RICS, N&B, Scanning FCS

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"How can the events in space and time which take place within the spatial boundary of a living organism be accounted for by physics and chemistry?"

(Erwin Schrödinger 1943)

Brief overview of Fluorescence Correlation in Microscopy:

Single point FCS

Elson, E.L., Schlessinger J, Koppel DE, Axelrod D, Webb WW. Measurement of lateral transport on cell surfaces. Prog Clin Biol Res. **1976**;9:137-47.

Scanning FCS

Berland et al, Two-photon fluorescence correlation spectroscopy: method and application to the intracellular environment. Biophys J. **1995** Feb;68(2):694-70)

Image Correlation Spectroscopy (ICS)

- **Petersen, N.O.** Scanning fluorescence correlation spectroscopy. I. Theory and simulation of aggregation measurements. Biophys J. **1986** Apr;49(4):809–815.
- **Wiseman, P. W. 1995**. Image correlation spectroscopy: development and application to studies of PDGF receptor distributions. Ph.D. thesis. The University of Western Ontario, London, ON.

Temporal Image Correlation Spectroscopy (tICS)

- **Brown, C. 1998**. and Petersen, N.O., An image correlation analysis of the distribution of clathrin associated adaptor protein at the plasma membrane, *Journal of Cell Science* 1998 (111):271-281

Spatio-Temporal Image Correlation Spectroscopy (STICS)

- **Hebert, B**., Constantino, S. and Wiseman, P.W. Spatiotemporal image correlation spectroscopy (STICS) theory, verification, and application to protein velocity mapping in living CHO cells. *Biophysical Journal* 2005(88):3601-14

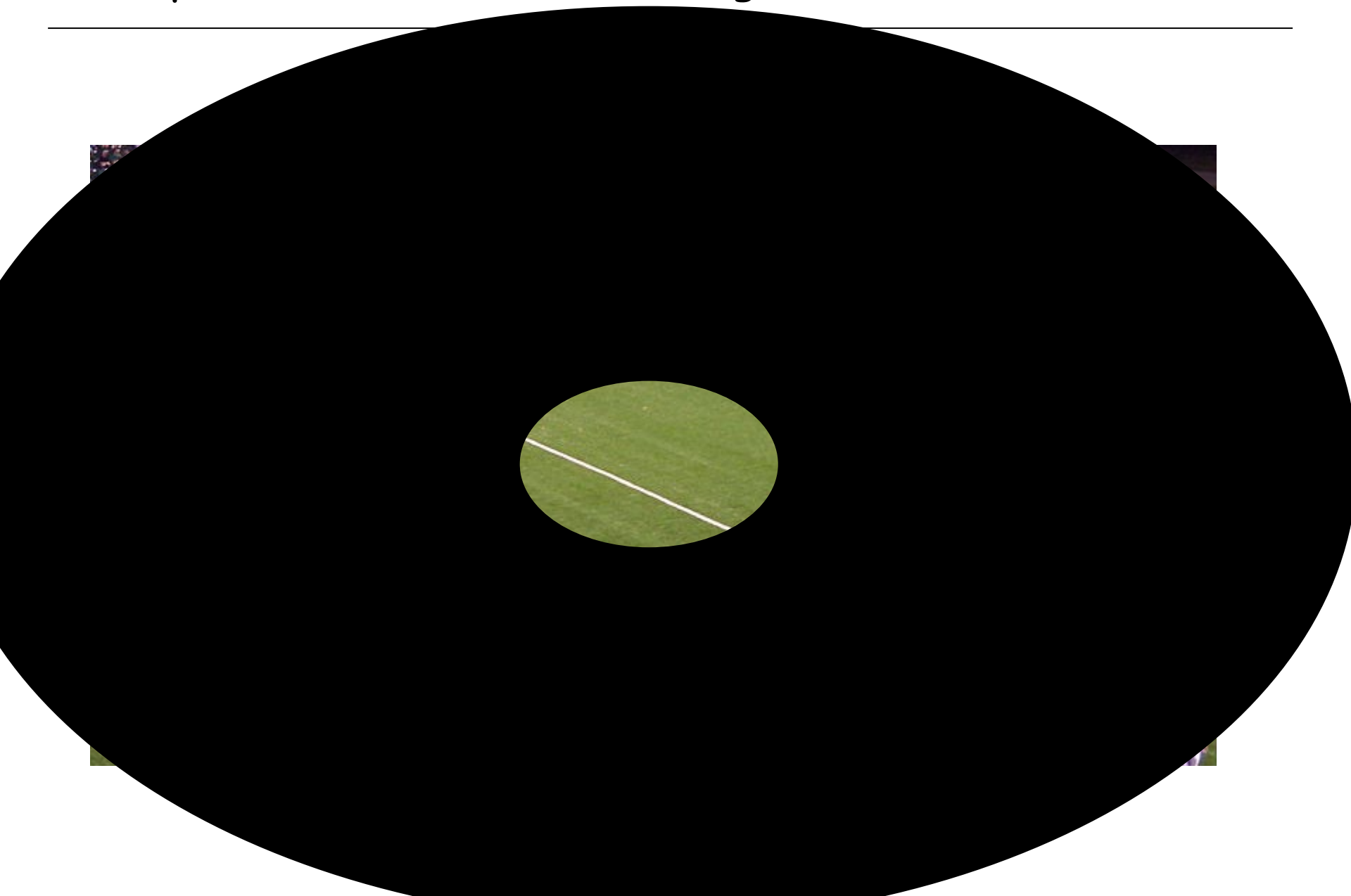
k-space ICS (kICS)

Kolin et al 2007 Biophysical Journal, k-Space Image Correlation Spectroscopy (kICS): A Method for Accurate Transport Measurements Independent of Fluorophore Photophysics

Drawbacks of Single Point FCS in the Cell

- Have to pick spatial location on the cell before making the measurement.
- Don't know what the cell is doing during the measurement.
- Can only measure one location at a time.

One point versus the entire image



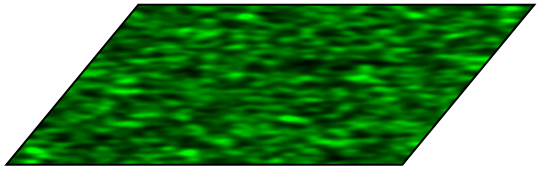
Raster Image Correlation Spectroscopy

Major benefits of RICS:

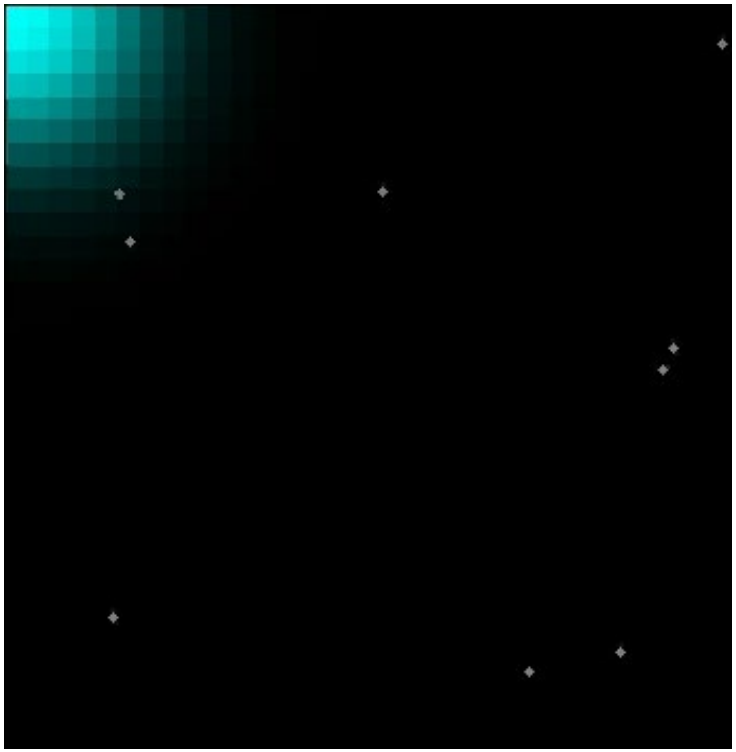
- It can be done with commercial laser scanning microscopes (either one or two photon systems).
- It can be done with analog detection, as well as with photon counting systems, although the characteristic of the detector must be accounted for (time correlations at very short times due to the analog filter).
- RICS provides an intrinsic method to separate the immobile fraction.
- It provides a powerful method to distinguish diffusion from binding.

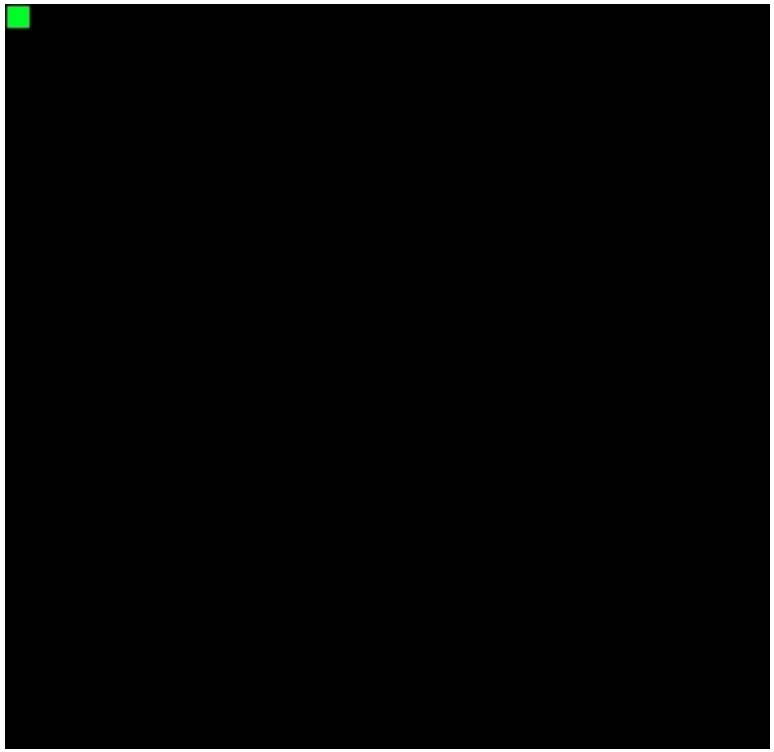
How does it work?

Raster Scanned Images



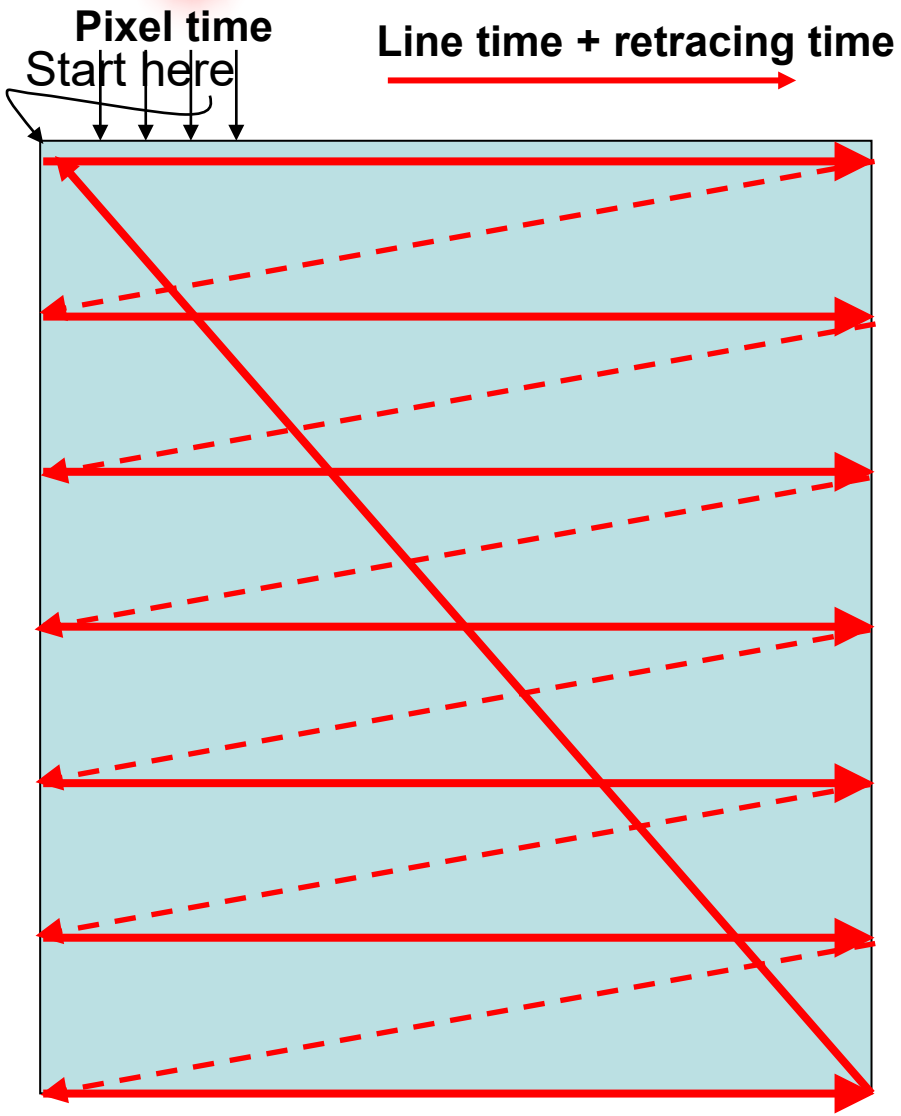
Fluorescence Microscopy Image from LSM

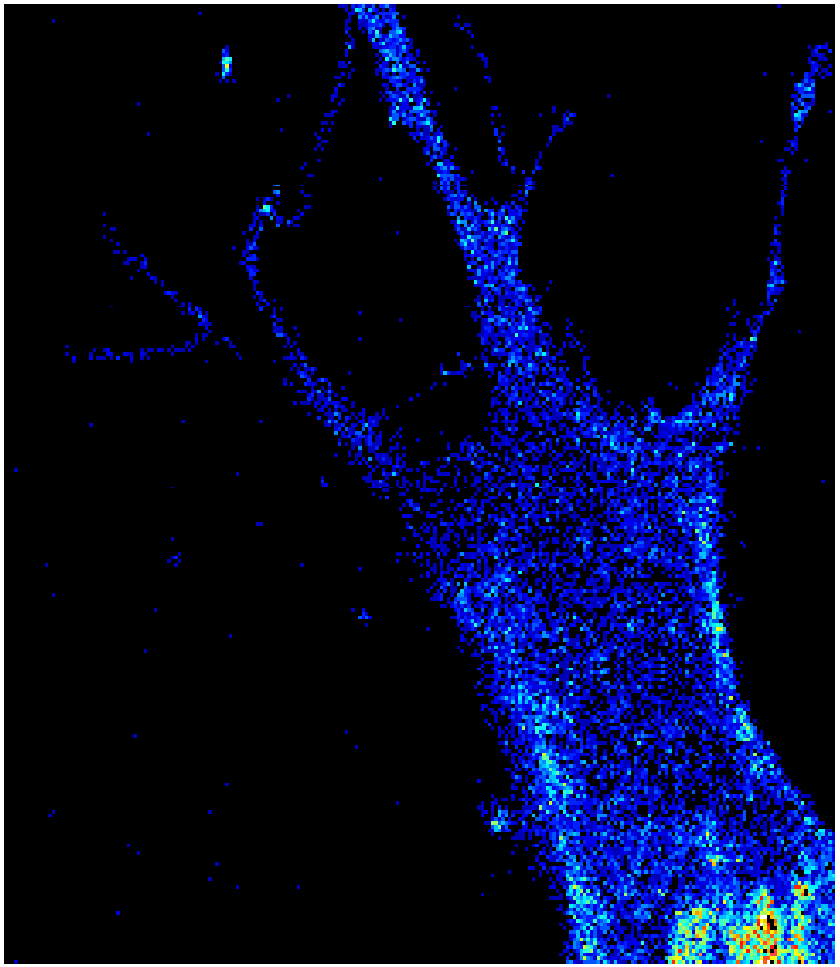




Movie courtesy of the Wiseman lab

Raster Scanning

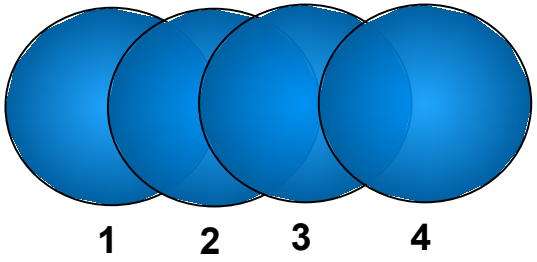




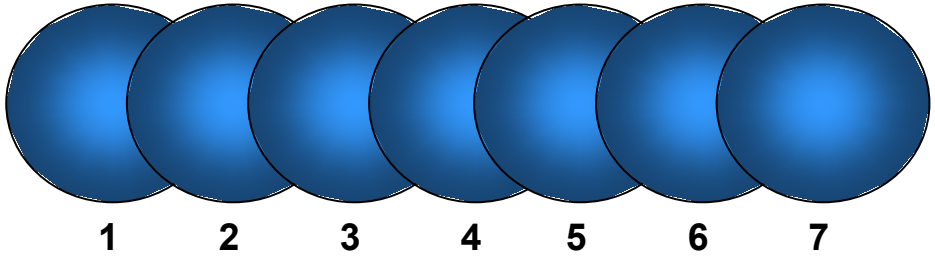
LSM image contains time structure.

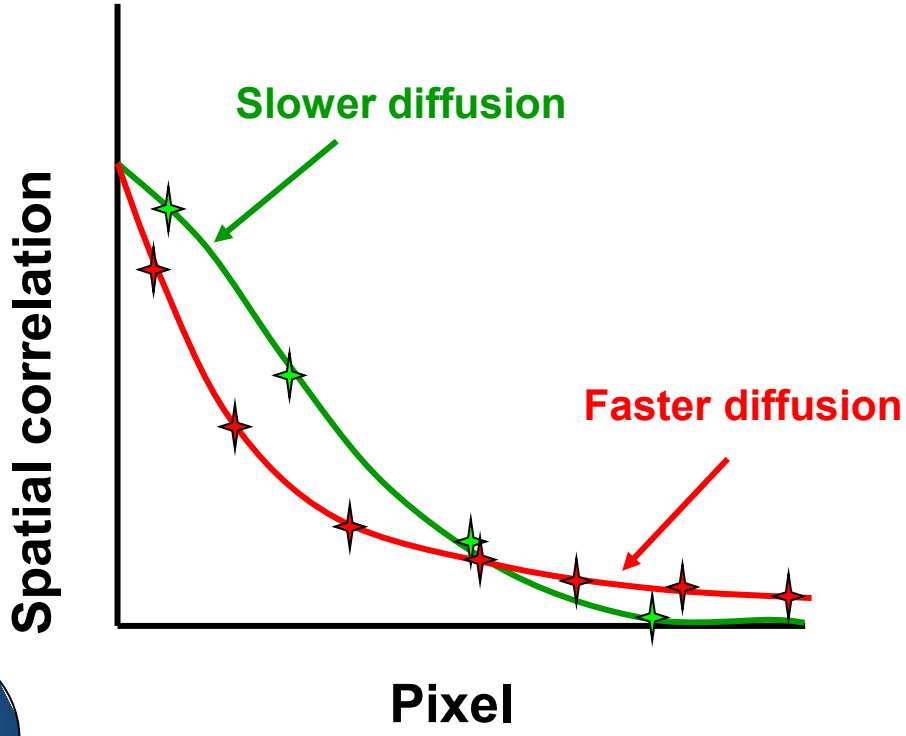
Using Temporal Information Hidden in the Raster-Scan Image: The RICS Approach

Situation 1:slow diffusion



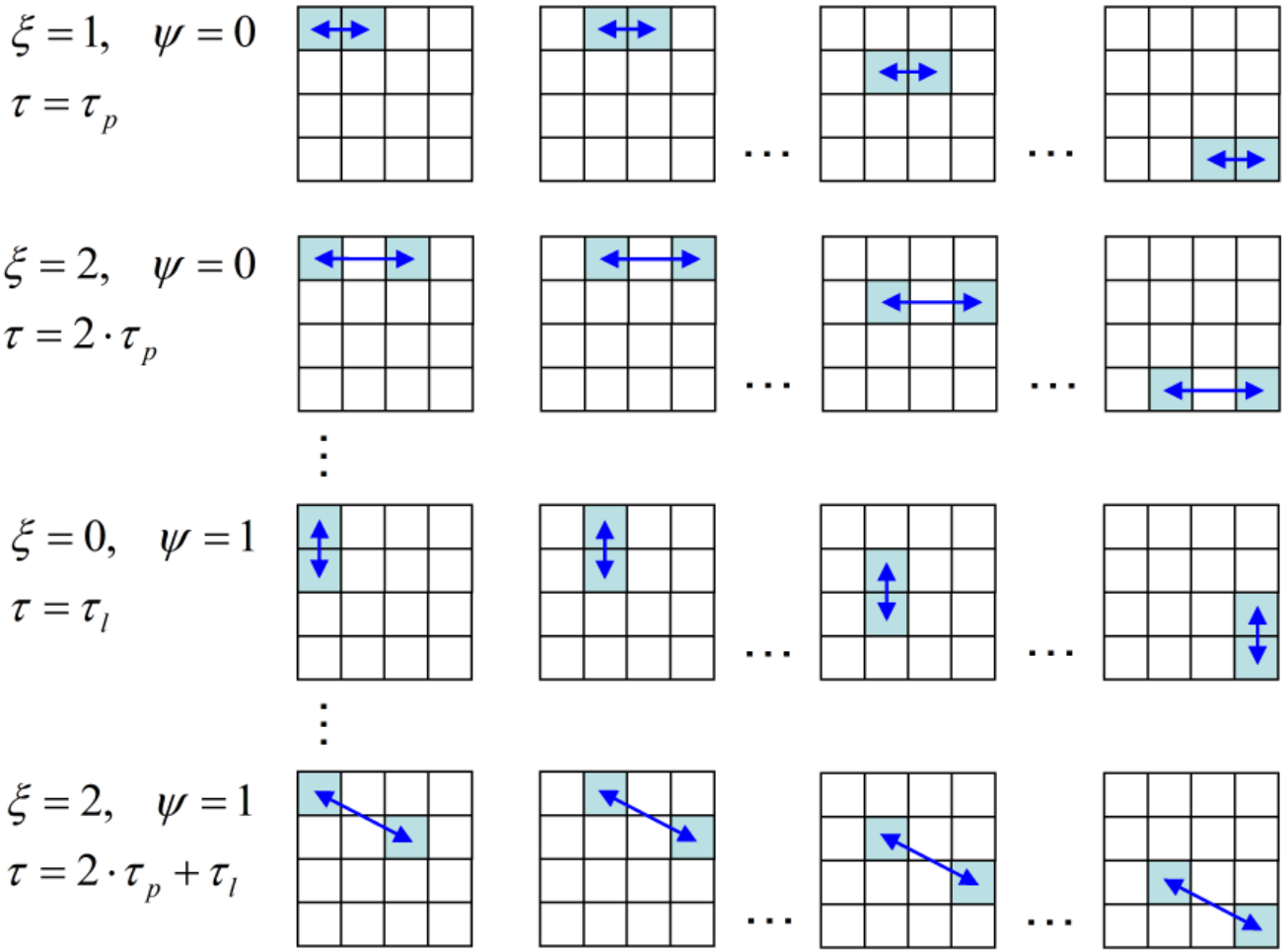
Situation 2: fast diffusion





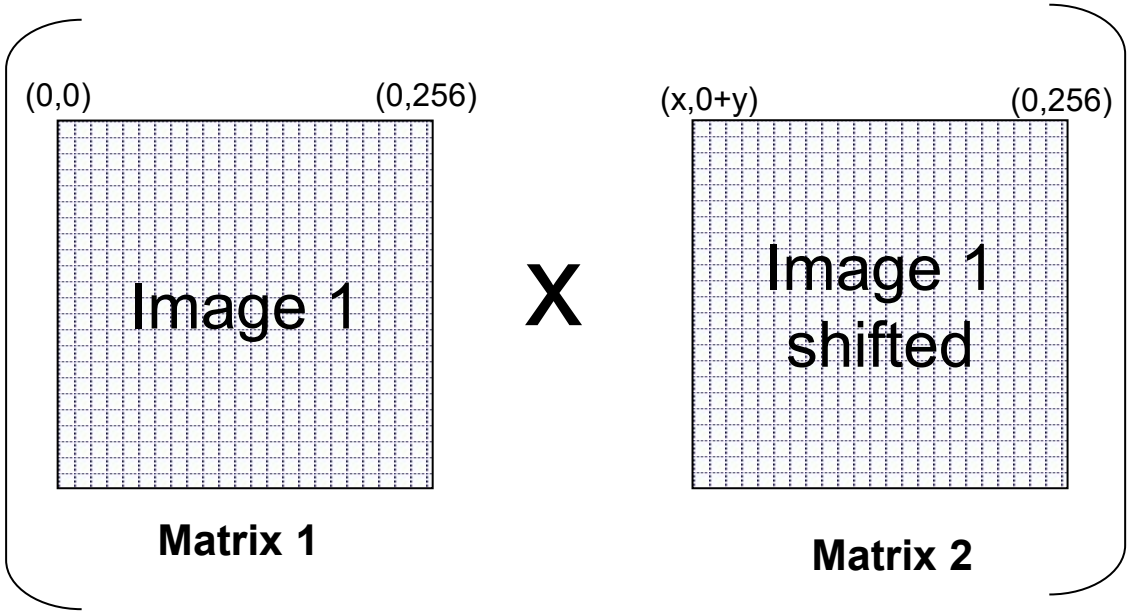
How is the Spatial Correlation Calculated?

This operation has to be repeated for all possible combinations of pixel and line shifts:



How is the Spatial Correlation Calculated?

Operation:

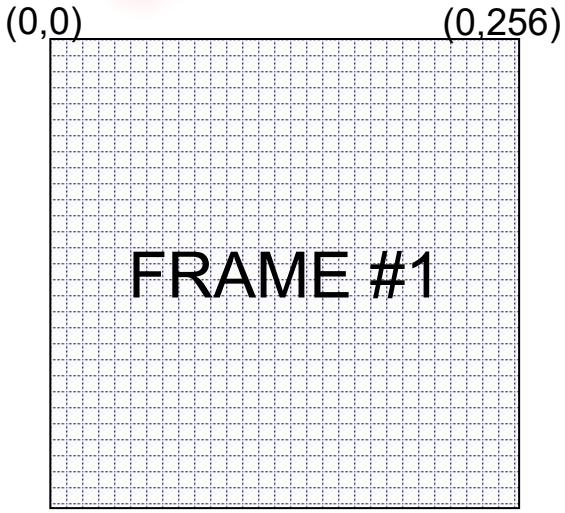


Shift in the x and y direction

$$(0,0\times0,0)+(0,1\times0,1)+(0,2\times0,2)\ldots(0,127\times0,127)$$
$$+(1,0\times1,0)+(1,1\times1,1)+(1,2\times1,2)\ldots(1,127\times1,127)$$
$$+\ldots$$

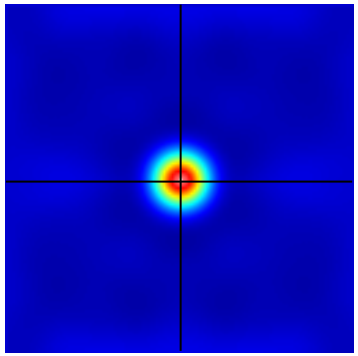
One number is obtained for each pixel delay normalized by the average intensity squared.

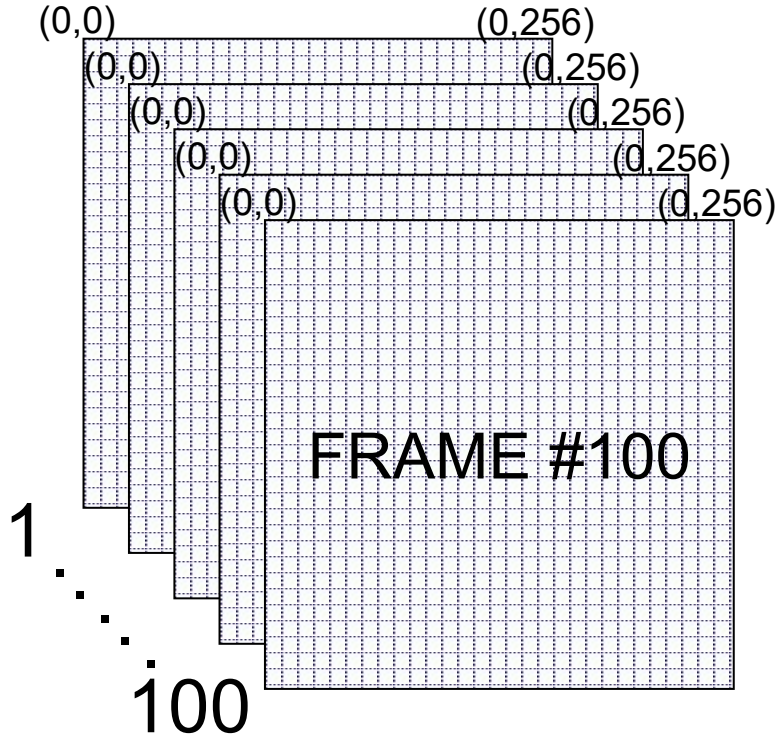
How to Use a Stack of Images?



Spatially correlate each frame Individually.

Then take the average of all the frames.



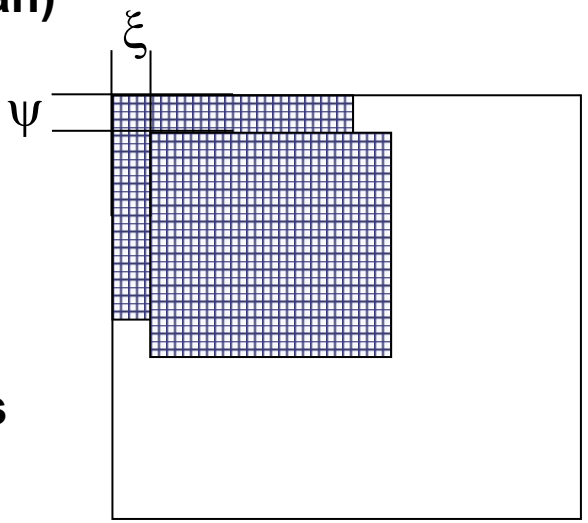


The RICS Approach: 2-D Spatial Correlations

- In a raster-scan image, points are measured at different positions and at different times simultaneously:
  - If we consider the pixel sequence, it is contiguous in space.
  - If we consider the time sequence, it is not continuous in time.

- In the RICS approach, we calculate the 2-D spatial correlation function (similarly to the ICS method of Petersen and Wiseman)

$$G_{RICS}(\xi,\psi) = \frac{< I(x,y)I(x+\xi,y+\psi) >}{< I(x,y) >^2} - 1$$



- The variables ξ and ψ represent spatial increments in the x and y directions, respectively.

- 2-D spatial correlation can be computed very efficiently using FFT methods.

- To use the "RICS concept" we must account for the relationship between time and position of the scanning laser beam.

The RICS Approach for Diffusion

- The dynamic at a point is independent on the scanning motion (S) of the laser beam:

$$G_{RICS}(\xi,\psi) = S(\xi,\psi)\mathrm{x}G(\xi,\psi)$$

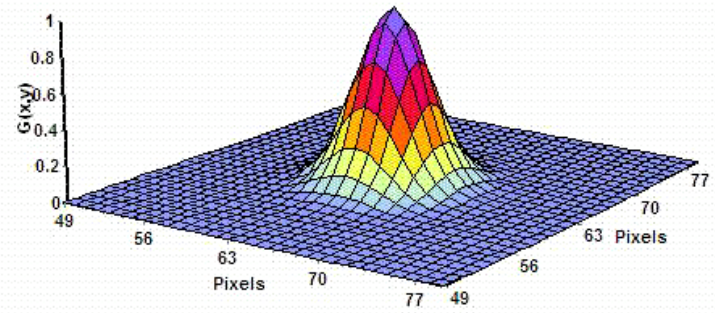
- Consider now the process of diffusion. The diffusion kernel can be described by the following expression:

$$P(r,t) = \frac{1}{(4\pi Dt)^{3/2}}\exp(-\frac{r^2}{4Dt})$$

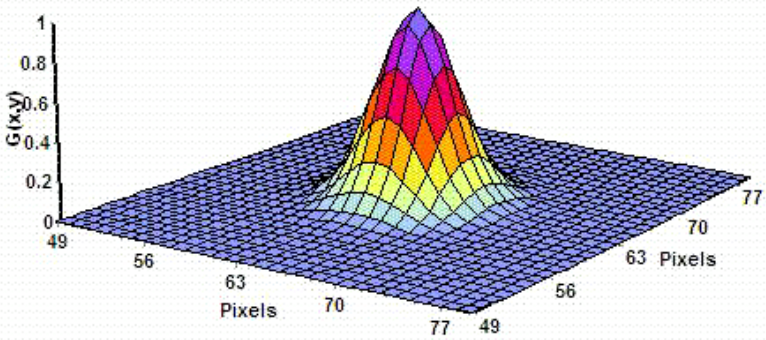
- There are two parts:
  - (1) The temporal term.
  - (2) The spatial Gaussian term.

- For any diffusion value the amplitude decreases as a function of time and the width of the Gaussian increases as a function of time.

FAST



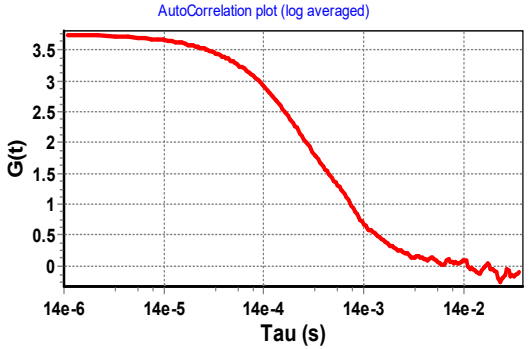
SLOW



RICS: Space and Time Relationships

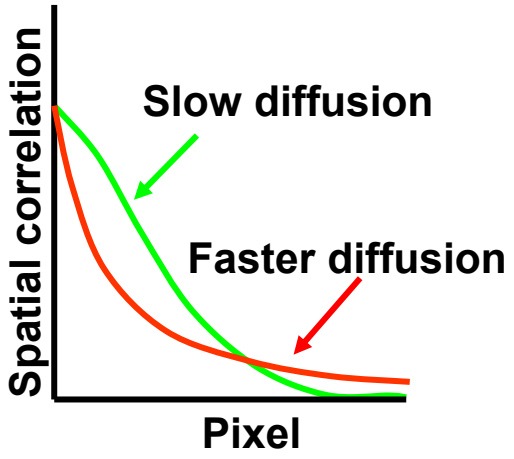
- **At any position, the ACF due to diffusion takes the familiar form:**

$$G(\xi,\psi)=\frac{\gamma}{N}\left(1+\frac{4D(\tau_p\xi+\tau_l\psi)}{w_0^2}\right)^{-1}\left(1+\frac{4D(\tau_p\xi+\tau_l\psi)}{w_z^2}\right)^{-1/2}$$



- **τ_p and τ_l indicate the pixel time and the line time. The correlation due to the scanner movement is:**

$$S(\xi,\psi)=\exp\left(-\frac{[(\frac{2\xi\delta r}{w_0})^2+(\frac{2\psi\delta r}{w_0})^2]}{(1+\frac{4D(\tau_p\xi+\tau_l\psi)}{w_0^2})}\right)$$



- **Where δr is the pixel size. For D = 0, the spatial correlation gives the autocorrelation of the PSF, with an amplitude equal to γ/N. As D increases, the correlation (G term) becomes narrower and the width of the S term increases.**

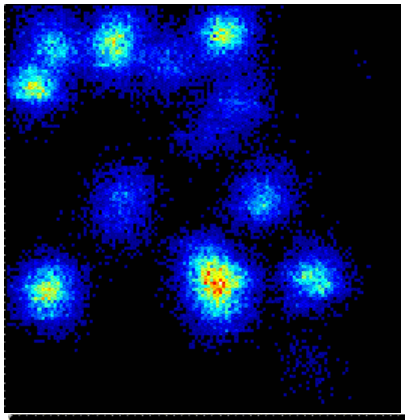
Digman et al. Biophys. J., 2005

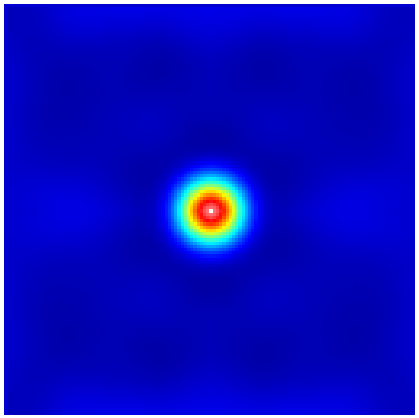
RICS Simulations of Three Different Diffusion Rates:

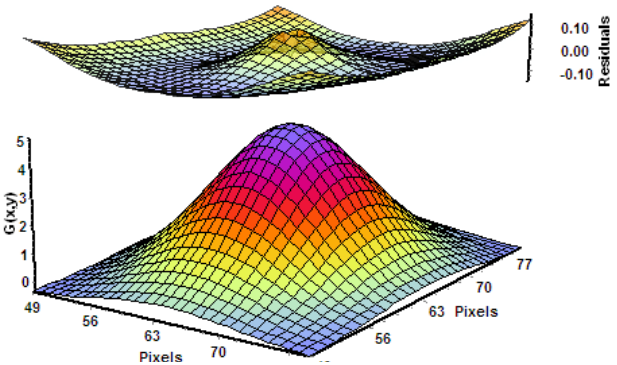
Box size = 3.4 µm sampling time: 1) 32 µs/pixel 2) 8 µs/pixel 3) 4 µs/pixel

D = 0.1 µm²/s

(membrane proteins)

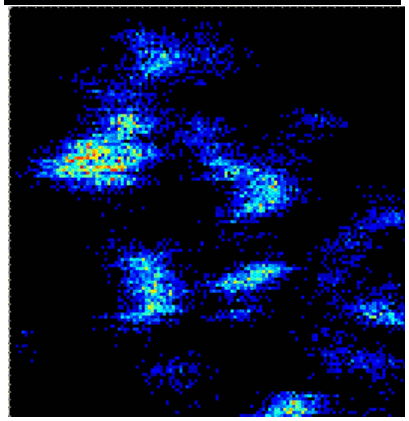


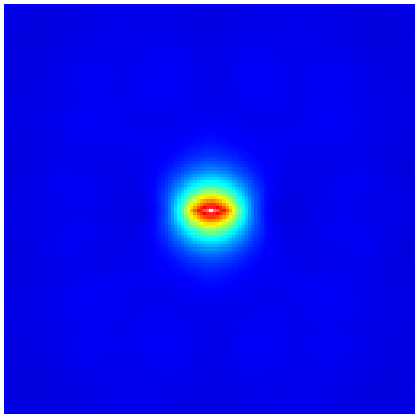


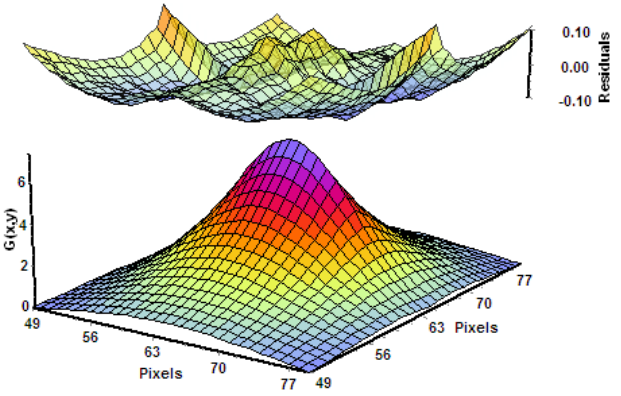


D = 5.0 µm²/s

(40 nm beads)

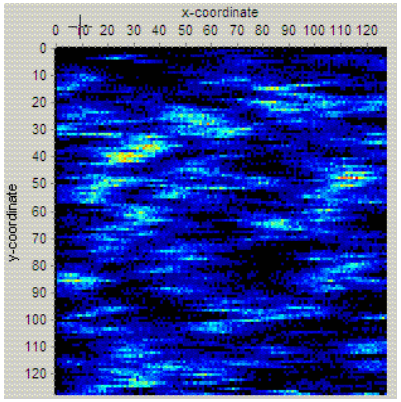


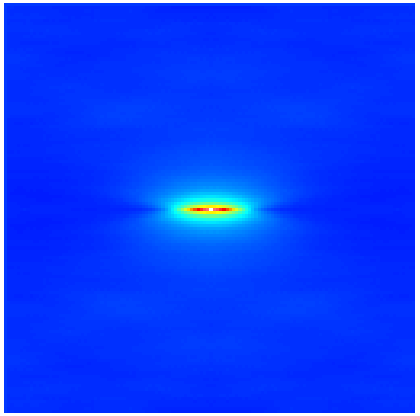


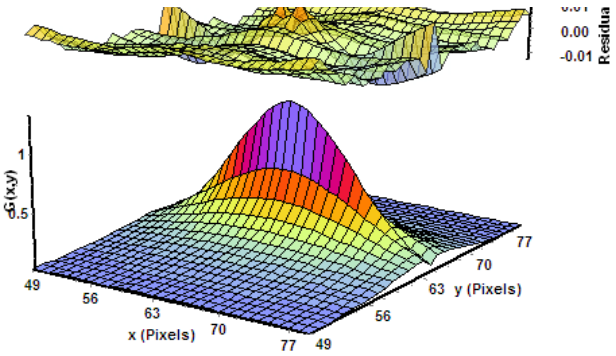


D = 90 µm²/s

(EGFP)



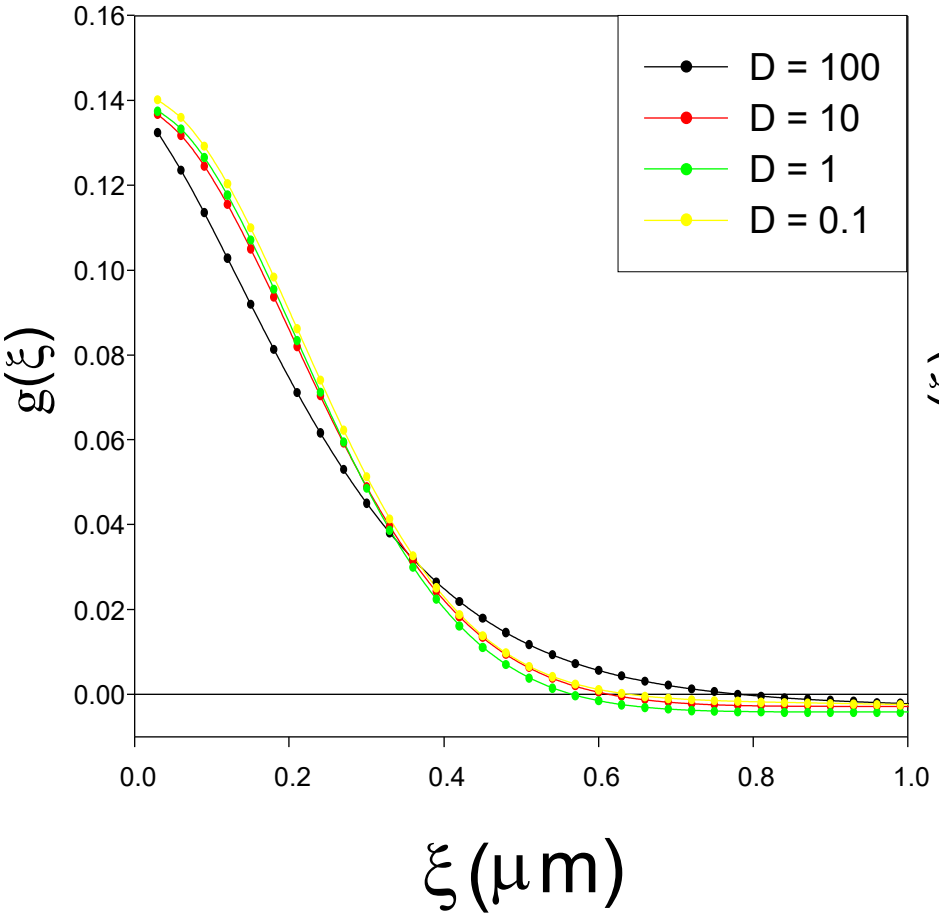




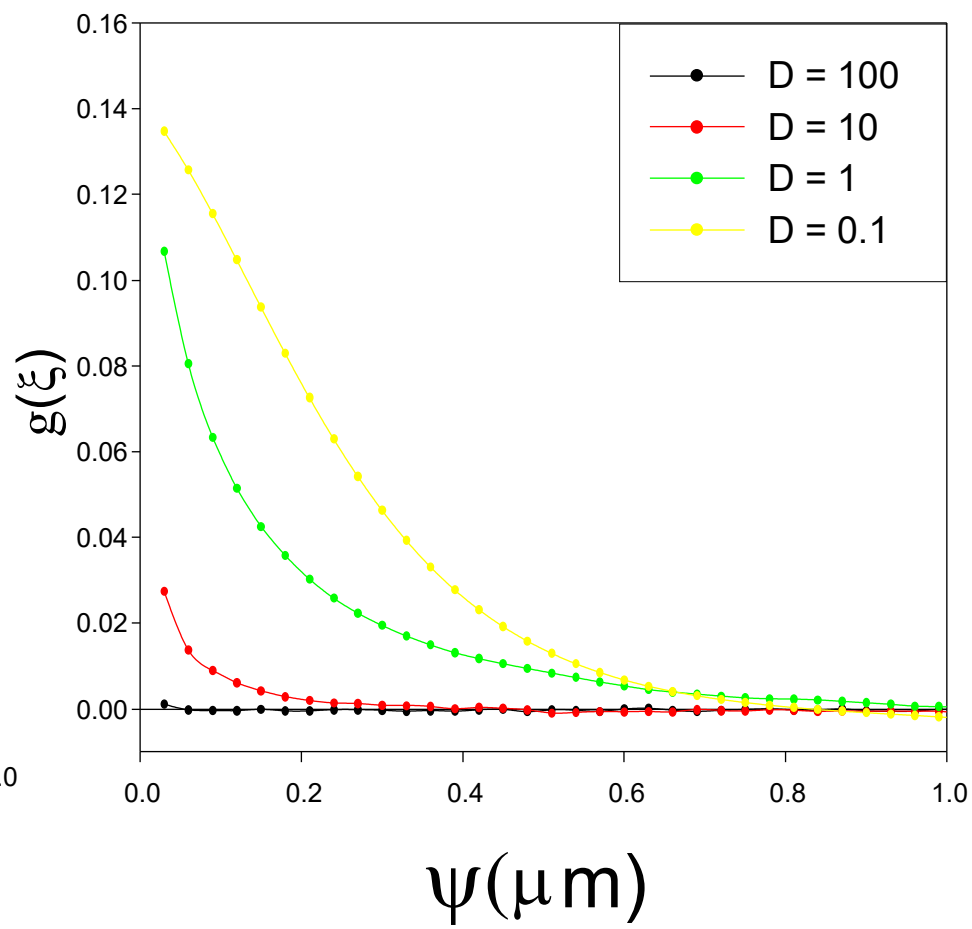
Horizontal and Vertical fits:

Simulations of beads 300 frames, 128 x 128 pixels, 8 μs/px, pixel size 30 nm

Horizontal ACF



Vertical ACF



Done using SimFCS.

Brown et al, JMI, 2007

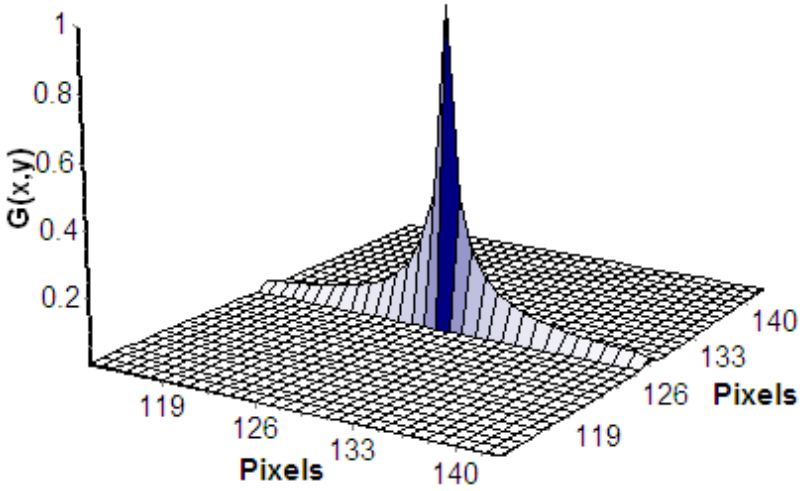
How to Set Up the Laser Scanning Microscope

- **Scan Speeds (μs/pixel):**
  - **4 μs for fast molecules (D > 100 $\mu m^2/s$).**
  - **8 – 32 μs for slower molecules (D = 1 $\mu m^2/s$ – 100 $\mu m^2/s$).**
  - **32 – 100 μs for slower molecules (D = 0.1 $\mu m^2/s$ – 10 $\mu m^2/s$).**
- **Pixel Size:**
  - **3 – 4x smaller than the Point Spread Function (PSF≈ 300nm).**
- **Molecular Concentrations**

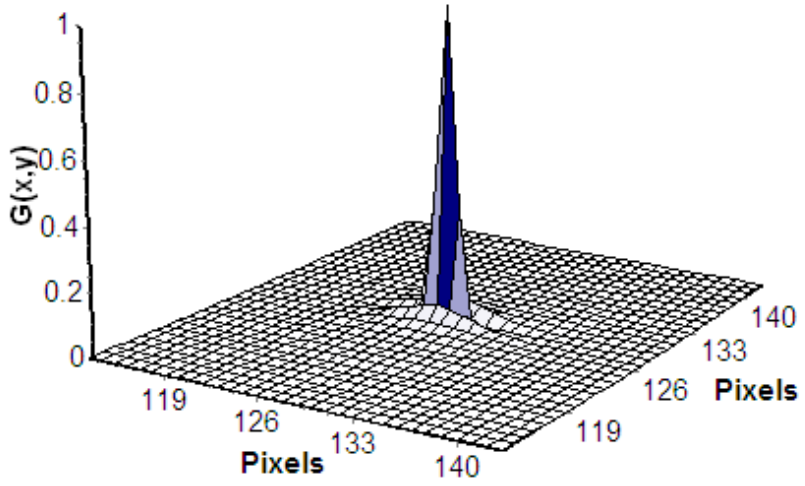
  Same conditions as conventional FCS methods.

Common Mistakes in RICS

Scanning speed too slow
(100 μs/pixel, D = 300 μm²/s)



Pixels are separated too much compared to the PSF
(pixel size = w_0 = 0.3 μm)

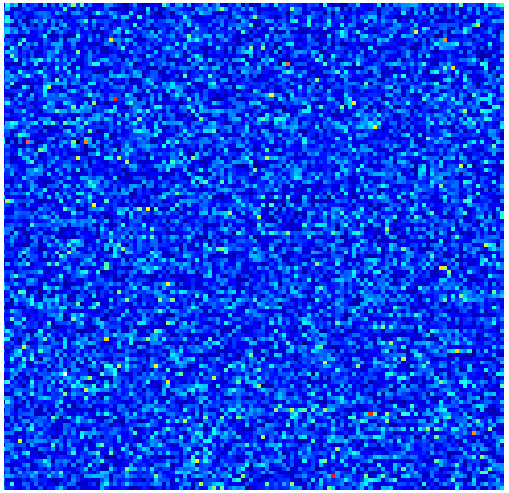


Courtesy of Jay Unruh

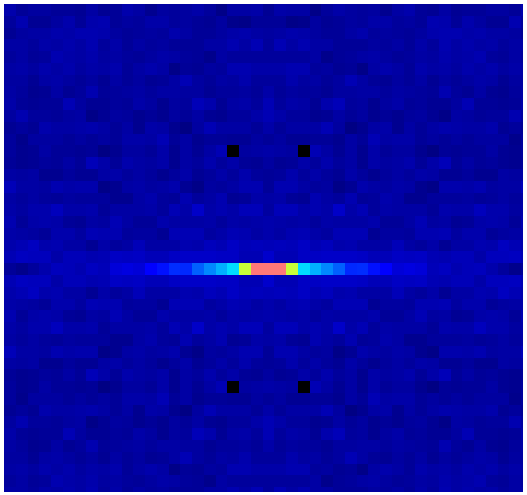
RICS: Fits to Spatial Correlation Functions

Olympus Fluoview300 LSM

EGFP in solution

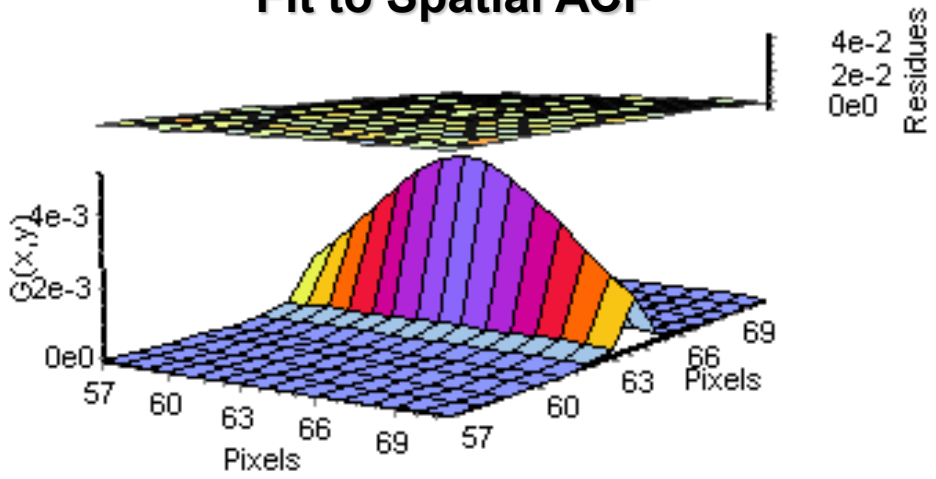


Spatial ACF



128 x 128, 4 μs/pixel, 5.4 ms/line, 0.023 μm/pixel

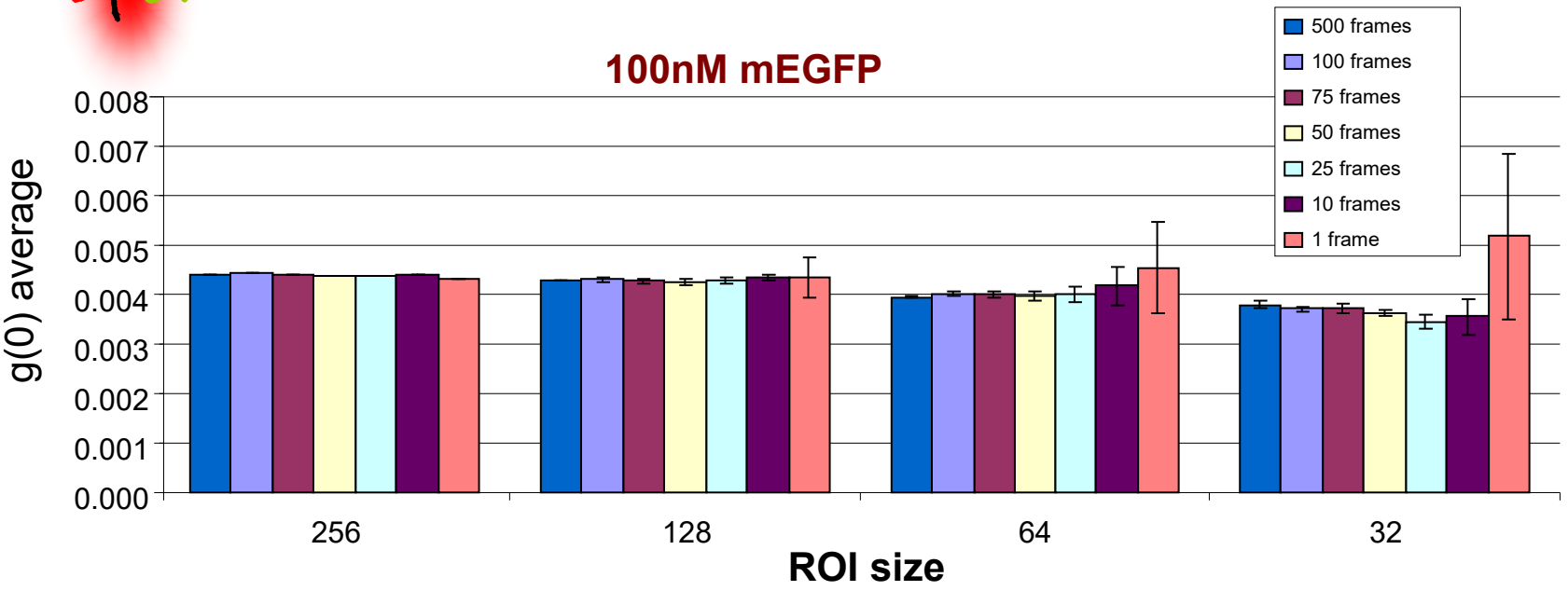
Fit to Spatial ACF

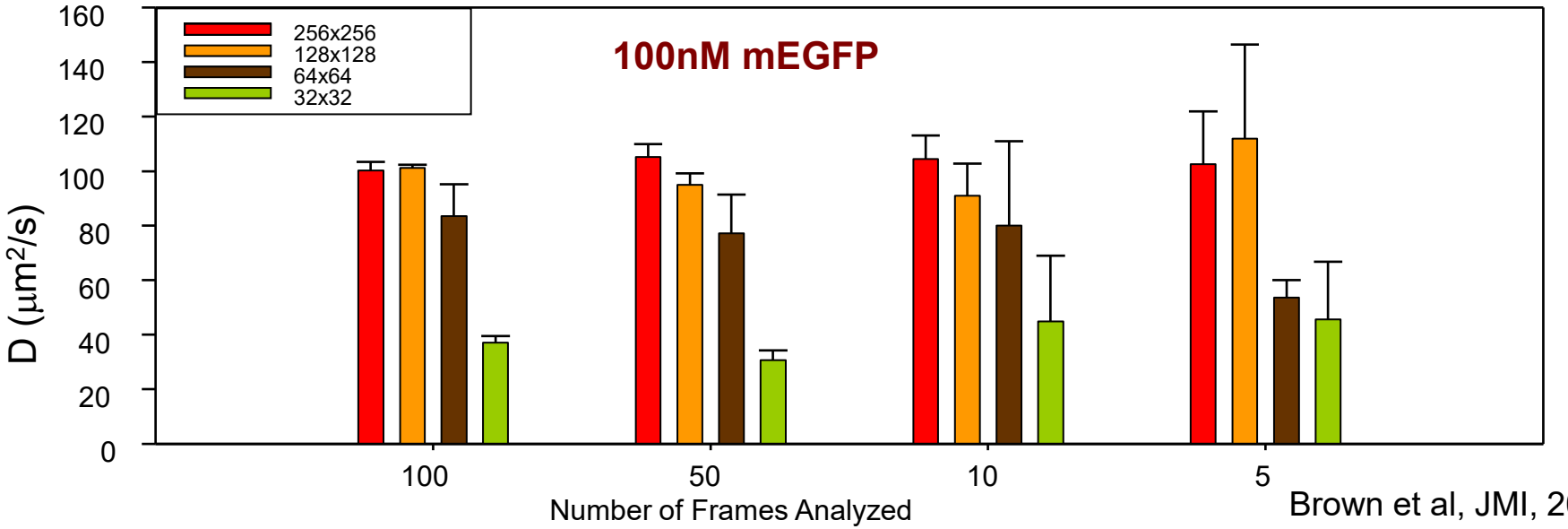


D = 105 ± 10 μm²/s

Digman et al. Biophys. J., 2005

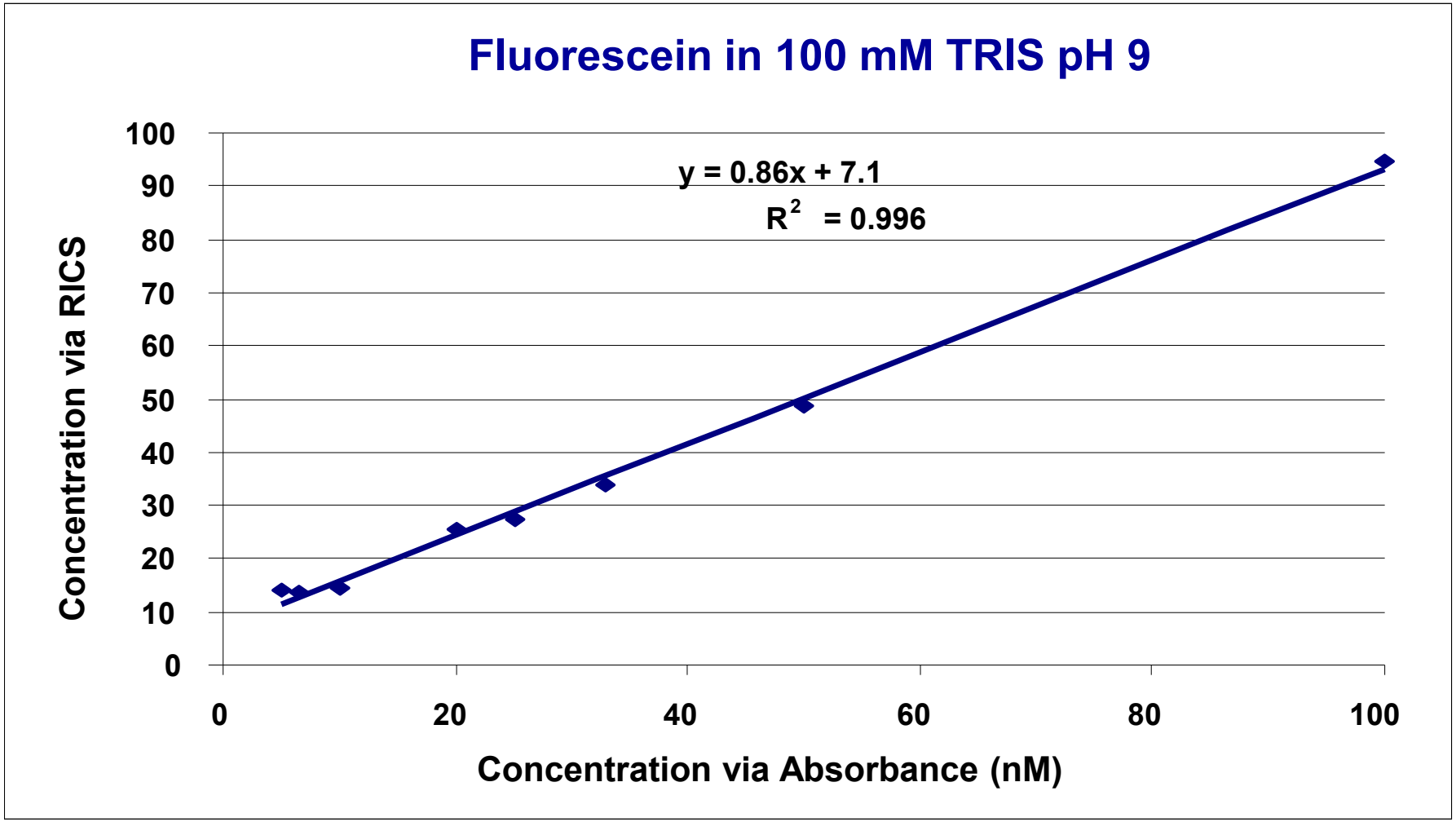
What ROI Size to Use? How Many Frames to Acquire?





Brown et al, JMI, 2007

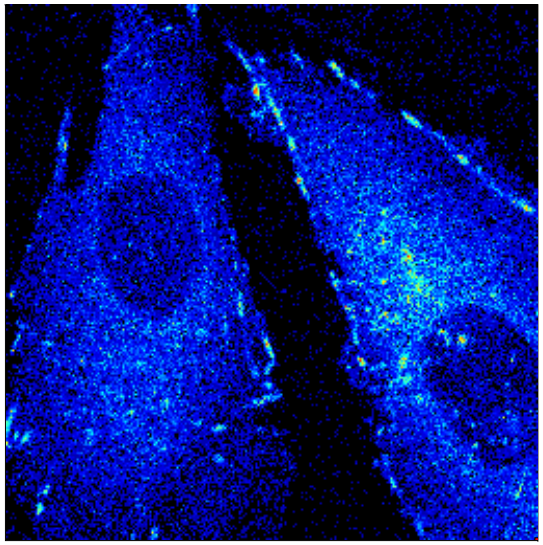
Obtaining Concentrations From RICS



Brown et al, JMI, 2007

How Do We Go from Solutions to cells?

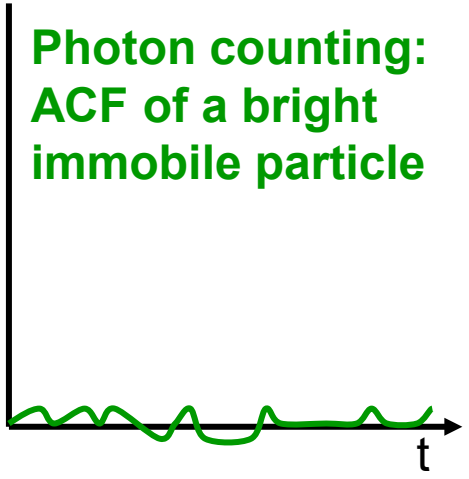
- In cells we have an immobile fraction.
- The 2-D spatial correlation of an image containing immobile features has a very strong correlation pattern.
- We need to separate this immobile fraction from the mobile part before calculating the transform.
- How is this achieved?

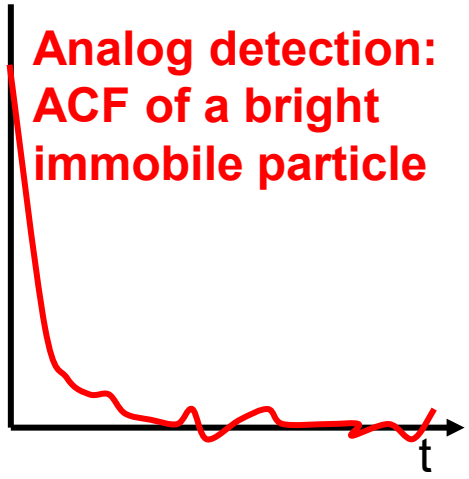


Does Noise from the Detector Correlate?

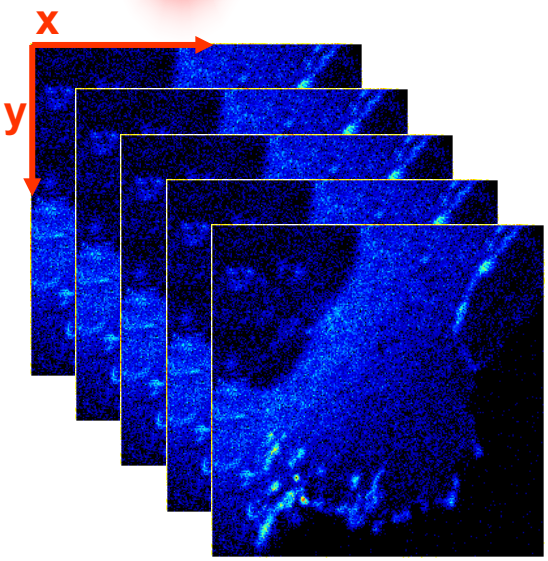
- In a “truly immobile” bright region, the intensity fluctuates according to the Poisson distribution due to shot noise.
- The time correlation of the shot noise is zero, except at time zero.
- The spatial correlation of the intensity at any two pixels due to shot noise is zero, even if the two points are within the PSF.
- If we subtract the average intensity and disregard the zero time-space point, the immobile bright region totally disappears from the correlation function.

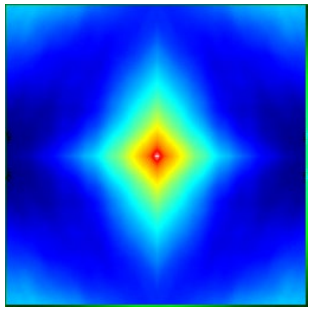
Attention!!!
This is not true for analog detection, not even in the first order approximation. For analog detection the shot noise is time (and space) correlated.



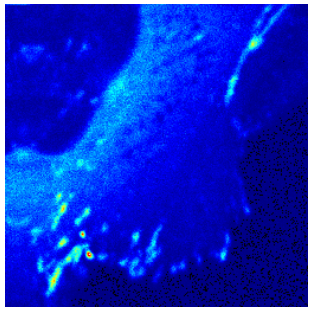


Formula Used to Subtract Background

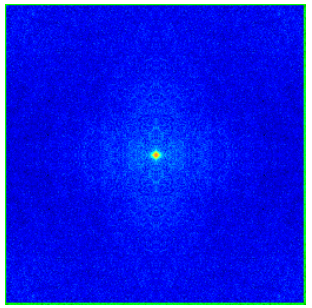




Spatial correlation before subtracting background.



Subtract the average.



Spatial correlation of entire image after subtracting average.

- Average intensity of each pixel on the overall stack: $\overline{I(x,y)}$
- The intensity of each pixel minus the average intensity from entire stack for each pixel:

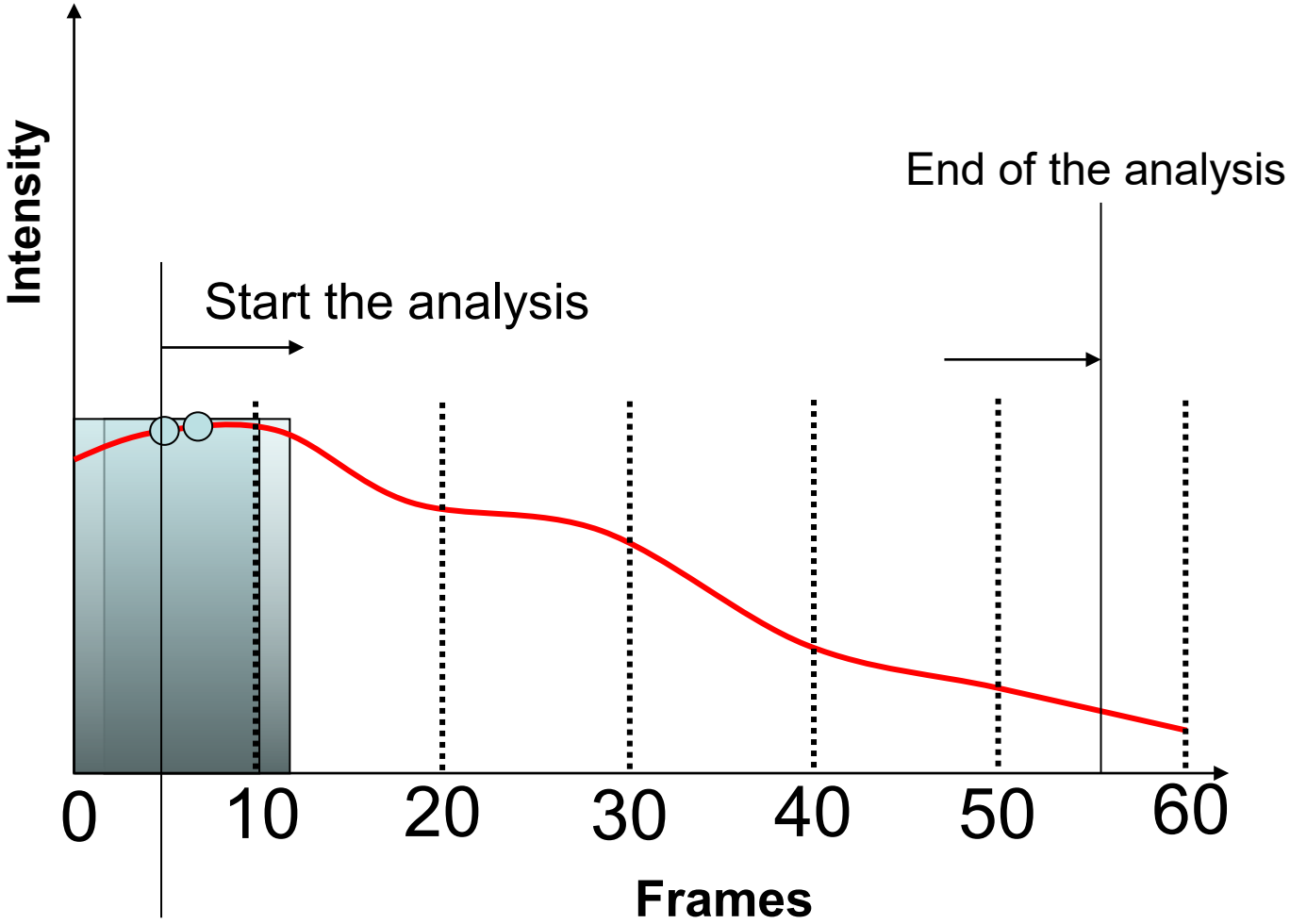
$$I_i(x,y) - \overline{I(x,y)}$$

- However, this yields negative values. A scalar must be added : $a = \overline{I}$

$$RICS(F_i(x,y)) \qquad where \qquad F_i(x,y) = I_i(x,y) - \overline{I(x,y)} + a$$

Subtraction of a Moving Average

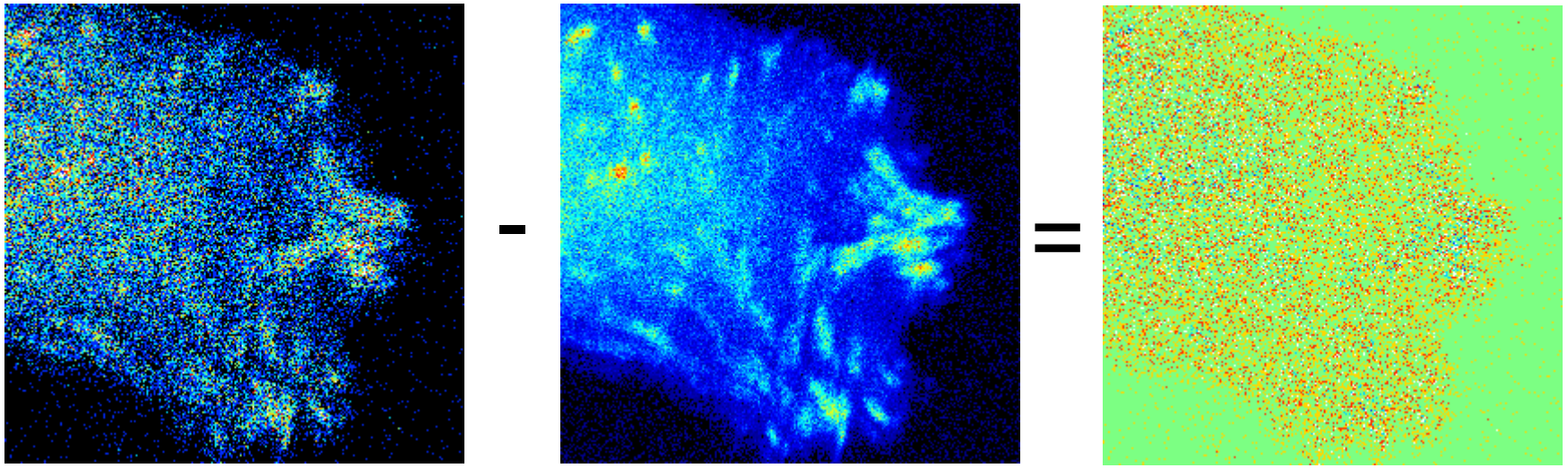
Problem: Bleaching during image acquisition



Moving Average Operation on Frames:

Frame #5

Average between 1-10



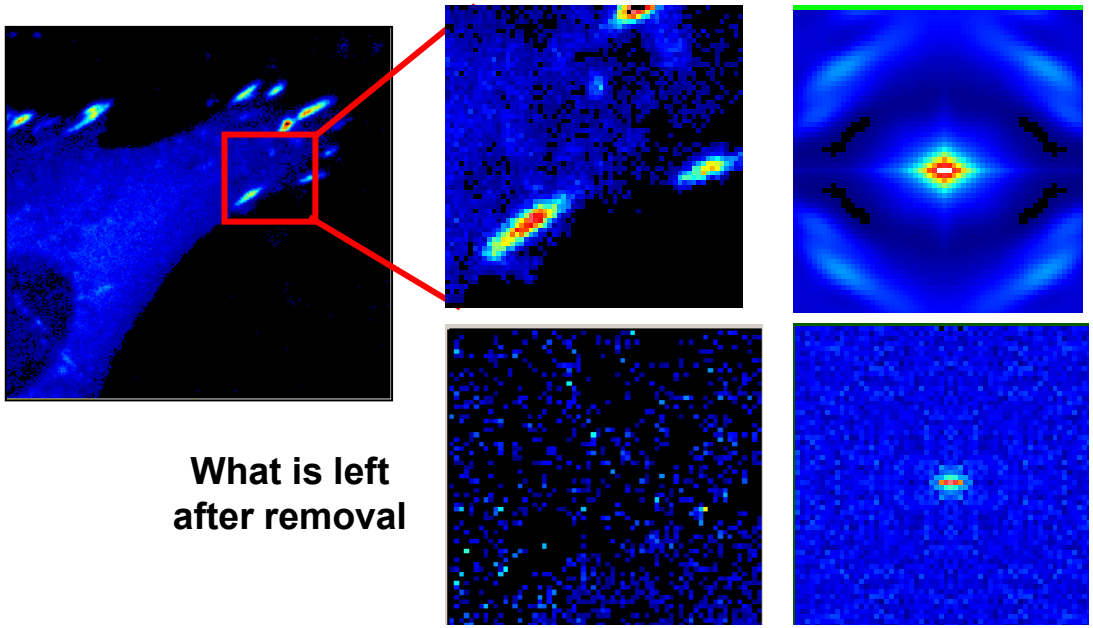
Matrix 1

Matrix 2

A scalar average is then added

Operation is repeated for frame #6 - average between frames 2-11
frame #7 - average between frames 3-12
…

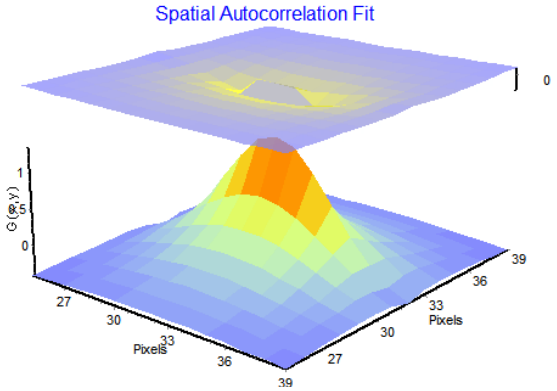
Example of the Removal of Immobile Structures and Slow Moving Features



Spatial ACF
no removal

What is left
after removal

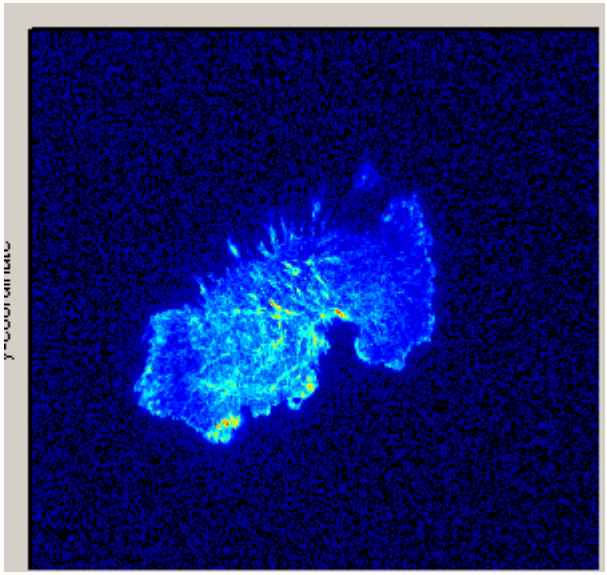
Spatial ACF
with removal



Fit using 3-D diffusion formula

Pixel size	=	0.092 µm	G1(0)	=	0.0062
Pixel time	=	8 µs	D1	=	7.4 µm²/s
Line time	=	3.15 ms	G2(0)	=	0.00023
w_o	=	0.35 µm	D2	=	0.54 µm²/s
			Bkg	=	-0.0012

Cell Migration



MEF cells expressing the Raichu biosensor
1 frame/30s. Total run time: 48 min.

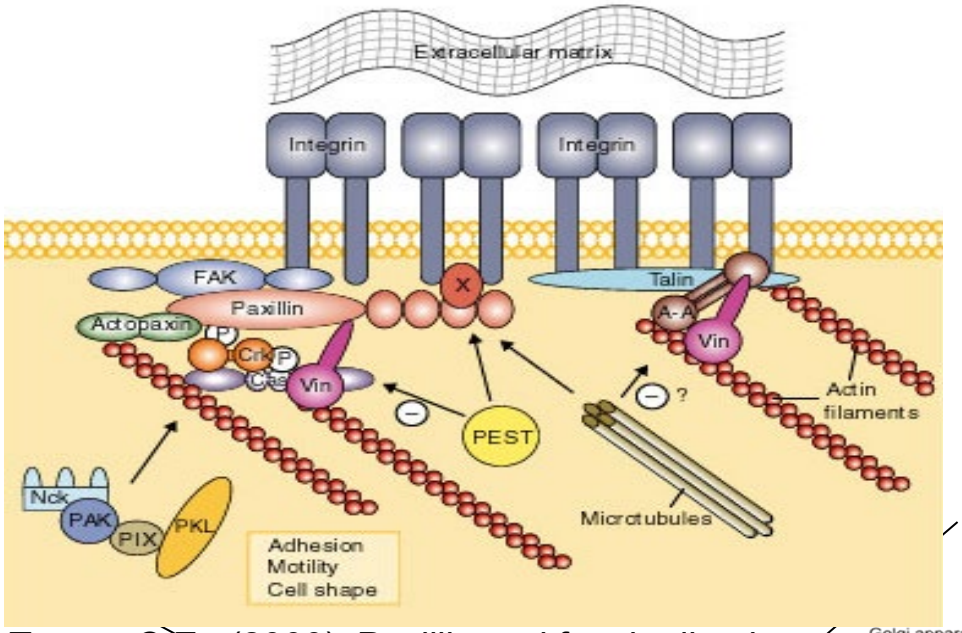
Cell migration is important for:

- Embryonic development
- Wound healing
- Immune responses
- Cancer metastasis

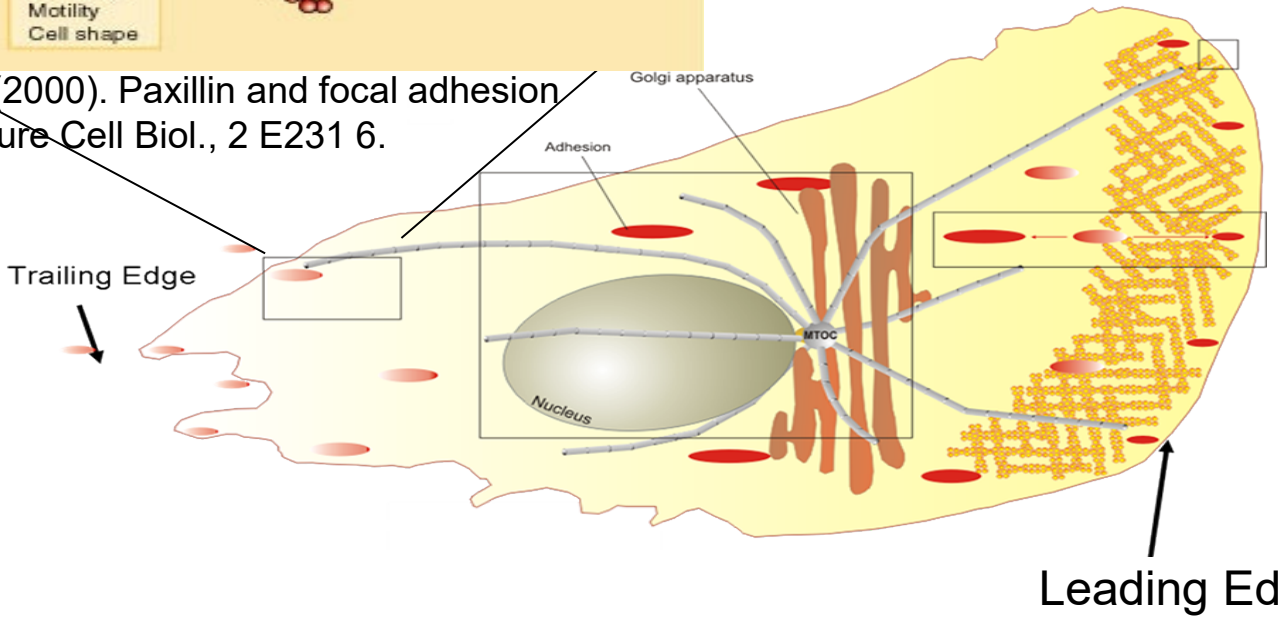
Lack of regulation of cell migration may lead to:

- Vascular disease
- Congenital brain defects
- Chronic inflammatory disease
- Tumor formation
- Metastasis

Cell Migration and Paxillin Function

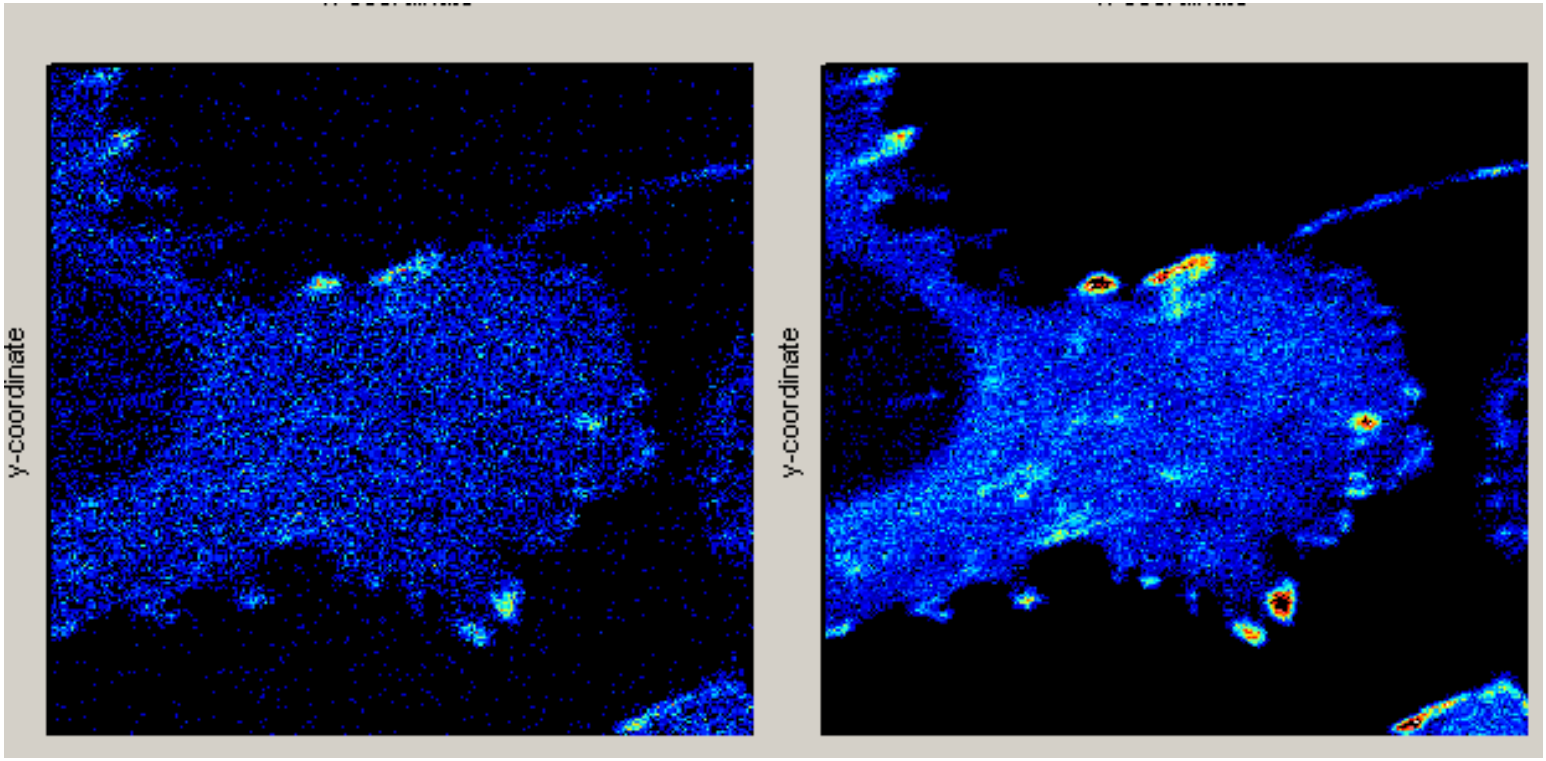


Turner, C.E., (2000). Paxillin and focal adhesion signaling. Nature Cell Biol., 2 E231 6.



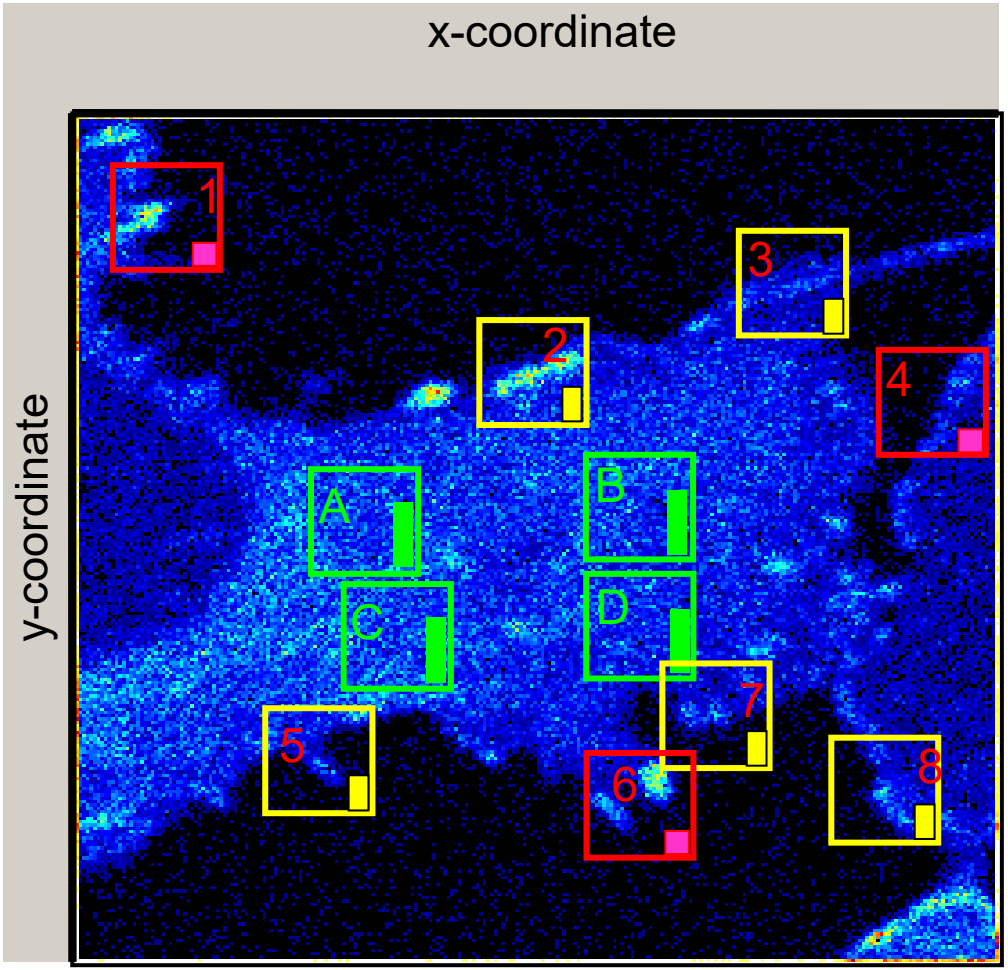
Vicente-Manzanares, M., Webb, D. J., and Horwitz, A. R. (2005) *J. Cell Sci.* **118,** 4917–4919

Confocal Cell imaging: CHO-K1 Cells Expressing Paxillin-EGFP



256 x 256 pixel (35.5 μm), 32 μs/pixel, 10.4 ms/line, ω_0 = 0.42, total run time 5.2 min.

Map of paxillin-EGFP diffusion rate

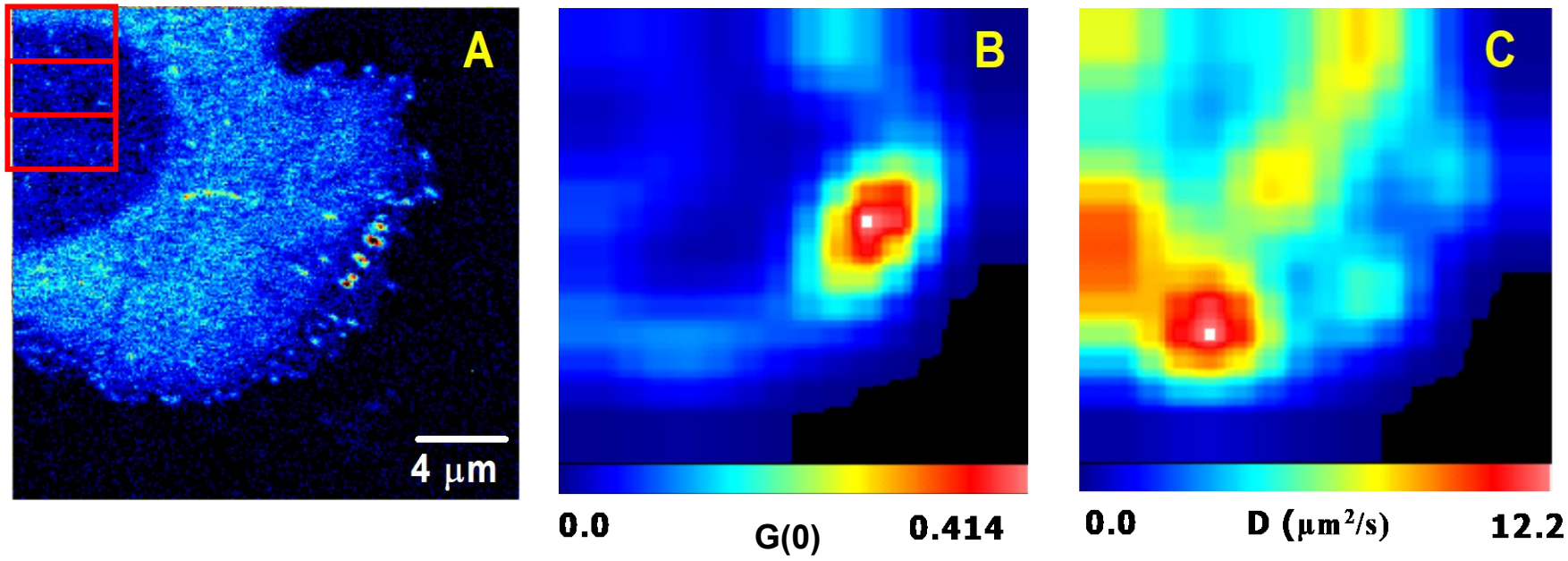


D = 10.2 μm²/s

D = 1.09 μm²/s

D = 0.20 μm²/s

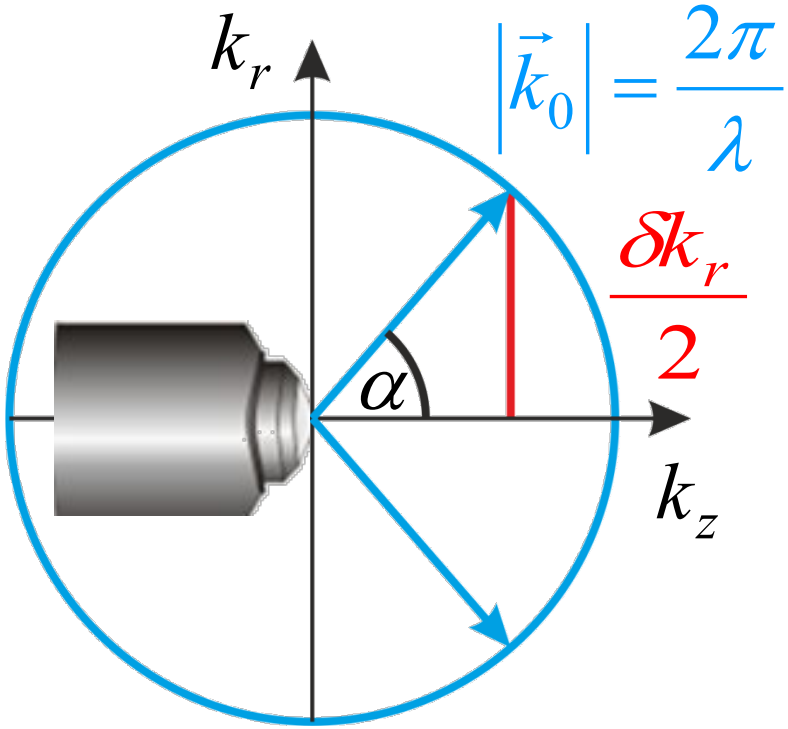
RICS analysis using auto-scan of a small area

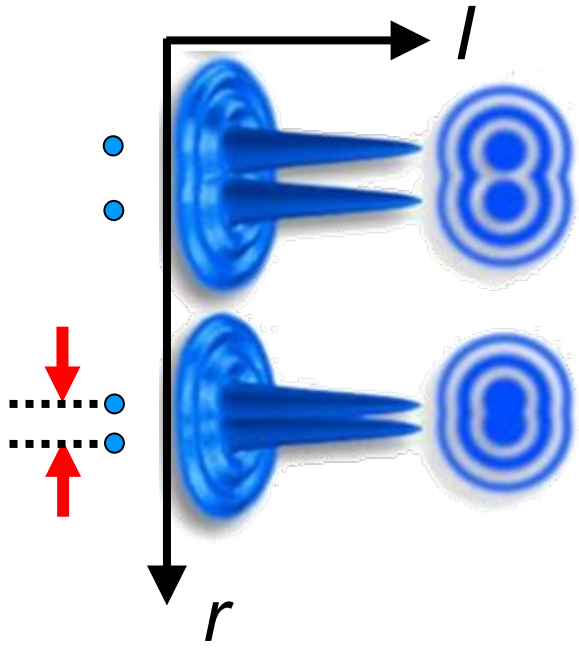


Spatial maps of the apparent diffusion coefficient of paxillin across the cell.

With diffraction limited microscopy, the spatial resolution is limited to ~2 μm.

Optical Resolution





$$\delta r_{\min} = \frac{\lambda}{2 \cdot n \cdot \sin \alpha}$$

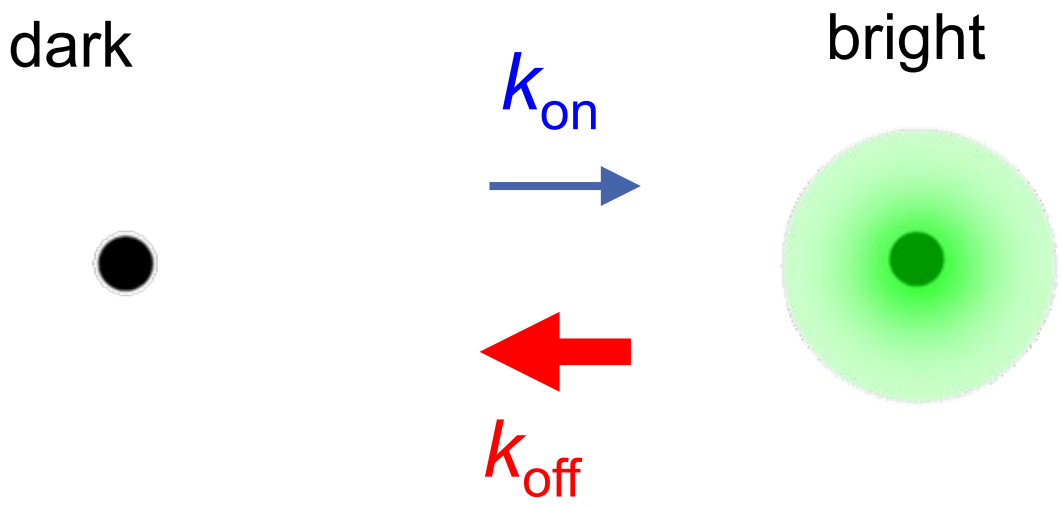
lateral ~200 nm

axial ~500 nm

wave vector of light is expressed as k=2pi/lambda

Stimulated emission Depletion (STED) Principle

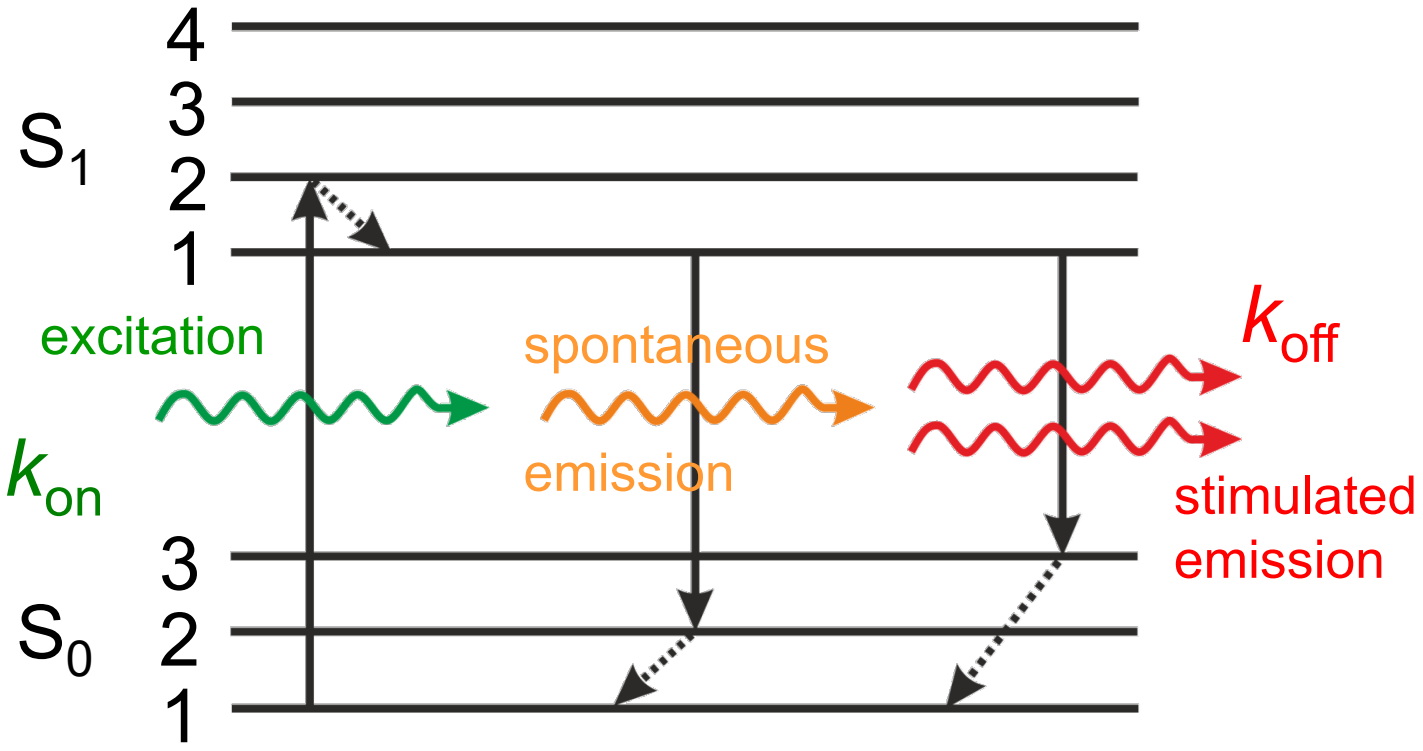
STED relies on fluorophore switching:



- STED functions by depleting fluorescence in specific regions of the sample while leaving a center focal spot active to emit fluorescence.
- The STED laser must possess zero-fields in the center and maximum in the periphery which exhibits a doughnut-shaped phase pattern

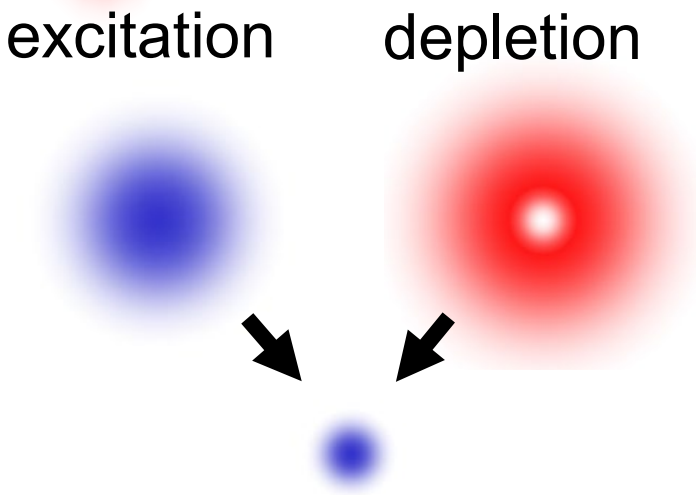
STED Principle

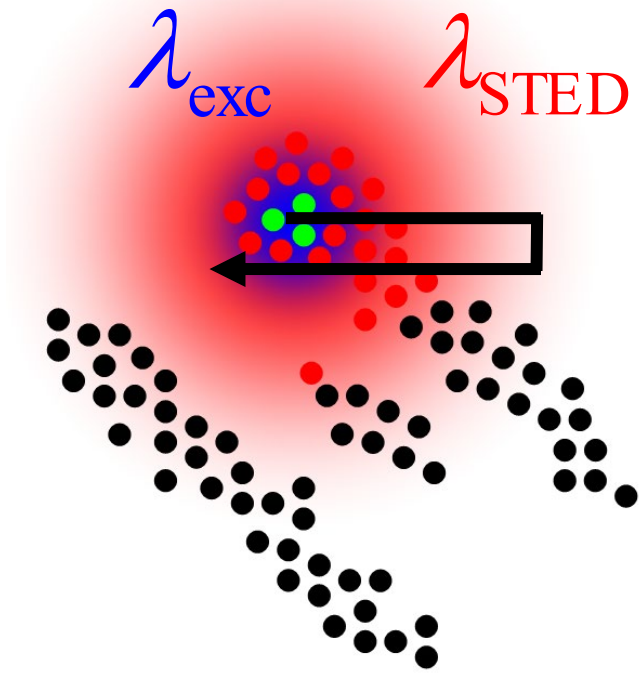
Stimulated emission:

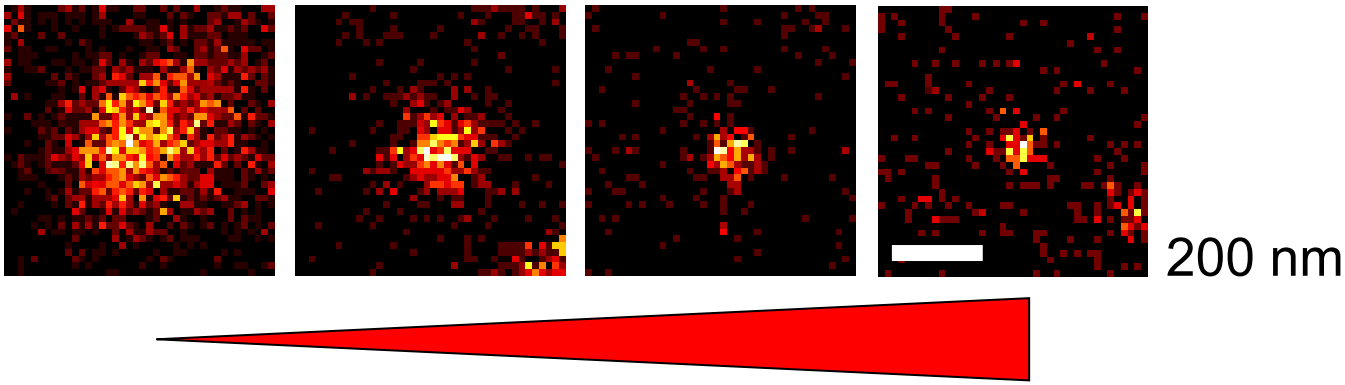


If a system is pumped by a second laser while still being in the excited state, the second laser kicks the electron back to the ground state without fluorescence. In this process known as the stimulated emission,

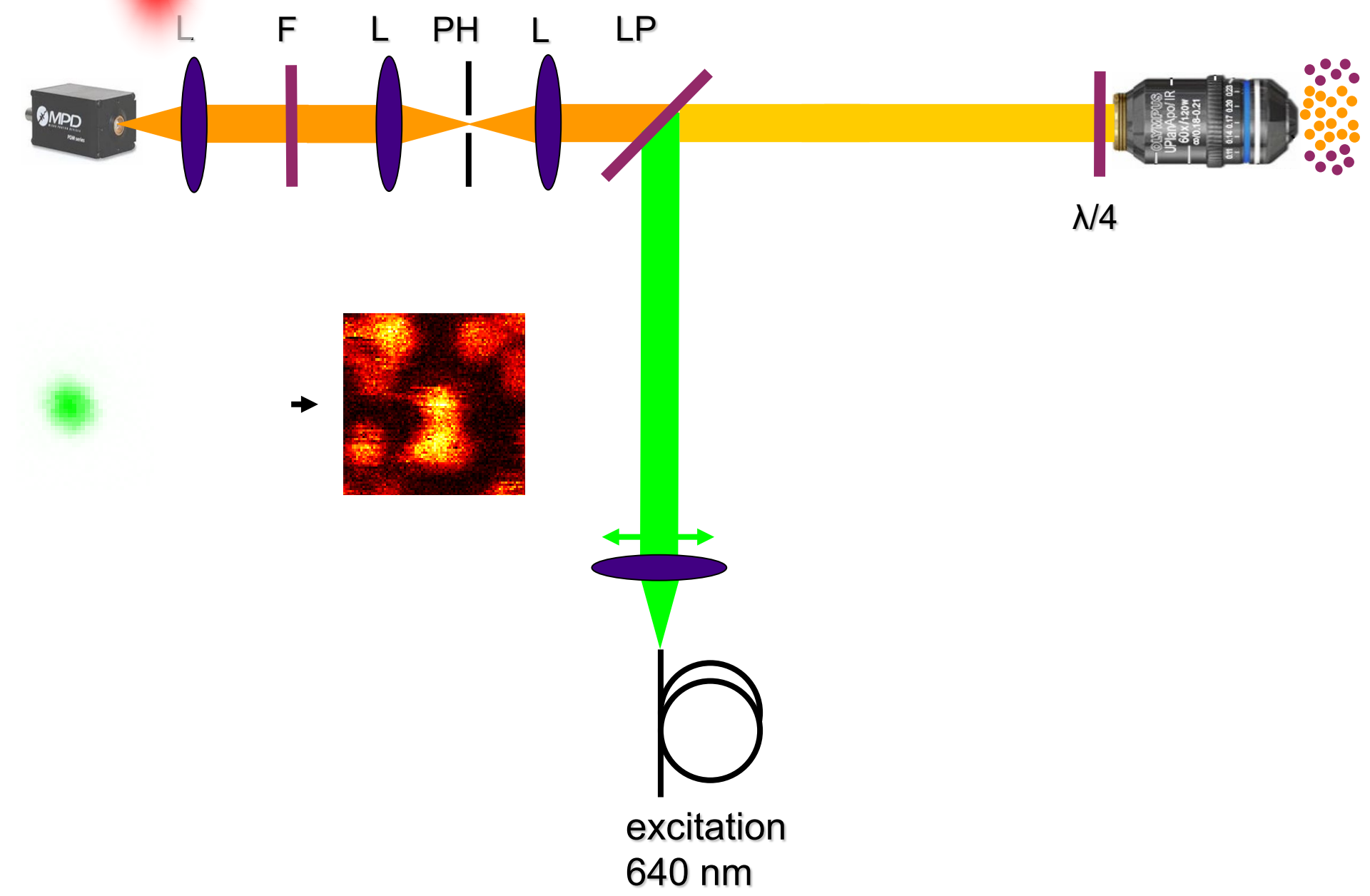
STED Principle



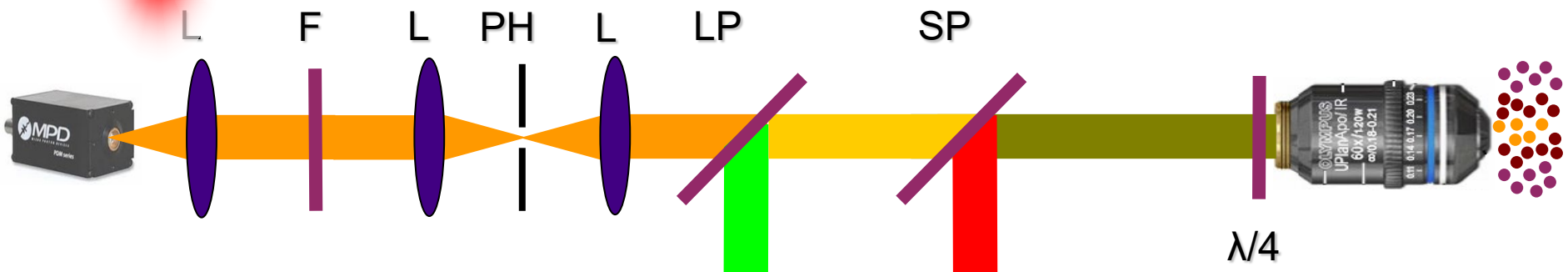


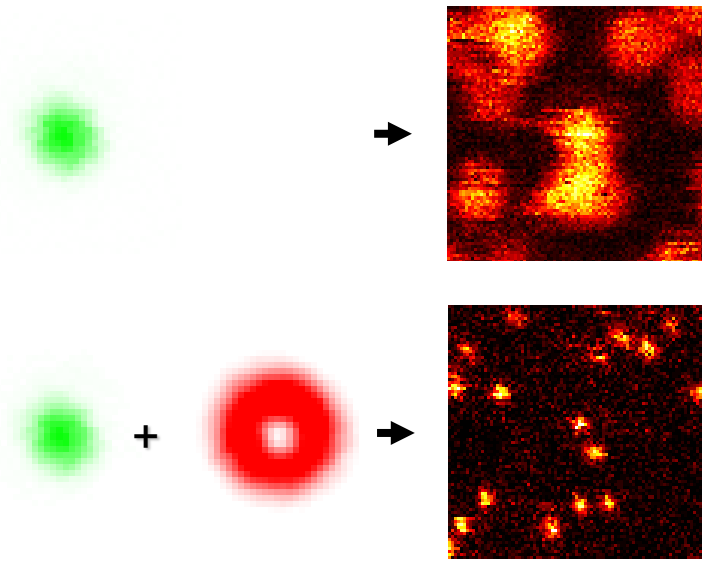


STED Setup

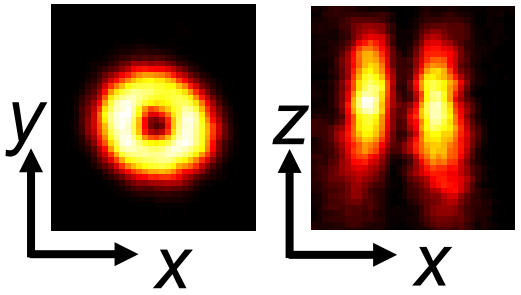


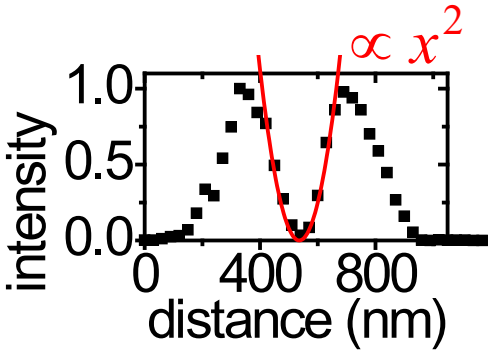
STED Principle





$0-2\pi$





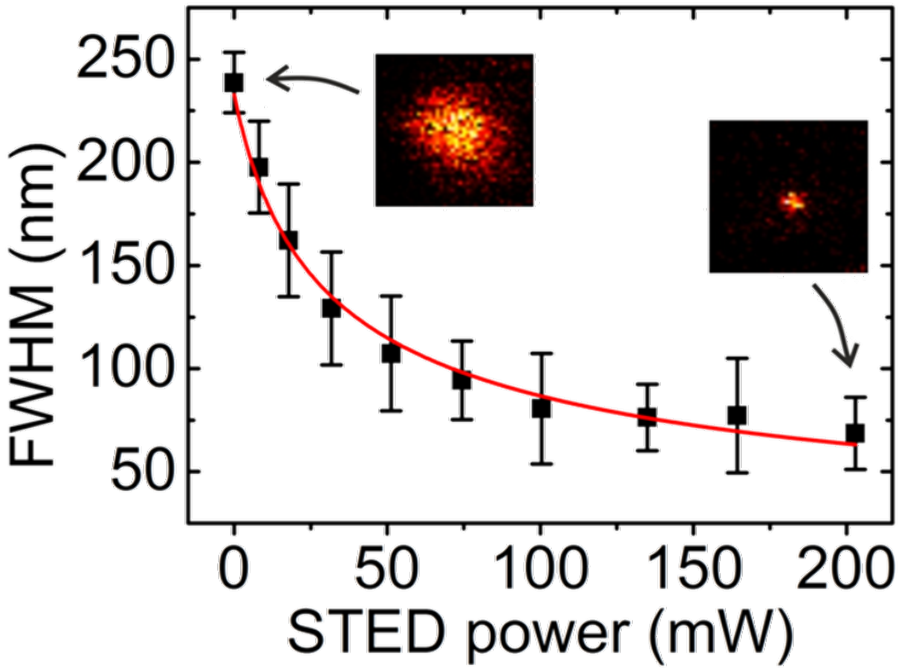
excitation
640 nm

STED
760 nm

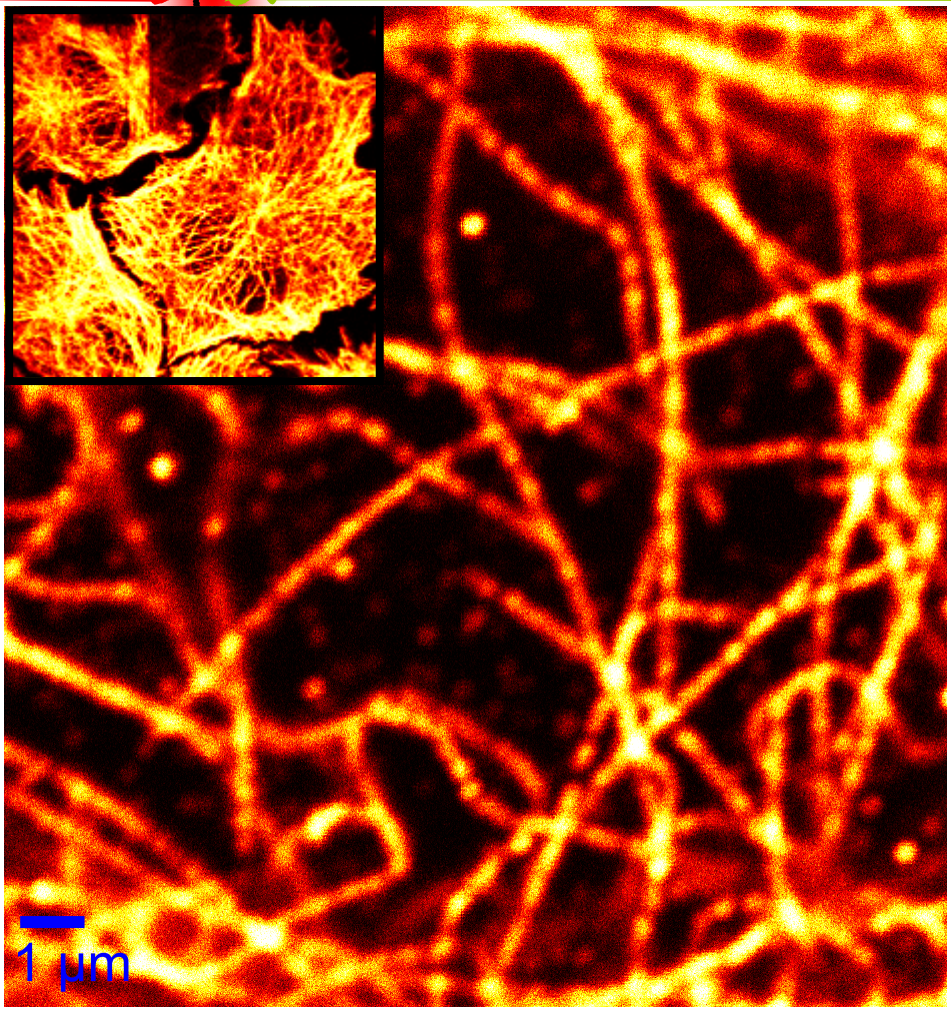
STED Resolution

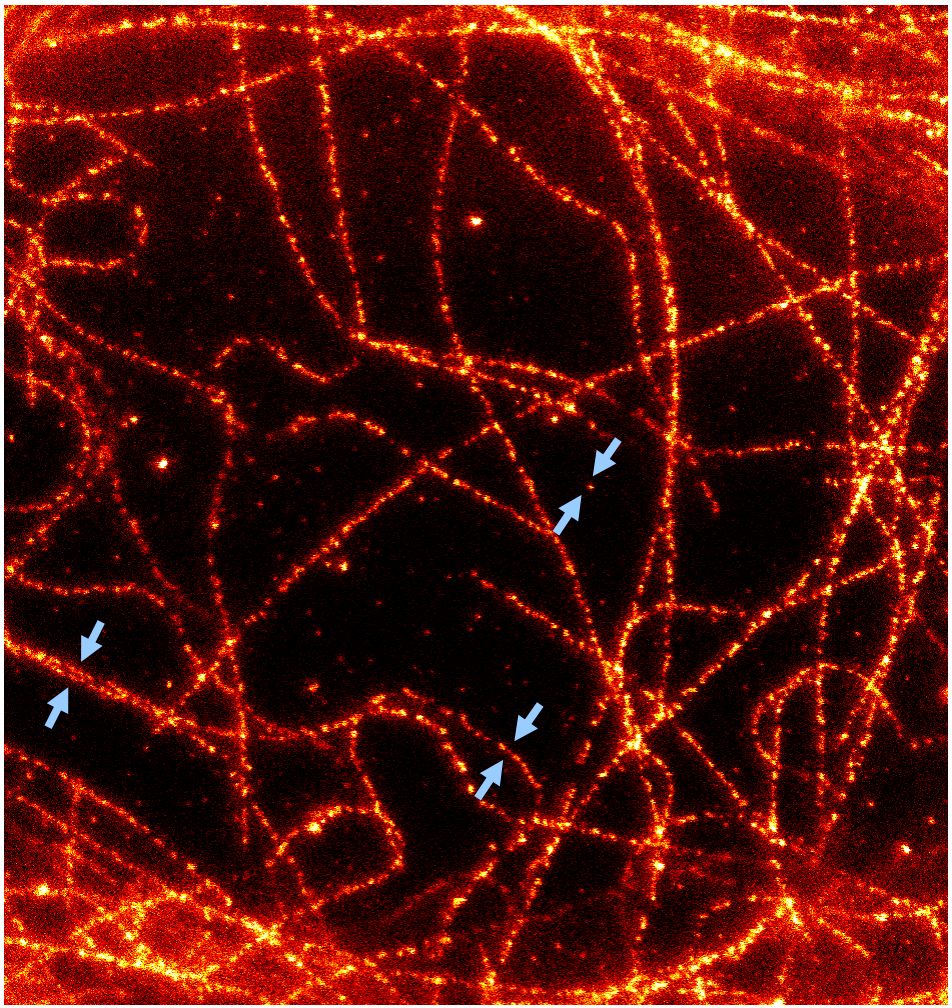
$$\delta r_{\min} = \frac{\lambda}{2 \cdot n \cdot \sin \alpha} \cdot \frac{1}{s}$$

$$s = \sqrt{1 + \frac{I_{\mathrm{STED}}}{I_{\mathrm{sat}}}}$$

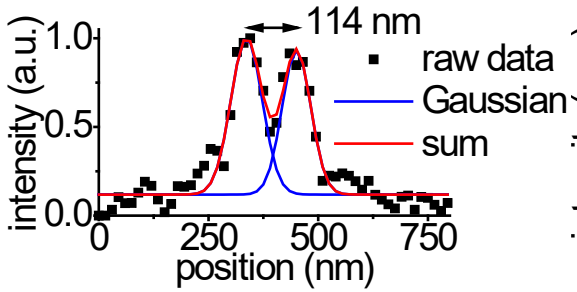


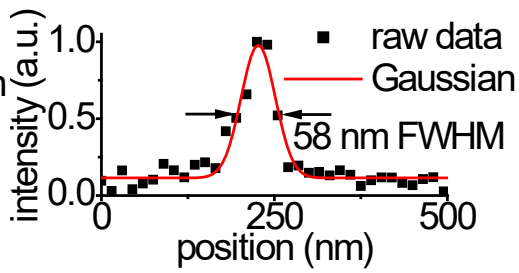
β-Tubulin-Alexa594 in Fixed HeLa Cells

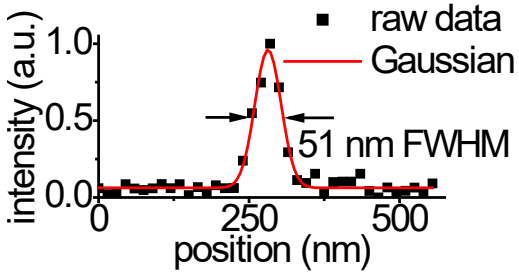




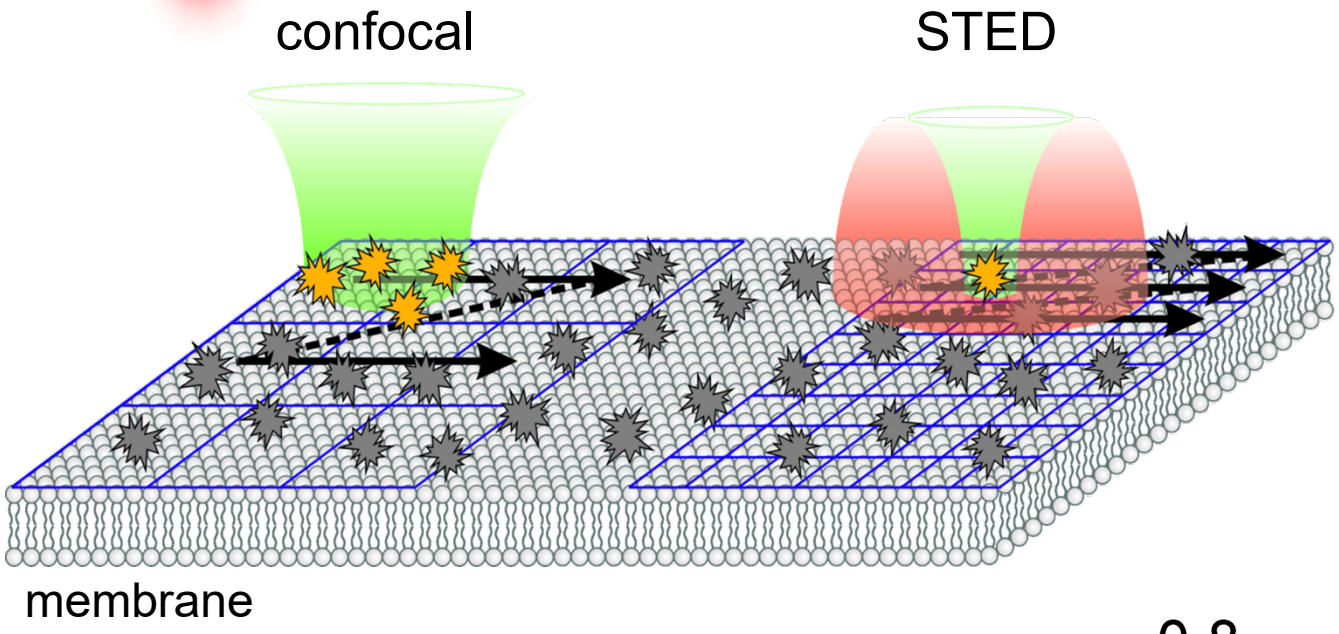
Hedde et al., in Springer Series: Physics and Biophysics, eds.: B. di Bartolo, J. Collins, Springer Science + Business Media B.V., Dordrecht (2013), pp. 47-71.



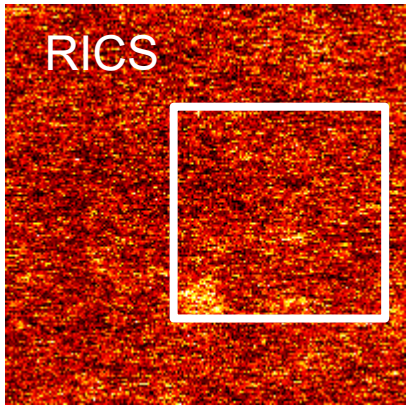




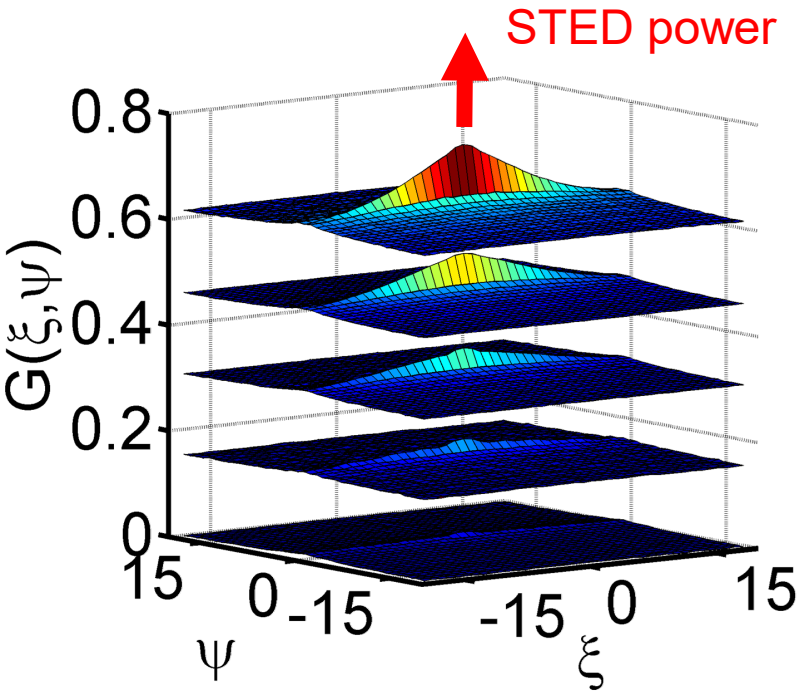
Application of STED to RICS



Tunable observation volume

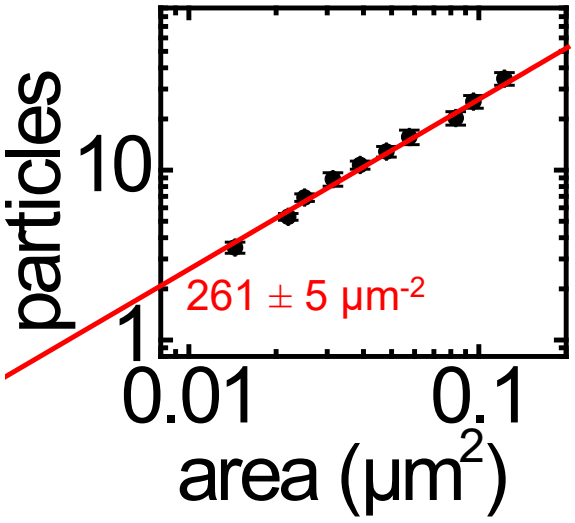


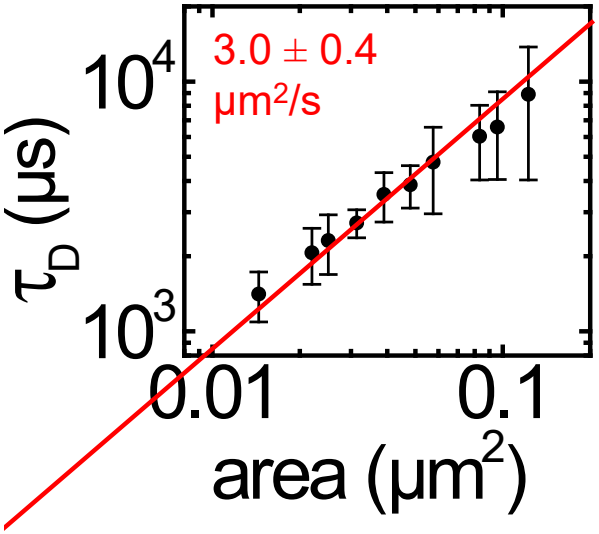
correlation

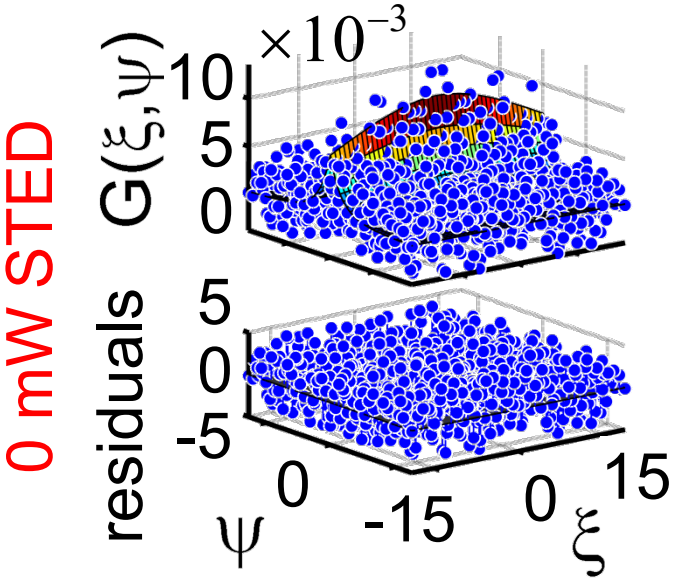


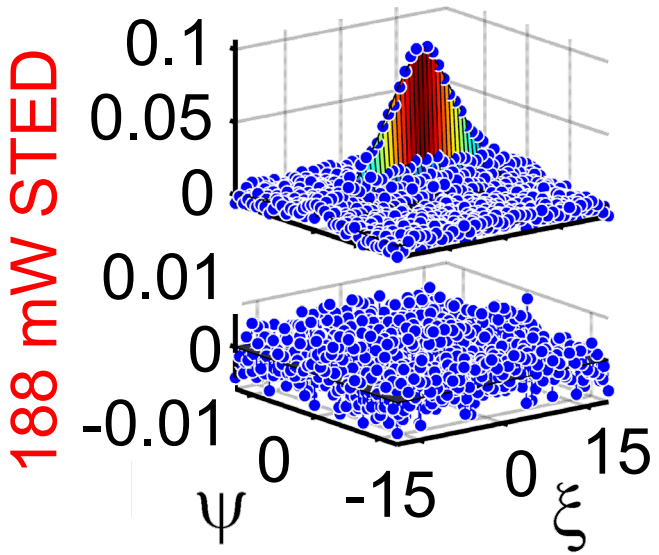
STED-RICS

Lipid bilayer labeled with Atto647N-DPPE:





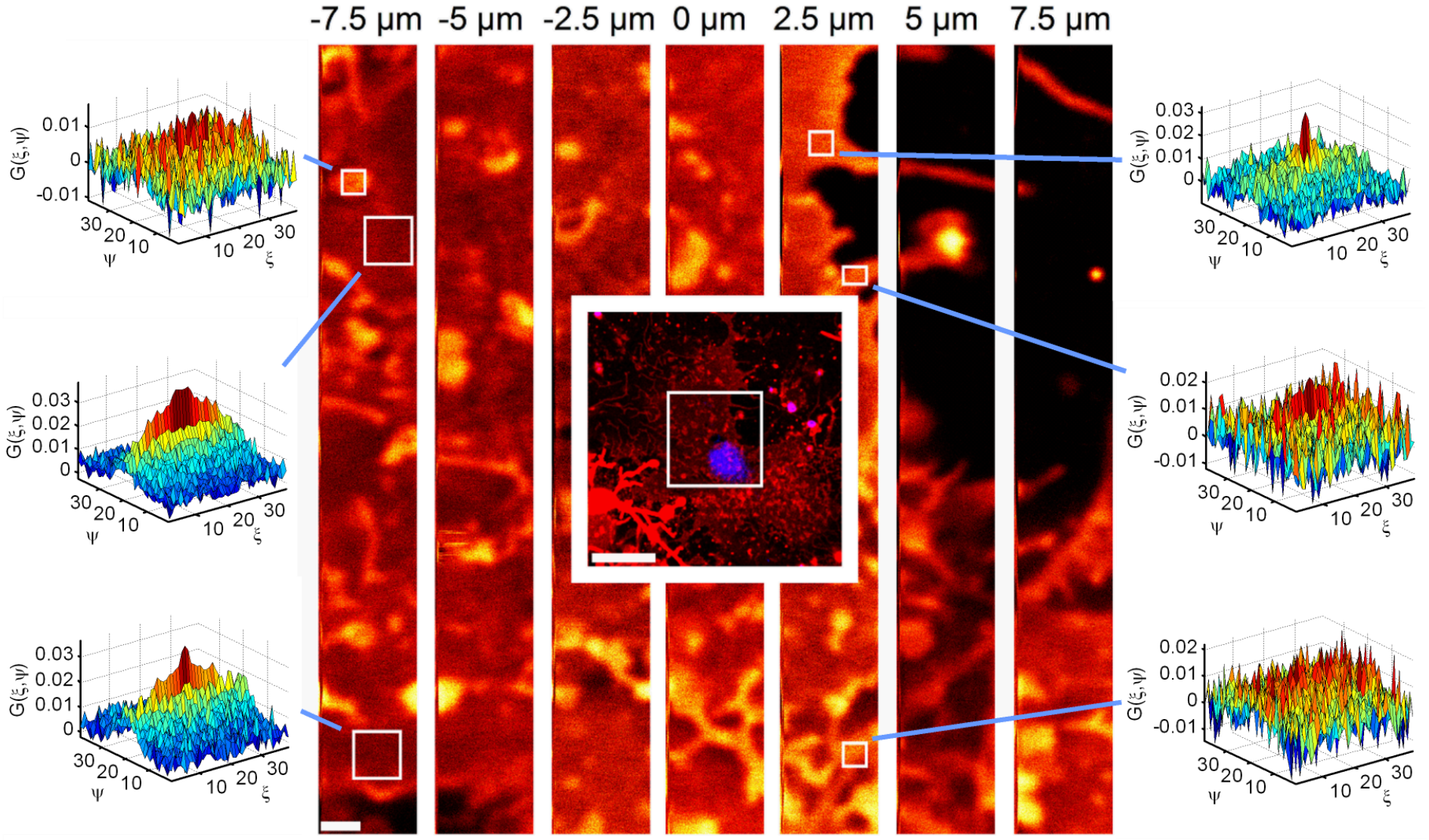




Hedde et al., Nature Commun. 4 (2013) 2093

STED-RICS

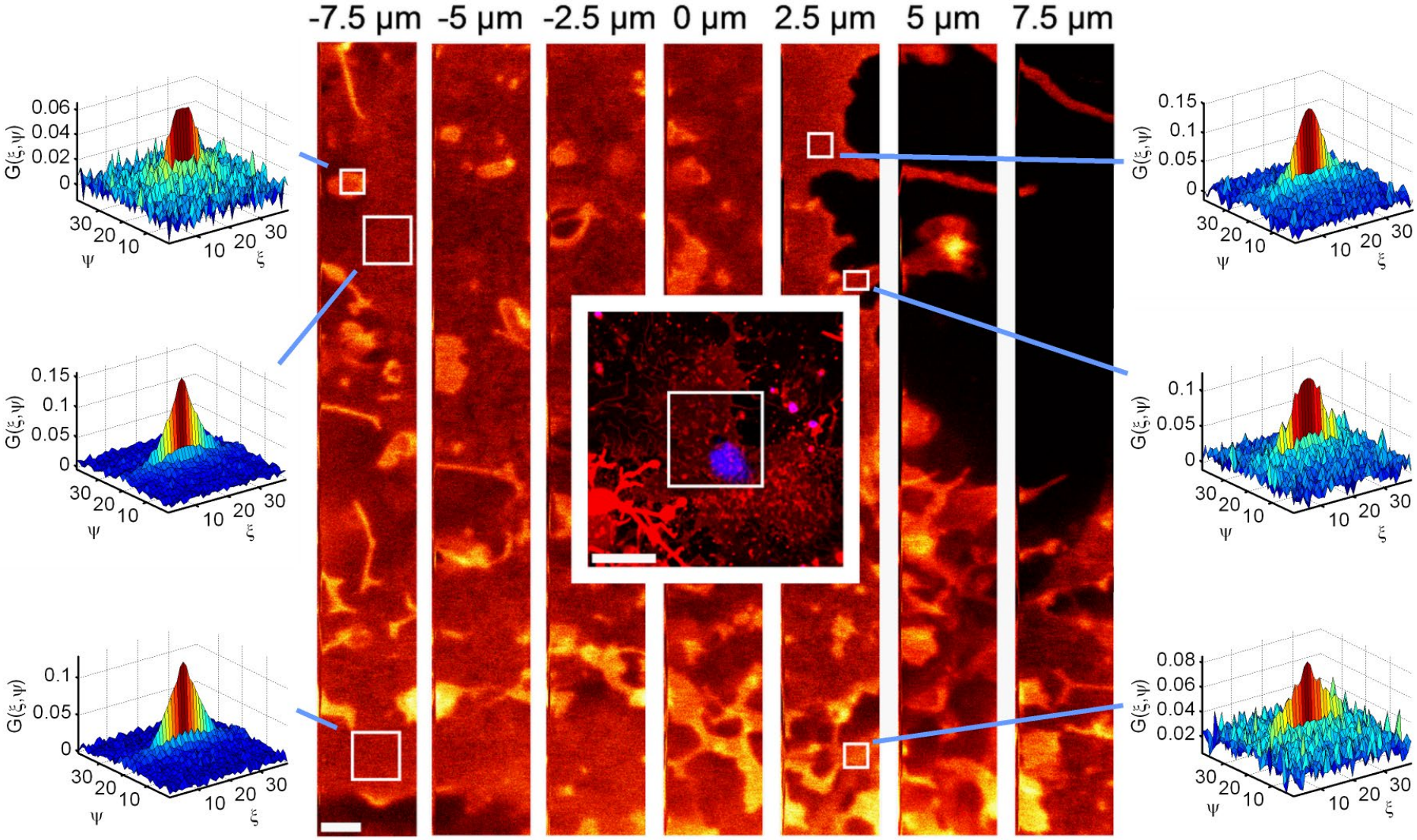
Atto647N-DPPE in the plasma membrane of XTC cells:



Hedde et al., Nature Commun. 4 (2013) 2093

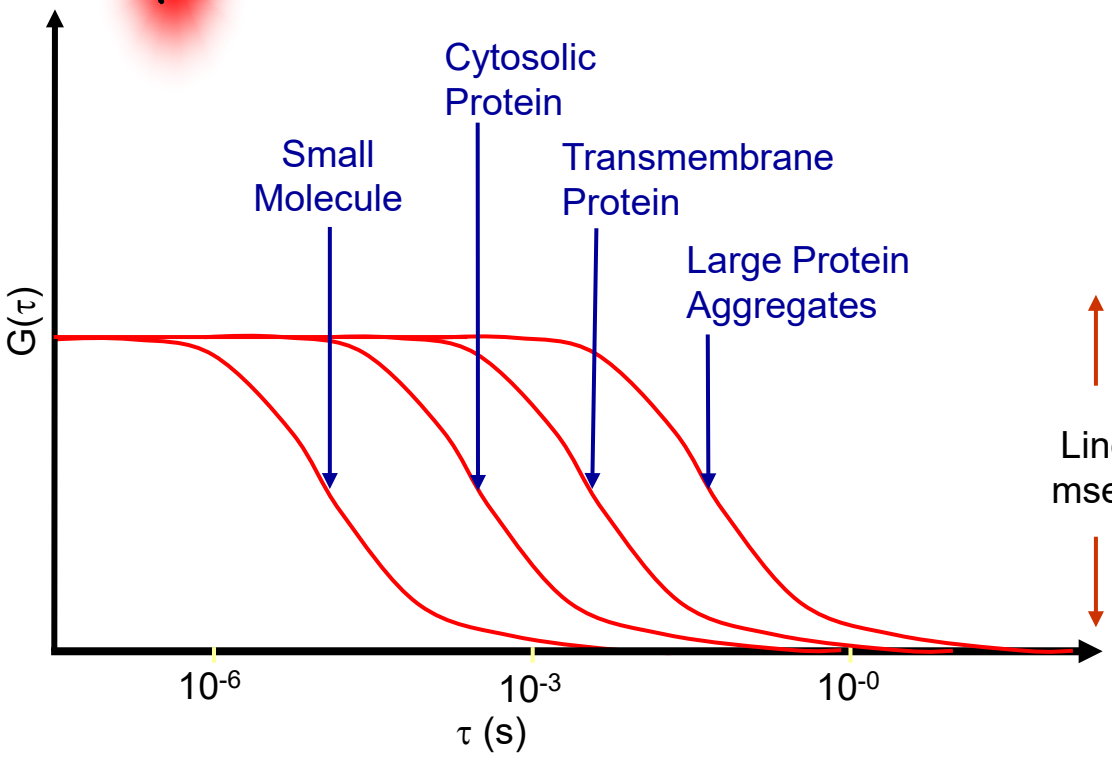
STED-RICS

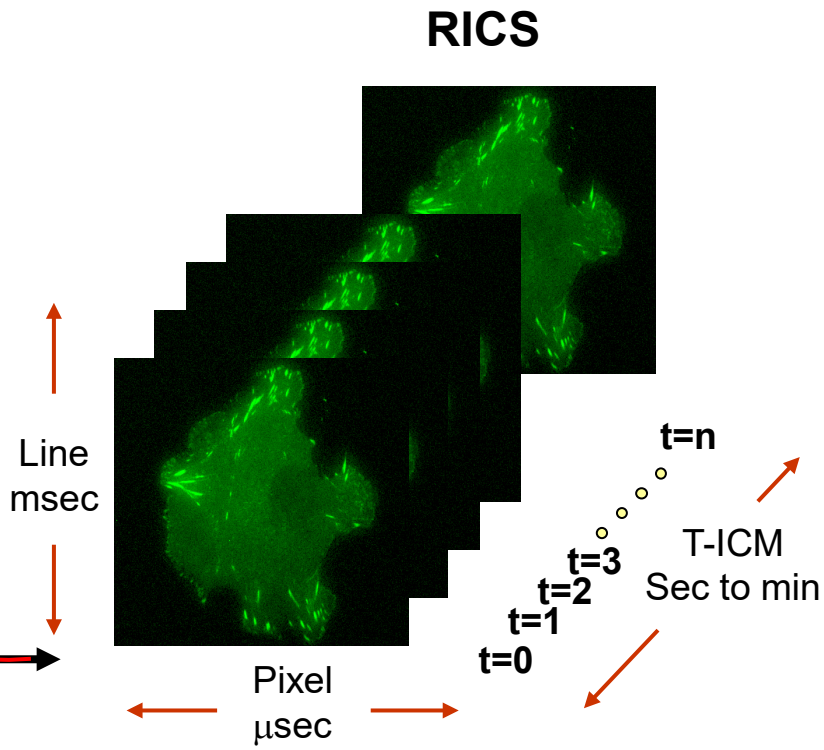
Atto647N-DPPE in the plasma membrane of XTC cells:



Hedde et al., Nature Commun. 4 (2013) 2093

Conclusions





RICS | **Temporal ICM**

Techniques	Time Res.	Spatial Res.	Used to Study
Temporal-ICM	sec	<0.5 μm	Protein aggregates Transmembrane proteins
RICS	μsec-msec	~2 μm	Soluble proteins Binding interactions
STED-RICS	μsec-msec	<0.5 μm	Soluble proteins Binding interactions

Acknowledgements

RICS/pCF

- Enrico Gratton, Department of Biomedical Engineering, UCI
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- Rick Horwitz, Department of Biophysics, University of Virginia
- Paul Wiseman, Department of Physics and Chemistry, McGill University
- Claire Brown, Core manager, McGill University
- LFD Lab members

National Center for Research Resources

UCIrvine University of California, Irvine

LFD

Cell Migration Consortium

National Institutes of Health

STED-RICS

- Gerd Ulrich Nienhaus, Institute of Applied Physics, Karlsruhe Institute of Technology.
- Rene Doerlich, Dietmar Gradl, Rosmarie Blomley, Emmanuel Oppong, Andrew Cato, Karlsruhe Institute of Technology.

The Number & Molecular Brightness (N&B) Method

Michelle Digman
Laboratory for Fluorescence Dynamics
University of California, Irvine

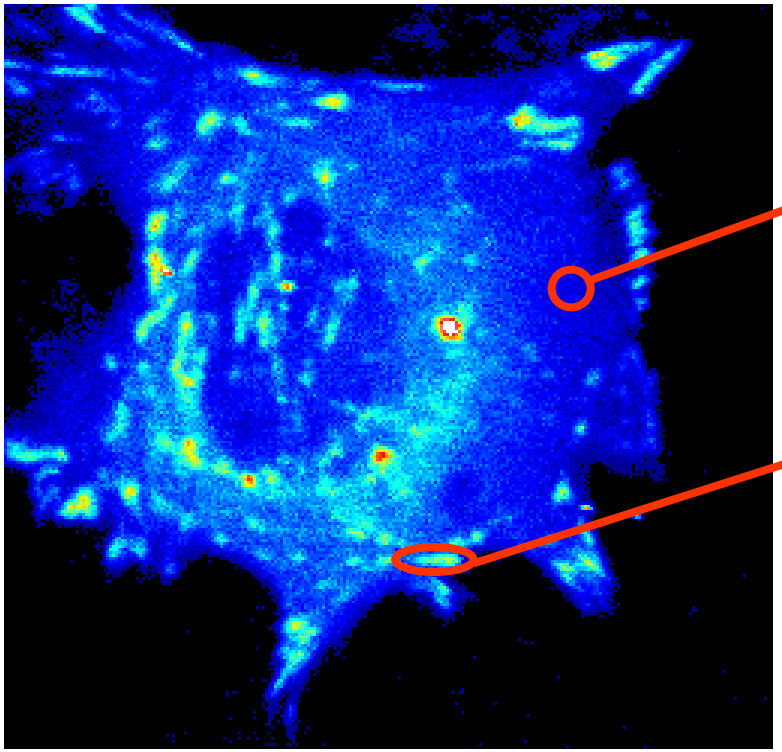
UCIrvine
University of California, Irvine

National Center for Research Resources

Cell Migration Consortium

Existing Methods to Determine Protein Concentration and Aggregation of Proteins in Cells

1. Calibration of the free fluorophore based on intensity



Average intensity of MEF cells expressing paxillin-EGFP.

INTENSITY

A
31,250 counts/s

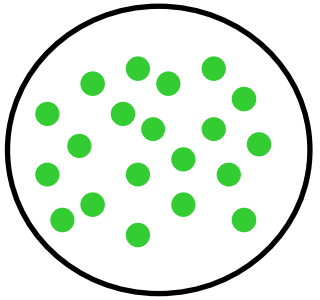
B
93,750 counts/s

If "free" EGFP at 10 nM gave 30,000 counts/s, the conclusion would be that:

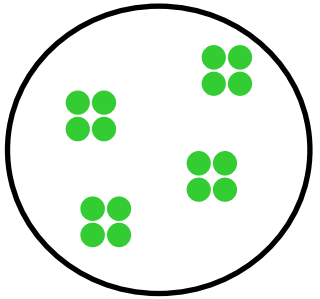
A **= 10 nM**

B **= 30 nM**

How can we determine if



or

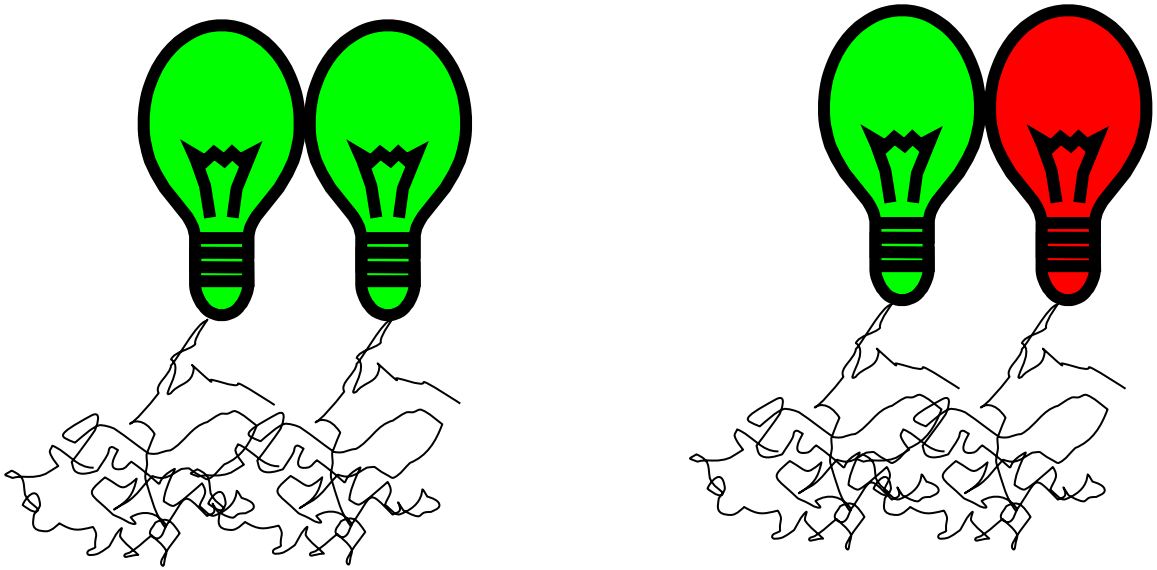


?

However, it doesn't give you the size distribution. Only concentration is given.

Existing Methods to Determine Protein Concentration and Aggregation of Proteins in Cells

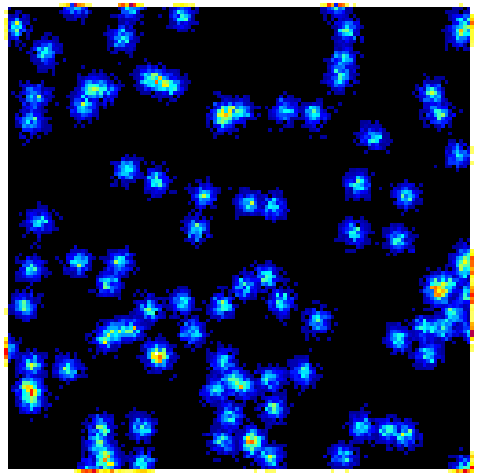
2. Förster Resonance Energy Transfer (FRET)

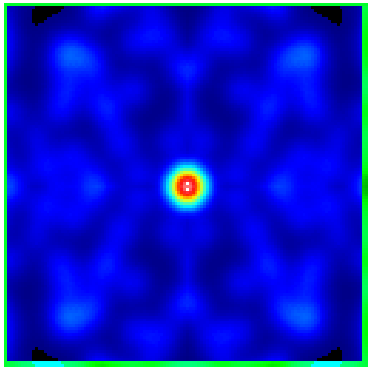


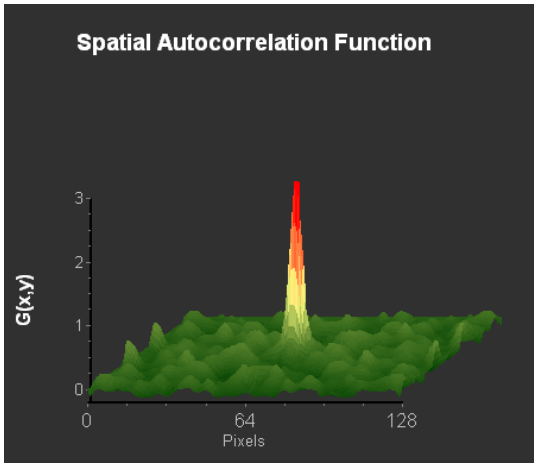
This method is very sensitive to detect the formation of pairs.

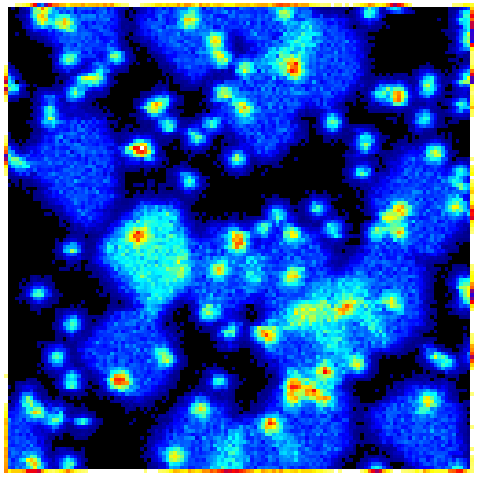
Existing Methods to Determine Protein Concentration and Aggregation of Proteins in Cells

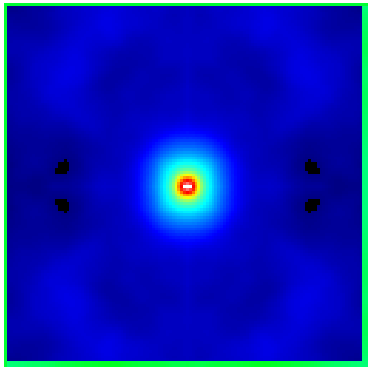
3. Image correlation Spectroscopy (ICS)

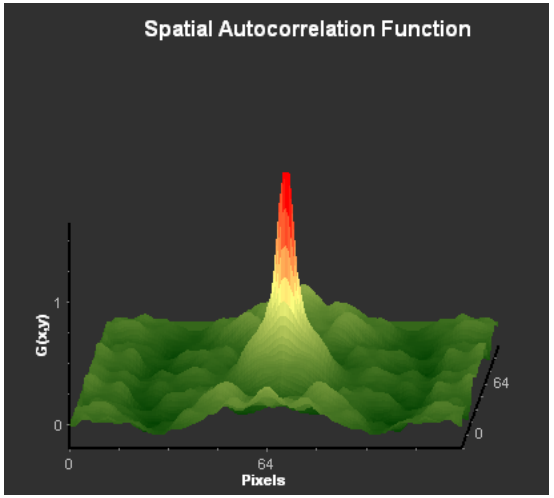












However, the events must be slow >1 s (no movement during one frame) and the aggregates must be large.

Petersen and Wiseman. Biophys. J. (1999)

The Number and Brightness (N&B) Analysis

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.

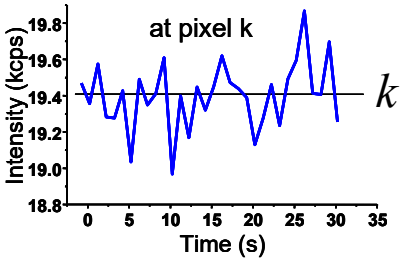
Tools: Cursor selection of pixel with similar brightness.
Quantitative analysis of center and std dev of the *e* and *n* distribution.
Tools for calibration of analog detectors.

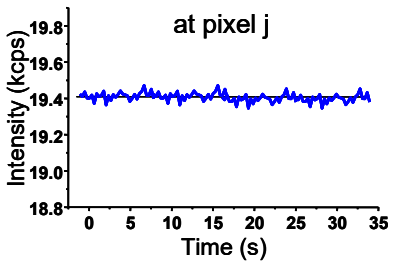
Tutorials: Mathematical background, data import, analysis examples (our web site).

How to Distinguish Pixels with Many Dim Molecules from Pixels with Few Bright Molecules?

Average (first moment)

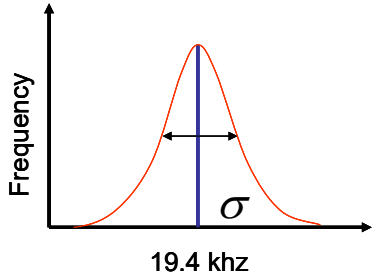
$$< k >= \frac{\sum_i k_i}{K}$$

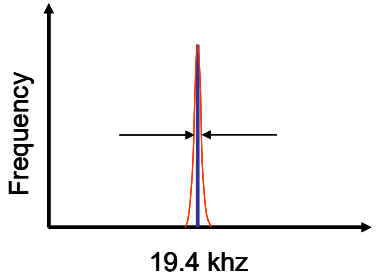


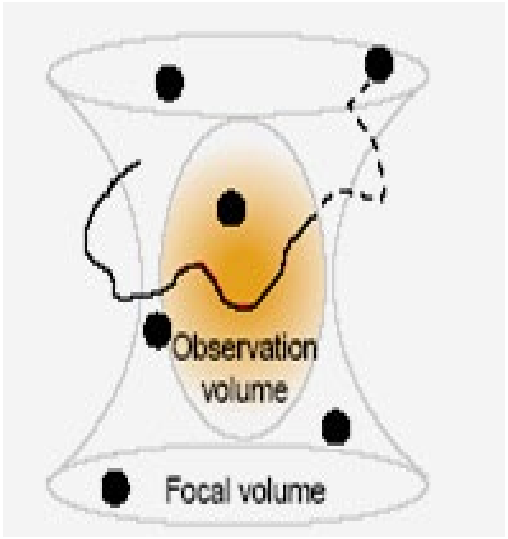


Variance (second moment)

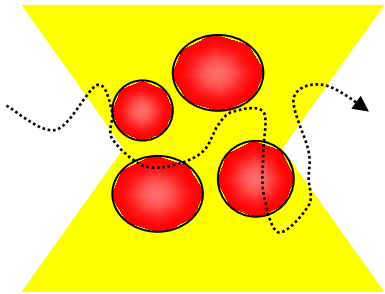
$$\sigma^2 = \frac{\sum_i (k_i - < k >)^2}{K}$$

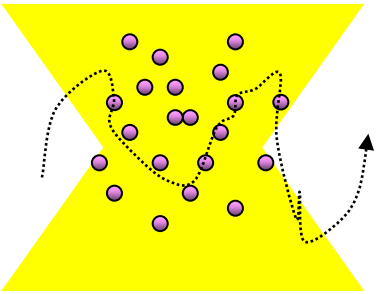






$G(0) = \sigma^2/\langle k \rangle^2 = 1/N$





Given two series of equal average, the larger is the variance, the less molecules contribute to the average. The ratio of the square of the average intensity ($\langle k \rangle^2$) to the variance (s^2) is proportional to the average number of particles $\langle N \rangle$.

Calculating Protein Aggregates from Images

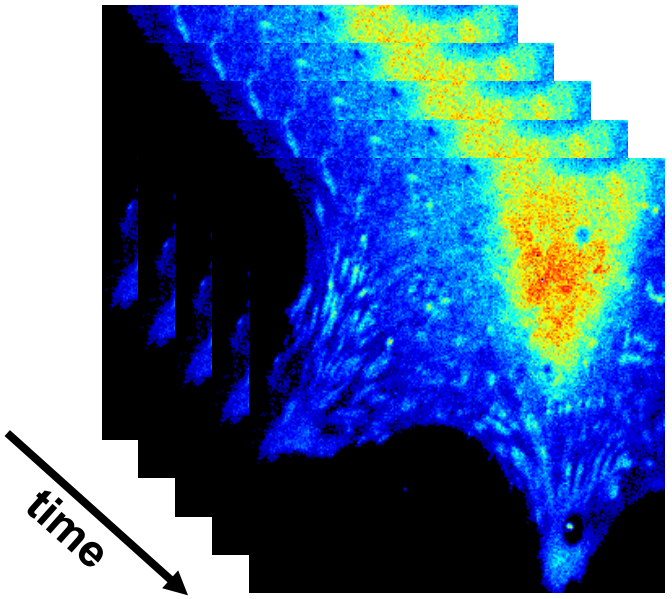
This analysis provides a map of <N> and brightness B for every pixel in the image. The units of brightness are related to the pixel dwell time and they are "counts/dwell time/molecule".

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

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

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

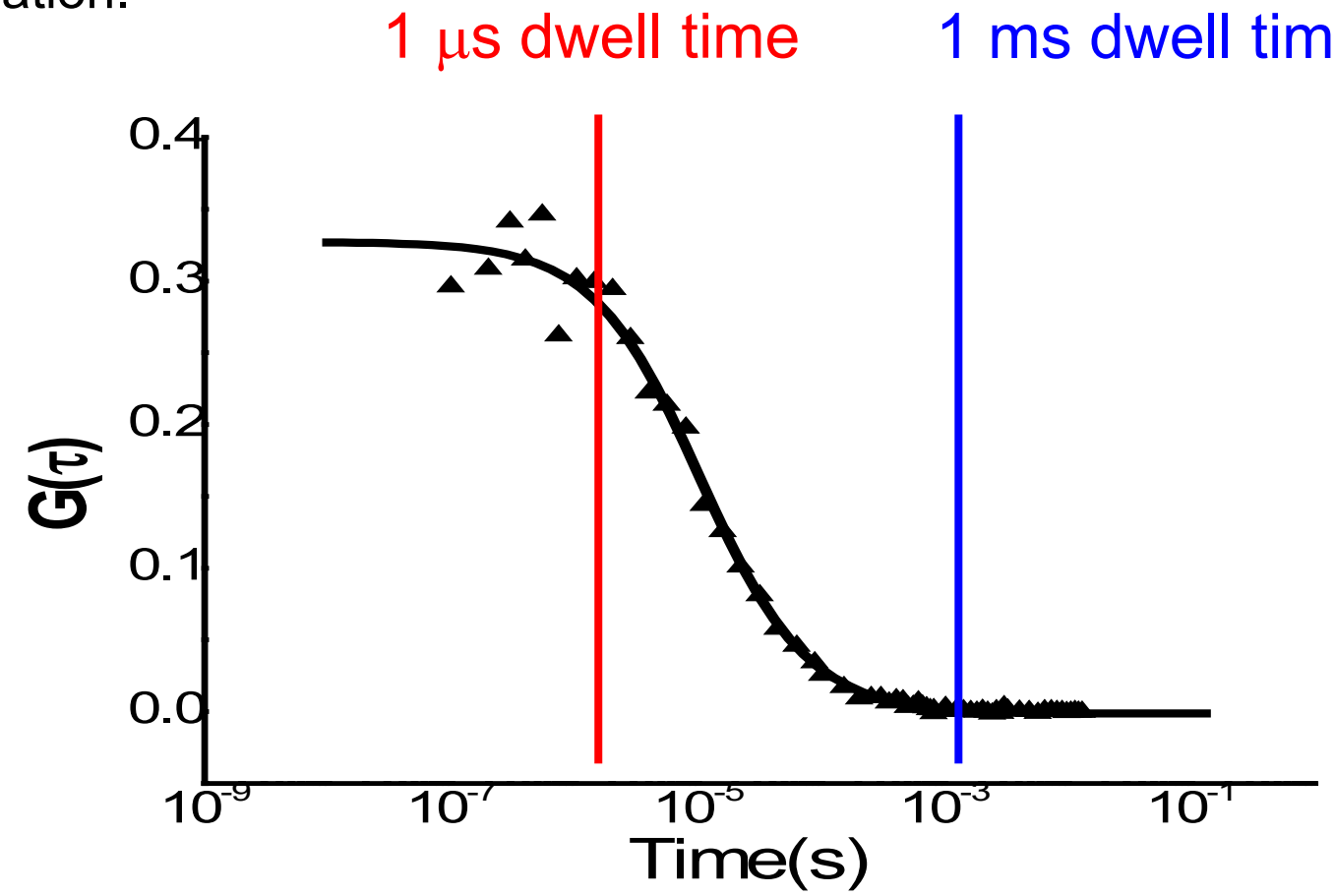
σ^2 = Variance
<k> = Average counts
N = Apparent number of molecules
B = Apparent molecular brightness
K = # of frames analyzed



Selecting the Dwell Time

To increase the apparent brightness we could increase the dwell time, since the brightness is measured in counts/dwell time/molecule.

However, increasing the dwell time decreases the amplitude of the fluctuation.



What Contributes to the Variance?

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

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

The measured variance contains two terms, the variance due to the particle number fluctuation and the variance due to the detector count statistics noise.

$$\boxed{\sigma^2 = \sigma_n^2 + \sigma_d^2}$$

These two terms have different dependence on the molecular brightness:

$\boxed{\sigma_n^2 = \varepsilon^2 n}$ $\boxed{\sigma_d^2 = \varepsilon n}$ (for the photon counting detector)

Both depend on the intrinsic brightness and the number of molecules.
We can invert the equations and obtain n and ε.

n is the true number of mol

ε is the true molecular brigh

How to Calculate n and ε

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

This ratio identifies pixels of different brightness due to mobile particles.

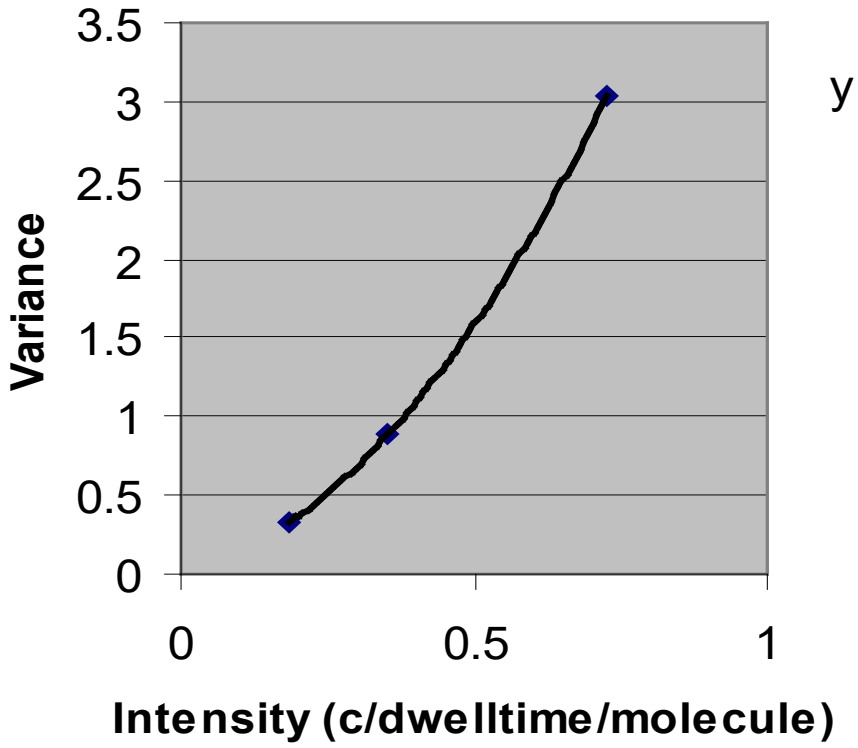
The “true” number of molecules n and the “true” molecular brightness for mobile particles can be obtained from

$$n = \frac{<k>^2}{\sigma^2 - <k>} \qquad \varepsilon = \frac{\sigma^2 - <k>}{<k>}$$

If there are regions of immobile particles, *n* cannot be calculated because for the immobile fraction the variance = <*k*>. For this reason, several plots are offered to help the operator to identify regions of mobile and immobile particles. Particularly useful is the plot of N *vs* B.

Quadratic Dependence of the Variance on Particle Brightness

20 nM EGFP in solution as a function of laser power:

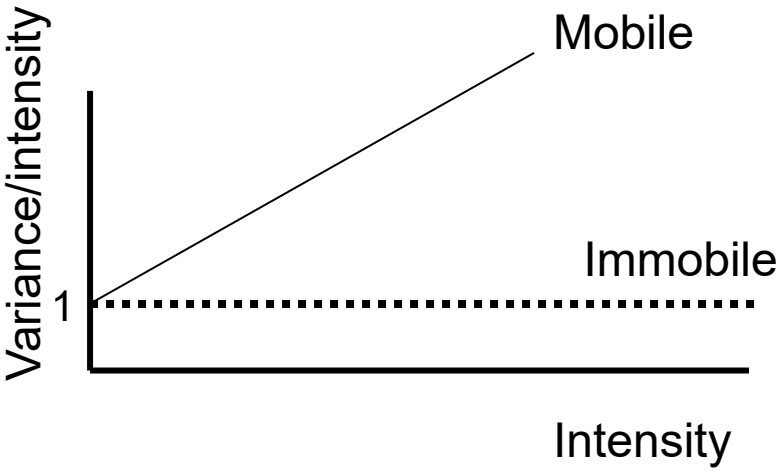


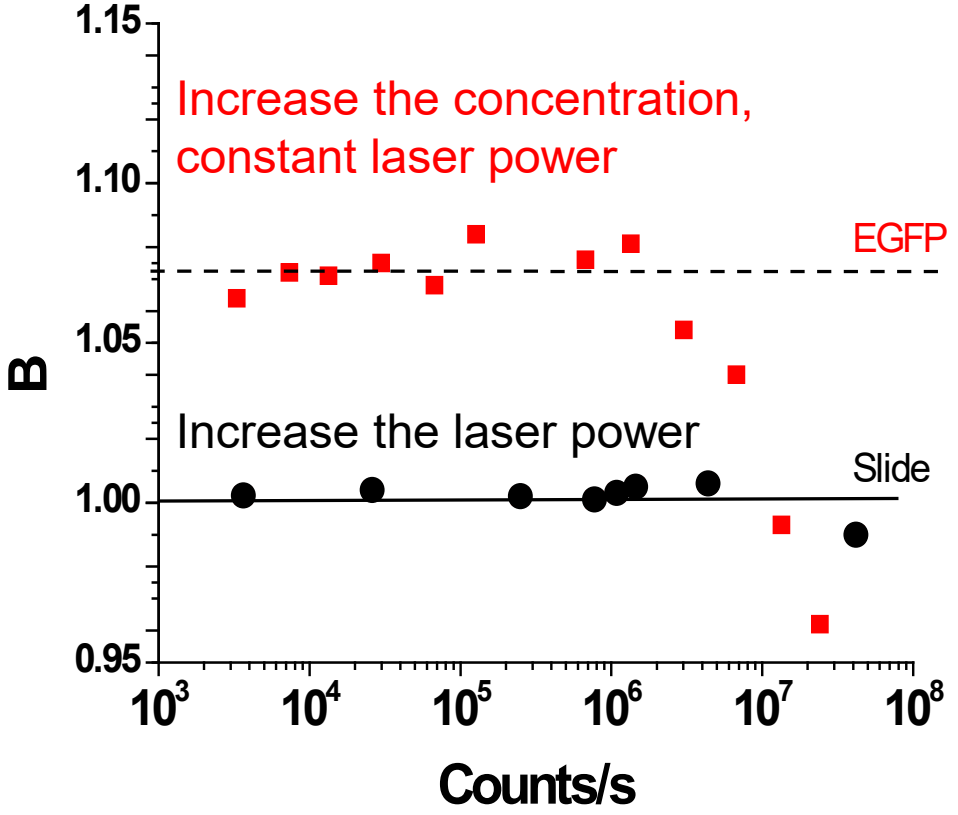
$y = 4.4321x^2 + 0.9438x$

$$\sigma_n^2 = \varepsilon^2 n$$

2-photon excitation using photon counting detectors.

Identification of Mobile and Immobile Molecules

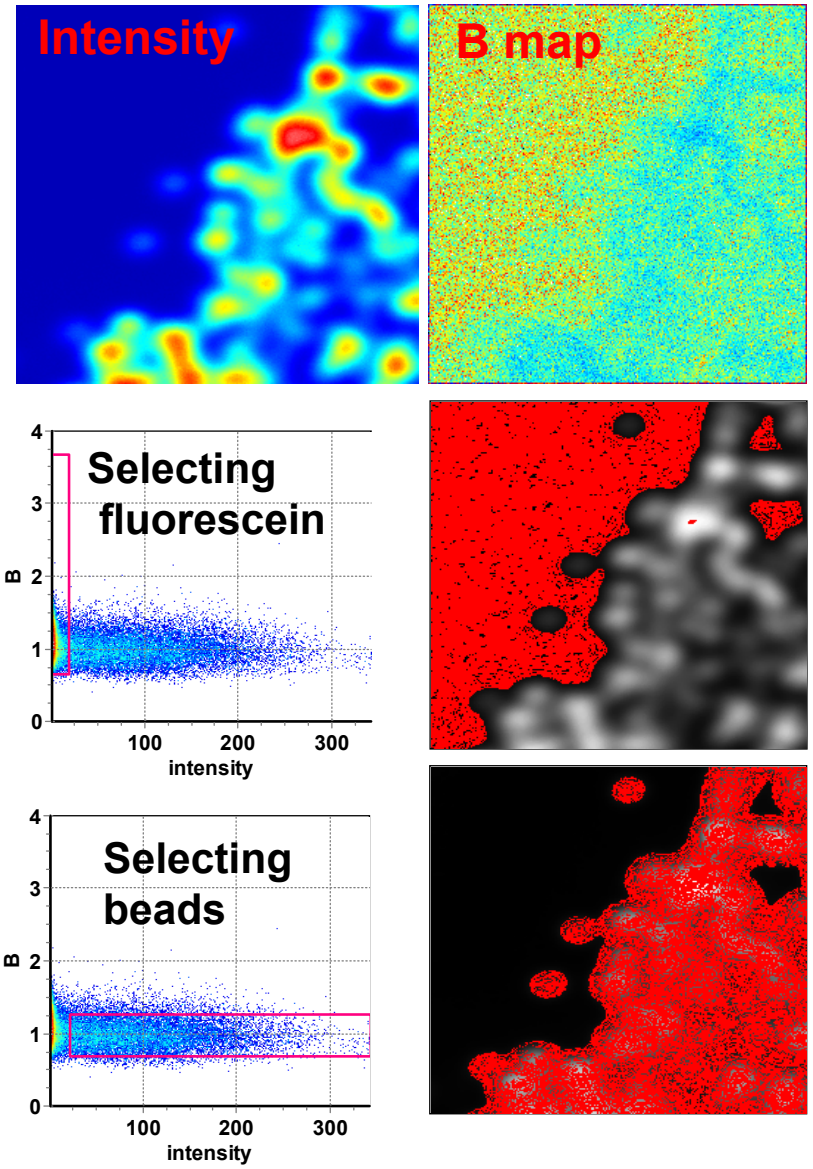


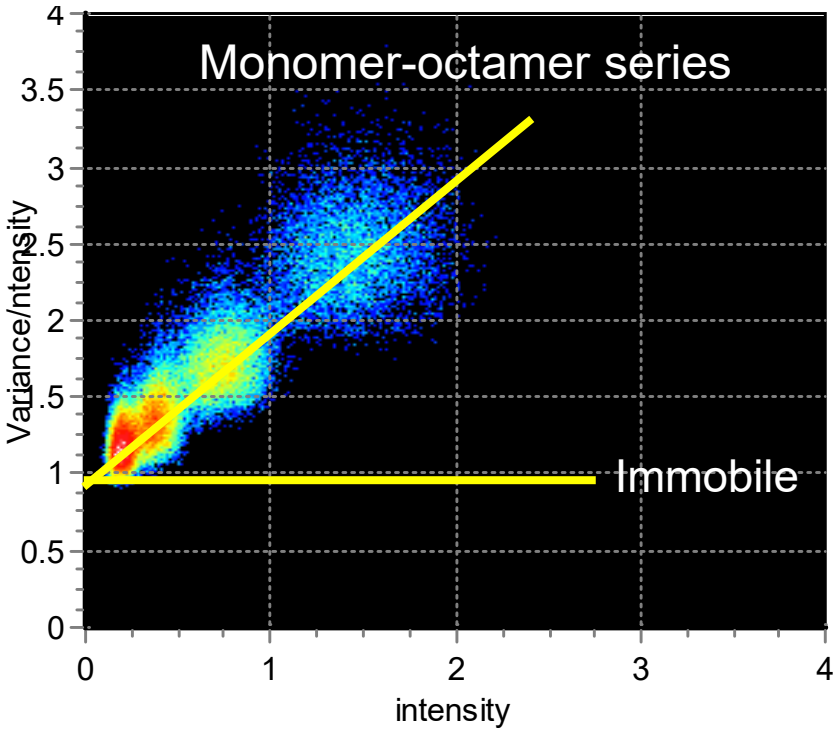


If we change the laser power, a plot of the ratio variance/intensity vs intensity can distinguish the mobile from immobile fraction. The two curves are for different pixel integration times.

The Effect of the Immobile Part

Fluorescent beads in a sea of 100nM Fluorescein:

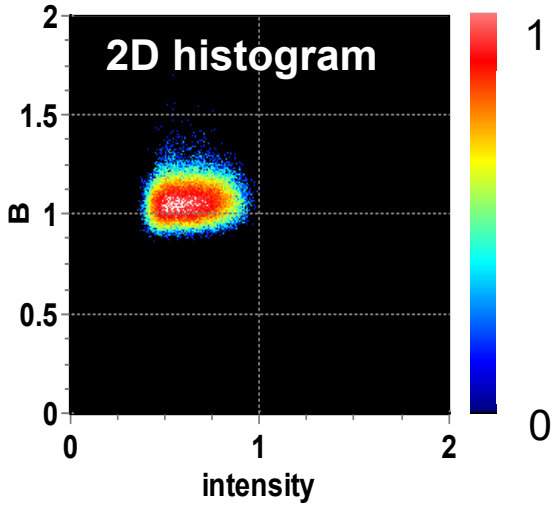


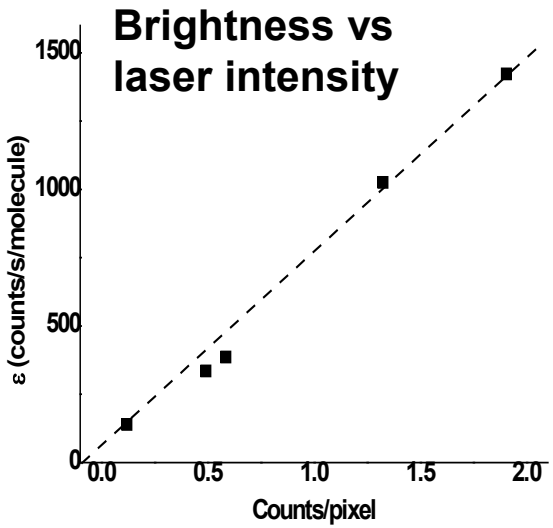


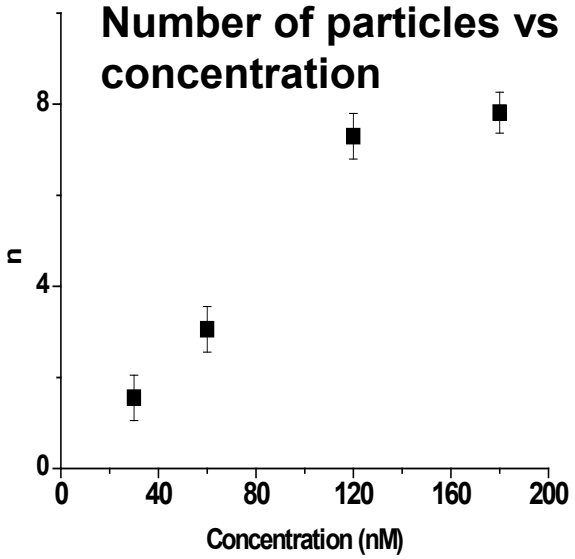
Measured with a photon counting detector.

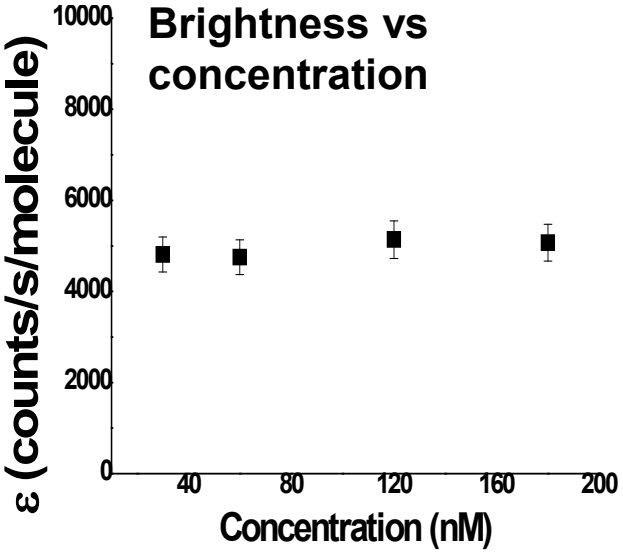
Brightness and Number of Molecules Can be Measured Independently

EGFP in solution









What are the parameters for analog systems?

Detector Noise in Analog Systems

Additional considerations with analog detection systems:

- **Digital levels are recorded (instead of photon counts).**
- **An offset is typically present.**
- **Additional detector variance at low currents.**

d_{offset} = analog offset

$$< k >= \varepsilon n + d_{offset}$$

S = digital levels per photon

σ_0^2 = variance of analog detector

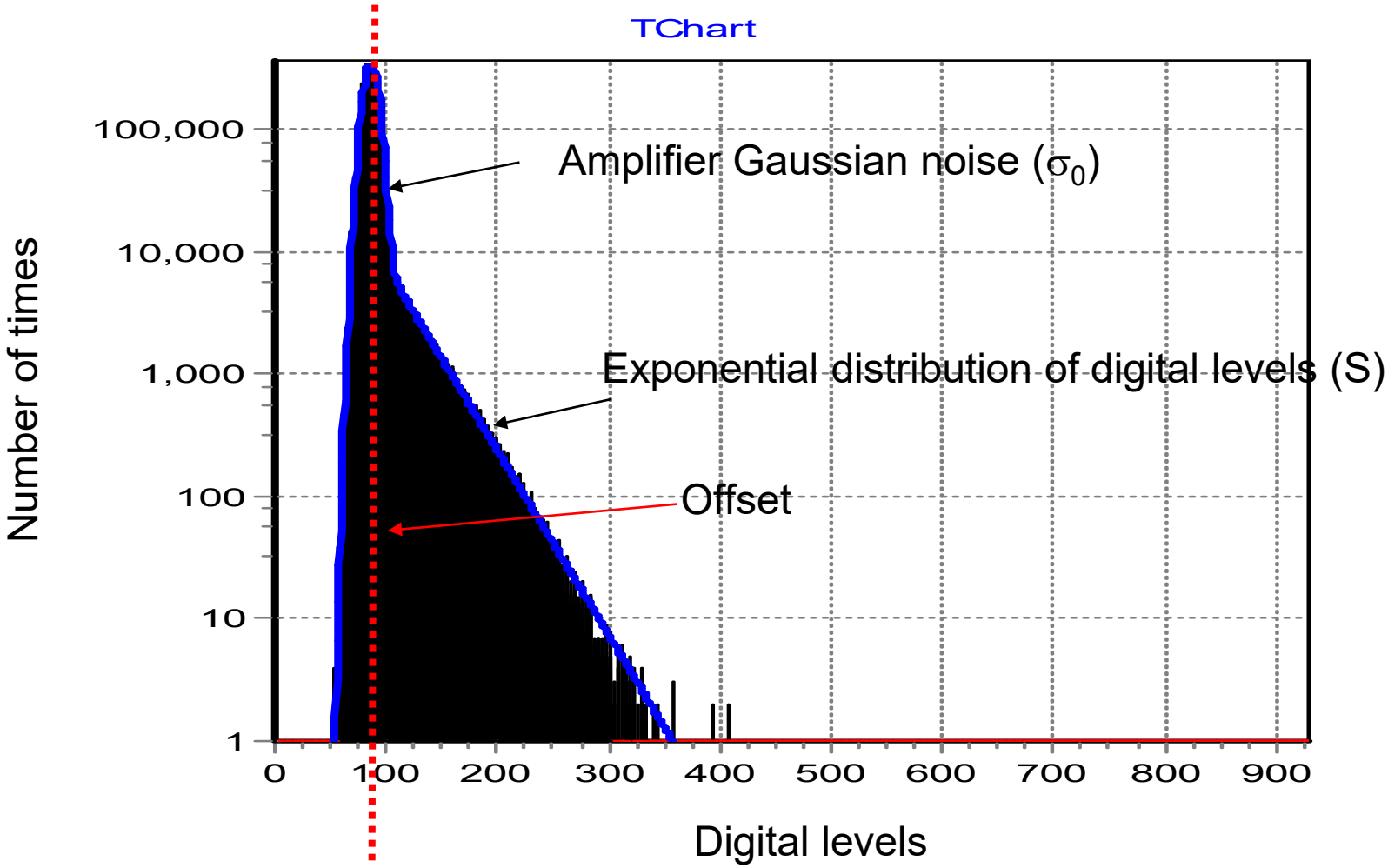
$$\sigma_d^2 = S\varepsilon n + \sigma_0^2$$

$$n = \frac{< N > B}{B - S}$$

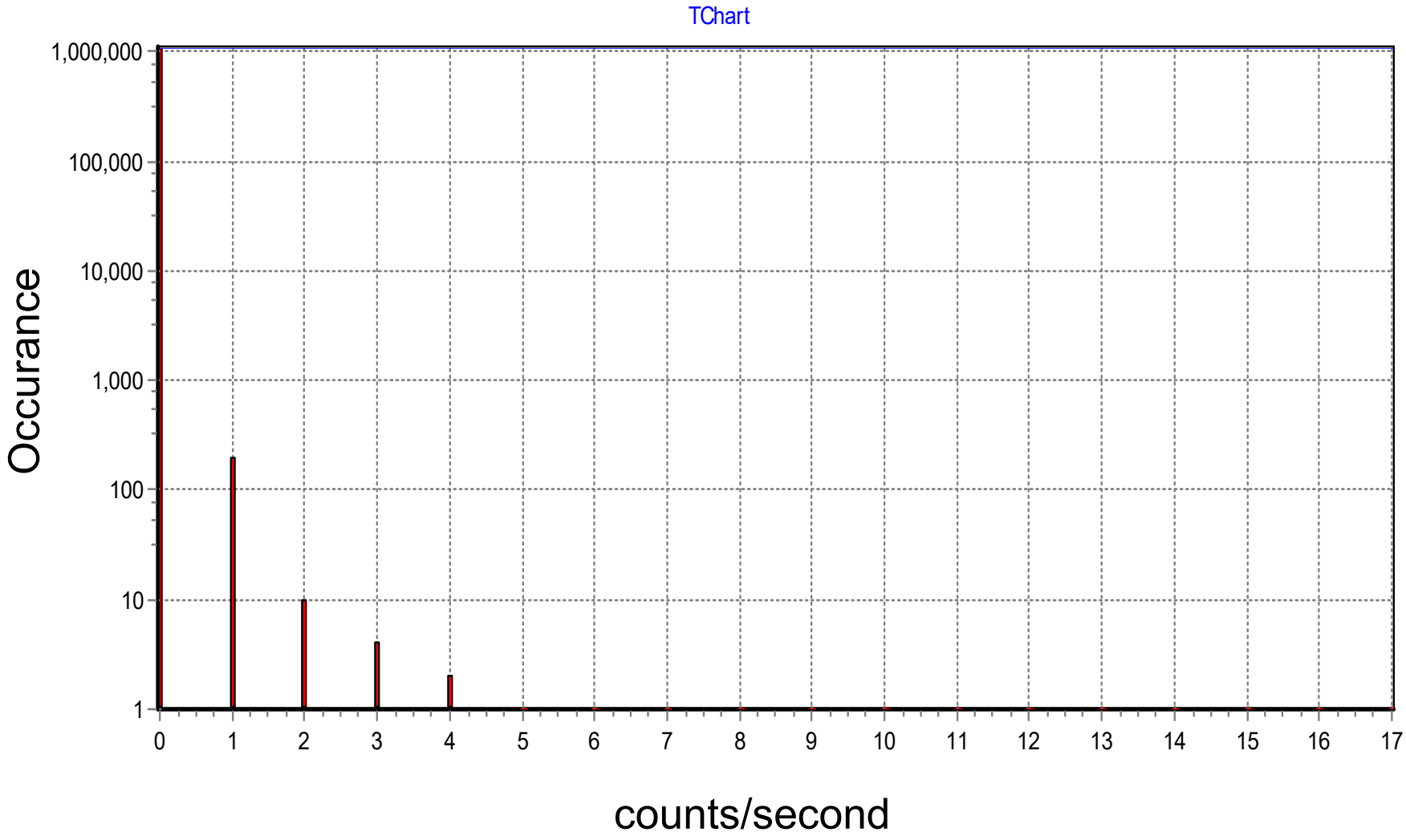
$$\varepsilon = B - S$$

If we fix the PMT settings (voltage and gain), then S and σ_0^2 should not change and need only be determined once.

Detector Characterization
Analog Detector Response (Dark Current)

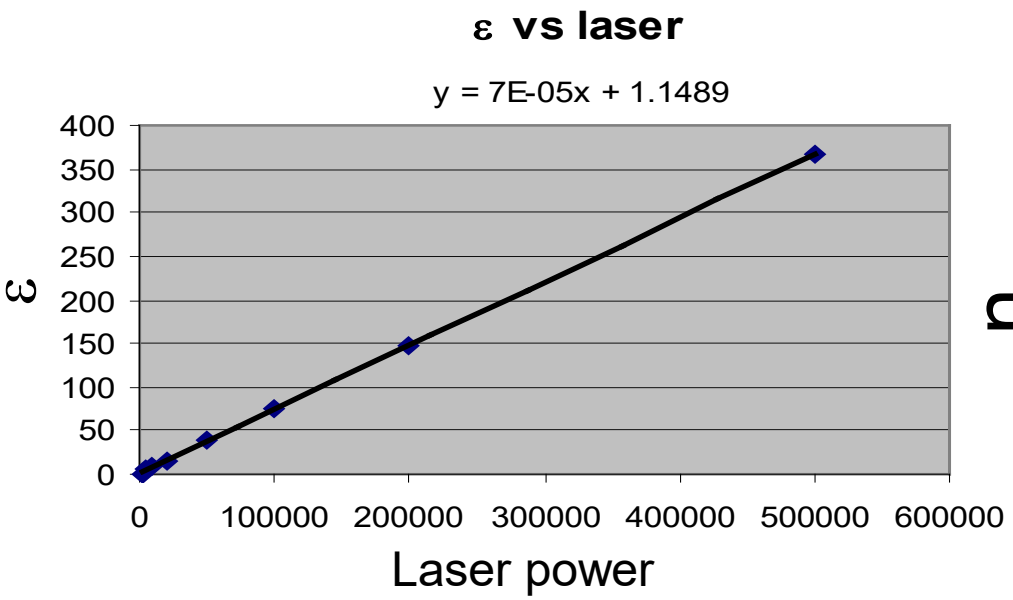


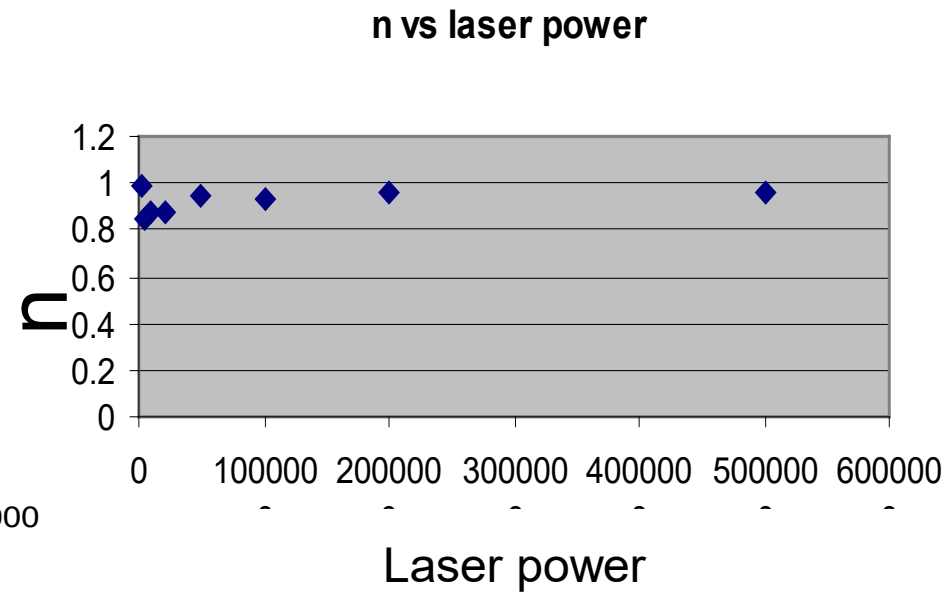
Detector Characterization
Photon Counting Detector Response (Dark Current)



Solution Experiments: Using Analog Detectors Recovery of *n* and ε in the Analog System

20 nM EGFP in solution



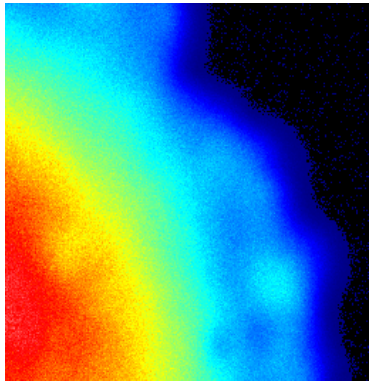


In the analog system, the recovery of relative values is good, for absolute values the calibration is more problematic. The best obtained so far is within a factor of two.

Courtesy of Valeria Vetri

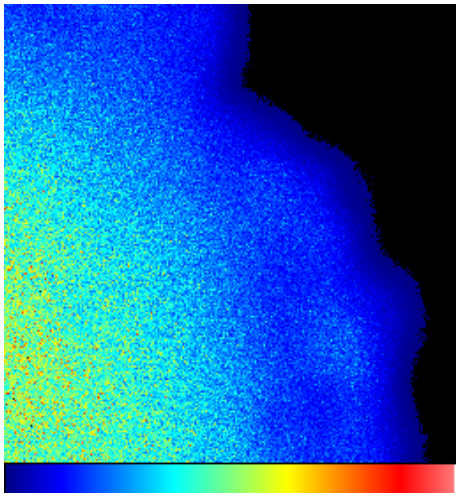
EGFP in CHO-K1 (1-Photon LSM)

Homogenous brightness & heterogeneous number of molecules:

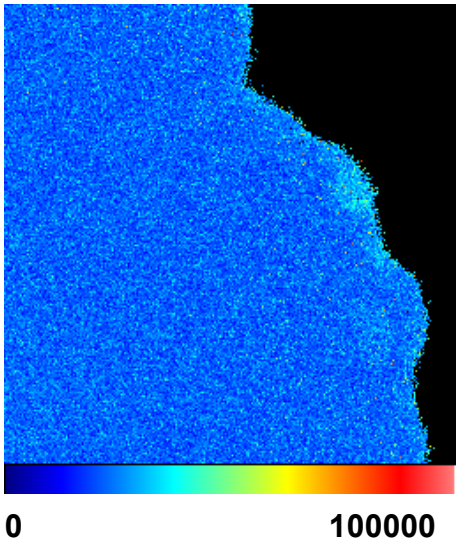


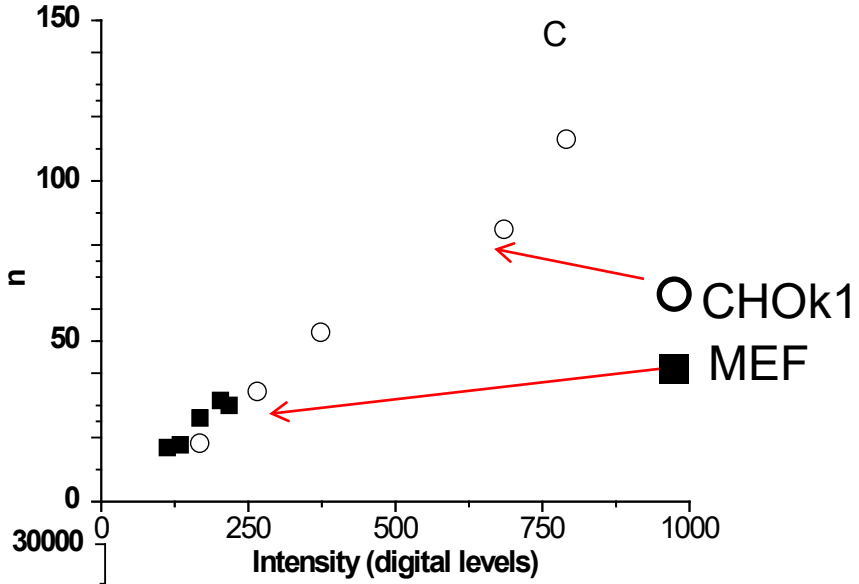
Average intensity

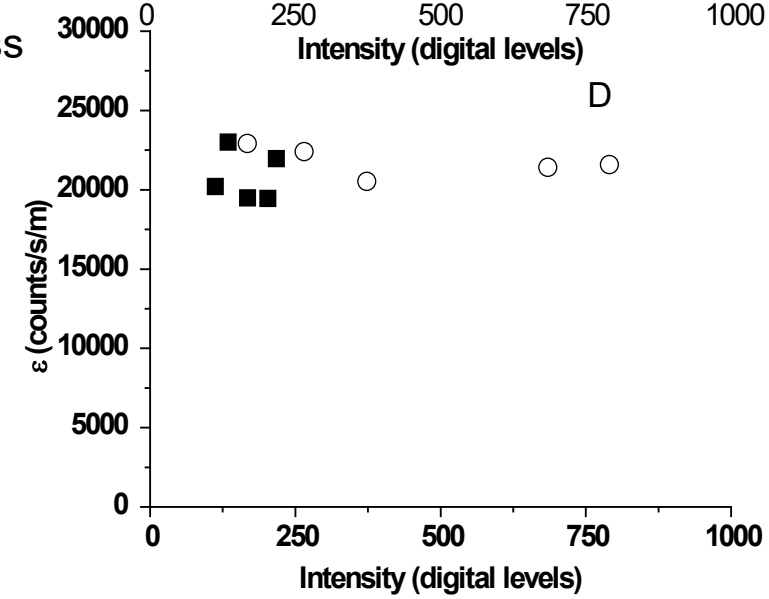
Map of number of molecules



Map of molecular brightness







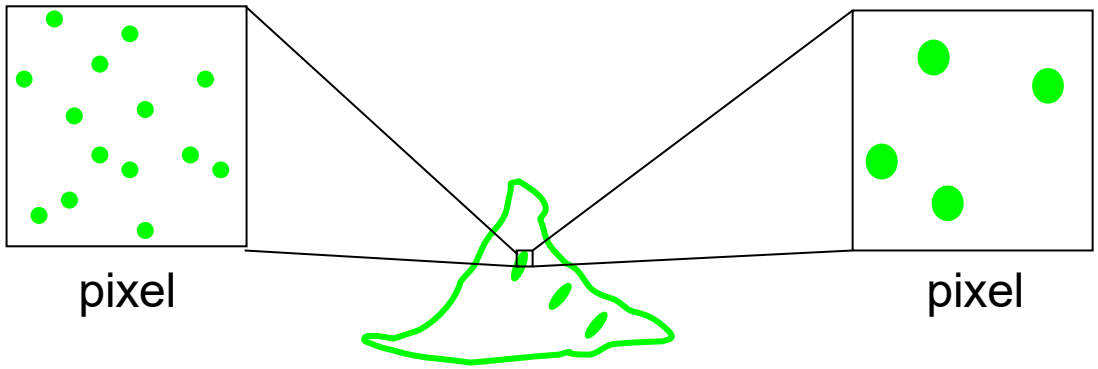
Paxillin in Live Cells

• A critical event during migration is **adhesion formation** and **disassembly**.

• **Hypothesis**: adhesions mature by increased associations.
→ This leads to increases in aggregation and protein density.

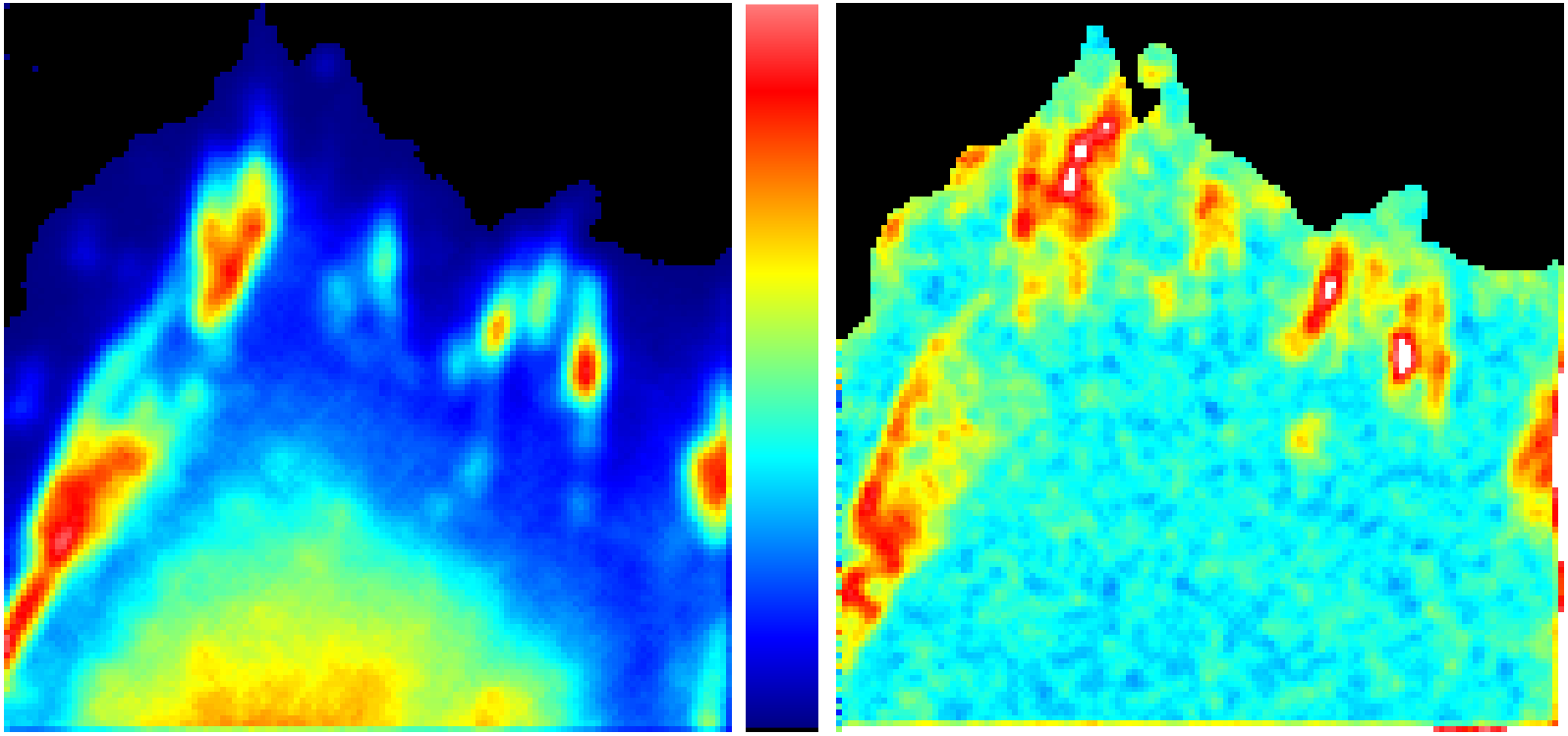
• **Goal:** Develop methods by which these features can be quantified at high spatial and temporal resolution in living cells.

• **Question:** Is the intensity in a given pixel of an image the result of many dim molecules or a few brighter aggregates?



Paxillin-mEGFP

Paxillin aggregation and dynamics

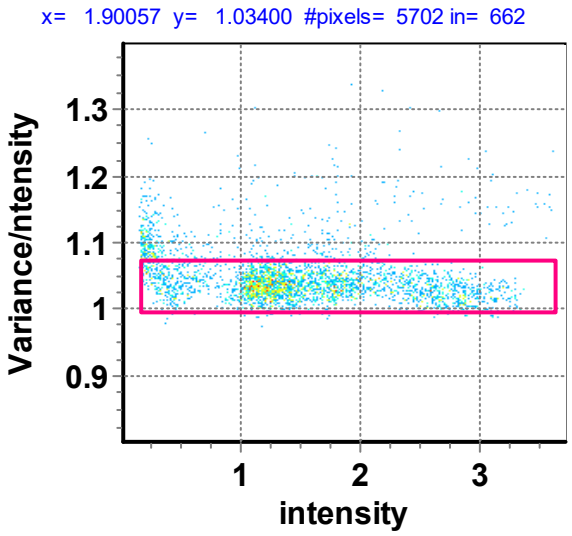


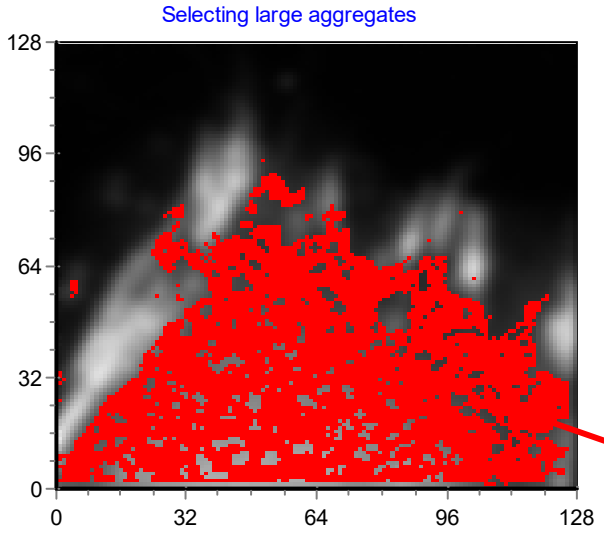
Average intensity (0-3.184c/s)

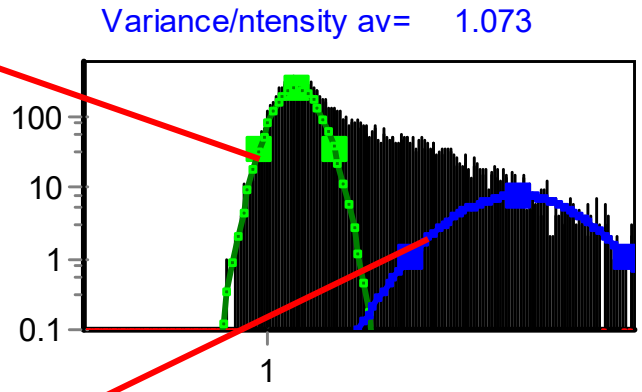
B map (1.01-1.3) (clustering)

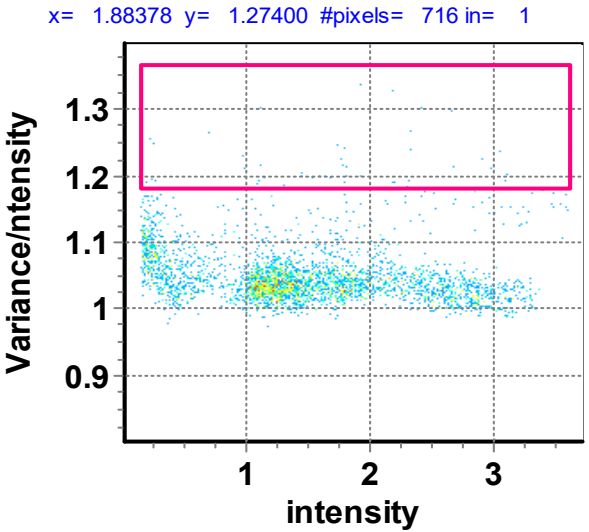
Calculated using 500 frames

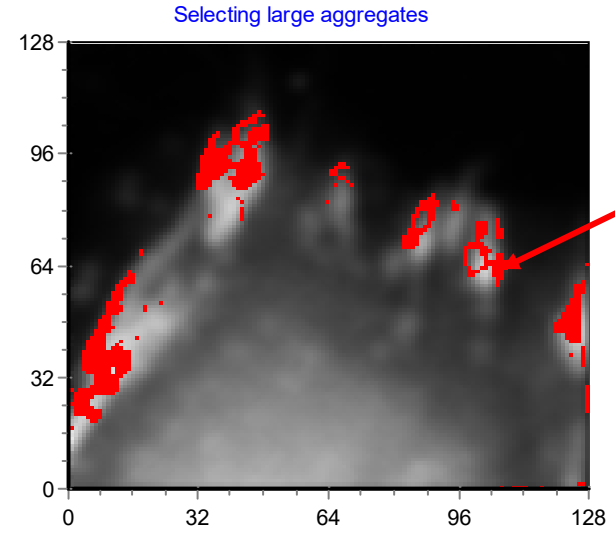
Selecting Monomers and Clusters











Green selects monomer centered at B = 1.034 ± 0.019

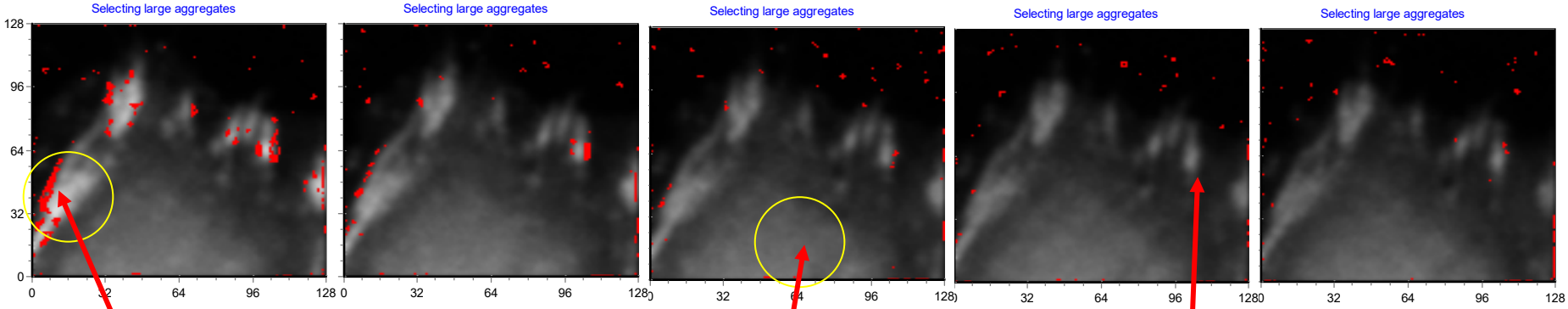
Blue selects aggregates centered at B = 1.274 ± 0.059

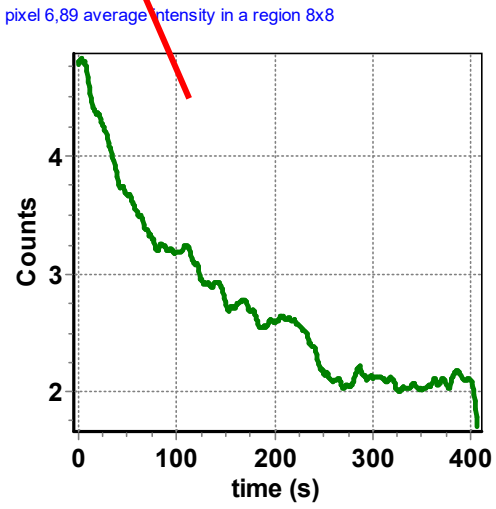
Paxillin clusters size ~8

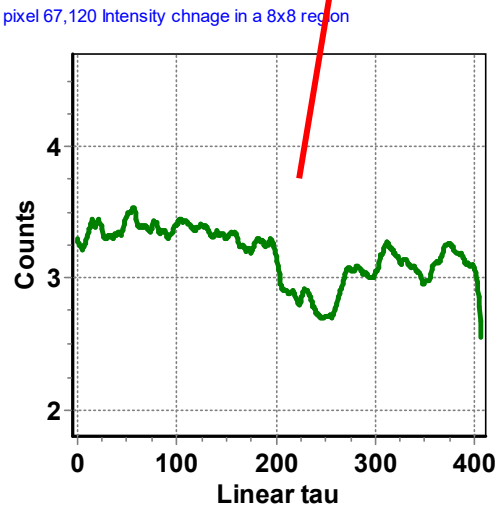
Time Sequences of Adhesions Assembling-Disassembling

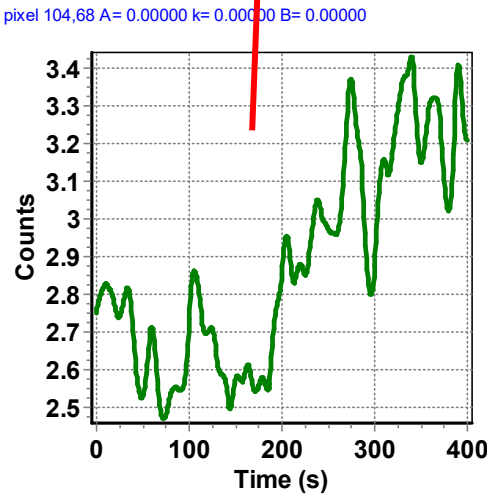
100 Frame averages

Selecting large aggregates

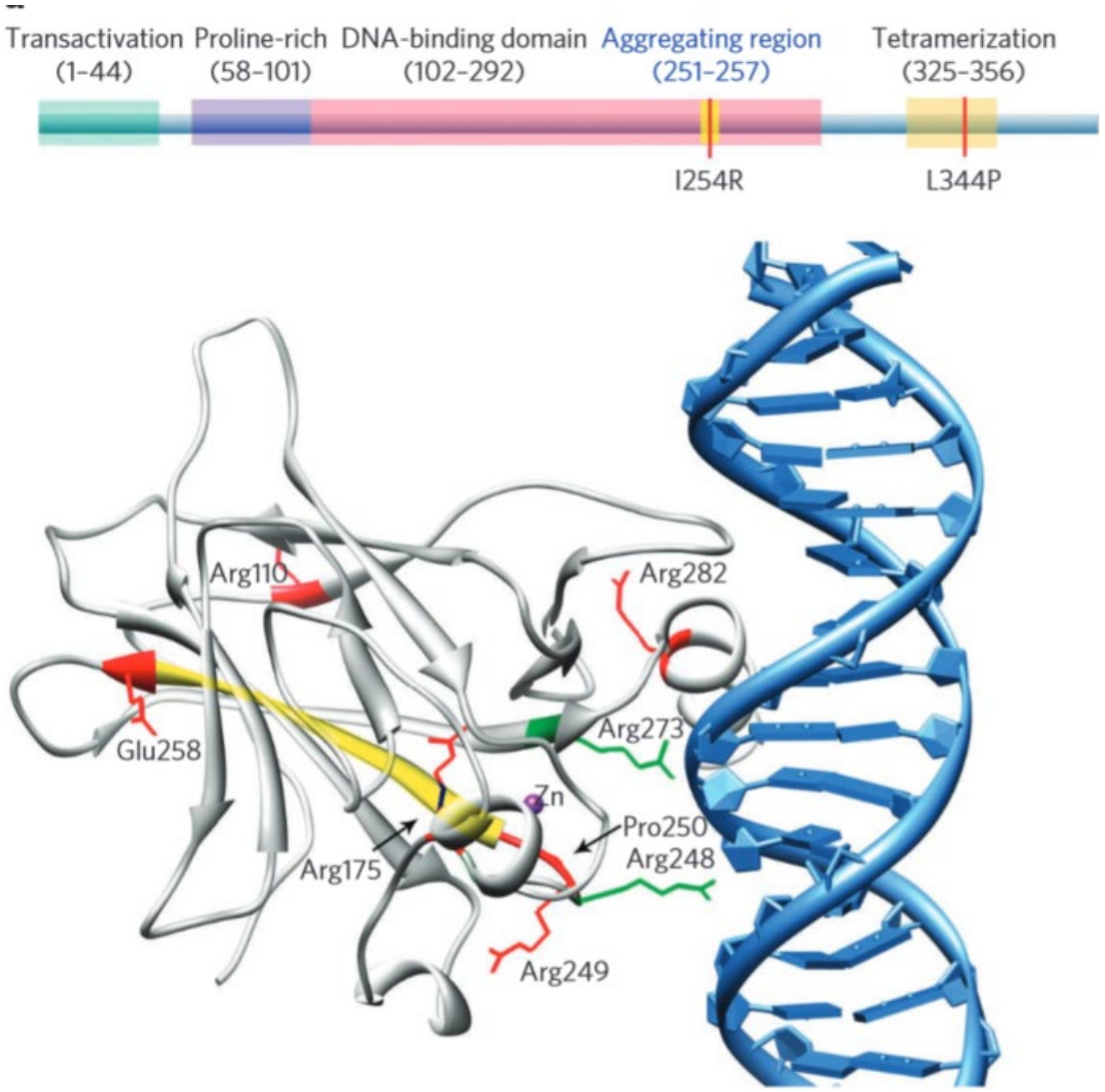








Spatio-temporal dynamics and metabolic alterations of p53 upon DNA damage

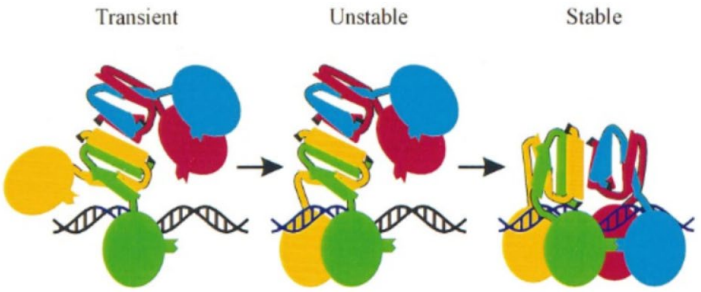


How is p53 activated?

- Under unstressed conditions, p53 exists at low concentrations in a monomeric state or in a dimeric state (or both).

- The protein functions most efficiently as a tetramer because tetramers have a higher binding affinity for DNA.

- Tetramerization-deficient p53 mutants exhibit much lower affinities for DNA than the wild-type protein (p53-WT).

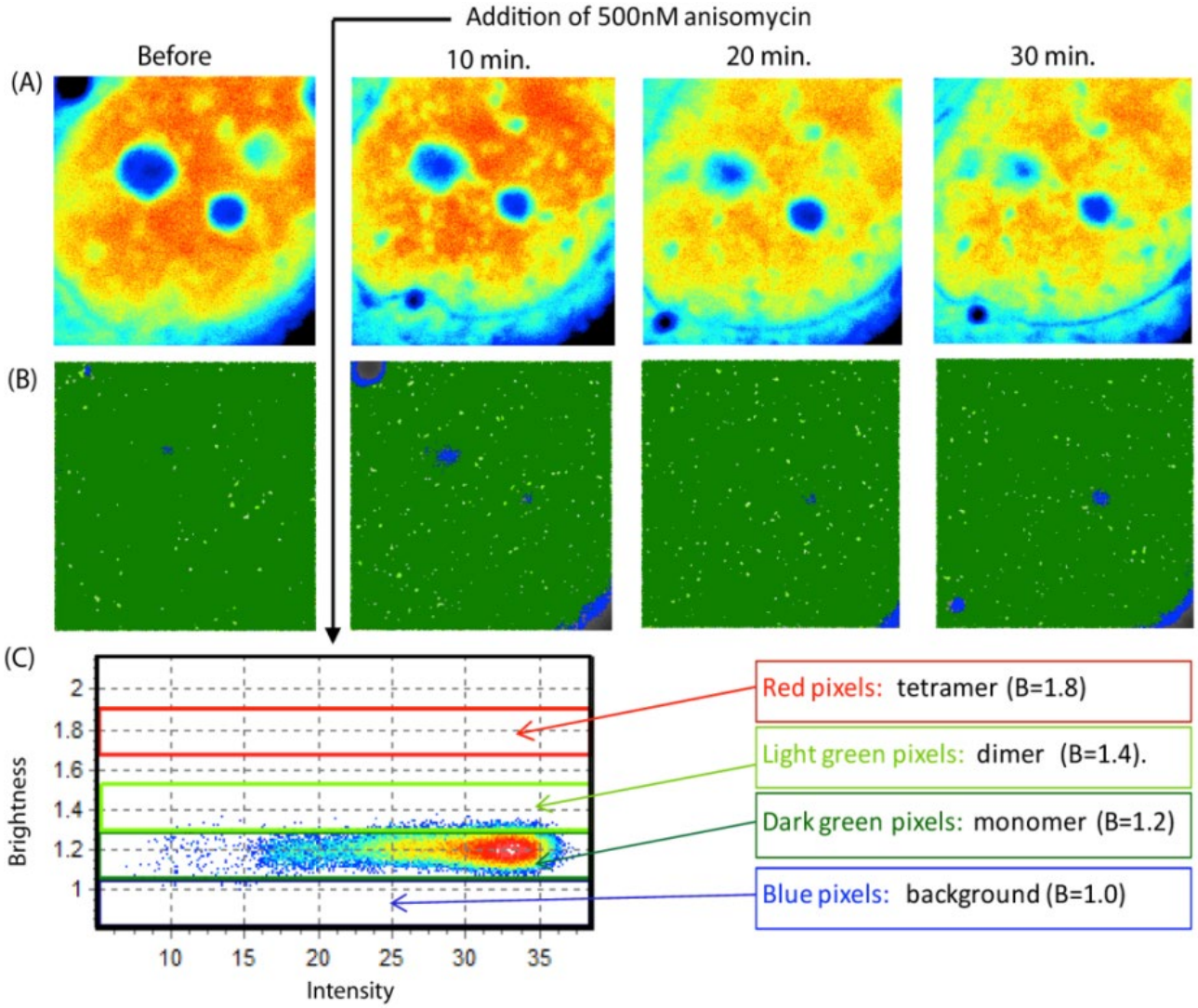


- DNA damag st-
translationa ation

Advanced Imaging technologies used to map p53 activity:

- Upon cell stress, how long does it take p53 to be activated?
  - Using standard confocal imaging we can calculate this time but how do we know its' activated?
- Can we provide real-time maps of p53 activation and concentration of protein expression?
  - Using standard confocal techniques we can calculate the molecular brightness and concentration
    - Number and Molecular Brightness
- At the binding region what are the overall time rates of p53 binding?
  - Using Line scanning we can calculate the binding rates
    - pCF analysis and Fluorescence Diffusing Tensor Imaging
- Can we predict if p53 will active the DNA response pathway or active cellular death?
  - Using fast imaging (SPIM, LSM880)
  - The Phasor approach to FLIM

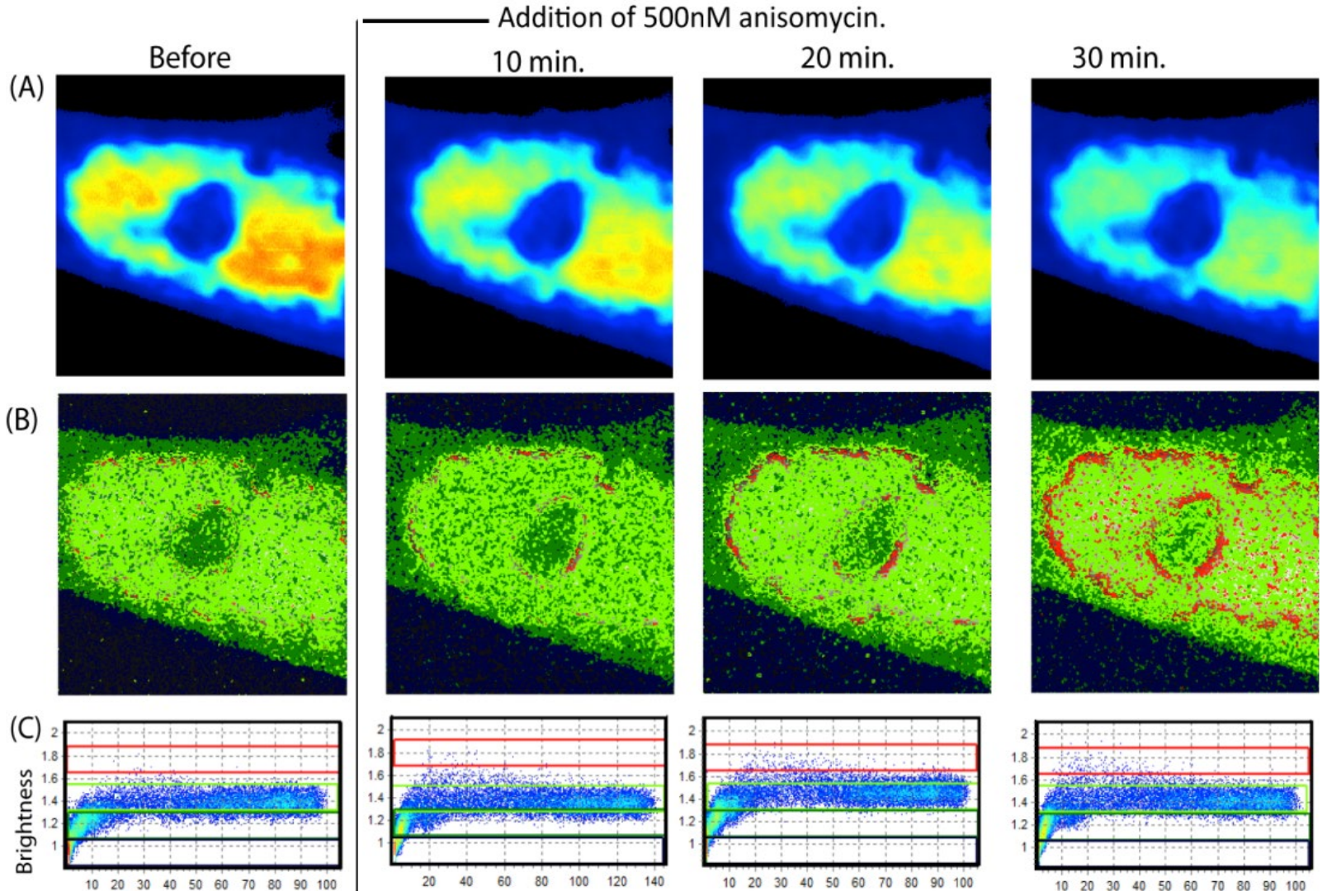
N&B: Treatment with Anisomyocin (protein synthesis inhibition) EGFP Control



Taken by Liz Hinde

N&B: Treatment with Anisomyocin

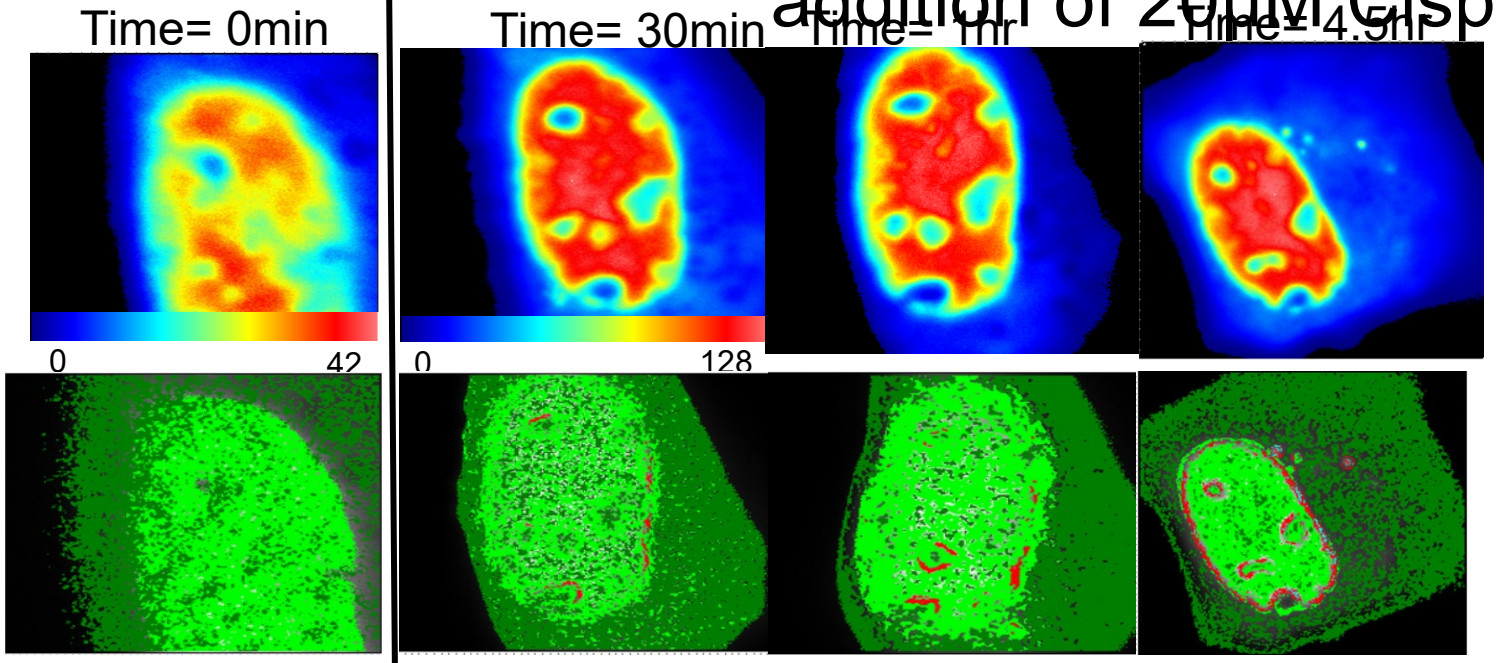
(protein synthesis inhibition) revels dimers & tetramers

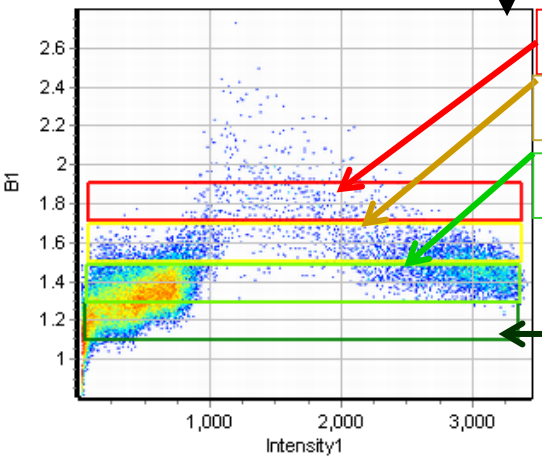


Taken by Liz Hinde

N&B: p53-EGFP aggregation with cisplatin reveals dimers and tetramers

addition of 20μM Cisplatin





Red Pixels: tetramers (B=1.8)

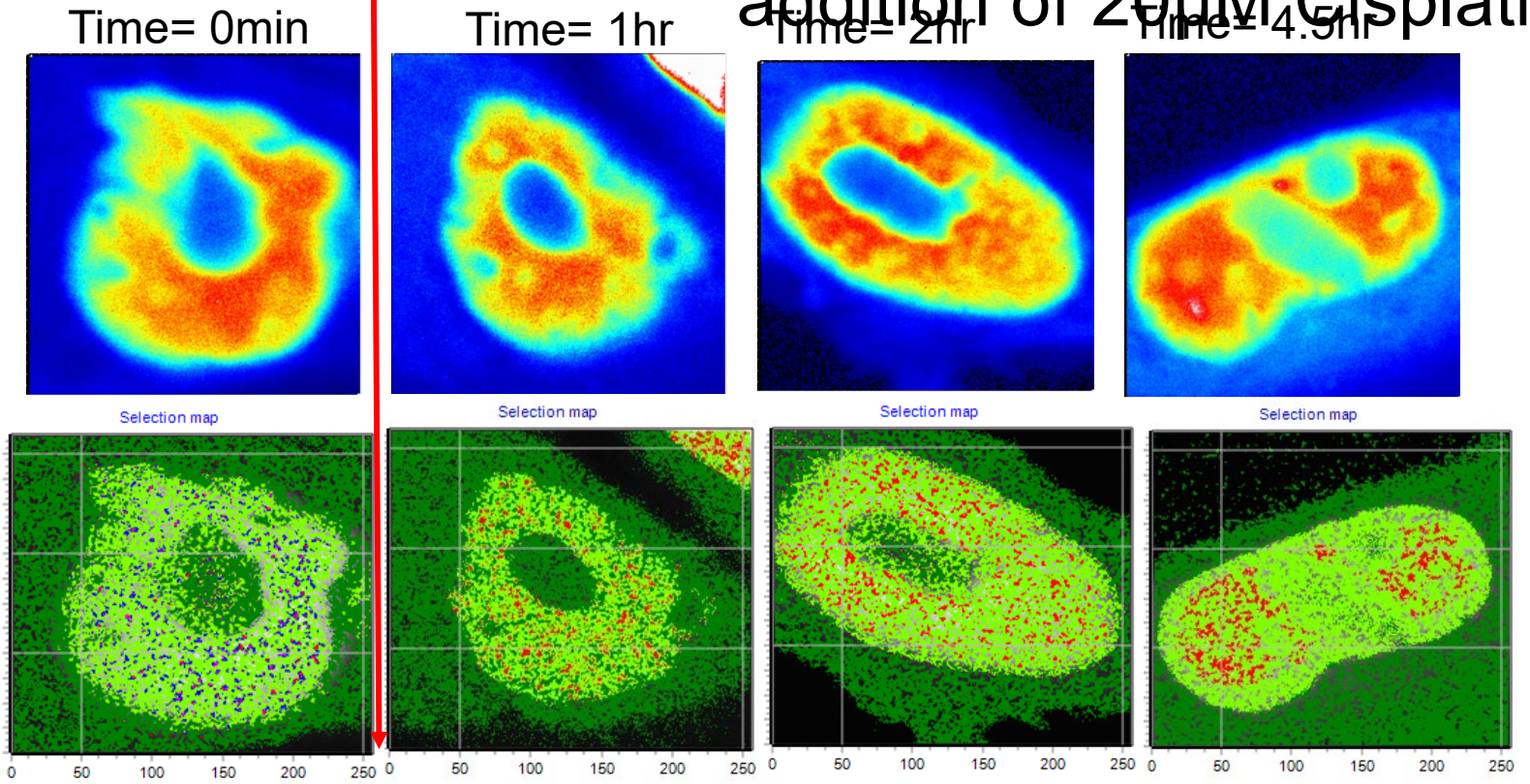
Yellow Pixels: trimers (B=1.6)

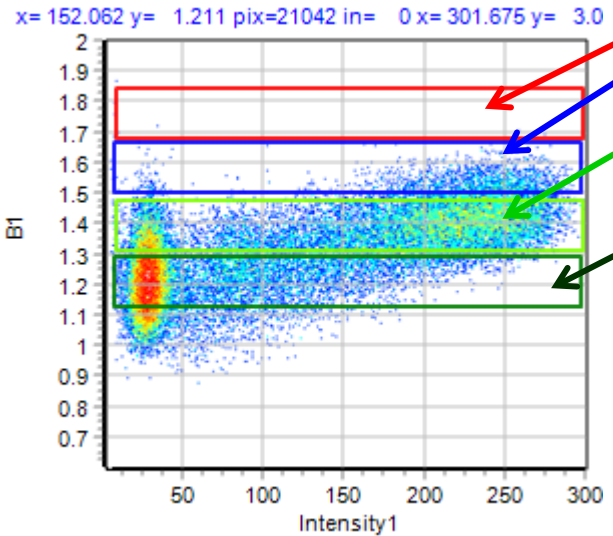
Light Green Pixels: dimers (B=1.4)

Dark Green Pixels: monomers (B=1.2)

	N	Concentration (μM)
Time=0	12	0.32
Time=30min	63	1.6
Time=1hr	47	1.1
Time=4.5hr	85	2.8

addition of 20uM Cisplatin





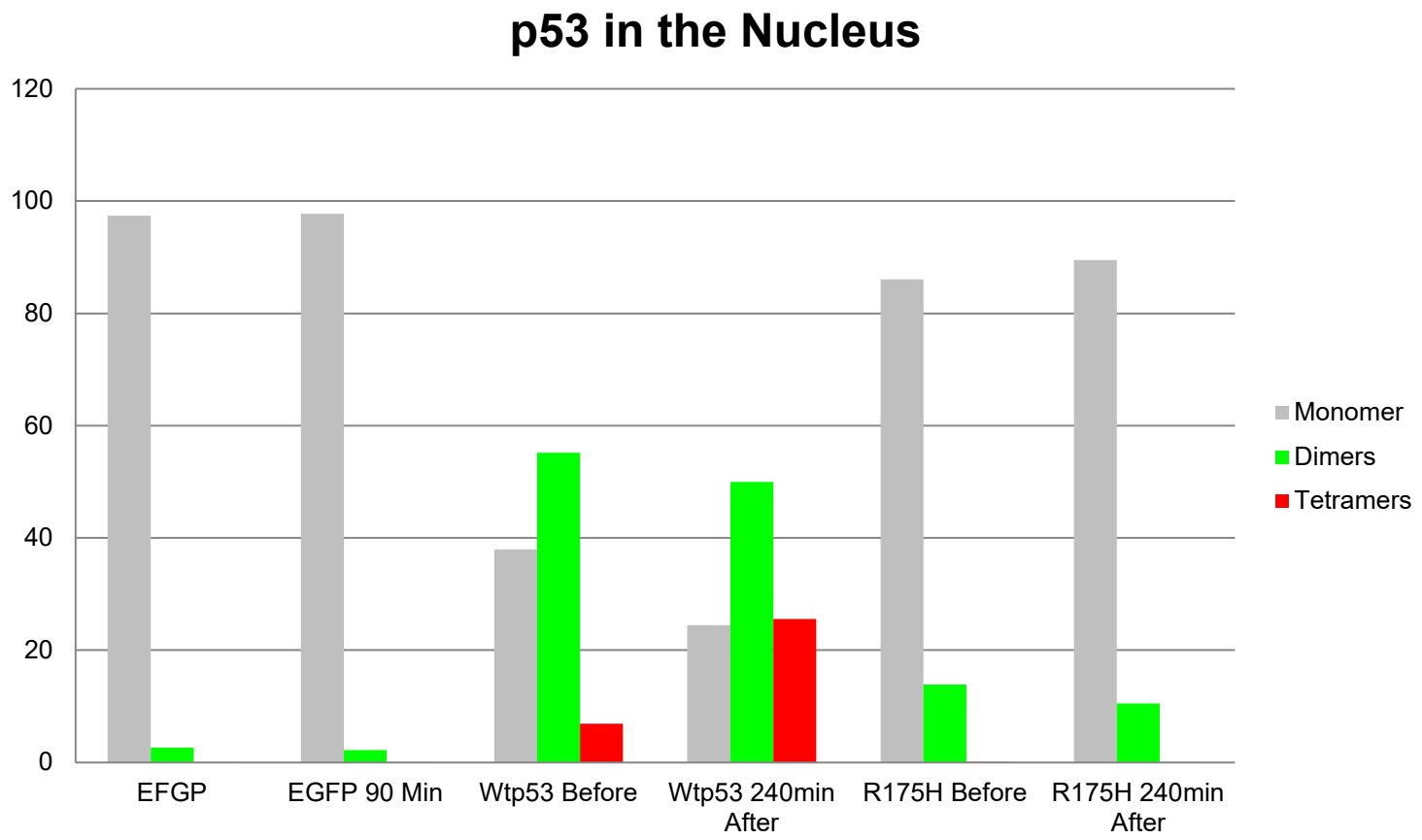
Red Pixels: tetramers (B=1.8)

Blue Pixels: trimers (B=1.6)

Light Green Pixels: dimers (B=1.4)

Dark Green Pixels: monomers (B=1.2)

Fraction of monomer, dimers and tetramers



Cross-RICS
and
Cross-N&B

UCIrvine
University of California, Irvine

dbc

LFD

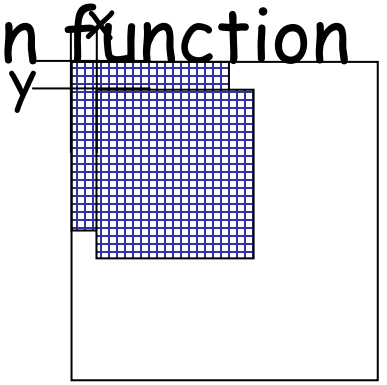
National Center for Research Resources

Cell Migration Consortium

We have expanded the RICS methods to do Cross-Correlation RICS (ccRICS)

The ccRICS approach

The spatial correlation function



$$G_{ccRICS}(\xi,\psi) = \frac{< I_1(x,y) I_2(x+\xi, y+\psi) >}{< I_1(x,y) >< I_2(x,y) >} - 1$$

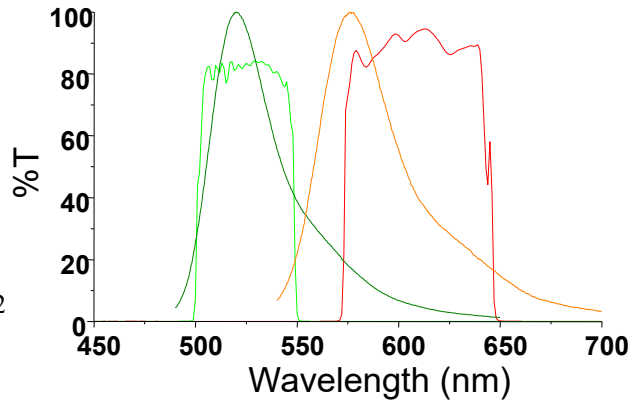
The variables ξ and ψ represent spatial increments in the x and y directions, respectively

The $G_{cc}(0,0)$ value and bleedthrough

$$G_{cc}(0,0) \propto \left[\frac{f_{11} f_{12} \langle N_1 \rangle + f_{21} f_{22} \langle N_2 \rangle}{f_{11} f_{12} \langle N_1 \rangle^2 + (f_{11} f_{22} + f_{21} f_{12}) \langle N_1 \rangle \langle N_2 \rangle + f_{21} f_{22} \langle N_2 \rangle^2} \right]$$

Ch1

$$F_1(t) = f_{11} N_1 + f_{21} N_2$$



Ch2

$$F_2(t) = f_{12} N_1 + f_{22} N_2$$

Experimental issues

- The volume of excitation and emission at the two excitation wavelengths must superimpose (we are using the Olympus FV1000 LSCM for these experiments)
- Bleedthrough of the green into the red channel must be small (<5%)
- FRET will strongly decrease the ccRICS signal
- High ratio of labeled to unlabeled molecules are needed (if you have only 10% labeled, in a complex of 1:1, you will only have 1% of the complexes labeled with both proteins)

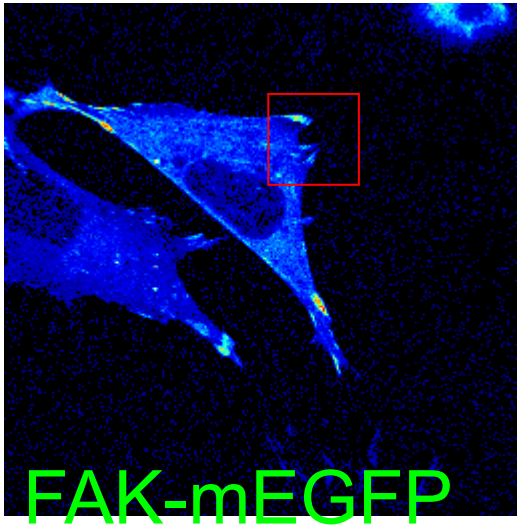
Cells. MEF transfected Vinculin, FAK and paxillin. cDNA were ligated to EGFP or mCherry at the C-terminal end.

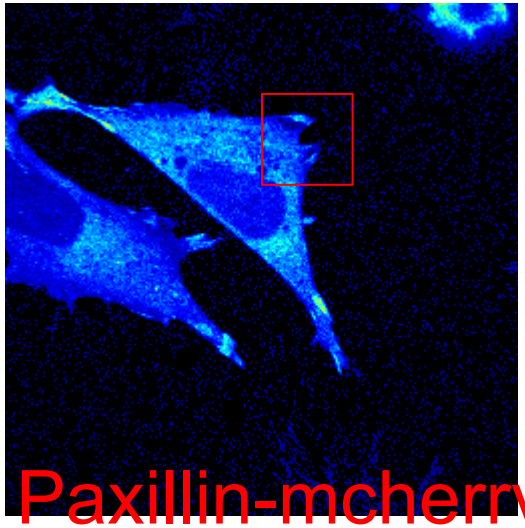
Microscopy. Olympus FV1000 with 60x 1.2NA water objective, 12.5 us/pixel, 256x256 pixels 12.5 µm square, 100 to 200 frames collected for each sample. 1frame/s.

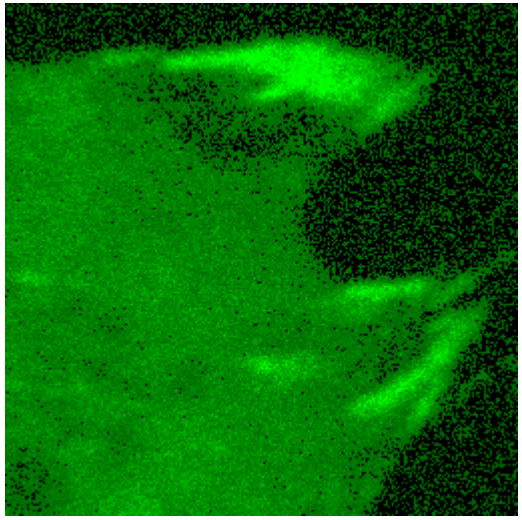
EGFP excitation at 488nm (0.5%) and mCherry at 559nm (adjusted to a max of 1.5%).
Emission filters at 505-540nm and 575-675 nm, for the green and red channels, respectively.

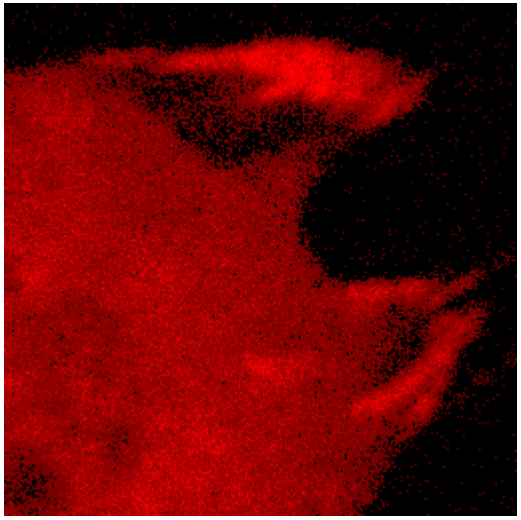
Overlap of the volume of observation was tested by imaging single 100 nm fluorescent beads carrying two colors simultaneously

Does paxillin bind to other proteins before and/or after assembly or disassembly of the focal adhesion?

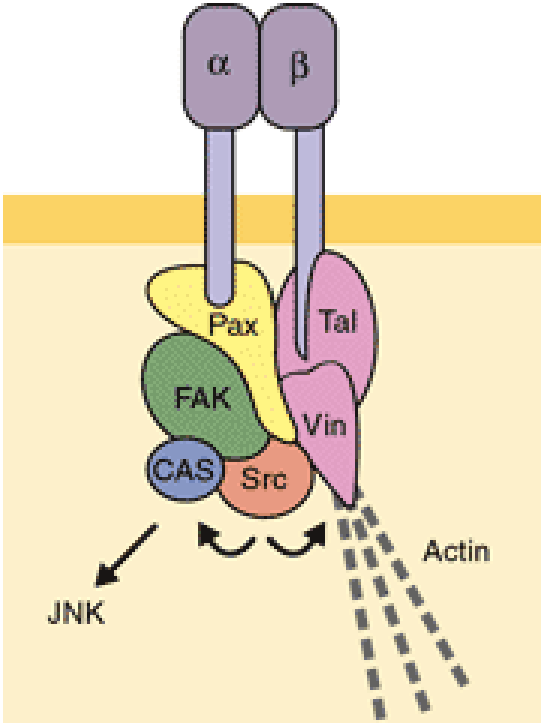






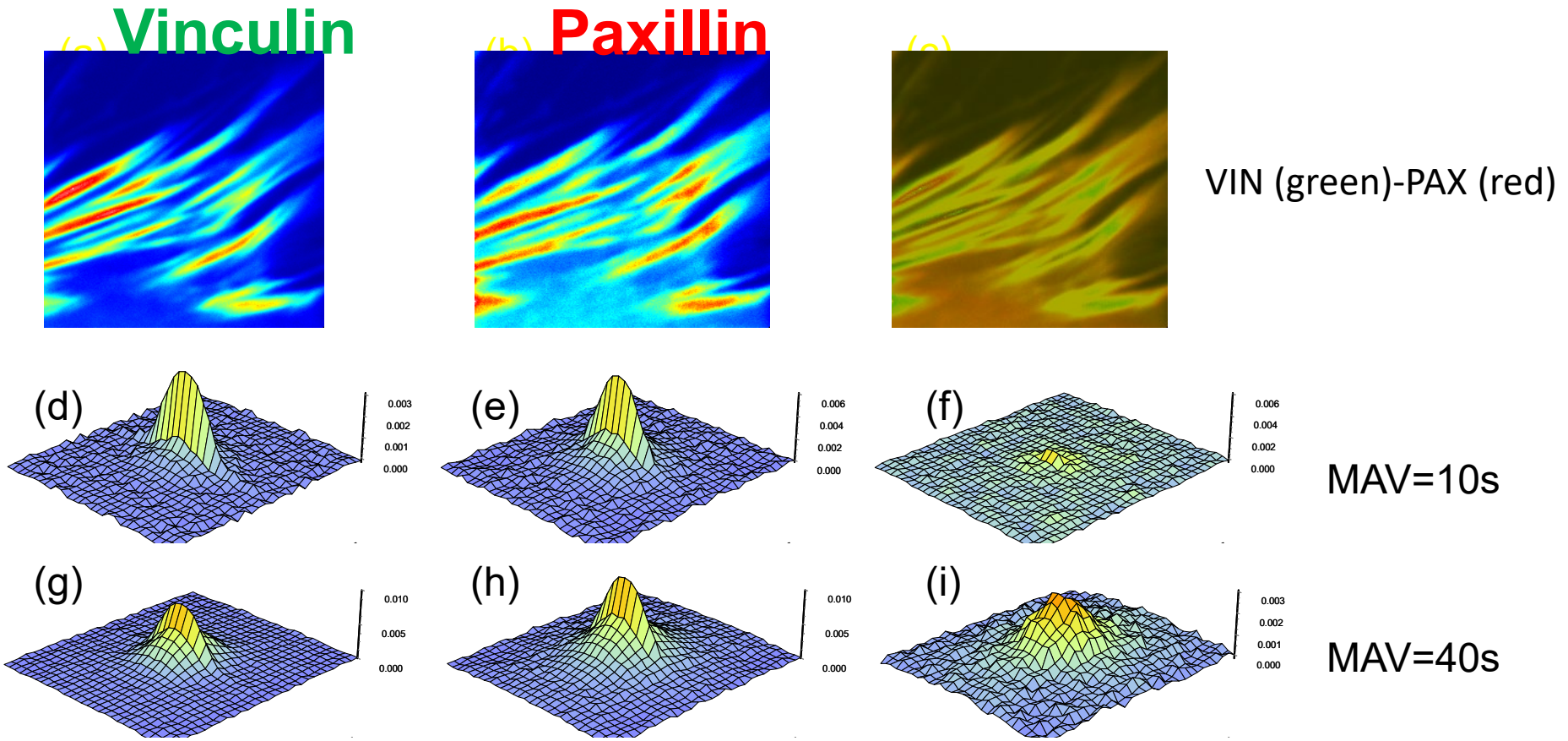


FAK
and
Vinculin



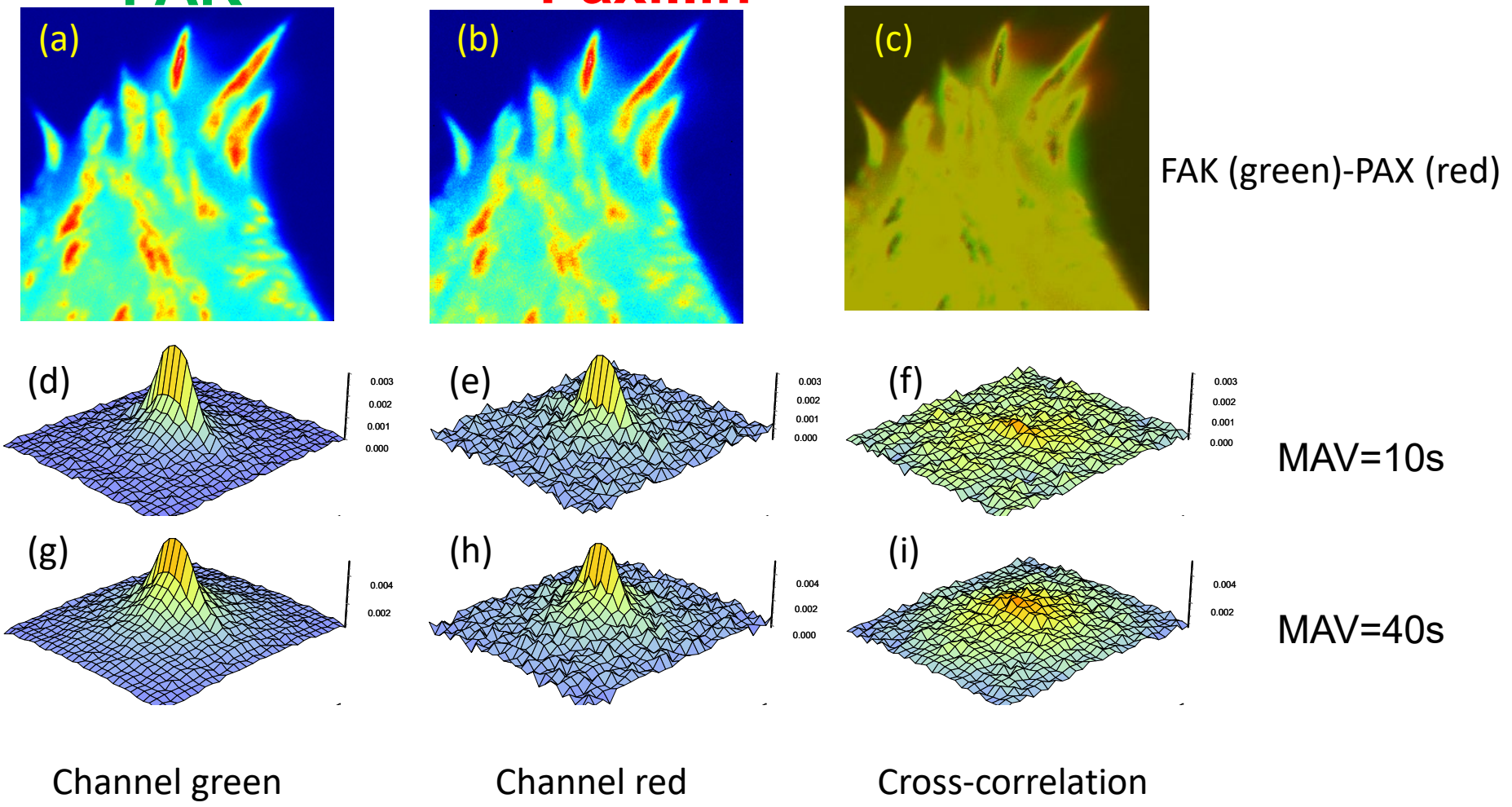
http://student.biology.arizona.edu/honors2001/group08/intro/integrin-actin2.jpg

Vinculin and Paxillin co-localize at adhesions but they are moving independently in the cytoplasm



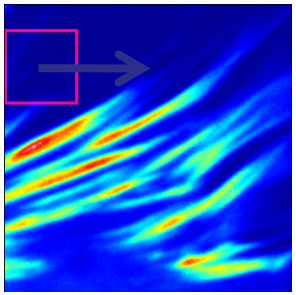
The cross-correlation increases for the slow fluctuations (at MAV=40s). It is round in shape indicating that it is generated at single locations.

FAK and Paxillin co-localize at adhesions but they are moving independently in the cytoplasm



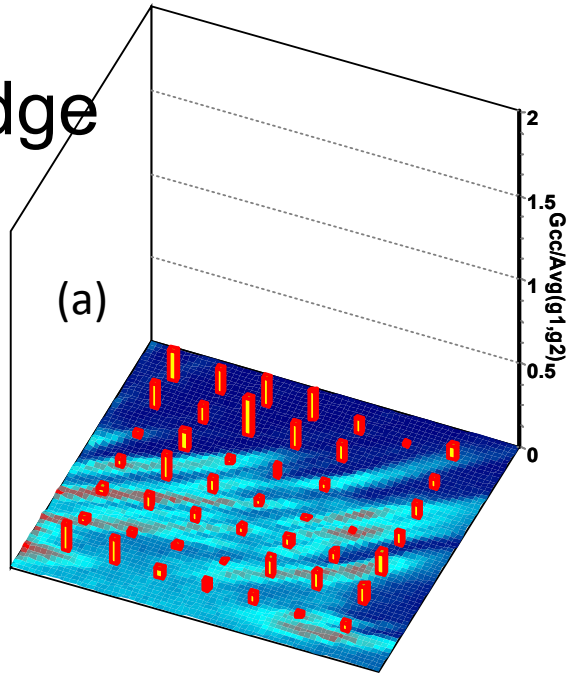
The cross-correlation increases for the slow fluctuations (at MAV=40s). It is round in shape indicating that it is generated at single locations and it is very small.

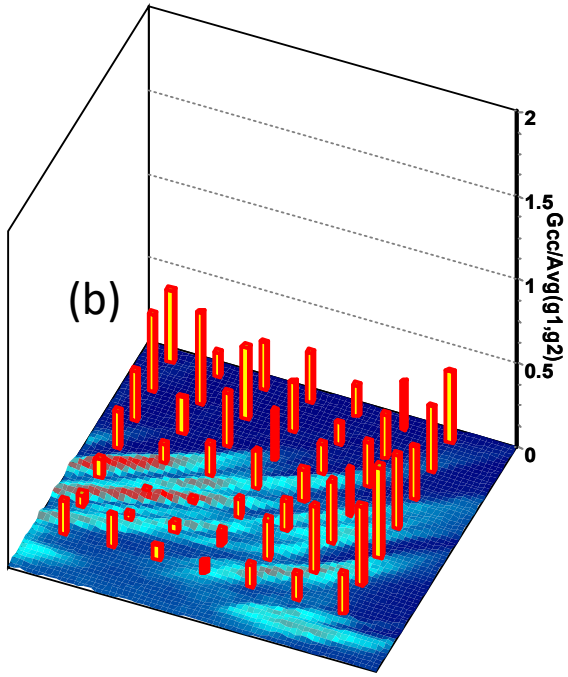
Distribution of fraction of cross-correlation in the cell. Correlation with adhesion disassembling



ccRICS by scanning a region of interest across the image
Calculating the ratio $G_{cc}/AV(G_1,G_2)$

Cell edge





VIN-PAX MAV=10s

VIN-PAX MAV=40s

There is "more" cross-correlation at the locations of adhesion disassembling

Digman, M.A. et al. *Biophys J.* 2009 Jan;96(2):707-16

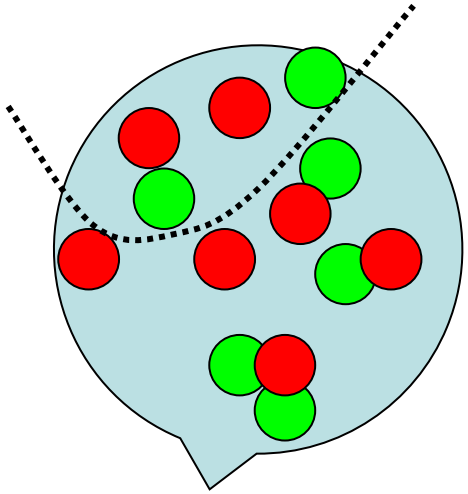
Summary of ccRICS

- We developed a **toolbox** for biophysicists and cell biologists to address common questions regarding the formation of protein complex, their spatial distribution and their stoichiometry

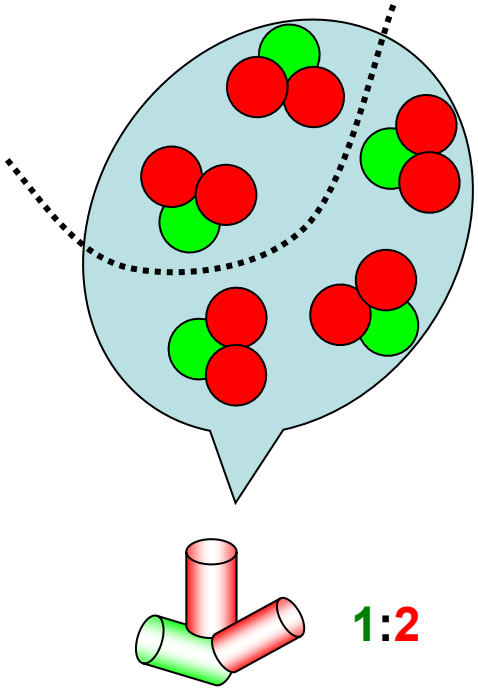
- **ccRICS** is extremely powerful at detecting joint diffusing proteins and in separating diffusion from binding processes

- The Paxillin, vinculin and FAK never crosscorrelate in the cytoplasm before binding to the focal adhesion. We only detect cross correlation due to dissociation of large clusters of proteins.

What is the stoichiometry of these clusters and is this stoichiometry crucial for the biological system?



Random



1:2

Cross N&B

The co-variance principle and the derivation of the ccN&B method

$$\sigma_{cc}^2 = \frac{\sum (G_i - < G >)(R_i - < R >)}{K}$$

Definition of **co-variance**. It is the average of product of the fluctuations in the Green and Red channel

$$N_{cc} = \frac{< G >< R >}{\sigma_{cc}^2}$$

Definition of the **cross-number** of molecules. It is the co-variance divided by the product of the intensity in the two channels

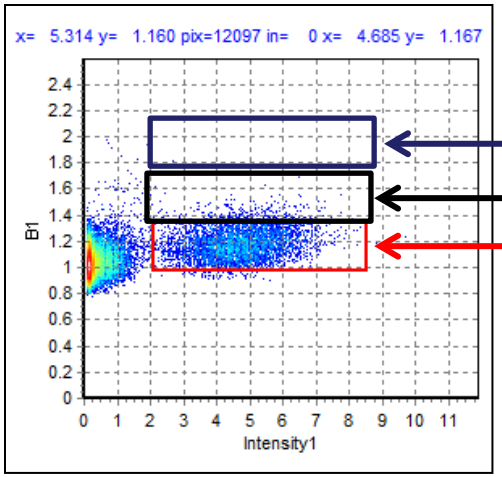
$$B_{cc} = \frac{\frac{\sum (G_i - \langle G \rangle) \times (R_i - \langle R \rangle)}{K}}{\langle G \rangle \times \langle R \rangle}$$

$$N_{cc(\,fractionofcross\,)} = \frac{\sum (G_i - \langle G \rangle) \times (R_i - \langle R \rangle)}{K}$$

K is the number of frames, σ^2 the variance and < > indicat

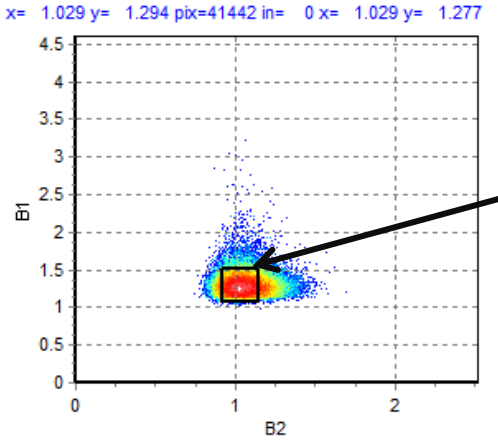
How to do the Cross-B

1. Calibrate the Molecular Brightness of each respective channel for the monomers. (Calculate the “S” factor if you are using an analog detector

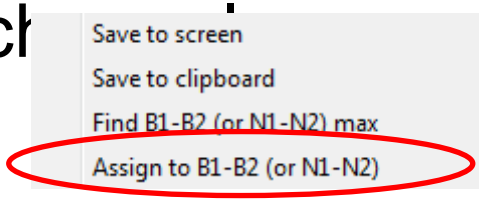


Theoretical Trimers B= 1.48
Theoretical Dimers B= 1.32
Monomer B=1.160
ε= (B-1)/pixel dwell time (sec)= 16,000

2. Plot all the possible Brightness of Ch1 and Ch2 on a B1 vs B2 plot:

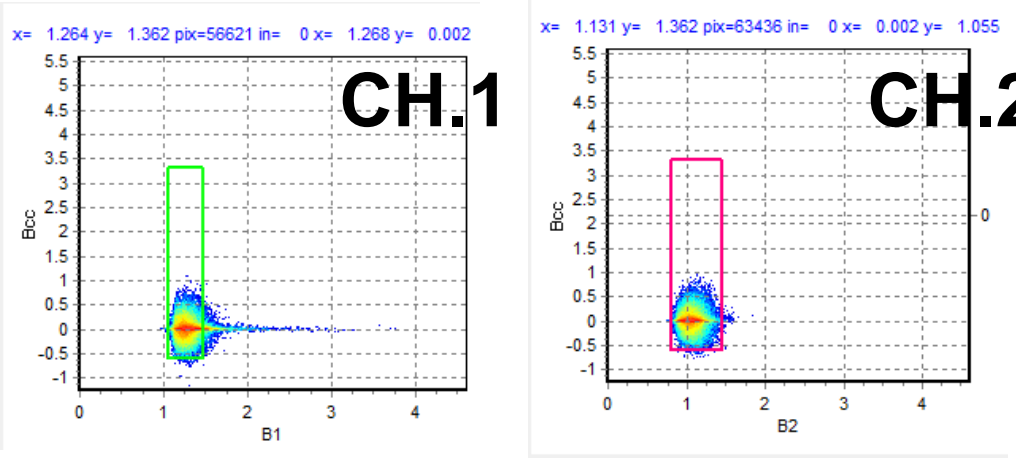


Assign these values to the Monomer
Respective ch



How to do the Cross-B Cont'

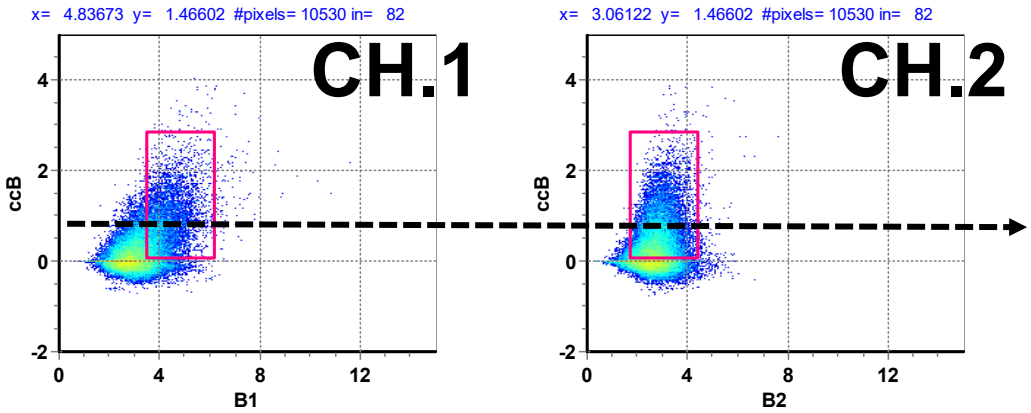
3. After selecting the B monomer values, calculate the Bcc plot As seen in CH1 and CH2



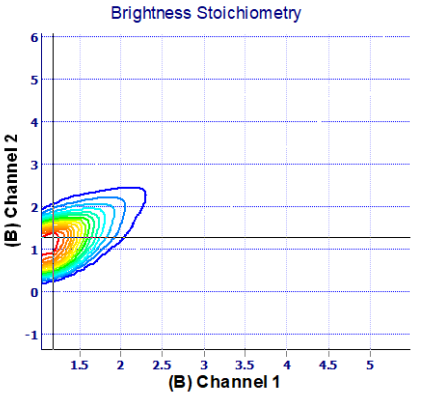
In this case the histogram of the Bcc plot is perfectly symmetrical!

3. If the plots are asymmetrical you can plot a histogram of the highest most probable complex these molecules form by "probing" the pixels that deviate from the average histogram on non-coincident brightnesses

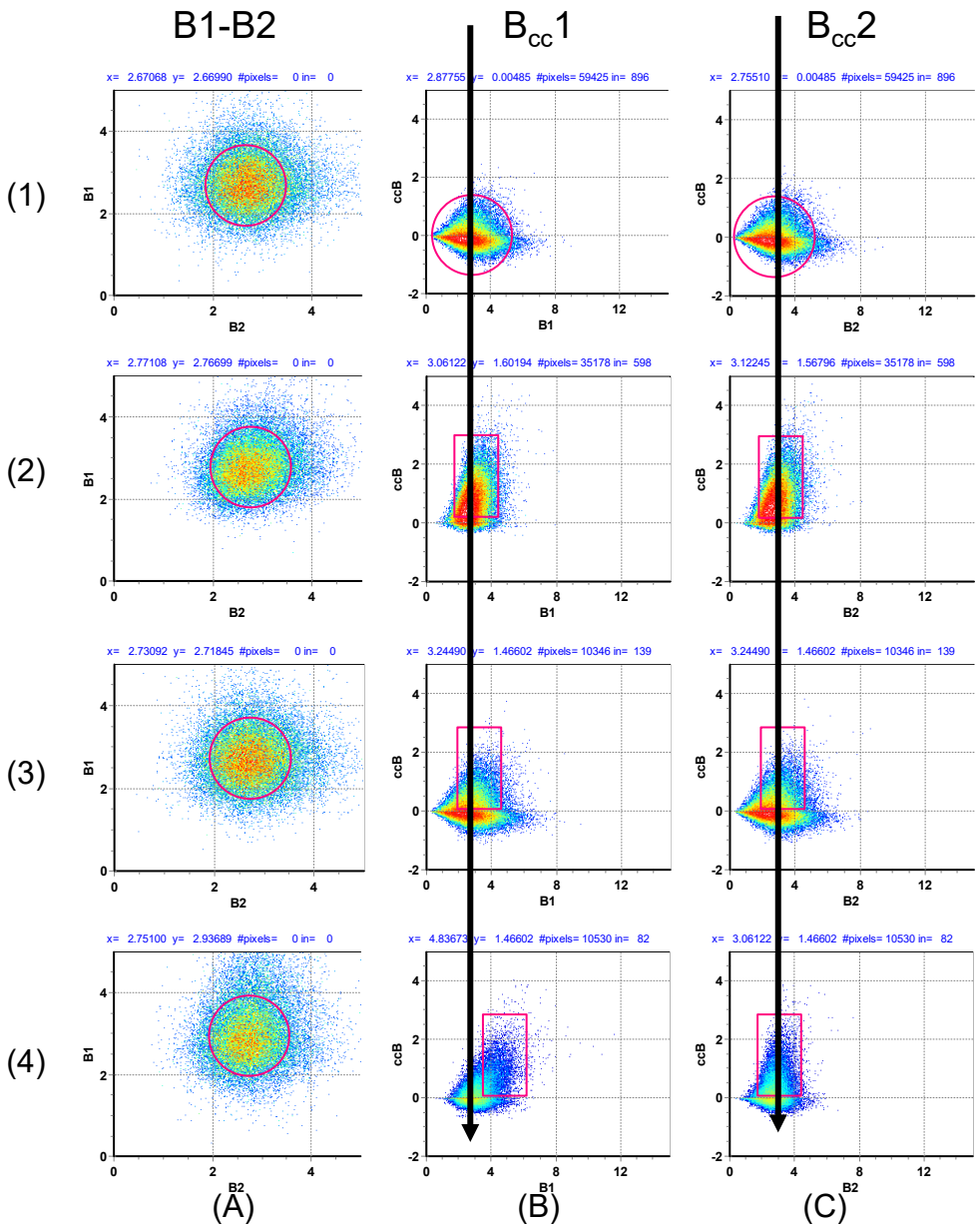
There is no Cross-variance!



"cutting the symmet



To calibrate the system we need to know the brightness of the monomers



0 % correlated

25 % correlated

5 % correlated

5 % correlated
intensity ratio 2:1

1) calibrate the monomers in both channels The lack of symmetry is due to Poissonian rather than Gaussian distribution of counts

2) Add correlated molecules (still all monomers)

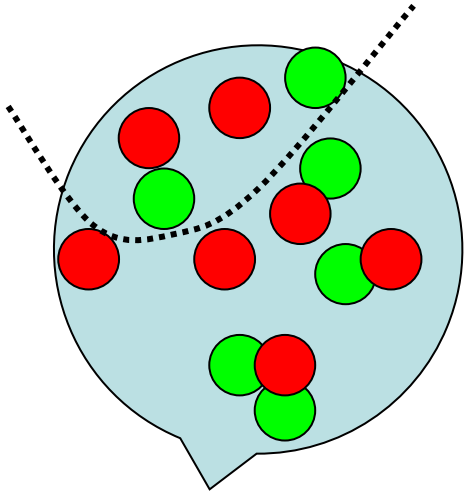
3) At 5% you can still distinguish the positive correlated fluctuations

4) Now we have 2:1 stoichiometry. We have more brightness in B1 but the same in B2

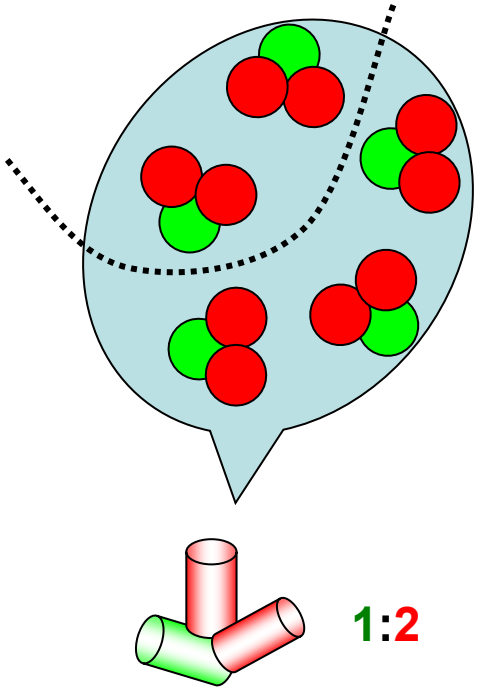
What to look for:

1) First we need to calibrate the monomers
2) We have to see if there is positive cross variance
3) We have to see where the cross variance occurs in respect to the brightness of Ch1 and Ch2

What is the stoichiometry of these clusters and is this stoichiometry at Focal Adhesions?



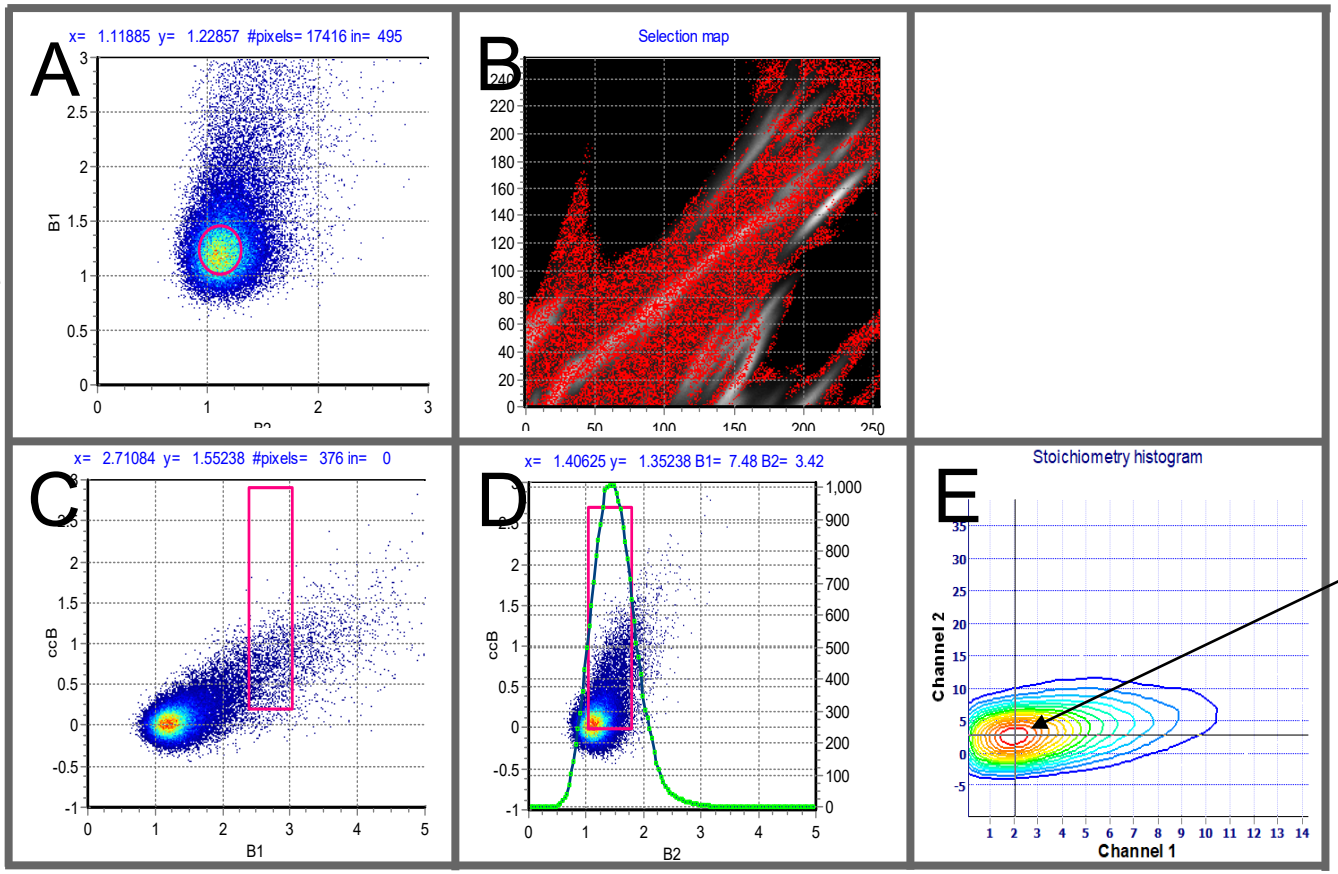
Random



Vinculin-EGFP and Paxillin-mcherry

Monomers
ε=0.118c/d/s

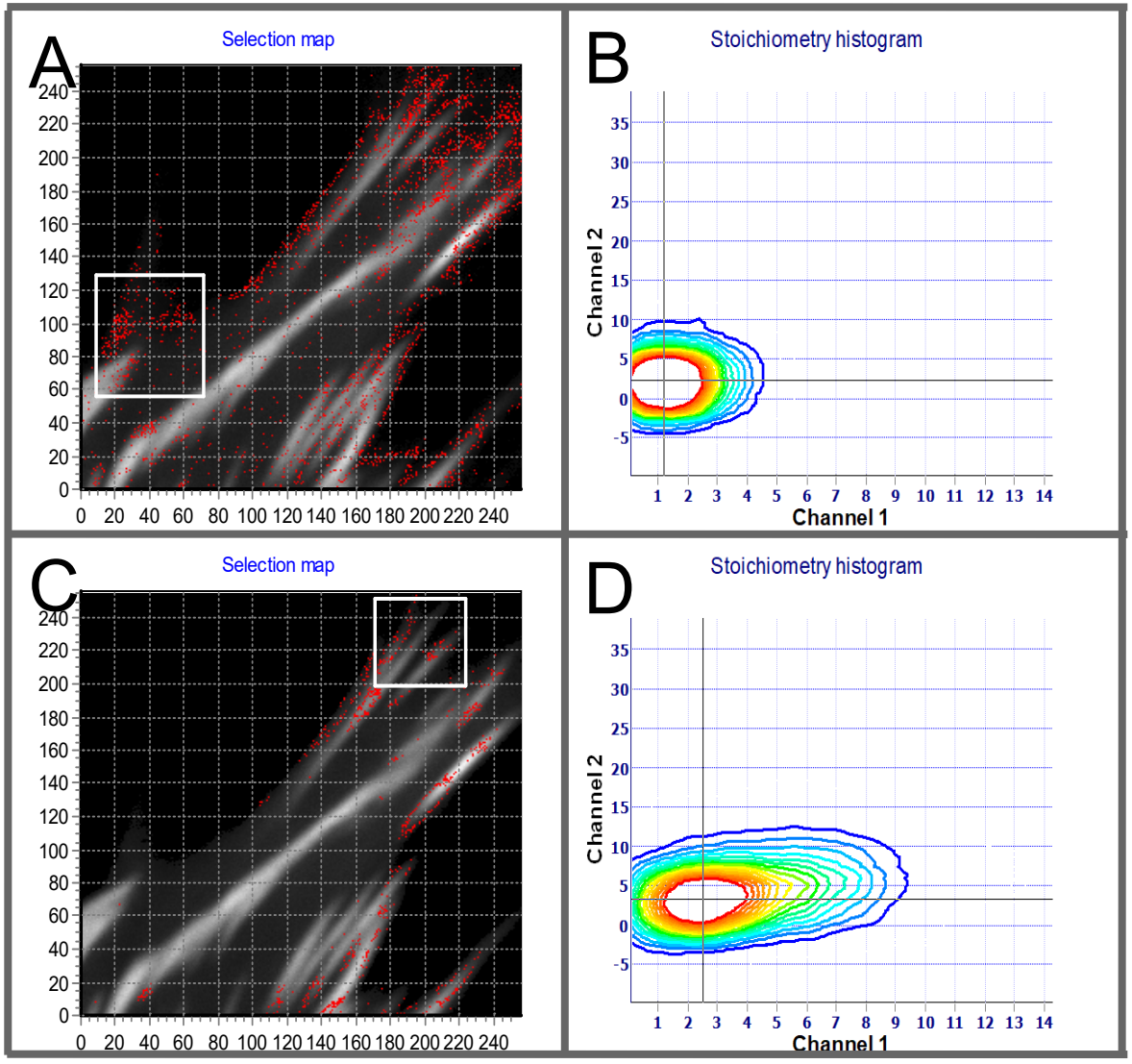
Aggregates



We must find for each value of B1 in one pixel, what is the value of B2 in the same pixel
The fluctuations must be correlated, so we only look at the positive cross-variance

Digman, M.A., et al, *PNAS* 2009

Selecting different regions of the image for vin-pax shows different compositions where large clusters come off at different times



In small adhesions smaller clusters come off

1:2

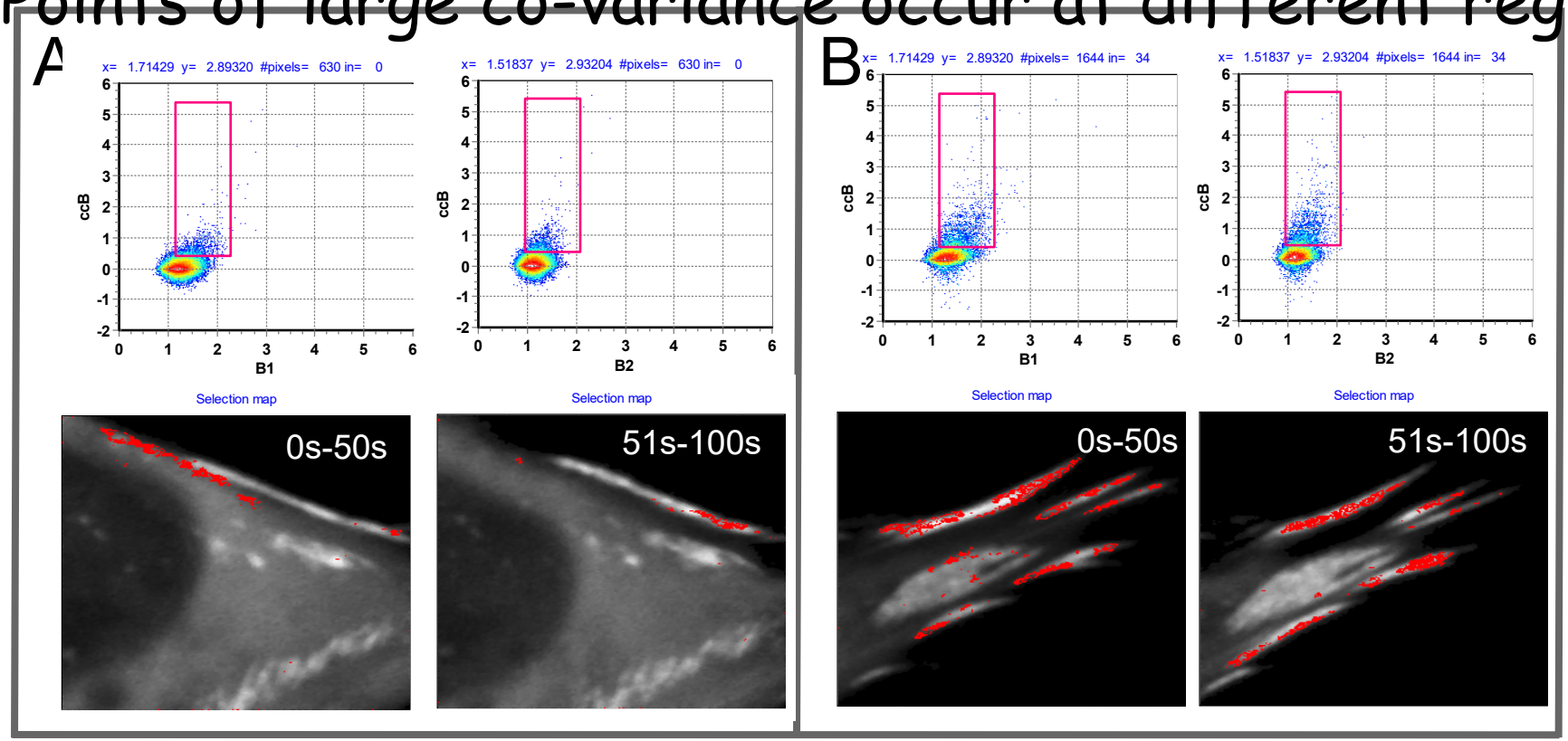
In larger adhesions large clusters come off

2:4

Digman, M.A., et al, *PNAS* ,2009

Cross –correlations occur at specific pixels at the adhesions
FAK-EGFP and Paxillin-mcherry

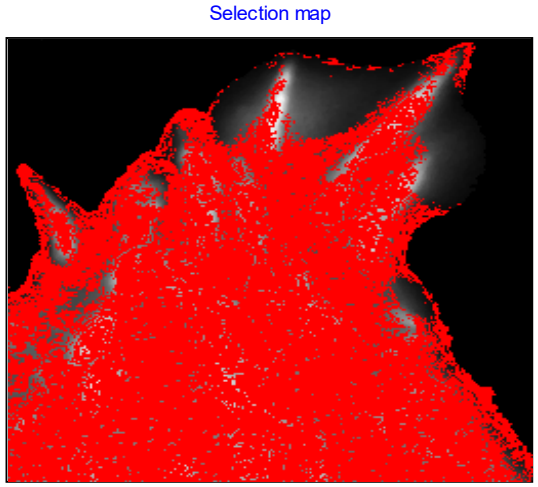
1. Large Cross variance is only seen at the adhesion
2. Points of large co-variance occur at different region

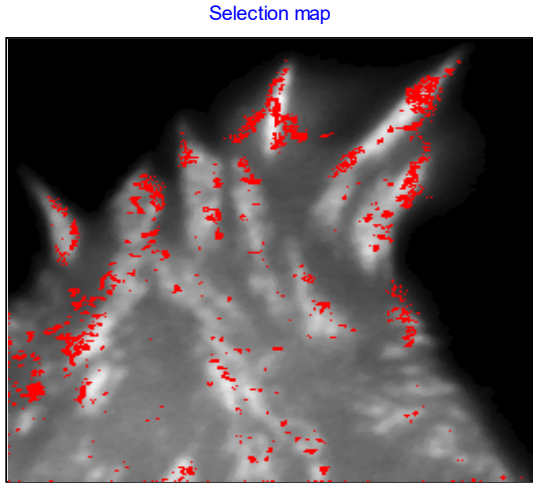


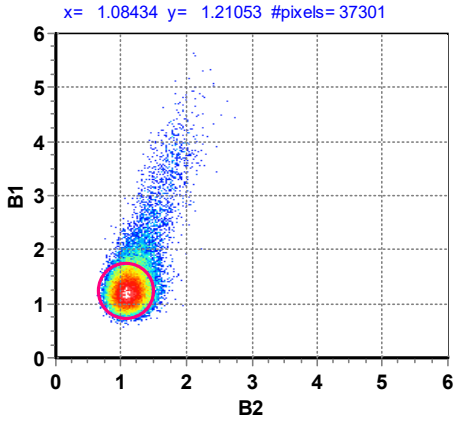
In larger adhesions large cluster come off
3:6

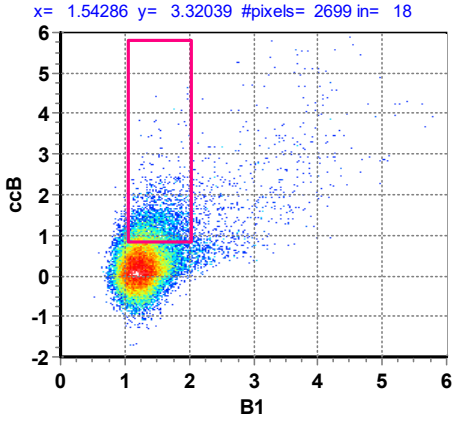
Digman, M.A., et al, *PNAS* ,2009

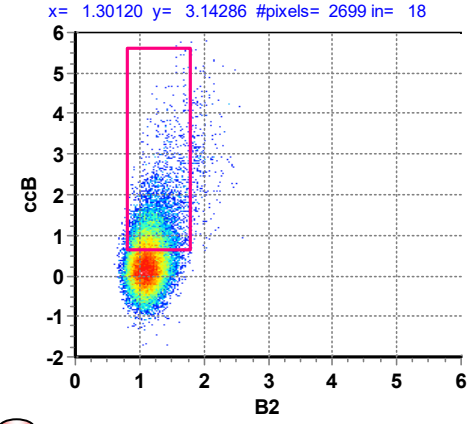
FAK and Paxillin









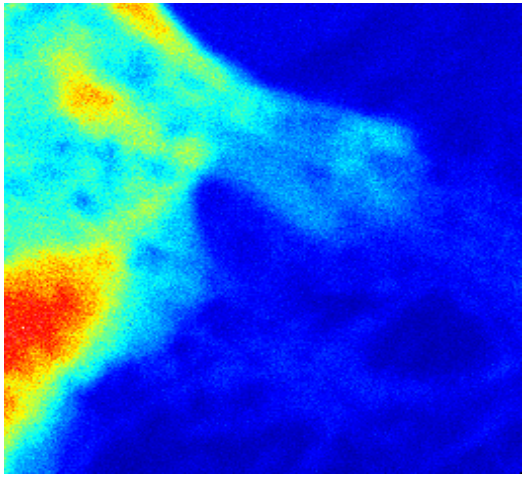


3:4

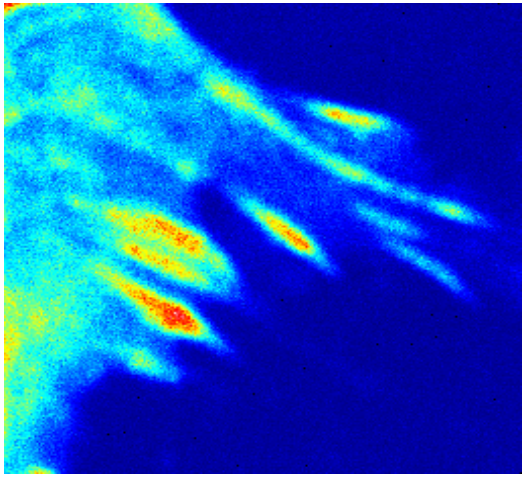
Digman, M.A., et al, *PNAS* ,2009

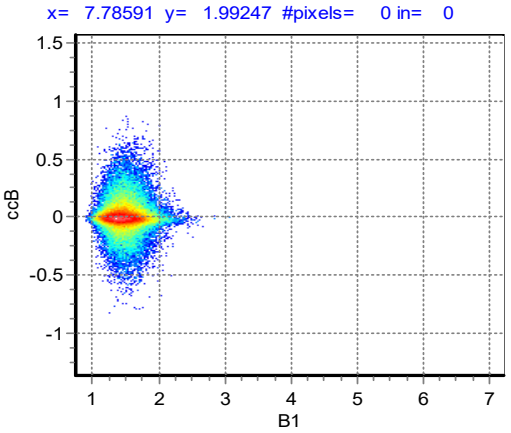
Testing for artifacts: FAK mutant does not form complexes

FAK-EGFP
I937E/I999E



Pax-mCherry



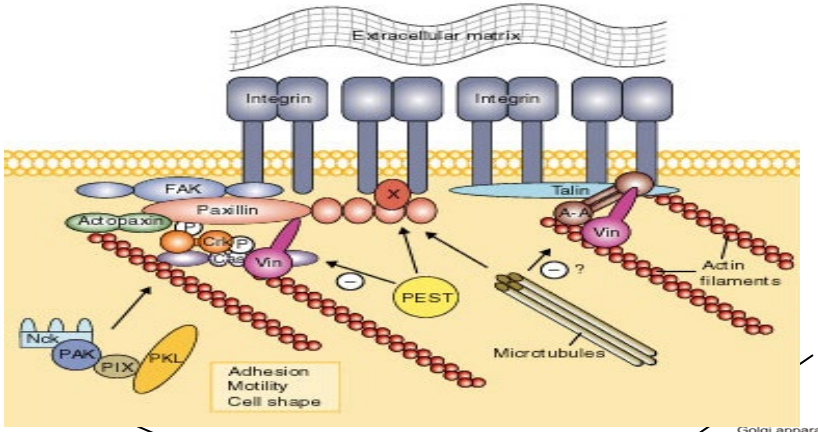


mutFAK-PAX cell shows no cross-correlation although the cell forms adhesion (endogenous FAK?)

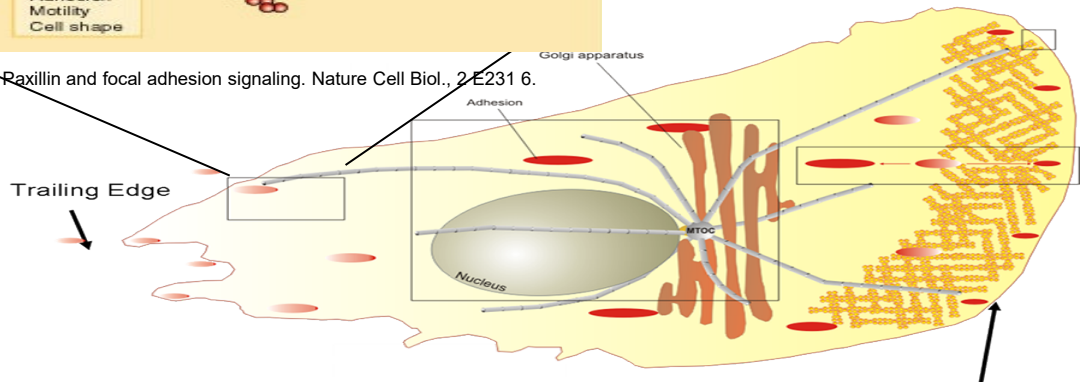
Physical motility

Focal Adhesion

(-) treadmilling



Turner, C.E., (2000). Paxillin and focal adhesion signaling. Nature Cell Biol., 2 E231 6.



Leading Edge

Vicente-Manzanares, M., Webb, D. J., and Horwitz, A. R. (2005) *J. Cell Sci.* 118, 4917–4919.

Additional Reading

1) Jay R Unruh and Enrico Gratton.Analysis of molecular concentration and brightness from fluorescence fluctuation data with an electron multiplied CCD camera.Biophys J. 2008; [epub ahead of print].

2) Michelle A Digman, Rooshin Dalal, Alan R Horwitz, and Enrico Gratton.Mapping the number of molecules and brightness in the laser scanning microscope.Biophys J. 2008; 94(6): 2320-2332.

3) Rooshin B Dalal, Michelle A Digman, Alan R Horwitz, Valeria Vetri, and Enrico Gratton. Determination of particle number and brightness using a laser scanning confocal microscope operating in the analog mode. Microsc Res Tech. 2008; 71(1): 69-81.

4) Yan Chen, Joachim D Müller, Qiaoqiao Ruan, and Enrico Gratton. Molecular brightness characterization of EGFP in vivo by fluorescence fluctuation spectroscopy. Biophys J. 2002; 82(1): 133-44.

5) Alberto Garcia-Marcos, Susana A Sánchez, Pilar Parada, John S Eid, David M Jameson, Miguel Remacha, Enrico Gratton, and Juan P G Ballesta.Yeast ribosomal stalk heterogeneity in vivo shown by two-photon FCS and molecular brightness analysis. Biophys J. 2008; 94(7): 2884-2890.

6) Michelle A Digman, Paul W Wiseman, Colin K Choi, Alan R Horwitz, and Enrico Gratton. Mapping the stoichiometry of molecular complexes at adhesions in living cells.Proc Natl Acad Sci USA. 2008; [submitted].

Acknowledgements

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Cell Migration Consortium

Scanning FCS

Introduction to scanning FCS

When we first applied FCS to cells, a series of problems arose:

- The cell could have moved, so that the volume of observation was not any more the chosen one

- The average intensity $\langle F(t) \rangle$ suddenly changed, perhaps due to the passage of a vesicle at the point of observation

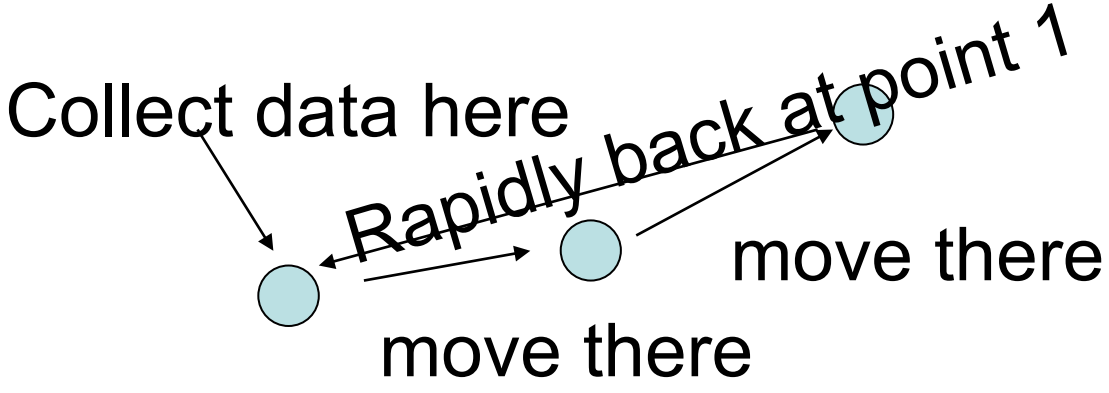
$\longrightarrow$ effect on the correlation function $G(\tau) = \frac{\langle \delta F(t) \delta F(t +$

$\delta \mathrm{F(t)} = \mathrm{F(t)} - \langle F(t) \rangle$ $\langle F(t) \rangle^2$

- Bleaching of the immobile fraction occurred, causing a large deviation of the apparent correlation curve

The principle of scanning FCS

If we can move the point at which we acquire FCS data fast enough to other points and then **return** to the original point "before" the particle has left the volume of excitation, then we can "multiplex the time" and collect FCS data at several points simultaneously!

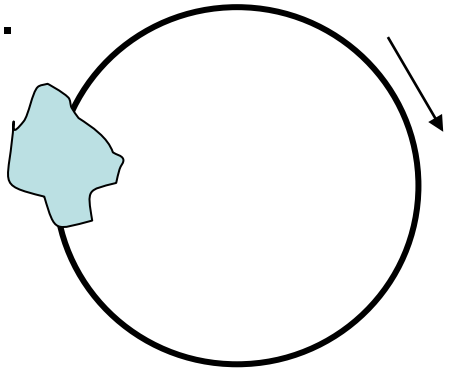


Why circular scanning? Circular scanning is faster!

The fastest way to scan several points and then return to the original point is to perform a circular orbit using the scanner galvo.

The x- and y-galvos are driven by 2 sine waves shifted by 90 degrees, thereby obtaining a projected orbit on the sample.

One orbit could be performed in less than 1 ms using conventional galvo drivers and in microseconds using acousto-optical beam deflectors.



Timing in scanning FCS

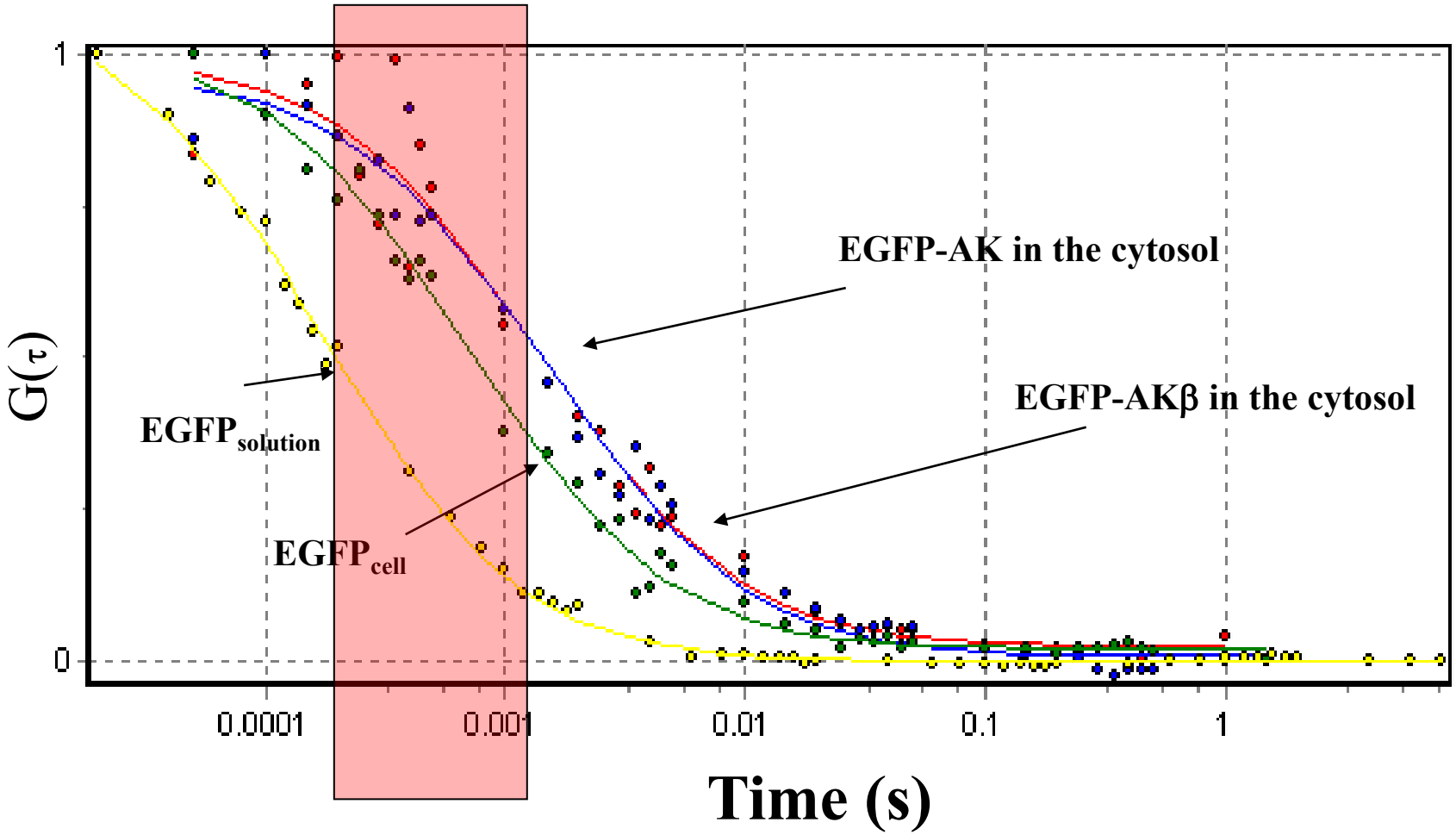
What is the maximum time required for an orbit so that we will not miss the “fastest” diffusion process in a cell?

EGFP diffuses in the cell with an apparent diffusion coefficient of approximately **20 $\mu m^2/s$**. The transit across the laser beam (assuming a w_0 of 0.35 μm) is about 1.5 ms!
(formula used: time=$w_o^2/4D$)

Therefore **0.5 to 1 ms** per orbit should catch the GFP diffusing in a cell. Faster diffusing molecules will be partially missed.

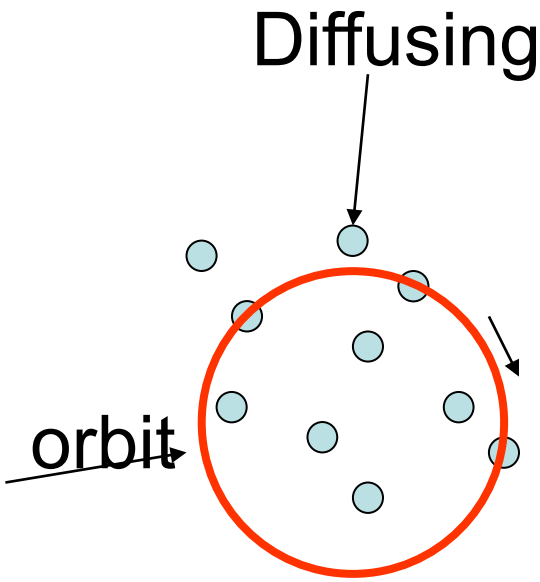
Instead, faster blinking and other fast intramolecular processes will not be missed!! (why?)

Autocorrelation of EGFP & Adenylate Kinase-EGFP



Normalized autocorrelation curve of EGFP in solution (•), EGFP in the cell (•), AK1-EGFP in the cell(•), AK1b-EGFP in the cytoplasm of the cell(•).

Acquiring scanning-FCS data



Light is collected along the orbit, generally at 64 or 128 points. If the orbit period is 1 ms, the dwell time at each point is about 16 μs (64 points) or 8 μs (128 points).

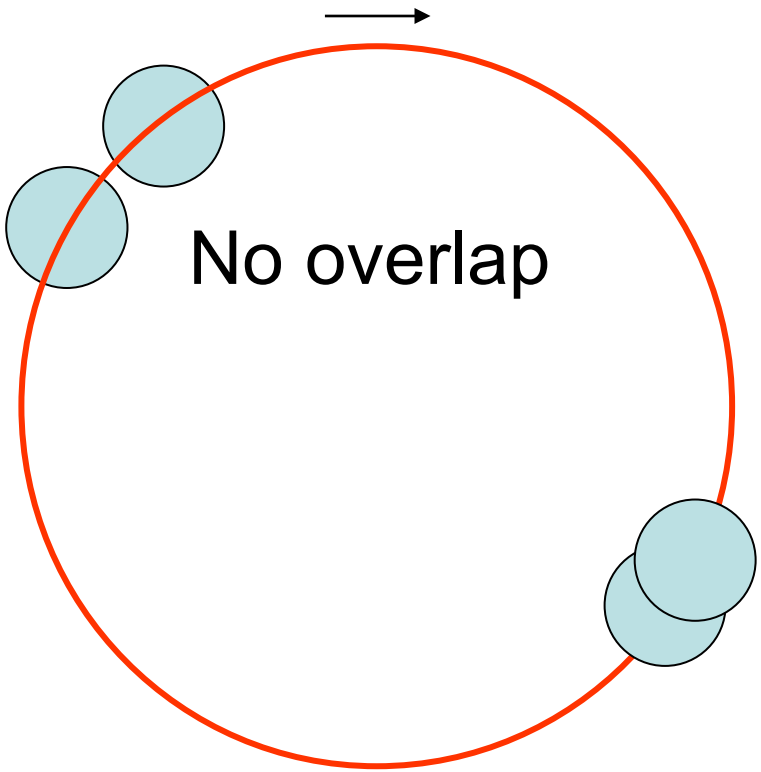
The separation between the points depends on the orbit radius:

For an orbit radius of 5 μm, the length of the orbit is about 32 μm. At 64 points per orbit the average distance is about 0.5 μm (0.25 μm at 128 points).

Why is the distance between points important?

Overlapping volumes in scanning FCS

If the orbit radius is larger than 5 μm, the points are separated by more than the width of the PSF
(assuming 64 points per orbit: $2\pi R/64 \sim 500nm$)

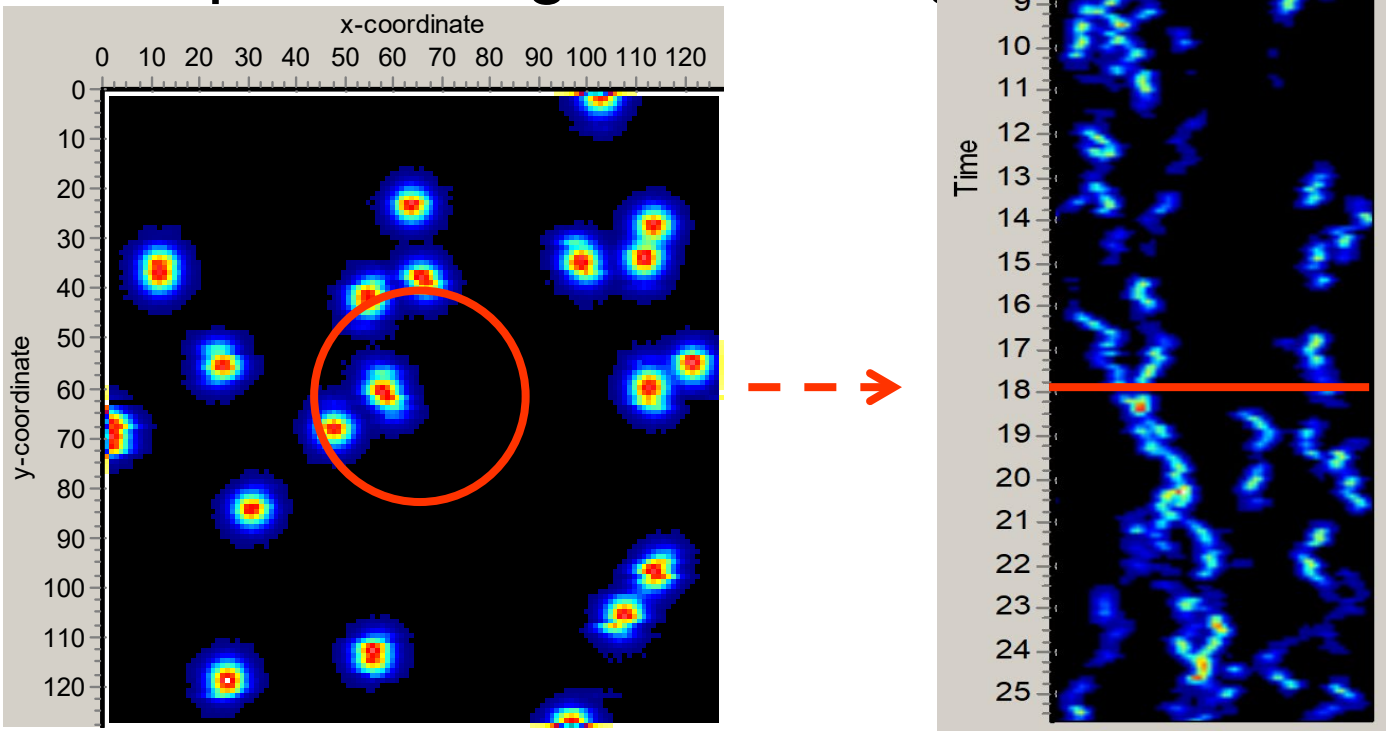


Setting the conditions of the instrument for **no-overlap** limits the capability of obtaining spatial correlations along the orbit

Data processing in scanning FCS

The data stream is presented as a **“carpet”** in which the **horizontal coordinate** represents data along the orbit and the **vertical coordinate** represents data at successive orbits (hyperspace).

Data processing in scanning



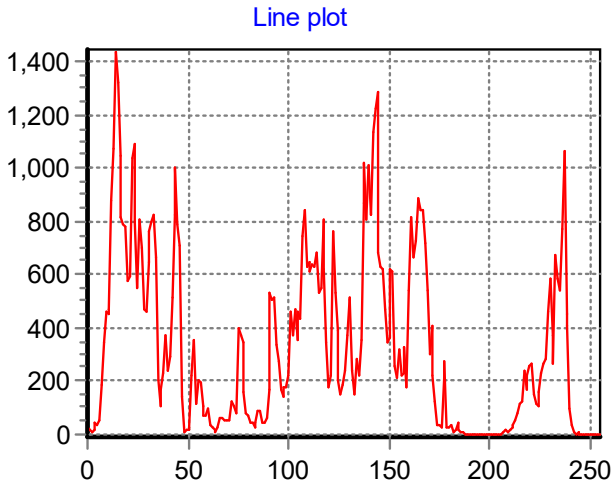
6 μm image side
1 μm orbit radius
D=0.1 μm^2/s

Analyzing data in scanning FCS

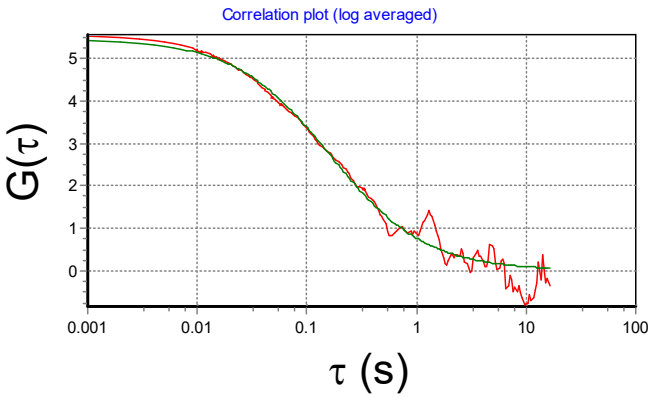
How do we proceed to determine the **diffusion** of particles, the **number** of particles and their **brightness**??

- Select a column of the carpet. It is a time sequence at a specific point of the orbit!
- Perform autocorrelation operation along a column
- What are we obtaining?
- What is the sampling time along one of these columns?
- What is the dwell time along one of these columns?

Intensity along a column



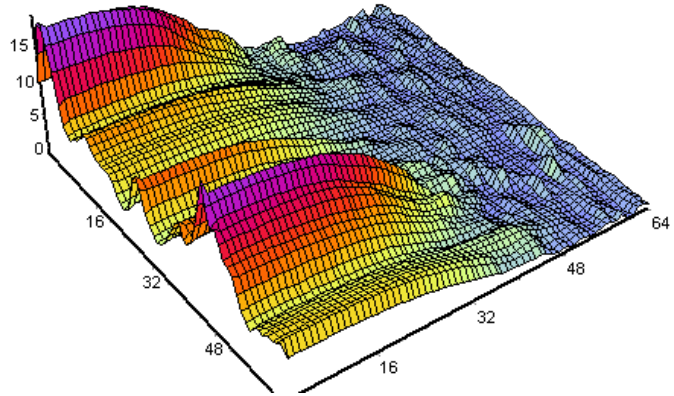
Perform the autocorrelation operation



Recovered value for $D=0.1\ \mu m^2/s$(= to the value input in the simulation!)

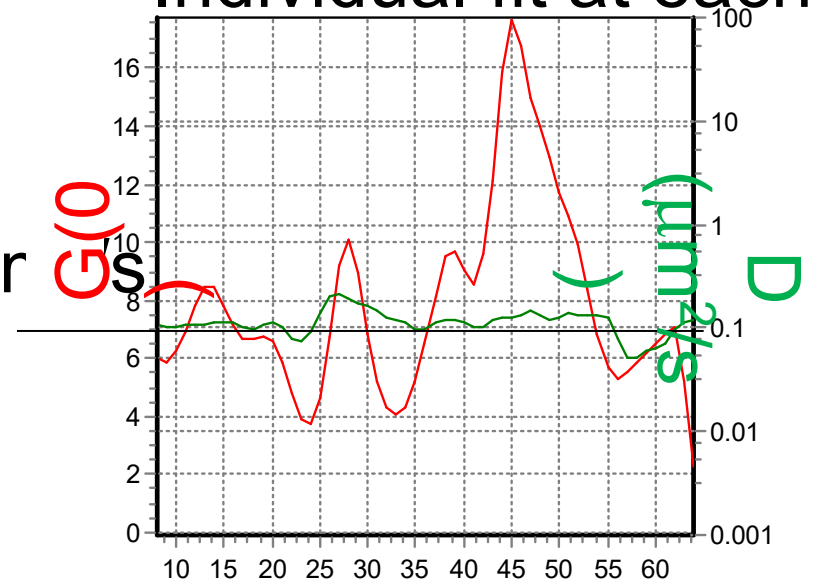
Carpet analysis

Every column should be equivalent for a homogeneous sample, so that we can calculate the ACF for every column and then fit all the columns either globally or individually.



ACF along each column:
the calculation takes few seconds

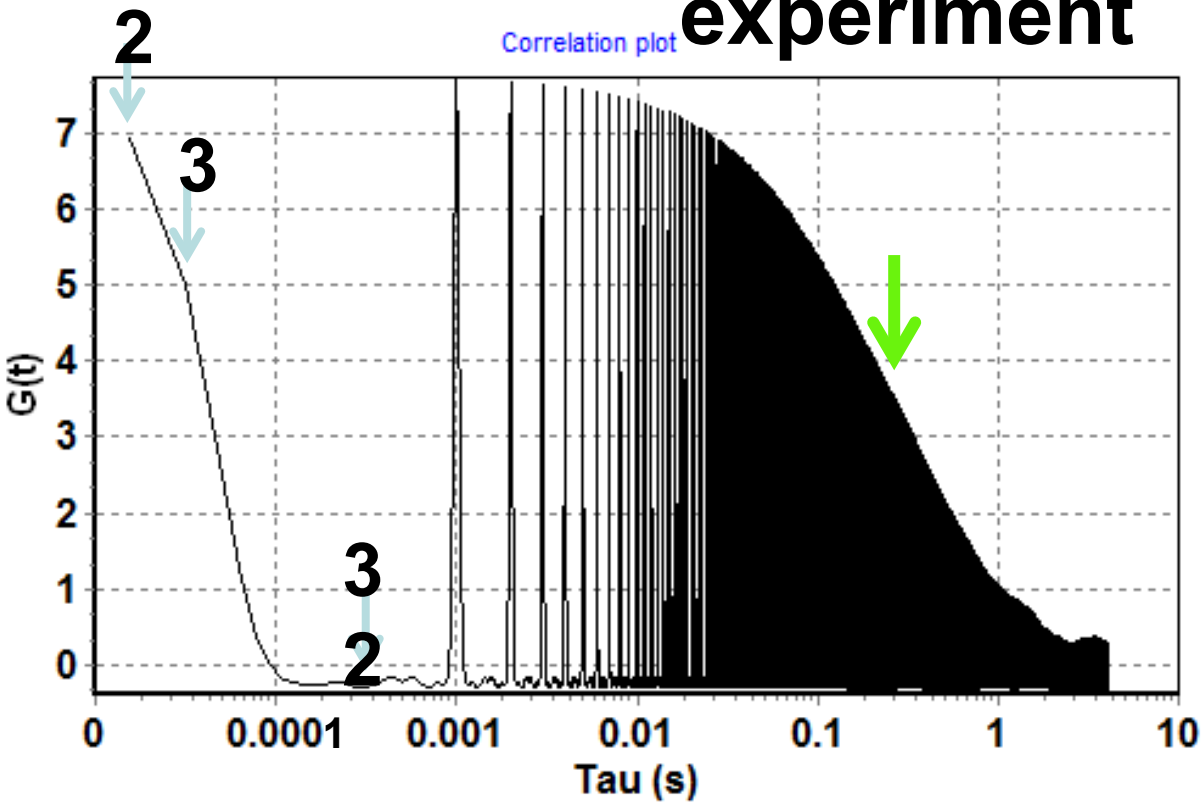
Individual fit at each c



D=0.1μr

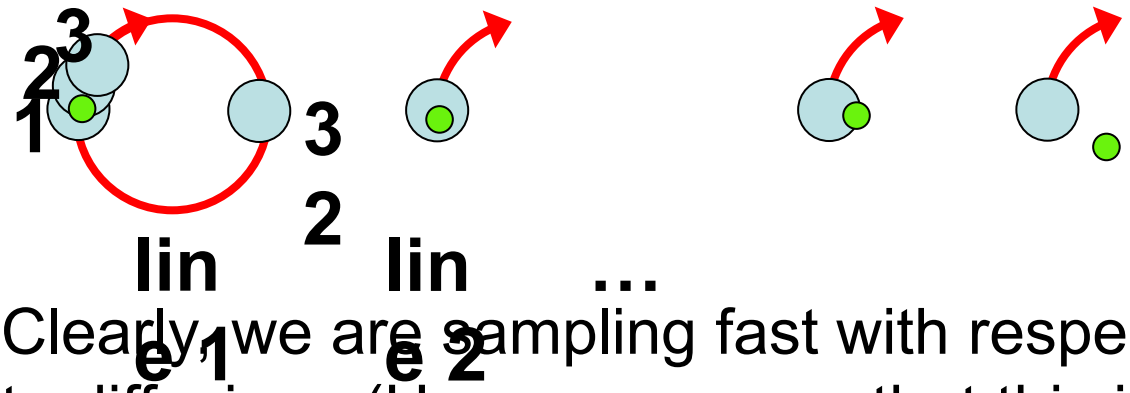
The G(0) changes from line to line, because the statistics is poor, but the D is pretty constant at the expected value of D=0.1um^2/s

Global correlation function for a solution experiment



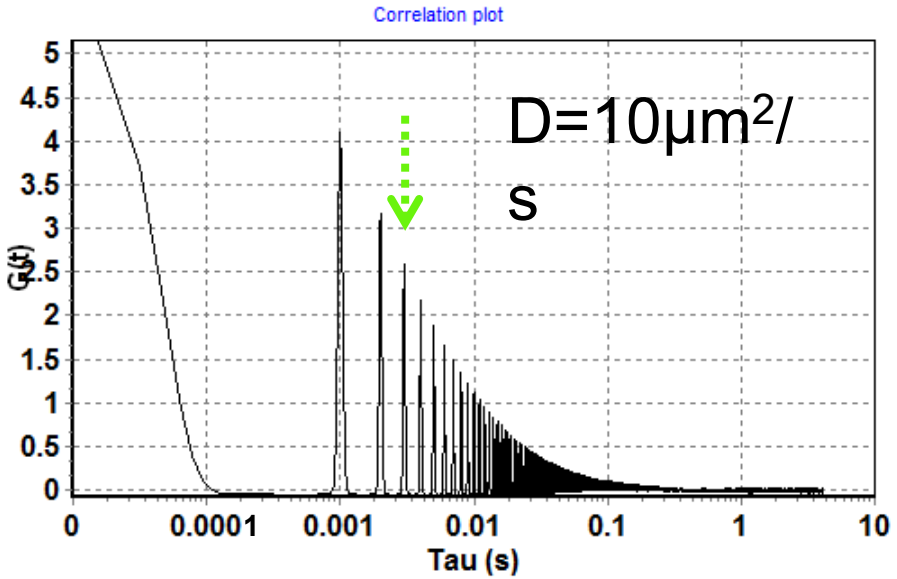
Global correlation function:
the periodicity is due to the scanning period which is 1 ms

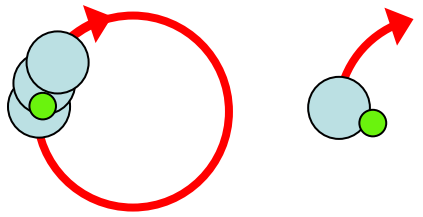
$D=0.1\mu m^2/s$
$R=1\mu m$



Clearly, we are sampling fast with respect to the relaxation due to diffusion. (How can we see that this is the case?)

Global correlation function for a solution experiment

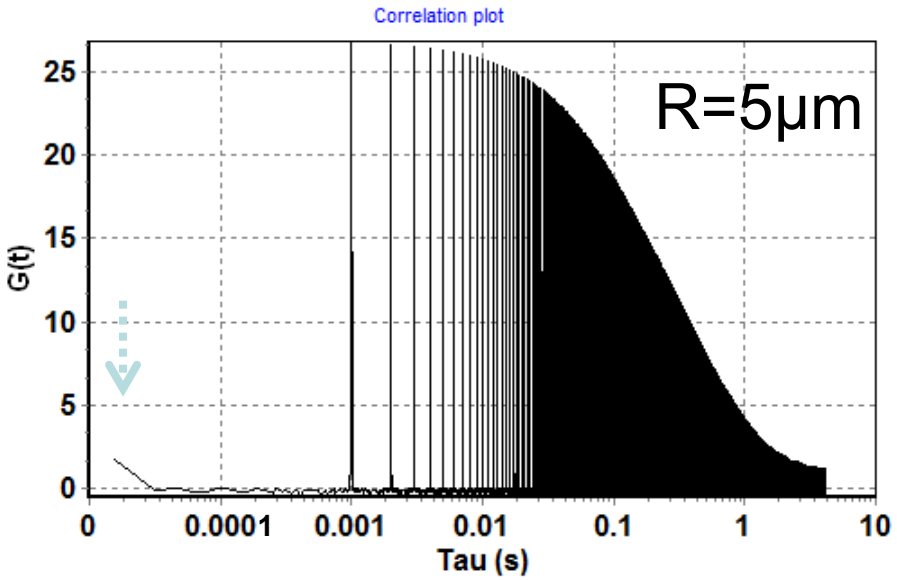


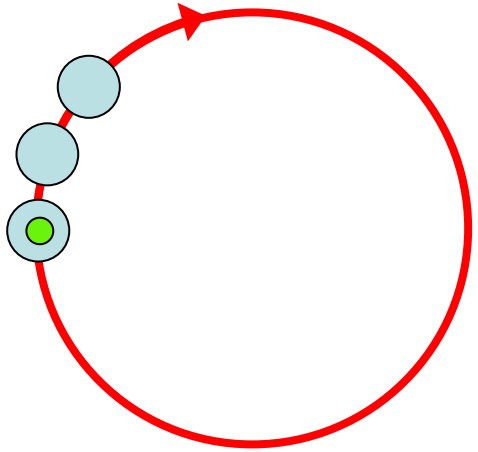


lin e 1

lin e 2

We are not scanning fast enough!

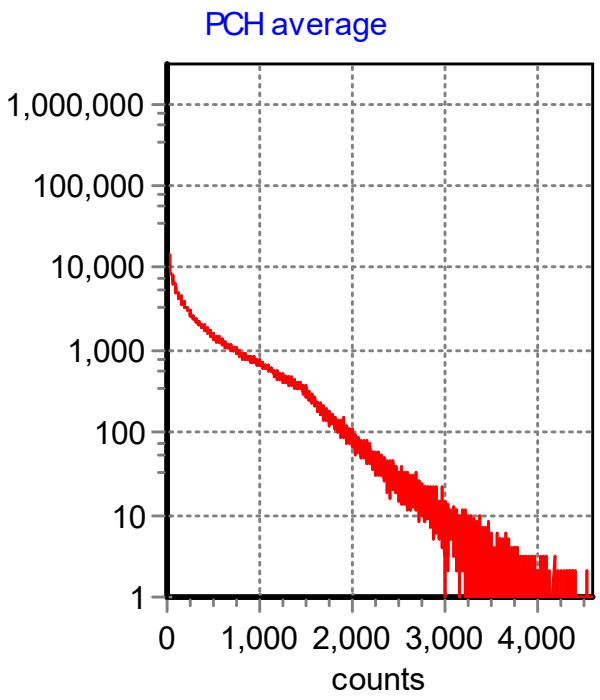




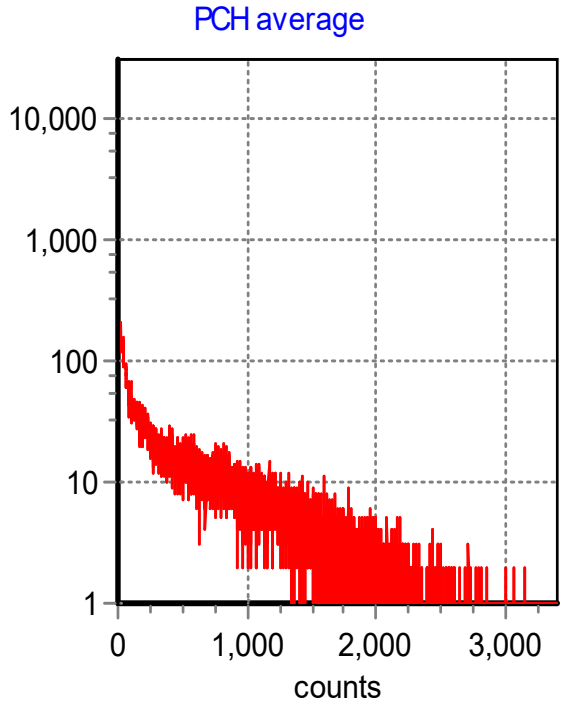
No spatial correlations!

PCH analysis at each column

What about the PCH analysis, can that be done?
Since we have a sequence, we can plot the histogram first globally and then individually for each column



Global histogram



Single histogram at one colu

Why scanning FCS in a homogeneous sample?

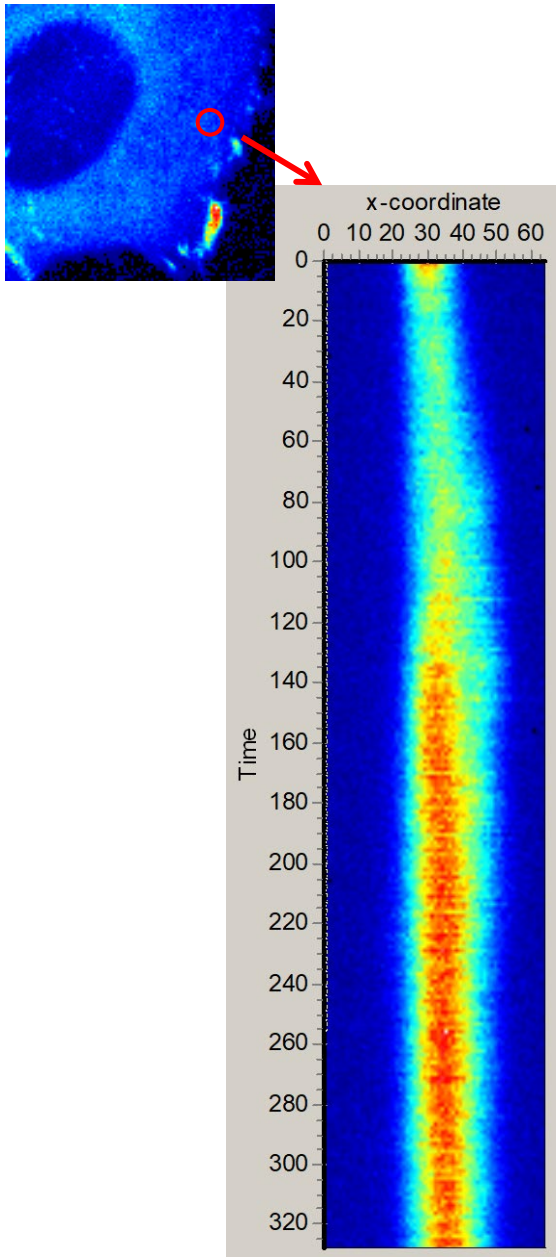
Is there any advantage to perform scanning FCS instead of single point FCS for a solution sample?

A major issue in FCS is that we need the volume of the PSF to calculate the diffusion coefficient.

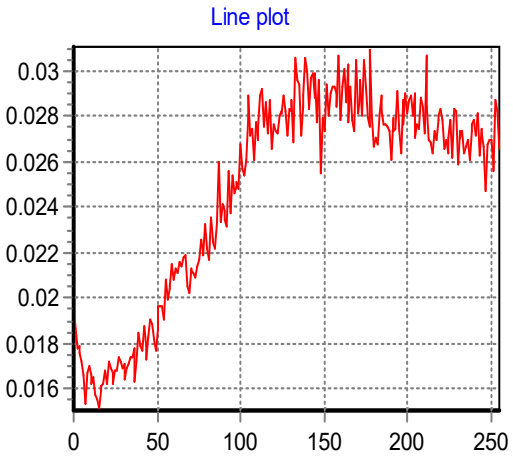
In scanning FCS we know the distance between points along the orbit. We can calculate the time for a molecule to diffuse between the two volumes.

What about cross-correlation between columns?

Scanning FCS in cells (some surprises!)



Example of scanning at an adhesion



64 points sampled along the orbit

Period of scanning is 1 ms

Radius of scanning is 2 μm

Distance between pixels is about 0.2 μm

Use "real world"

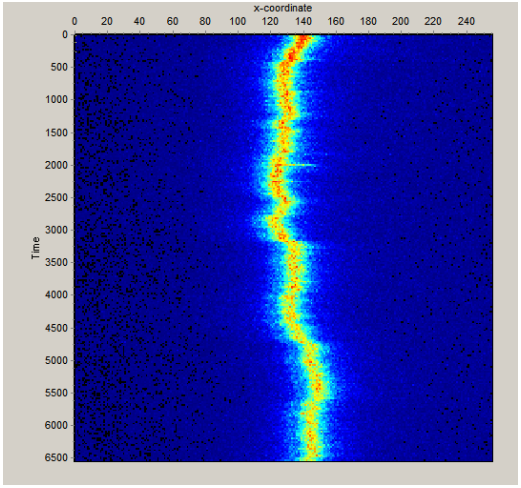
What do we do with the changes in intensity?

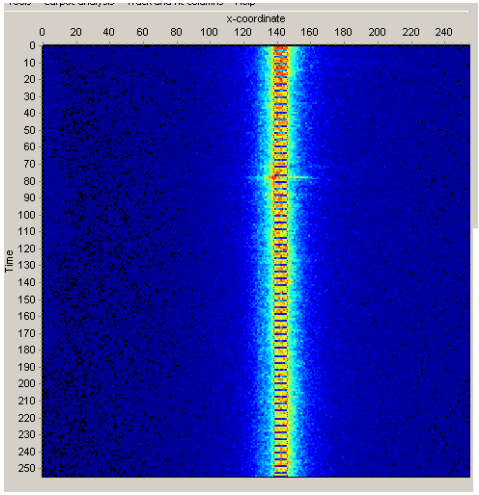
There is some fast initial bleaching followed up by a slow increase in intensity.

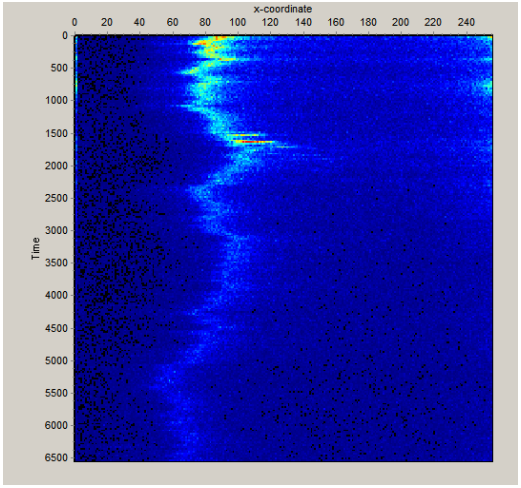
What are the questions?

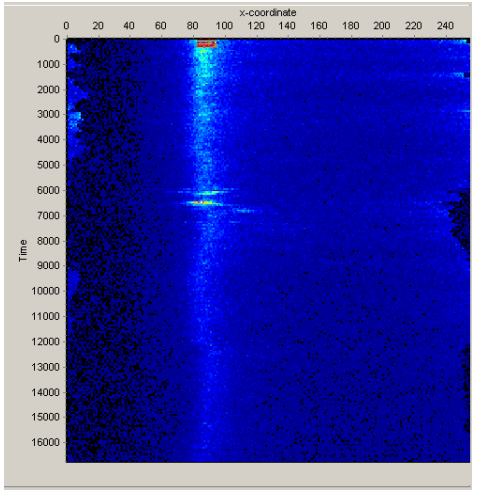
- What is the apparent "diffusion" coefficient of paxillin ?
- Is the diffusion coefficient homogeneous?
- Is paxillin monomeric (i.e., what is the brightness)?
- What is the number of particles in the different parts of the adhesion?

Welcome to the real world!







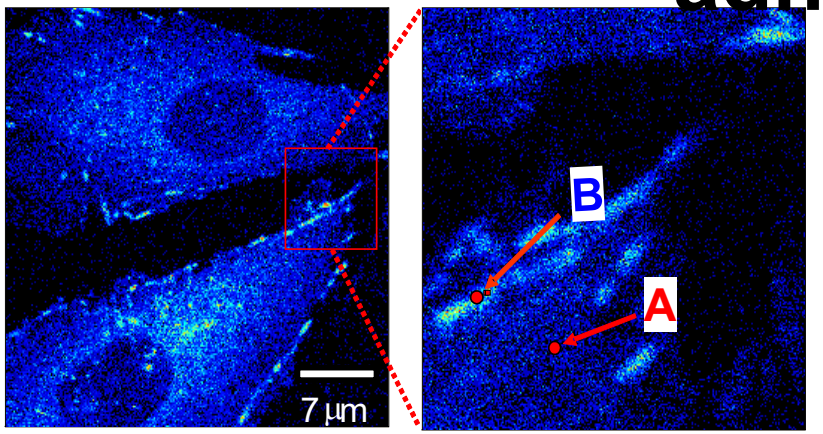


Detrend?
Centering?

Data from Pierre Moens

Scanning a moving target: GUV. How to determine the diffusion in the membrane?

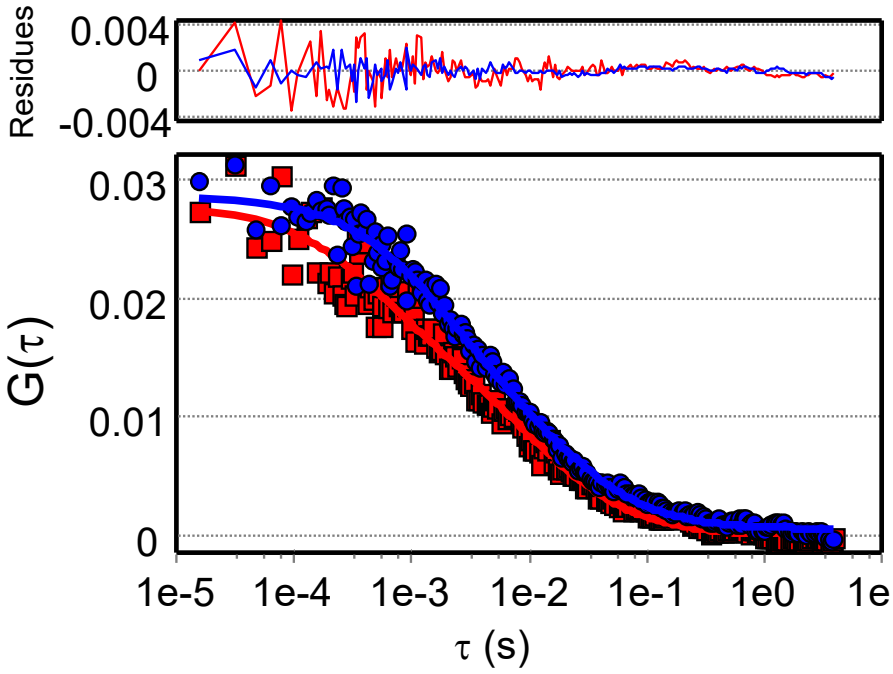
Scanning FCS in cells: heterogeneity along adhesions



CHO-k1 cells expressing Paxillin-EGFP

Single point FCS depicts two-species

Paxillin-EGFP	Diffusion ($\mu m^2/s$)	Fractional Contribution Cytosol (%)	Fractional Contribution Adhesions(%)
D1 Monomers	19.6	61	44
D2 Aggregates?	1.43	39	56



What are these diffusion rates due to?

~~1. Differences in cell viscosity~~

2. Paxillin complexed to other proteins

3. Large aggregates of paxillin

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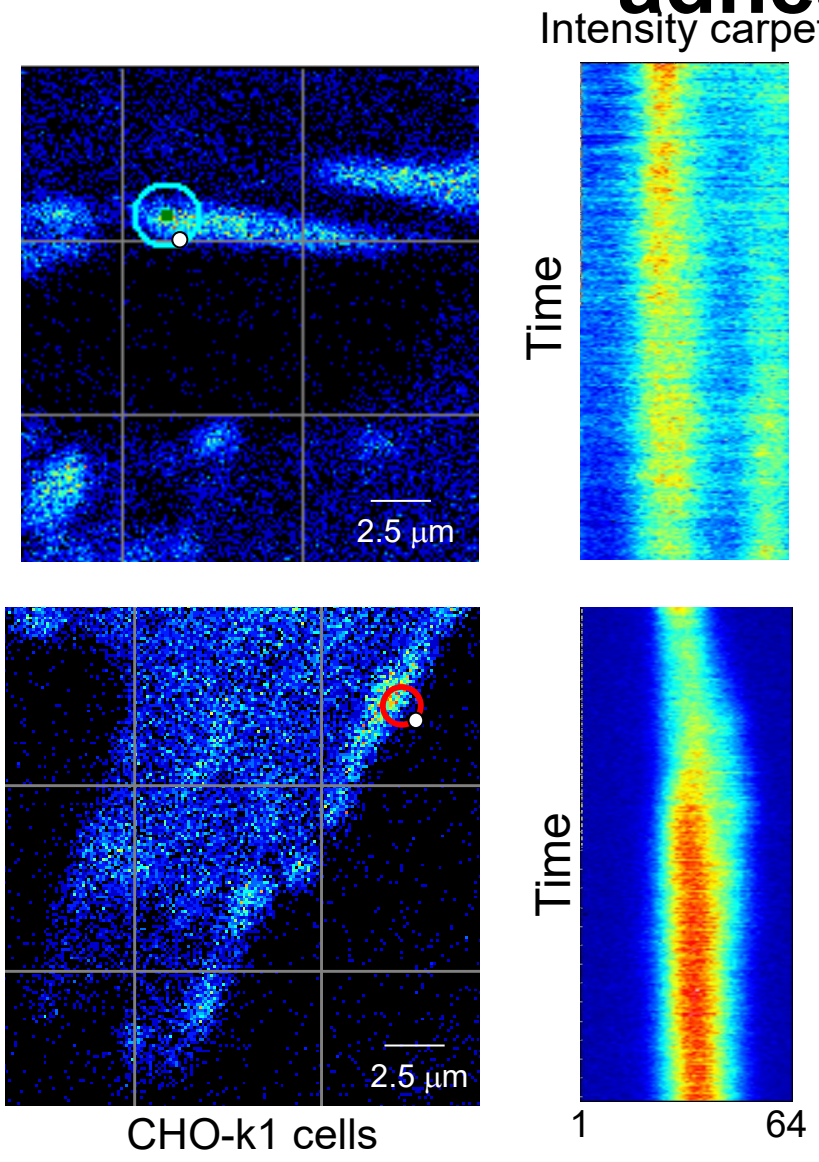
Scanning FCS in cells: heterogeneity along adhesions



Data were sampled at 64kHz (1ms/orbit, 64 points per orbit).

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Scanning FCS in cells: heterogeneity along adhesions

FCS and Scanning FCS results:

• Paxillin moves differently at an adhesion with respect to the cytosol

• Adhesions are heterogeneous

• At the assembling side of the adhesion the fluctuation dynamics is faster and the number of molecules is larger than in stable cellular adhesions

Are the adhesions assembling and disassembling in synchrony?
Can we map out protein dynamics in a larger area?

We need a method where we can analyze the entire cell:
THE RICS APPROACH

Described so far

- Circular versus line scanning
- Line scanning can be performed with any confocal microscope
- Line scanning is not as fast as circular scanning (few ms versus a fraction of a ms)
- For homogeneous samples, is there any advantage in performing scanning-FCS (either circular or line) with respect to single point FCS??
- Filtering operations on the data and integrity of the original statistics

Observations

- Even in the “simplest” implementation, FCS in cells requires precautions in data analysis and interpretation
- The user must set up the instrument parameters (line period, dwell time, etc) for the particular experiment
- The software for data analysis must offer a series of tools to the user for data filtering, analysis and presentation. It is not enough to collect line scanning data!
- Maps of diffusion coefficients, number of particles and brightness can be obtained if we can deal with slowly varying fluctuations

What is next?

This was an “introduction” to scanning FCS

We discussed the analysis of the carpet columns as individual time traces at separate points

We have not considered the correlation between adjacent columns or between distant columns

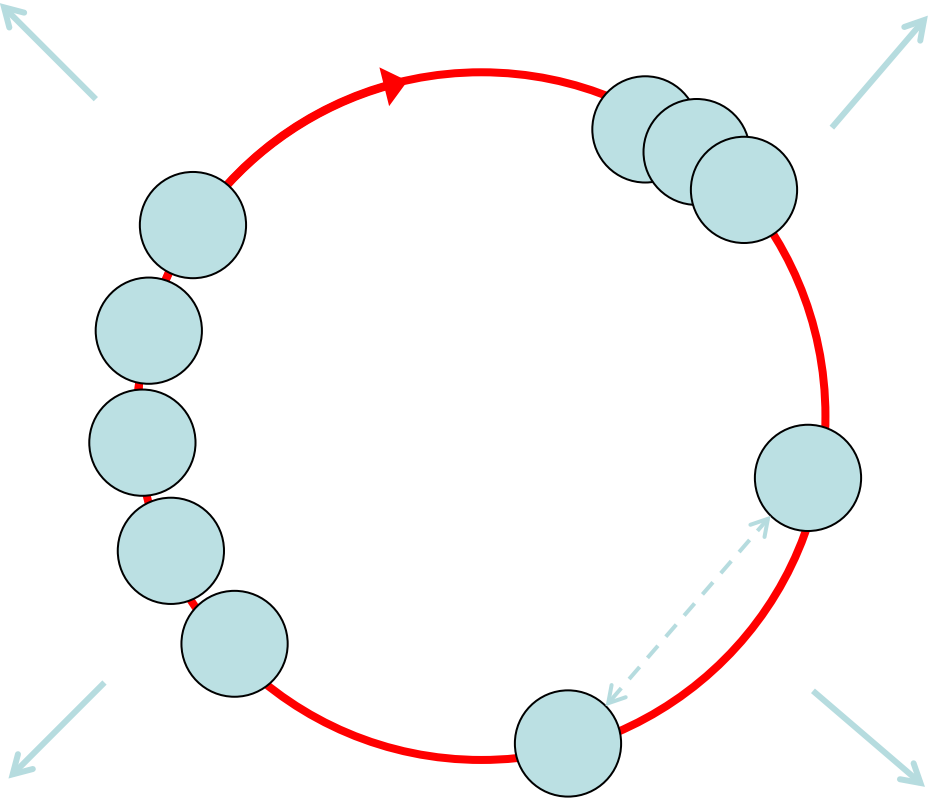
We need to develop new concepts and mathematical tools to account for these spatial correlations

As we understand the scanning experiment we discover a new world about fluctuation methods that was not possible to explore with single point FCS

What is next?

Spatial Resolution

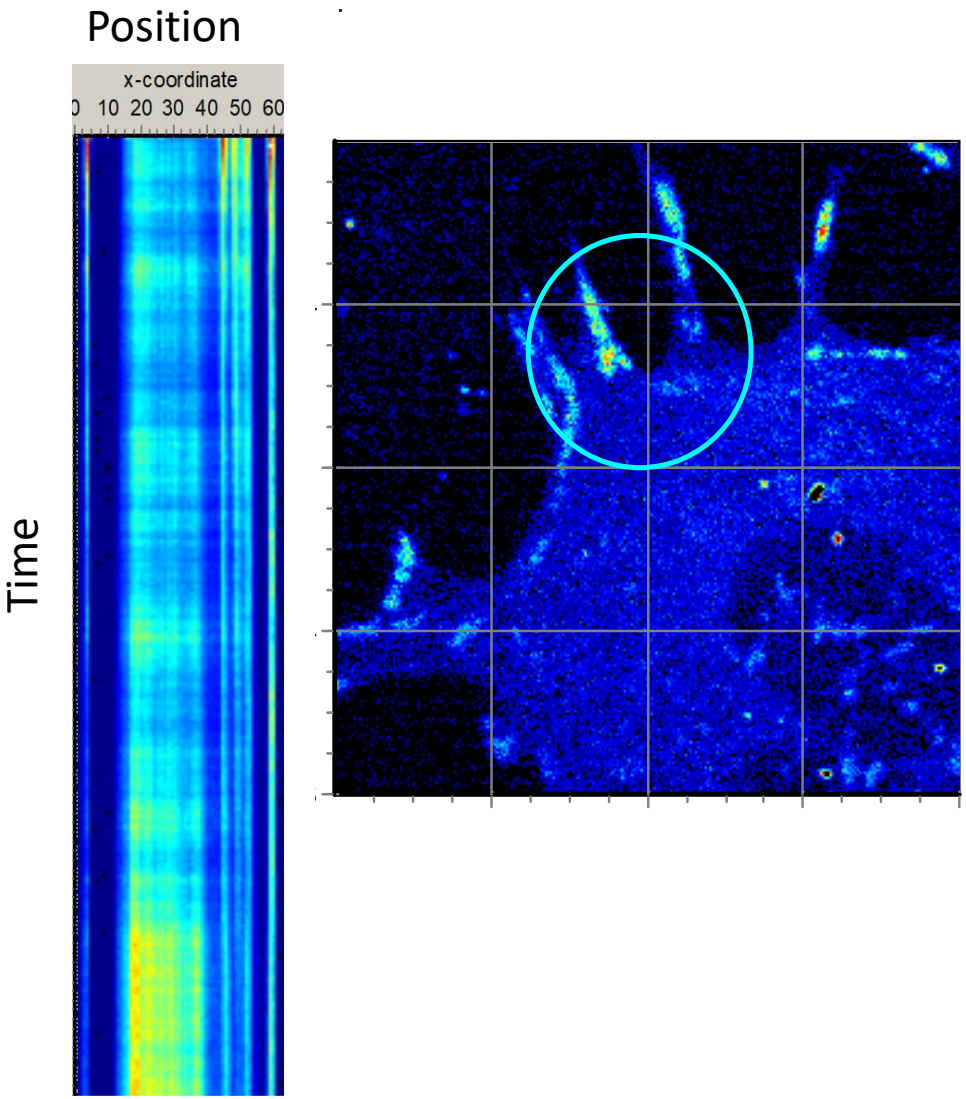
RICS

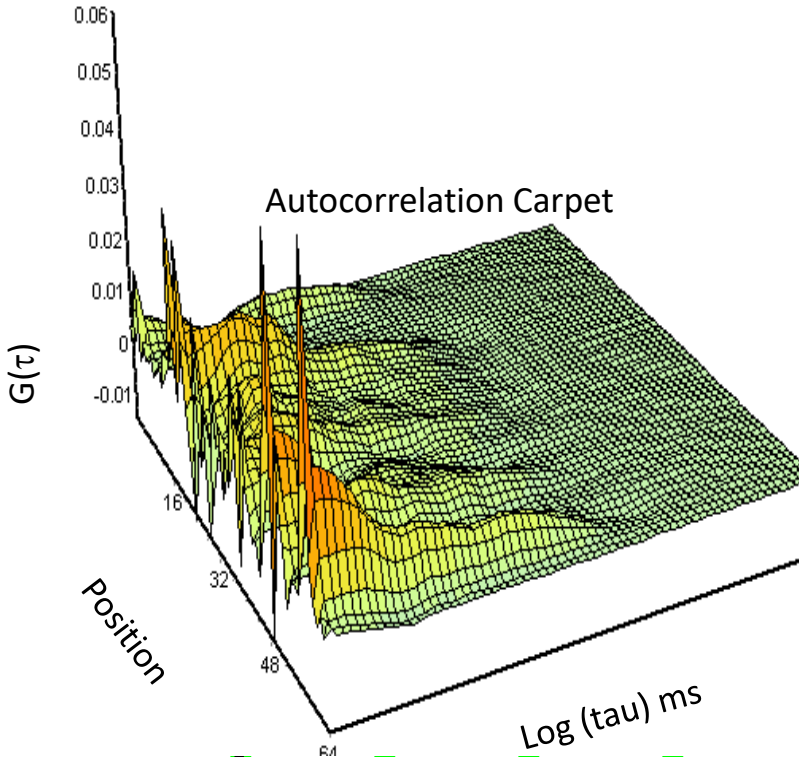


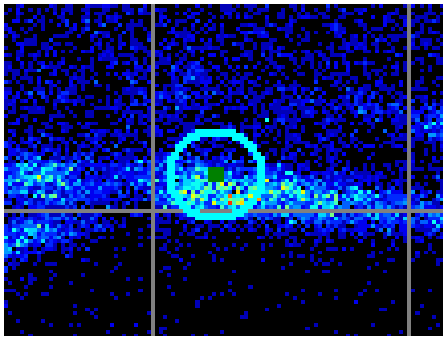
STICS - iMSD

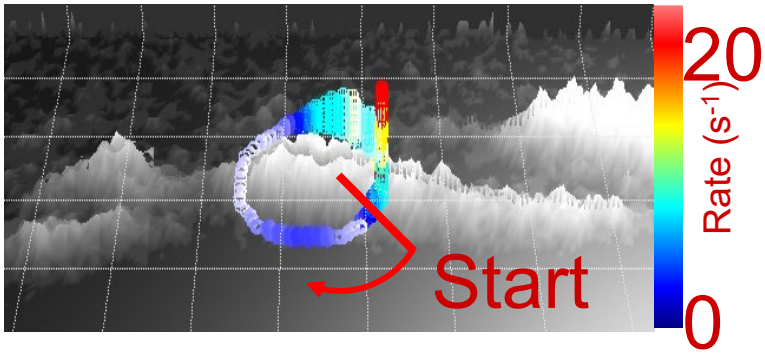
Pair Correlation

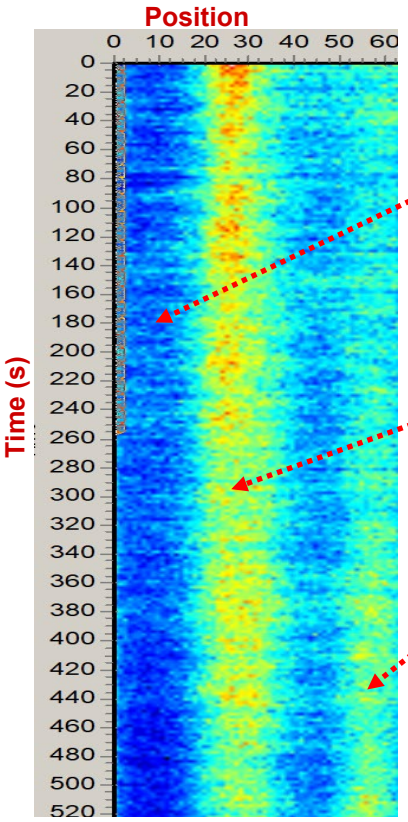
Scanning FCS measurements:
Obtaining molecular dynamics at many points along the adhesions







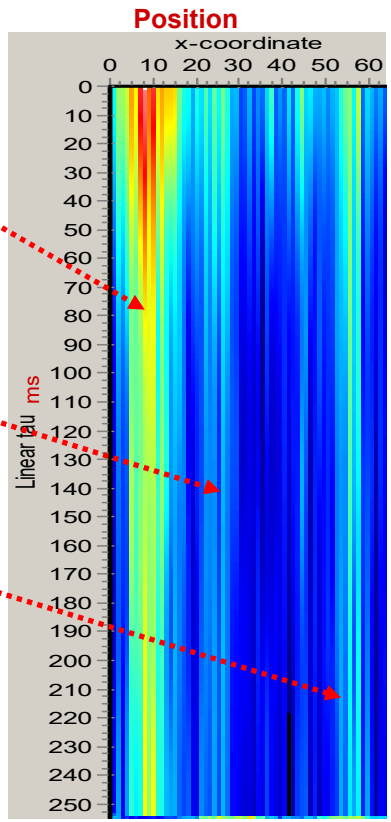




Relatively fast
Diffusing Paxillin

Slower process
at Adhesion border

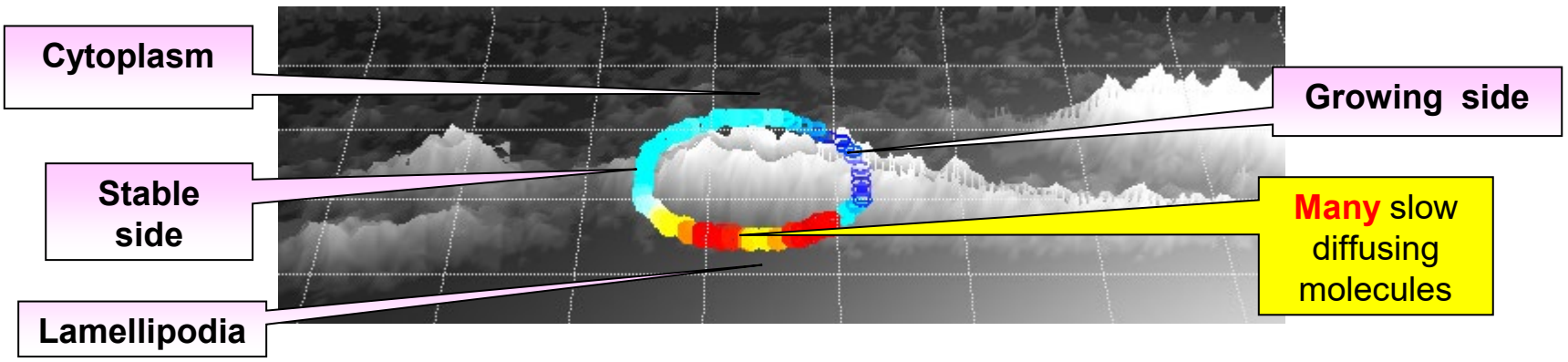
growing
adhesion



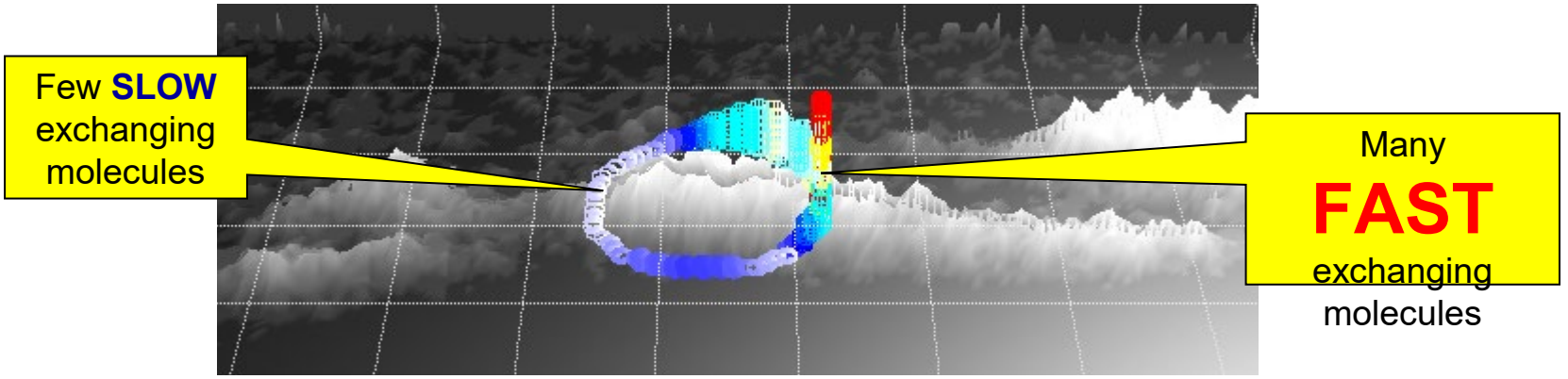
Auto Correlation carpet

2-D plot of the autocorrelation Functions along the orbital scan

Map of the Number of diffusing molecules

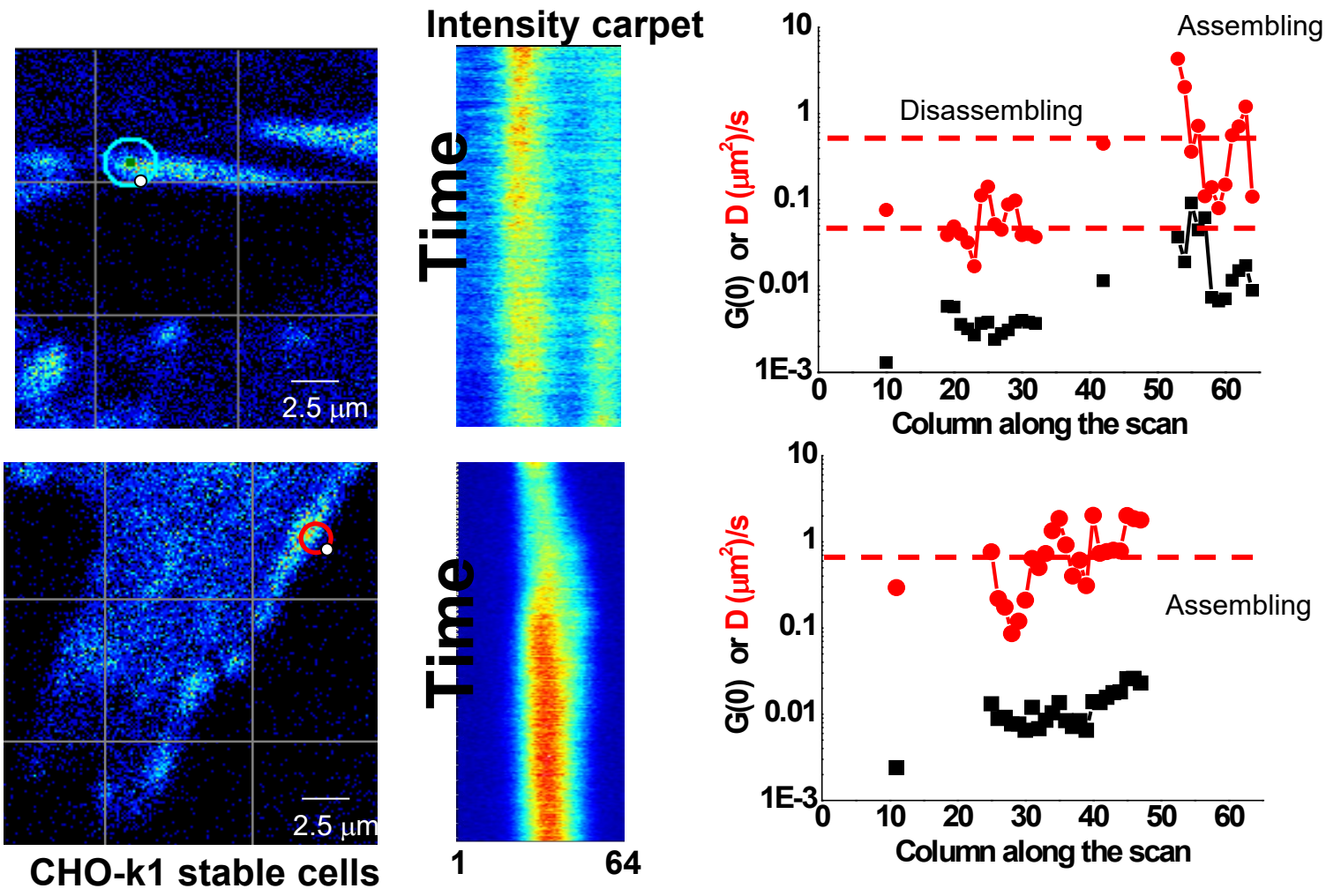


Map of the concentration of binding-unbinding molecules and rates



(Max is 20 s^{-1})

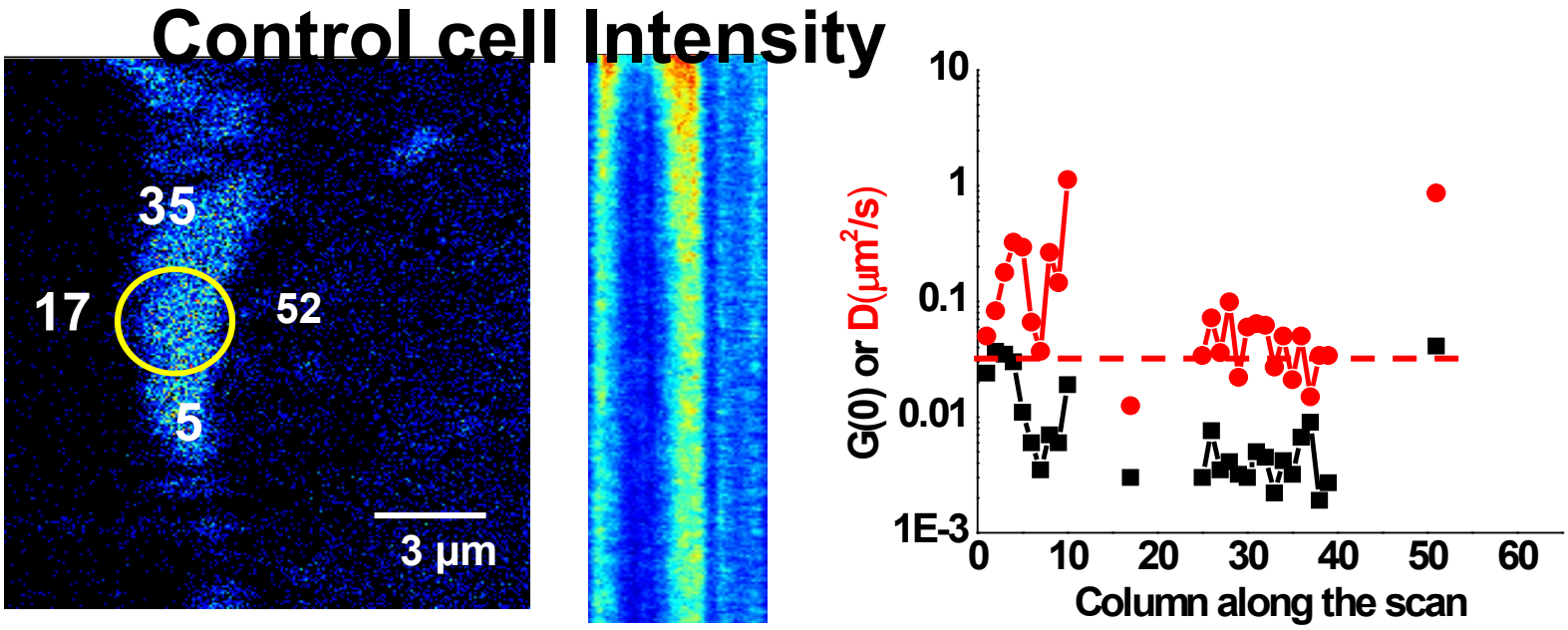
Heterogeneity along adhesions



Data were sampled at 64kHz (1ms/orbit, 64 points per orbit).

Digman et al." *Biophys J.* 94(7):2819-31, 2008

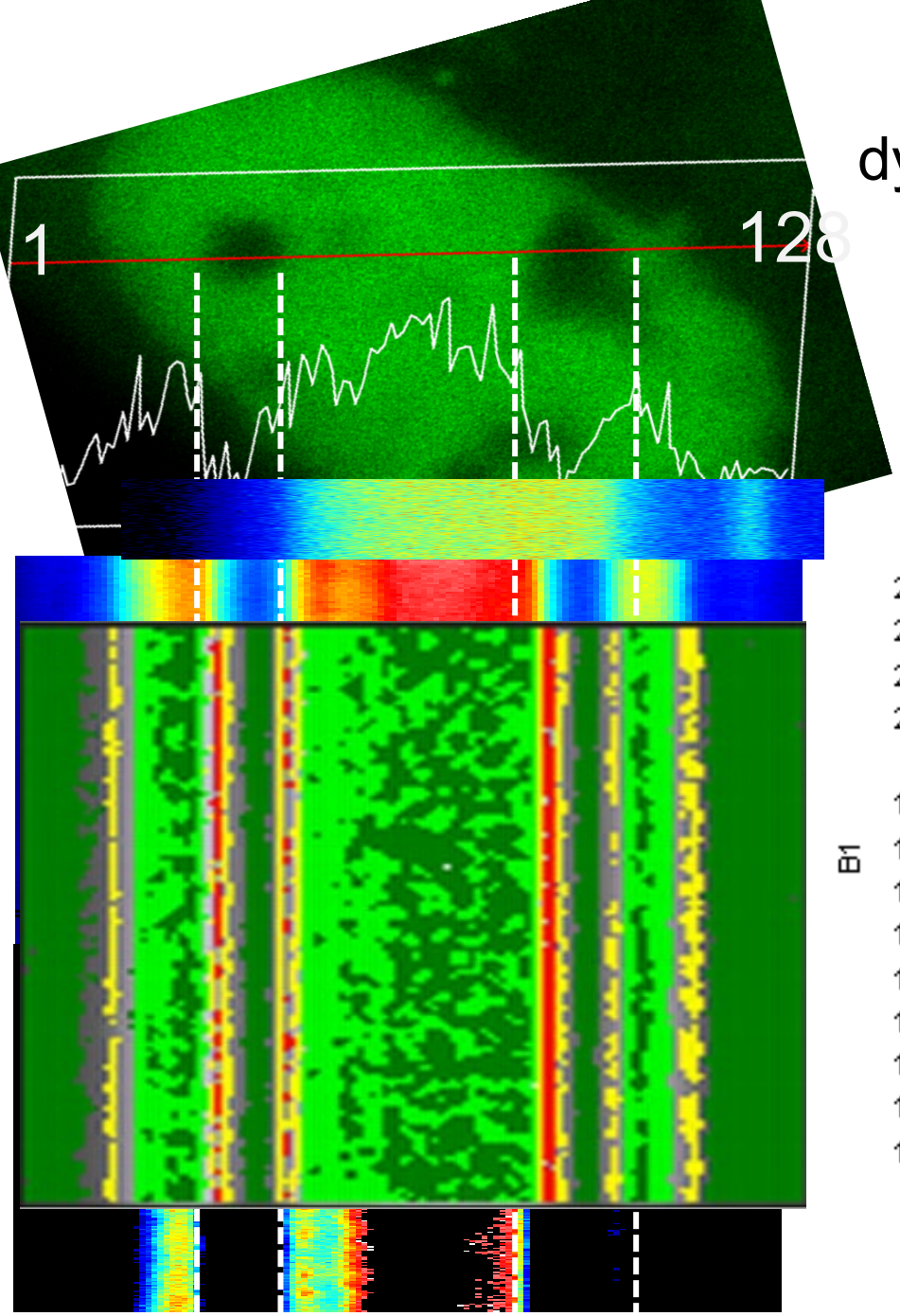
Stable Adhesion: only disassembly is detected



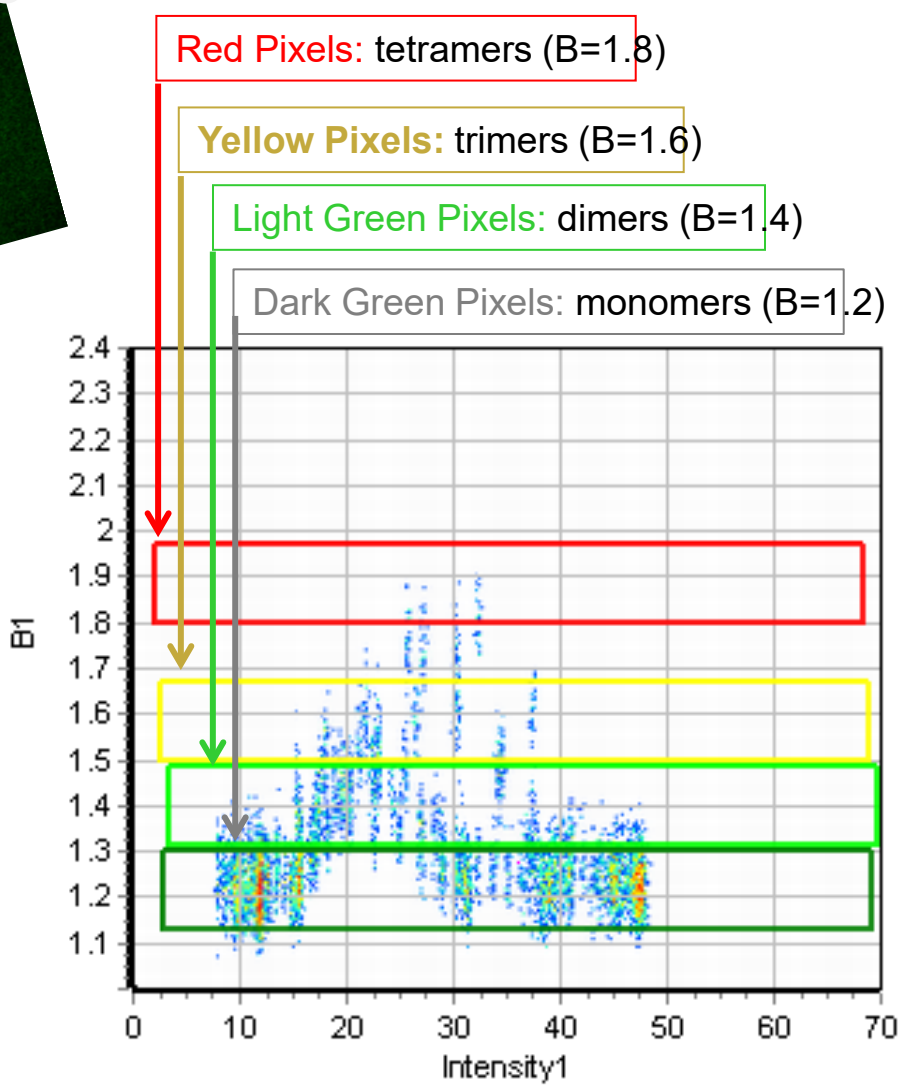
Stable Adhesion in MEF pax(-/-) cells

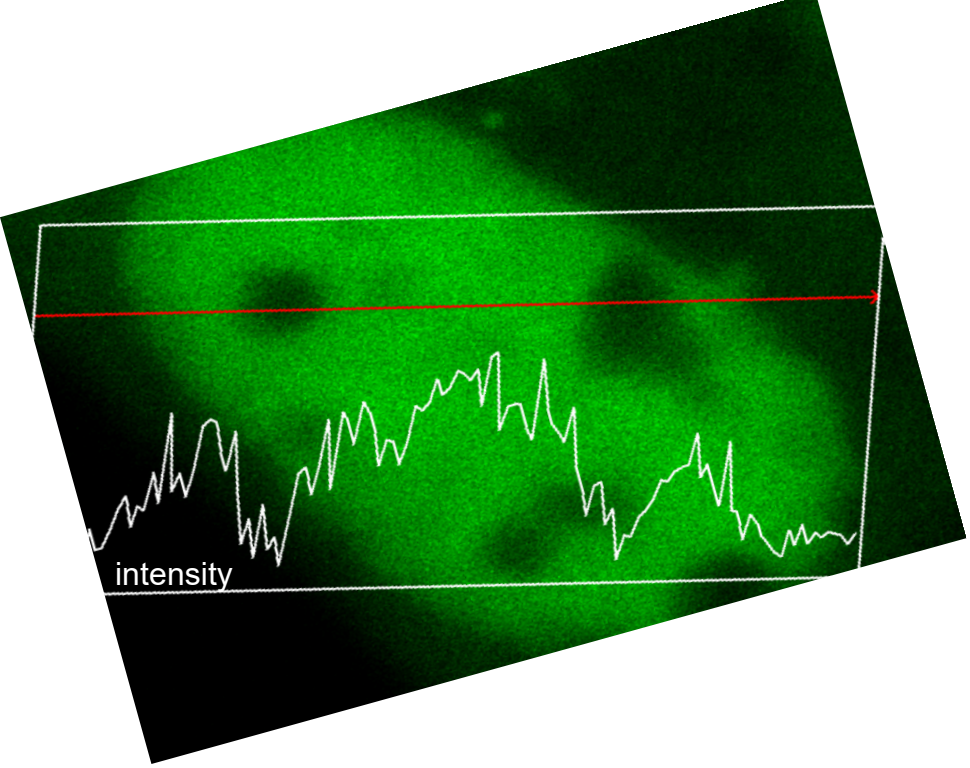
Digman et al." *Biophys J.* 94(7):2819-31, 2008

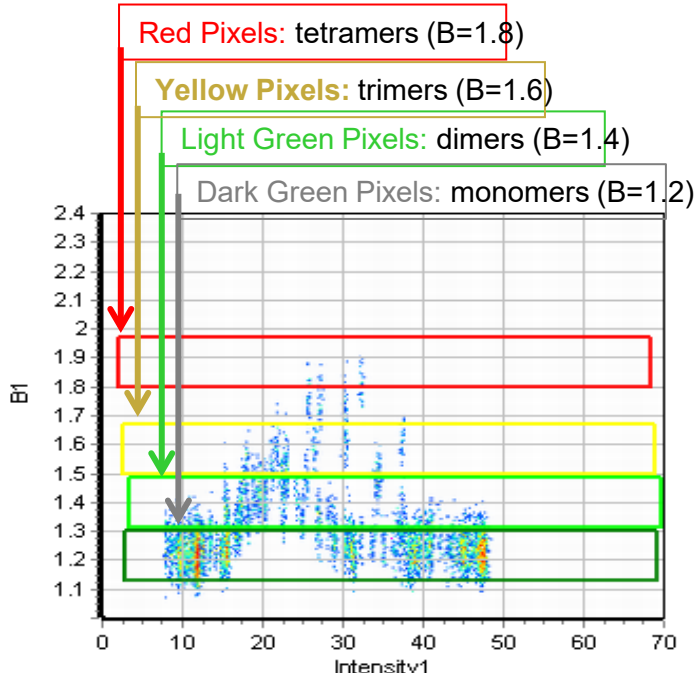
Line Scanning Reveals protein dynamic assembly at the chromatin structures as a function of time

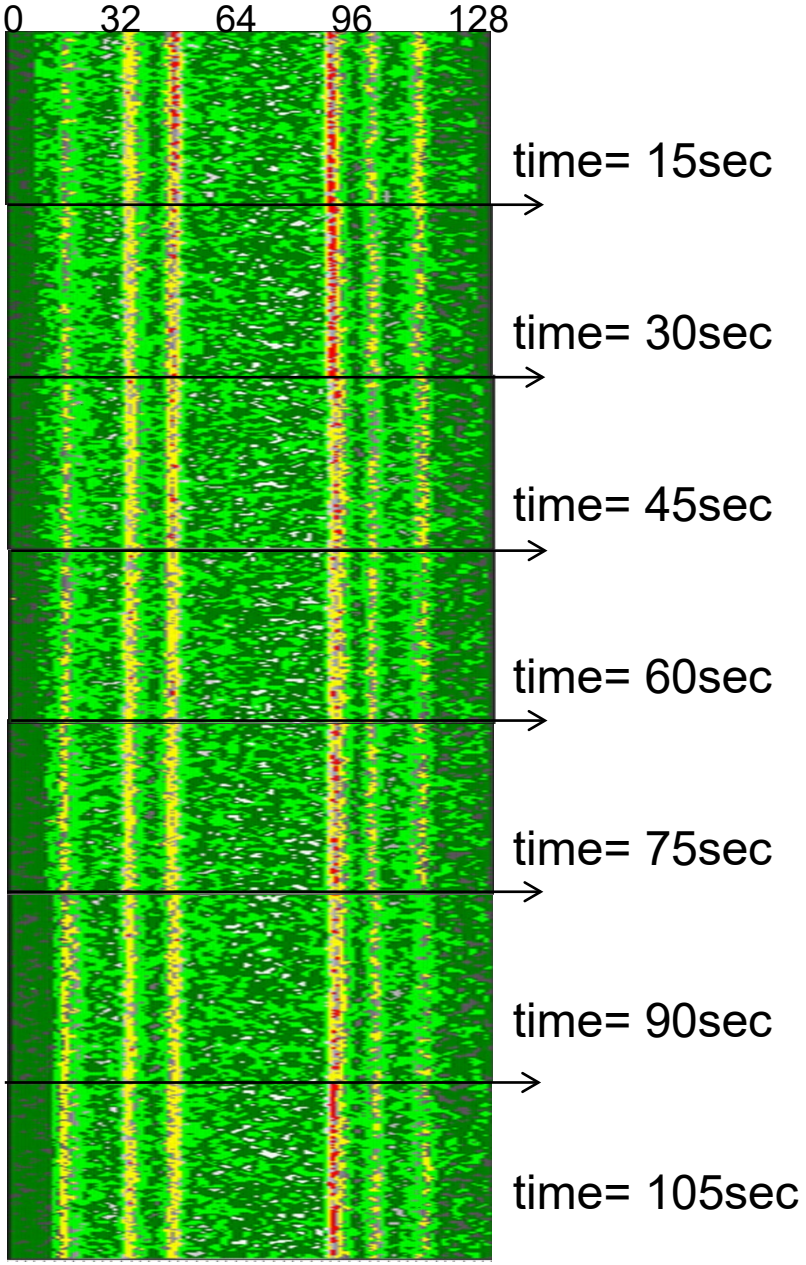


Line time: 1ms after 4.5 hrs



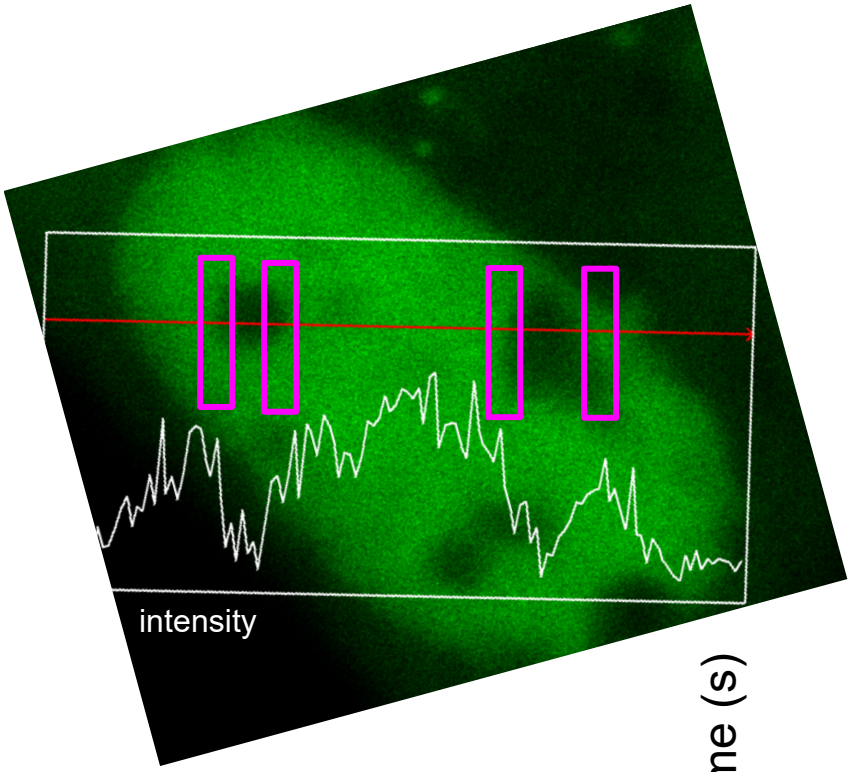


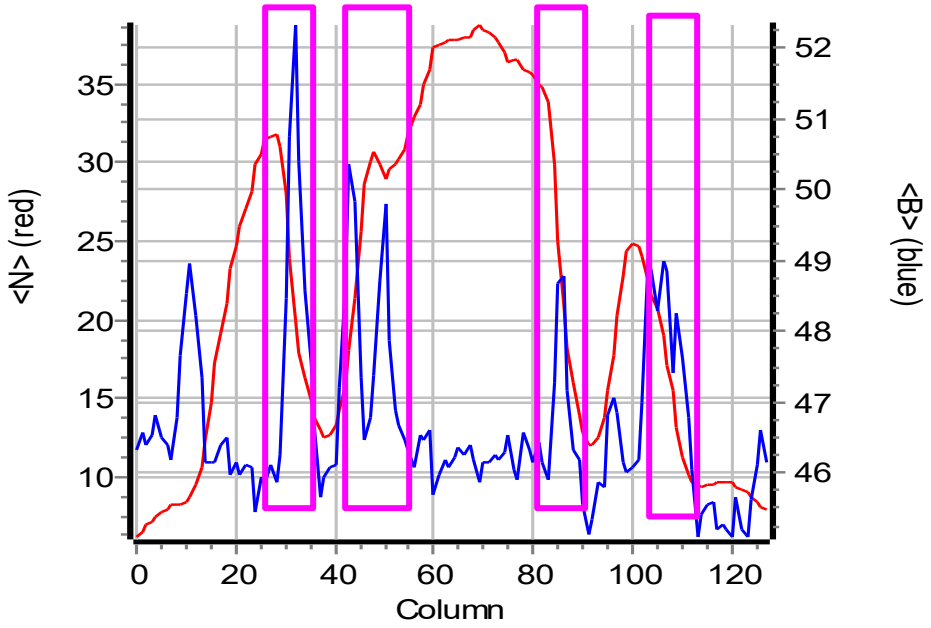


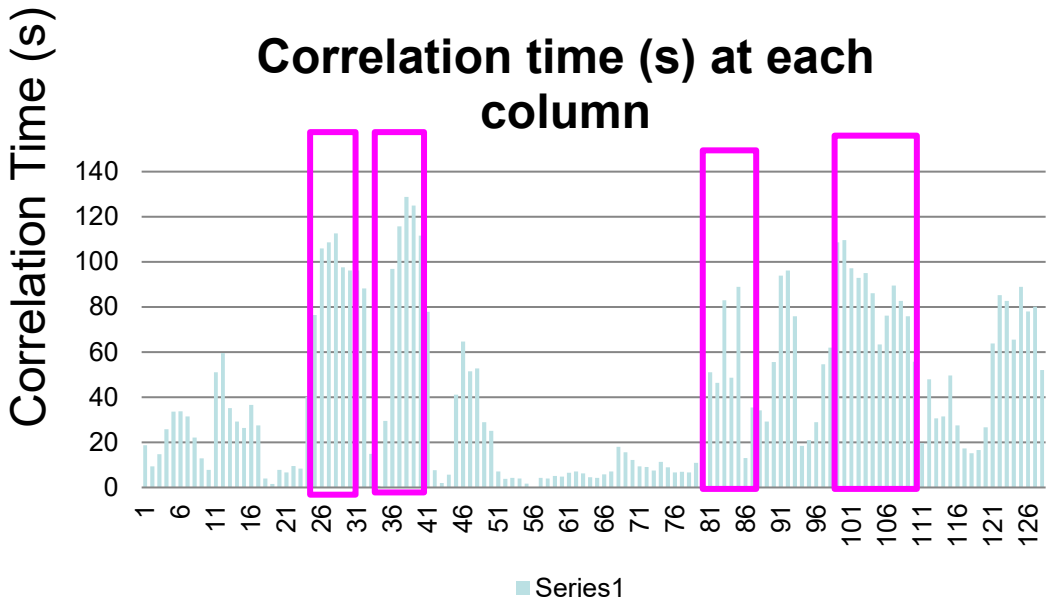


3.15μs/pixel or 1ms/line

Brightness and Correlation time along a line

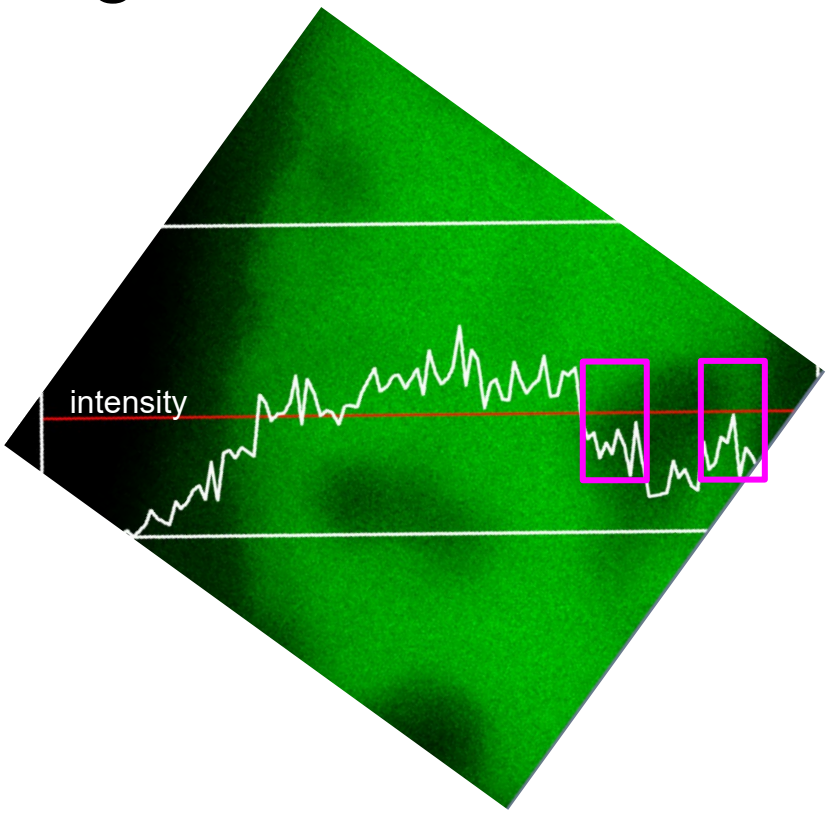


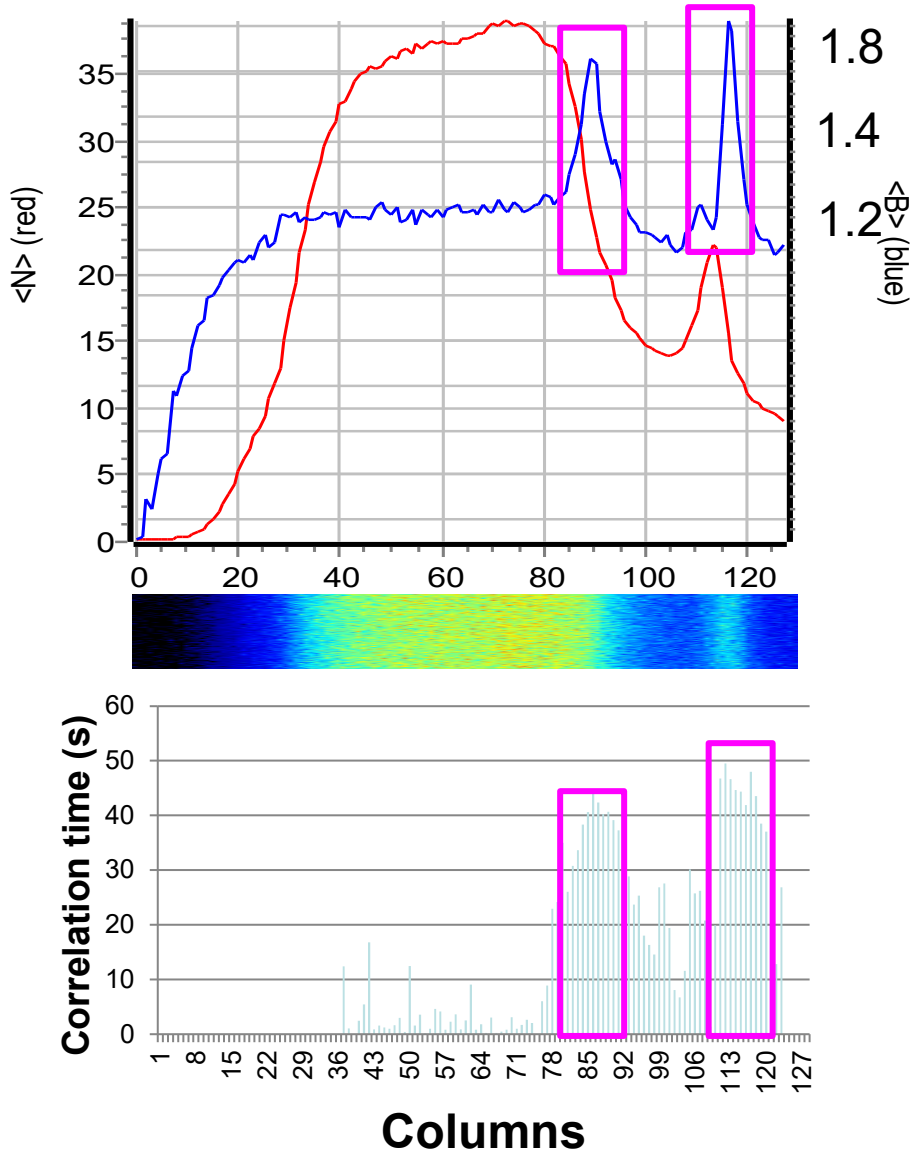




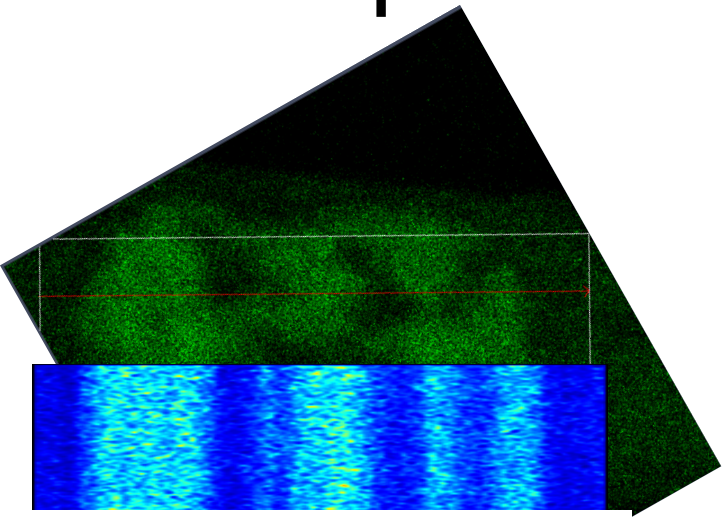
- The binding interaction of p53 5 hours after Cisplatin damage occurs in regions near the nucleous.

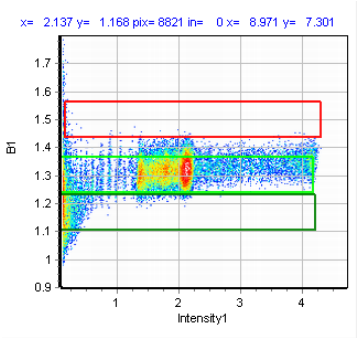
Brightness and Correlation time along a line

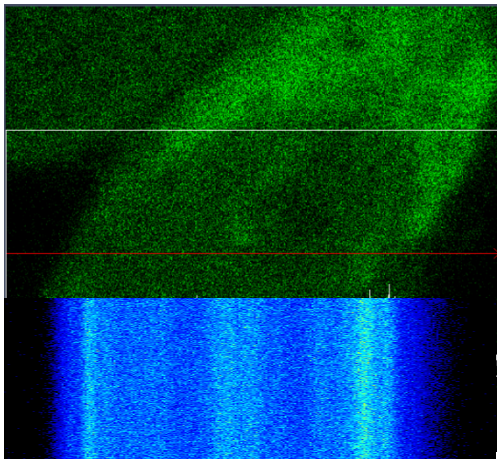


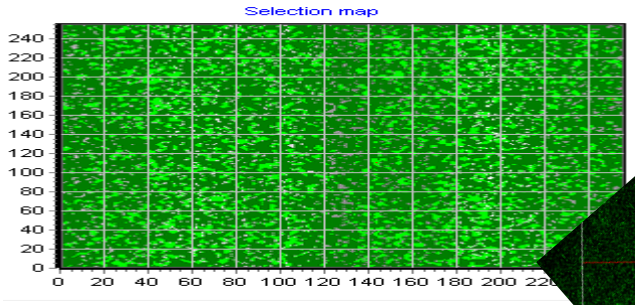


p53 cancer mutant R175H

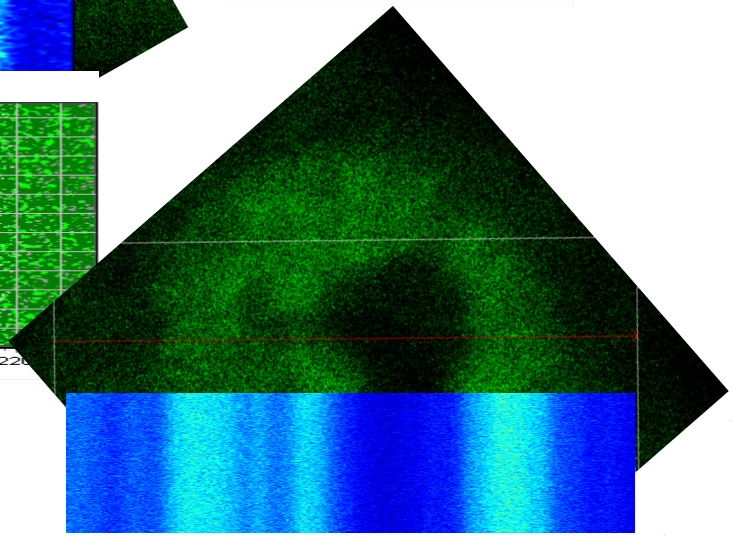


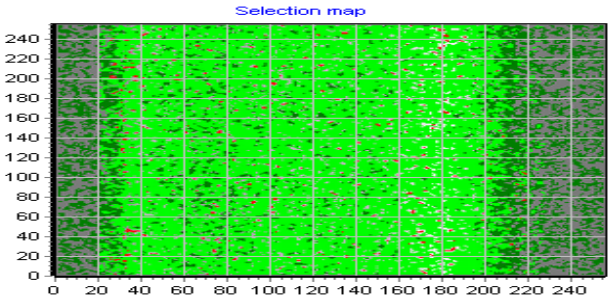




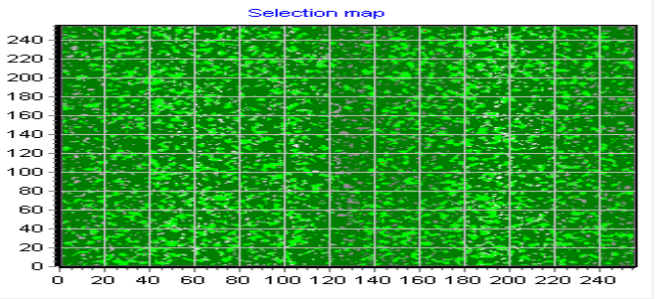


Before Damage





4hr after Damage



1hr after Damage