

## **Lecture 8: RICS and FLIM**

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## **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

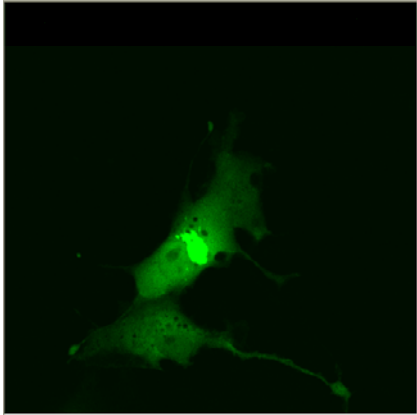
**N&B in transfected COS 7 cells using human Htt exon 1 with varying lengths of polyglutamine fused to GFP (Httex1 97QP-GFP, Httex1 46Qp-GFP and Httex1 25QP-GFP)**

Giulia Ossato, Elisa Frasnelli, Luca Lanzano, Michelle Digman, J. Lawrence Marsh\* and Enrico Gratton

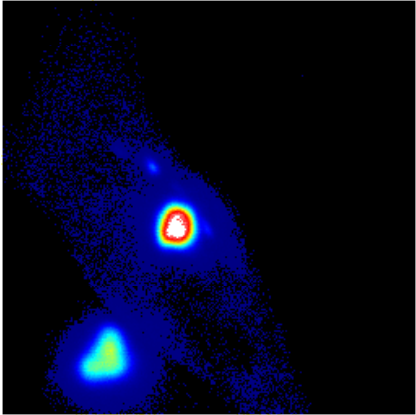
Laboratory for Fluorescence Dynamics, University of California, Irvine,

\*Development Biology Center, UCI

**97QP after 24 hours**  
 COS7: Zeiss 510 META 1-photon and 2-photon system



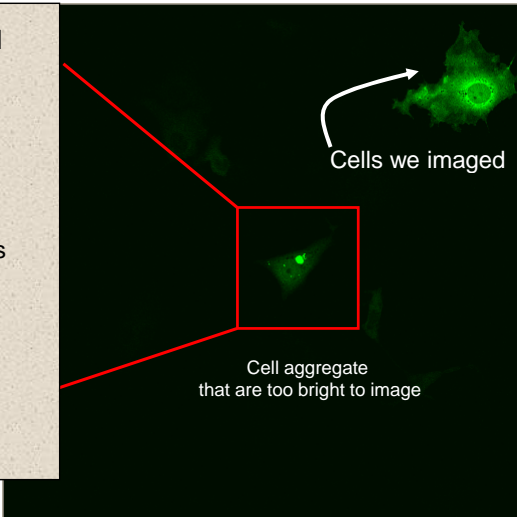
1-photon



2-photon

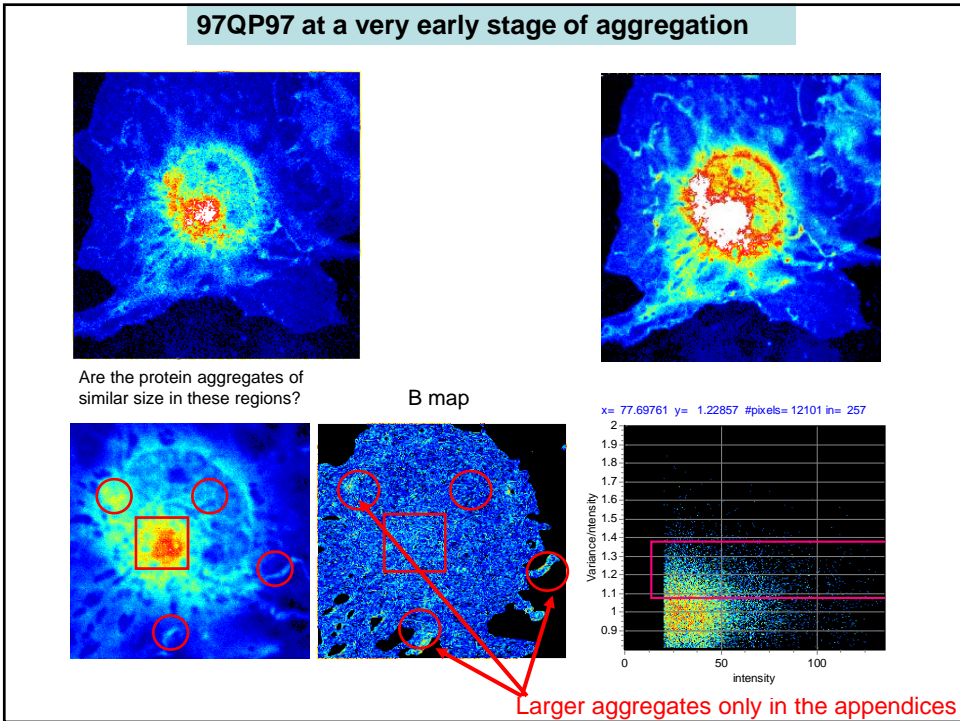
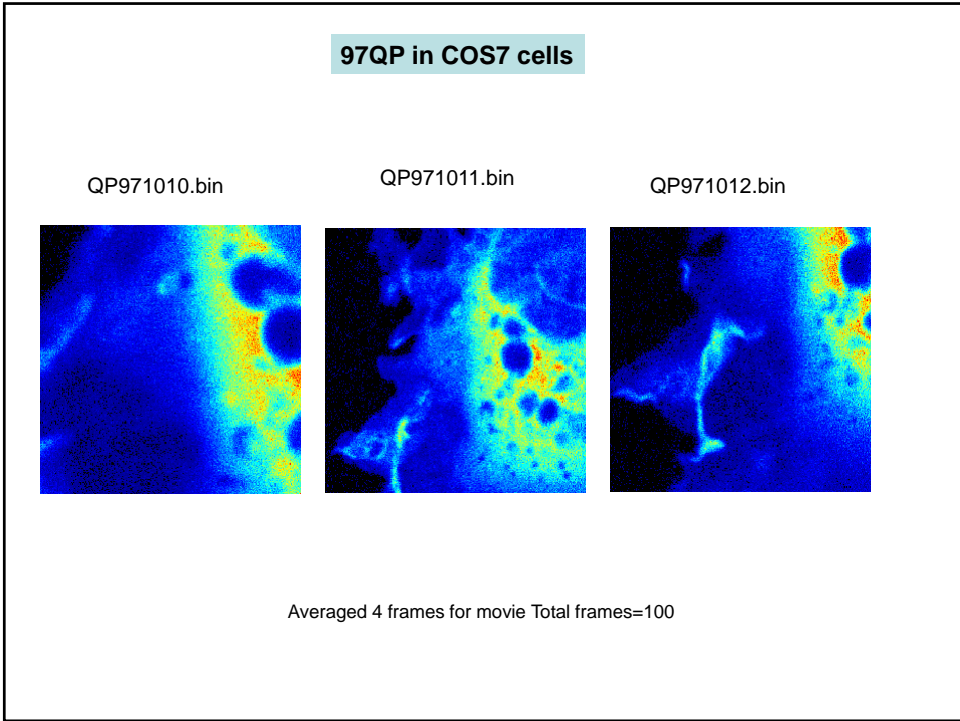
**What are the Biological questions?**

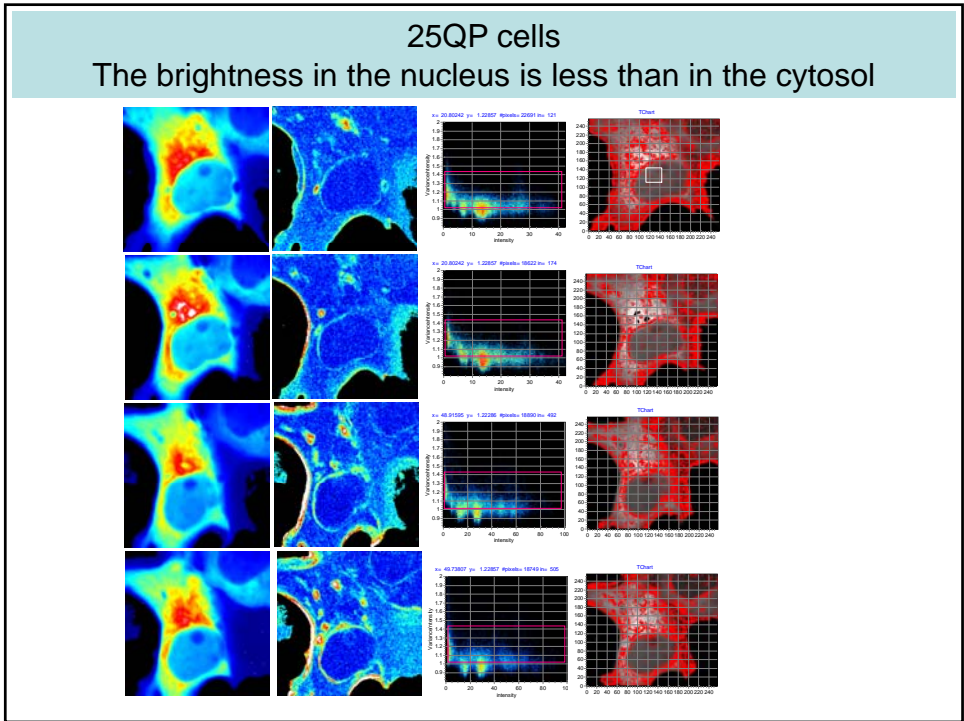
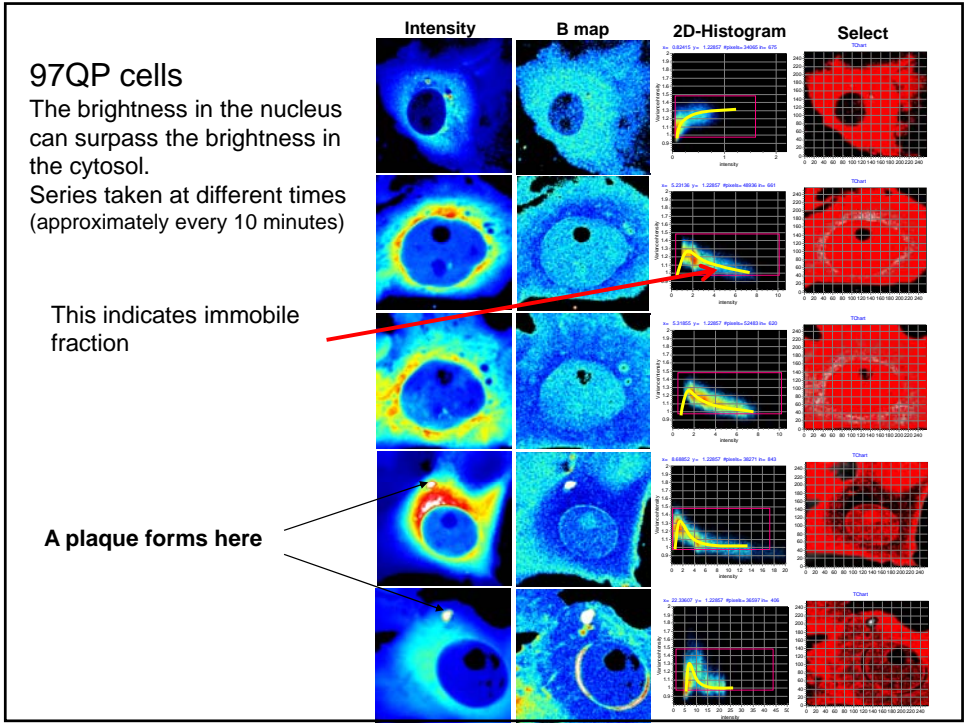
- Are there precursor aggregates before the plaque stage?
- Are there smaller aggregates (precursors to the large aggregate)?
- Can we follow aggregation?
- Can we detect aggregates in the nucleus?



Cells we imaged

Cell aggregate that are too bright to image





## 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<sup>2+</sup> Calmodulin

Hugo Sanabria<sup>\*</sup>, Michelle A. Digman<sup>†</sup>, Enrico Gratton<sup>†</sup>, and M. Neal Waxham<sup>\*</sup>.

<sup>\*</sup> Department of Neurobiology and Anatomy, University of Texas Health Science Center at Houston, Houston, TX 77030

<sup>†</sup> Laboratory of Fluorescent Dynamics, University of California at Irvine, Irvine, CA 92697

### Questions

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

What is the spatial distribution of Cam in the cell?

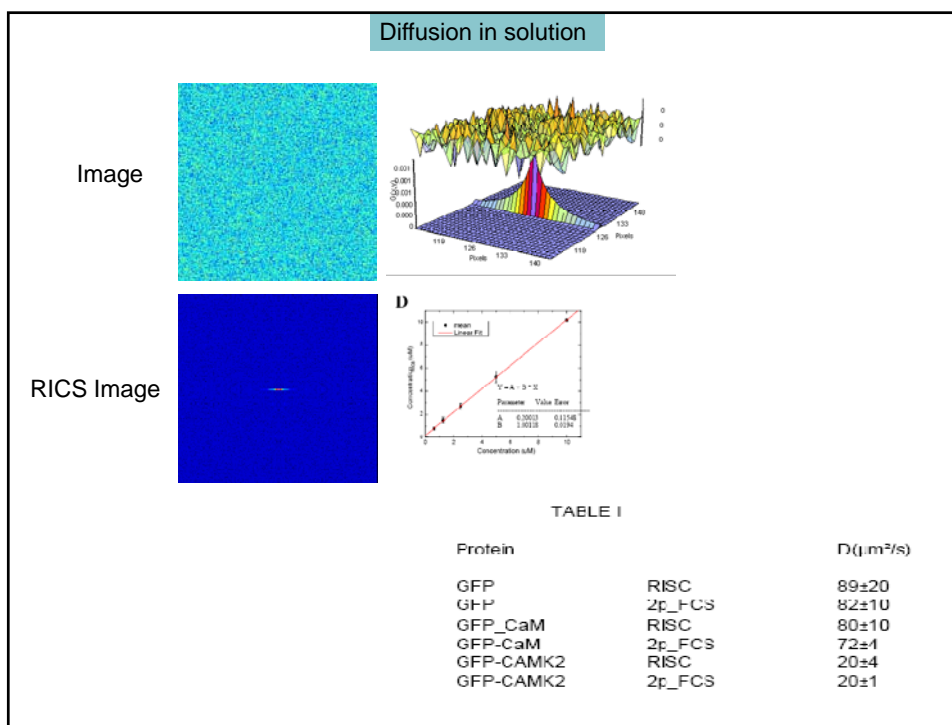
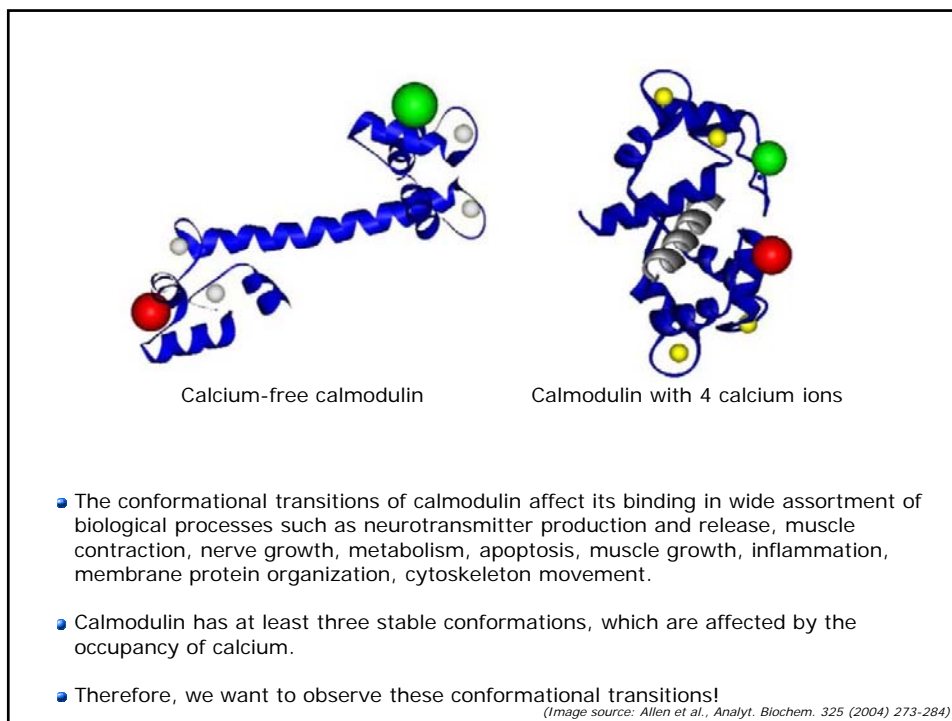
Does the spatial distribution of Cam changes at different extracellular Ca levels?

### 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

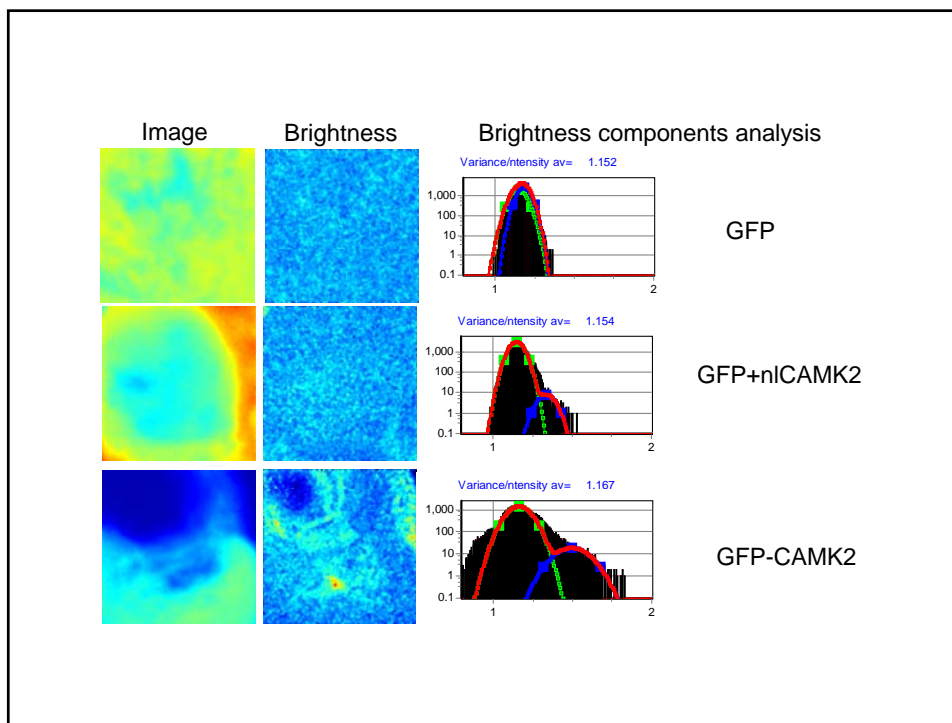


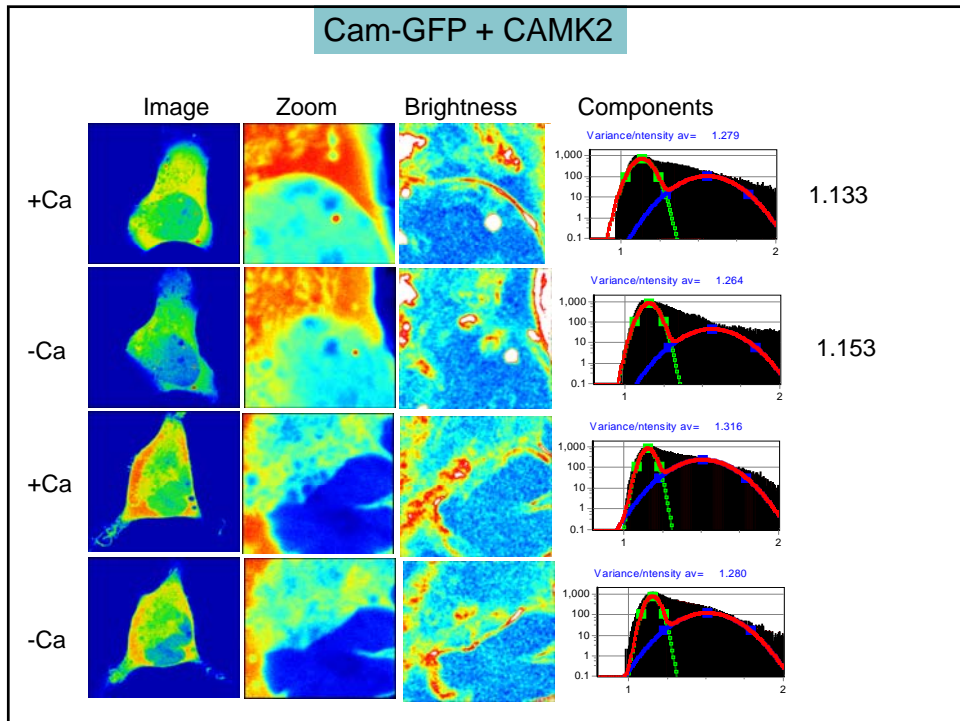
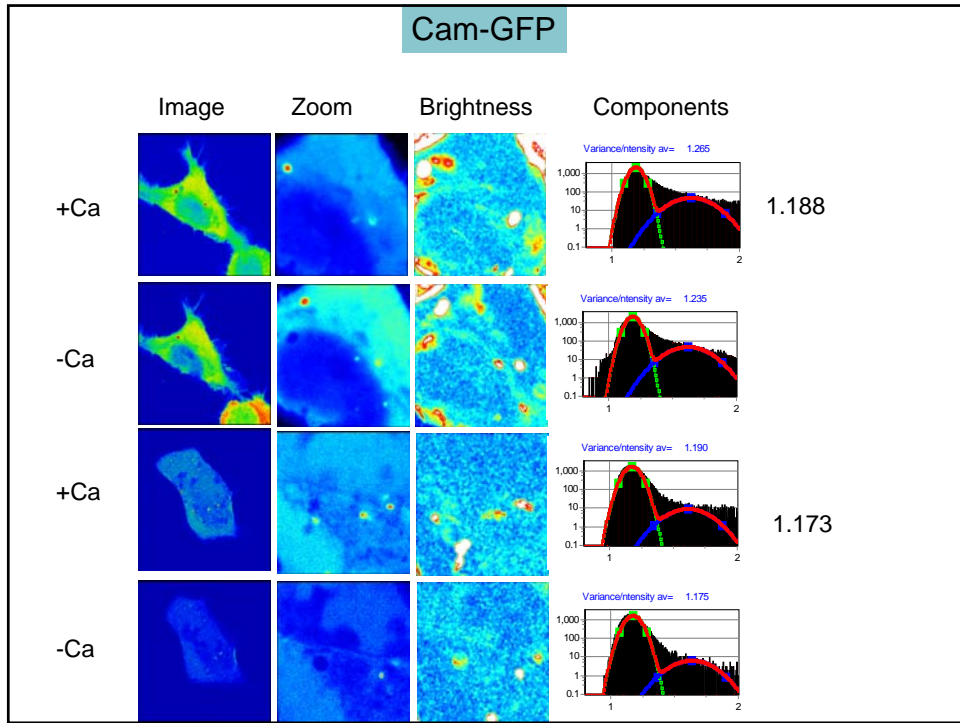
## Diffusion in cells

TABLE 2

Protein	Condition		D( $\mu\text{m}^2/\text{s}$ )	% of fast
GFP			$20 \pm 5$	$90 \pm 9$
GFP+nICAMK2			$21 \pm 8$	$86 \pm 13$
GFP-CAMK2			$1.6 \pm 0.9$	$74 \pm 11$
GFP-CaM	basal	nucleus	$10 \pm 5$	$80 \pm 19$
	Ca+		$10 \pm 4$	$85 \pm 16$
	Ca-		$10 \pm 7$	$75 \pm 12$
GFP-CaM	basal	Cyt	$11 \pm 6$	$60 \pm 17$
	Ca+		$11 \pm 4$	$68 \pm 17$
	Ca-		$10 \pm 9$	$64 \pm 26$
GFP-CaM+nICAMK2	basal	nucleus	$7 \pm 5$	$52 \pm 28$
	Ca+		$12 \pm 2$	$58 \pm 23$
	Ca-		$6 \pm 2$	$67 \pm 11$
GFP-CaM+nICAMK2	basal	Cyt	$7 \pm 4$	$29 \pm 27$
	Ca+		$8 \pm 1$	$67 \pm 10$
	Ca-		$4 \pm 3$	$34 \pm 10$

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





## 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  $\epsilon$  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  $\langle N \rangle$  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".

$$\langle k \rangle = \frac{\sum_i k_i}{K} \quad \sigma^2 = \frac{\sum_i (k_i - \langle k \rangle)^2}{K}$$

$$B = \frac{\langle k \rangle}{\langle N \rangle} = \frac{\sigma^2}{\langle k \rangle}$$

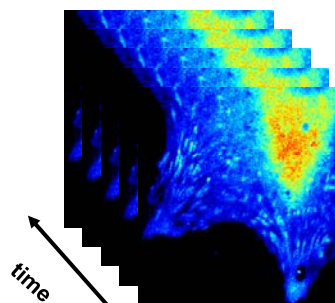
$$\langle N \rangle = \frac{\langle k \rangle^2}{\sigma^2}$$

$\sigma^2$  = Variance

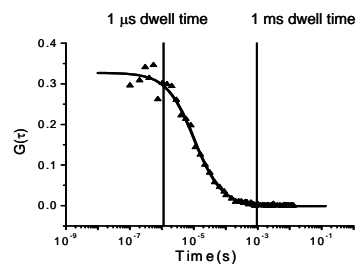
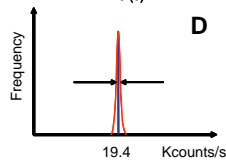
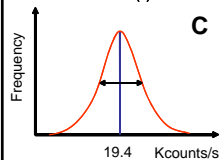
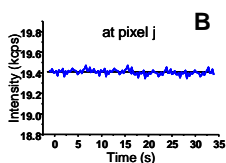
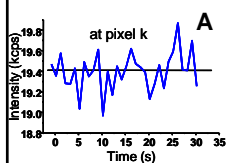
$\langle k \rangle$  = Average counts

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

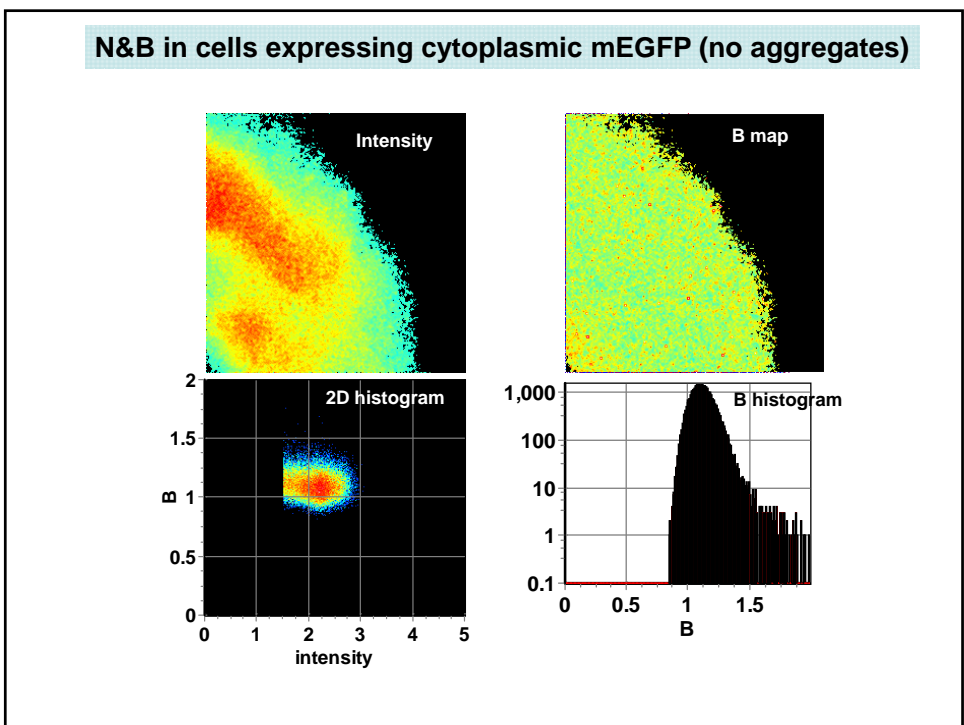
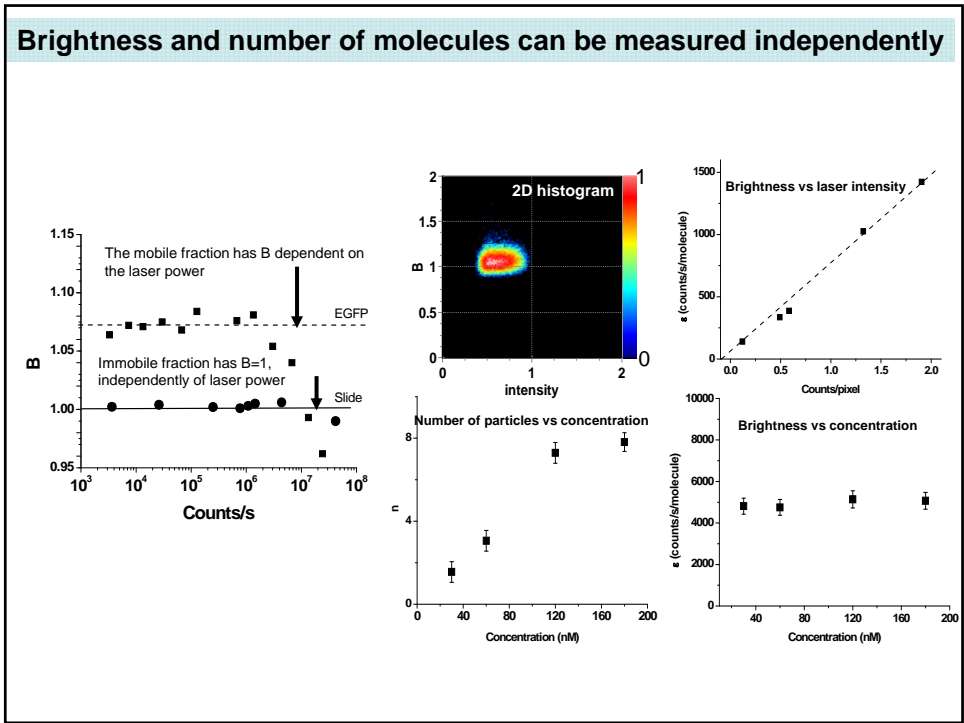
$$\sigma_d^2 = \varepsilon n$$

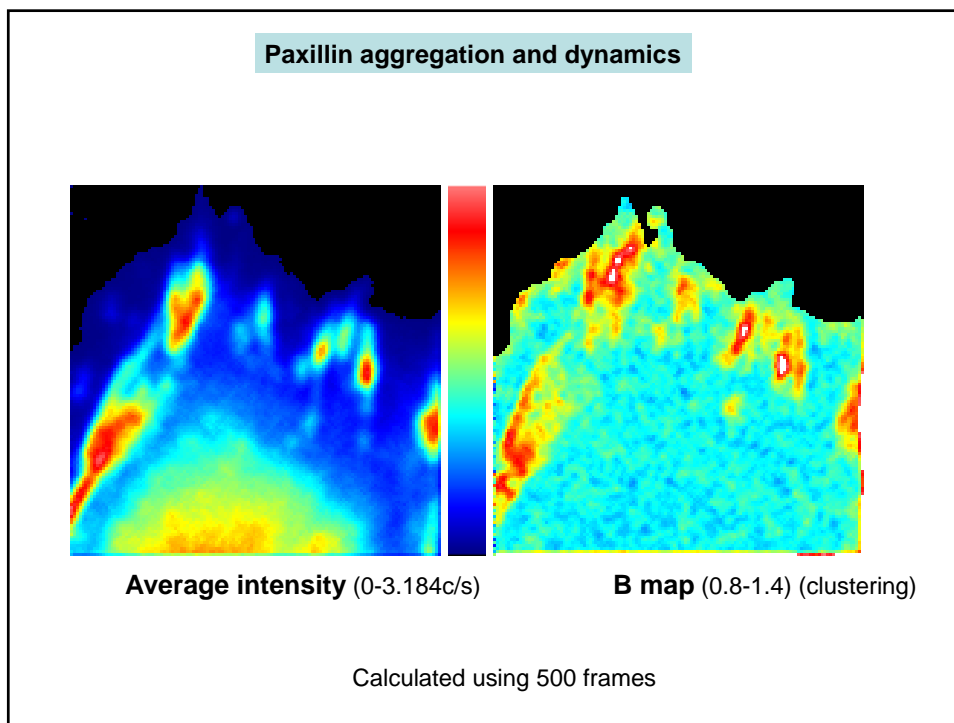
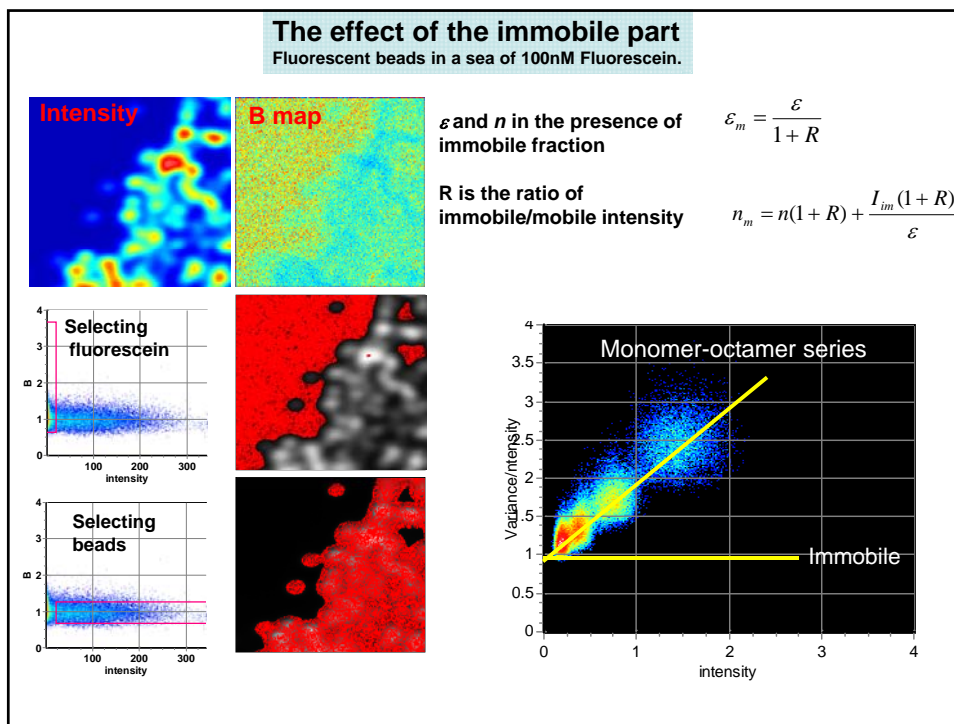
Average intensity in one pixel

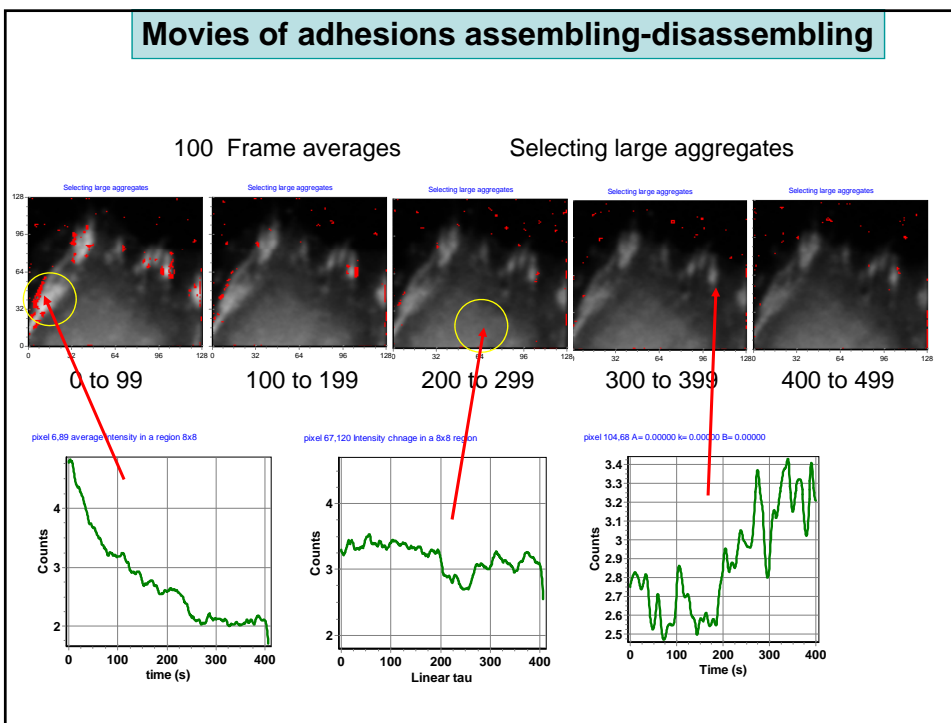
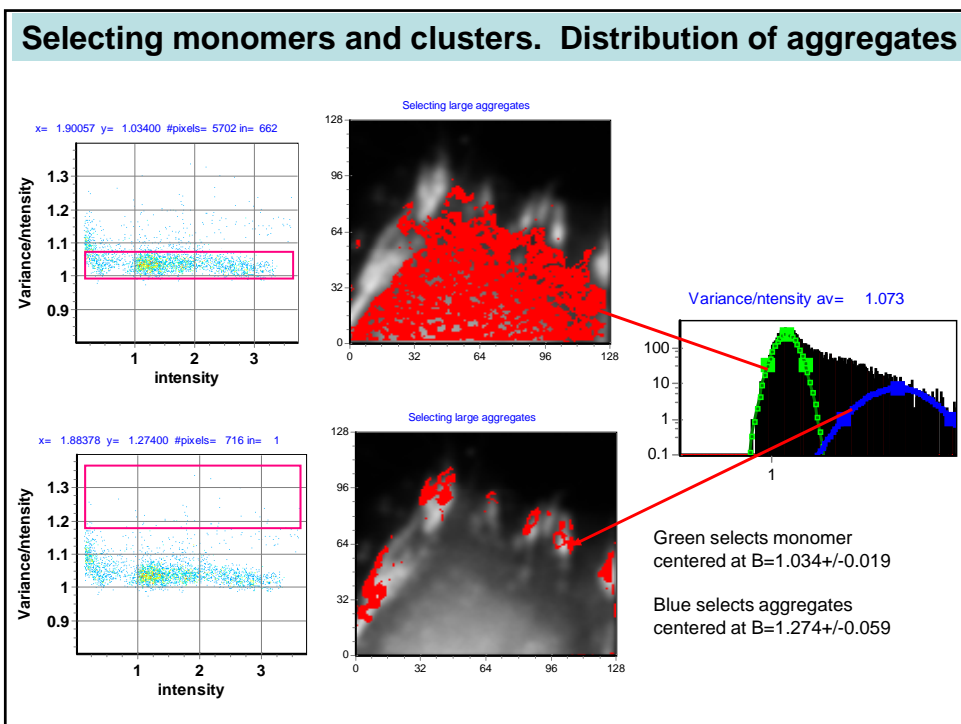
$$\langle k \rangle = \varepsilon n$$

$$B = \frac{\sigma^2}{\langle k \rangle} = \varepsilon + 1$$

$$N = \frac{\langle k \rangle^2}{\sigma^2} = \frac{\varepsilon n}{\varepsilon + 1}$$







## 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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## 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	<p><math>\lambda</math> Excitation <math>\lambda</math> emission</p>	<p>Lifetime Components</p>
2) Demixing of multiple species in a pixel	<p>Spectral demixing</p>	<p>Multiexponential analysis</p>
3) Identification of processes: FRET	<p>Ratio of acceptor/donor spectral intensity</p>	<p>Quenching of donor lifetime</p>

## The challenges of FLIM

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

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

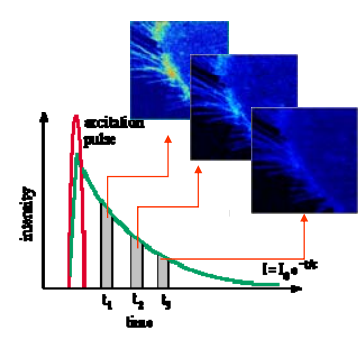
## Major issues with FLIM

- Rather difficult technique
  - Fitting is slow
  - Results depend on initial conditions
  - Interpretation requires expertise
- Can we avoid all these problems?

**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.

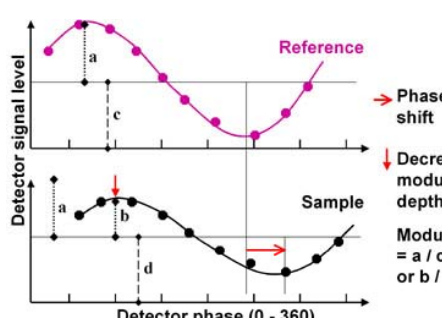
- No expertise necessary
- Instantaneous results
- Independent of initial choices
- Quantitative results
- Intuitive simple interface

## 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 1<sup>st</sup> 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. Verwee. **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 assumed that a heterogeneous emitting population, in the absence of excited state reactions, will demonstrate a phase lifetime which is always less than the modulation lifetime. The significant demonstration of this fact is mathematical (see eq. 11, 12). We present here a brief and more intuitive demonstration of the phenomenon.

One may make a simple geometrical construction of the phase delay and relative modulation as shown in Fig. 11. Here we depict a vector of length M making an angle  $\phi$  with the x-axis where  $\phi$  represents the phase delay and M the relative modulation. Here for a single exponential decay we have the relation  $M < \cos \phi$ . The amplitude 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 of course  $\cos \phi$ . This intercept is referred to as single exponential systems in respect to the lifetime or modulation frequency. We note that the M and  $\phi$  components of the vector correspond to our previously defined G and  $\theta$  functions (page 2) -  $M \cos \phi$  and  $\theta = M \sin \phi$ .

Figure 12 represents the case of two exponential decays with phase delays and relative modulations of  $\phi_1, \phi_2$  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  $\phi$ . Again we note that the intercept of the extension of the M vector with the  $x = 1$  line corresponds to  $\cos \phi$  (since  $\cos \phi = \cos^2 \phi$ ). The value of  $\cos^2 \phi$ , however, corresponds to the real component BD. This observation follows from the fact that the triangle OAB,

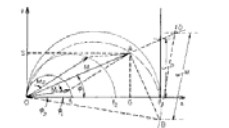


FIG. 11. Geometrical representation of phase delay ( $\phi$ ) and modulation ratio ( $M$ ) for a double exponential decay.

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:

$$\sin \phi / \cos \phi = \sin \theta / \cos \theta$$

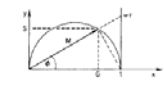
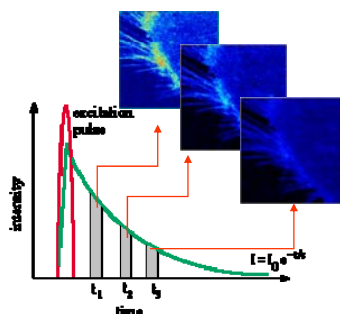


FIG. 12. Geometrical representation of phase delay ( $\phi$ ) and modulation ratio ( $M$ ) for a single exponential decay.

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

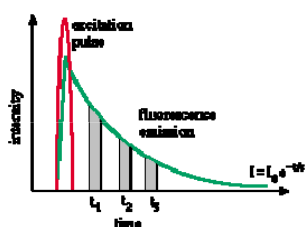


A sample is flashed many times by a short duration laser source

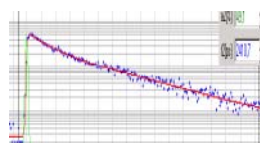
The interval between the excitation flashes, and 1<sup>st</sup> excited photon is measured

## How to obtain the lifetime distribution?

From decay data

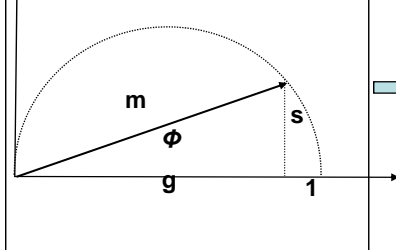


Fitting of exponentials

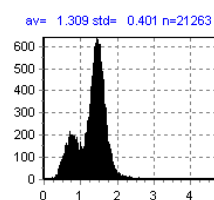


Multiexponential analysis

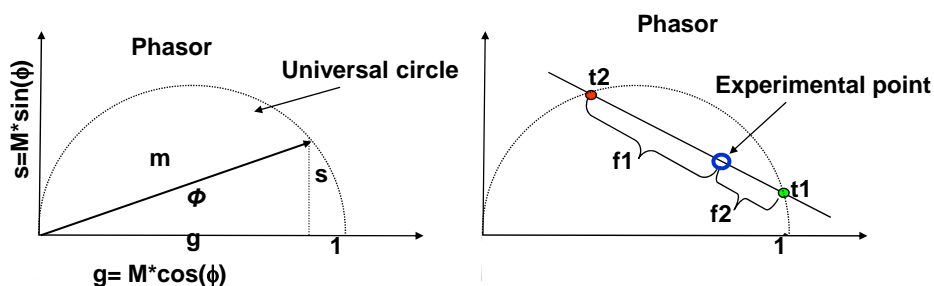
Phasor



Lifetime distribution



## The algebra of phasors

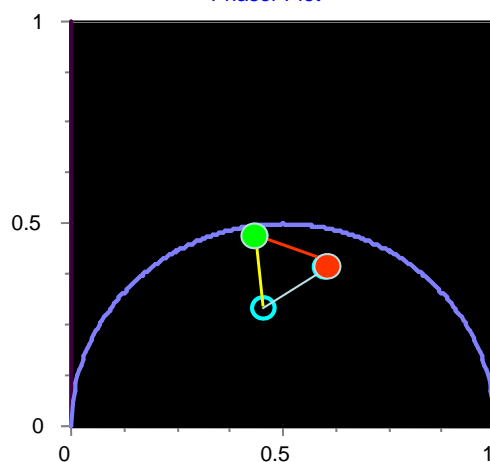


Simple rules to the Phasor plot:

- 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

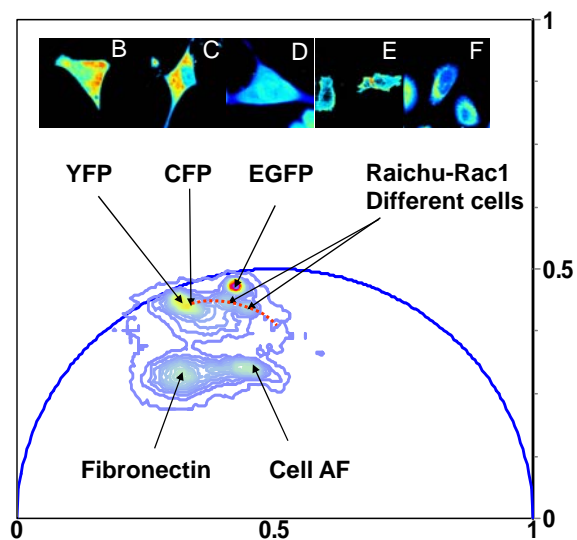
## How to identify components?

Phasor Plot



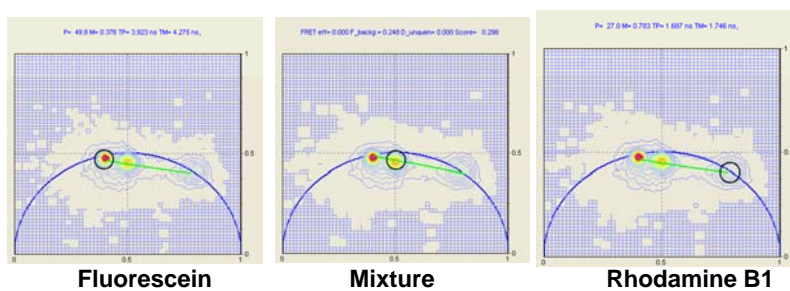
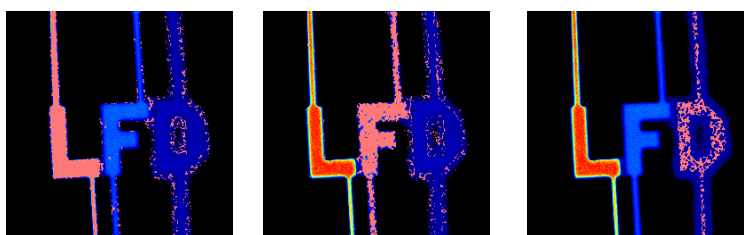
Phasors for common fluorophores. EGFP (green), autofluorescence (blue) and mRFP1 (red) (at 880 nm -2-photon excitation). In any given pixel, mixture of EGFP and autofluorescence must be on the yellow line, mixtures of EGFP and mRFP1 must be on the red line. Mixtures of three of them must be inside the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

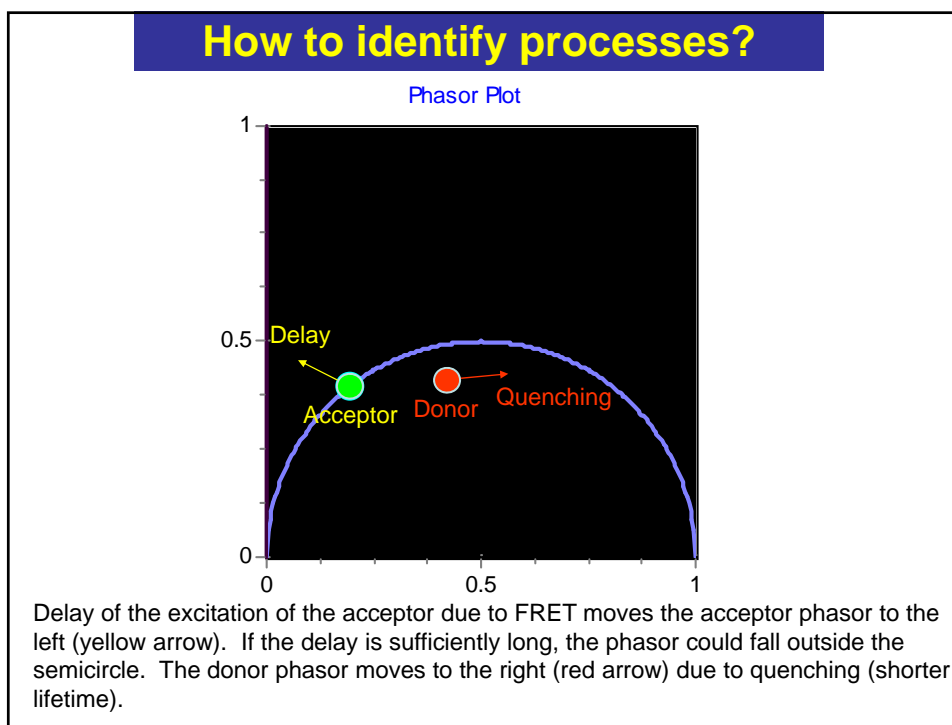
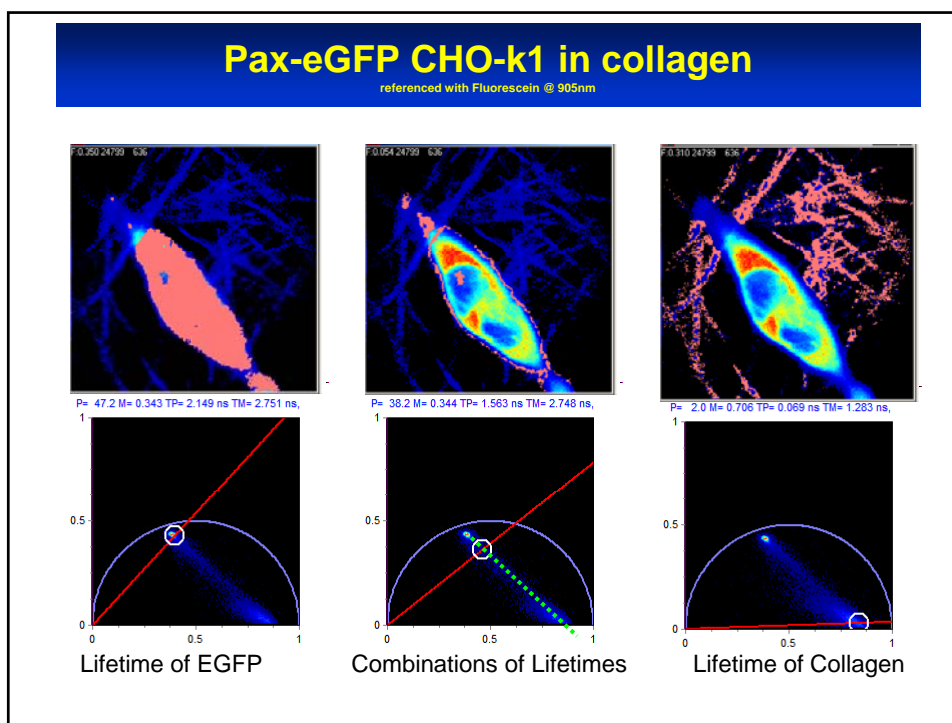
## Examples of phasors identification



Digman et al, Biophys. J. 2007

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





## The principle of the Calculator

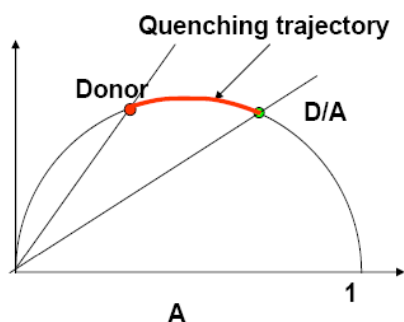
Purpose: to generate trajectories in the phasor plot corresponding to different processes

At present, there are 4 functions programmed

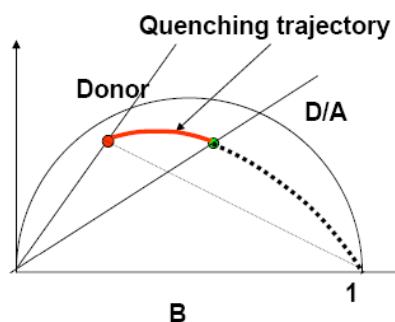
1. Fractional contribution of two species give the phasor of the two individual species
2. Ion concentration given the phasor of the bound and free form and the pK of the indicator
3. FRET efficiencies from the observation of the donor only with background and fraction of donor unquenched
4. FRET efficiency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



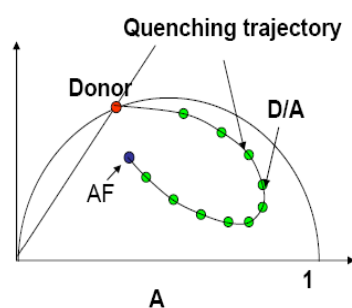
The lifetime of the donor is along a  
different "trajectory", Why is the  
trajectory an arc rather than a line to the  
(1,0) point?

## The FRET Calculator

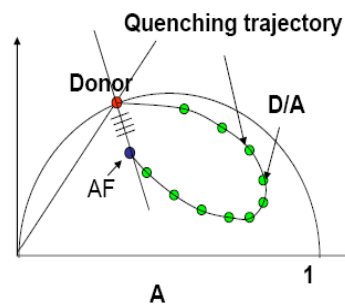
- Can we quench up to zero lifetime?
- Even if we quench all the DONOR, we still are left with the autofluorescence.
- The final point is not at zero but at the autofluorescence phasor!!!

## The FRET Calculator

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

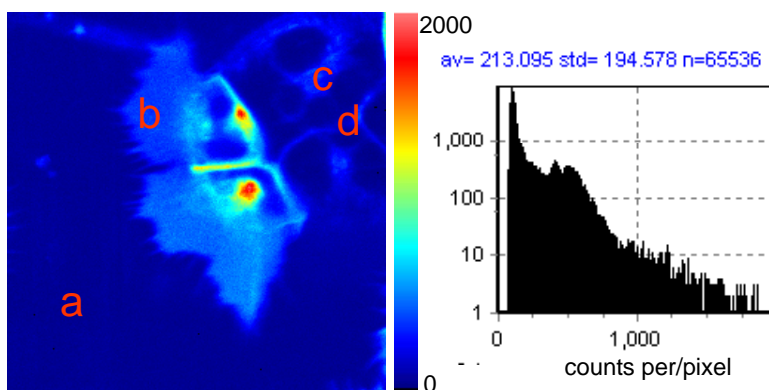


As the lifetime of the Donor is quenched, the phasor of the quenched Donor is added to the phasor of the autofluorescence



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

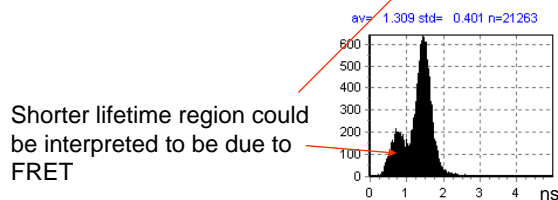
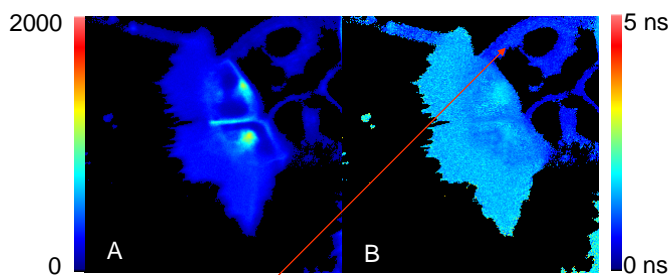


Several regions the image can be identified corresponding to **a**) background (2 exponentials) **b**) cell 1 bright (2 exponentials) **c**) cell 2 dim, **d**) cell junctions dim.

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

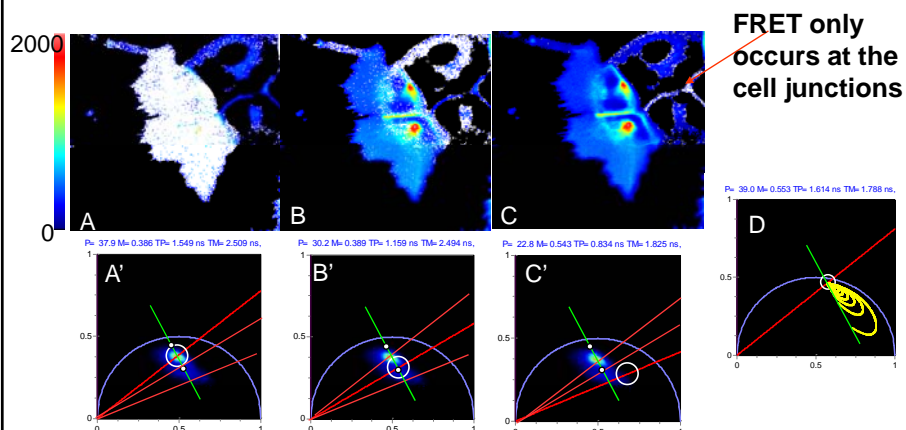
## The pitfall of “conventional” FLIM analysis

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



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**.

## 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