Raster Image Correlation Spectroscopy
RICS

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Raster Image Correlation Spectroscopy

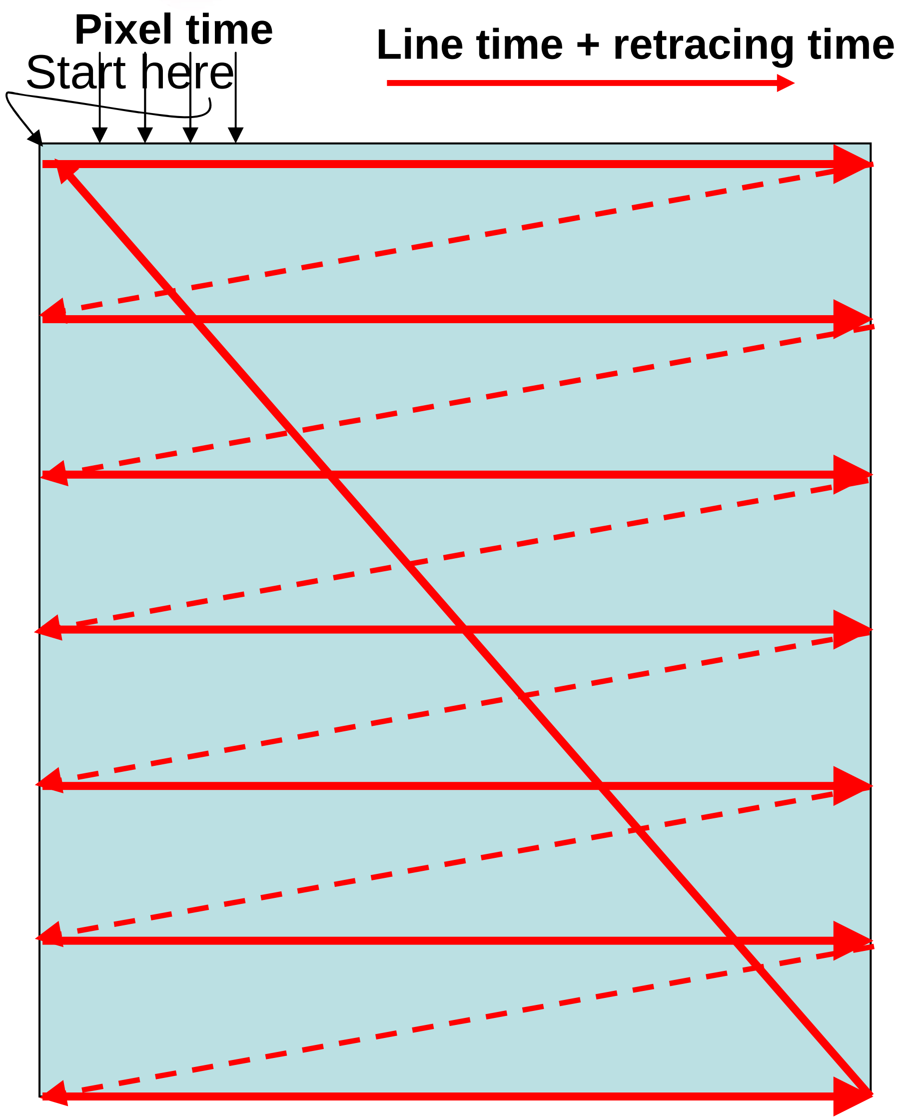
We can have a combination of very high time resolution with sufficient spatial resolution.

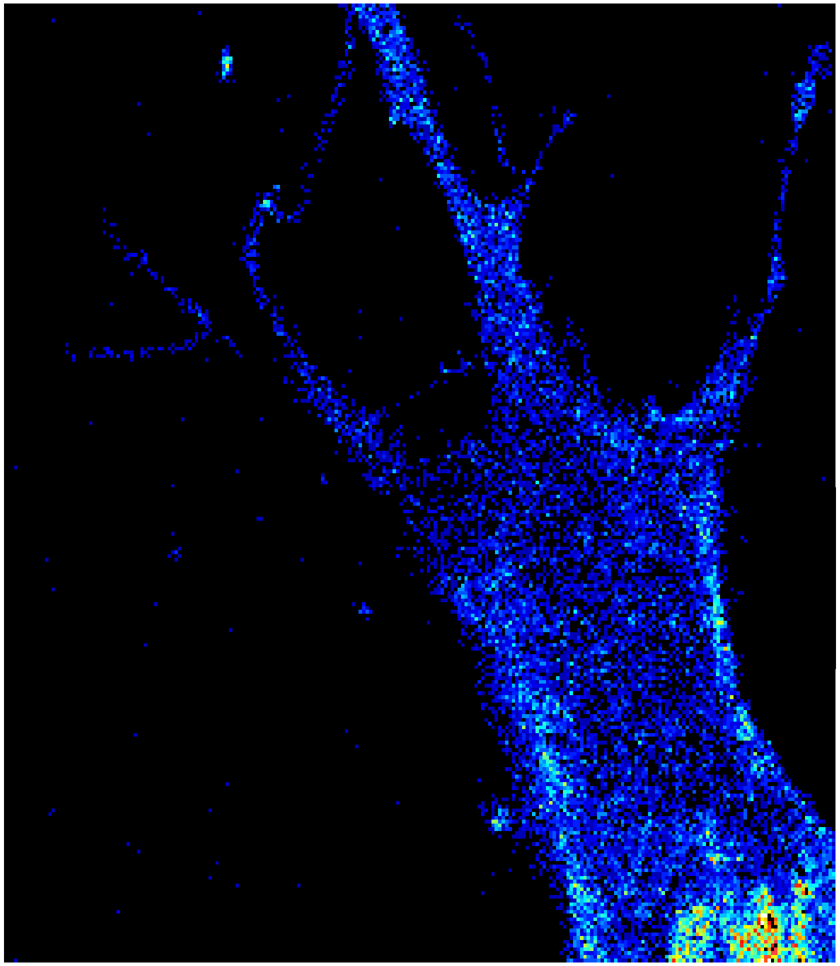
Major benefits of RICS:

- 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?

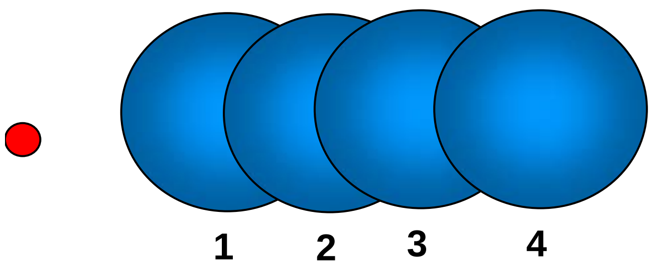
Raster Scanning



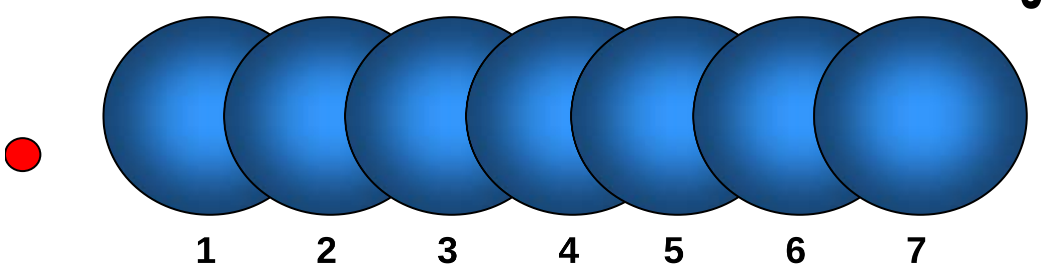


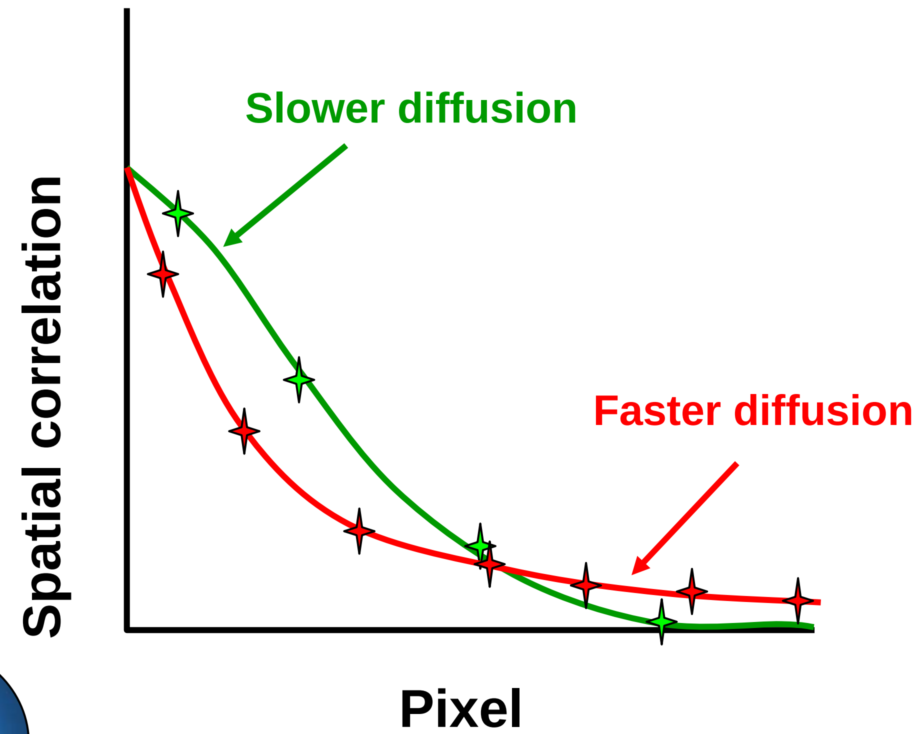
Temporal information hidden in the raster-scan image: the RICS approach

Situation 1:slow diffusion

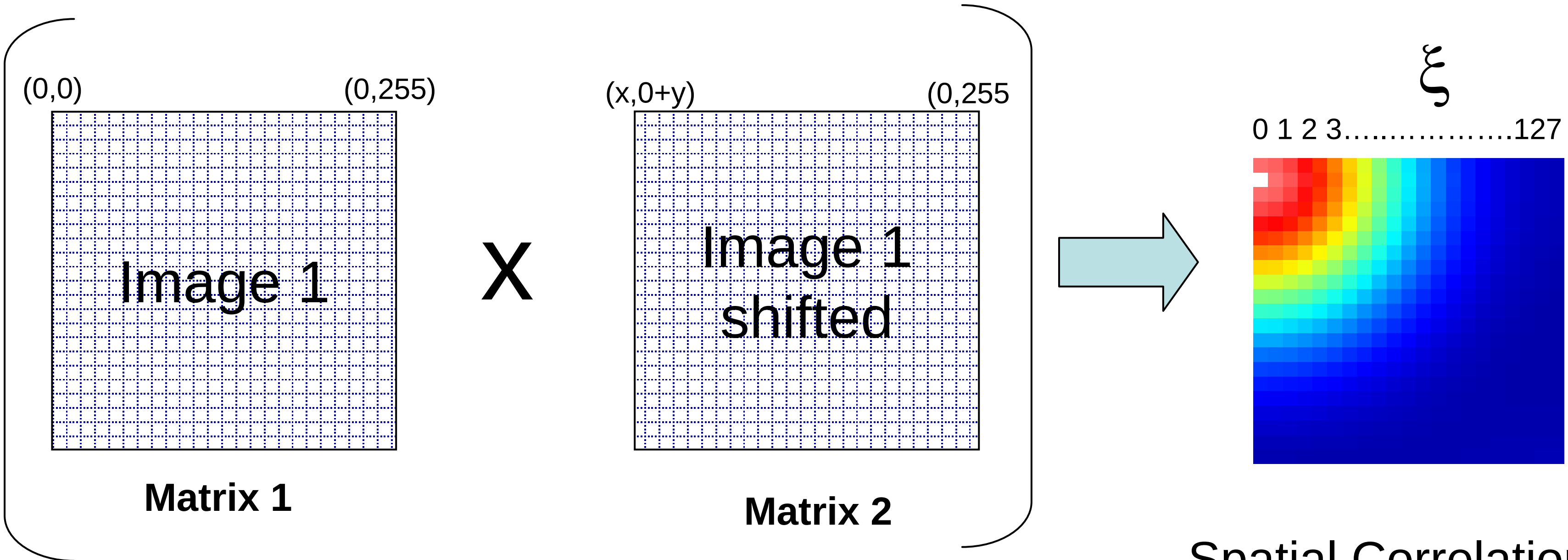


Situation 2: fast diffusion





How is the spatial correlation done?



Spatial Correlation Results

Operation:

In the x direction

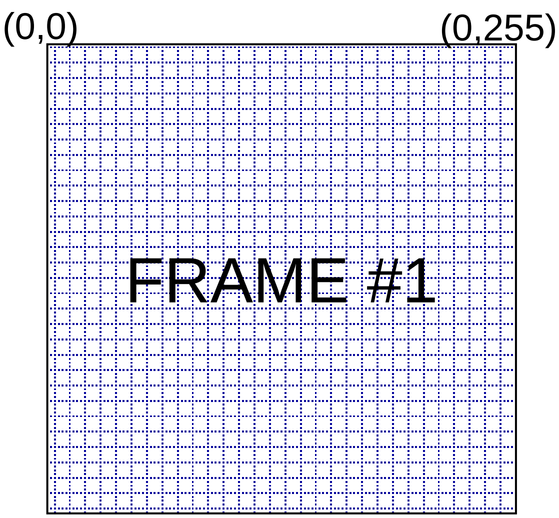
PLUS In the y direction

$$(0,0\times 0,0)+(0,1\times 0,1)+(0,2\times 0,2)\ldots(0,127\times 0,127)$$

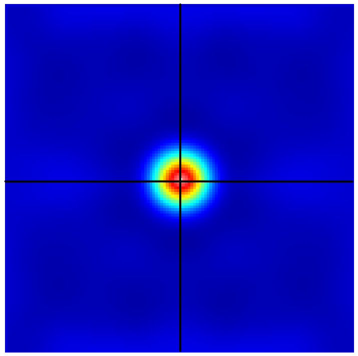
$$+(1,0\times 1,0)+(1,1\times 1,1)+(1,2\times 1,2)\ldots(1,127\times 1,127)$$

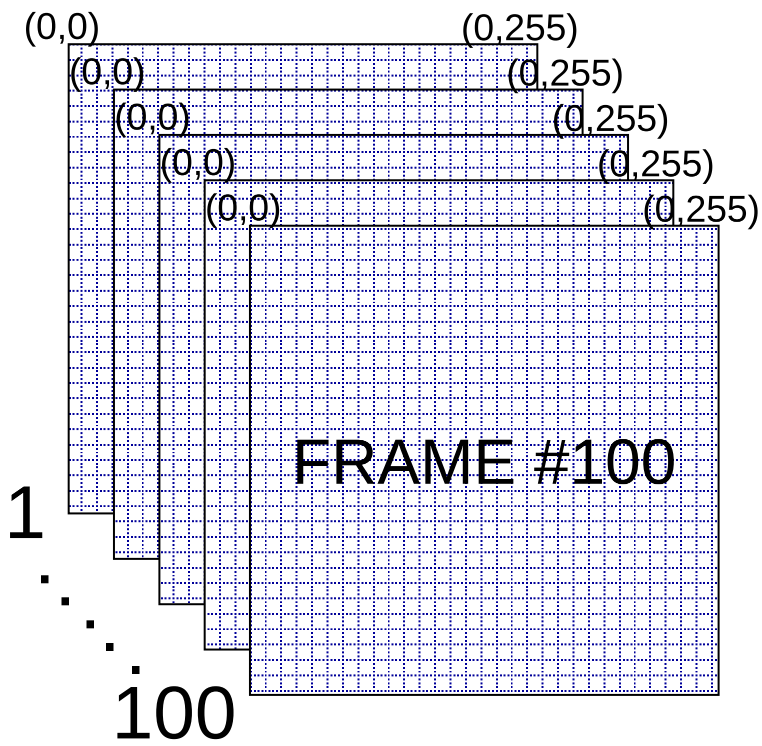
One number is obtained for x and y and is
divided by the average intensity squared

How to use a stack of images?



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





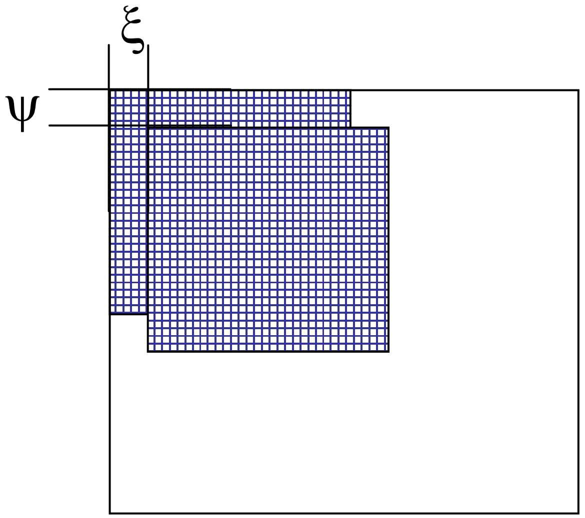
The RICS approach: 2-D spatial correlations

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)

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



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

The RICS approach for diffusion

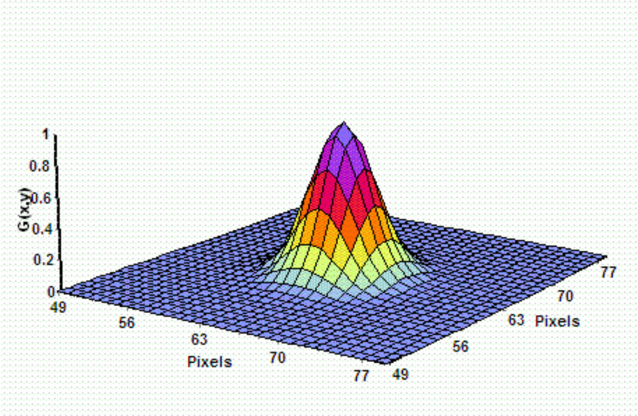
The dynamic at a point is independent on the scanning motion of the laser beam

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

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

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

FAST

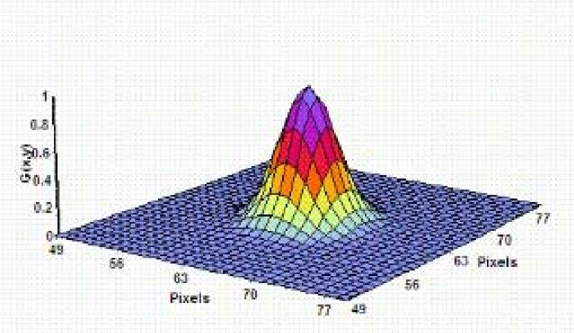


There are two parts:

(1) the temporal term,

(2) the spatial Gaussian term

SLOW

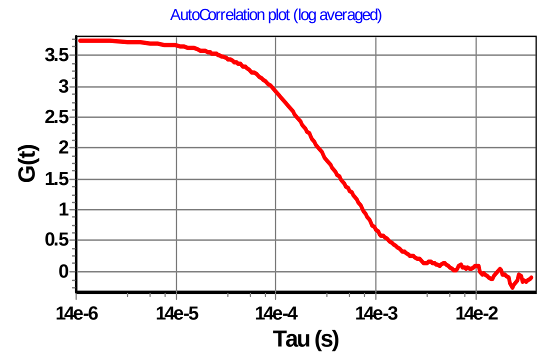


For any diffusion value the amplitude decreases as a function of time and the width of the Gaussian increases as a function of time

RICS: space and time relationships

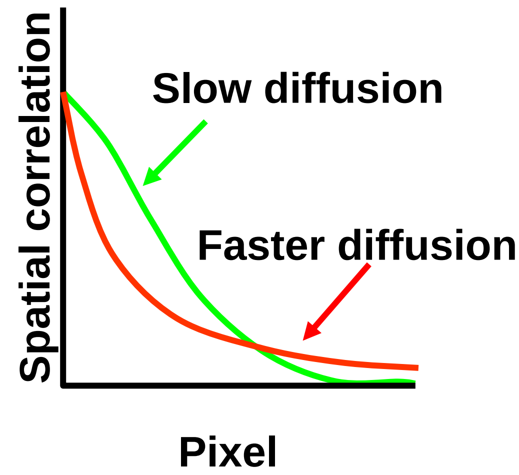
At any position, the ACF due to diffusion takes the familiar form:

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



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

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



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.

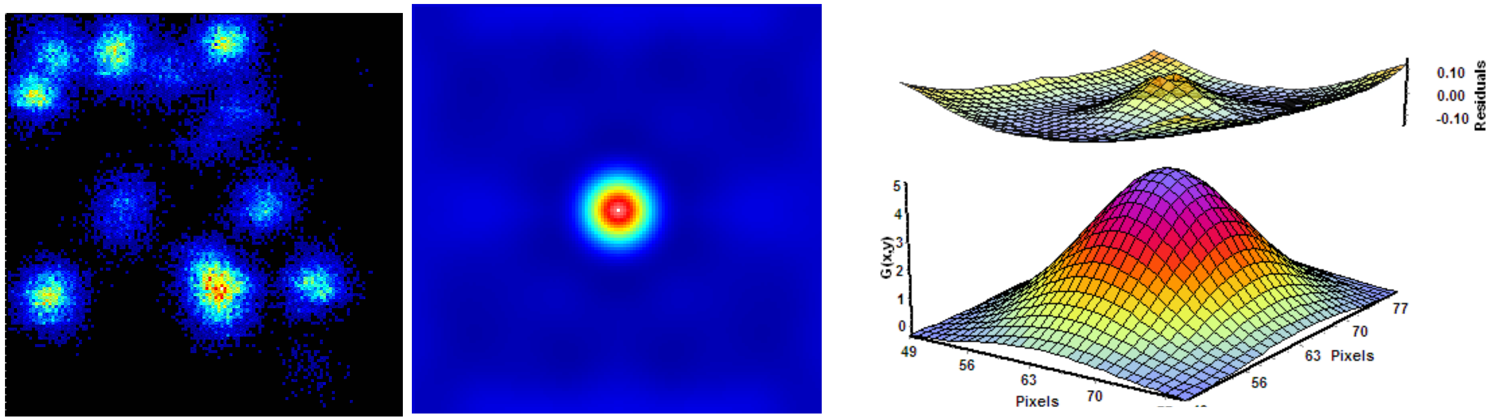
Digman et al. Biophys. J., 2005

RICS Simulations of three different diffusion rates:

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

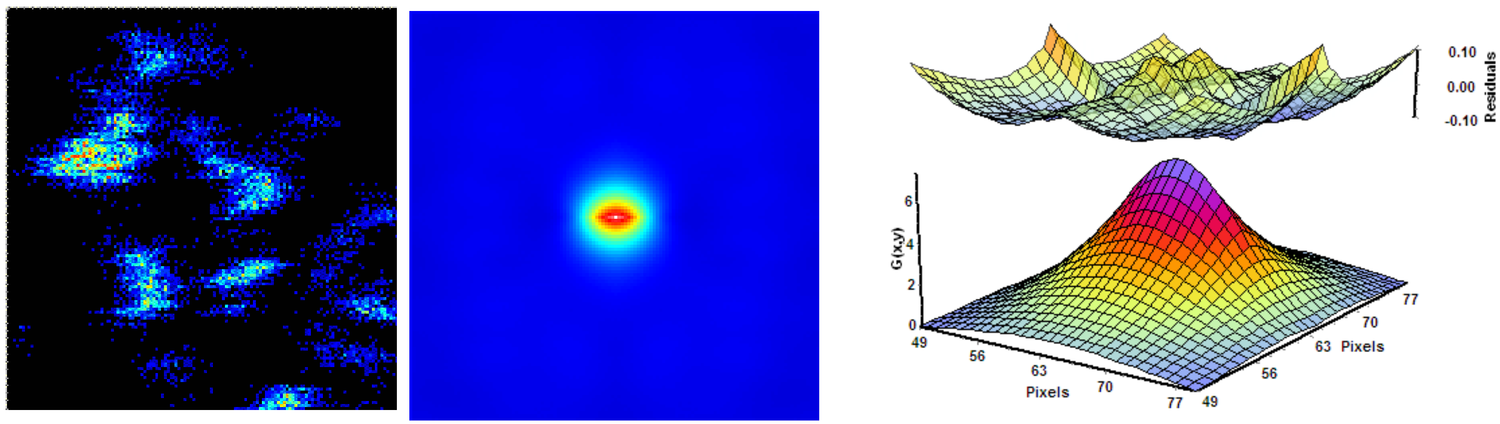
D = 0.1 µm²/s

(membrane proteins)



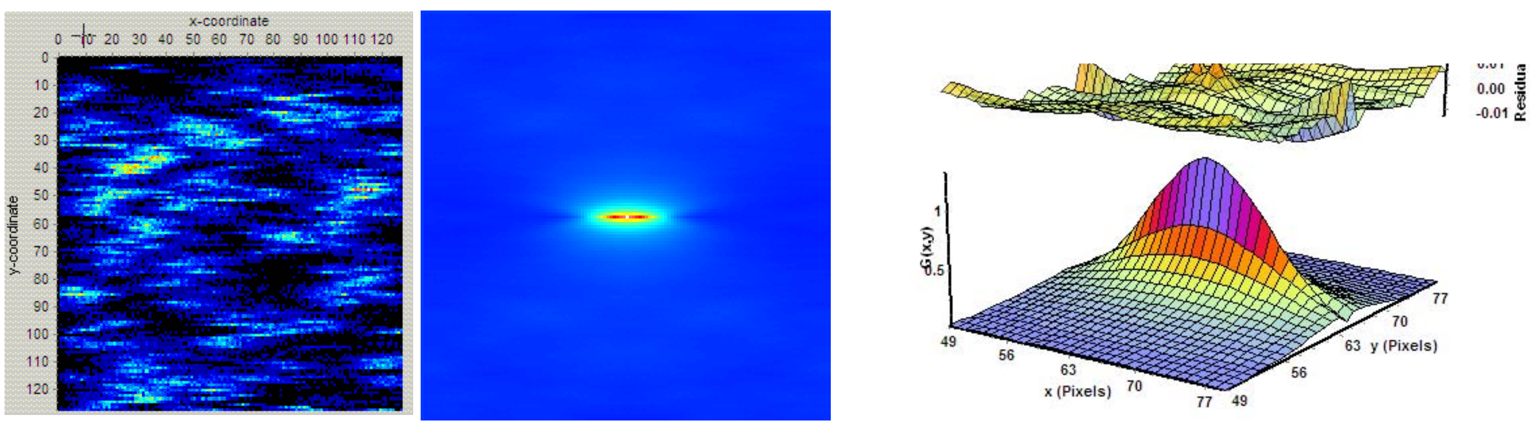
D = 5.0 µm²/s

(40 nm beads)



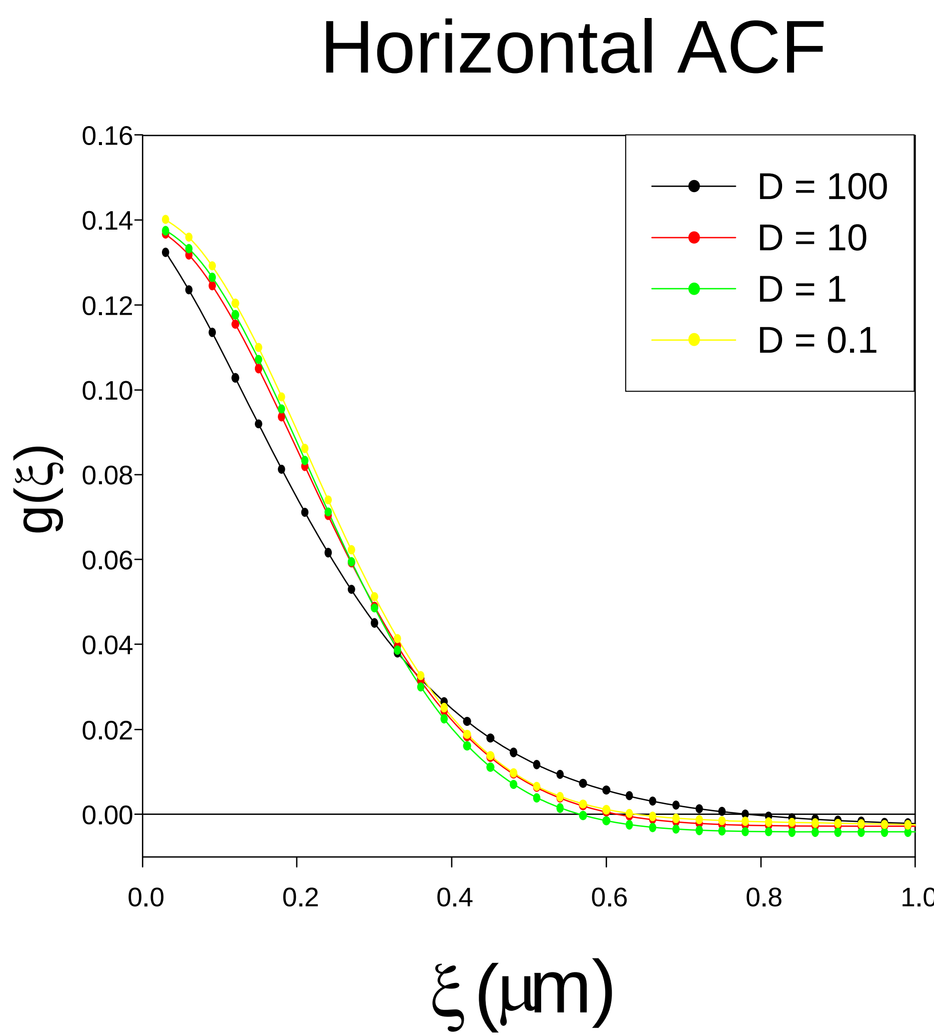
D = 90 µm²/s

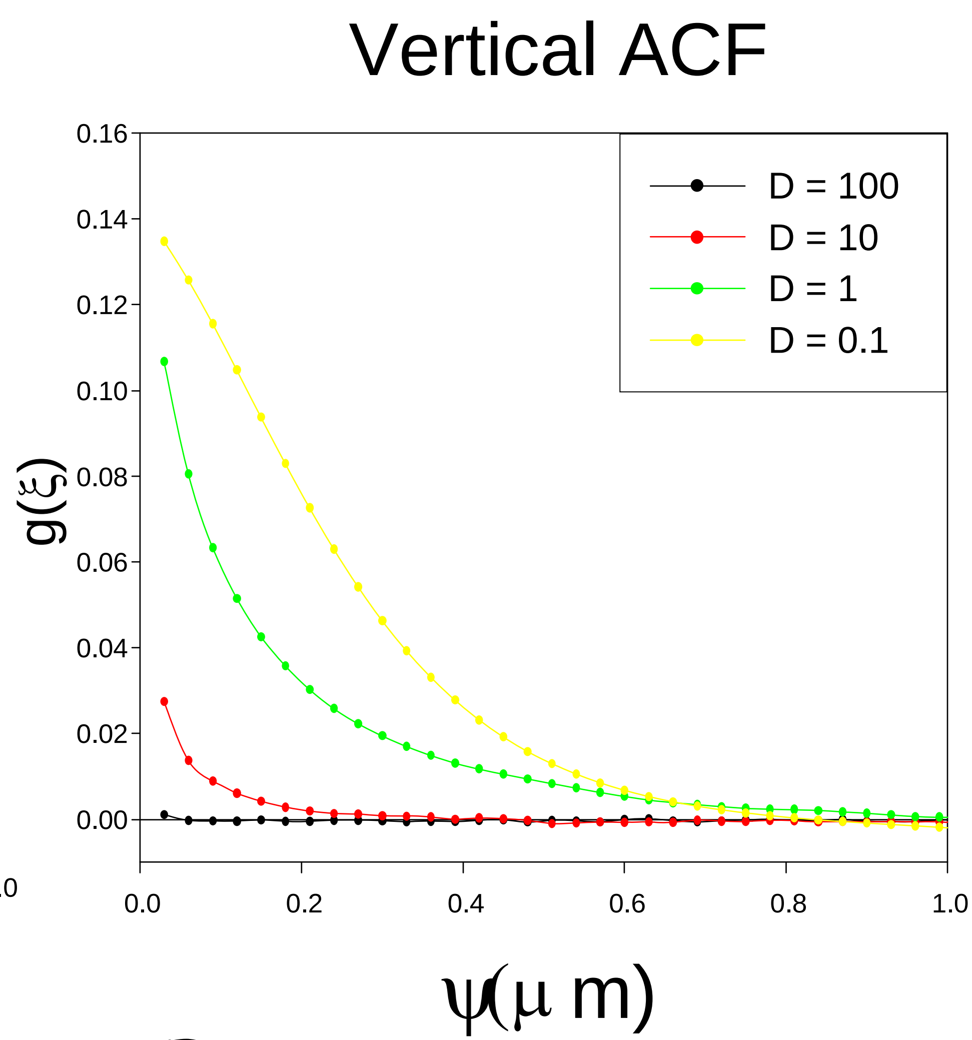
(EGFP)



Horizontal and Vertical fits:

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





In SIMFCS

Brown et al, JMI, 2007

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:

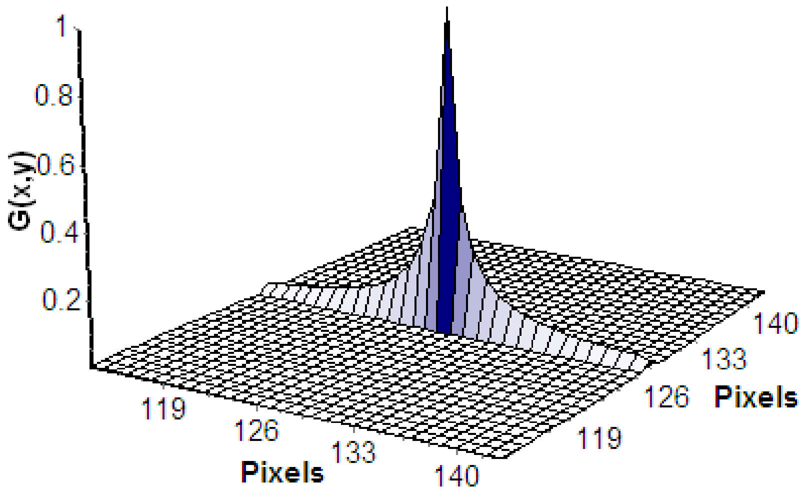
- 3-4x smaller than the Point Spread Function (PSF~300nm)

Molecular Concentrations

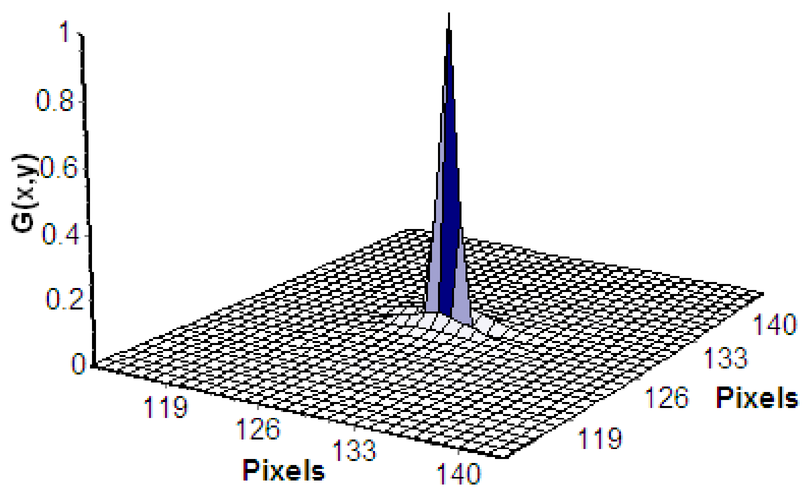
- Same conditions as conventional FCS methods

Common Errors in RICS

Scanning Too Slow
(100 us/pixel, D = 300 um²/s)



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

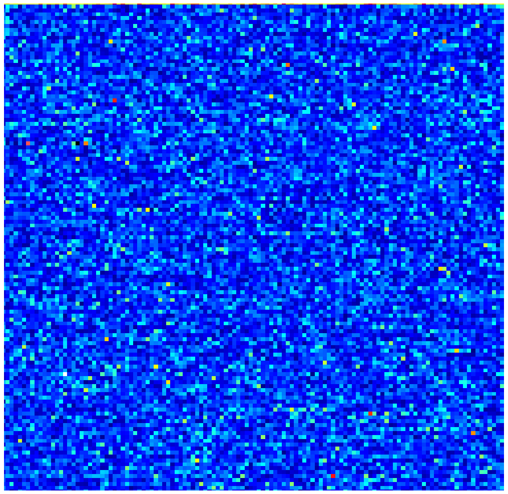


Courtesy of Jay Unruh

RICS: Fits to spatial correlation functions

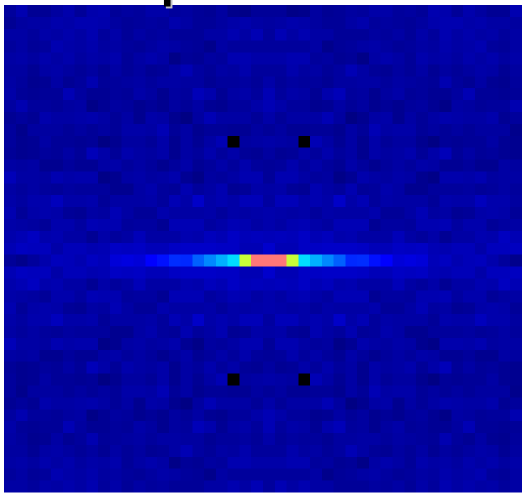
Olympus Fluoview300 LSM

EGFP in solution

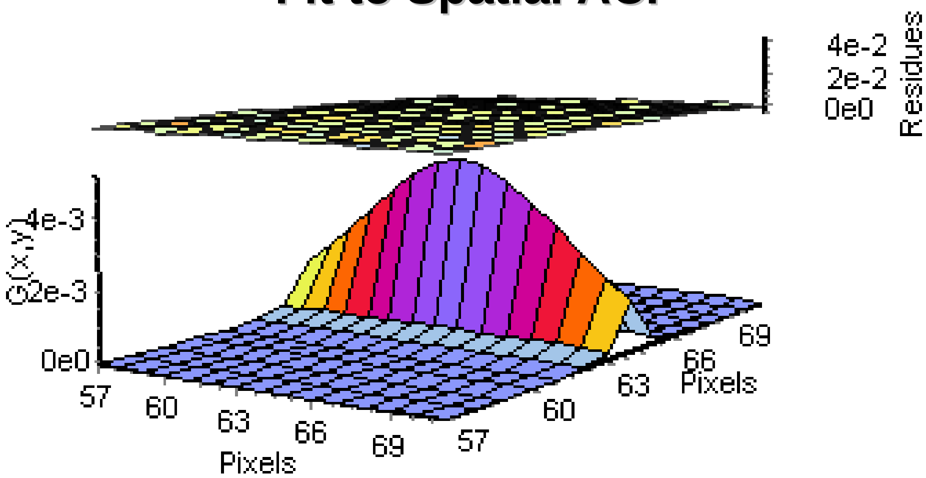


128x128, 4 μs/pixel, 5.4 ms/line, 0.023 μm/pixel

Spatial ACF



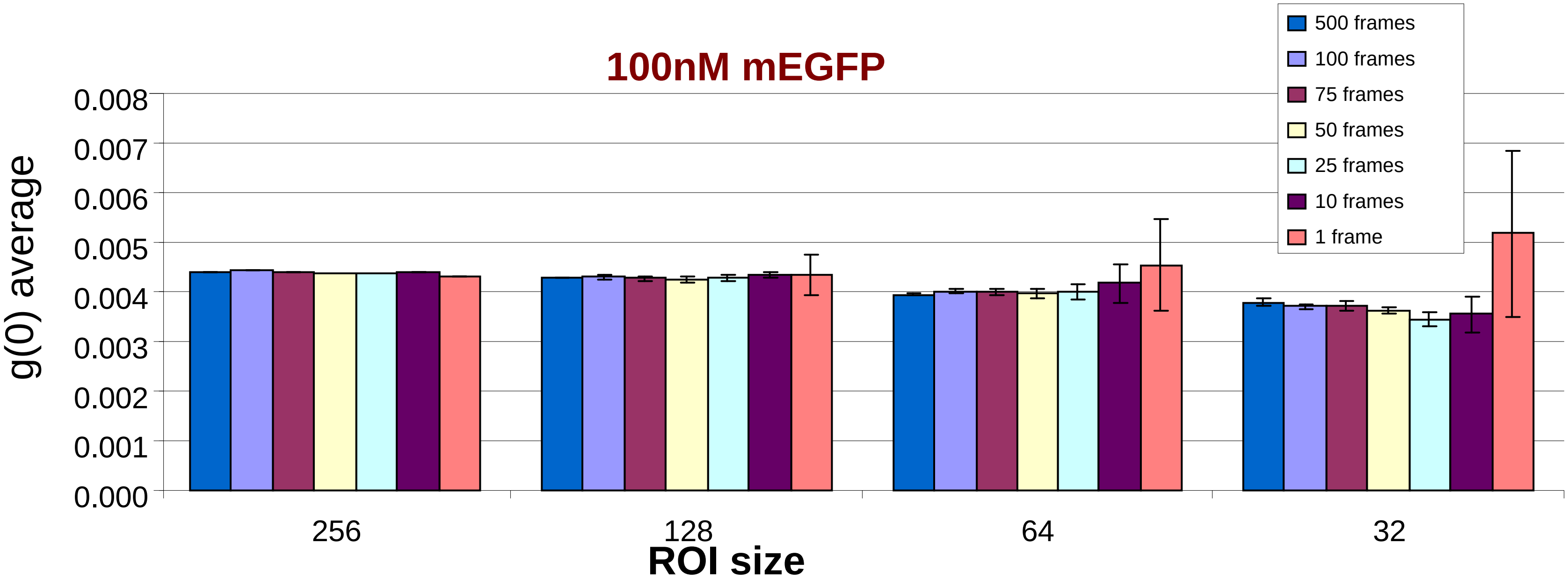
Fit to Spatial ACF

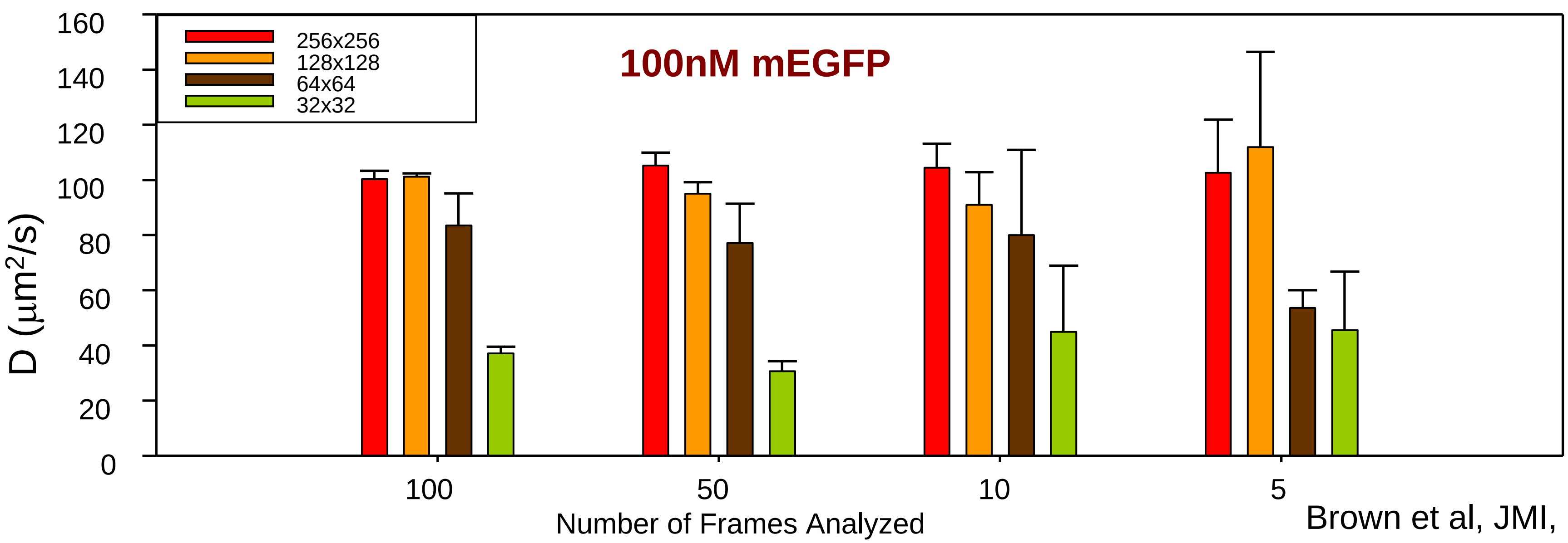


D = 105 ± 10 $\mu m^2/s$

Digman et al. Biophys. J., 2005

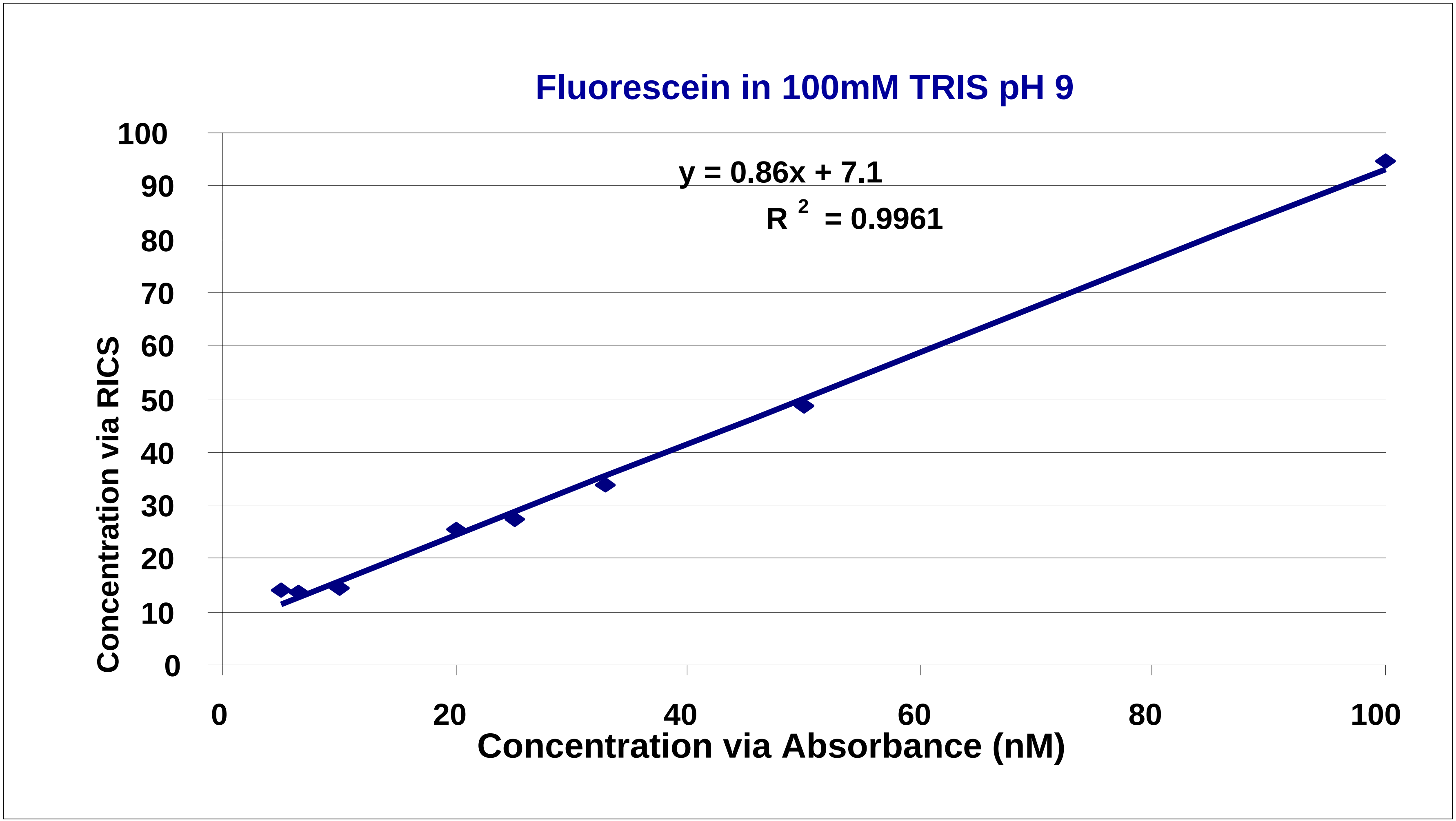
What ROI size to use? How many frames to acquire?





Brown et al, JMI, 2007

Obtaining concentration from RICS

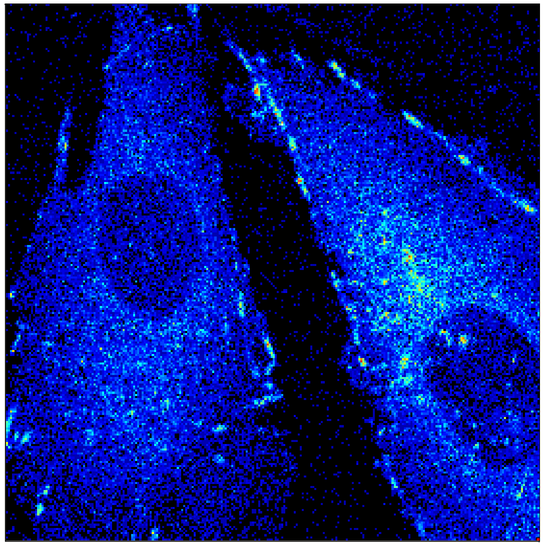


Brown et al, JMI, 2007

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.

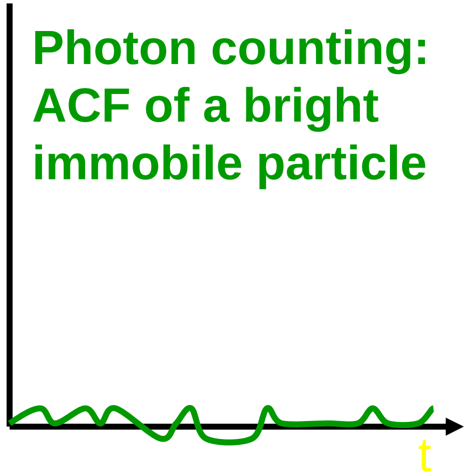
The time correlation of the shot noise is zero, except at time zero.

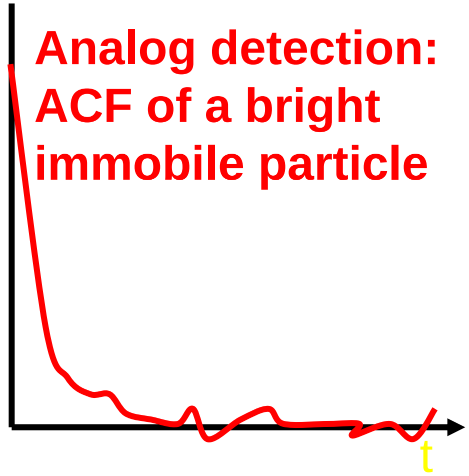
The spatial correlation of the intensity at any two pixels due to shot noise is zero, even if the two points are within the PSF.

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

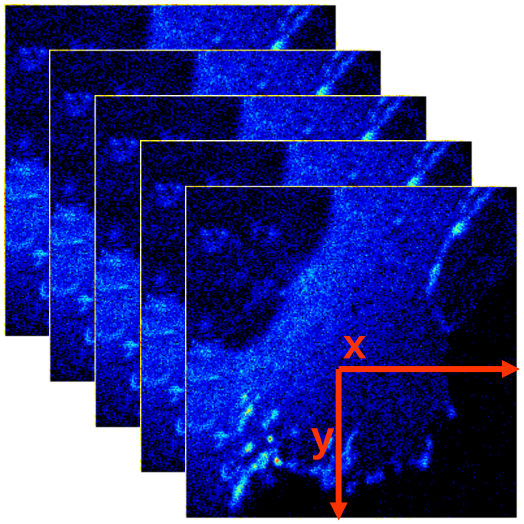
Attention!!!!

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.

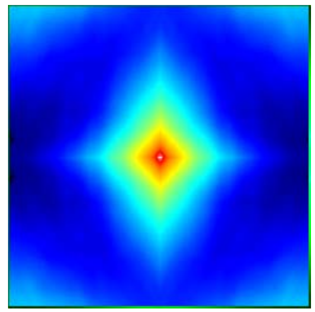




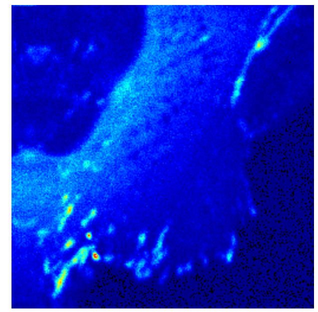
Formula used to subtract background:



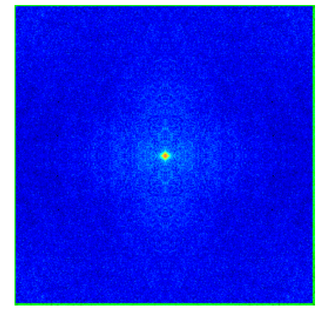
Spatial Correlation



Spatial correlation before subtracting background



Subtract the average



Spatial Correlation of entire image After subtracting image

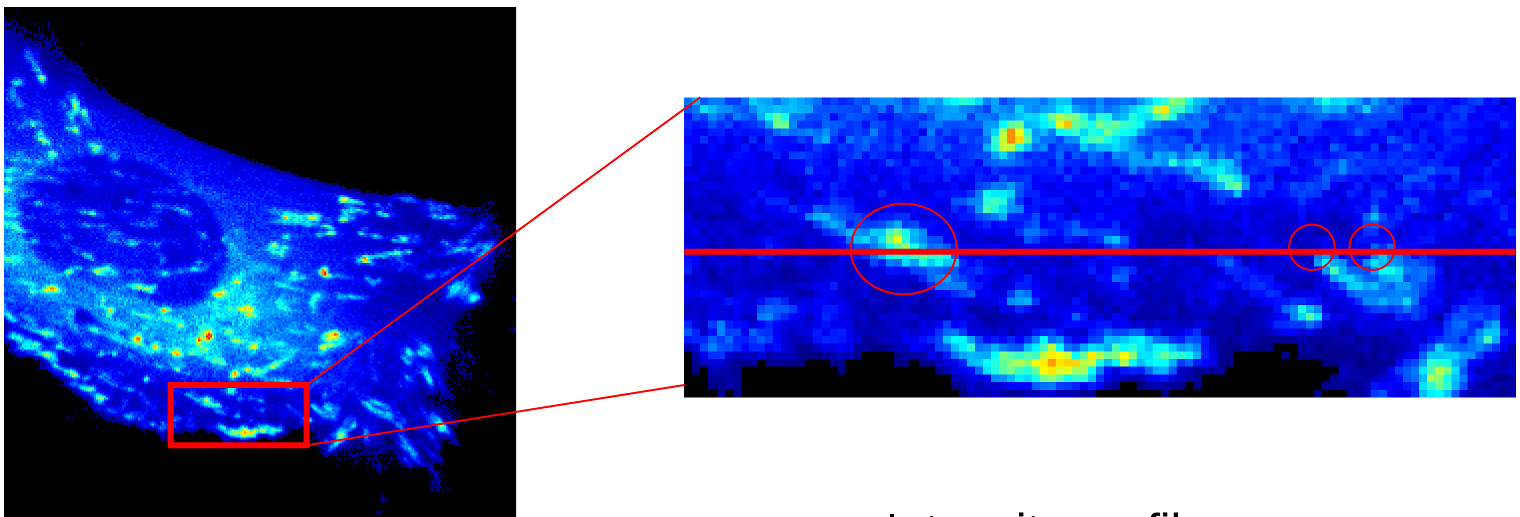
Average intensity of each pixel on the overall stack: $\overline{I(x,y)}$

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

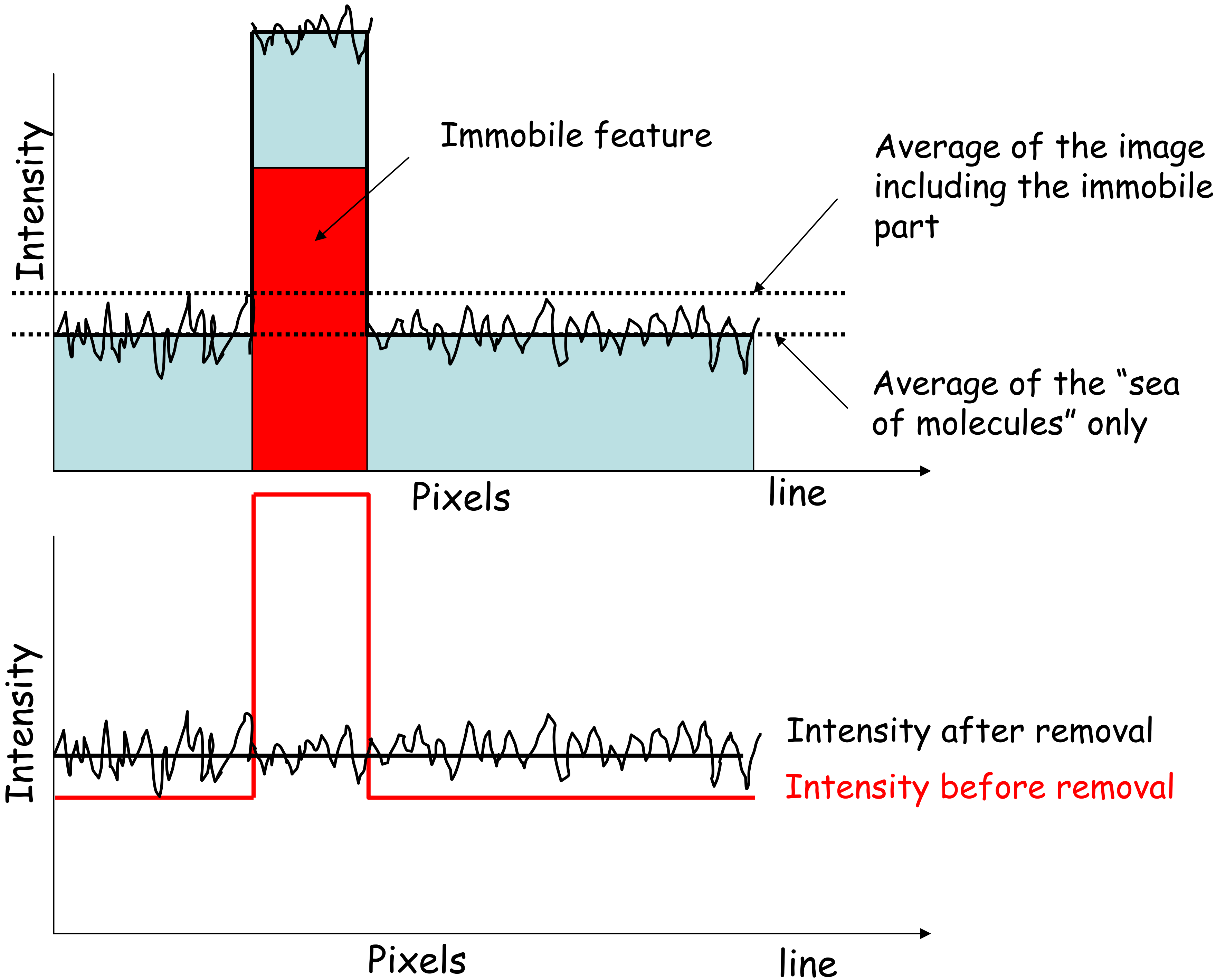
A scalar must be added : $a = \overline{I}$

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

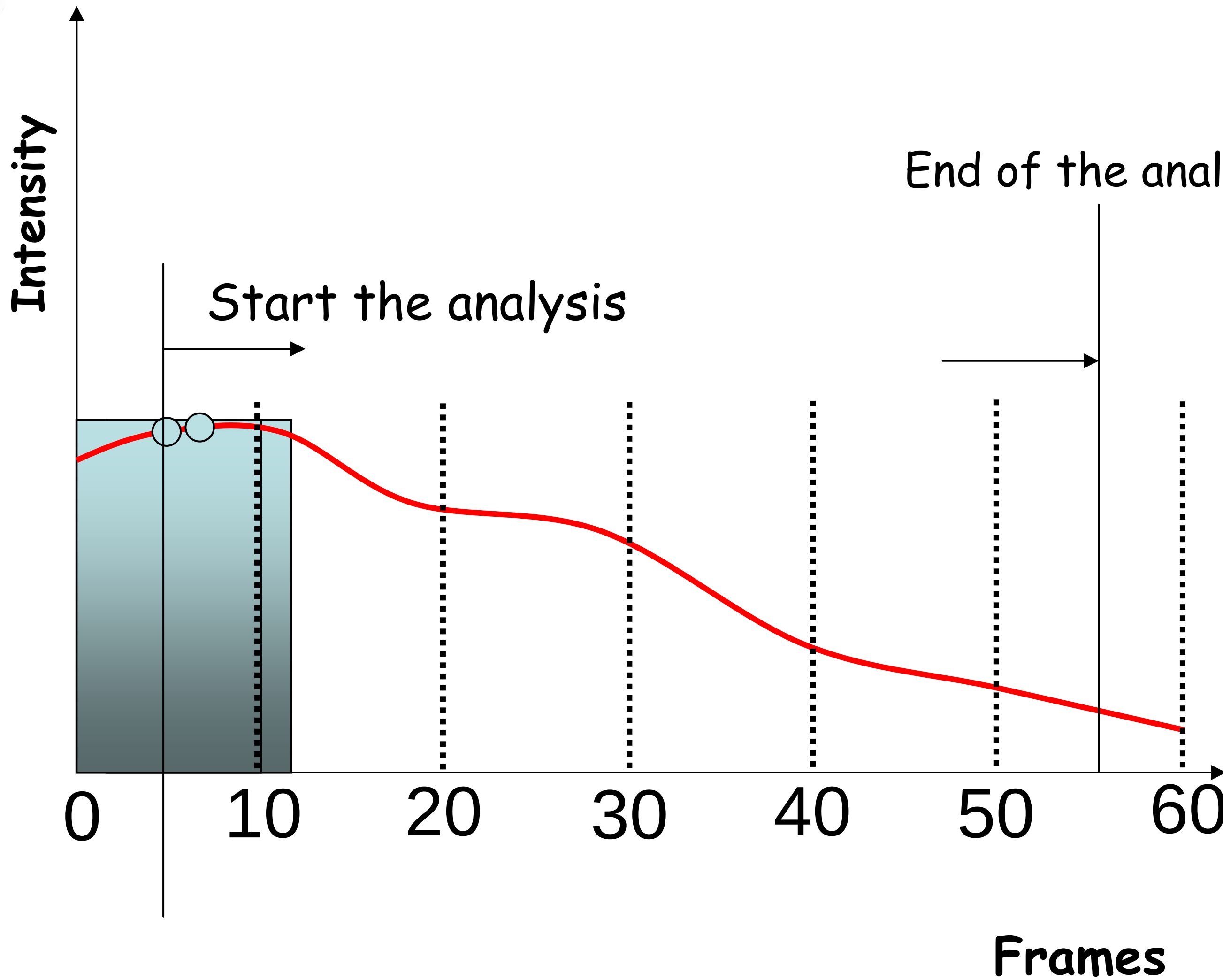
How to subtract immobile features from images?



Intensity profile

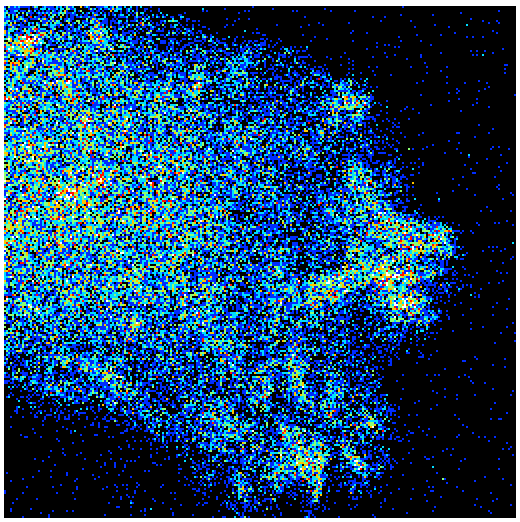

Intensity
Immobile feature
Average of the image including the immobile part
Average of the "sea of molecules" only
Pixels
line
Intensity
Intensity after removal
Intensity before removal
Pixels
line

Subtraction of moving average



Moving average operation on frames:

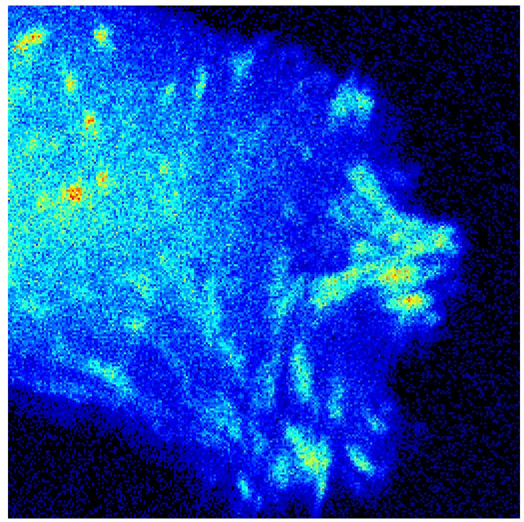
Frame #5



Matrix1

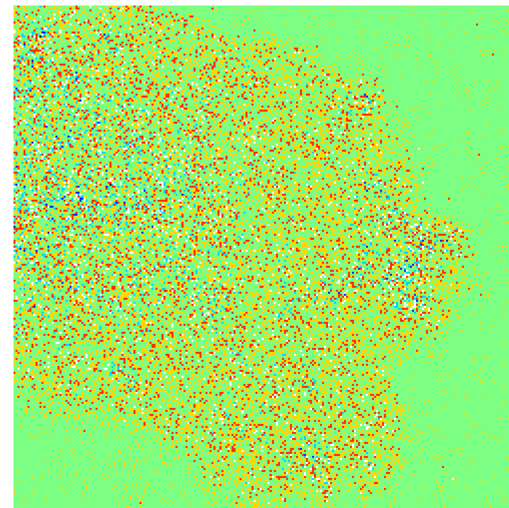
-

Average
between 1-10



Matrix 2

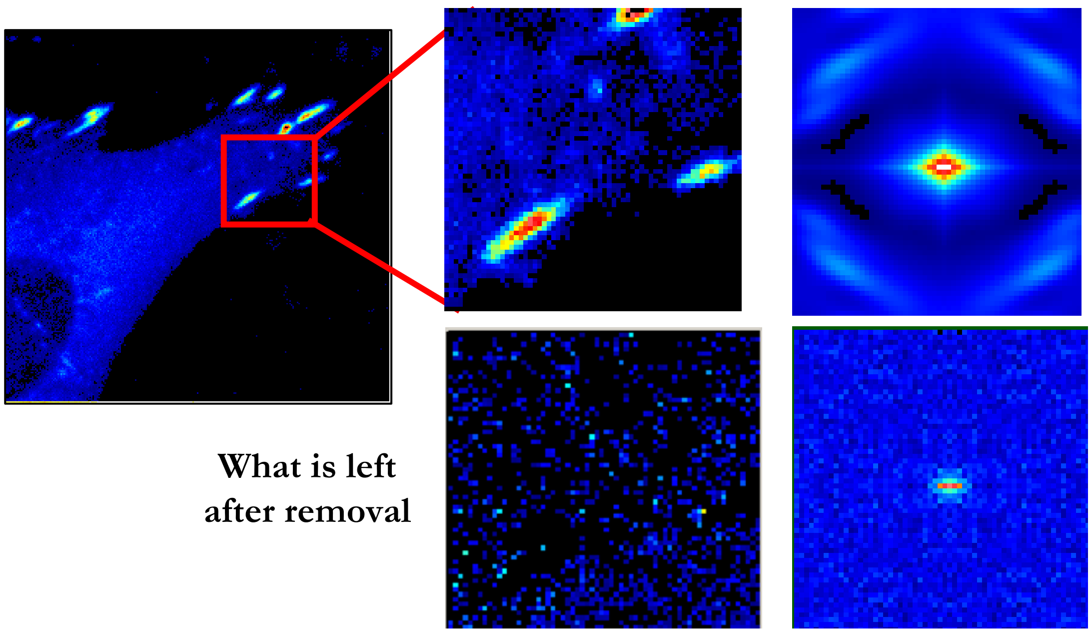
=



A scalar average is then added

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

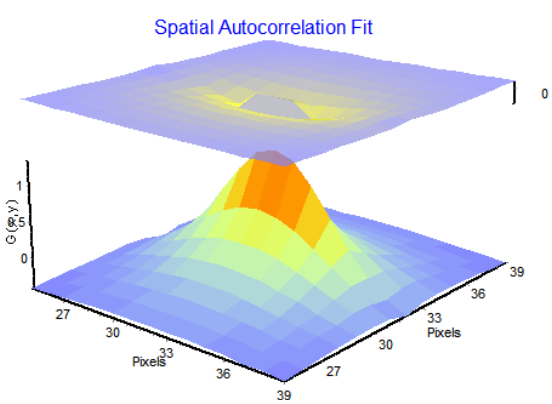
Example of the Removal of Immobile Structures and Slow Moving Features



Spatial ACF
No removal

What is left
after removal

Spatial ACF
With removal

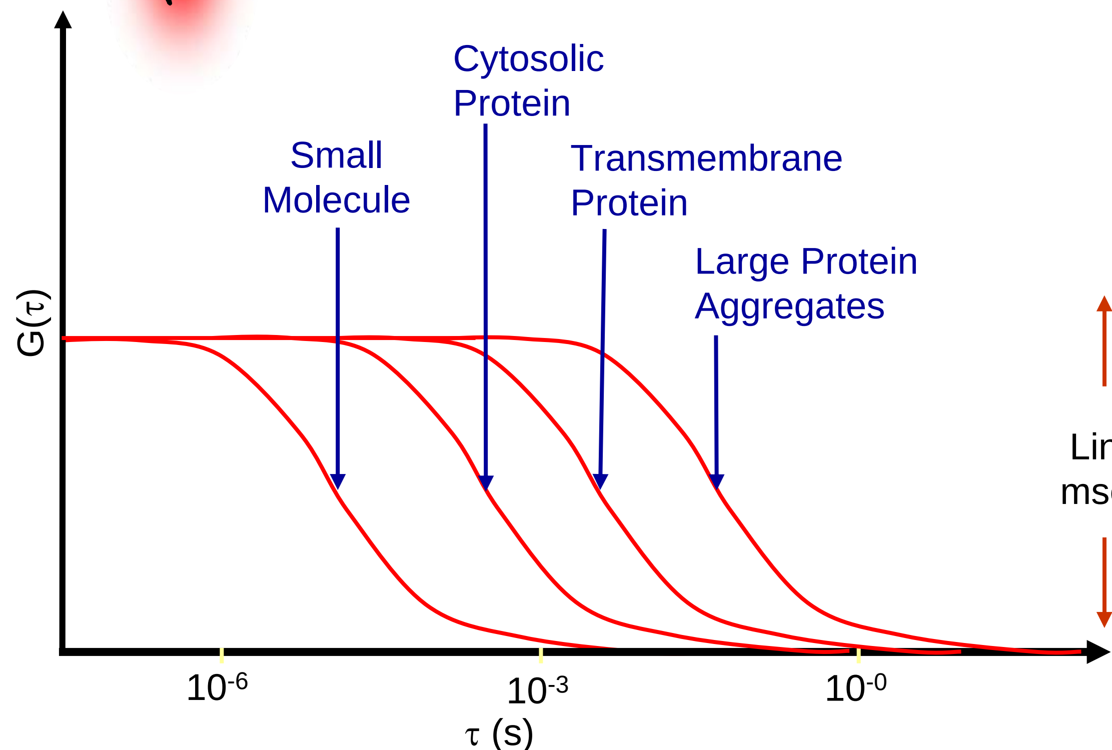


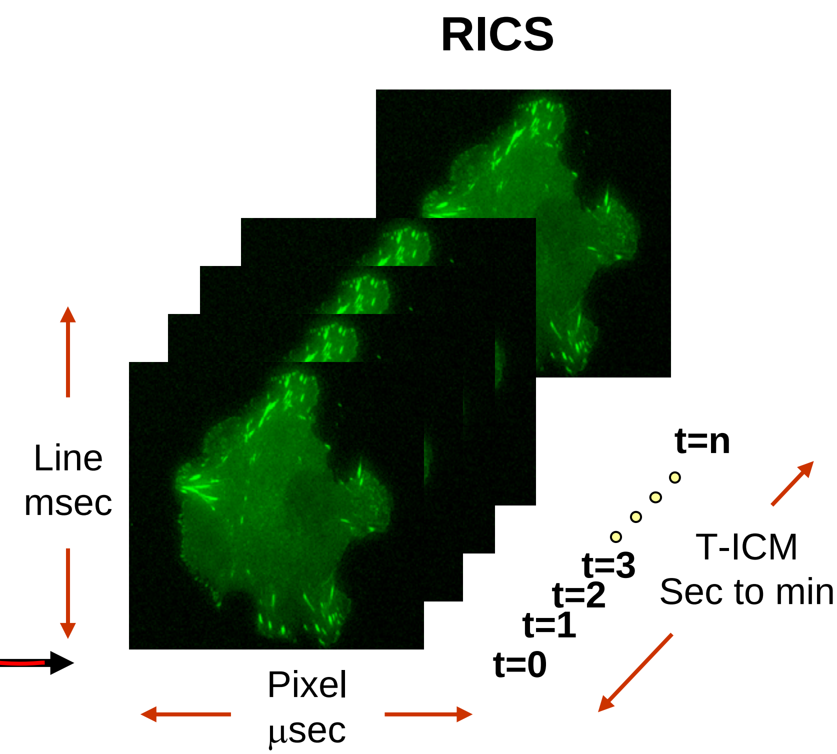
Fit using 3-D diffusion formula

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

G1(0) = 0.0062
$\boxed{\text{D1} = 7.4\ \mu\text{m}^2/\text{s}}$
G2(0) = 0.00023
$\boxed{\text{D2} = 0.54\ \mu\text{m}^2/\text{s}}$
Bkgd = -0.00115

Conclusions





FCS

Line Scan

Temporal correlation

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
Line-RICS	msec	<0.5 μm	Soluble proteins Binding interactions

Summary of RICS

- Measures dynamic rates from the μsec-sec 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 (0.001-300um^2/s)
- Other types of processes and interactions are also measured
- Line scanning is essential for determination of binding process and complements the RICS analysis