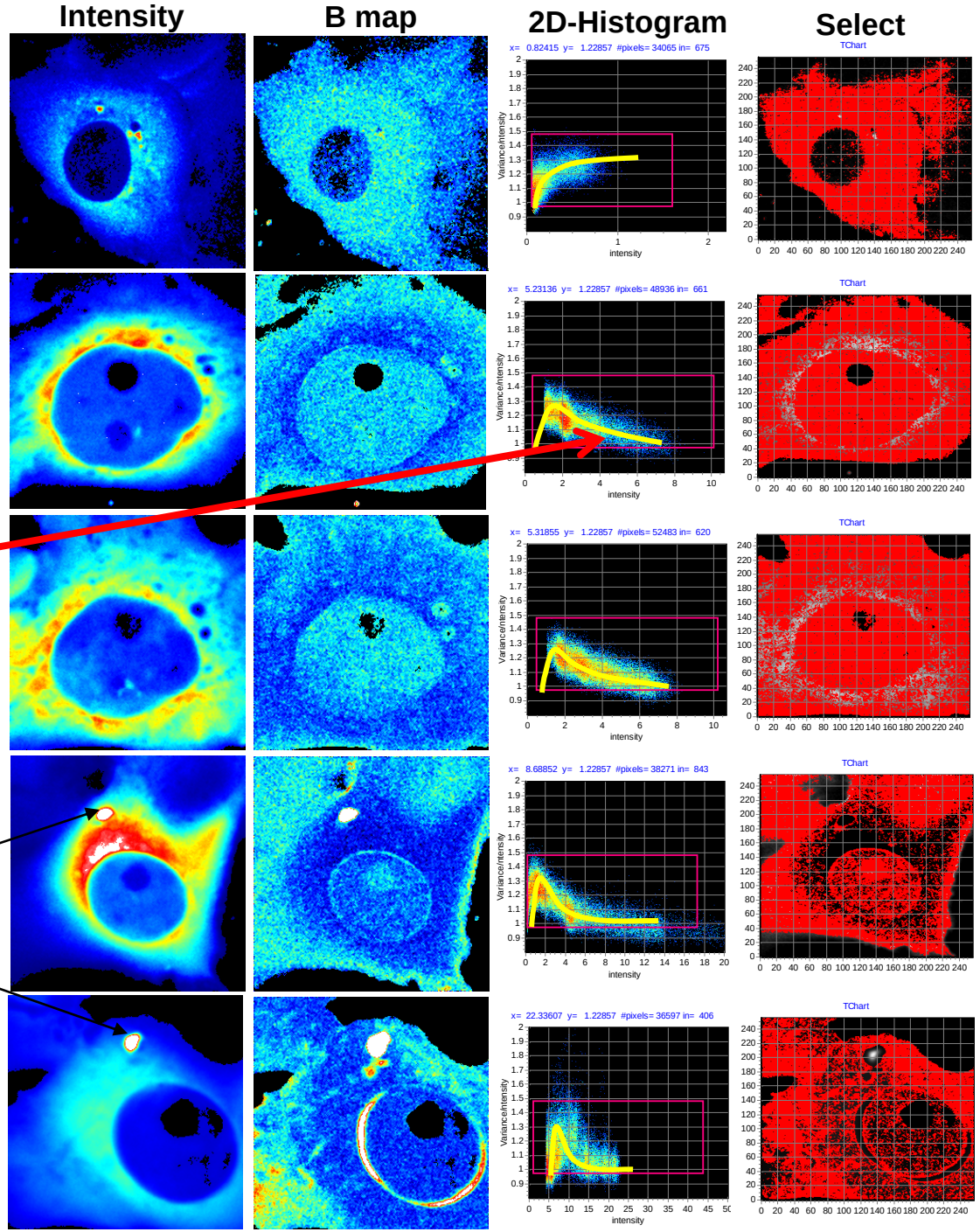
97QP cells

The brightness in the nucleus can surpass the brightness in the cytosol.

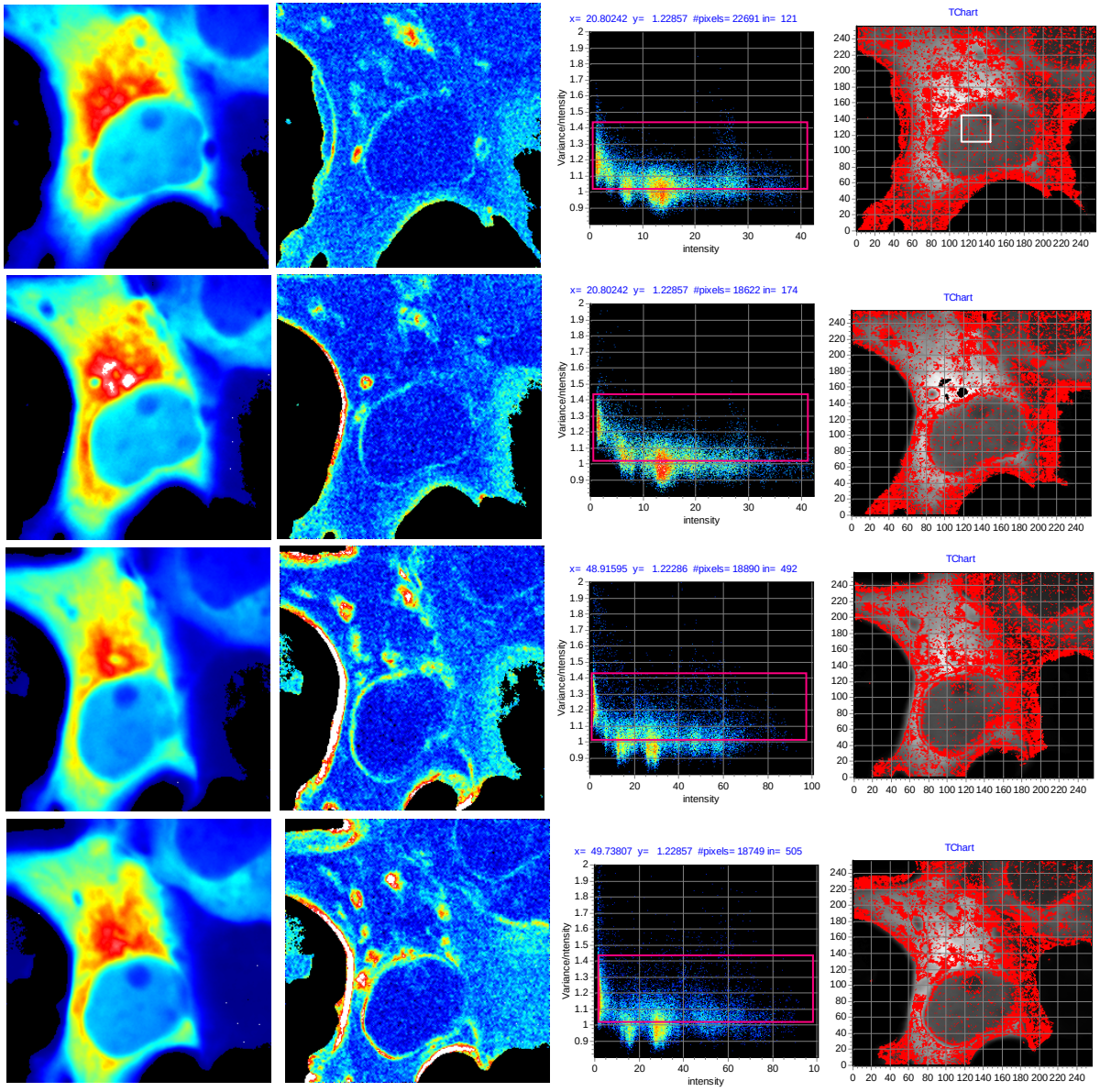
Series taken at different times (approximately every 10 minutes)

This indicates immobile fraction

A plaque forms here



25QP cells
The brightness in the nucleus is less than in the cytosol



Conclusions from the N&B analysis

- There are htt proteins every where
- Aggregation starts earlier in the cytoplasm and later inside the nucleus.
- Probably only monomers or small aggregates can migrate to the nucleus
- In the cytoplasm, as the aggregates become larger, the B value starts to decrease
- In the nucleus the aggregation occurs at a slower rate and brighter aggregates are observed after many hours. Eventually also in the nucleus the aggregates become too large and start to decrease in apparent brightness
- When a plaque forms, the intensity in the cytosol decreases and also the brightness decreases

Spatial Diffusivity and Availability of Intracellular Ca^{2+} Calmodulin

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Questions

How much Cam is “free” to respond to external Ca changes?

What is the spatial distribution of Cam in the cell?

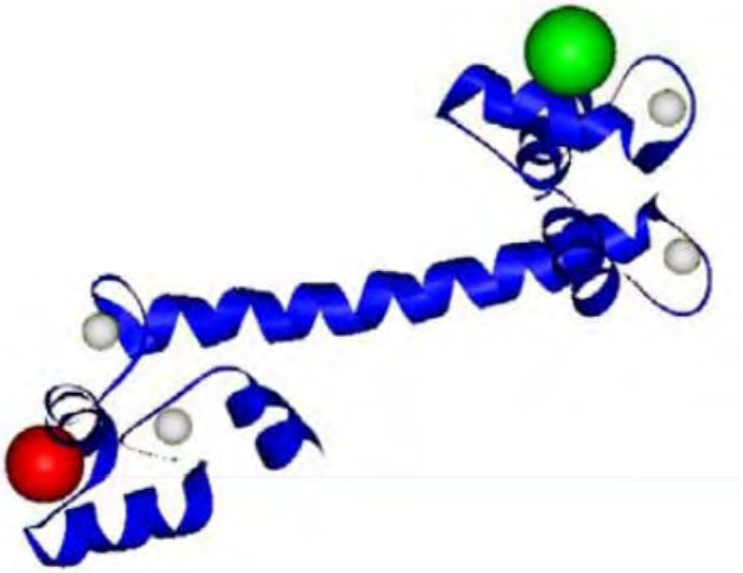
Does the spatial distribution of Cam changes at different extracellular Ca levels?

Methods

RICS, to measure the local diffusion coefficient of Cam

N&B, to measure the state of aggregation of Cam

COS-7 cells transfected with different GFP constructs



Calcium-free calmodulin

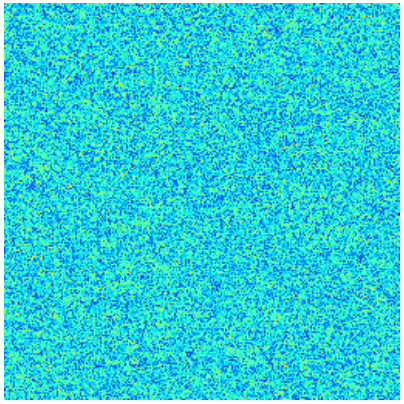
Calmodulin with 4 calcium ions

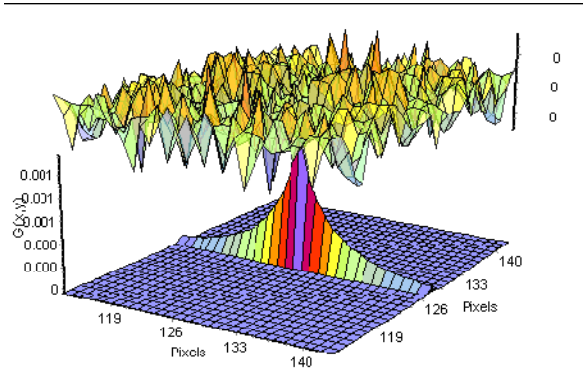
- The conformational transitions of calmodulin affect its binding in wide assortment of biological processes such as neurotransmitter production and release, muscle contraction, nerve growth, metabolism, apoptosis, muscle growth, inflammation, membrane protein organization, cytoskeleton movement.
- Calmodulin has at least three stable conformations, which are affected by the occupancy of calcium.
- Therefore, we want to observe these conformational transitions!

(Image source: Allen et al., Analyt. Biochem. 325 (2004) 273-284)

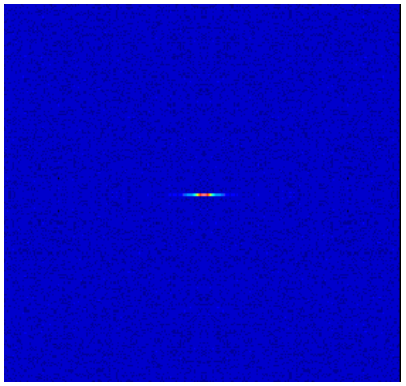
Diffusion in solution

Image





RICS Image



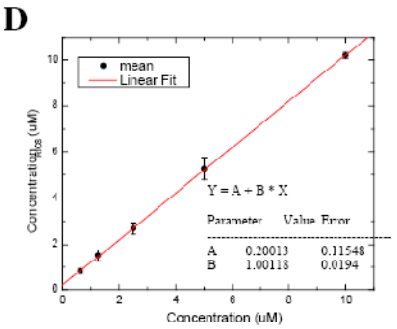


TABLE I

Protein		D(μm²/s)
GFP	RISC	89±20
GFP	2p FCS	82±10
GFP_CaM	RISC	80±10
GFP-CaM	2p_FCS	72±4
GFP-CAMK2	RISC	20±4
GFP-CAMK2	2p_FCS	20±1

Diffusion in cells

TABLE 2

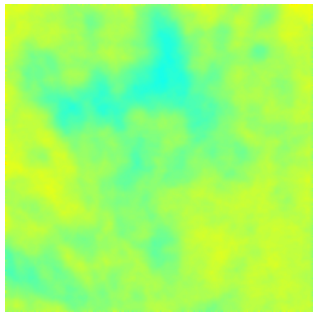
Protein	Condition		D(μm²/s)	% of fast
GFP			20 ± 5	90 ± 9
GFP+nlCAMK2			21 ± 8	86 ± 13
GFP-CAMK2			1.6 ± 0.9	74 ± 11
GFP-CaM	basal	nucleus	10 ± 5	80 ± 19
	Ca+		10 ± 4	85 ± 16
	Ca		10 ± 7	75 ± 12
GFP-CaM	basal	Cyt	11 ± 6	60 ± 17
	Ca+		11 ± 4	68 ± 17
	Ca-		10 ± 9	64 ± 26
GFP-CaM+nlCAMK2	basal	nucleus	7 ± 5	52 ± 28
	Ca+		12 ± 2	58 ± 23
	Ca		6 ± 2	67 ± 11
GFP-CaM+nlCAMK2	basal	Cyt	7 ± 4	29 ± 27
	Ca+		8 ± 1	67 ± 10
	Ca		4 ± 3	34 ± 10

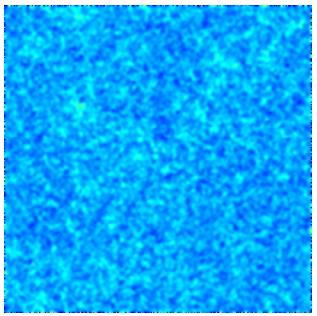
There are at least two components that diffuse with different rates. The slow component is associated with the protein in vesicles. There is also an immobile component, which is always subtracted from the RICS measurement

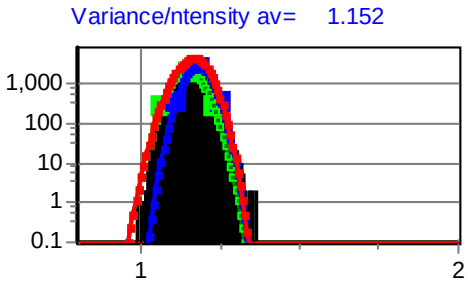
Image

Brightness

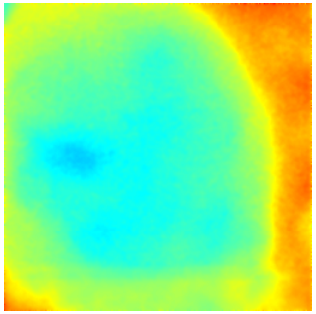
Brightness components analysis

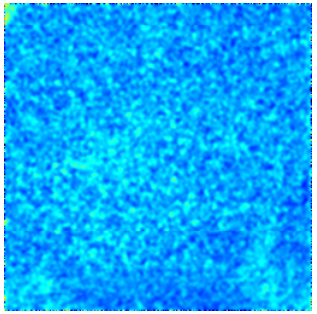


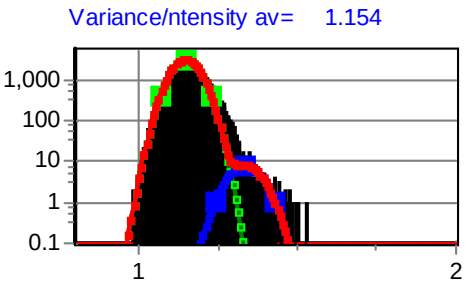




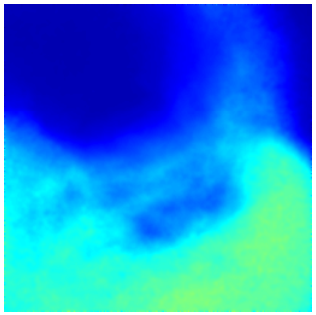
GFP

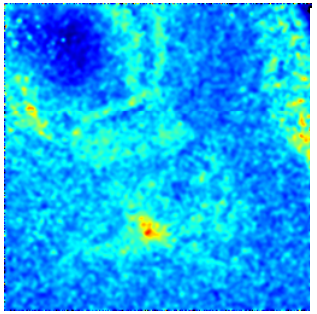


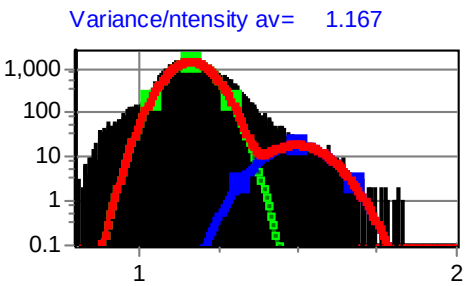




GFP+nlCAMK2







GFP-CAMK2

Cam-GFP

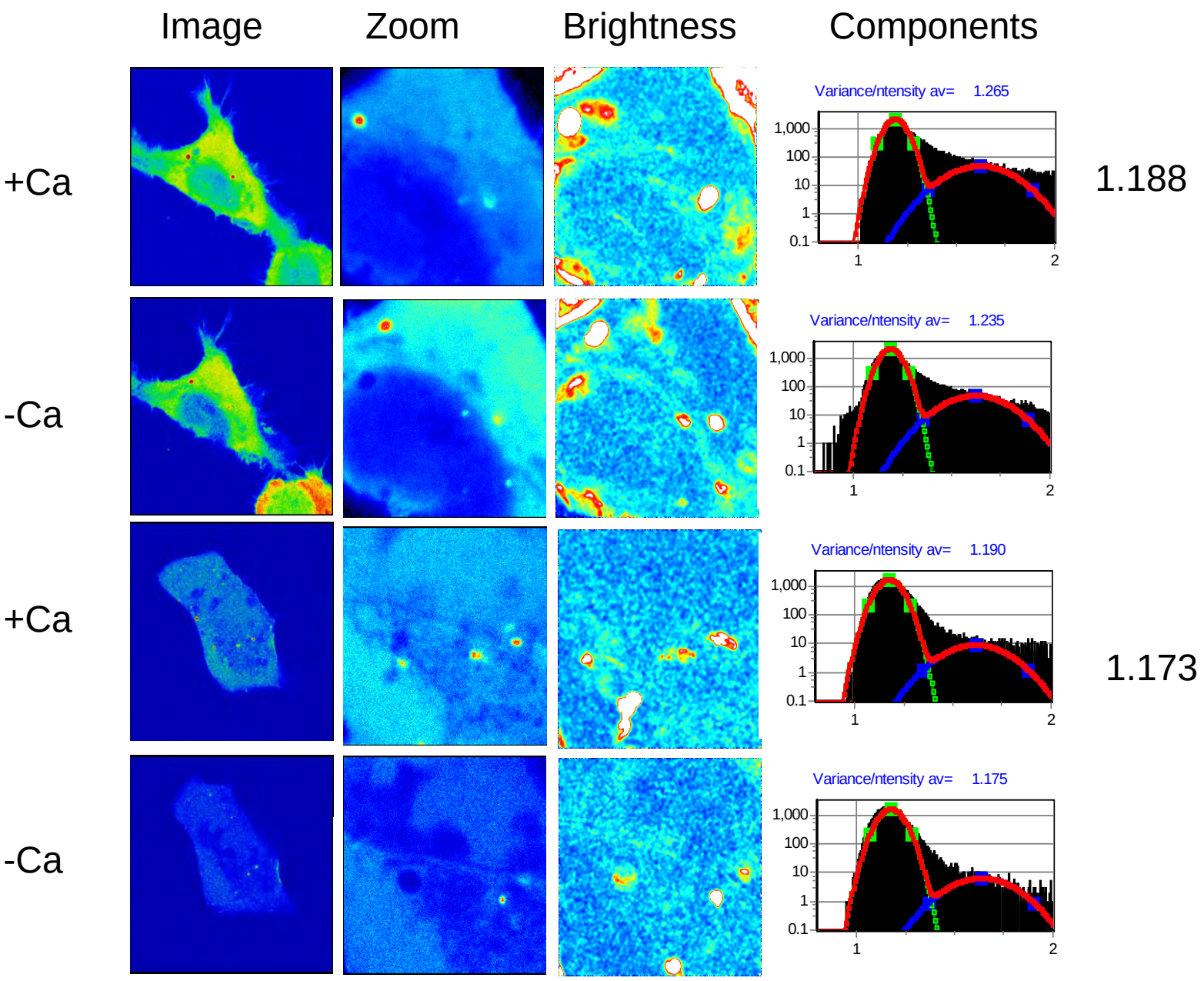
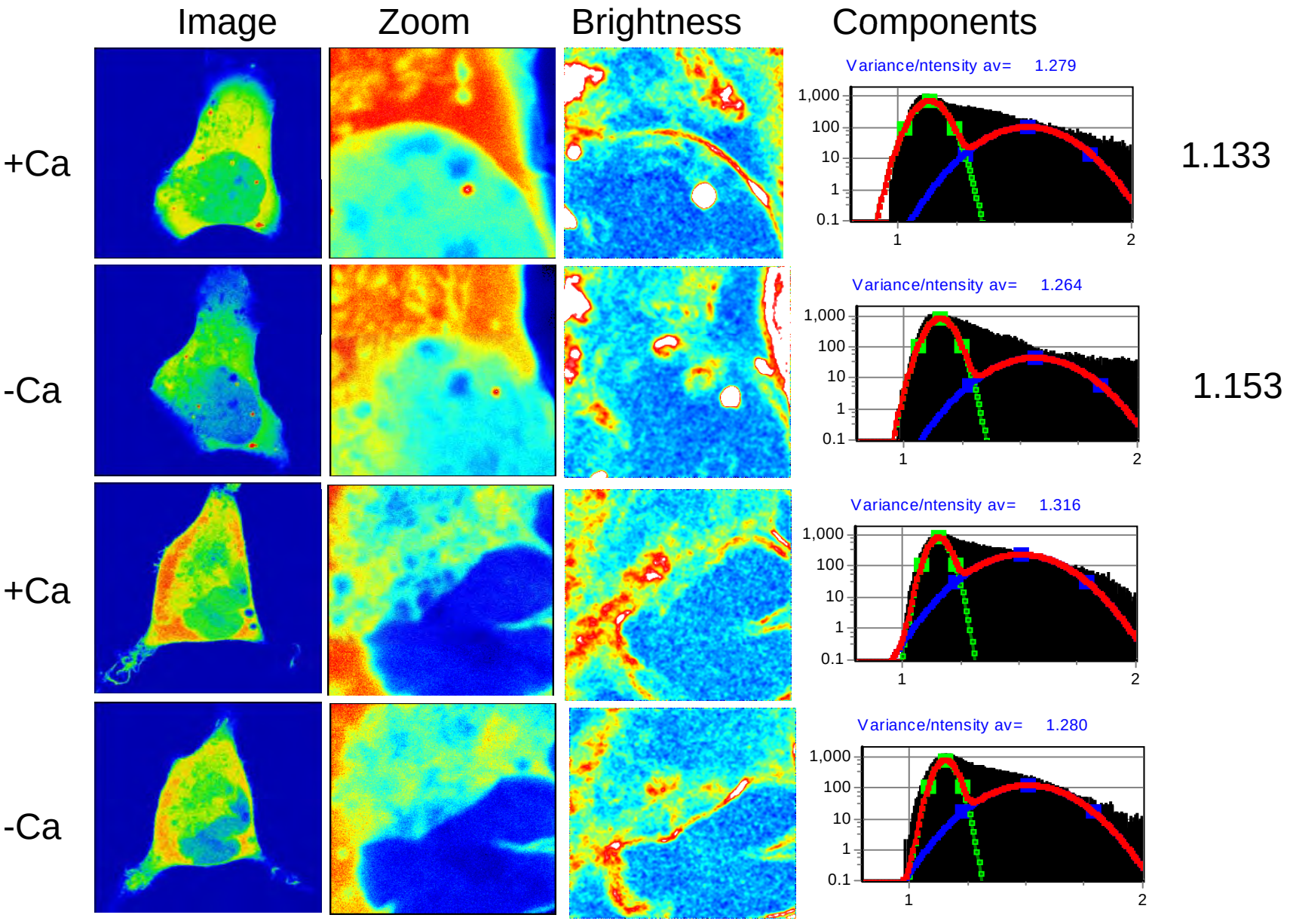


Image
Zoom
Brightness
Components
+Ca
-Ca
+Ca
-Ca
Variance/ntensity av= 1.265
Variance/ntensity av= 1.235
Variance/ntensity av= 1.190
Variance/ntensity av= 1.175
1,000
100
10
1
0.1
1
2
1.188
1.173

Cam-GFP + CAMK2



Conclusions From the N&B and RICS analysis

Only a small fraction of Cam is free

Cam is associated with other protein partners both in the calcium free and calcium bound form

There is a map of cam concentration in the cell

In the nucleus, Cam is less concentrated and it appears to have the brightness of a monomer

However, the diffusion in the nucleus indicates that Cam is bound to some other protein

We needed both the RICS and the N&B analysis to respond to the initial question

The N&B project

Purpose: Provide a pixel resolution map of molecular number and aggregation in cells

Method: First and second moment of the fluorescence intensity distribution at each pixel

Source: Raster scanned image obtained with laser scanning microscopes
TIRF with fast cameras
Spinning disk confocal microscope

Output: The N and B maps, B vs intensity 2D histogram

Tools: Cursor selection of pixel with similar brightness
Quantitative analysis of center and std dev of the ε and n distribution
Tools for calibration of analog detectors

Tutorials: mathematical background, data import, analysis examples (our web site

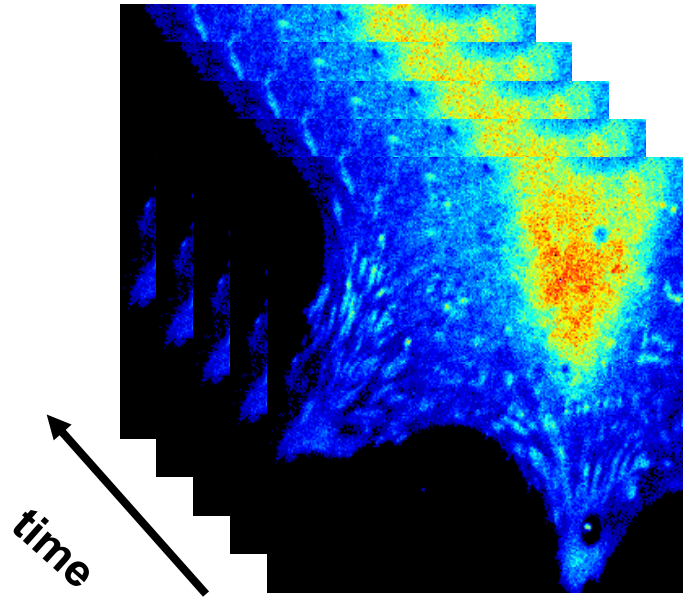
Calculating protein aggregates from images

This analysis provides a map of <N> and brightness (B) for every pixel in the image. The units of brightness are related to the pixel dwell time and they are "counts/dwell time/molecule".

$$< k >= \frac{\sum_i k_i}{K} \qquad \sigma^2 = \frac{\sum_i (k_i - < k >)^2}{K}$$

$$B = \frac{< k >}{< N >} = \frac{\sigma^2}{< k >}$$

$$< N >= \frac{< k >^2}{\sigma^2}$$



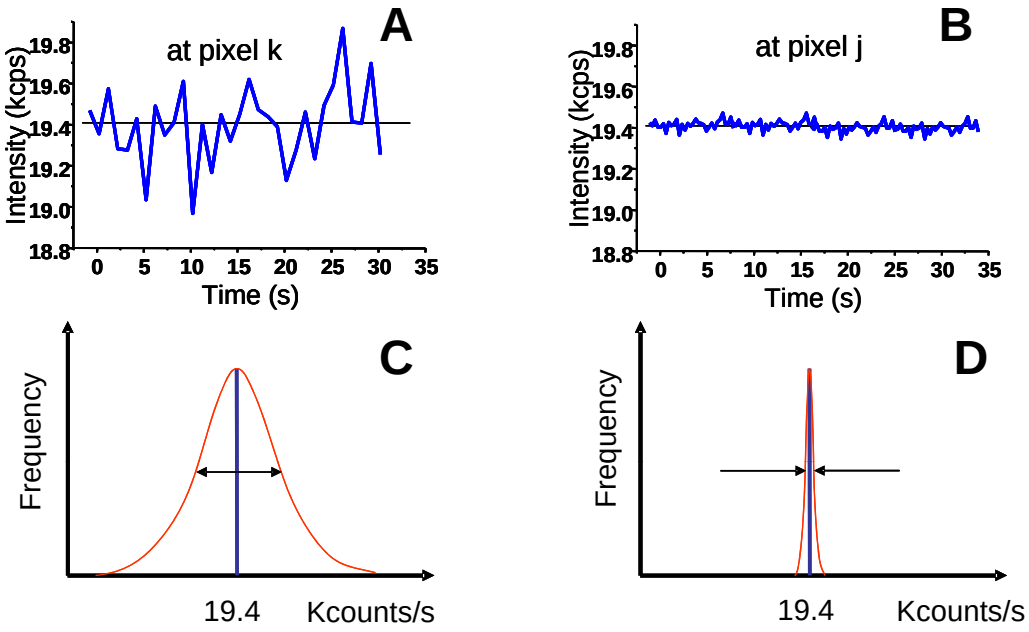
σ^2 = Variance

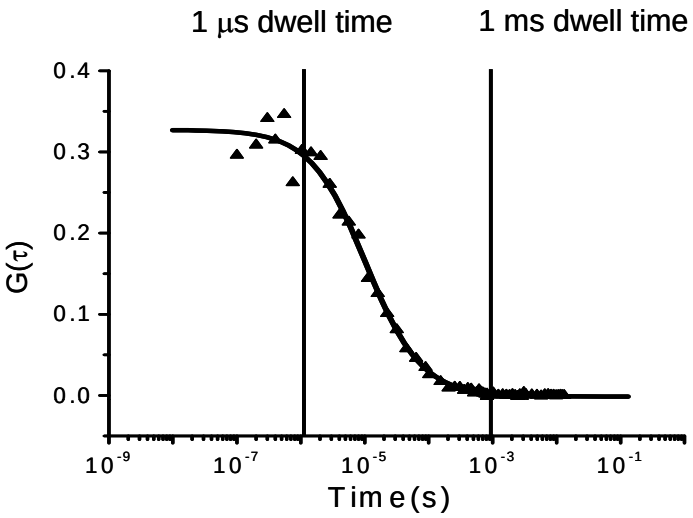
<k>= Average counts

N = Apparent number of molecules

B = Apparent molecular brightness

The Basic Idea





Variance due to particle fluctuations $\sigma_n^2 = \varepsilon^2 n$

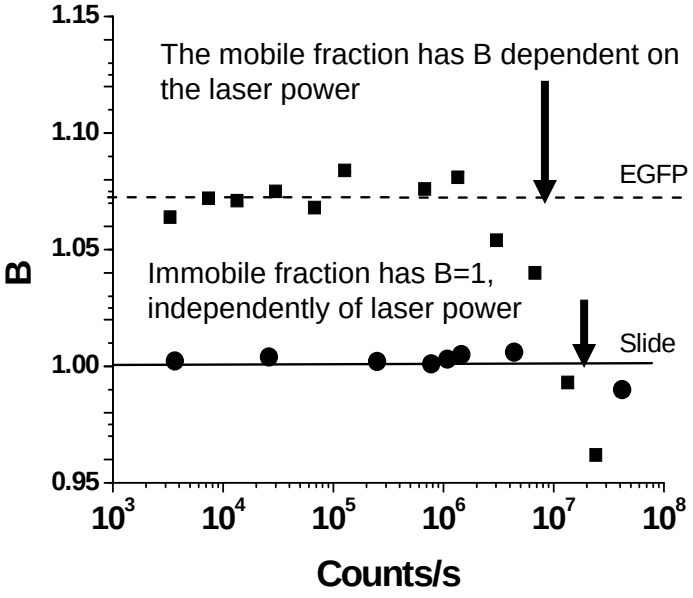
Variance due to detector shot noise $\sigma_d^2 = \varepsilon n$

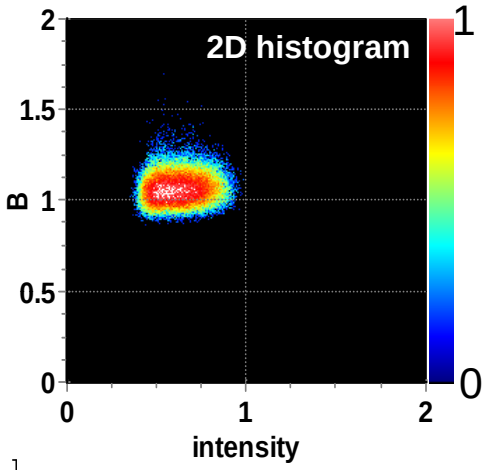
Average intensity in one pixel $< k >= \varepsilon n$

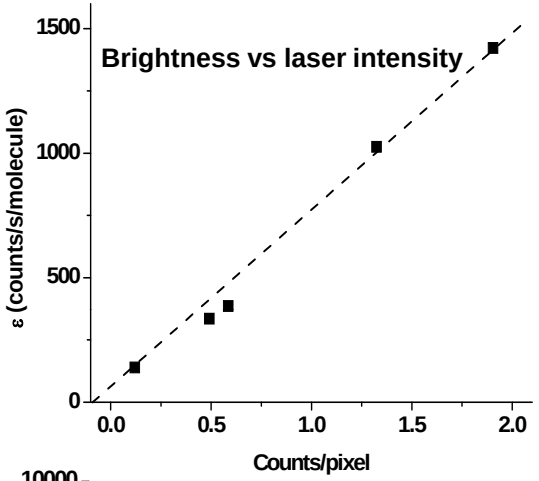
$$B = \frac{\sigma^2}{< k >} = \varepsilon + 1$$

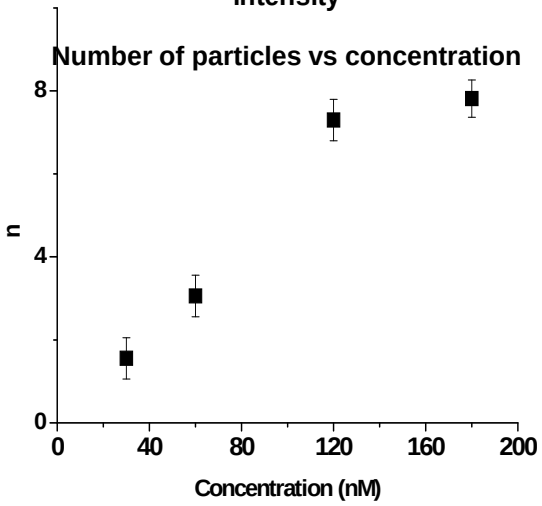
$$N = \frac{< k >^2}{\sigma^2} = \frac{\varepsilon n}{\varepsilon + 1}$$

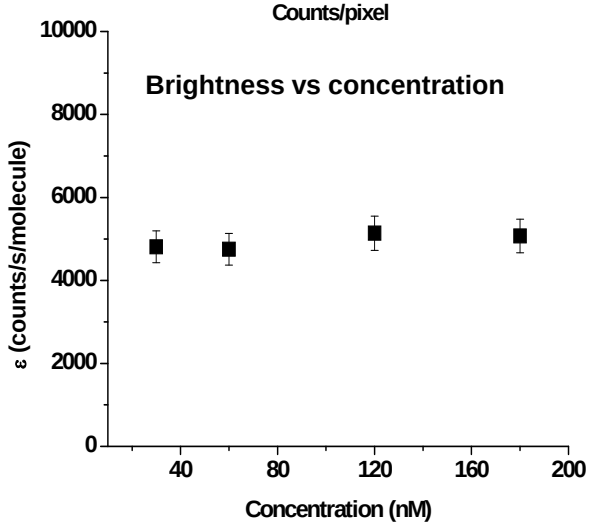
Brightness and number of molecules can be measured independently



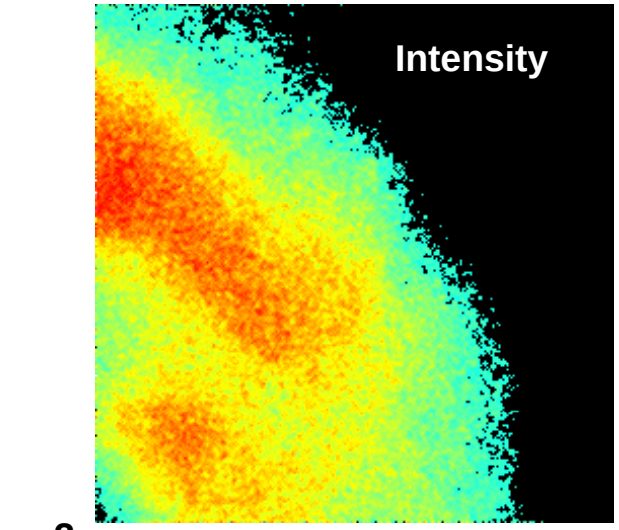


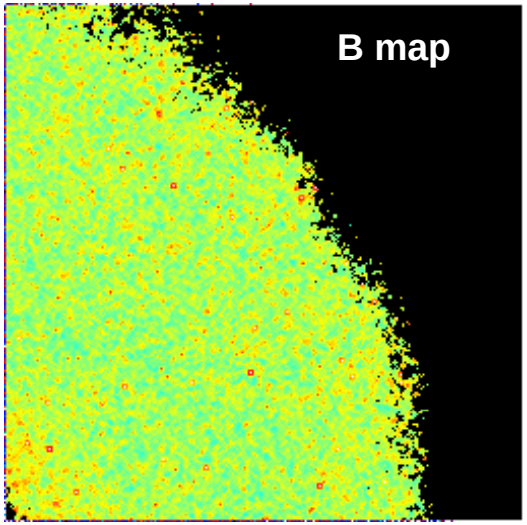


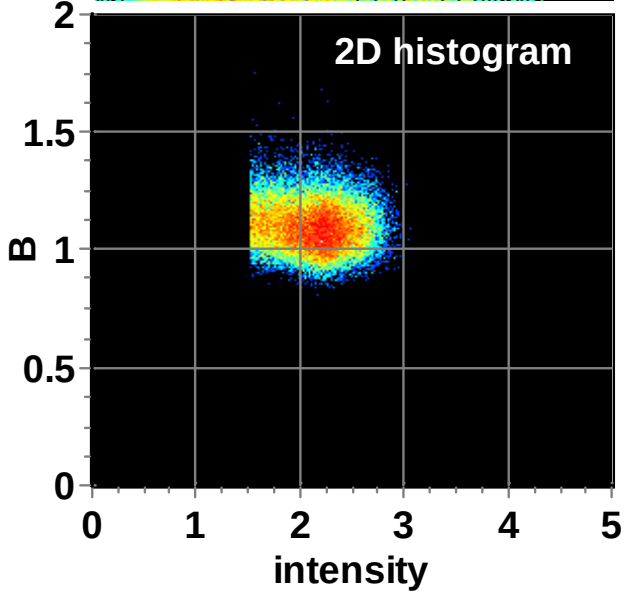


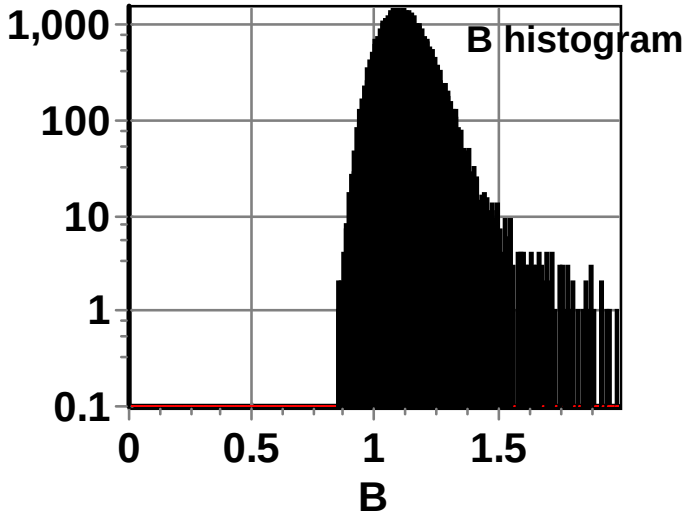


N&B in cells expressing cytoplasmic mEGFP (no aggregates)



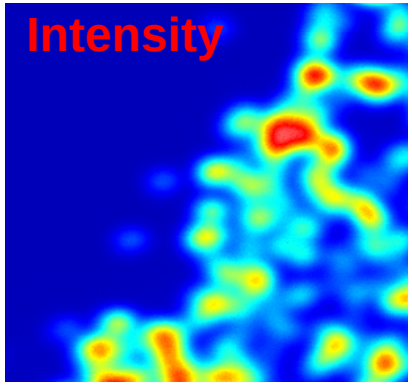


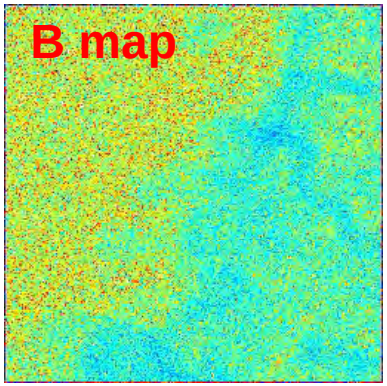




The effect of the immobile part

Fluorescent beads in a sea of 100nM Fluorescein.



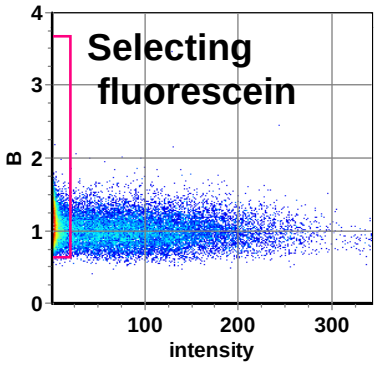


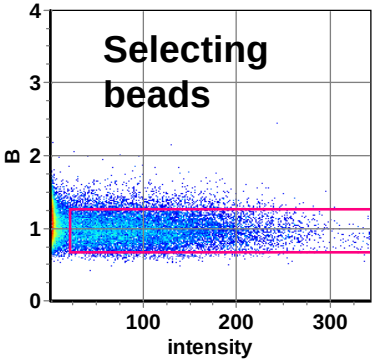
ε and *n* in the presence of immobile fraction

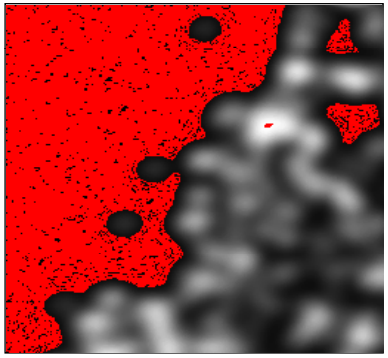
$$\varepsilon_m = \frac{\varepsilon}{1+R}$$

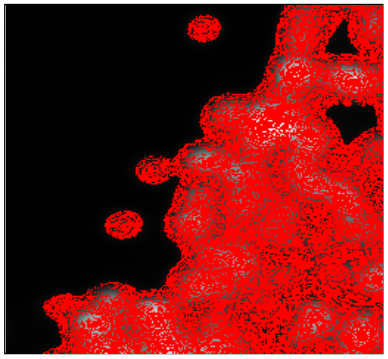
R is the ratio of immobile/mobile intensity

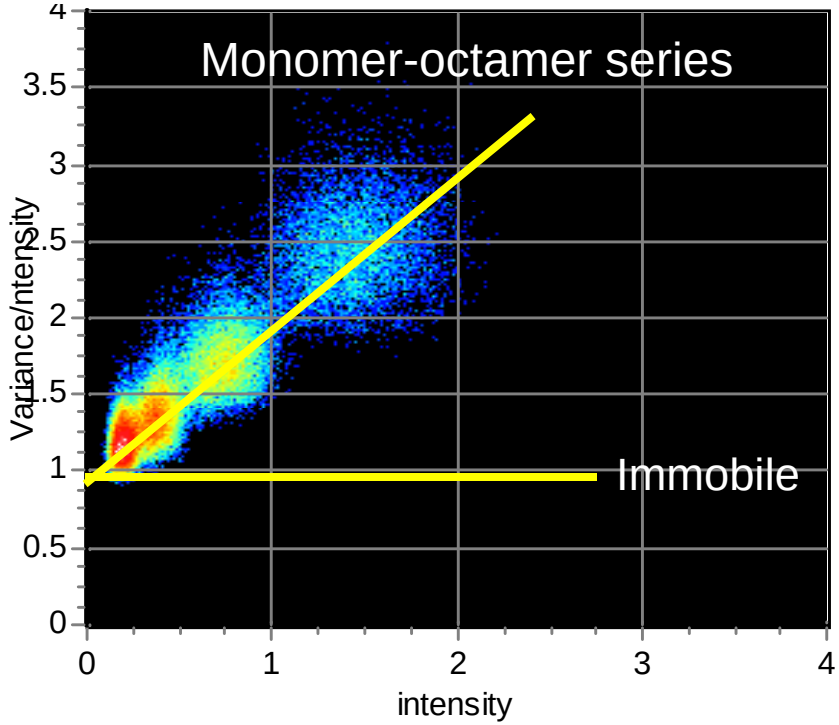
$$n_m = n(1+R) + \frac{I_{im}(1+R)}{\varepsilon}$$



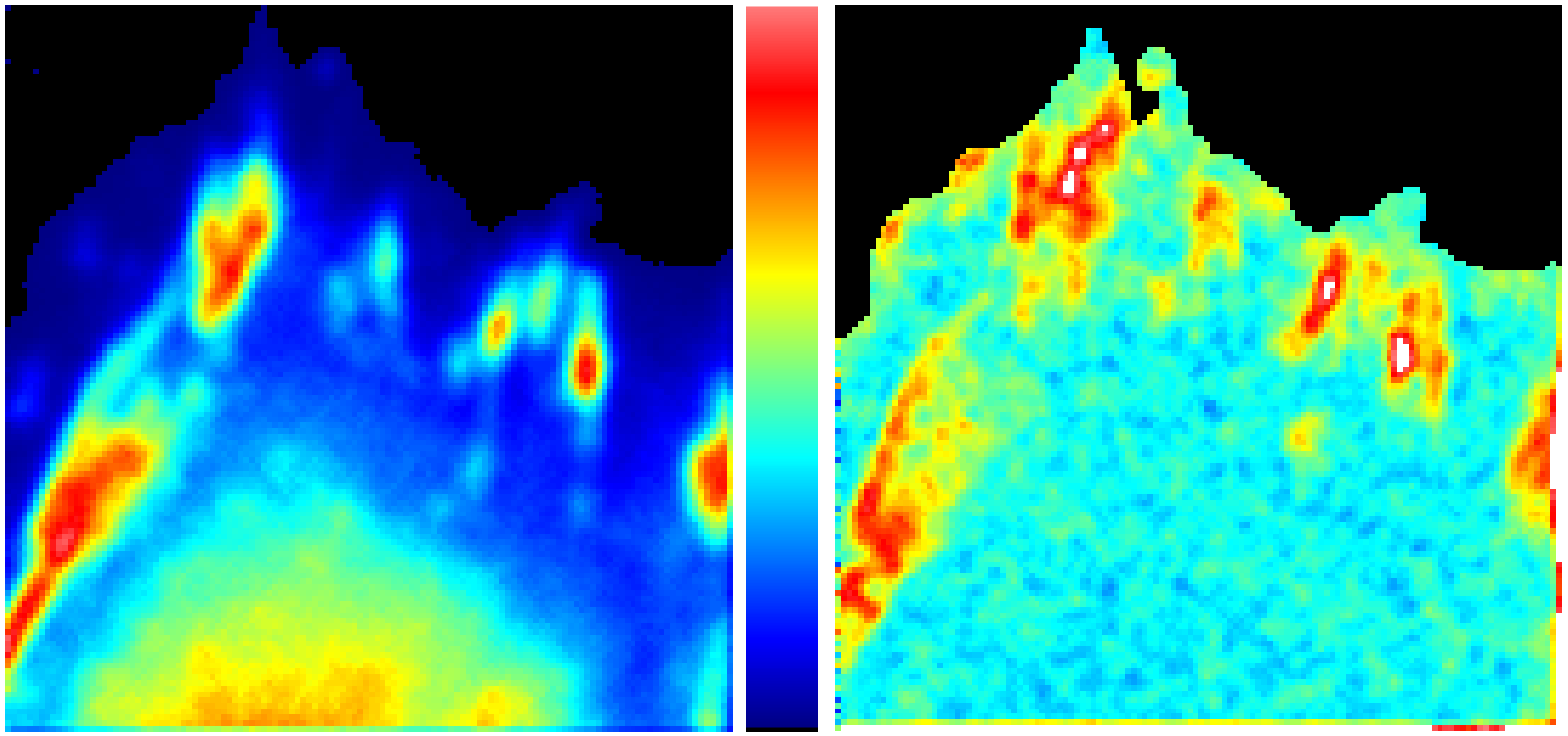








Paxillin aggregation and dynamics

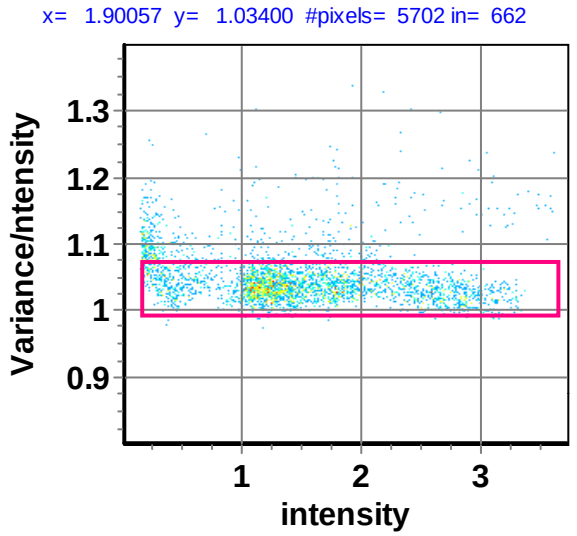


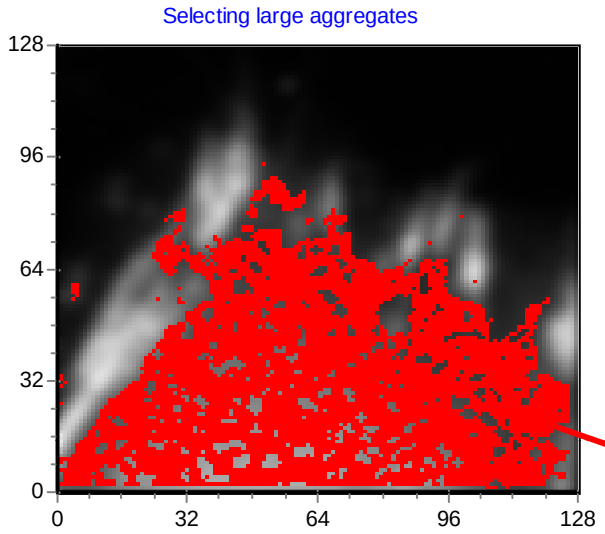
Average intensity (0-3.184c/s)

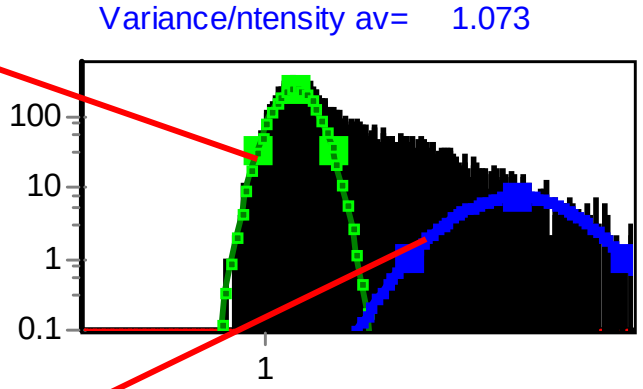
B map (0.8-1.4) (clustering)

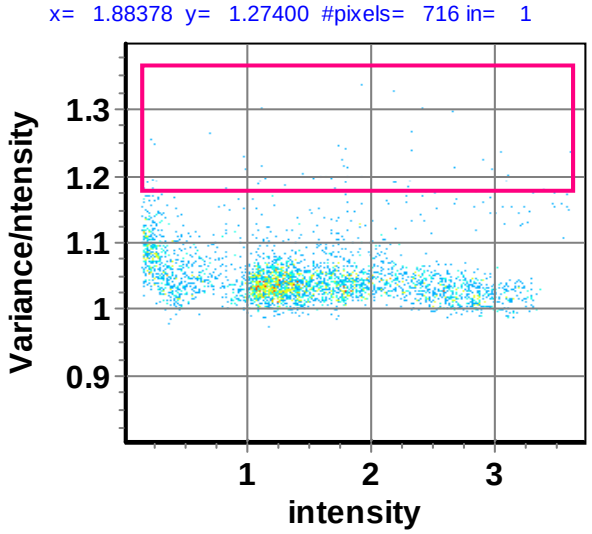
Calculated using 500 frames

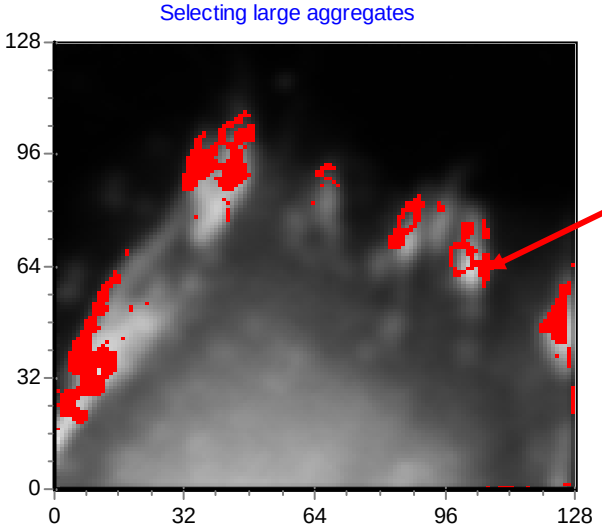
Selecting monomers and clusters. Distribution of aggregates











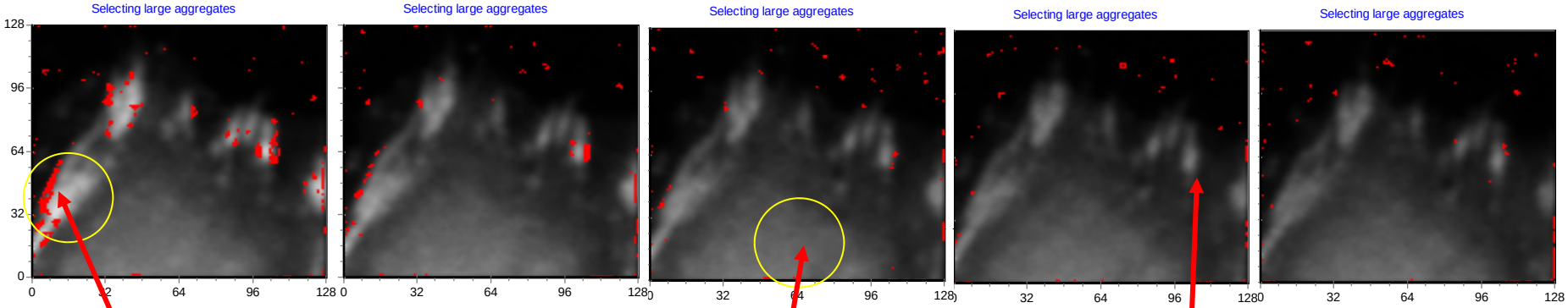
Green selects monomer
centered at B=1.034+/-0.019

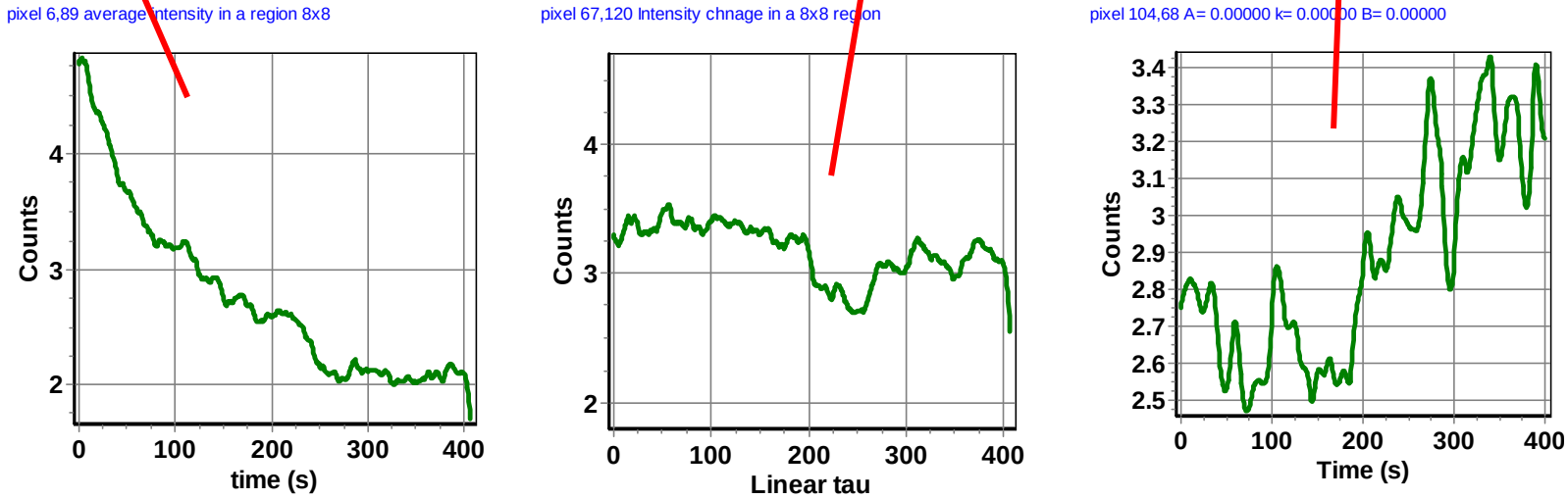
Blue selects aggregates
centered at B=1.274+/-0.059

Movies of adhesions assembling-disassembling

100 Frame averages

Selecting large aggregates





Summary

- Spatio-temporal correlations are needed to describe the dynamics and interactions in cells
- RICS gives us an approach to obtain fast dynamics in an image without introducing new hardware.
- The RICS approach allows us to perform FCS experiments even in the presence of slowly moving and immobile structures
- N and B analysis can be done at every point of the image, thereby providing a new contrast method in microscopy

Questions and discussion

- Where we stand?
- What is needed?
- Current limits of optics and fluorescence
- New optical instruments
- New fluorescent probes
- Other methods to study dynamics in cells

The Phasor Approach and Digital Frequency Domain FLIM

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University of California at Irvine

Outline

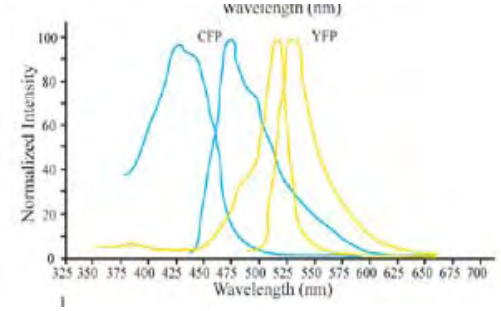
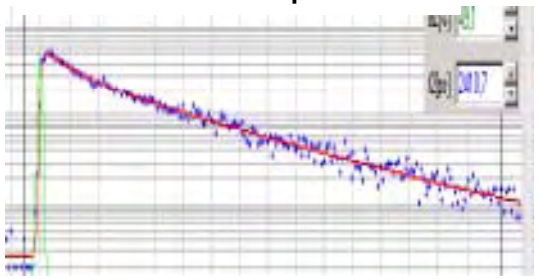
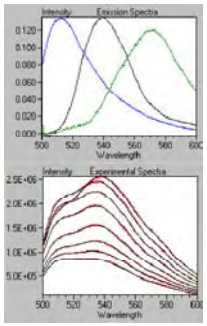
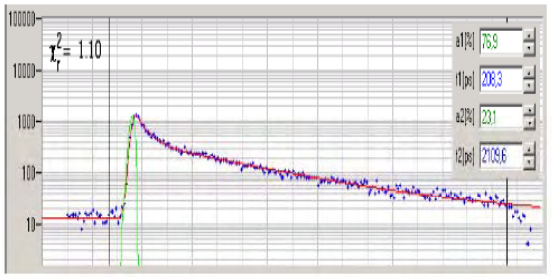
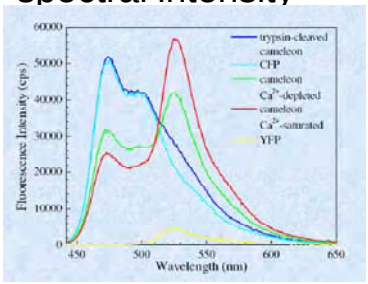
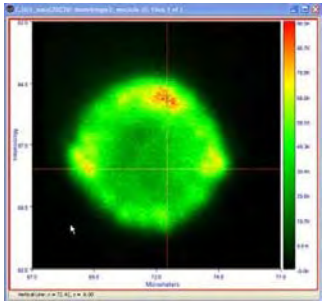
- Background: Lifetime
- Intro to Fluorescence Lifetime Imaging Microscopy
- Motivation for FLIM
- The Phasor approach

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**

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

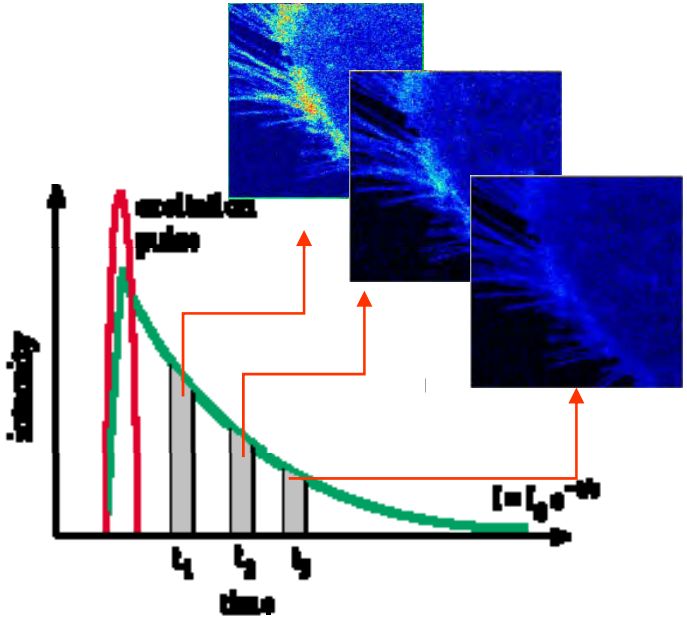
- Rather difficult technique
- Fitting is slow
- Results depend on initial conditions
- Interpretation requires expertise

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.

Can we avoid all these problems?

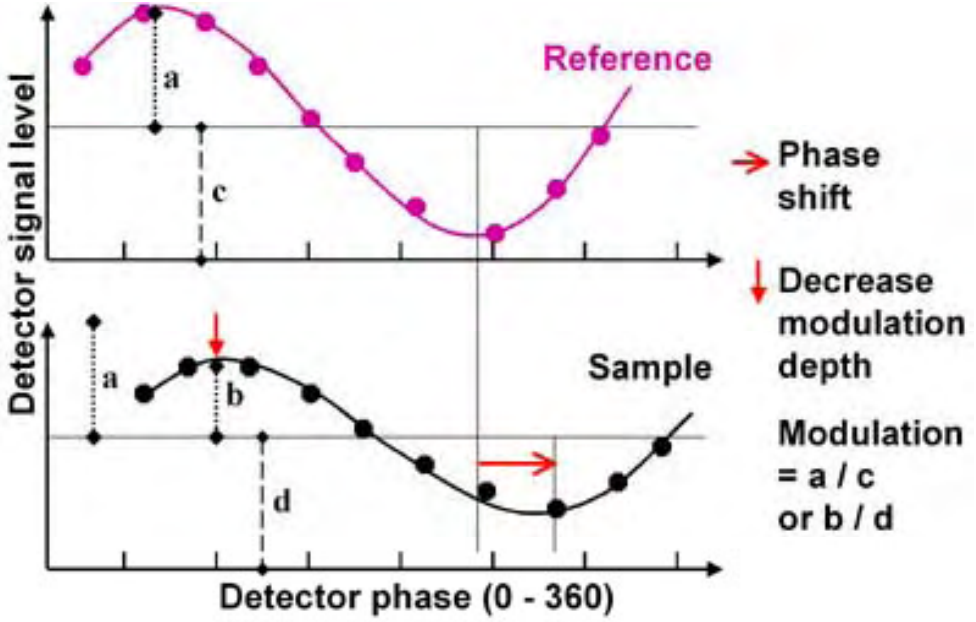
- No expertise necessary
- Instantaneous results
- Independent of initial choices
- Quantitative results
- Intuitive simple interface

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

*Jameson, D. M., E. Gratton and R. Hall. **1984**. The measurement and analysis of heterogeneous emissions by multifrequency phase and modulation fluorometry. App. Spec. Rev. 20:55-106*.

Clayton, A. H., Q. S. Hanley, and P. J. Verveer. **2004**. Graphical representation and multicomponent analysis of single-frequency fluorescence lifetime imaging microscopy data. J Microsc 213:1-5.

Redford, G. I. and R. M. Clegg. **2005**. Polar plot representation for frequency-domain analysis of fluorescence lifetimes. J Fluoresc 15:805-815.

APPENDIX 2., PHASE AND MODULATION LIFETIME RELATIONS

We have asserted that a heterogeneous emitting population, in the absence of excited state reaction, will demonstrate a phase lifetime which is always less than the modulation lifetime. The algebraic demonstration of this fact is somewhat cumbersome [11, 68]. We present here a brief and more intuitive demonstration of the phenomenon.

One may make a simple geometrical representation of the phase delay and relative modulation as shown in Fig. 11. Here we depict a vector of length M making an angle ϕ with the x-axis where ϕ represents the phase delay and M the relative modulation. Since for a single exponential decay we have the relation $M = \cos \phi$, the endpoint of the vector is constrained to be on the circle of radius 1/2 with a center at (1/2, 0). The intercept of the extension of this vector with the line through x = 1 equals ωt (since $\tan \phi = \omega t$). This circle is universal for single exponential systems irrespective of the lifetime or modulation frequency. We note that the X and Y intercepts of the vector correspond to our previously defined G and S functions (since $G = M \cos \phi$ and $S = M \sin \phi$).

Figure 12 represents the case of two exponential decays with phase delays and relative modulations of ϕ_1, ϕ_2 and M_1, M_2, respectively. These decays contribute to the total emission intensity decay with fractional weights of f_1 and f_2, respectively. The total fluorescence observed is represented by the vector sum, M, of the two components and gives an observed phase delay of ϕ. Again we see that the intercept of the extension of the M vector with the x = 1 line corresponds to $\omega\tau^P$ (since $\tan \phi = \omega\tau^P$). The value of $\omega\tau^M$, however, corresponds to the line segment BD. This observation follows from the fact that the triangle OAB,

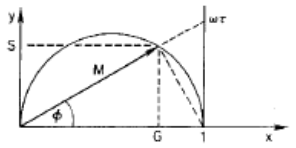


FIG. 11. Geometrical representation of phase delay (ϕ) and modulation ratio (M) for a single exponential decay.

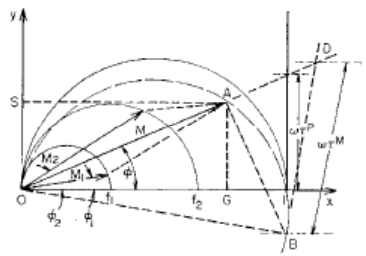
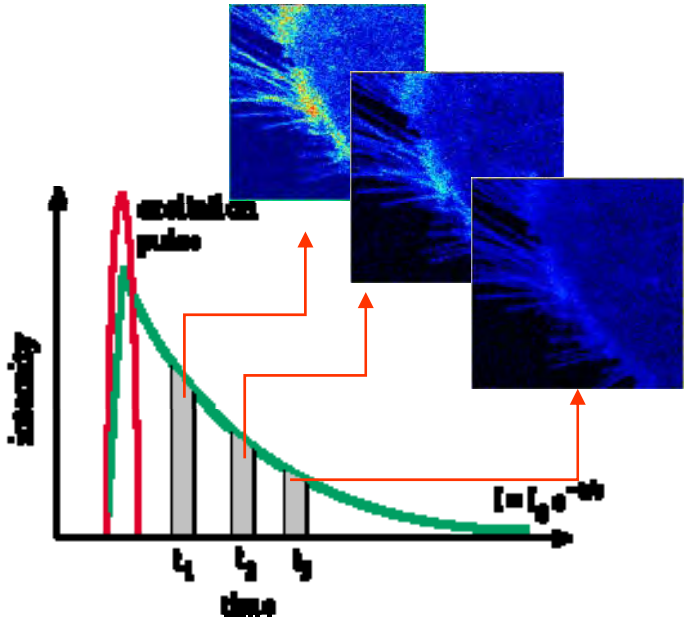


FIG. 12. Geometrical representation of phase delay (ϕ) and modulation ratio (M) for a double exponential decay.

which has a right angle, is congruent to the triangle ODB, which also has a right angle. Hence, from the ratios of the sides we have:

$$DB/BO = AB/OA \qquad (A13)$$

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

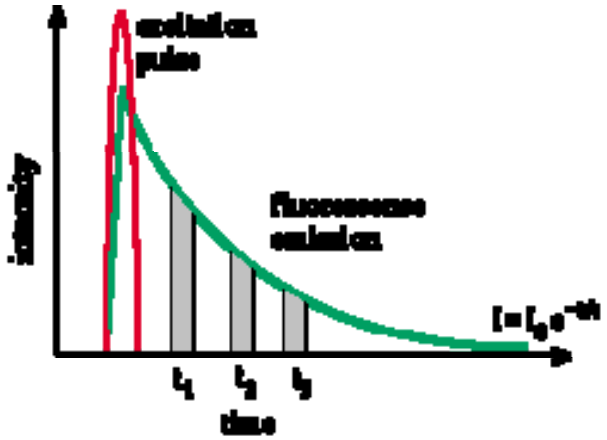


A sample is flashed many times by a short duration laser source

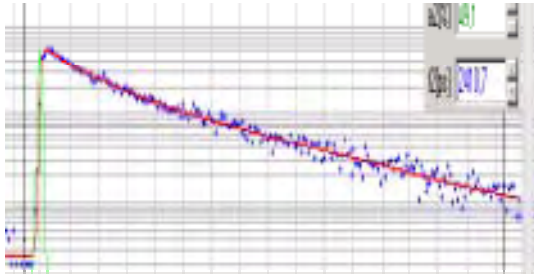
The interval between the excitation flashes, and 1st excited photon is measured

How to obtain the lifetime distribution?

From decay data

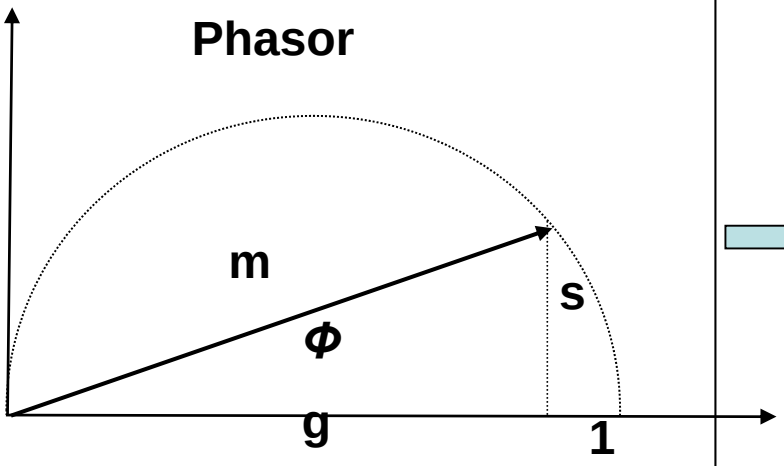


Fitting of exponentials

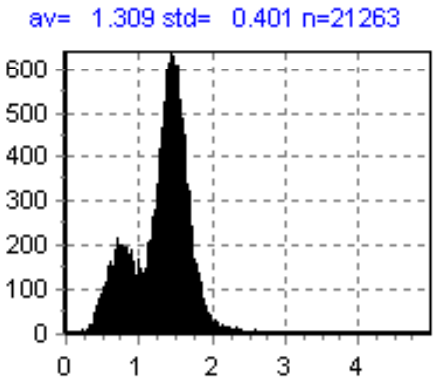


Multiexponential analysis

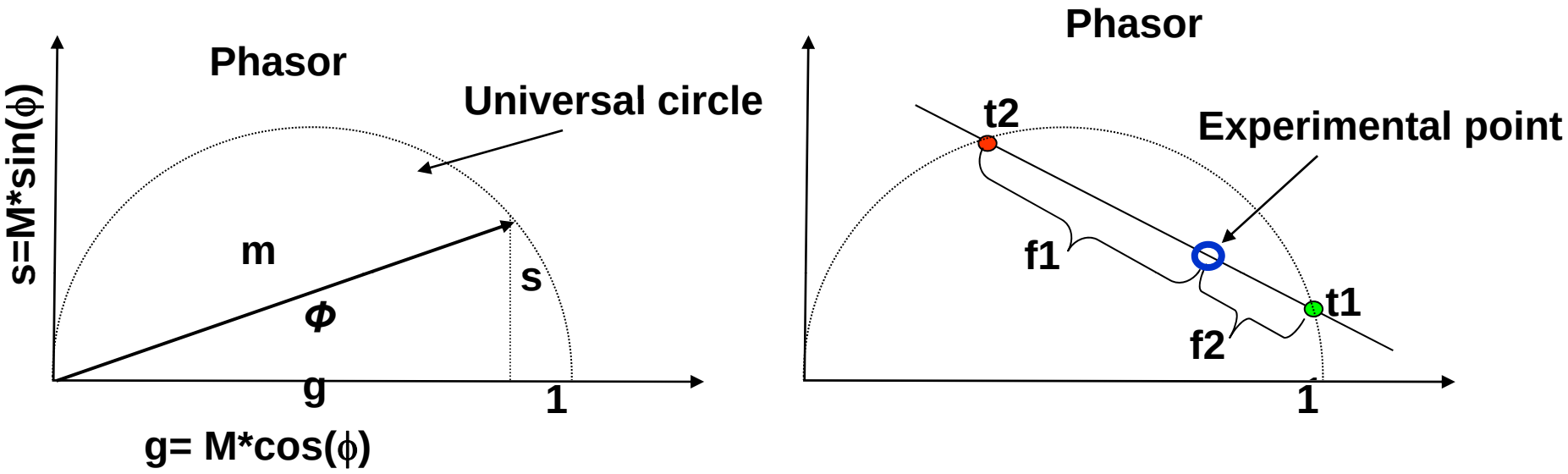
Phasor



Lifetime distribution



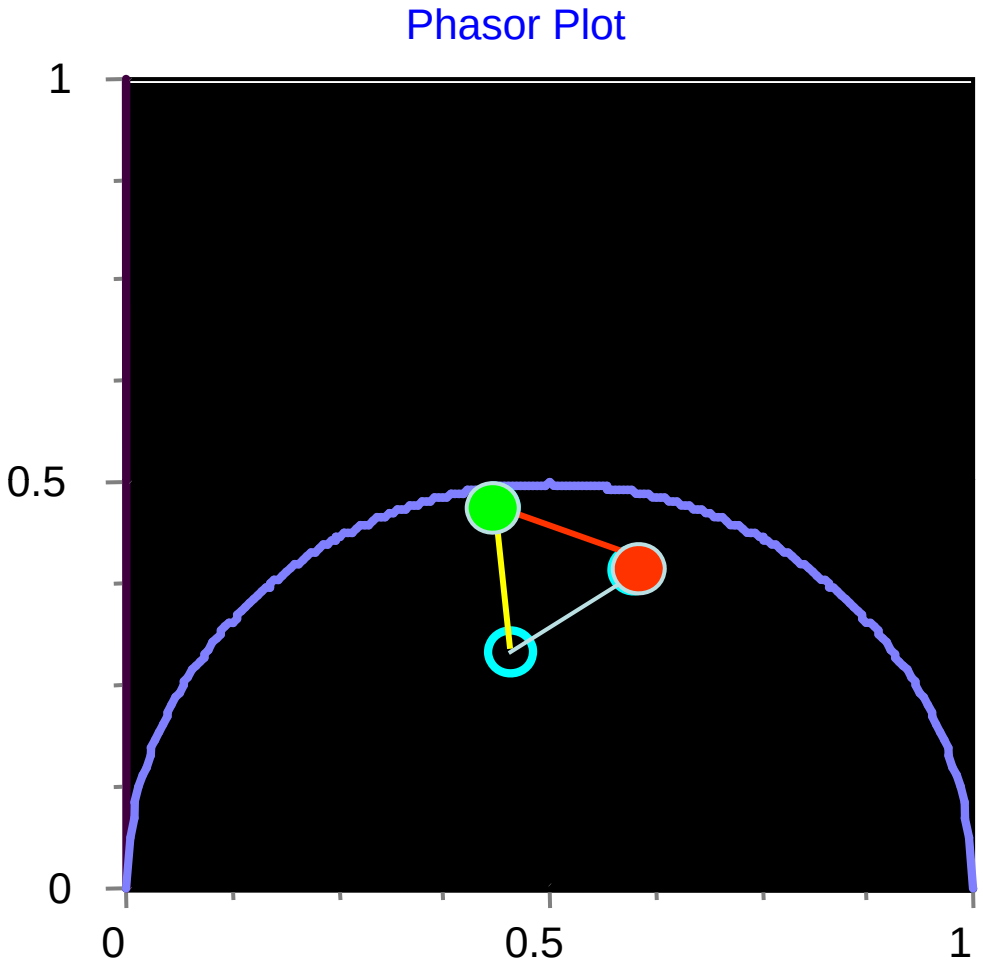
The algebra of phasors



Simple rules to the Phasor plot:

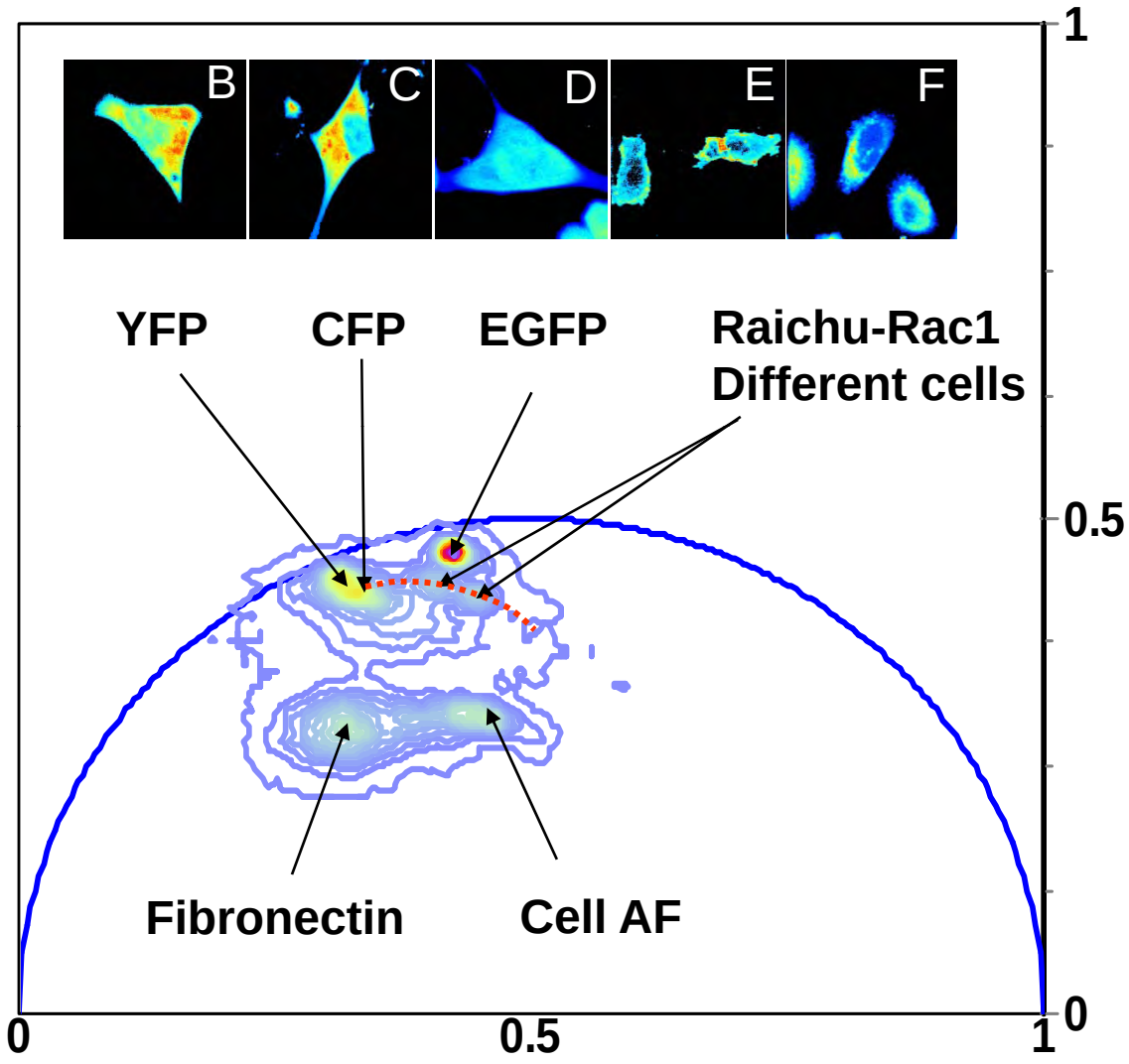
1) All single exponential lifetimes lie on the “universal circle”
2) Multi-exponential lifetimes are a linear combination of their components
3) The ratio of the linear combination determines the fraction of the components

How to identify components?



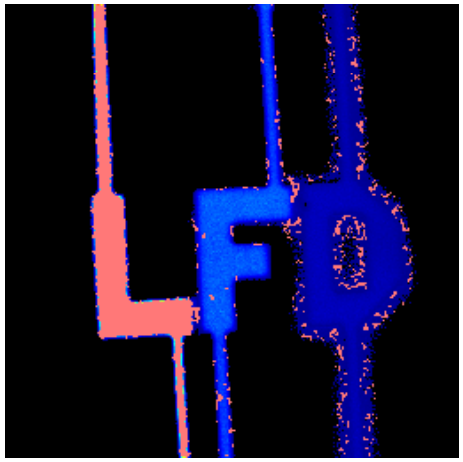
Phasors for common fluorophores. EGFP (green), autofluorescence (blue) and mRFP1 (red) (at 880 nm -2photon excitation) . In any given pixel, mixture of EGFP and autofluorescence must be on the yellow line, mixtures of EGFP and mRFP1 must be on the red line. Mixtures of three of them must be inside the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

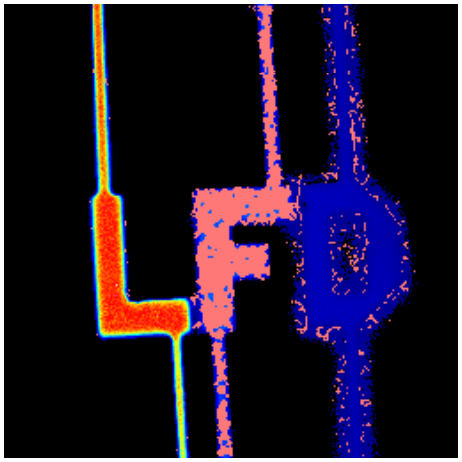
Examples of phasors identification

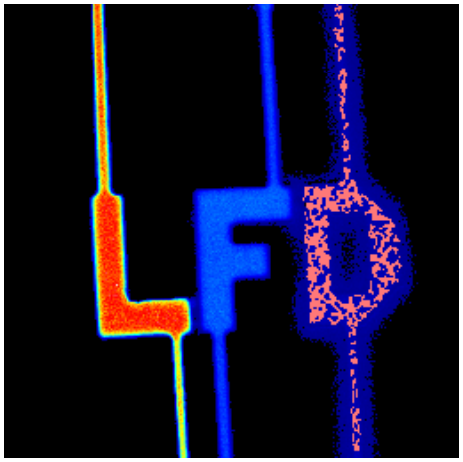


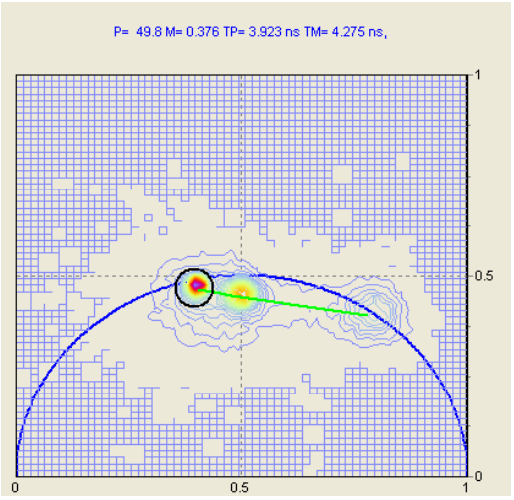
Digman et al, Biophys. J. 2007

Separating Different Single exponential lifetimes using the ISS Fast FLIM system

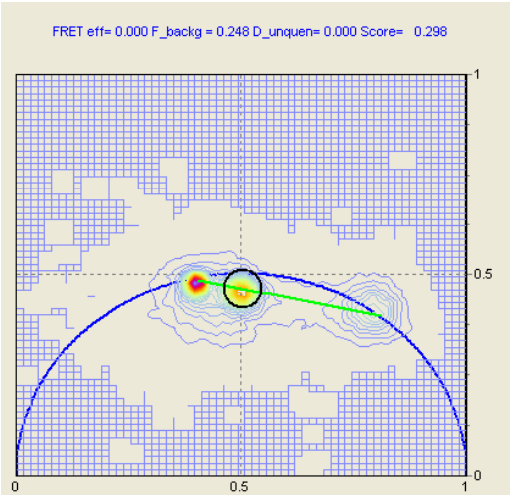




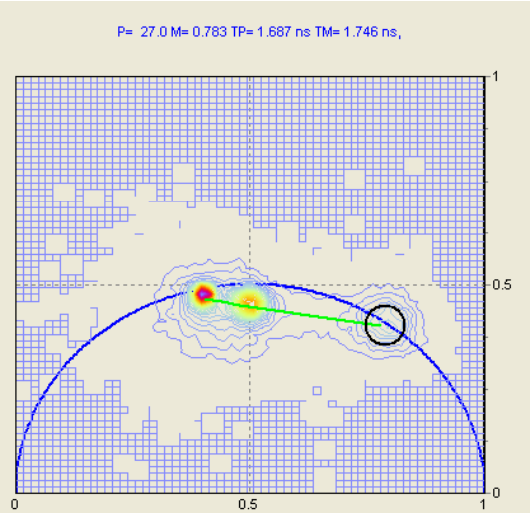




Fluorescein



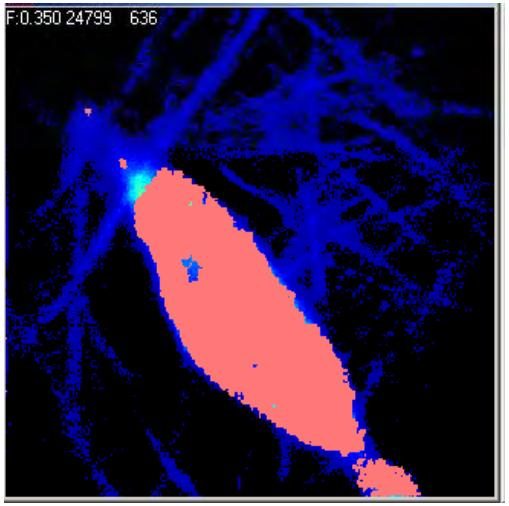
Mixture



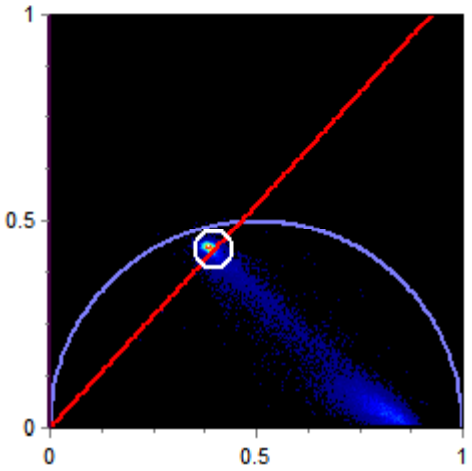
Rhodamine B1

Pax-eGFP CHO-k1 in collagen

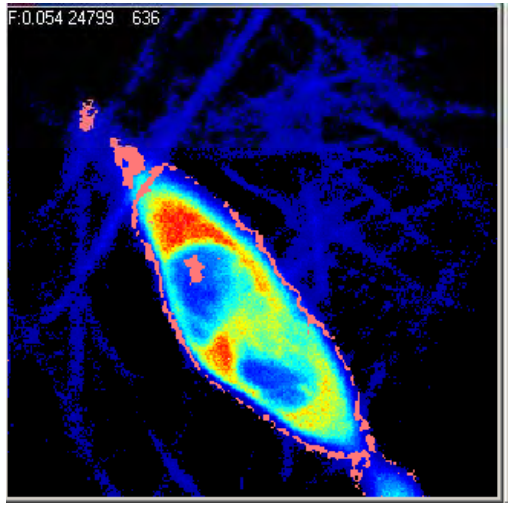
referenced with Fluorescein @ 905nm



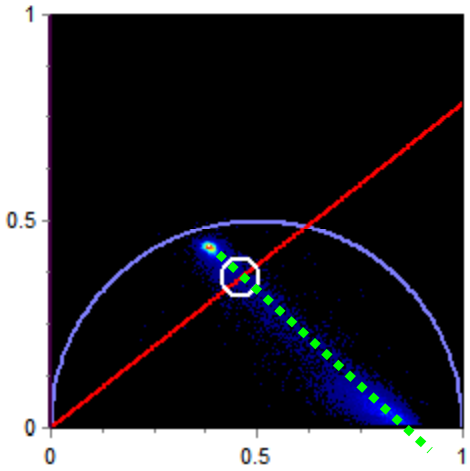
P= 47.2 M= 0.343 TP= 2.149 ns TM= 2.751 ns,



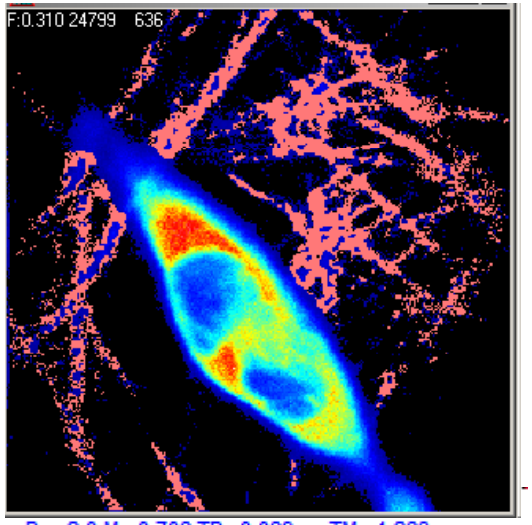
Lifetime of EGFP



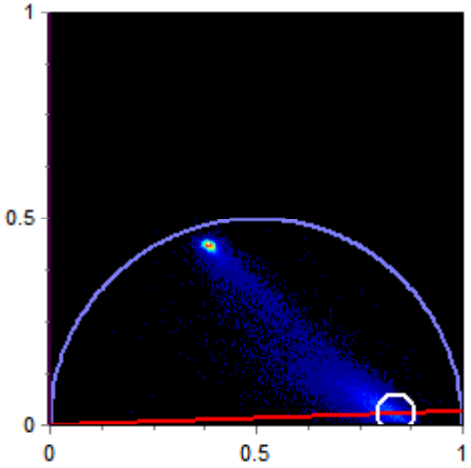
P= 38.2 M= 0.344 TP= 1.563 ns TM= 2.748 ns,



Combinations of Lifetimes

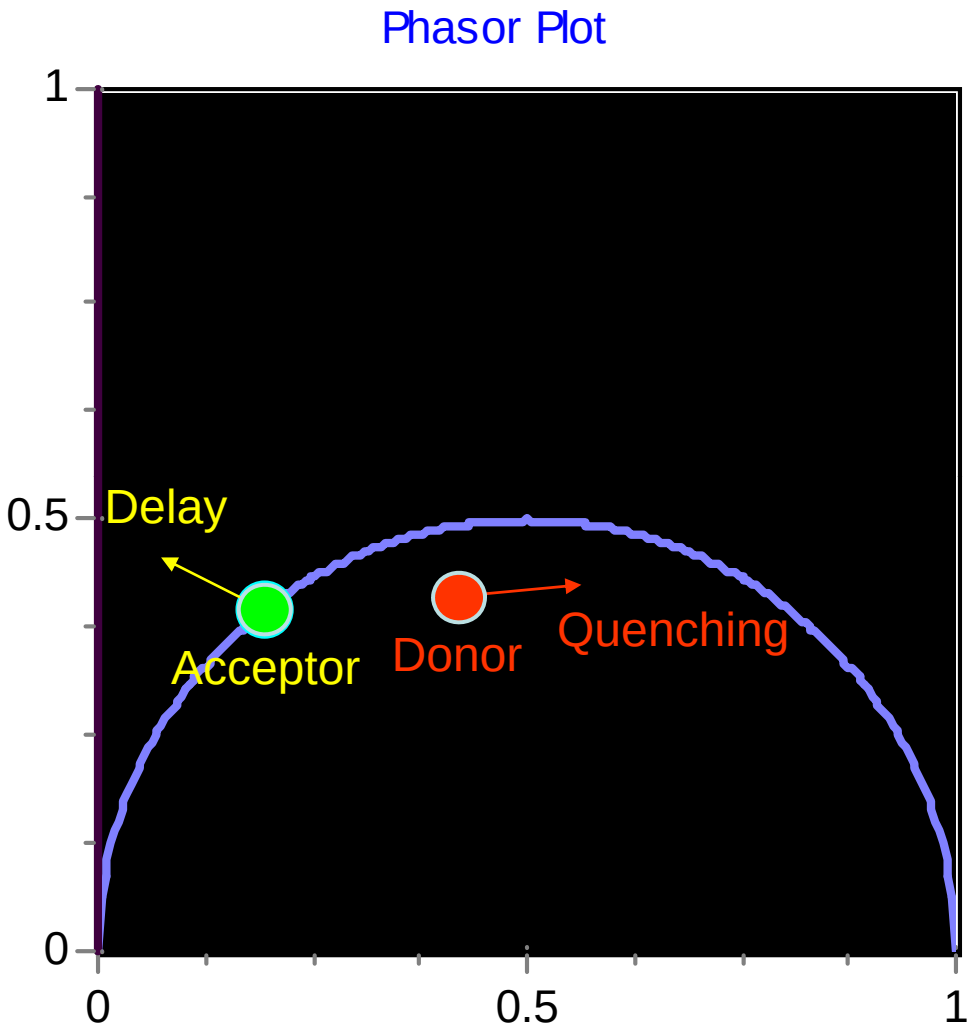


P= 2.0 M= 0.706 TP= 0.069 ns TM= 1.283 ns,



Lifetime of Collagen

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

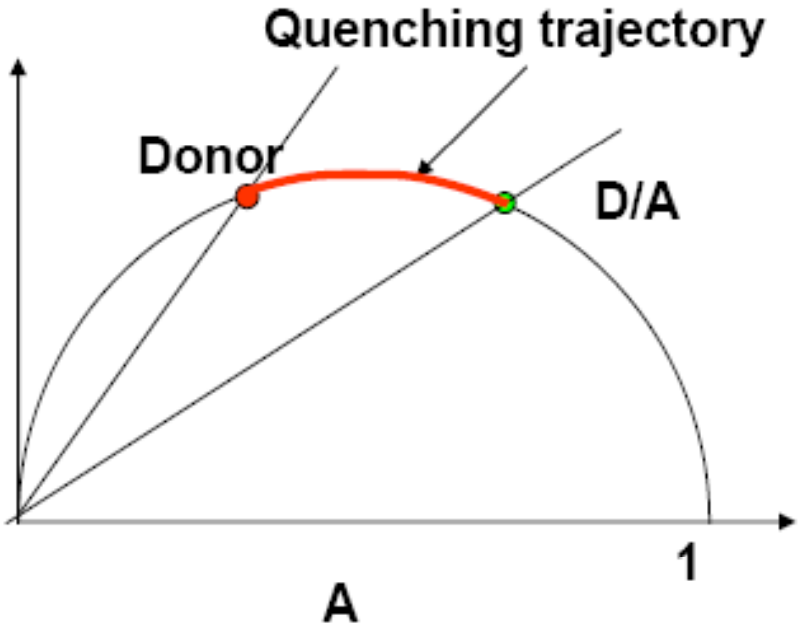
Purpose: to generate trajectories in the phasor plot corresponding to different processes

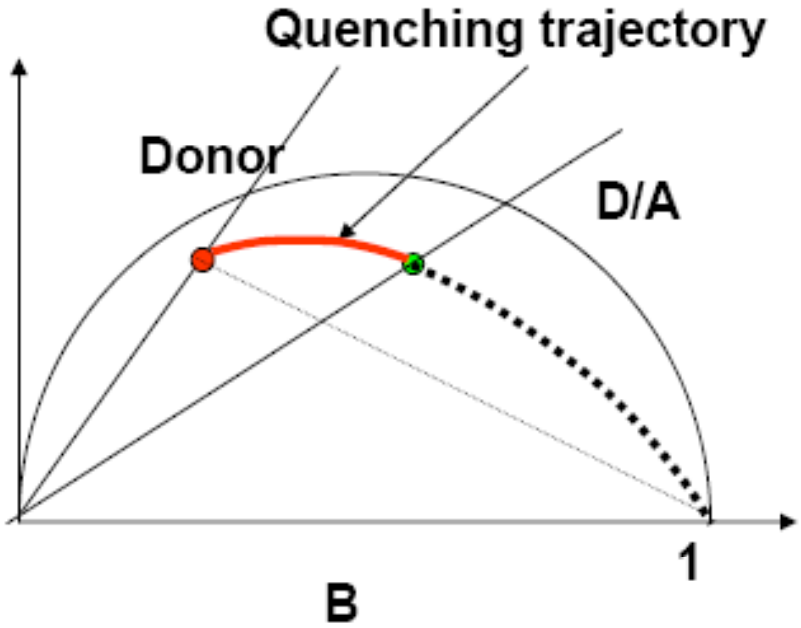
At present, there are 4 functions programmed

1. Fractional contribution of two species give the phasor of the two individual species
2. Ion concentration given the phasor of the bound and free form and the pK of the indicator
3. FRET efficiencies from the observation of the donor only with background and fraction of donor unquenched
4. FRET efficiency using the observation of both donor and acceptor with given background and fraction of donor unquenched and acceptor excitation

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

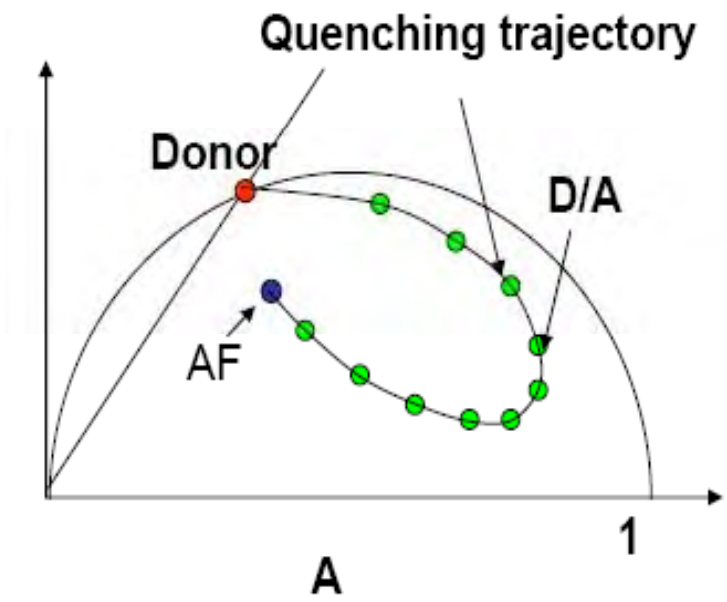
The lifetime of the donor is along a different “trajectory”, Why is the trajectory an arc rather than a line to the (1,0) point?

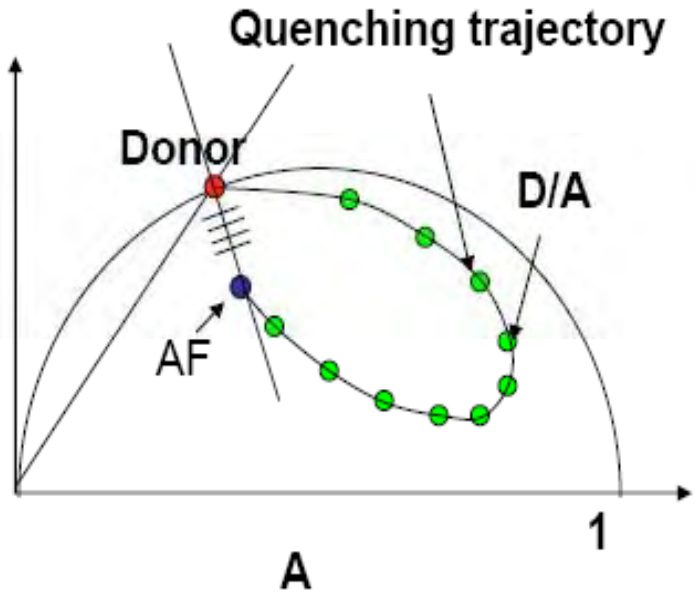
The FRET Calculator

- 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 autofluorescence phasor!!!

The FRET Calculator

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

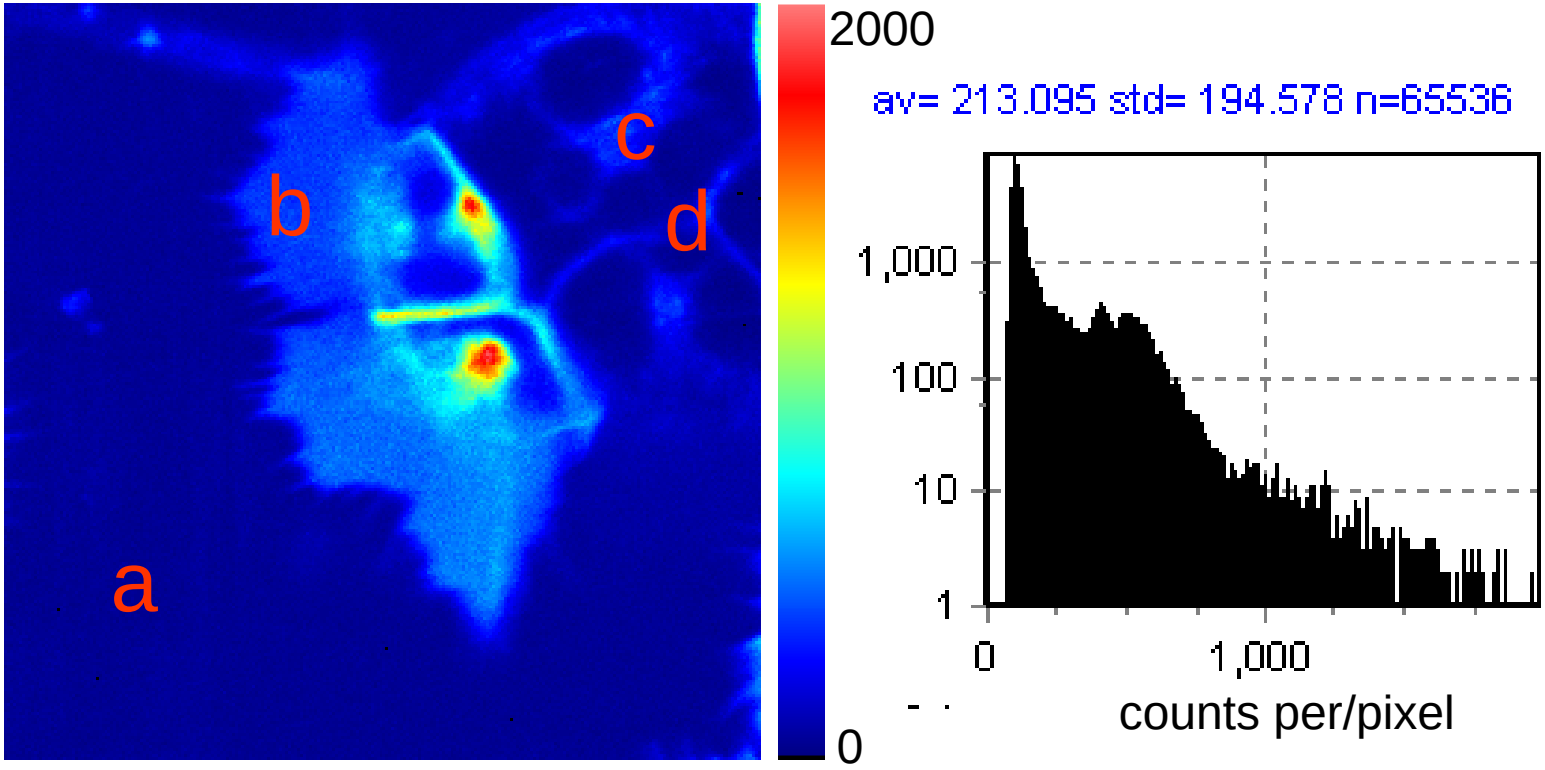




As the lifetime of the Donor is quenched, the phasor of the quenched Donor is added to the phasor of the autofluorescence

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

Example of FLIM analysis using phasors

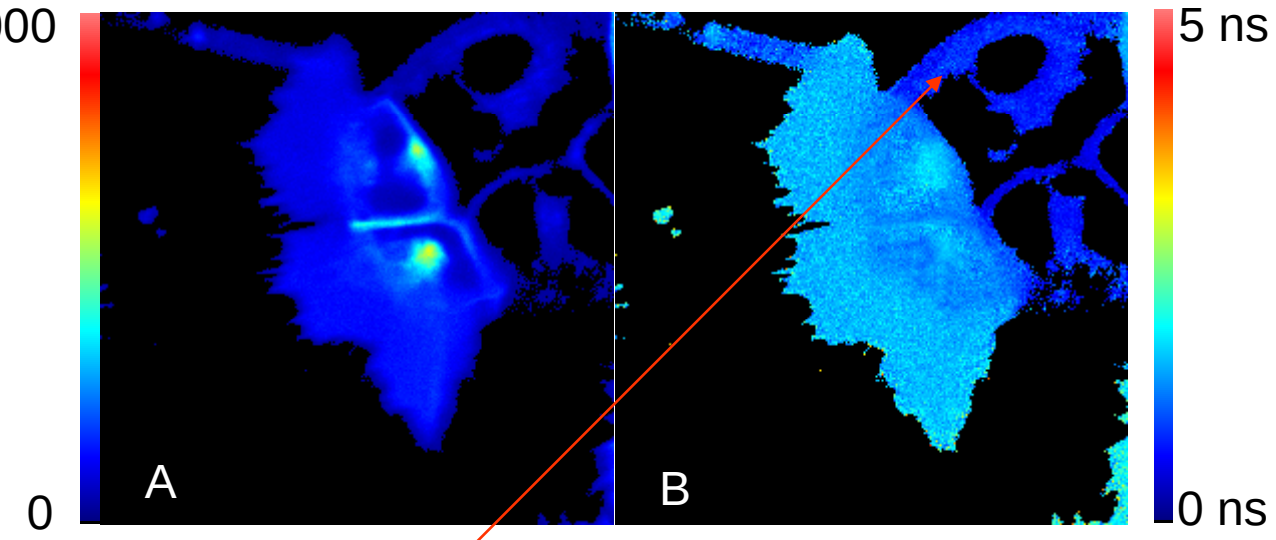


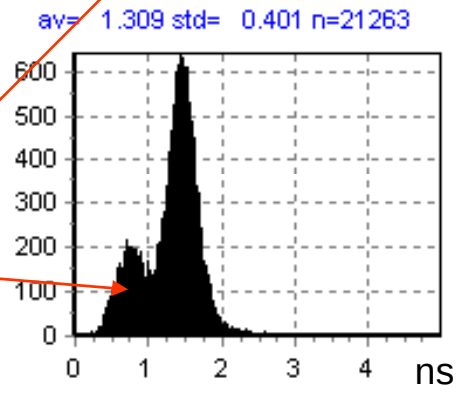
Several regions the image can be identified corresponding to **a)** background (2 exponentials) **b)** cell 1 bright (2 exponentials) **c)** cell 2 dim, **d**) cell junctions dim.

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

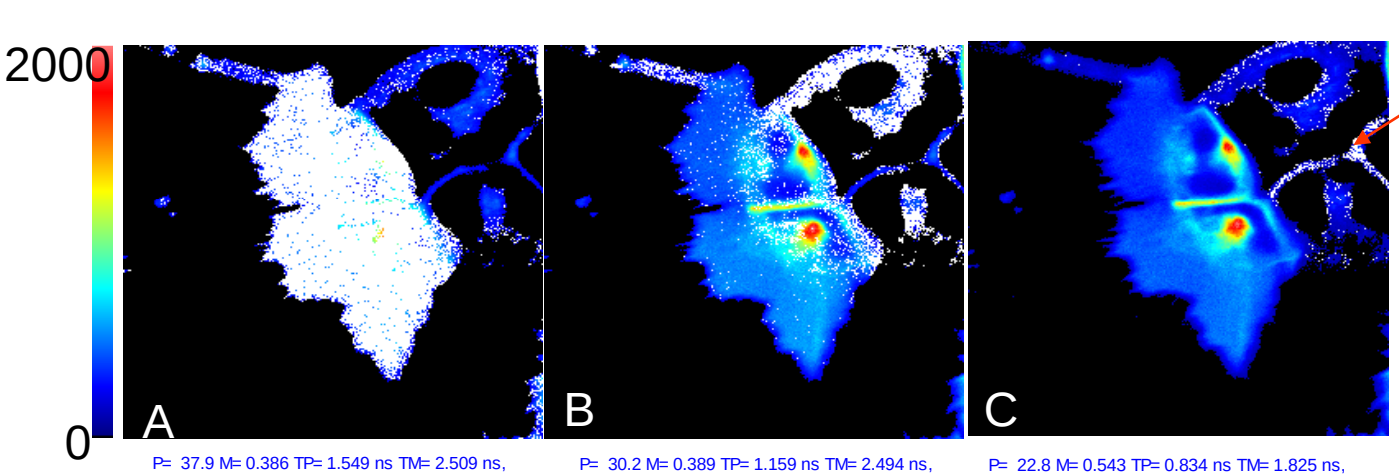




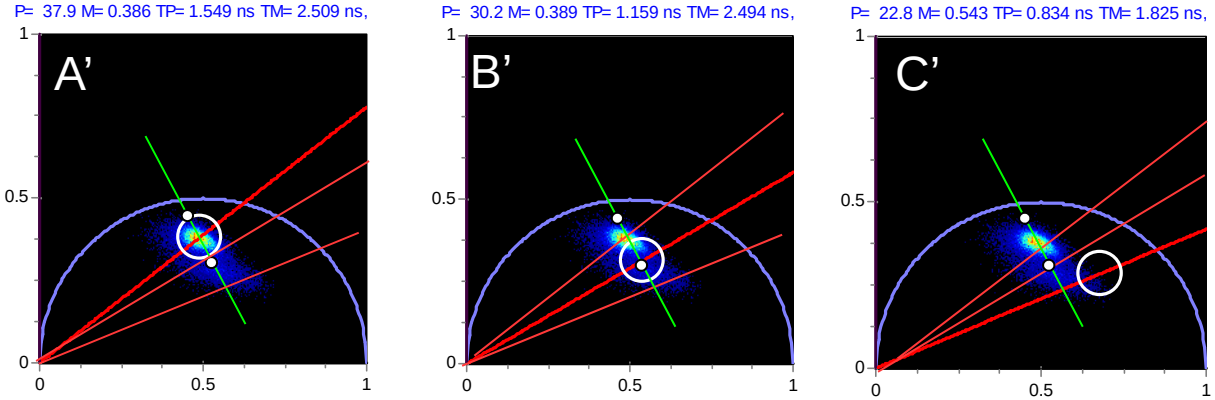
Shorter lifetime region could be interpreted to be due to FRET

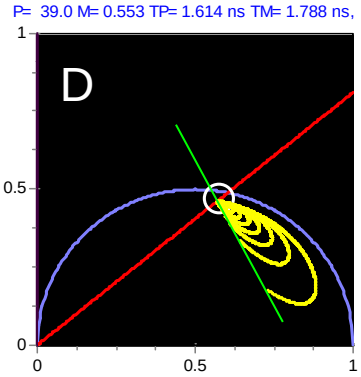
Donor+acceptor+ligand. A) intensity image after background subtraction, B) τ_p image

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**.

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