National Center for Research Resources

UCIrvine
University of California, Irvine

Raster Image Correlation Spectroscopy

RICS

Novel Idea: Raster Image Correlation Spectroscopy

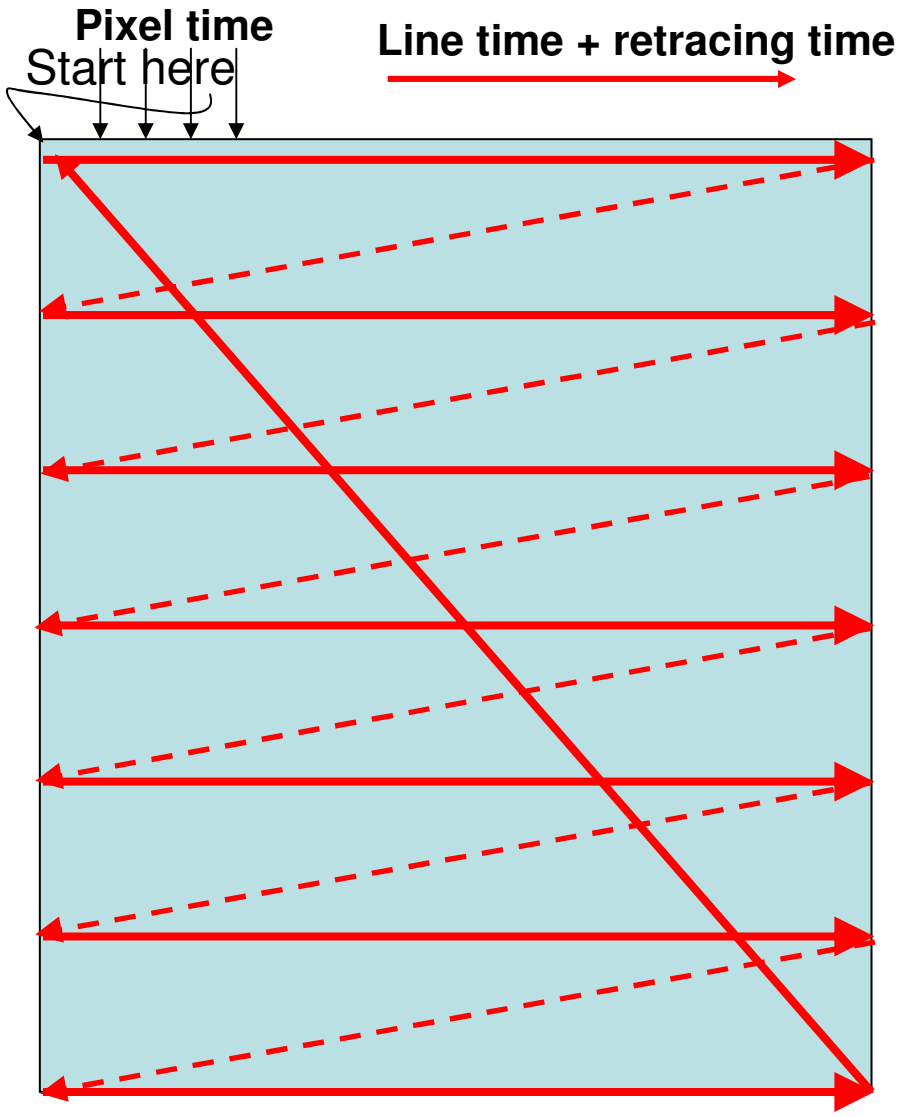
We can have a combination of very high time resolution with sufficient spatial resolution.

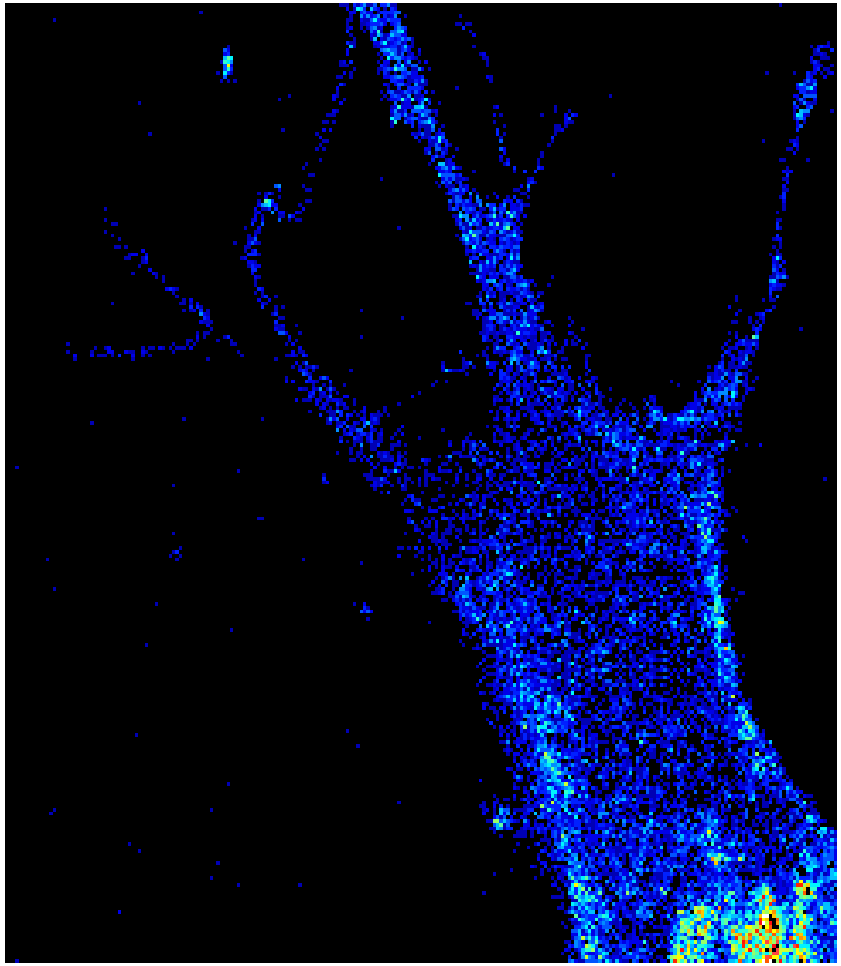
Major benefits of RICS:

- **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?

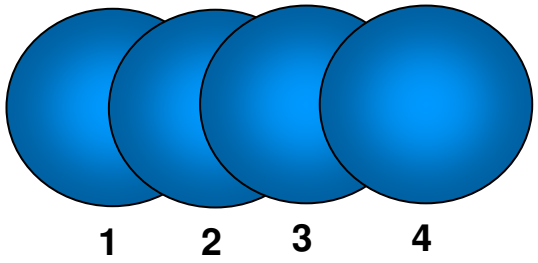
Raster Scanning



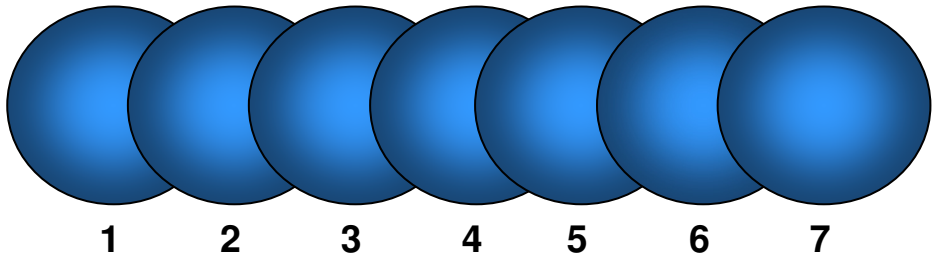


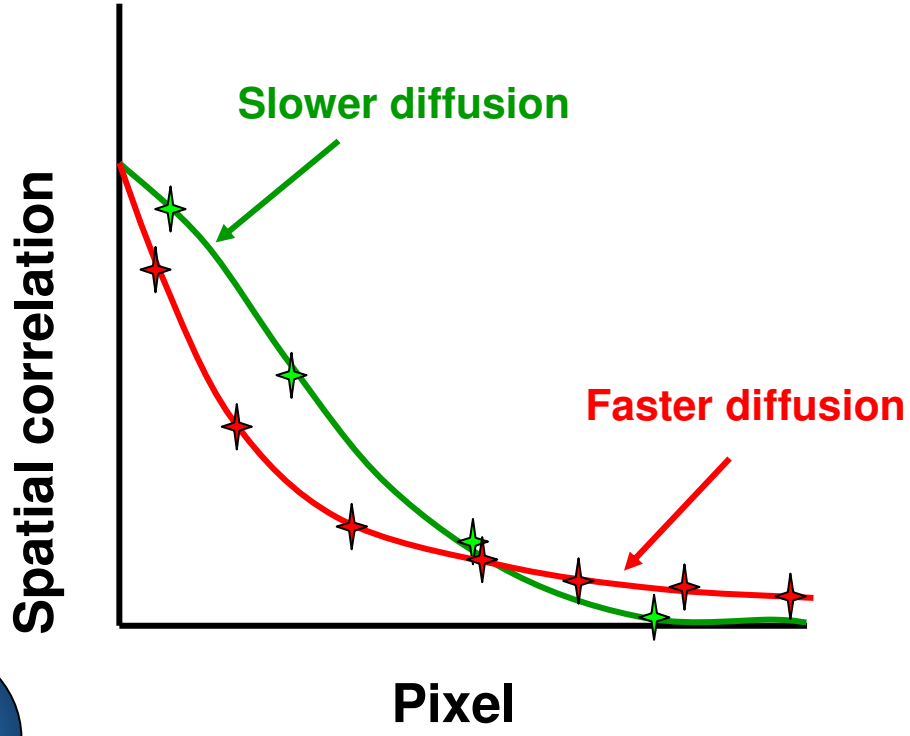
Temporal information hidden in the raster-scan image: the RICS approach

Situation 1:slow diffusion

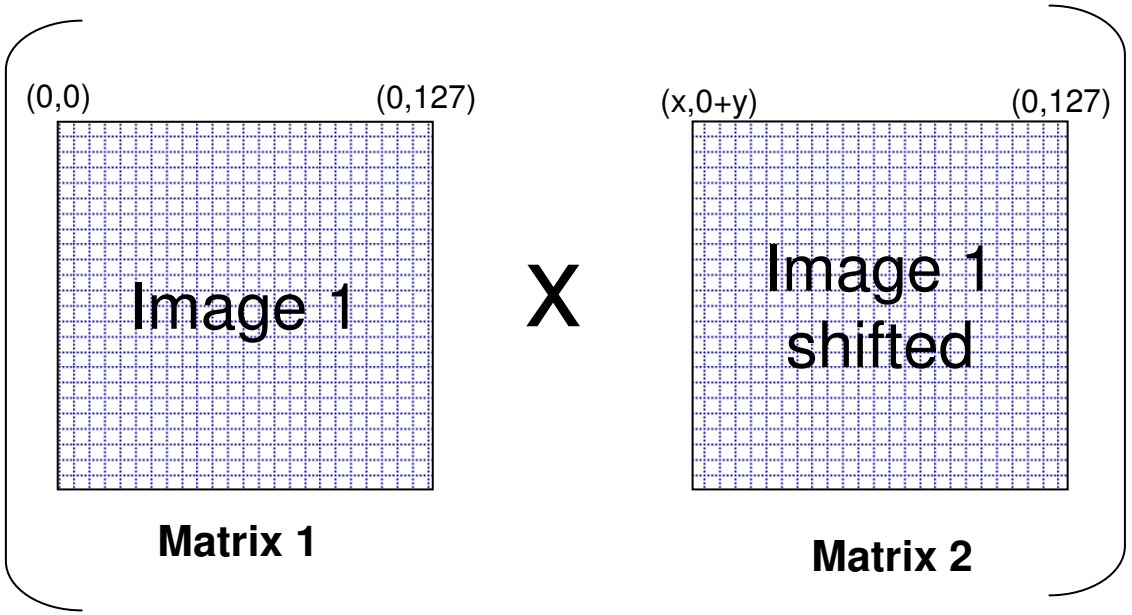


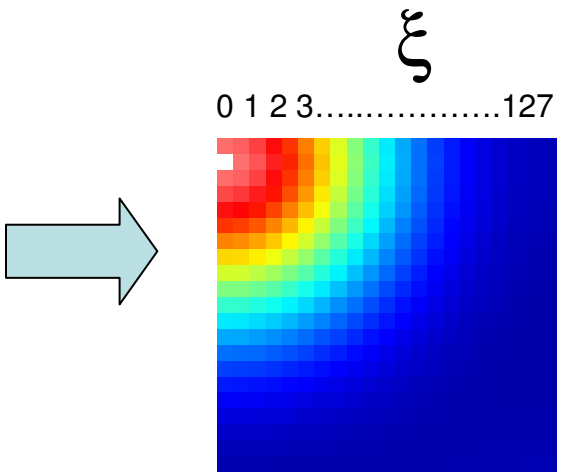
Situation 2: fast diffusion





How is the spatial correlation done?





Spatial Correlation Results

Operation:

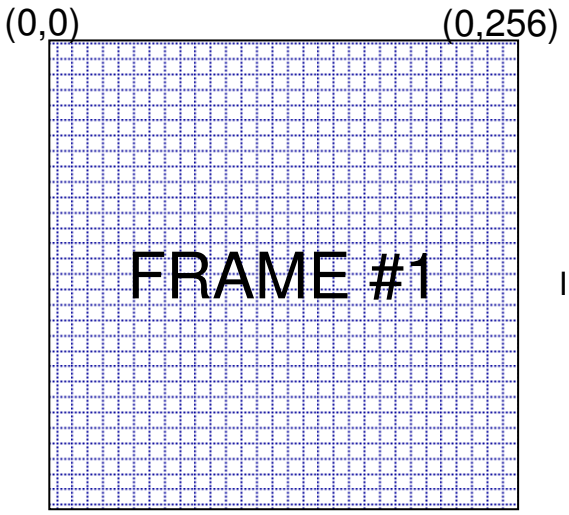
In the x direction

PLUS In the y direction

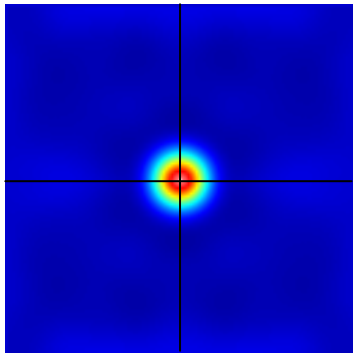
$$(0,0\times0,0)+(0,1\times0,1)+(0,2\times0,2)...(0,127\times0,127)$$
$$+(1,0\times1,0)+(1,1\times1,1)+(1,2\times1,2)...(1,127\times1,127)$$

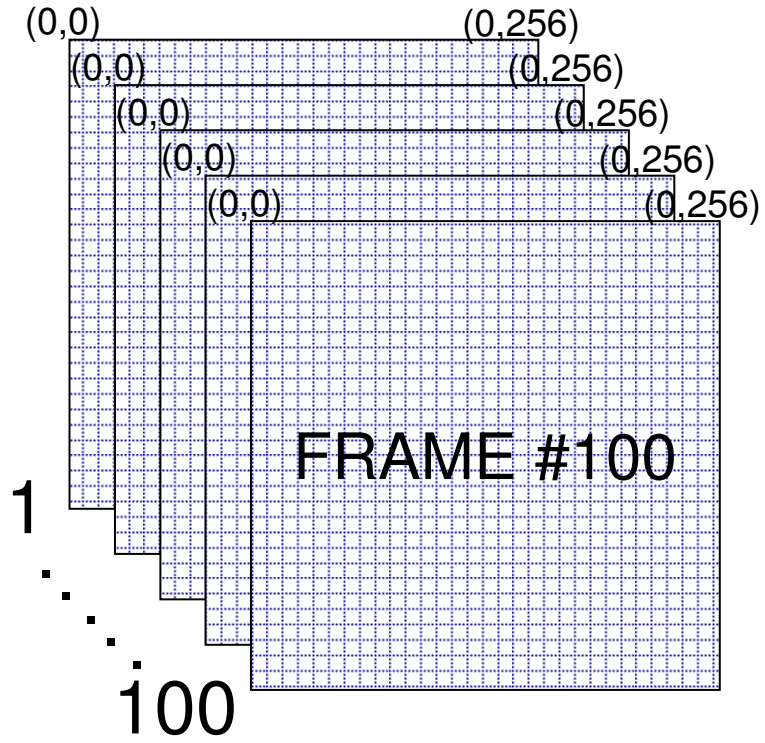
One number is obtained for x and y and is divided by the average intensity squared

How to use a stack of images?



Spatially correlate each frame
Individually then take the average of all the frames





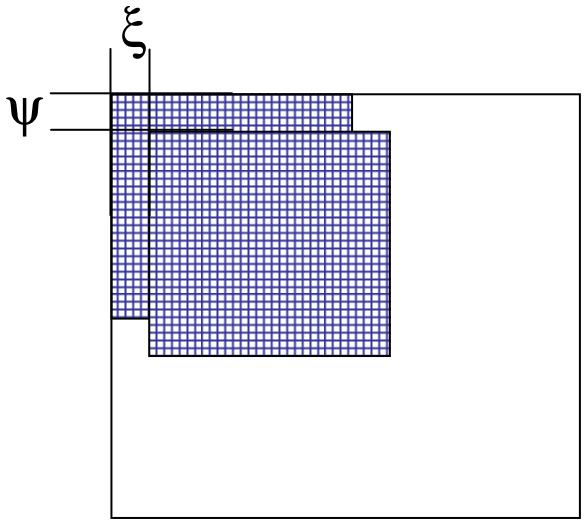
The RICS approach: 2-D spatial correlations

In a raster-scan image, points are measured at different positions and at different times simultaneously

If we consider the time sequence, it is not continuous in time
If we consider the pixel sequence, it is contiguous in space

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

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



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

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

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

The RICS approach for diffusion

The the dynamics at a point is independent on the scanning motion of the laser beam

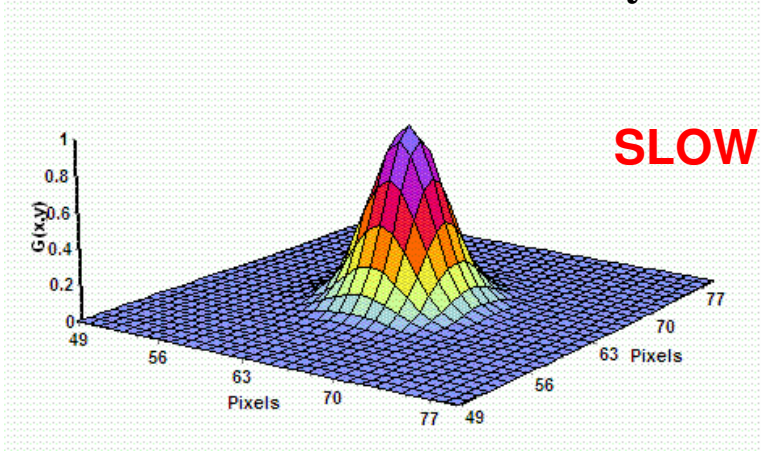
$$G_{RICS}(\xi,\psi) = S(\xi,\psi) \times G(\xi,\psi)$$

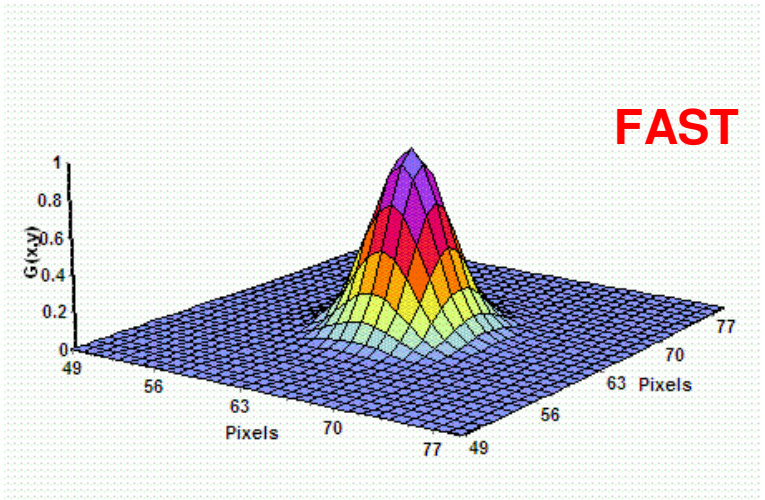
Consider now the process of diffusion. The diffusion kernel can be described by the following expression

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

There are two parts:
(1) the temporal term,
(2) the spatial Gaussian term

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

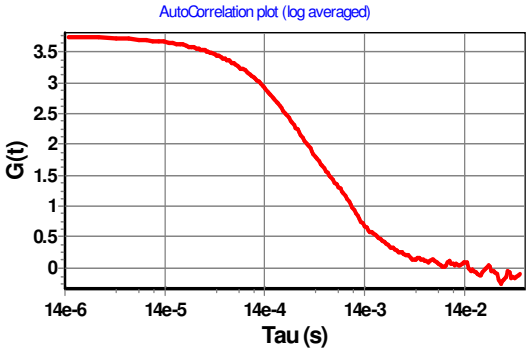




RICS: space and time relationships

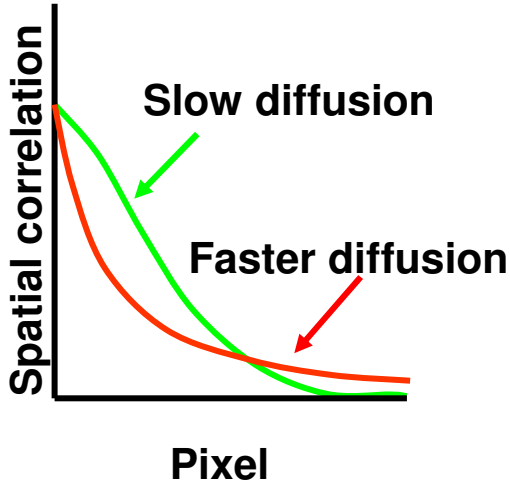
At any position, the ACF due to diffusion takes the familiar form:

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



t_p and t_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.

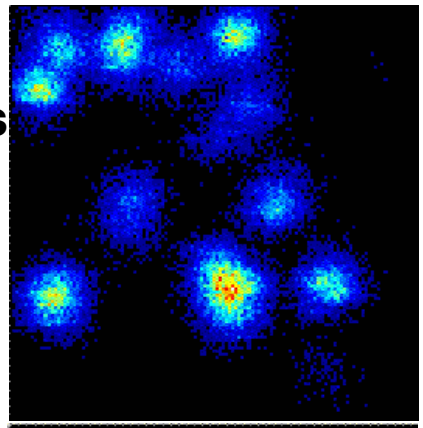
Digman et al. Biophys. J., 2005

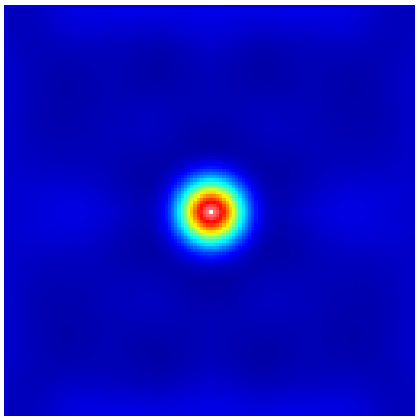
RICS Simulations of three different diffusion rates:

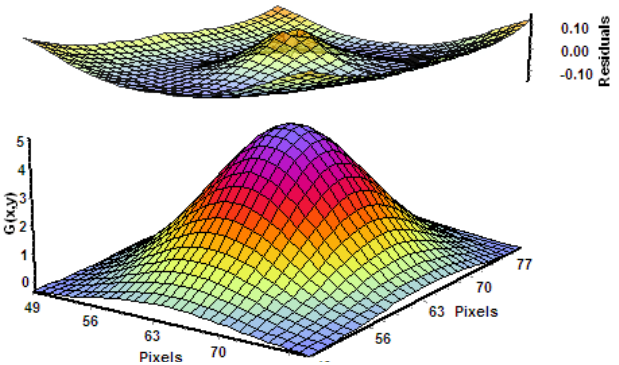
Box size=3.4µm sampling time: 1) 32µs/pixel 2) 8µs/pixel 3) 4µs/pixel

D = 0.1 µm²/s

(membrane proteins)

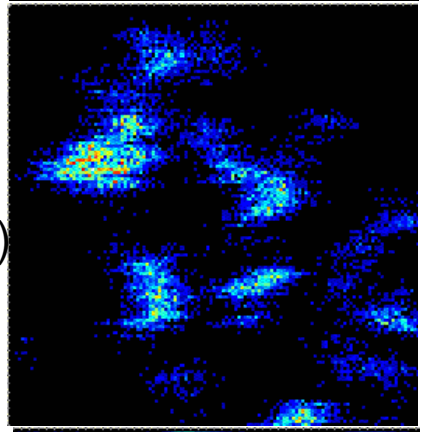


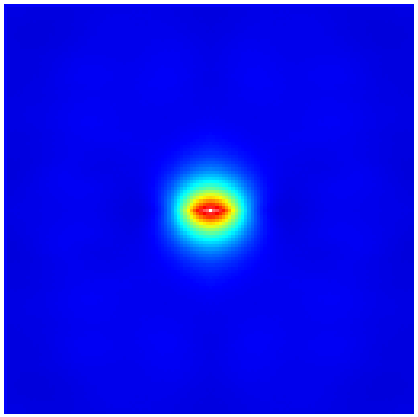


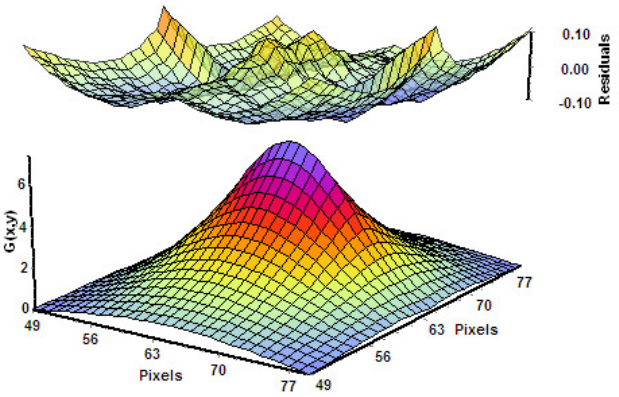


D = 5.0 µm²/s

(40 nm beads)

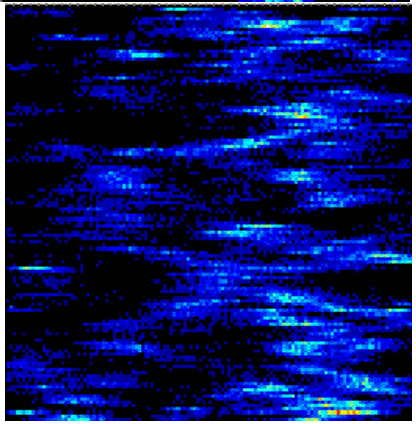


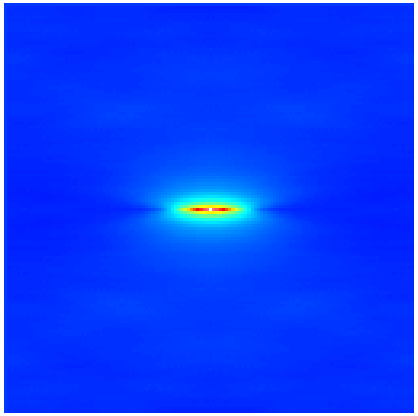


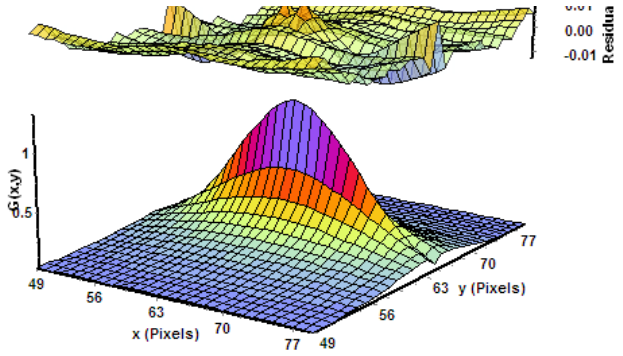


D = 90 µm²/s

(EGFP)



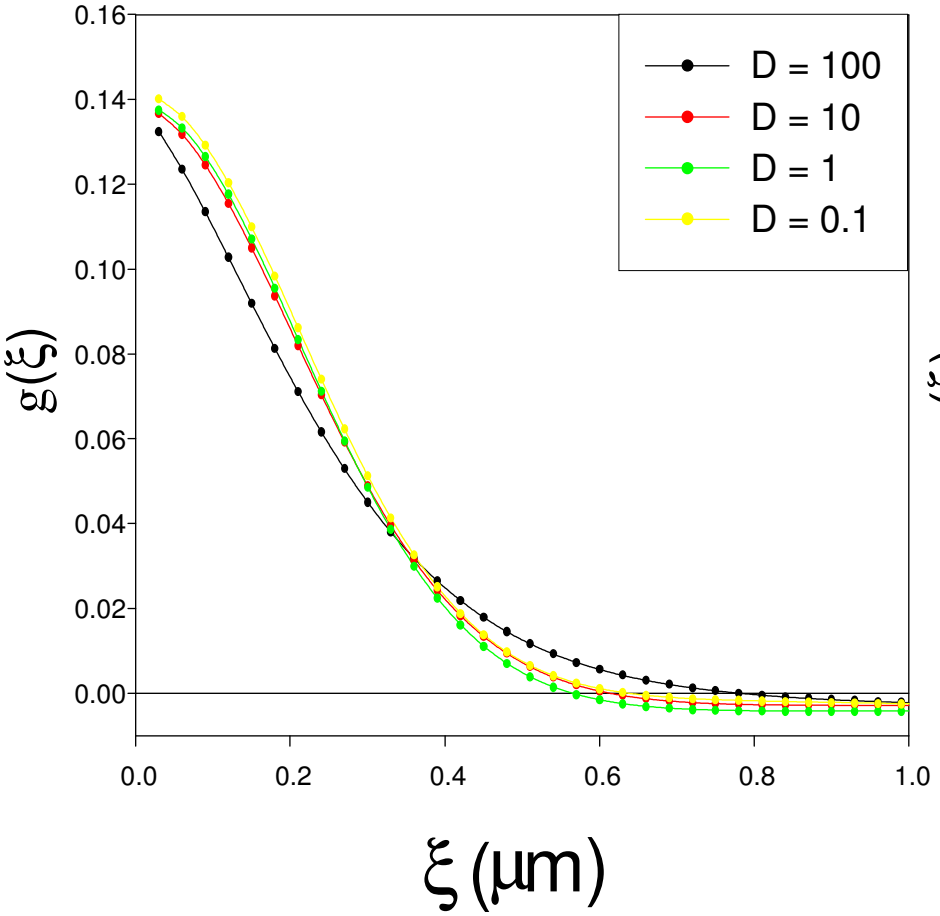




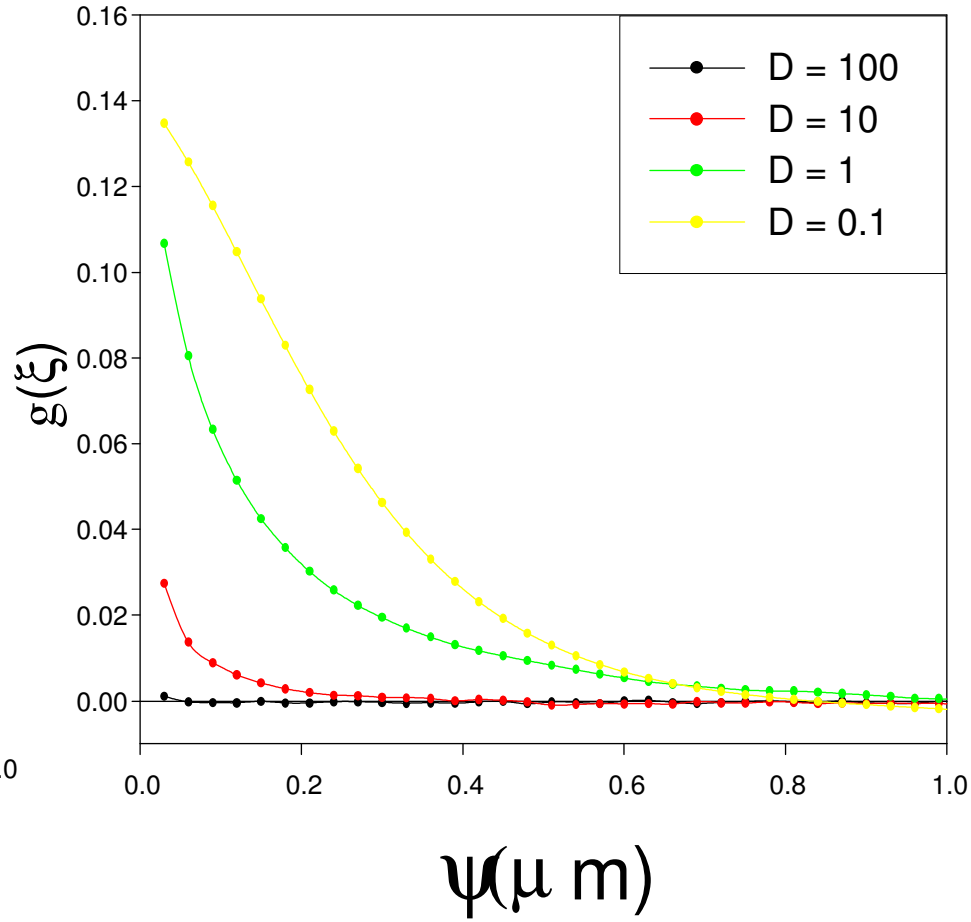
Horizontal and Vertical fits:

Simulations of beads 300 frames, 128x128pixels, 8µs/pix, size of pixels=30nm

Horizontal ACF



Vertical ACF



In SIMFCS

Brown et al, JMI, 2007

How to Setup the Laser Scanning Confocal Microscope

➢ Scan Speeds (μs/pixel):

- 4μs for fast molecules D >100μm²/s
- 8 -32μs for slower molecules D= 1 μm²/s-100μm²/s
- 32-100μs for slower molecules D= 0.1 μm²/s-10μm²/s

➢ Pixel Size:

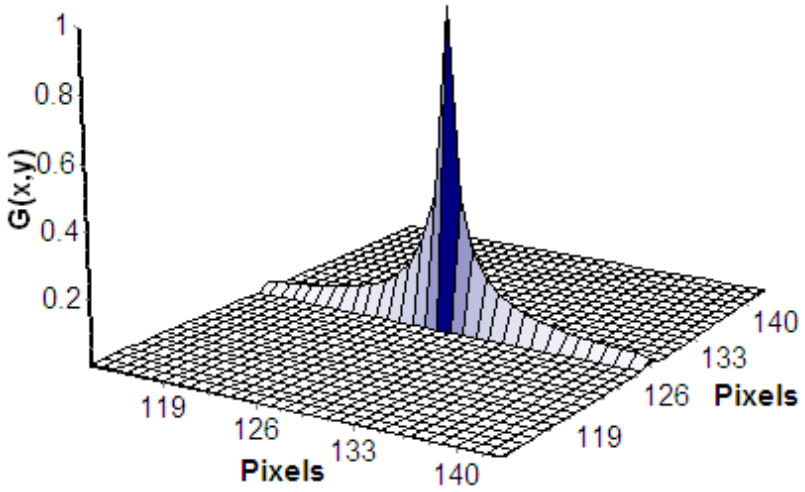
- 3-4x smaller than the Point Spread Function (PSF≈ 300nm)

➢ Molecular Concentrations

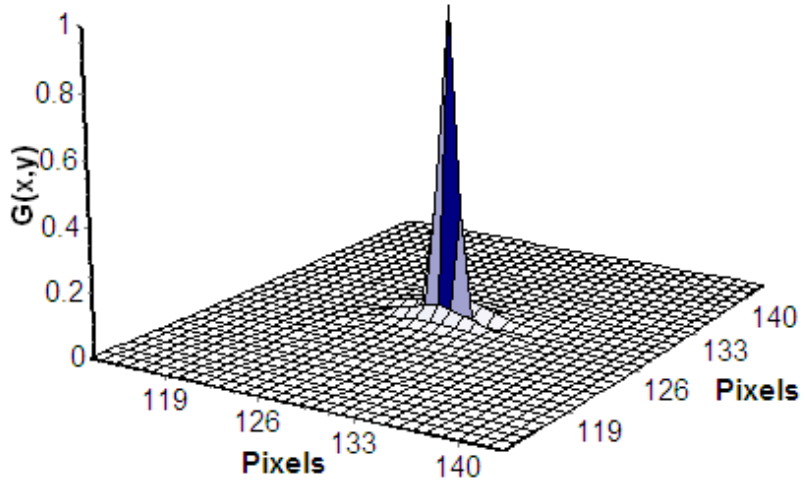
- Same conditions as conventional FCS methods

Common Errors in RICS

Scanning Too Slow
(100 us/pixel, D = 300 um^2/s)



Pixels are separated too much relative to PSF
(pixel size = w_0 = 0.3 um)

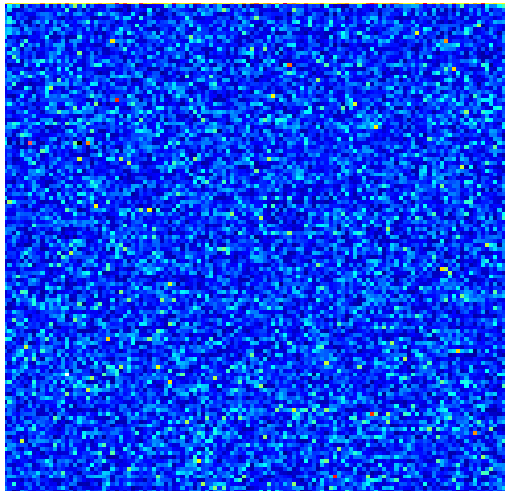


Courtesy of Jay Unruh

RICS: Fits to spatial correlation functions

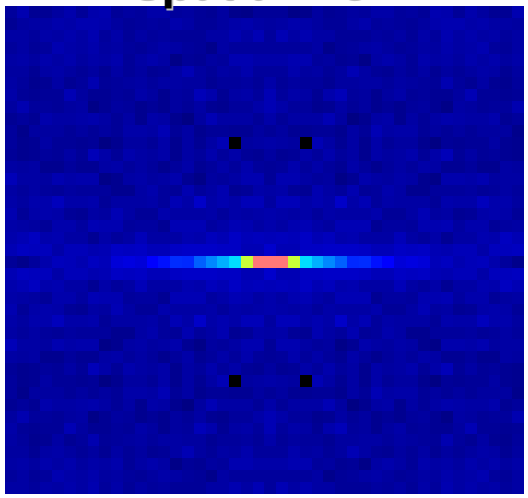
Olympus Fluoview300 LSM

EGFP in solution

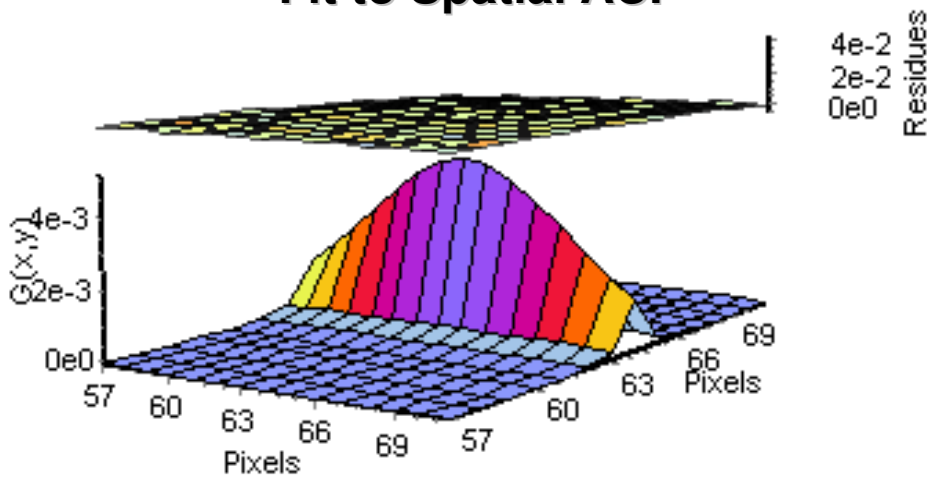


128x128, 4 µs/pixel, 5.4 ms/line, 0.023 µm/pixel

Spatial ACF



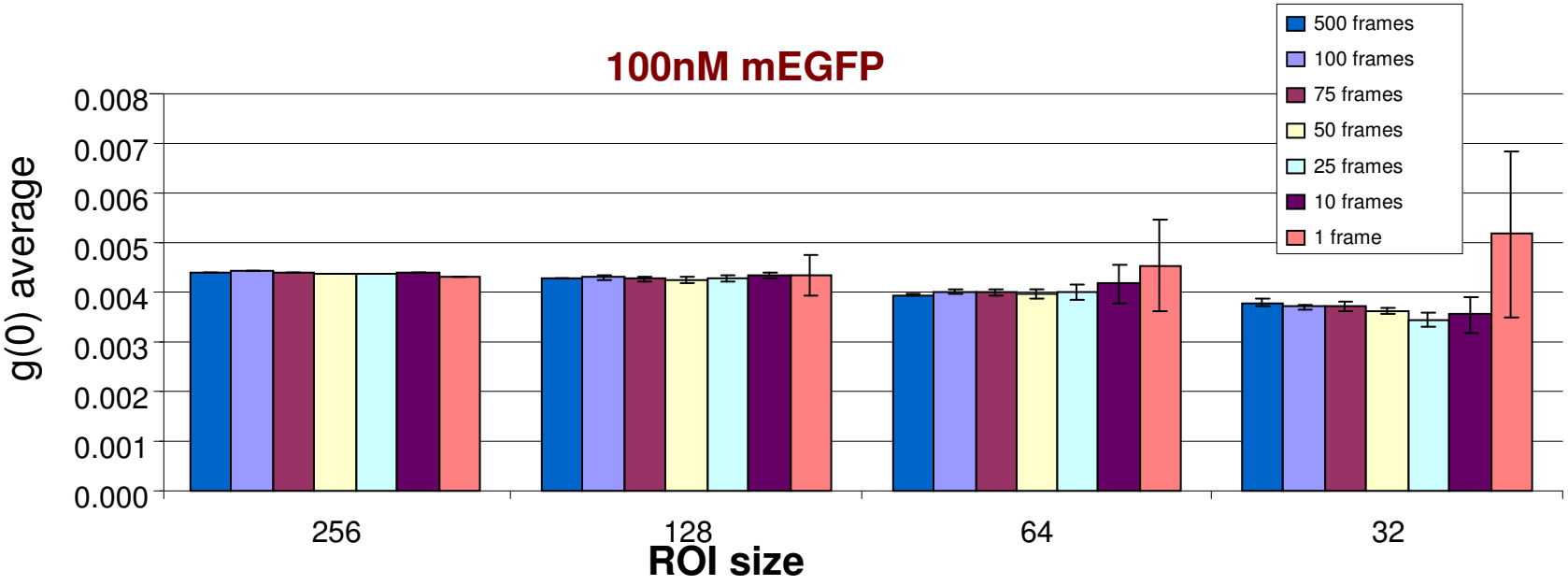
Fit to Spatial ACF

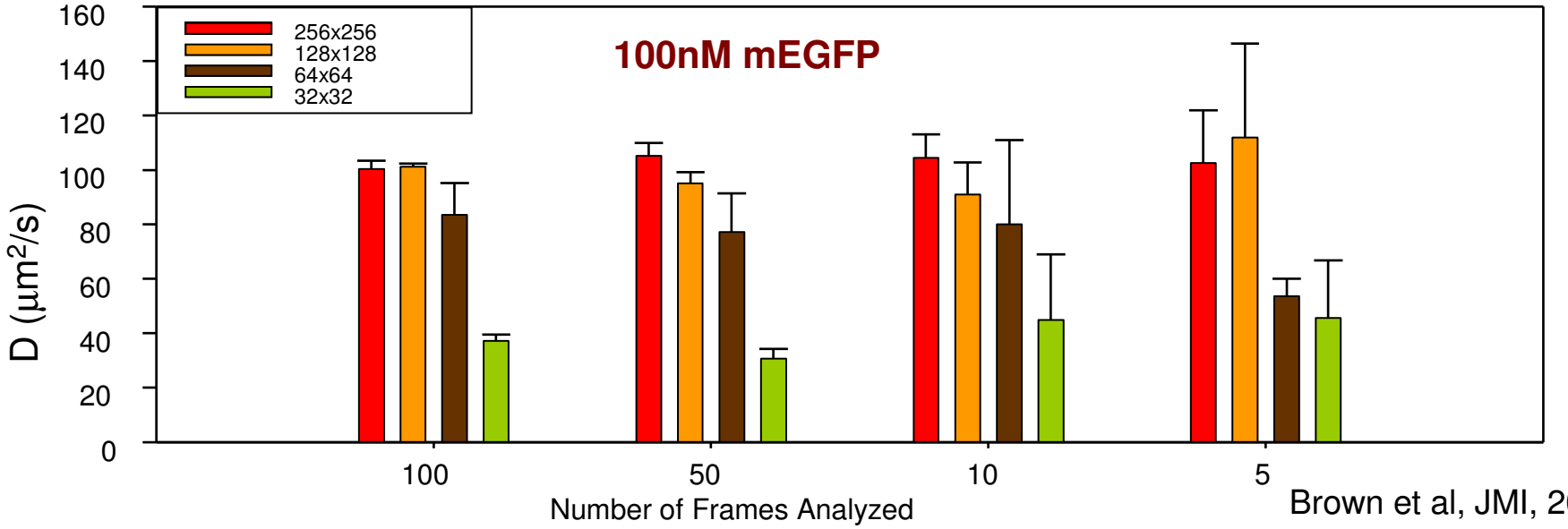


D = 105 ± 10 µm²/s

Digman et al. Biophys. J., 2005

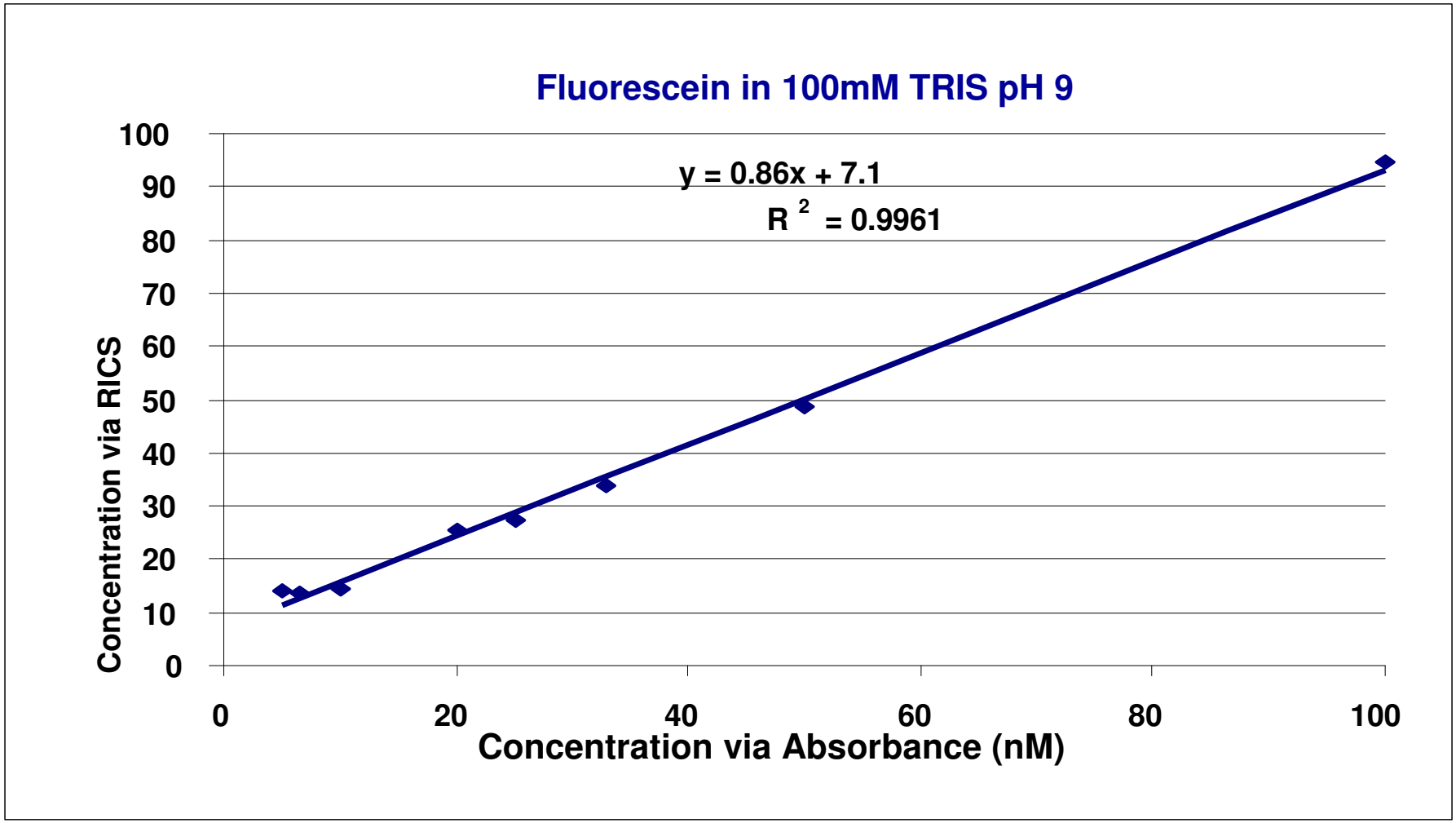
What ROI size to use? How many frames to acquire?





Brown et al, JMI, 2007

Obtaining concentration from RICS

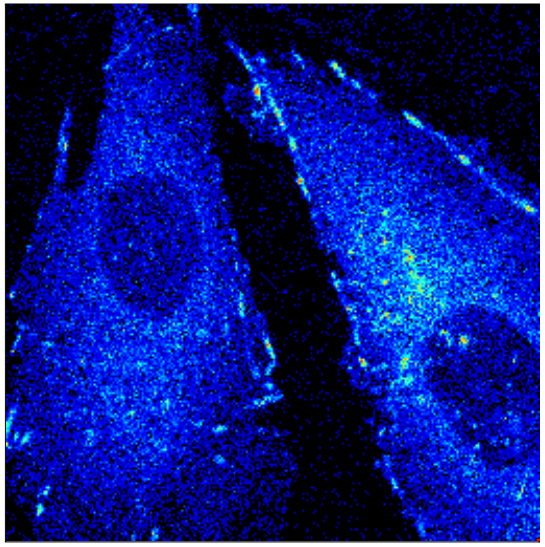


Brown et al, JMI, 2007

How 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 detectors correlate?

In a "truly immobile" bright region, the intensity fluctuates according to the Poisson distribution due to shot noise.

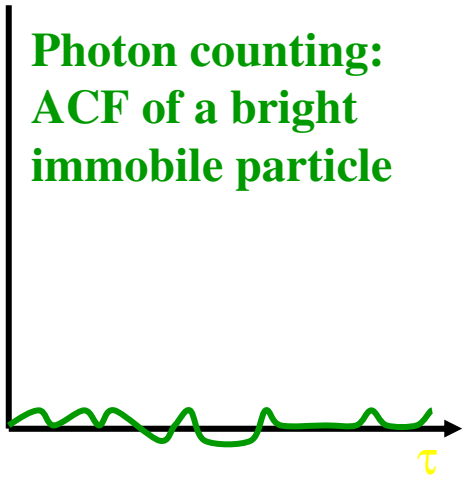
The time correlation of the shot noise is zero, except at time zero.

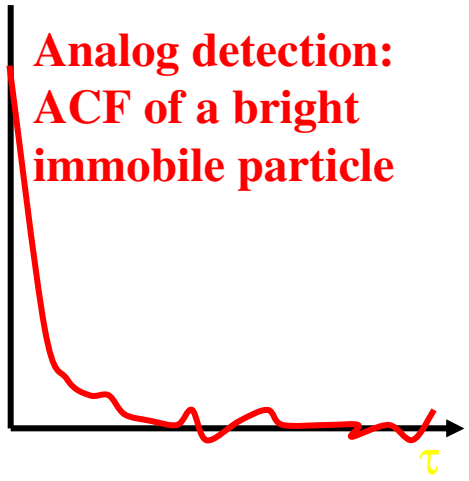
The spatial correlation of the intensity at any two pixels due to shot noise is zero, even if the two points are within the PSF.

If we subtract the average intensity and disregard the zero time-space point, the immobile bright region totally disappear from the correlation function

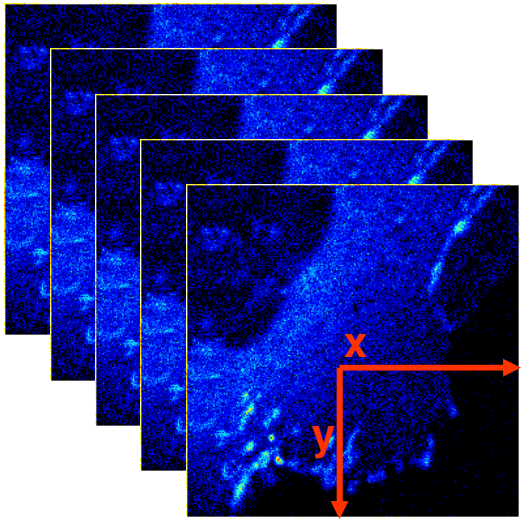
Attention!!!!

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.

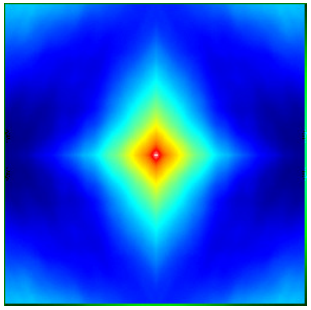




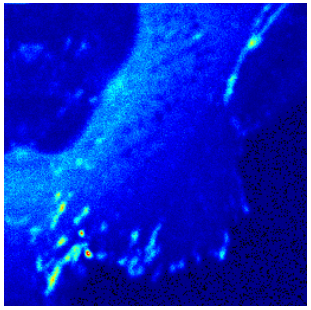
Formula used to subtract background:



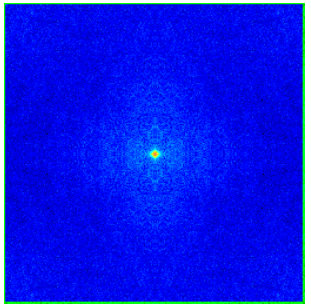
Spatial Correlation



Spatial correlation before subtracting background



Subtract the average



Spatial Correlation of entire image After subtracting image

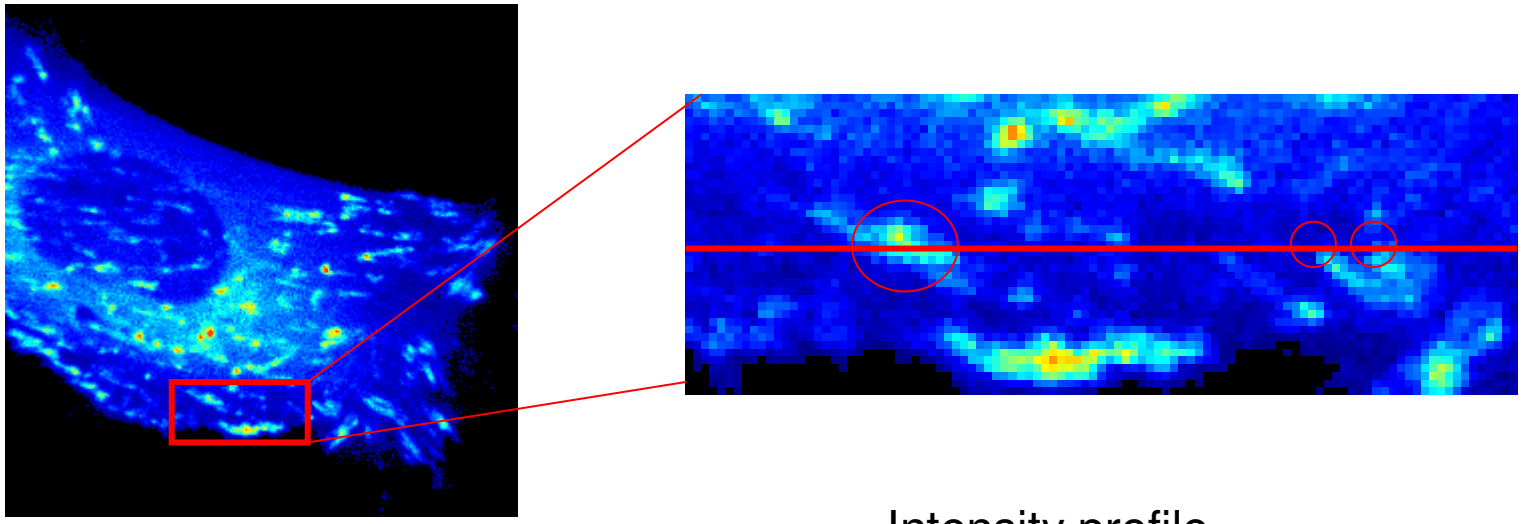
Average intensity of each pixel on the overall stack: $\overline{I(x,y)}$

$I_i(x,y) - \overline{I(x,y)}$ The intensity of each pixel minus the average intensity from entire stack for each pixel: However, this yields negative values.

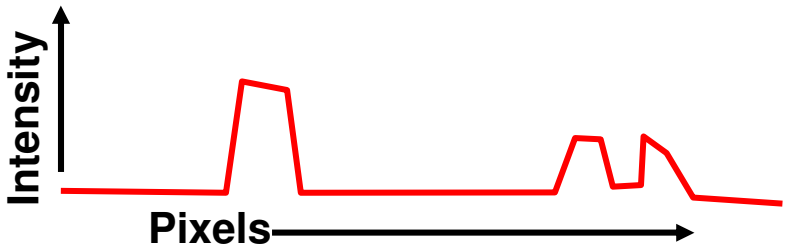
A scalar must be added : $a = \overline{I}$

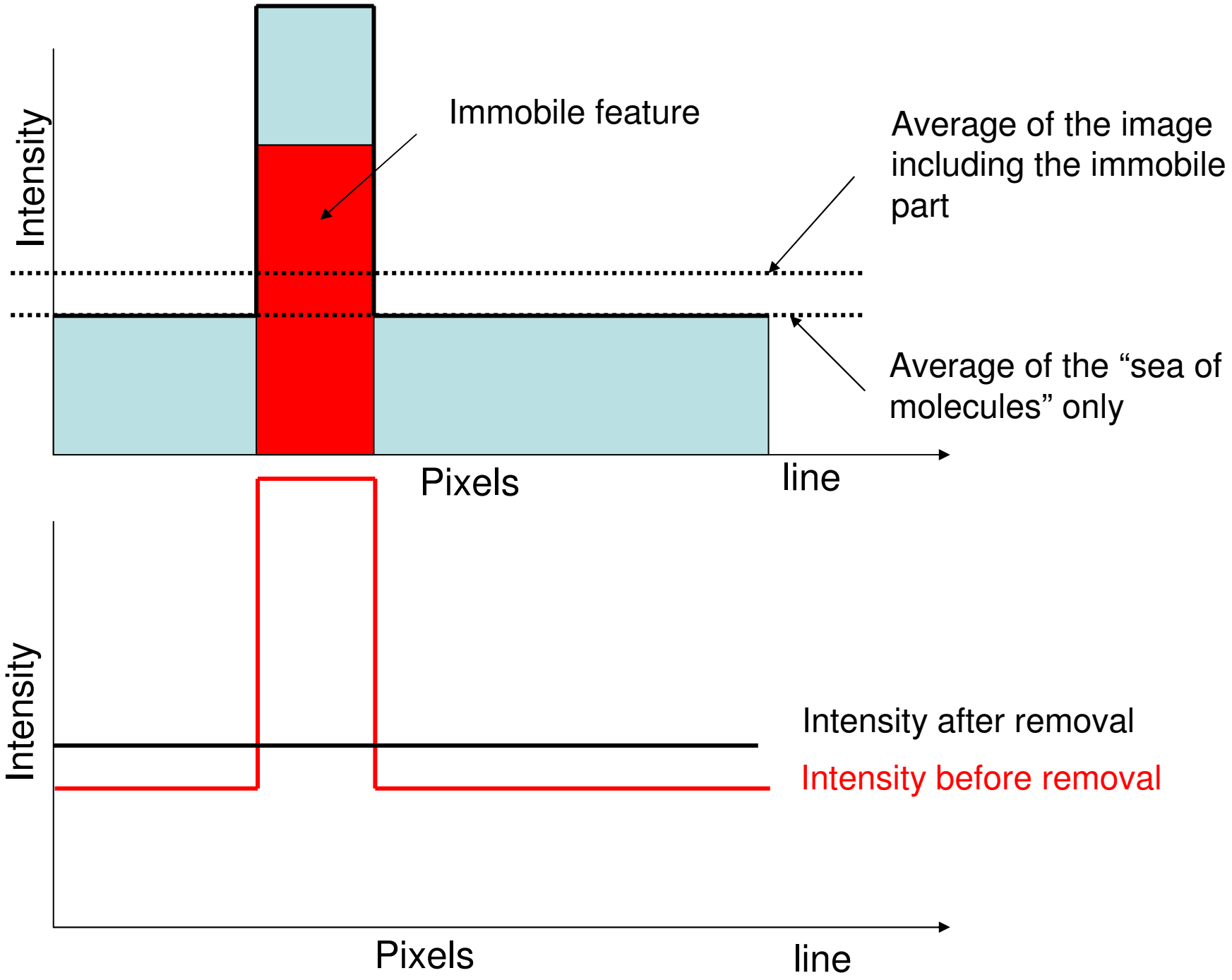
$$ICS(F_i(x,y)) \qquad where \qquad F_i(x,y) = I_i(x,y) - \overline{I(x,y)} + a$$

How to subtract immobile features from images?

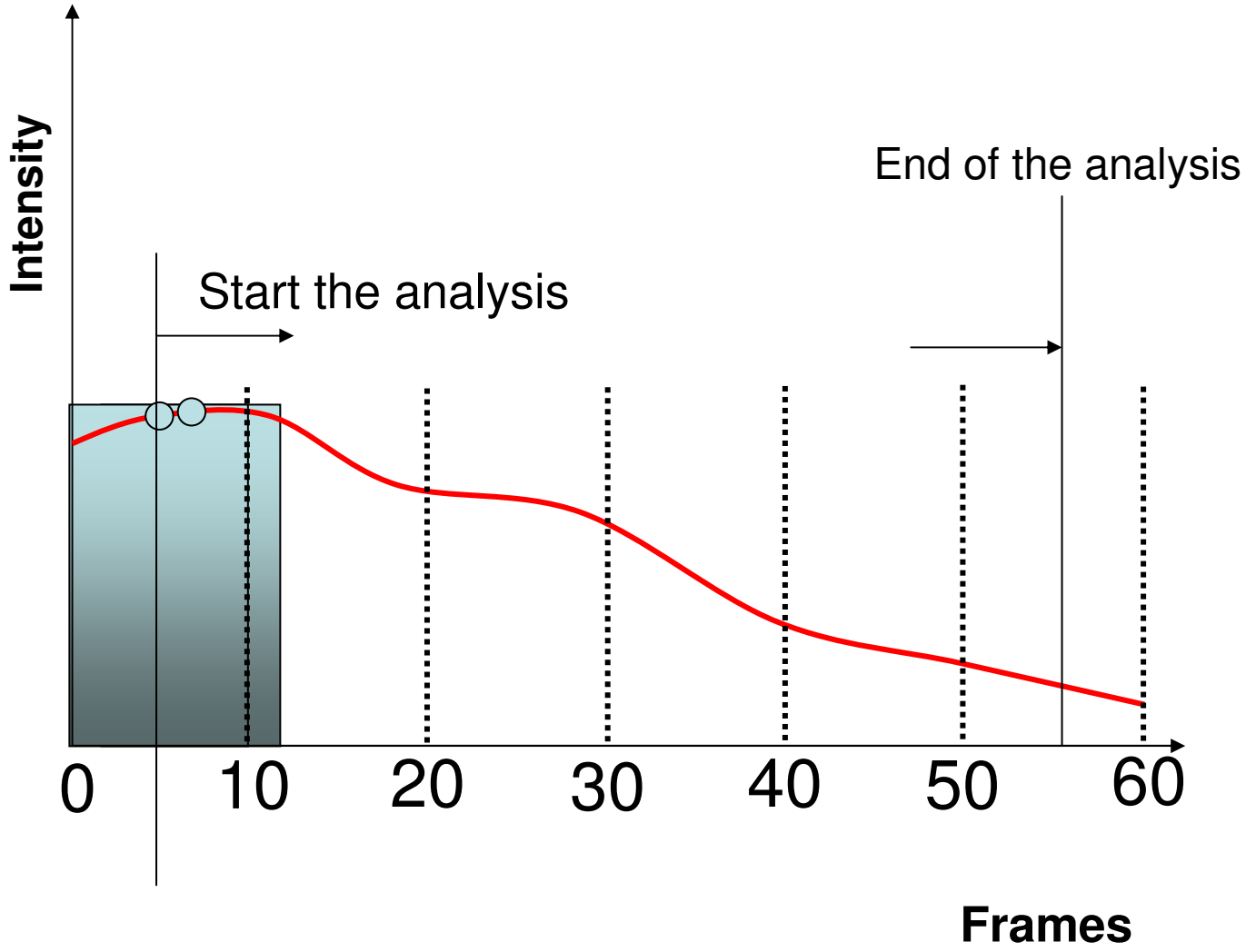


Intensity profile



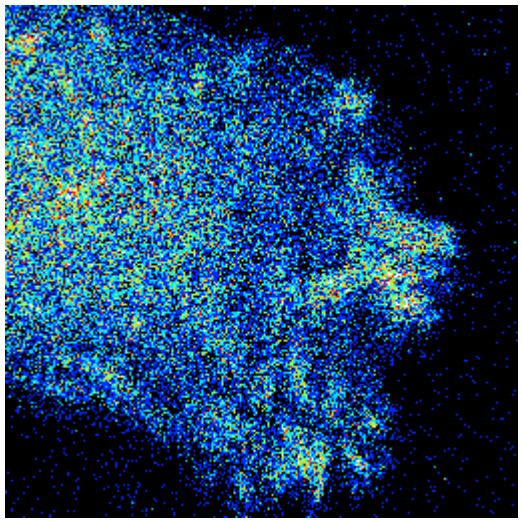

Intensity
Immobile feature
Average of the image including the immobile part
Average of the “sea of molecules” only
Pixels
line
Intensity
Intensity after removal
Intensity before removal
Pixels
line

Subtraction of moving average



Moving average operation on frames:

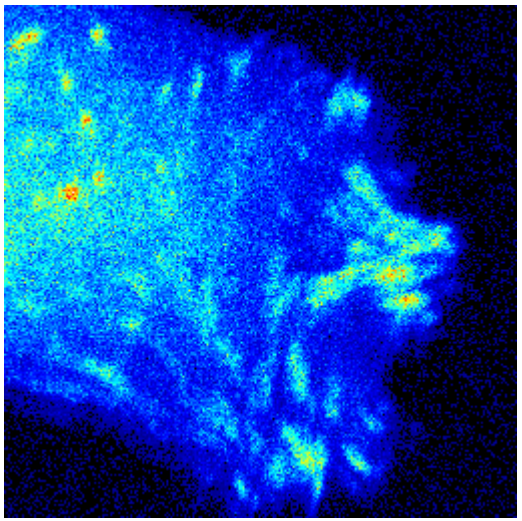
Frame #5



Matrix1

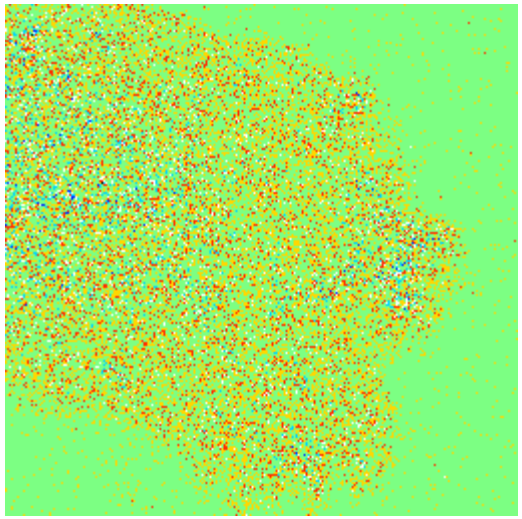
-

Average
between 1-10



Matrix 2

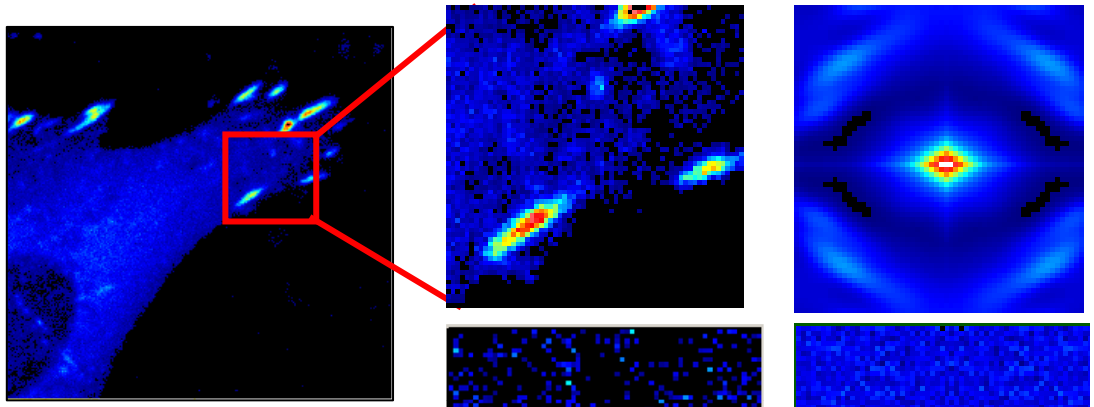
=



A scalar average is then added

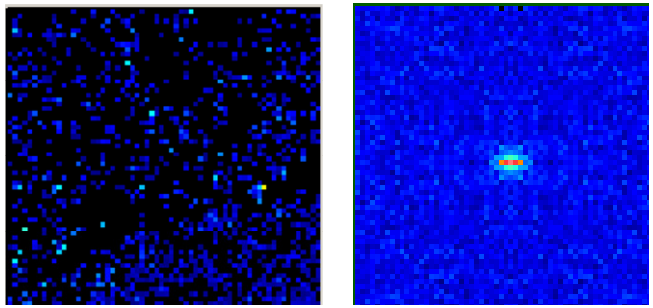
Operation is repeated for frame #6 - average between 2-11
frame #7 - average between 3-12

Example of the Removal of Immobile Structures and Slow Moving Features

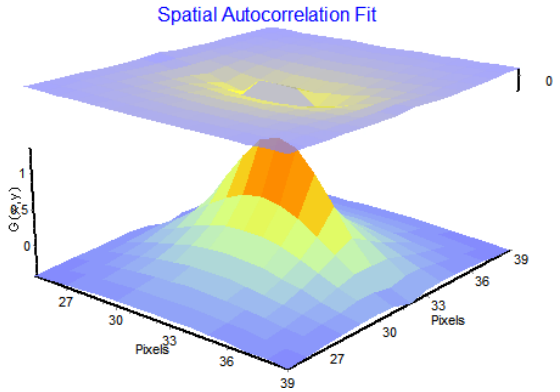


Spatial ACF
No removal

What is left
after removal



Spatial ACF
With removal

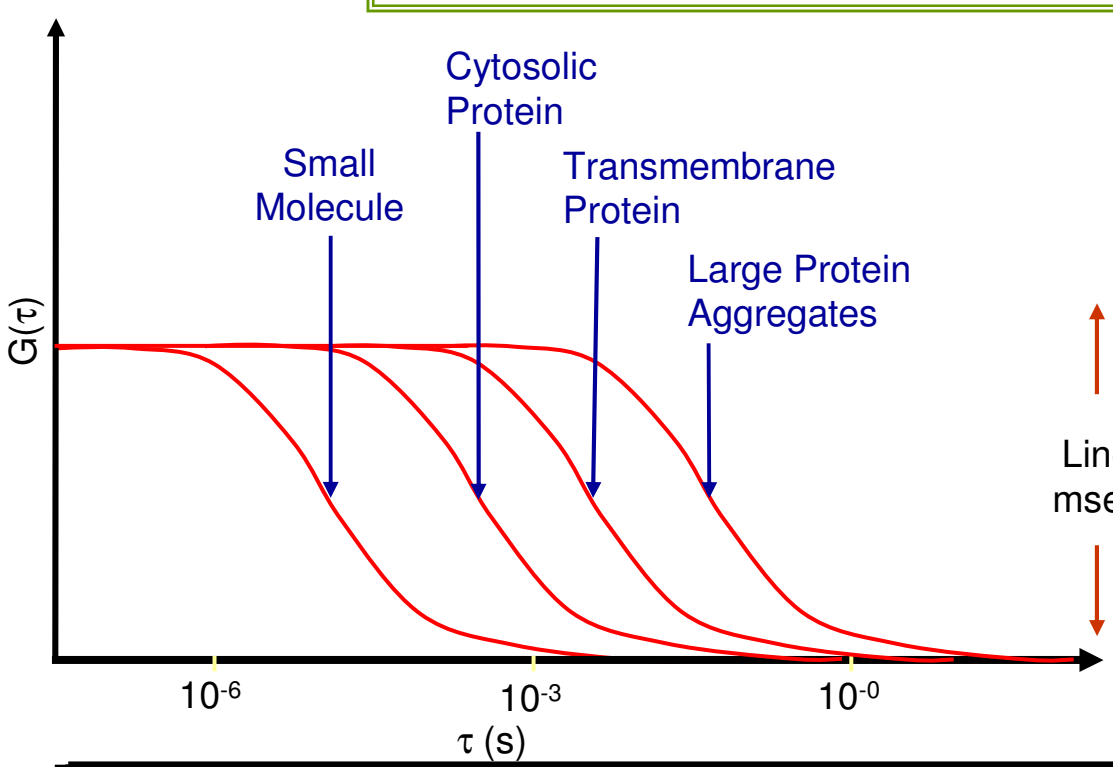


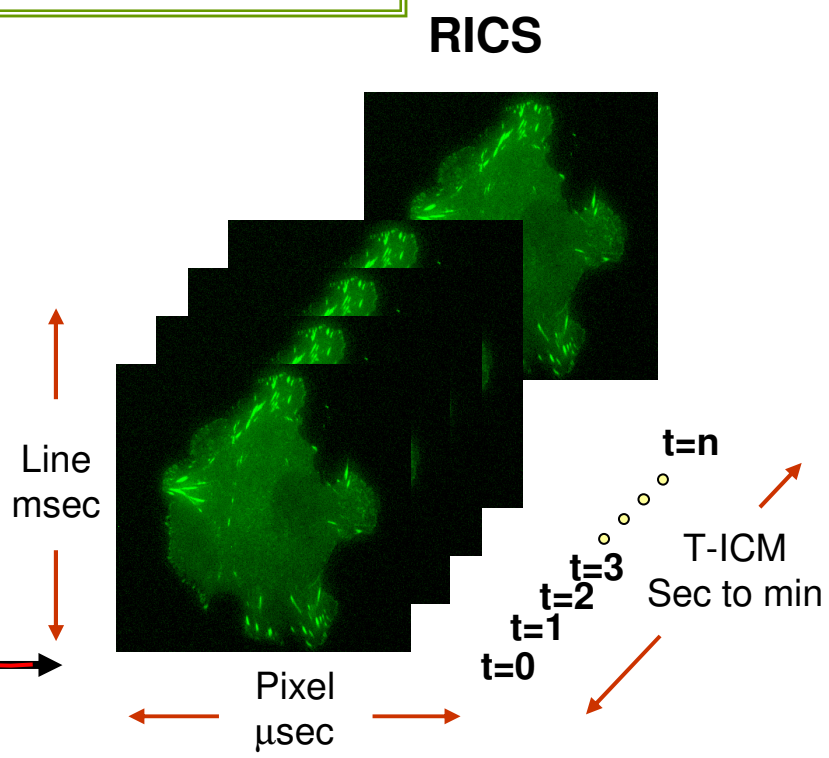
Fit using 3-D diffusion formula

Pixel size = 0.092µm
Pixel time= 8 µs
Line time = 3.152 ms
Wo = 0.35 µm

G1(0) = 0.0062
D1 = 7.4 µm²/s
G2(0) = 0.00023
D2 = 0.54 µm²/s
Bkgd = -0.00115

Conclusions





Techniques	Time Res.	Spatial Res.	Used to Study
FCS / Temporal ICM	Line Scan / Temporal correlation	<0.5 µm	Protein aggregates Transmembrane proteins
RICS / Temporal ICM	µsec-msec	~2 µm	Soluble proteins Binding interactions
Line-RICS	msec	<0.5 µm	Soluble proteins Binding interactions

Summary

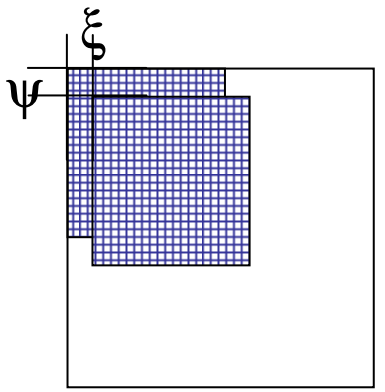
- **Measures dynamic rates from the μsec-msec time scale**
- **Anyone with a commercially available instrument can use it**
- **Immobile structures can be filtered out and fast fluctuations can be detected**
- **RICS has high spatial and temporal resolution**
- **The range of these dynamic rates covers a wide range from immobile to cytosolic diffusions (0.2-12um2/s)**
- **Other types of processes and interactions are also measured**
- **Line scanning is essential for determination of binding process and complements the RICS analysis**

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

The ccRICS approach

The spatial correlation function

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



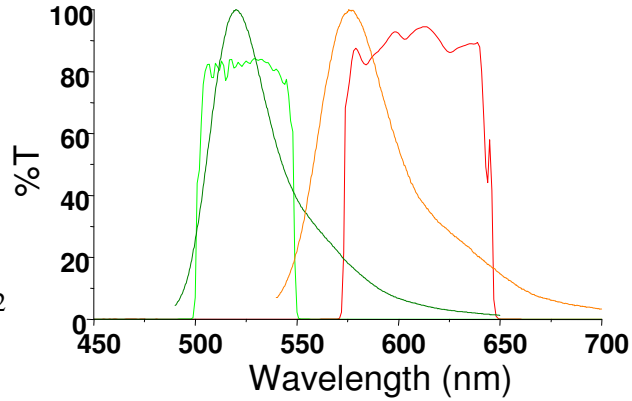
The variables ξ and ψ represent spatial increments in the x and y directions, respectively

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)

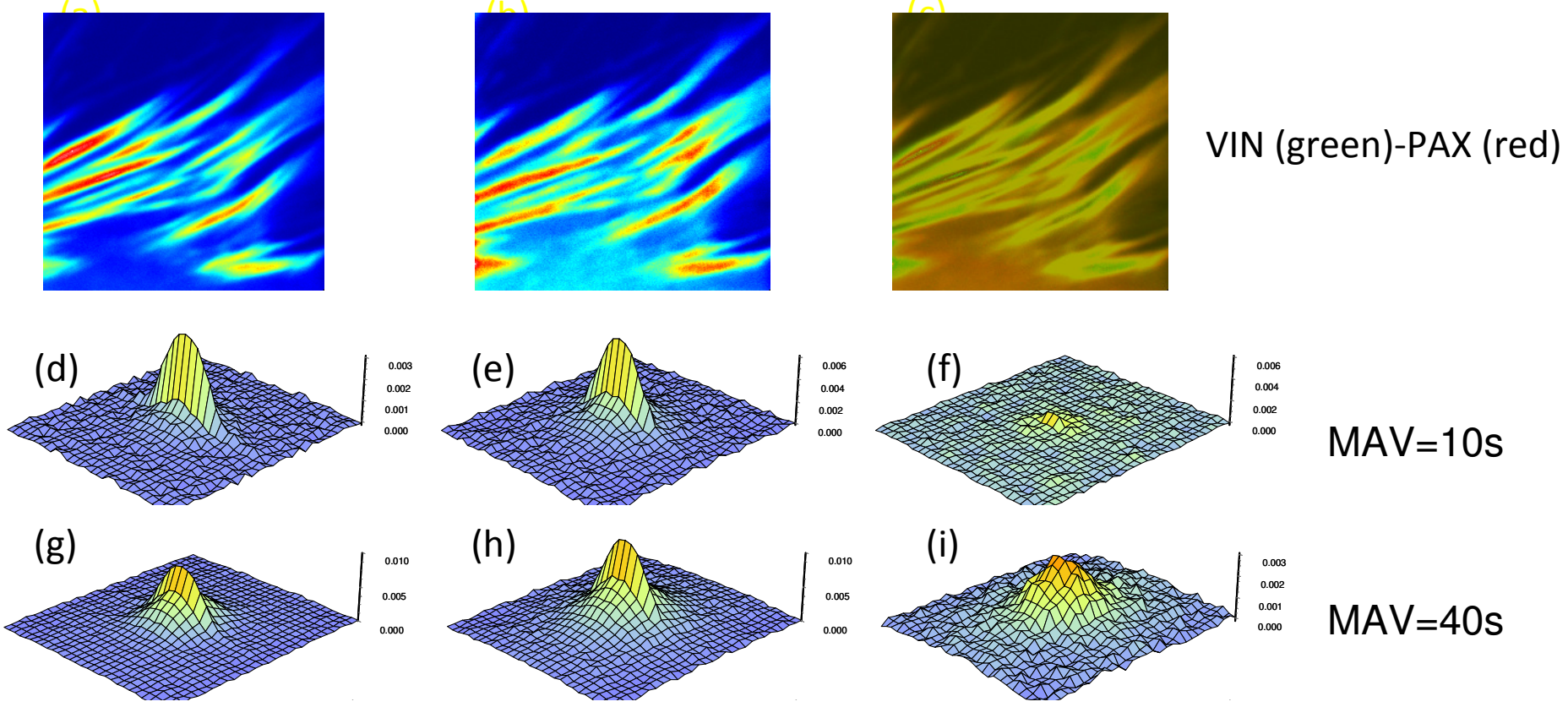
Cells. MEF transfected Vinculin, FAK and paxillin. cDNA were ligated to EGFP or mCherry at the C-terminal end.

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.

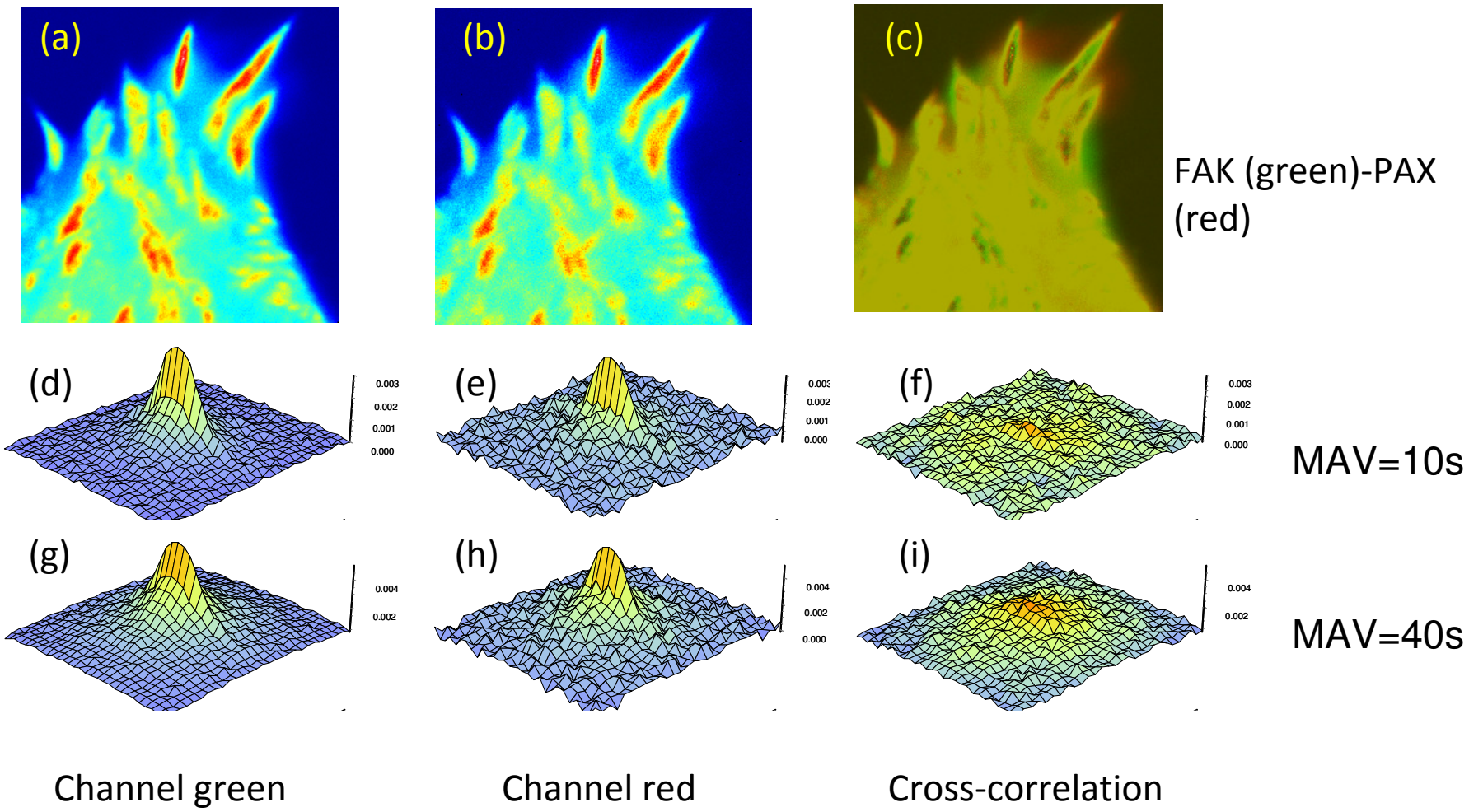
Overlap of the volume of observation was tested by imaging single 100 nm fluorescent beads carrying two colors simultaneously

VIN and PAX co localize at adhesions but they are moving independently in the cytoplasm



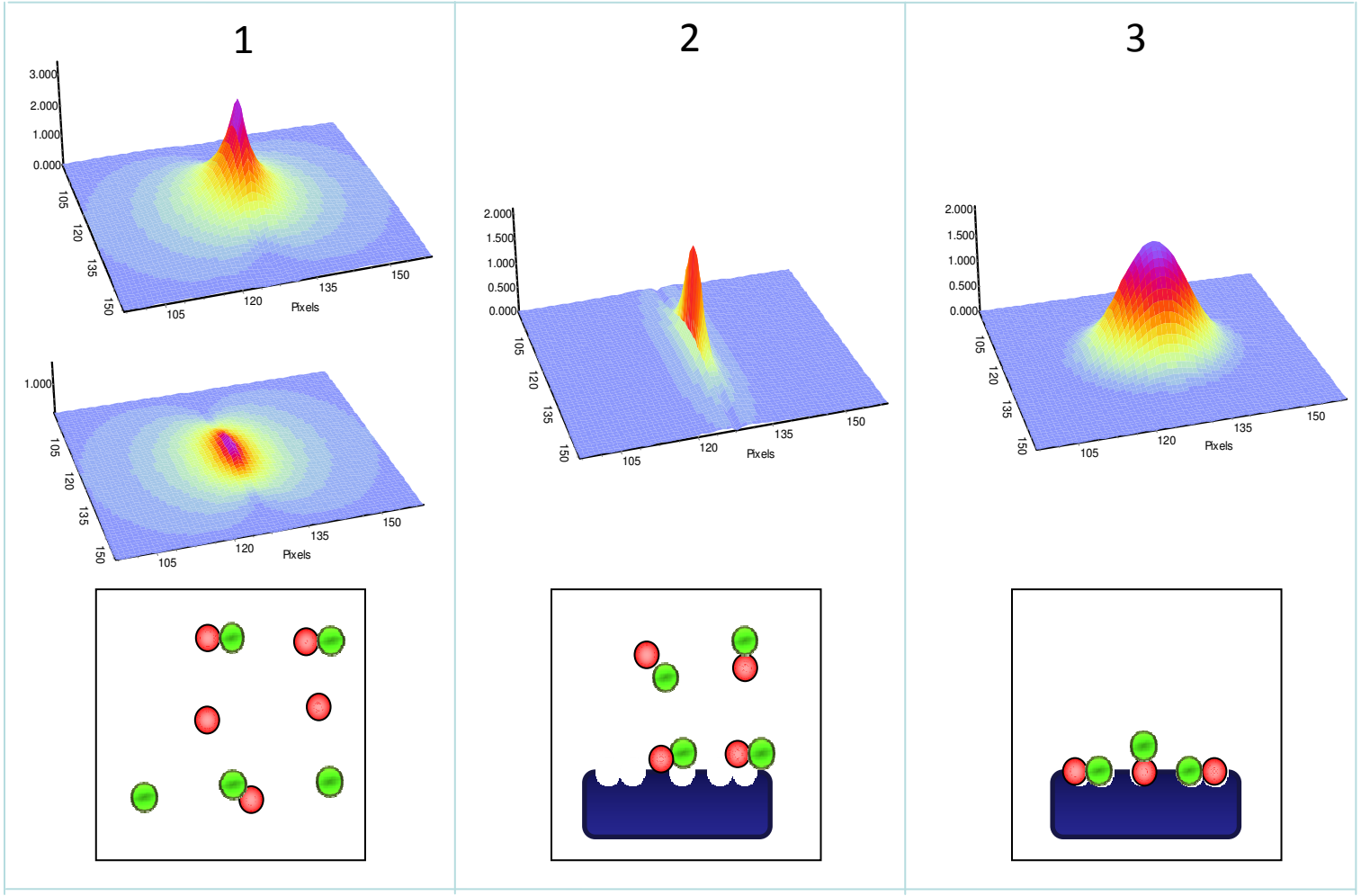
The cross-correlation increases for the slow fluctuations (at MAV=40s). It is round in shape indicating that it is generated at single locations.

FAK and PAX 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.

Schematic representation for the interpretation of the ccRICS experiment. Simulation of binding and diffusion

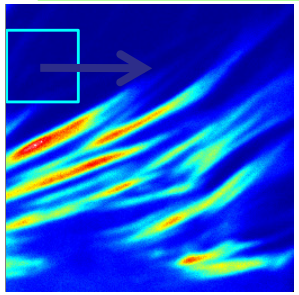


Diffusion
Few complexes

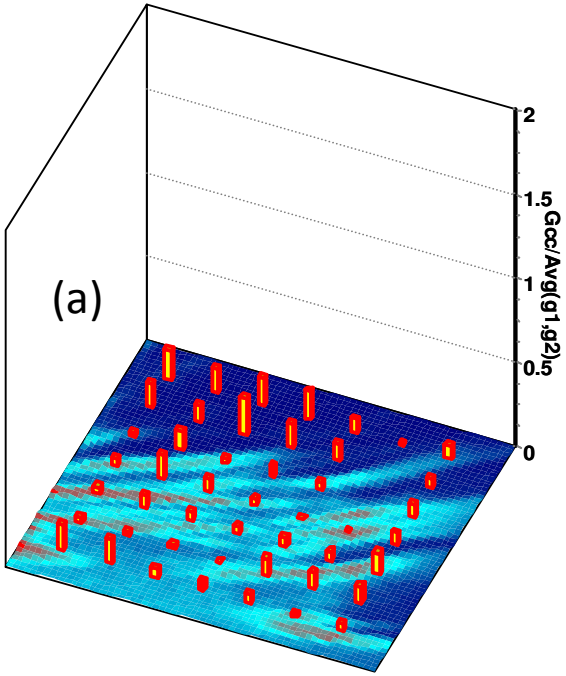
Fast binding
Different shape
Smaller than PSF

Slow binding
Round shape

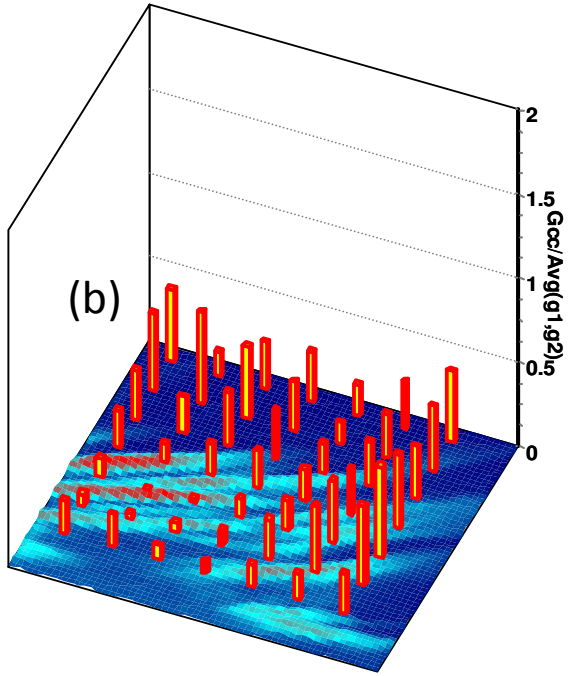
Distribution of fraction of cross-correlation in the cell. Correlation with adhesion disassembling



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



VIN-PAX MAV=10s



VIN-PAX MAV=40s

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

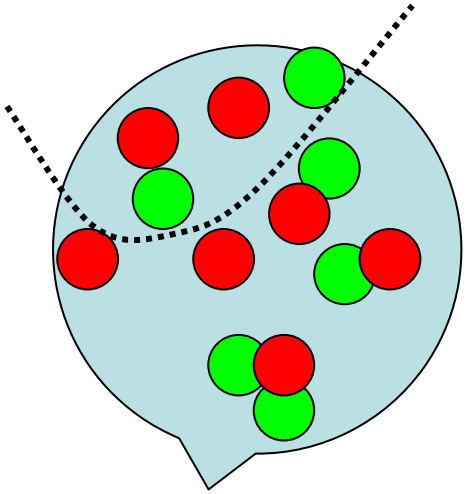
Summary of ccRICS

- 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

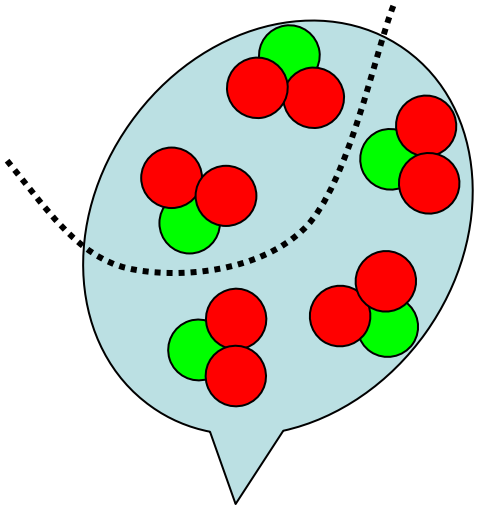
- **ccRICS** is extremely powerful at detecting joint diffusing proteins and in separating diffusion from binding processes

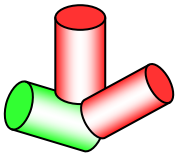
- The Paxillin, vinculin and FAK never crosscorrelate in the cytoplasm before binding to the focal adhesion. We only detect cross correlation due to dissociation of large clusters of proteins.

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



Random





1:2

The Number & Molecular Brightness (N&B) Method

UCIrvine
University of California, Irvine

dbc

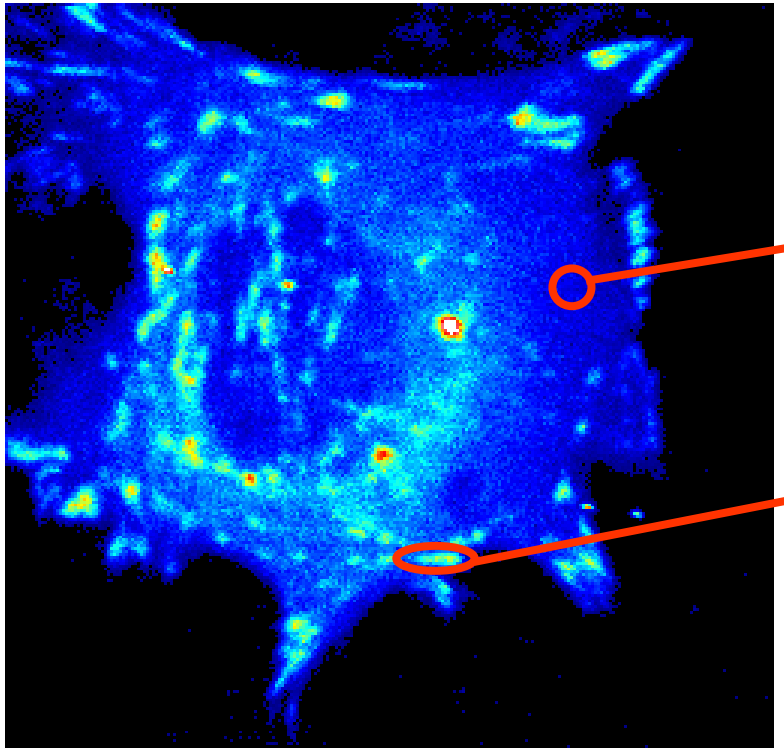
LFD

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

A INTENSITY

31,250counts/sec

B

93,750 counts/sec

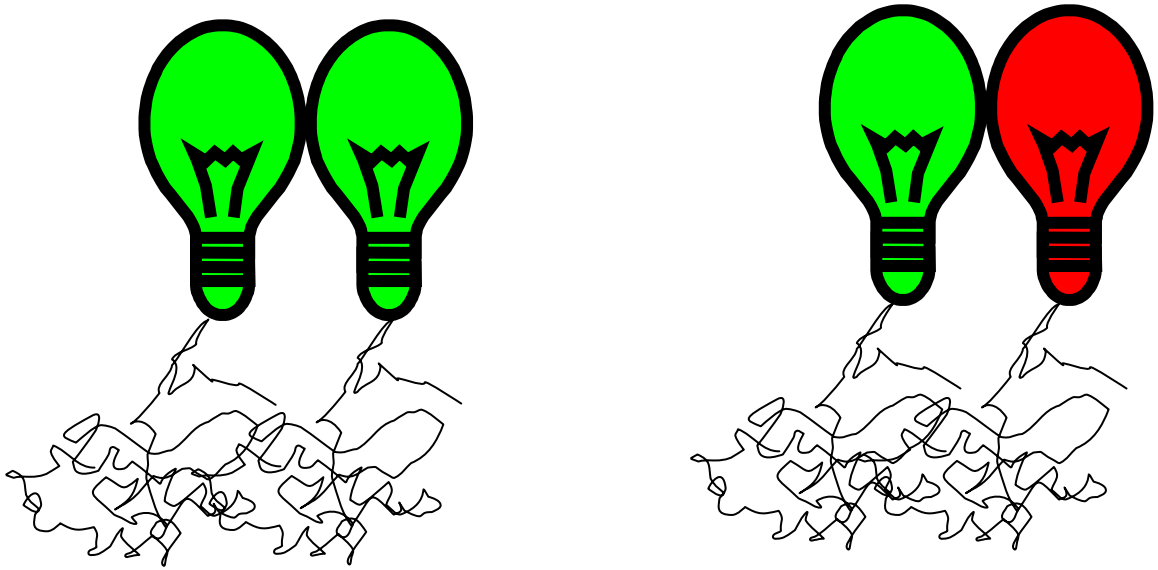
If "free" EGFP at 10nM gave 30,000 counts/sec then the conclusion would be that :

A =10nM

B = 30nM

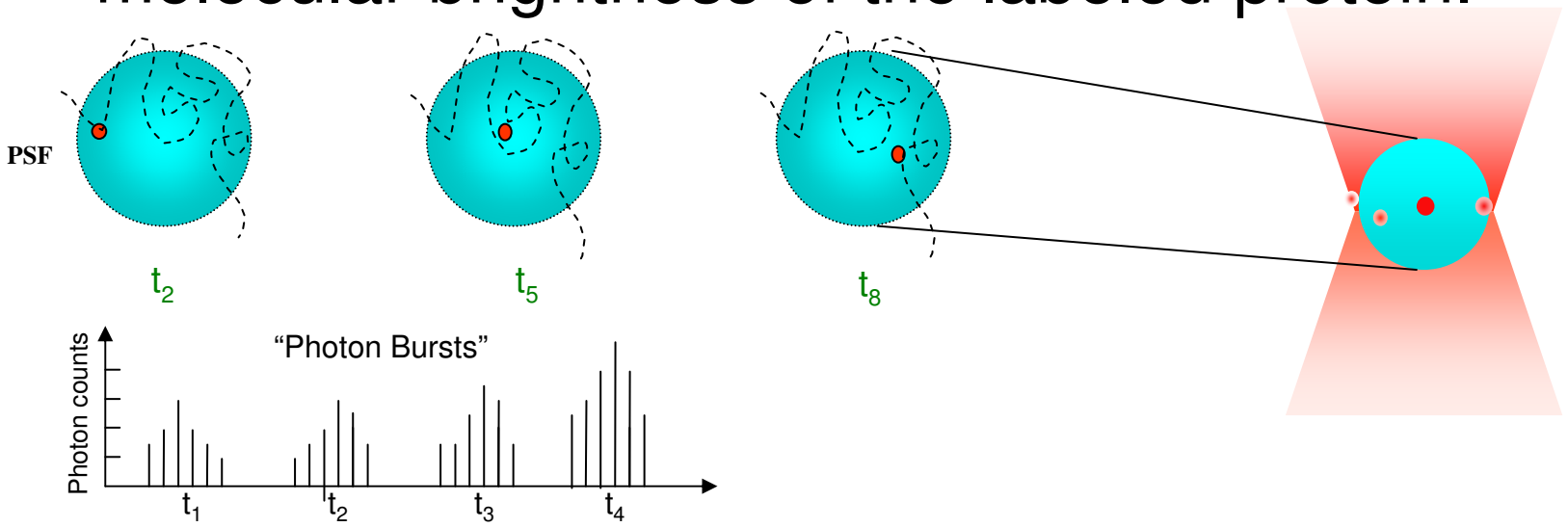
However, it doesn't give you the size distribution
Only concentration is given

2. Förster resonance energy transfer (FRET)

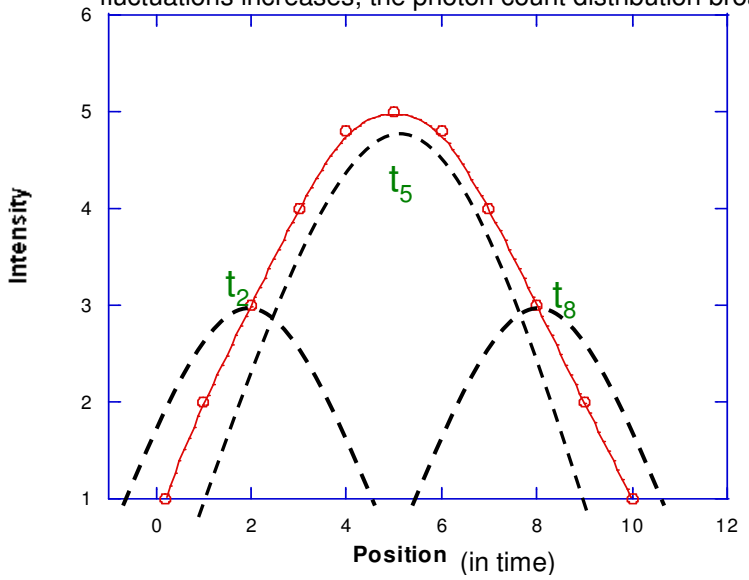


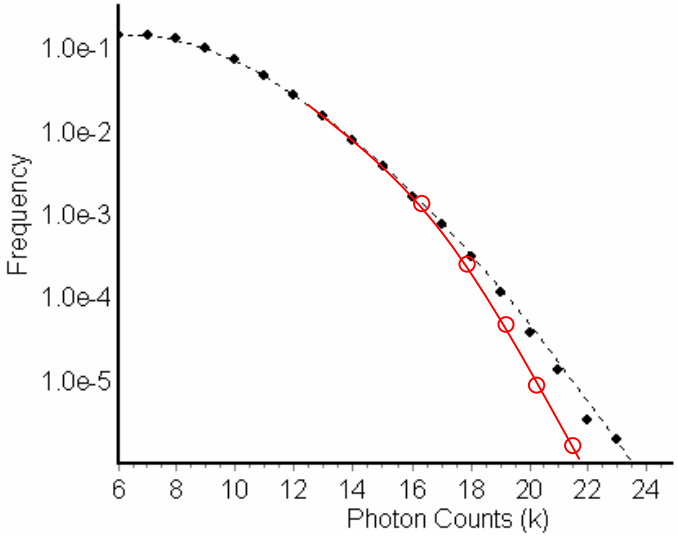
This method is very sensitive to detect the formation of pairs.

The average photon count rate of bursts determines the molecular brightness of the labeled protein.

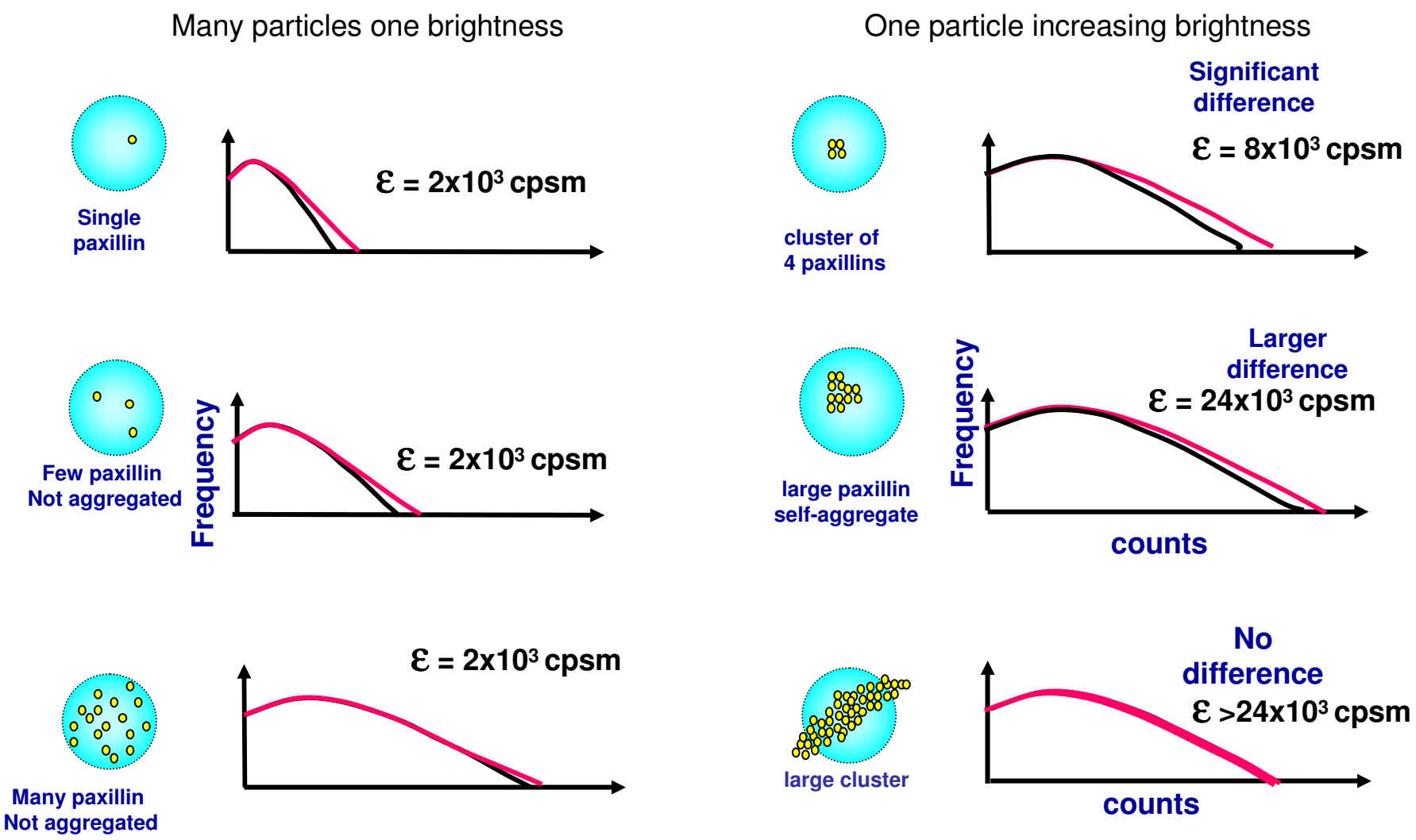


The intensity distribution accounts for the fluctuations of photons from the molecule freely diffusing through the exitation profile. Thus, the overall photon counting count distrubution is the weighted superposition of individual Poissonian distributions for each intensity values with a scaling amplitude. The fluctuations light intensity results in a broadeing of photon count distribution with respect to a pure poisson distribution. As the fluctuations increases, the photon count distribution broadens.



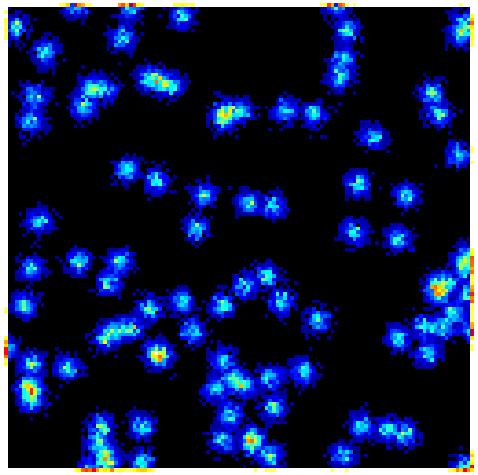


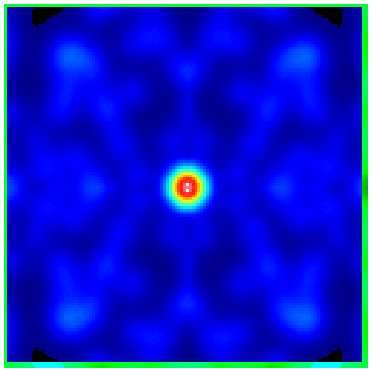
4. Photon Counting Histogram Analysis

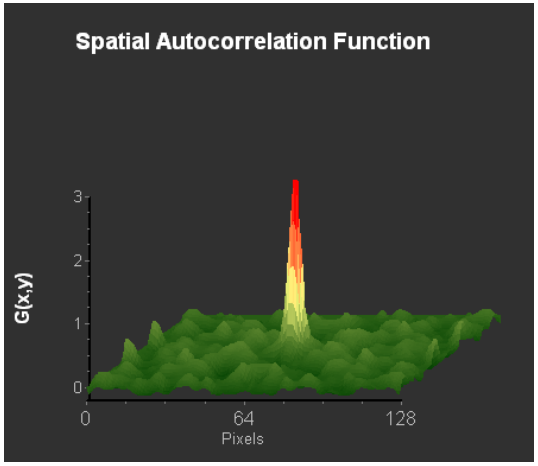


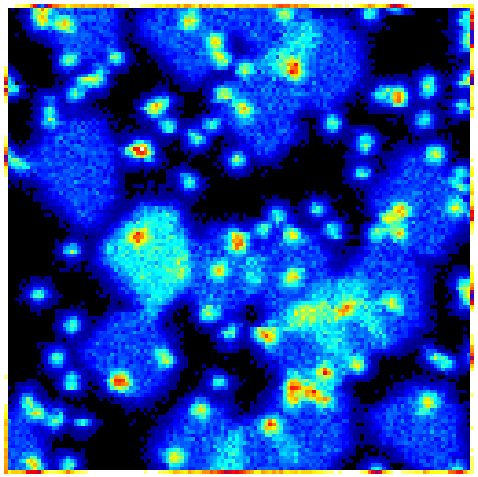
Red is PCH
Black is Poisson

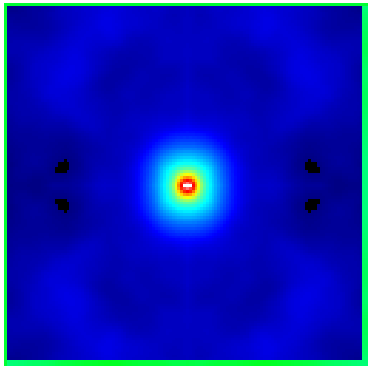
3. Image correlation Spectroscopy (ICS)

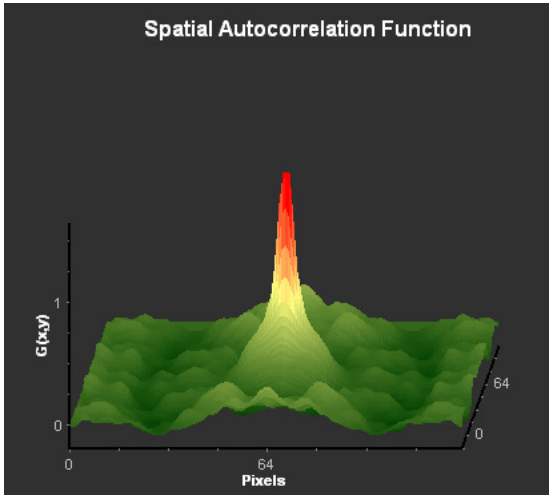












However, the events must be slow >1sec (no movement during one frame) and the aggregates must be large.

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

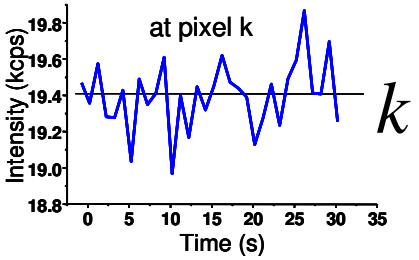
Tools: Cursor selection of pixel with similar brightness
Quantitative analysis of center and std dev of the *e* and *n* distribution
Tools for calibration of analog detectors

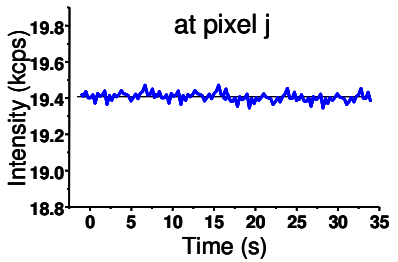
Tutorials: mathematical background, data import, analysis examples (our web site

How to distinguish pixels with many dim molecules from pixels with few bright molecules?

Average (first moment)

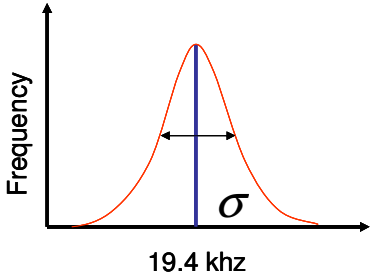
$$< k >= \frac{\sum_i k_i}{K}$$

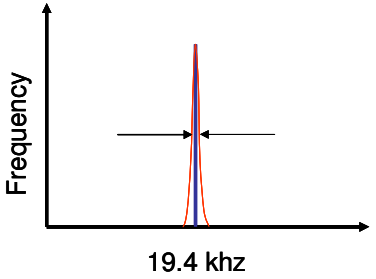


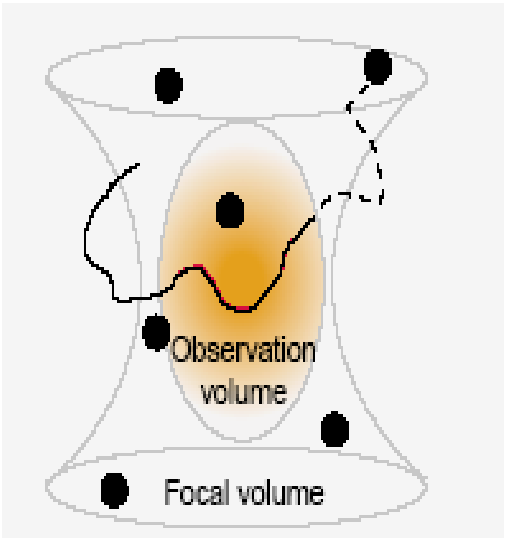


Variance (second moment)

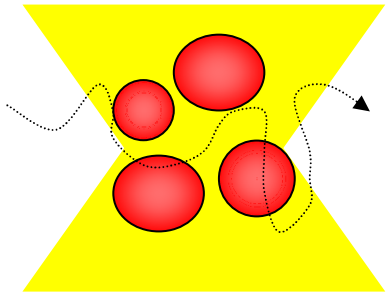
$$\sigma^2 = \frac{\sum_i (k_i - < k >)^2}{K}$$

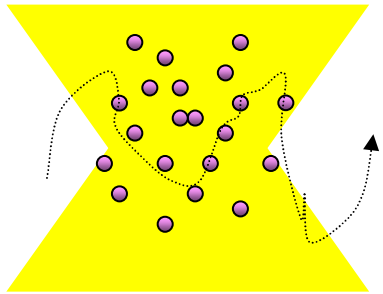






G(0) = $\sigma^2/<k>^2$ = 1/N





- **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 ($<k>^2$) to the variance (σ^2) is proportional to the average number of particles <N>.**

* Originally developed by Qian and Elson (1990) for solution measurements.

Calculating protein aggregates from images

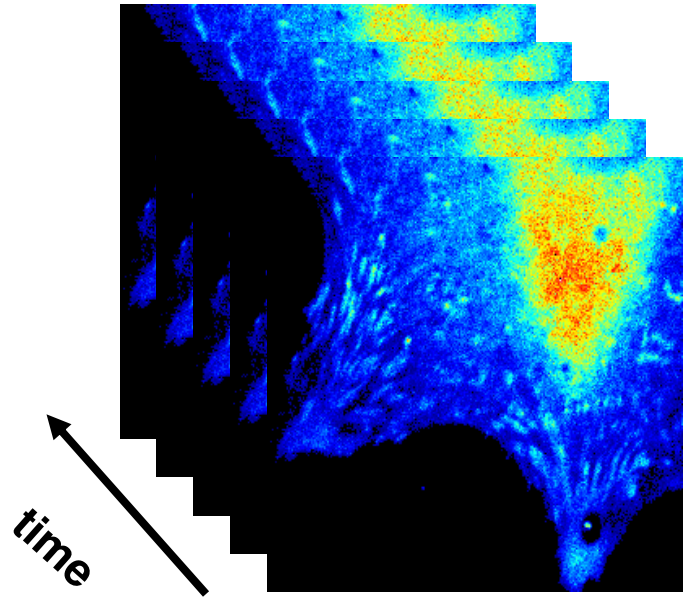
This analysis provides a map of <N> and brightness (B) for every pixel in the image. The units of brightness are related to the pixel dwell time and they are “counts/dwell time/molecule”.

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

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

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

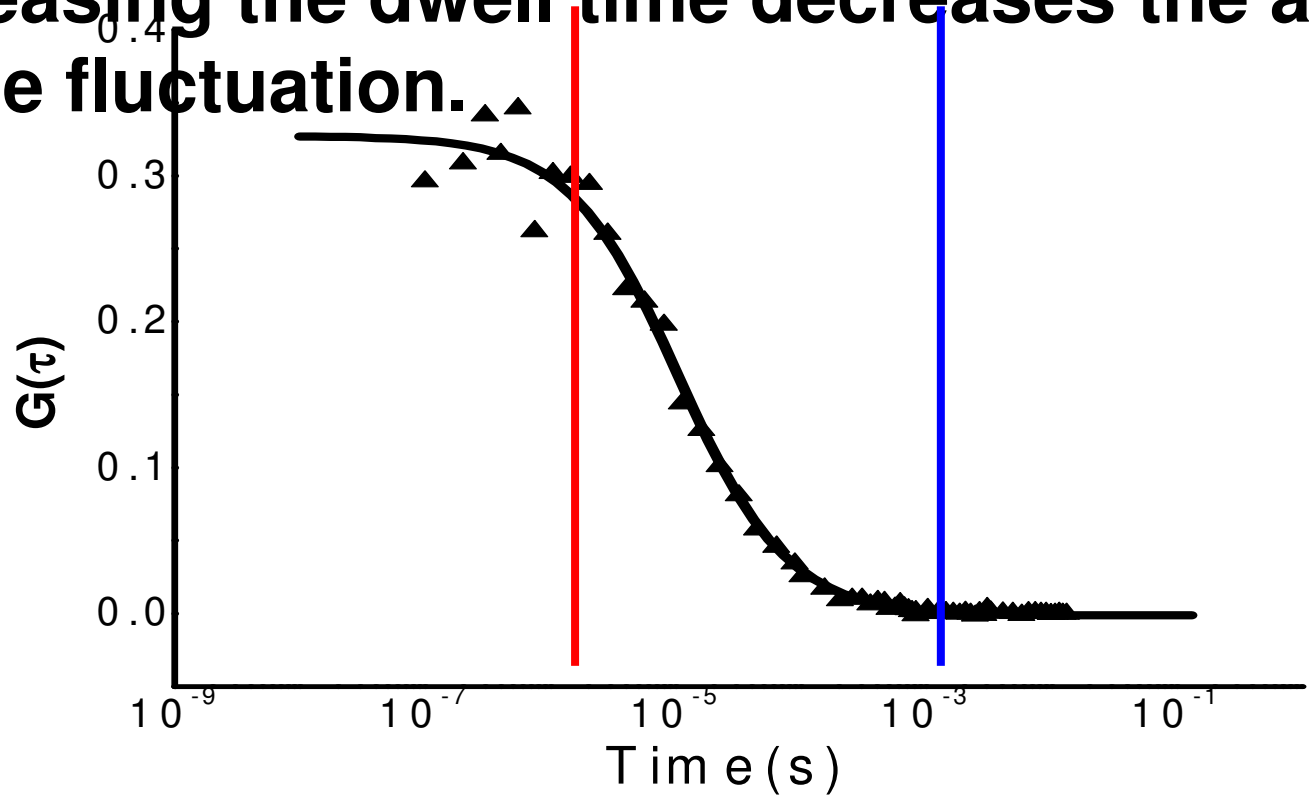
σ^2 = Variance
<k>= Average counts
N = Apparent number of molecules
B = Apparent molecular brightness
K = # of frames analyzed



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.

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 s

$$\sigma^2 = \sigma_n^2 + \sigma_d^2$$

These two terms have different dependence on the

$\sigma_n^2 = \varepsilon^2 n$ $\sigma_d^2 = \varepsilon n$ (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 m

ε is the true molecular br

How to Calculate n and ε

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

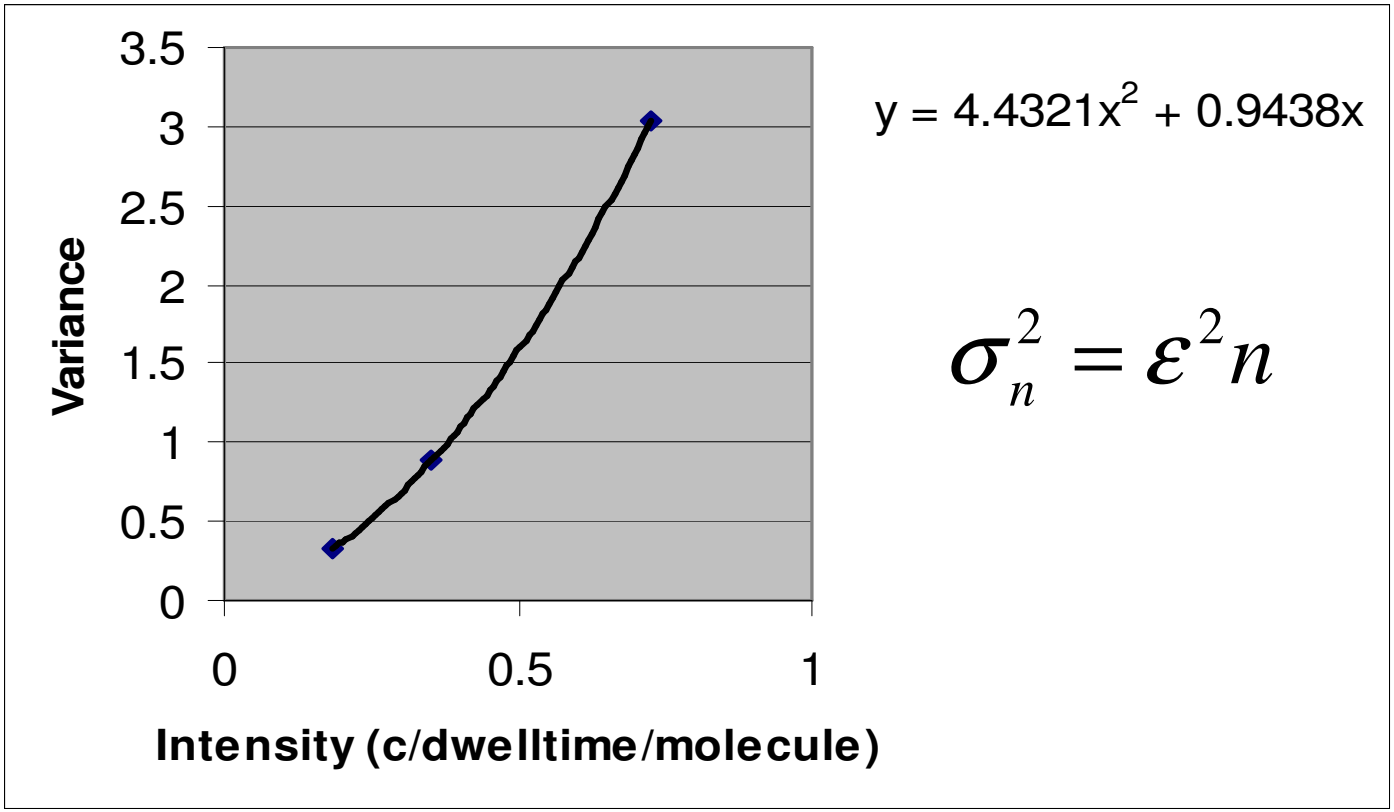
This ratio identifies pixels of different brightness due to mobile particles.

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

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

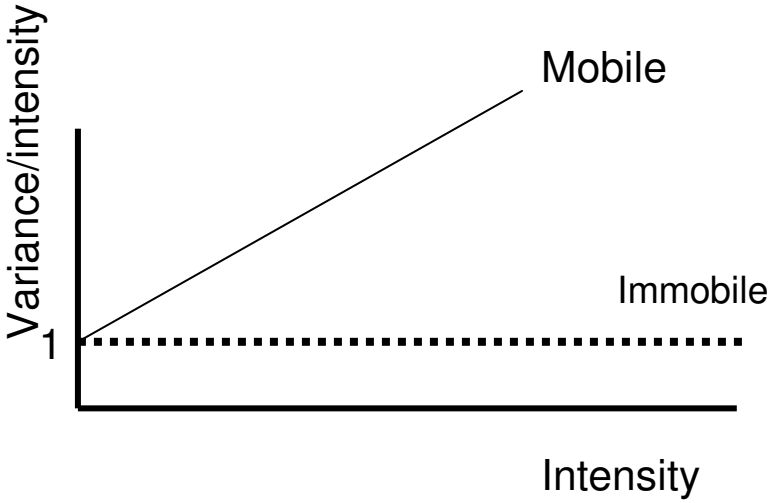
If there are regions of immobile particles, n cannot be calculated because for the immobile fraction the variance is = <k>. For this reason, several plots are offered to help the operator to identify regions of mobile and immobile particles. Particularly useful is the plot of N*vs*B.

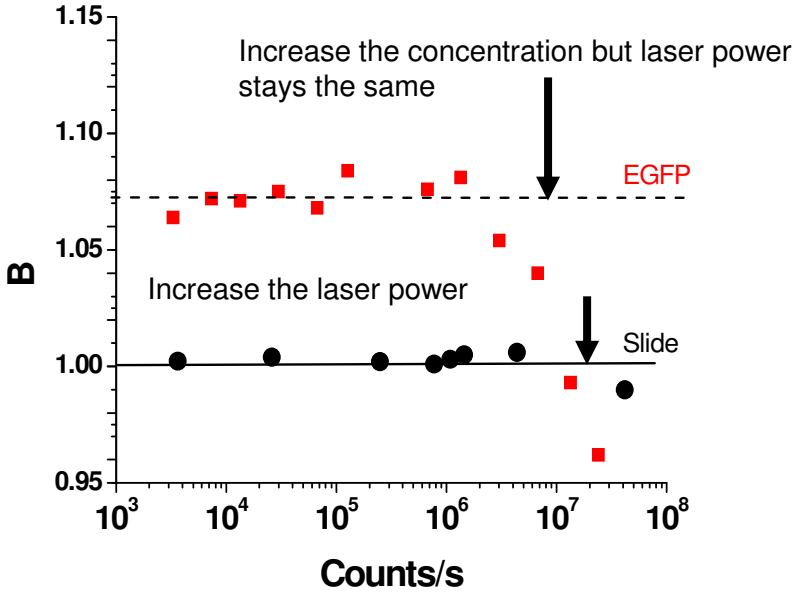
Quadratic dependence of the variance on particle brightness
20nM EGFP in solution as a function of laser power



2-photon excitation using photon counting det

Identification of mobile and immobile molecules

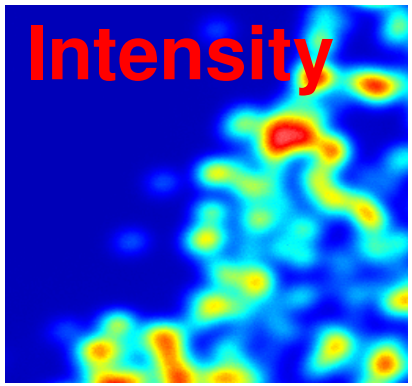


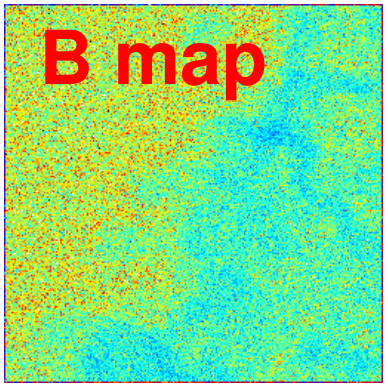


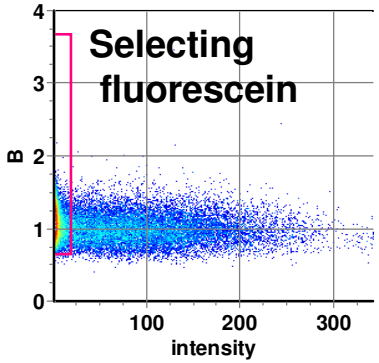
If we change the laser power, a plot of the ratio variance/intensity vs intensity can distinguish the mobile from immobile fraction. The two curves are for different pixel integration times.

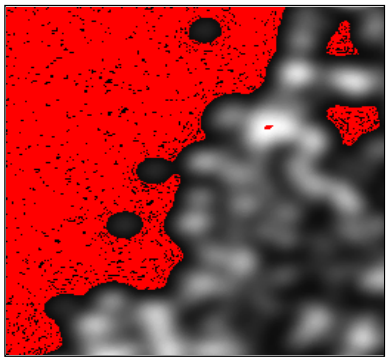
The effect of the immobile part: with photon counting detectors

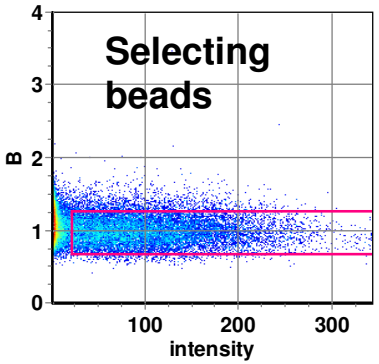
Fluorescent beads in a sea of 100nM Fluorescein.

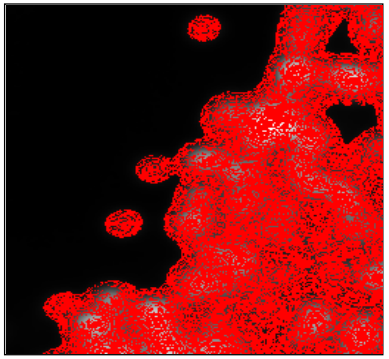


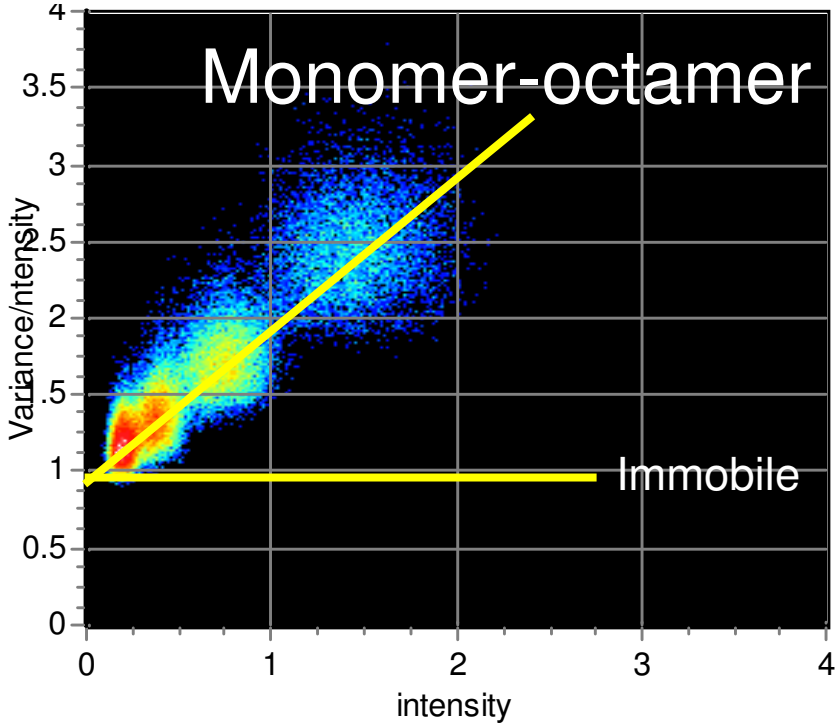






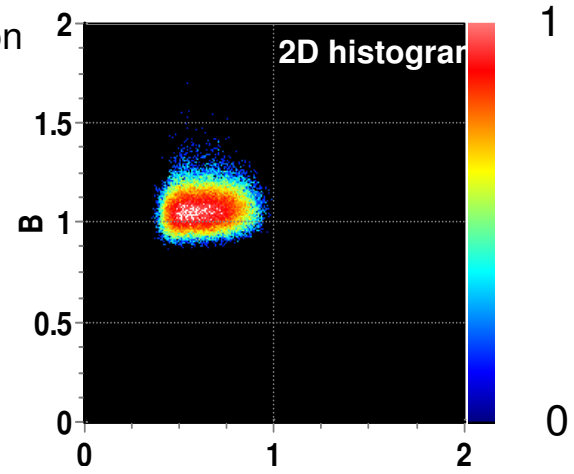


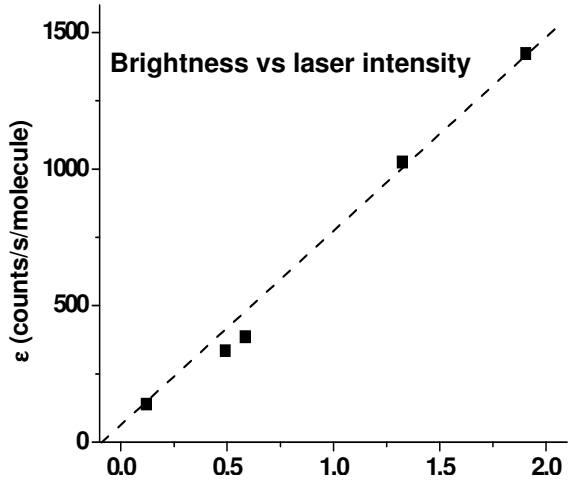


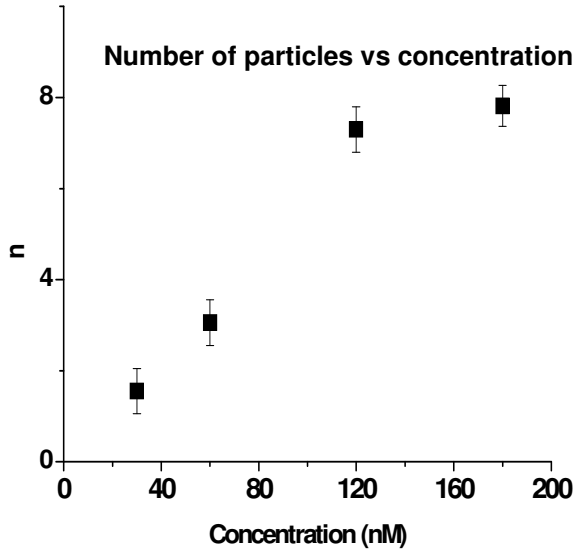


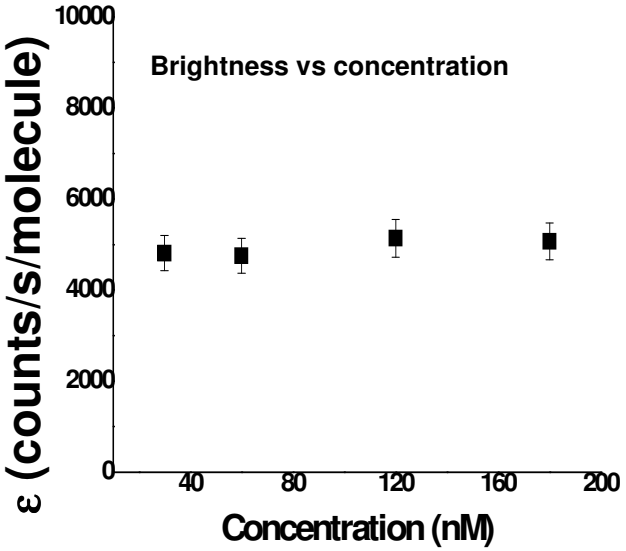
Brightness and number of molecules can be measured independently

EGFP in solution



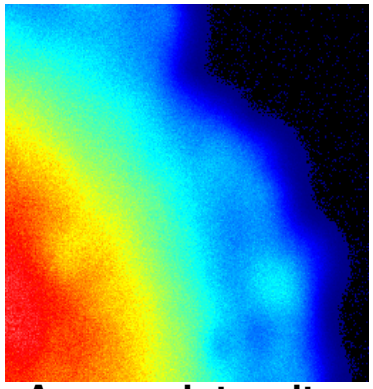




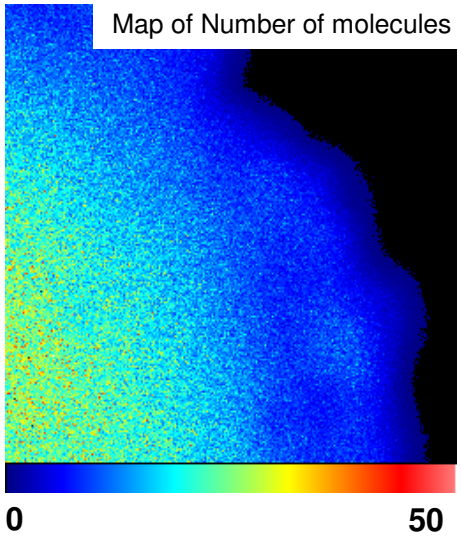


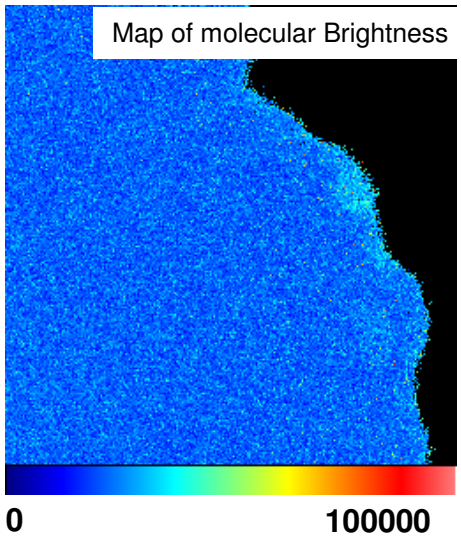
EGFP in CHO-k1 (1-Photon LSM)

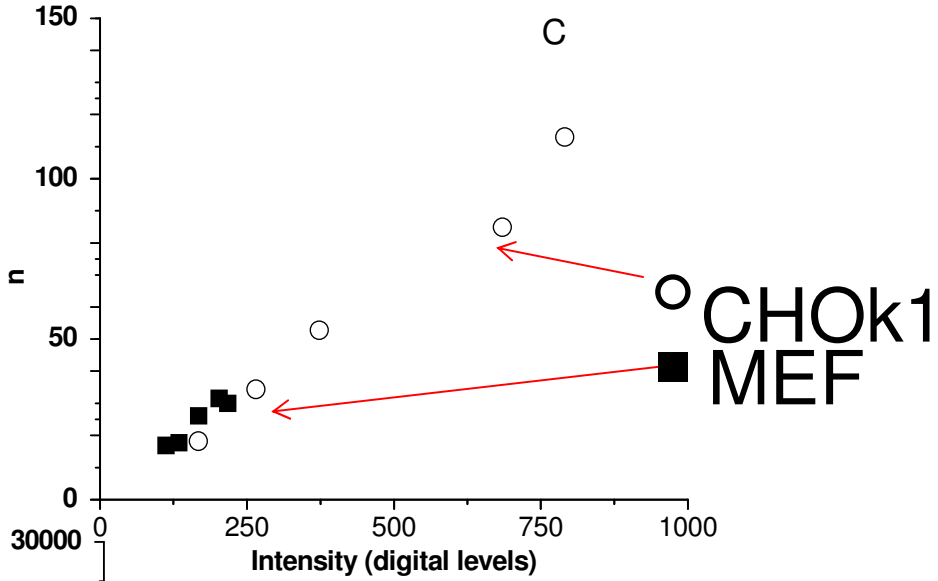
homogenous Brightness & heterogeneous Number of Molecules

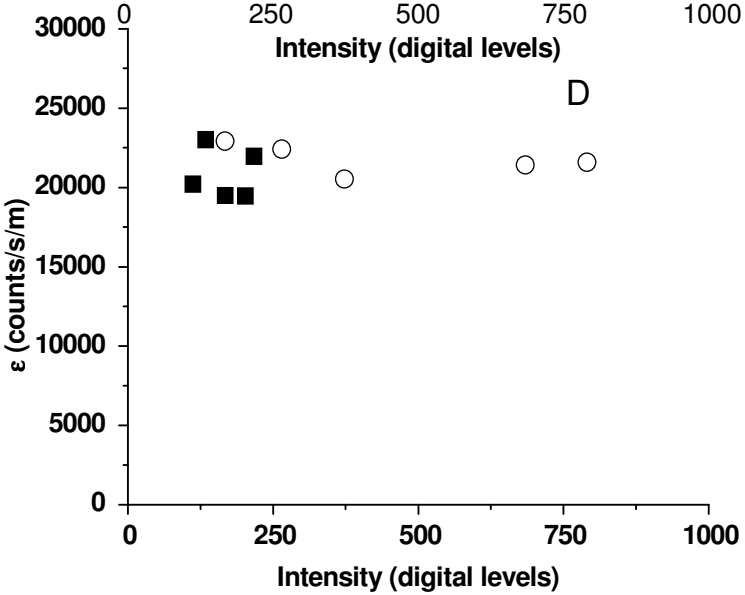


Average intensity

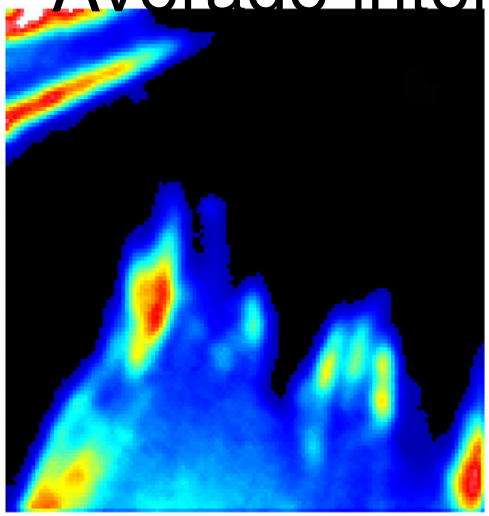


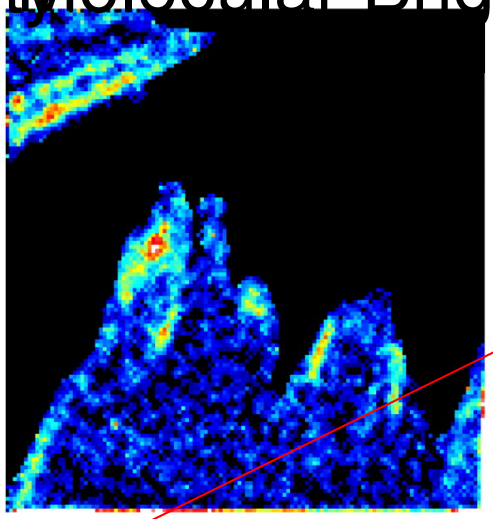


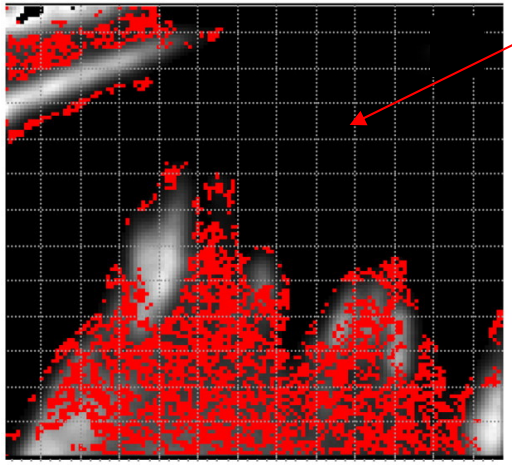


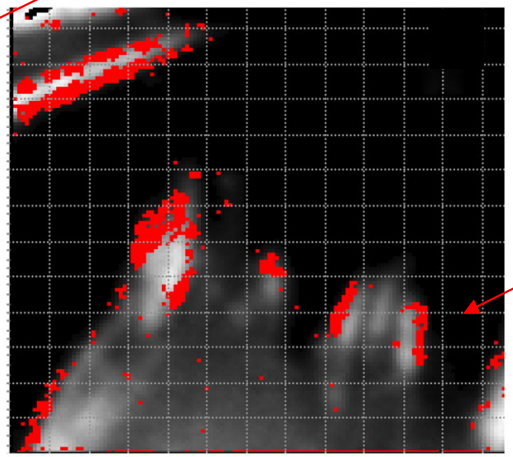


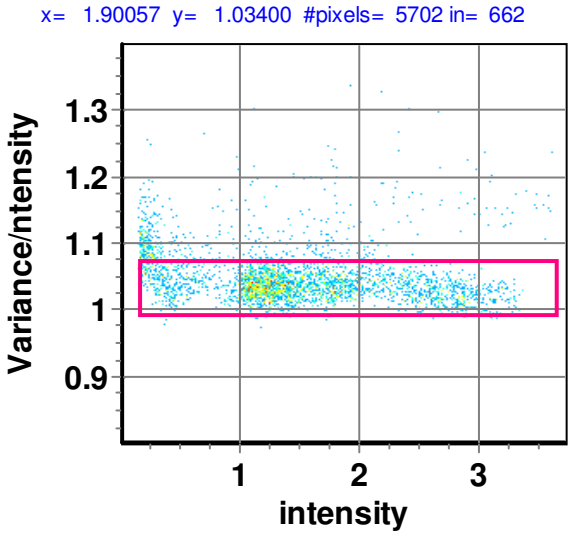
Paxillin assembles as monomers and disassembles as aggregates as large as 8-12

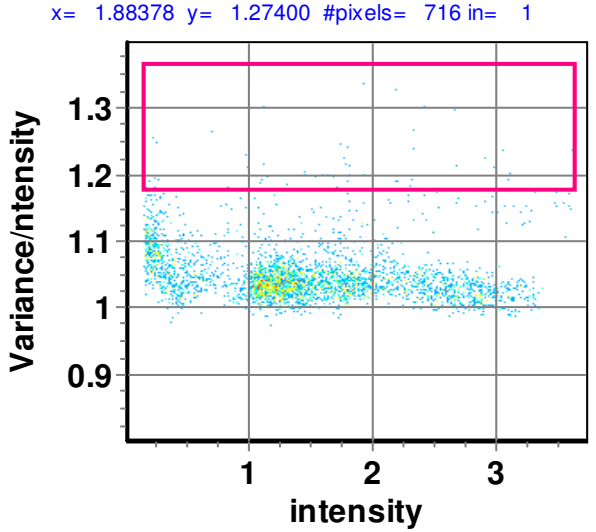












Average intensity

Molecular Brightn

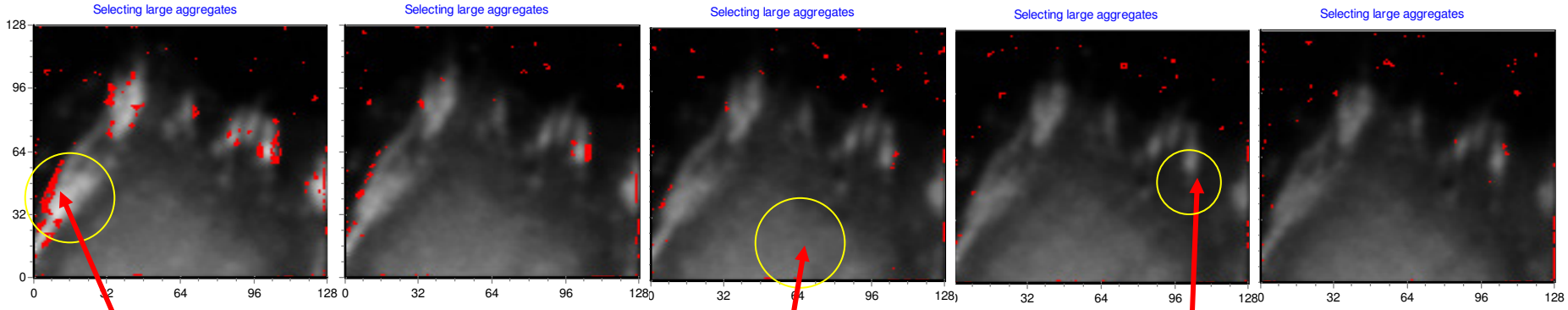
Selected Monomers

Selected >5mers

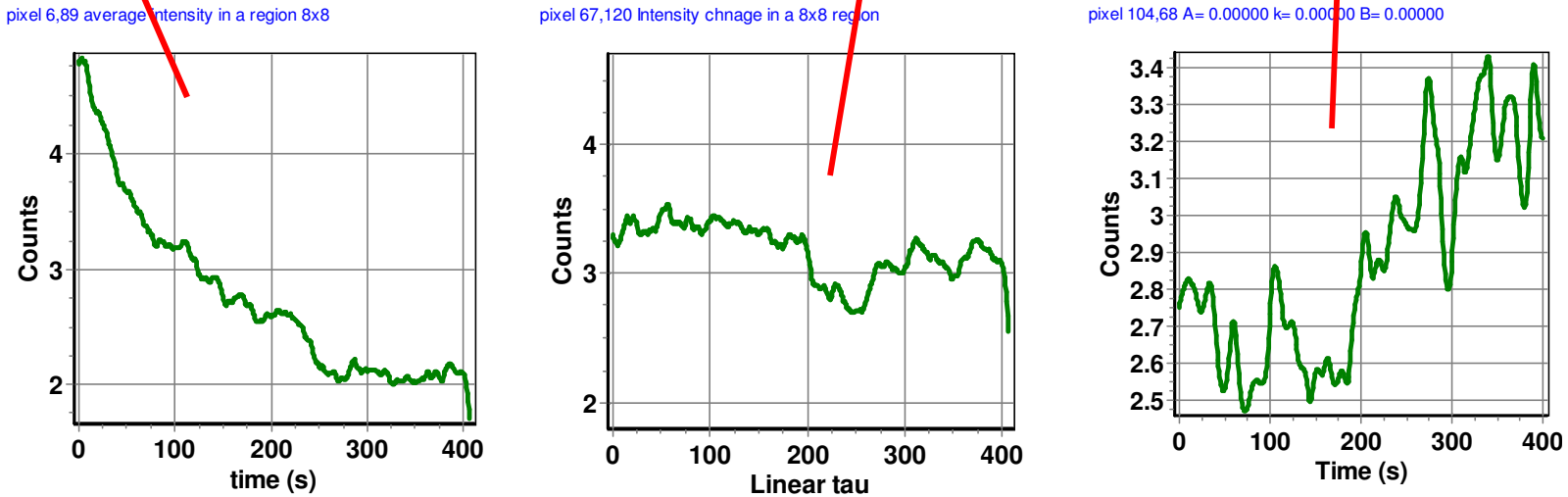
Digman, M.A., et al, *Biophys J.* 2008 Mar 15;94(6):2320-32

Assembly and disassembly occurs in the order of minutes

100 Frame averages Selecting large aggregates



0 to 99 100 to 199200 to 299 300 to 399400 to 499

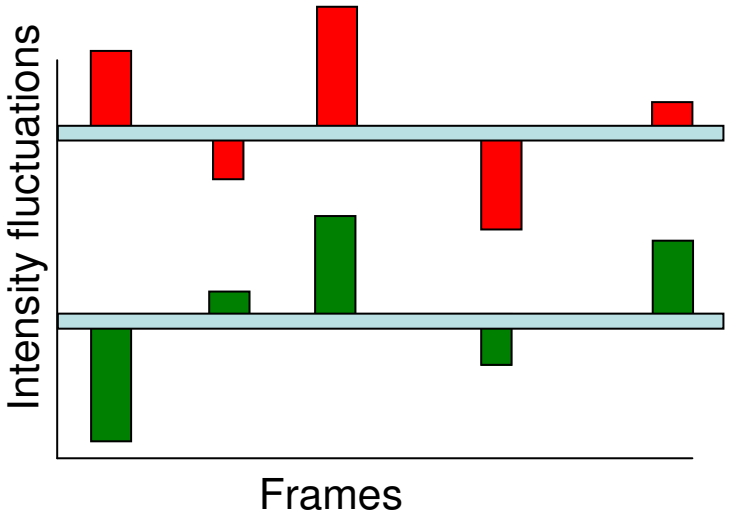


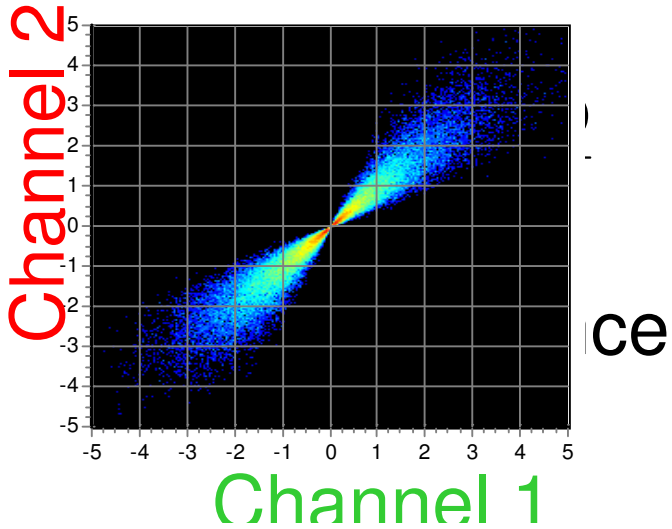
Digman, M.A., et al, *Biophys J.* 2008 Mar 15;94(6):2320-32

Cross N&B

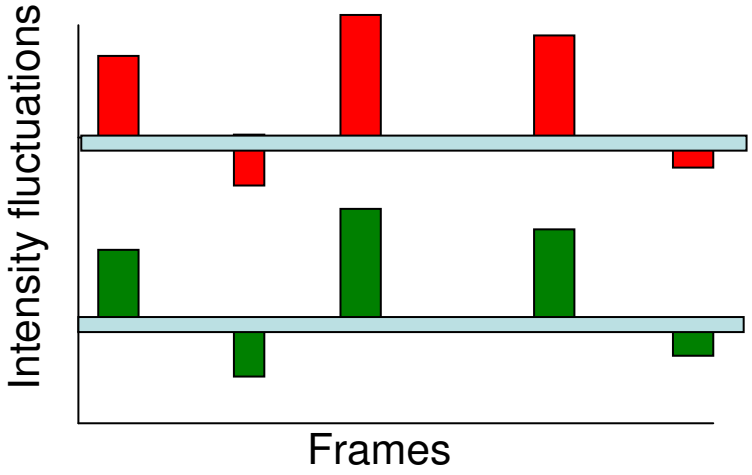
Conceptual illustration of Cross N&B

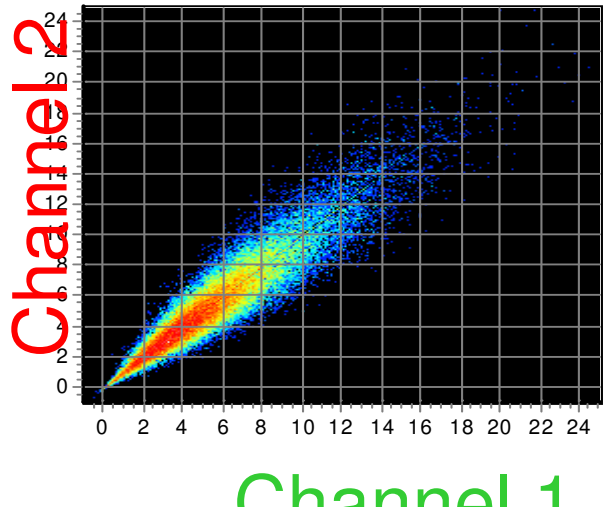
Uncorrelated



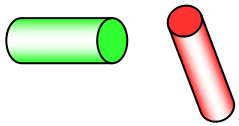


Correlated

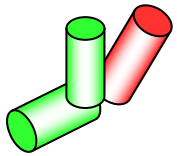




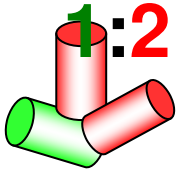
Cross N&B Analysis determines stoichiometry

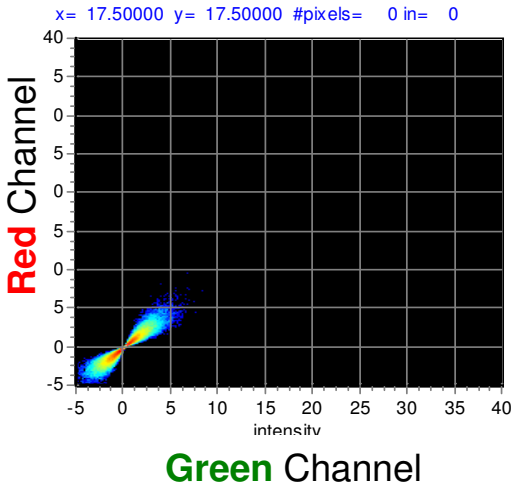


1:1
2:1

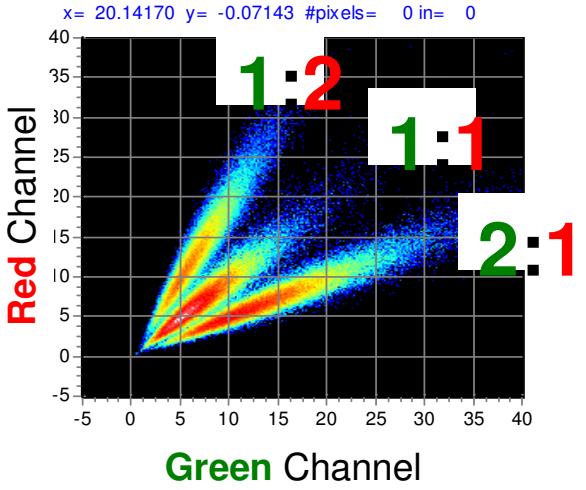


1:2





No Cross-Brightness



Positive Cross-Variance

This example is only for ideal systems where the brightness is calibrated for both channels.

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

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

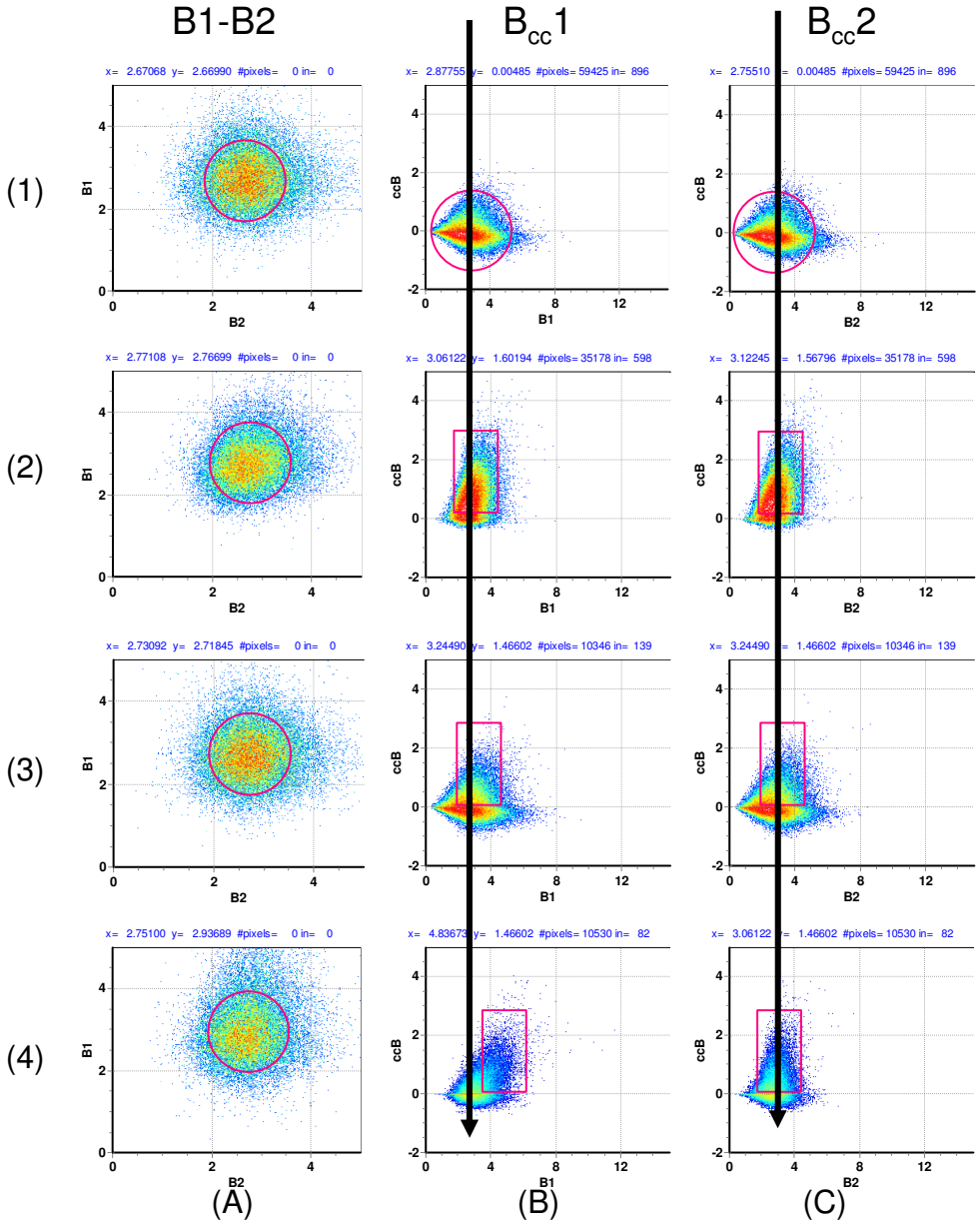
Definition of **co-variance**. It is the average of product of the fluctuations in the Green and Red channel

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

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

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

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)

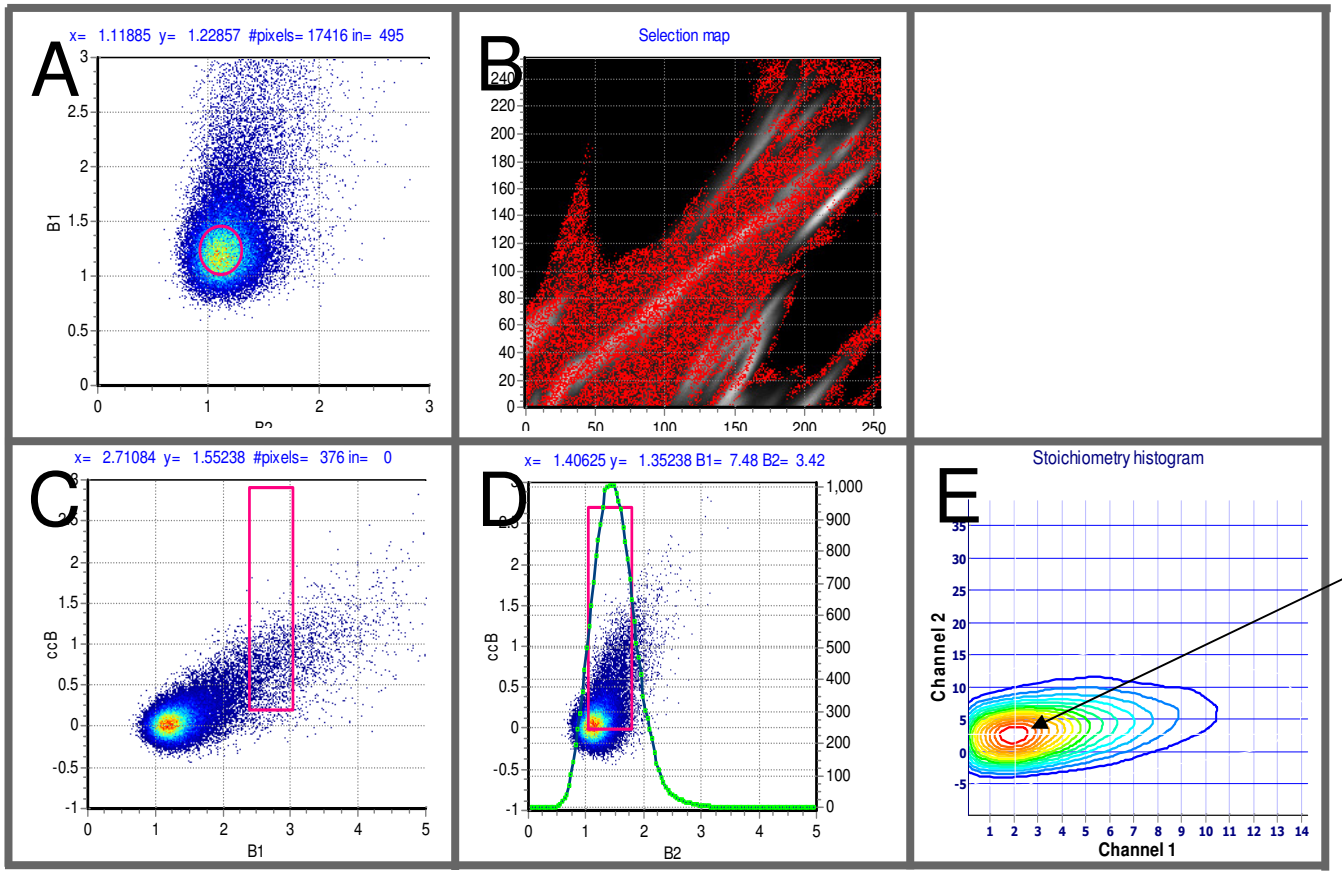
3) At 5% you can still distinguish the positive correlated fluctuations

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

The unknown sample: Vinculin-EGFP and Paxillin-mcherry

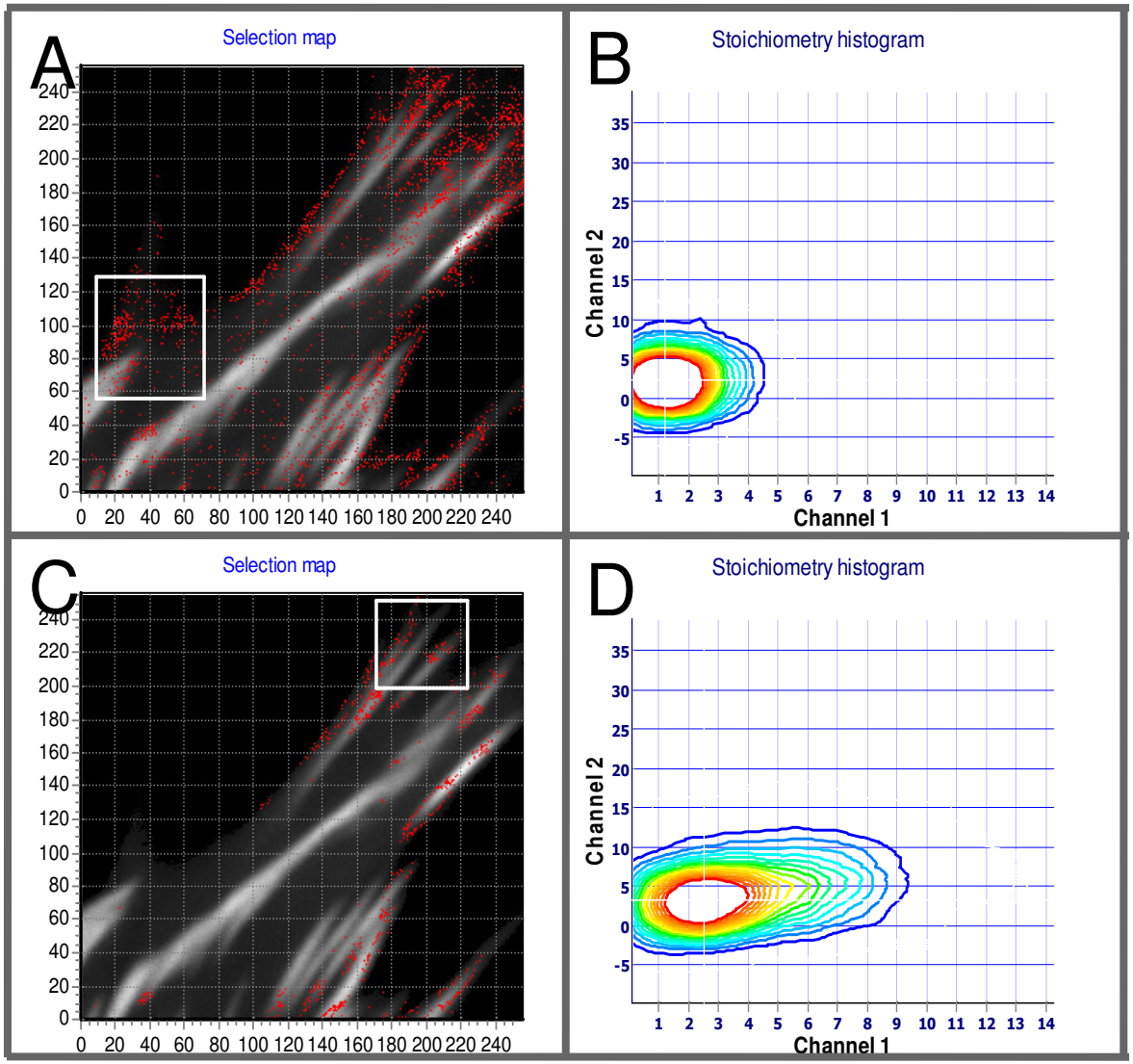


Look at the brightnesses that coincide for Ch1 a

We must find for each value of B1 in one pixel, what is the

The fluctuations must be correlated , so we only look at th

Selecting different regions of the image



AFH1

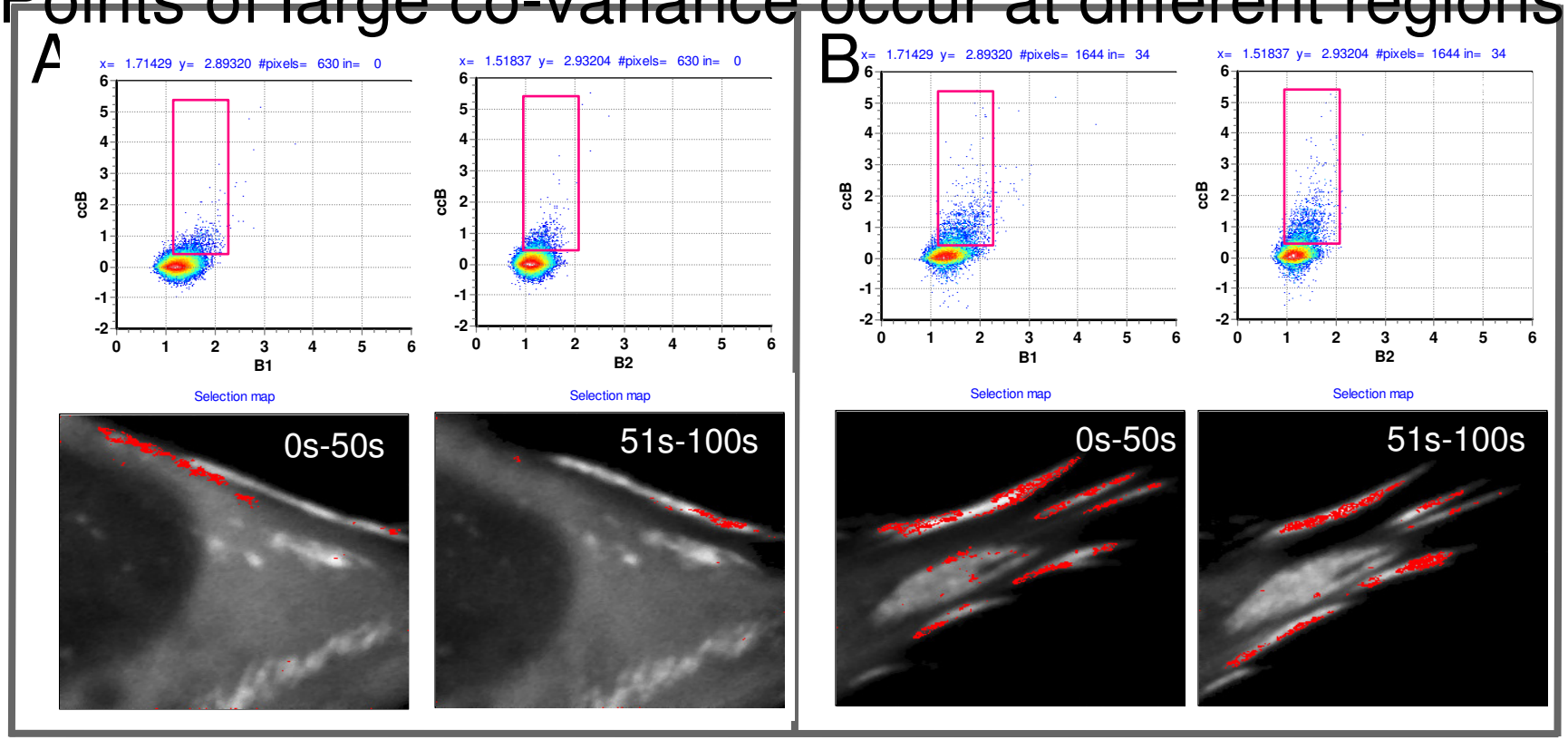
Slide 62

AFH1 Make sure I didnt mess this up.
Rick Horwitz, 6/18/2008

Cross –correlations occur at specific pixels at the adhesions

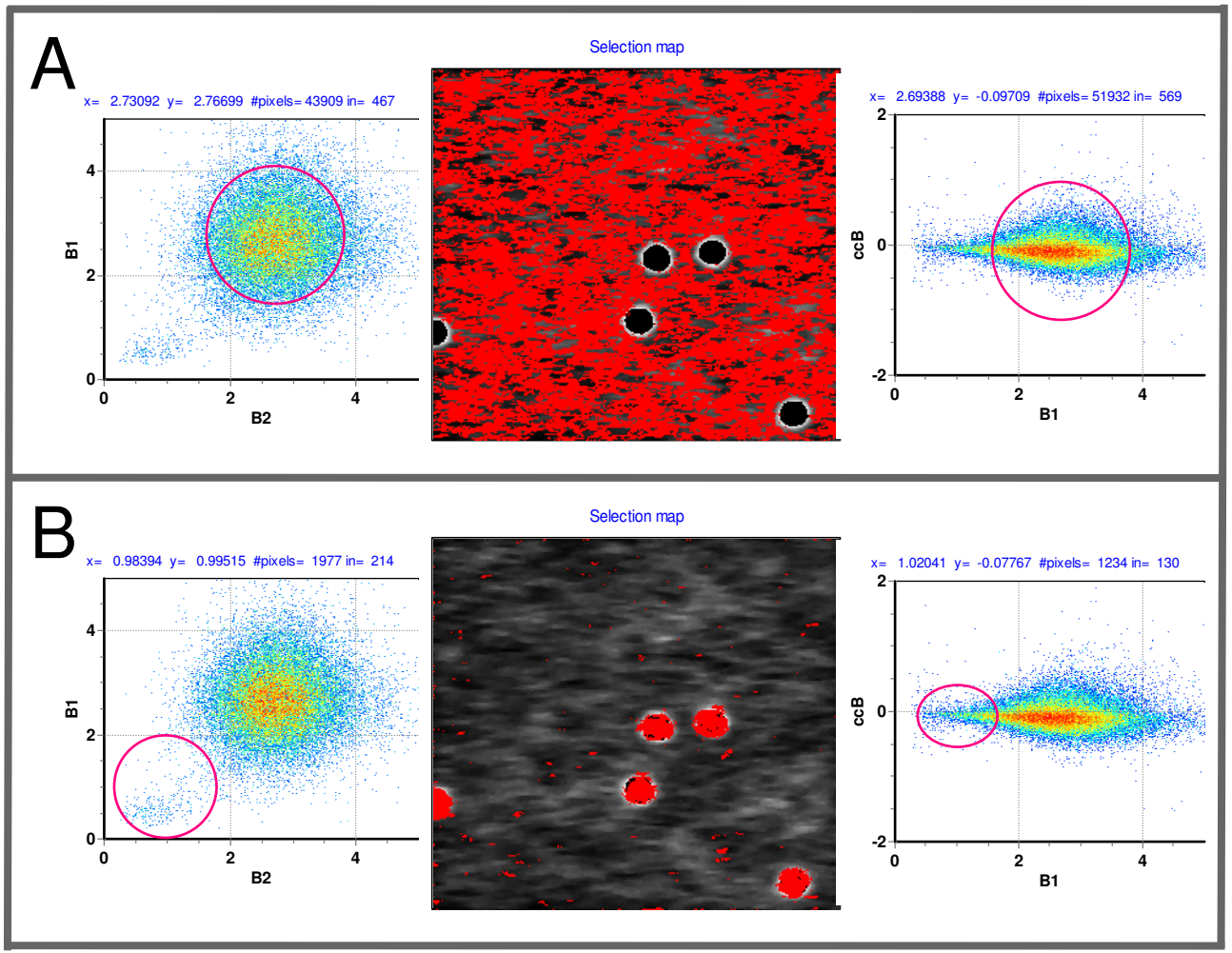
Vinculin-EGFP and Paxillin-mcherry

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

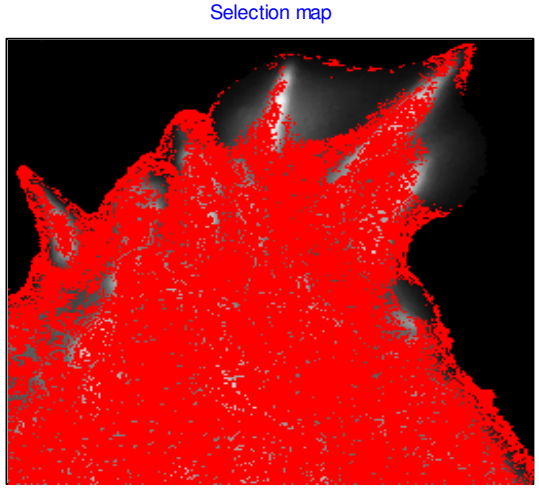


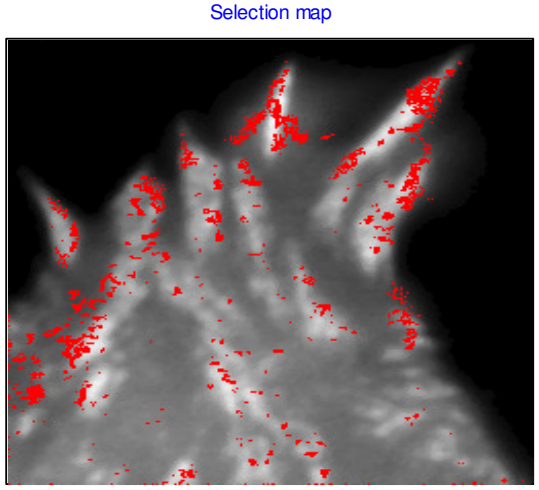
We determined that the brightness for monomers B1= 1.118 and B2=1.22. Thus the ccB1= 6x monomer and for ccB2= 3 x the monomer

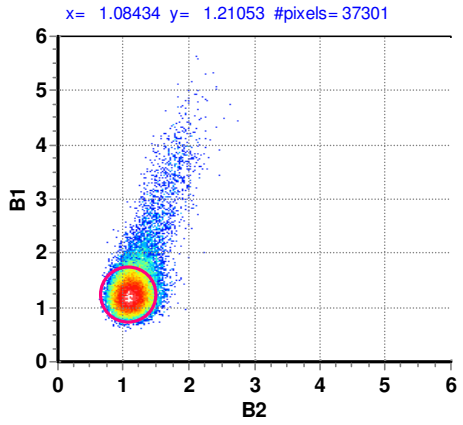
Simulations: effect of bright immobile features

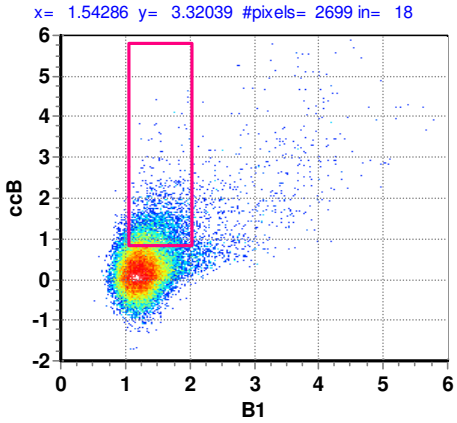


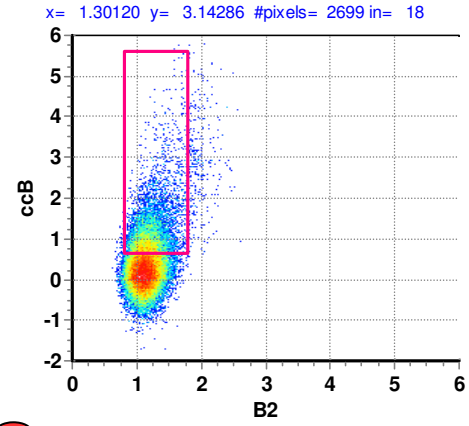
FAK and Paxillin









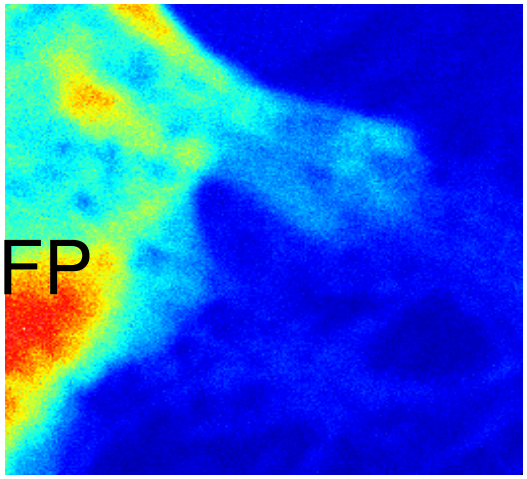


3:4

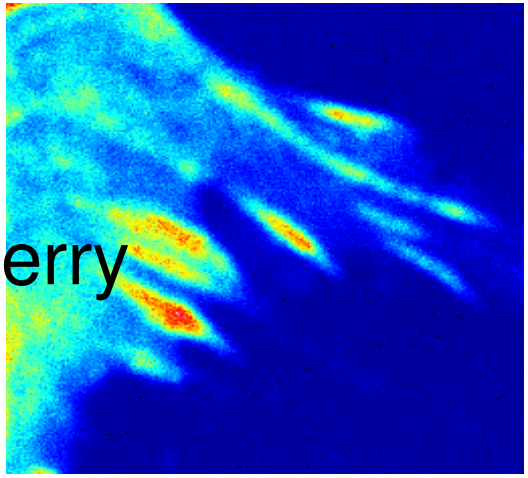
Digman, M.A., et al, *PNAS* Jan.23, 2009 Ahead of print

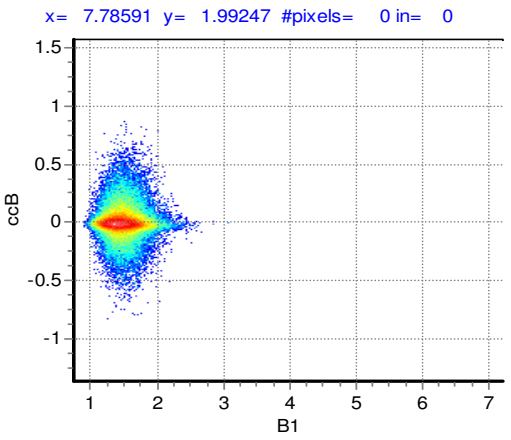
Testing for artifacts: FAK mutant does not form complexes

FAK-EGFP



Pax-mCherry





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

Summary

- N&B distinguishes between number of molecules and molecular brightness in the same pixel
- The acquisition for the N&B can be done with a commercial Laser Scanning Microscope (LSM) and the same data used for RICS can be used to map N and B.
- The Immobile fraction can be separated since it has a Brightness value =1
- The N&B analysis of paxillin at adhesions shows large aggregates of protein during disassembly.
- Cross N&B allows us to determine the stoichiometry of the complexes.

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].