Fluctuation Spectroscopy
Making signal out of noise

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CIPSM

BIN LMU
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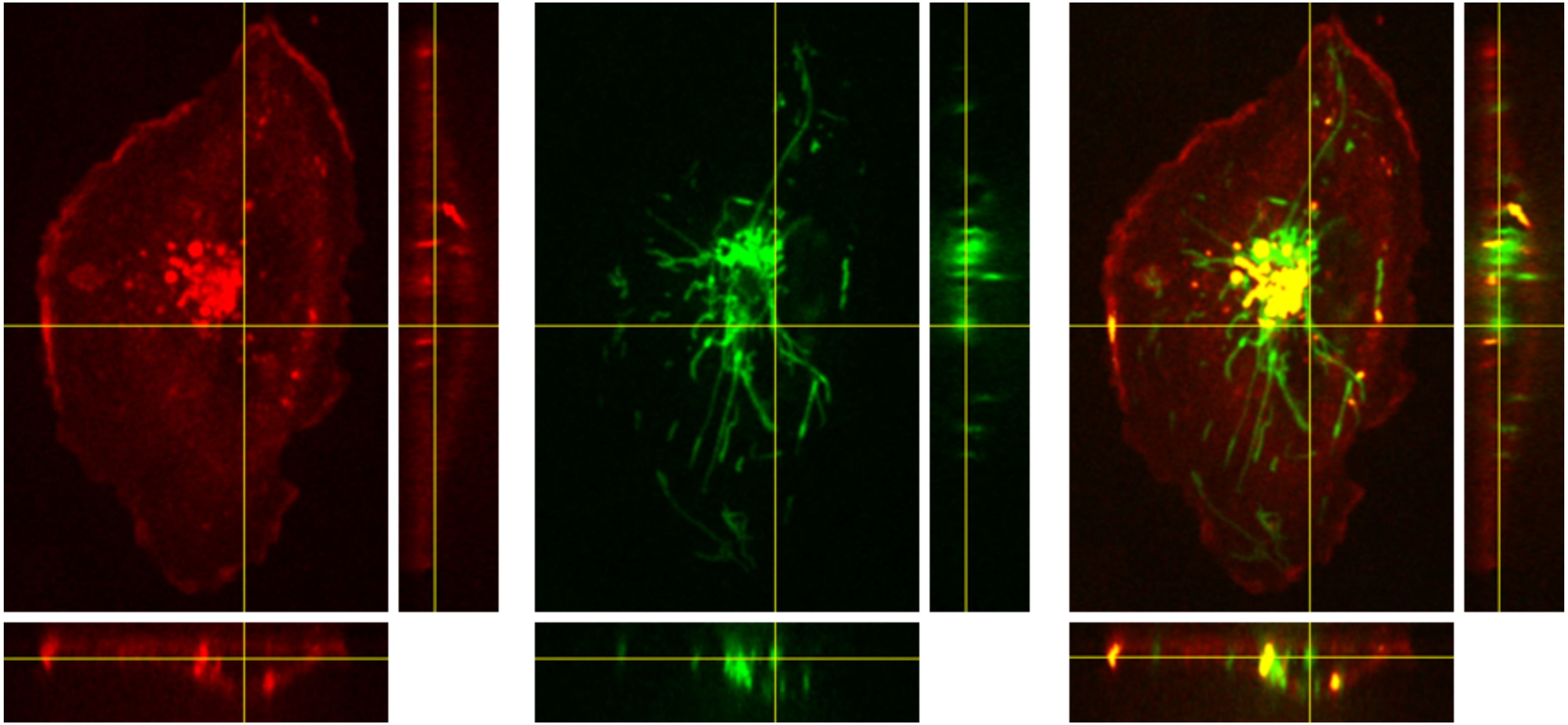
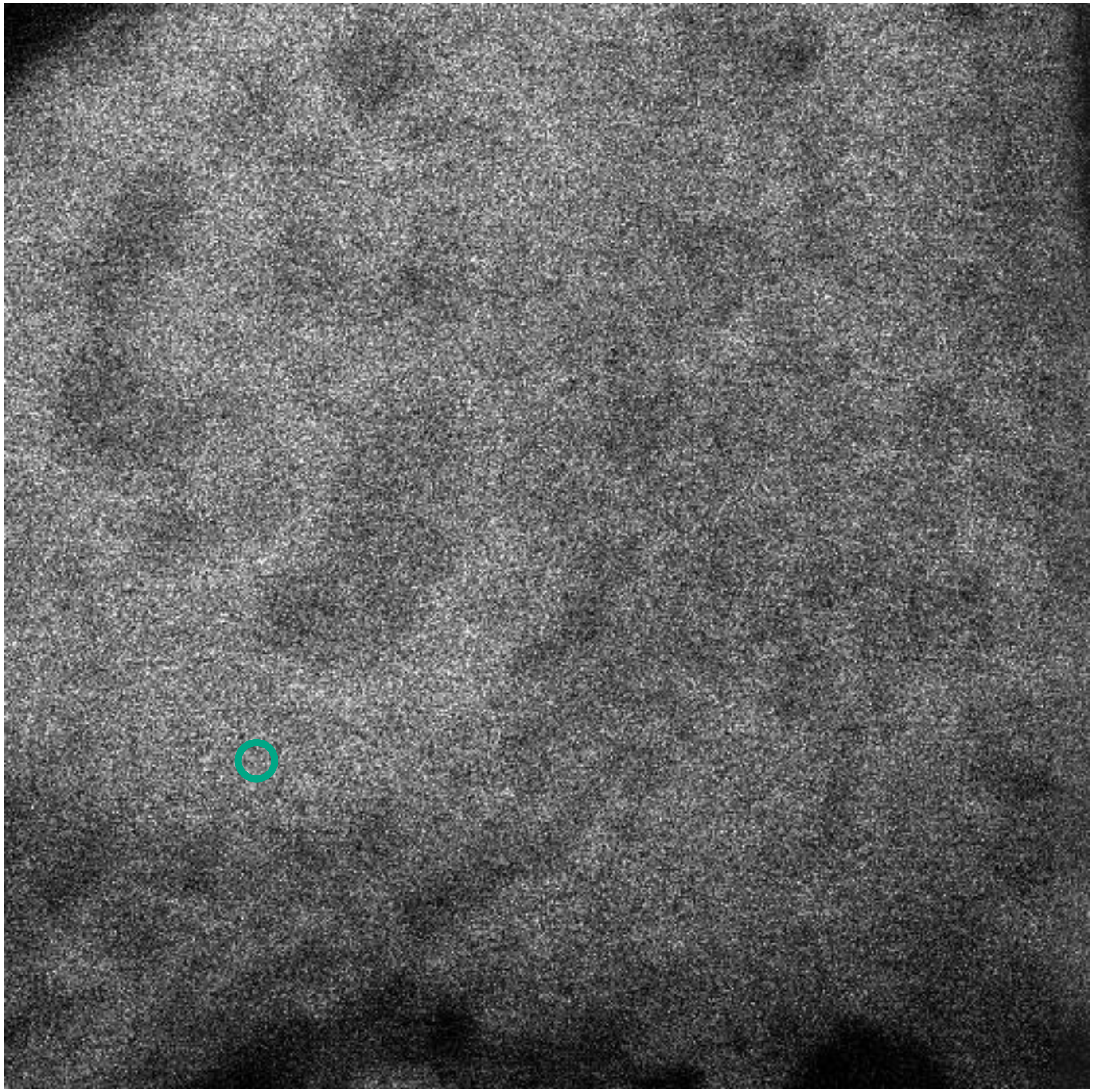
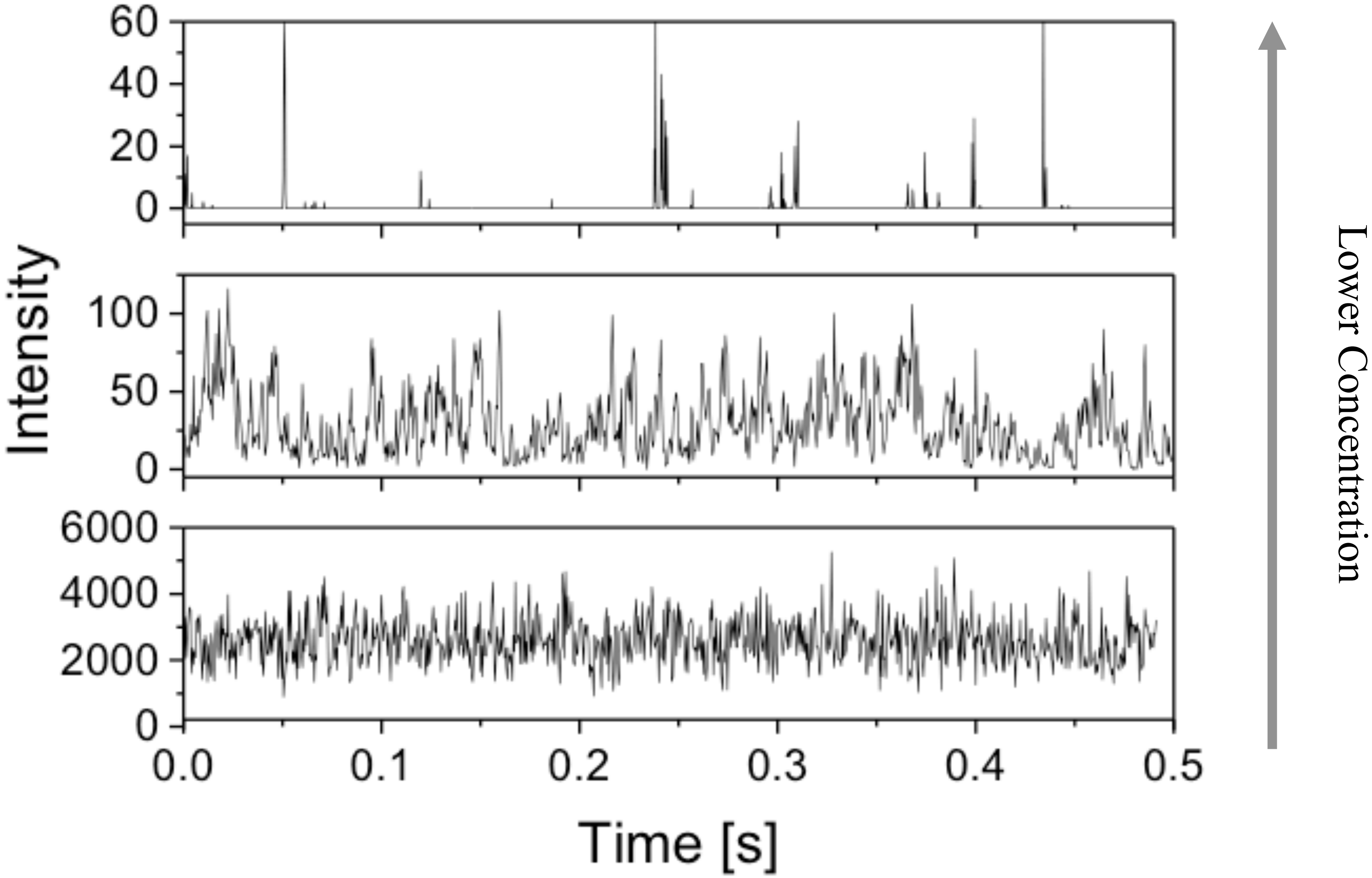
Microscopy: What we show you



Microscopy: What we measure in living cells

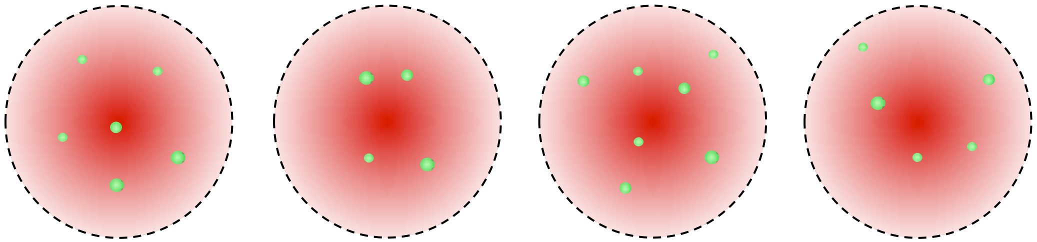


Fluctuation Measurements



Measurements at Low Concentration

Fluctuations in molecular number in a small volume.



***n* = 6 4 7 5**

$$P(N) = \frac{\langle N \rangle^{N} e^{-\langle N \rangle}}{N!}$$

$$\langle \Delta N^2 \rangle = \langle N \rangle$$

A Poissonian Process

Experiments that result in counting the number of events in a given time or in a given object can be described by a Poisson process provided:

a) Number changes on nonoverlapping intervals are independent.

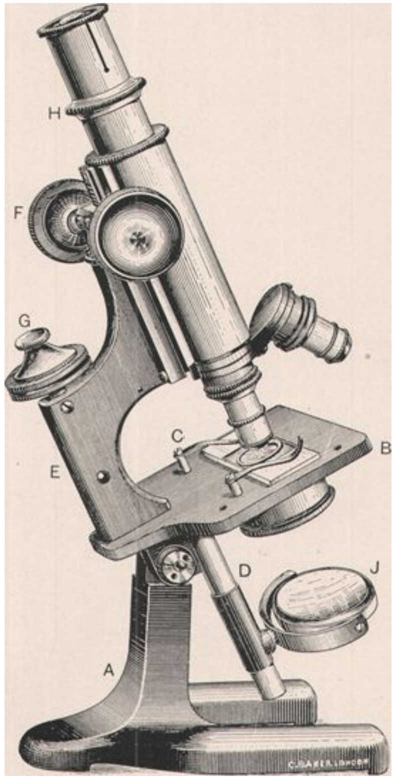
b) The probability of exactly one change occurring in a sufficiently short interval of length h is approximately λh

c) The probability of two or more changes in a sufficiently short interval is essentially zero.

Fluctuations

Observation of gold colloids using an ultra-microscope (Svedberg and Inouye, *Zeitschr f. Physik Chemie* **1911**, 77:145-119)

Measurement of the Equilibrium Thermodynamic Fluctuations in Molecular Number



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42345241141311423100100421123123201111000111-21100
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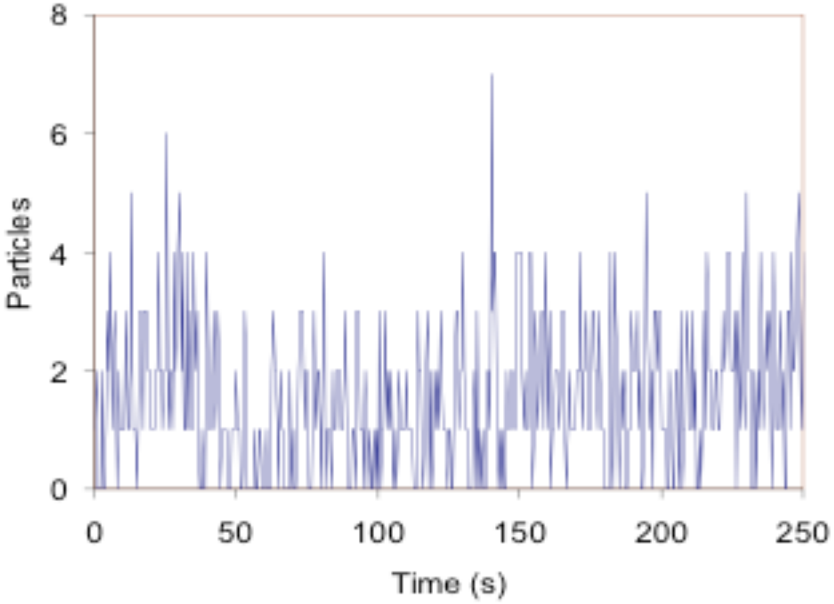
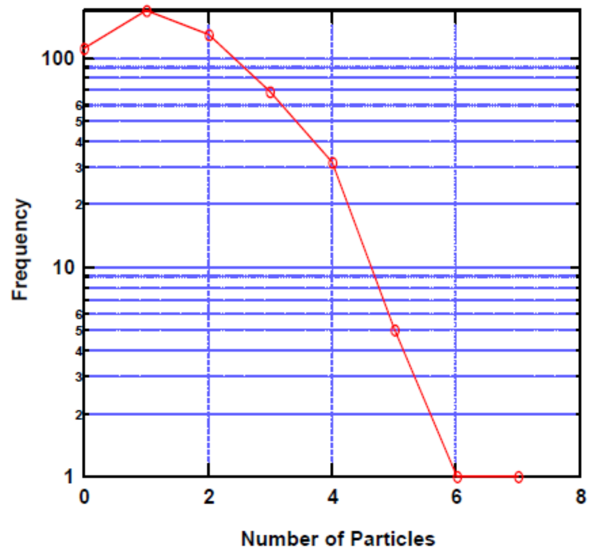
21024011102-1222112310001103311102101001010301131212 1
010121111211-1000322101230201212132111011002331224211
00012030101002217344101010021122114444212114401321233 14
31301122212331012111222412231113322132110000410432012 1
20011322231200-25321203323311110021002201301132 11
31200101314322112211223234422230321421532200202142123 23
204311231200331422345213411041232222 0221

http://www.1911encyclopedia.org/Microscope

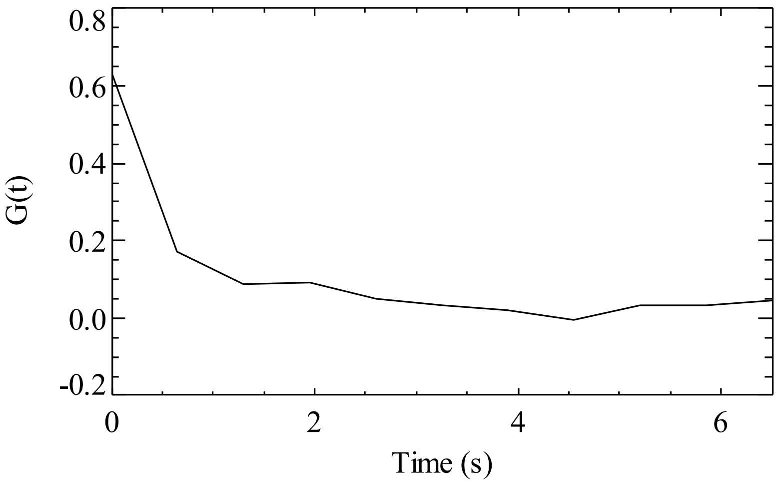
Autocorrelation Analysis



PCH



Autocorrelation Analysis



$\langle N \rangle$ = 1.55 particles

$\tau \sim$ 1.5 s

Autocorrelation Analysis

The normalized autocorrelation function (ACF) is given by:

$$G(\tau) = \frac{\langle F(t)F(t+\tau)\rangle - \langle F(t)\rangle^2}{\langle F(t)\rangle^2}$$

$$= \frac{\langle \delta F(t)\delta F(t+\tau)\rangle}{\langle F(t)\rangle^2}$$

where

$$\delta F(t) = F(t) - \langle F(t)\rangle$$

For processes that are:

Stationary: i.e. the average parameters do not change with time

the ACF is independent of the absolute time

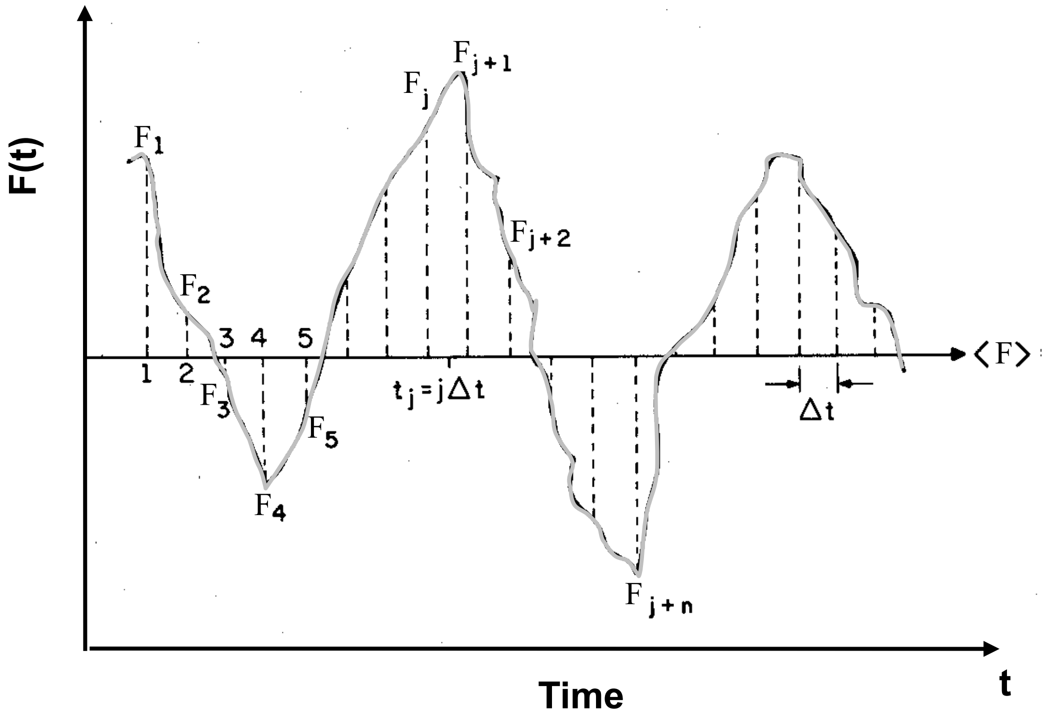
Ergodic: i.e. every sizeable sampling of the process is representative of the whole

the time average is equal to the ensemble average

$$\frac{\langle \delta F(t)\delta F(t+\tau)\rangle}{\langle F(t)\rangle^2} = \frac{\langle \delta F(0)\delta F(\tau)\rangle}{\langle F\rangle^2}$$

Properties of the Autocorrelation Function

The autocorrelation function (ACF) measures the self similarity of the observable F as a function of t



$$G(\tau) = \frac{\langle \delta F(0)\delta F(\tau)\rangle}{\langle F\rangle^2} = \frac{\sum_{i=1}^{\ell} \delta F_i \delta F_{i+\tau} \Big/ \ell}{\left(\sum_{i=1}^{\ell} F_i \Big/ \ell\right)^2}$$

$$(\delta F_i)^2 \geq 0$$

$\delta F_i \delta F_{i+\tau}$ can be < 0

$G(\tau)$ has maximum at $G(0)$

Properties of the Autocorrelation Function

The amplitude is proportional to the size of the fluctuations

$$G(0)=\frac{\langle \delta F(t)\delta F(t)\rangle}{\langle F\rangle^2}=\frac{\langle \delta F(t)^2\rangle}{\langle F\rangle^2}=\frac{\left\langle \left(F(t)-\langle F(t)\rangle\right)^2\right\rangle}{\langle F\rangle^2}=\frac{\sum_{i=1}^{\ell}\left(F_i-\langle F\rangle\right)^2/\ell}{\left(\sum_{i=1}^{\ell}F_i/\ell\right)^2}$$

$$G(0)=\frac{\sigma^2}{\mu^2}=\frac{\langle N\rangle}{\langle N\rangle^2}=\frac{1}{\langle N\rangle}$$

For a Poissonian process

For non-conserved, non-periodic signals

$G(t)\rightarrow 0$ as $t\rightarrow\infty$

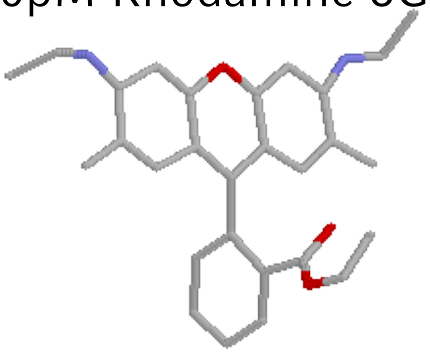
Alternately, the ACF is sometimes given as:

$$g(\tau)=\frac{\langle F(t)F(t+\tau)\rangle}{\langle F(t)\rangle^2}=G(\tau)+1$$

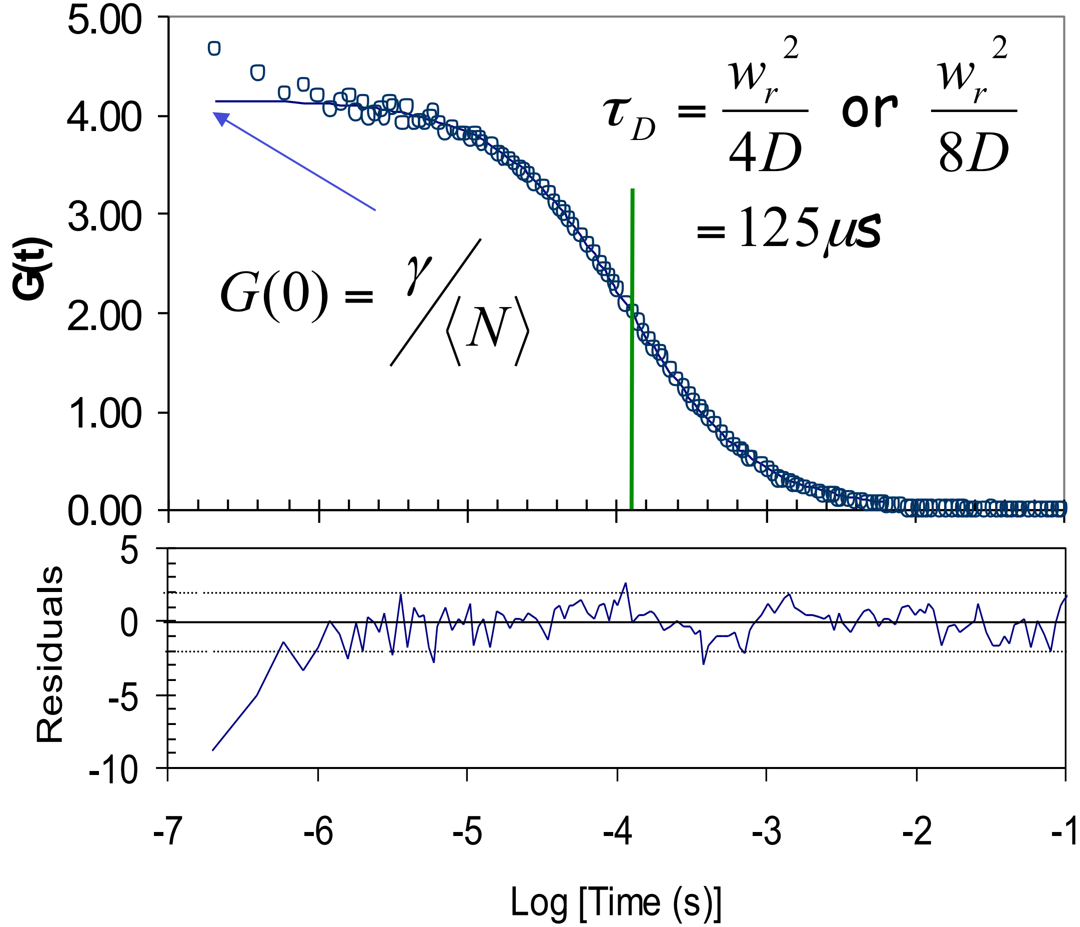
$g(\tau)$ can be interpreted as being proportional to the probability of detecting a photon at delay τ when a photon was detected at $\tau=0$

ACF of Freely Diffusing Molecules

230pM Rhodamine 6G in buffer



$$G_D(\tau, N, \tau_D) = \frac{\gamma}{\langle N \rangle}\left(\frac{1}{1+\tau/\tau_D}\right)\left(\frac{1}{1+\left(w_r/w_z\right)^2 \tau/\tau_D}\right)^{1/2}$$



Amplitude ACF ⇒

Concentration

$\langle N \rangle = 0.085$ molecules

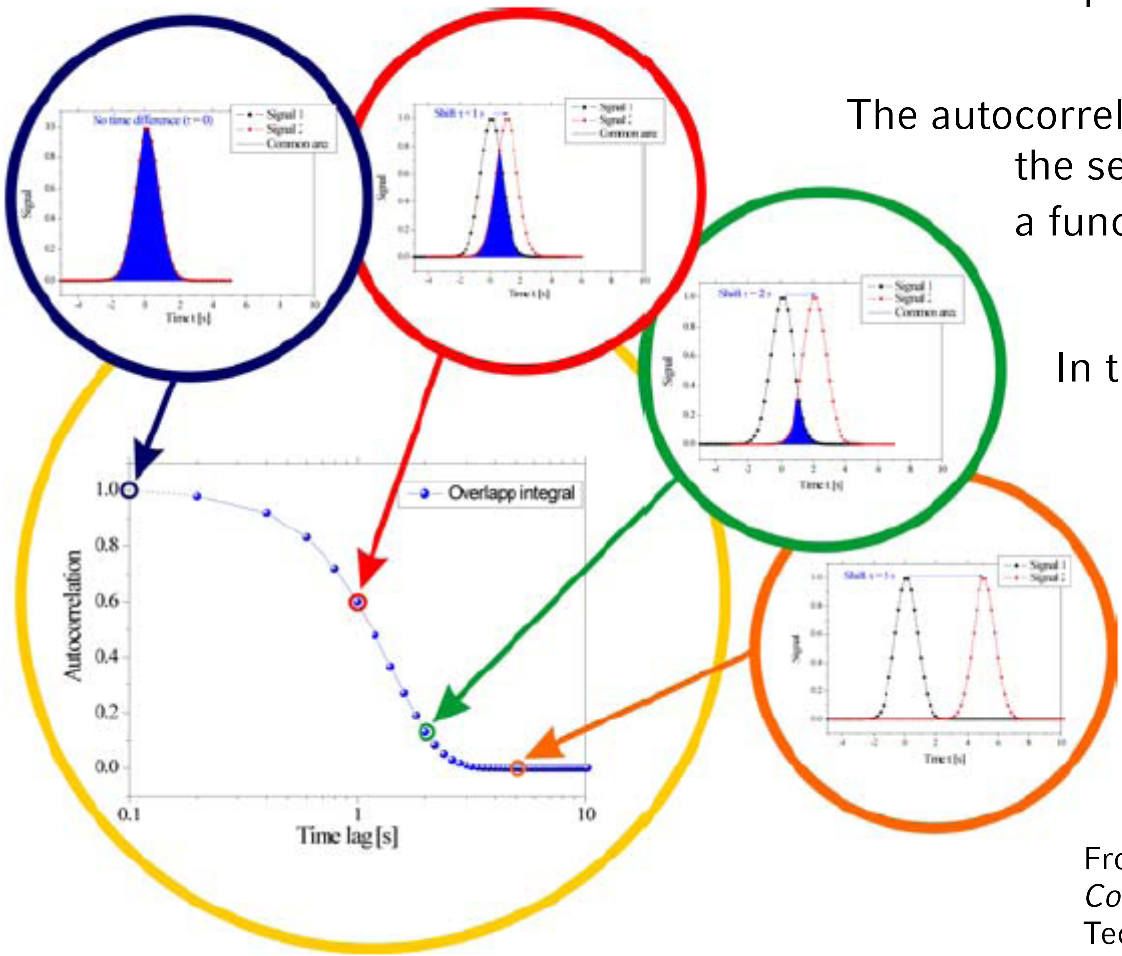
Decay ACF ⇒

Diffusion Constant

$D = 415\ \mu m^2/s\ (w_r = 456\ nm)$

Autocorrelation Function

$$G(\tau) = \frac{\langle F(t)F(t+\tau)\rangle - \langle F(t)\rangle^2}{\langle F(t)\rangle^2}$$



Photon are not detected stochastically, but in bursts when a molecule transverses the probe volume

The autocorrelation function (ACF) measures the self similarity of the time trace as a function of the shift τ

In this case, the ACF measures the averaged duration of a burst of photons

Why Spectroscopy?

Spectroscopy of the Fluctuations

From: Schwille, Haustein, *Fluorescence Correlation Spectroscopy*, In: Single Molecule Techniques, Biophysics Textbook Online

Signal-to-Noise Considerations

High Intensity Limit:

Uncertainty dominated by number of fluctuations:

$$\frac{S}{N} \approx \left(\frac{t_{\exp}}{\tau_C}\right)^{1/2}$$

where $t_{\exp}$ is the measurement time of the experiment and τ_C is the correlation time of the fluctuations

Low Intensity Limit:

Uncertainty dominated by number of photons:

$$\frac{S}{N} \approx \left(t_{\exp}\right)^{1/2} I_T \frac{\gamma}{\langle N \rangle}$$

$$I_T = \varepsilon \langle N \rangle$$

$$\frac{S}{N} \approx \left(t_{\exp}\right)^{1/2} \varepsilon\gamma$$

Only possibilities to improve the S/N ratio are:

- extend the measurement time
- increase the counts per molecule second
- change the geometry

S/N is independent of sample concentration!!!

Limitations

Time Scale: ns/μs → ms/s/hrs

Early time limit:

- Detector afterpulsing: (100 ns - 5 μs)
- Detector deadtime: (2 ns - 30 ns)
- Numbers of available photons: (10 ns - 100 ns)

Long time limit:

- Time molecule remains in the excitation volume (typically ~ 1 ms)

Increase the long time limit by:

- Increasing the excitation volume: (10 ms)
- Placing sample in viscous solvents or gels: (s)
- Slow reactions can be measured by changes in the ACF with time. (hrs)

Concentration Limits: ~ 200nM → 1pM

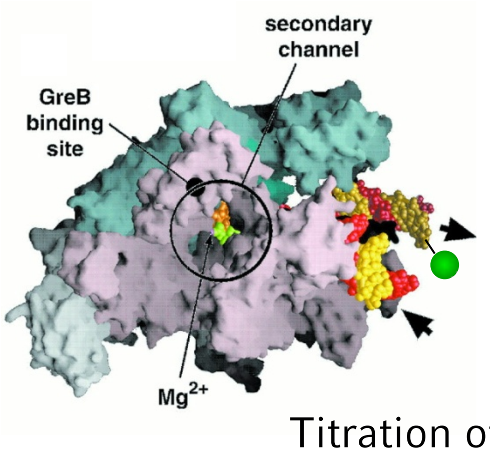
Maximum Concentration: (200nM)

- Detector Saturation
- Other noise sources become comparable to the signal

Minimum Concentration: (1pM)

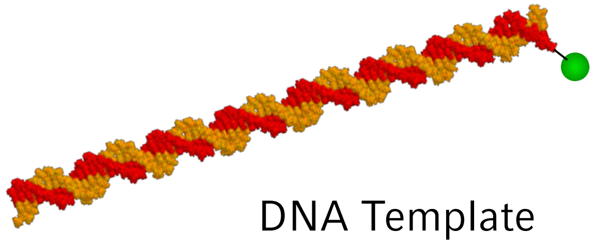
- Limit statistics
- Impurities

Molecular Interactions



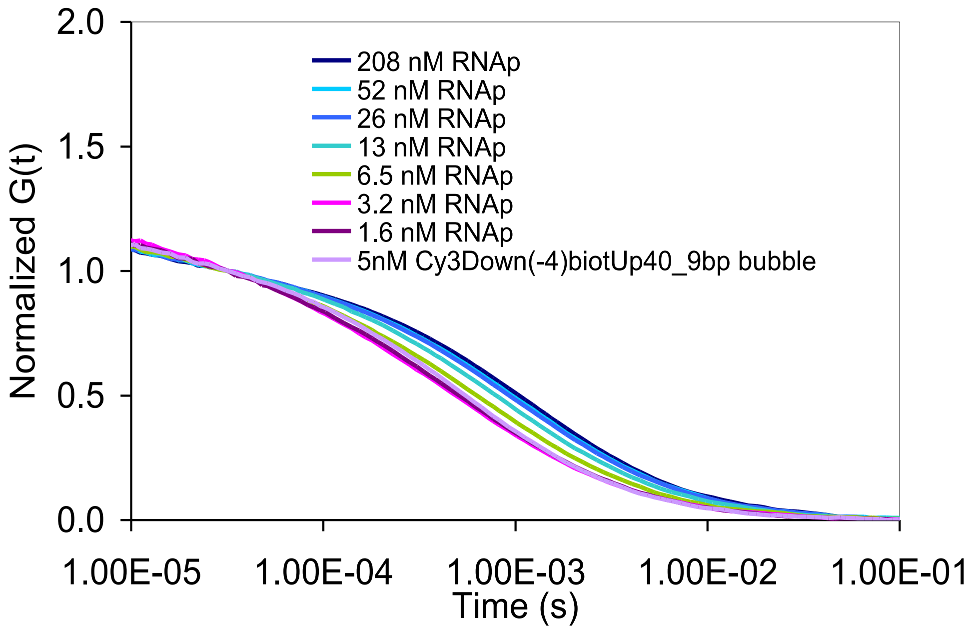
Titrations and slow reactions resulting in a change in the diffusion constant can be followed using FCS

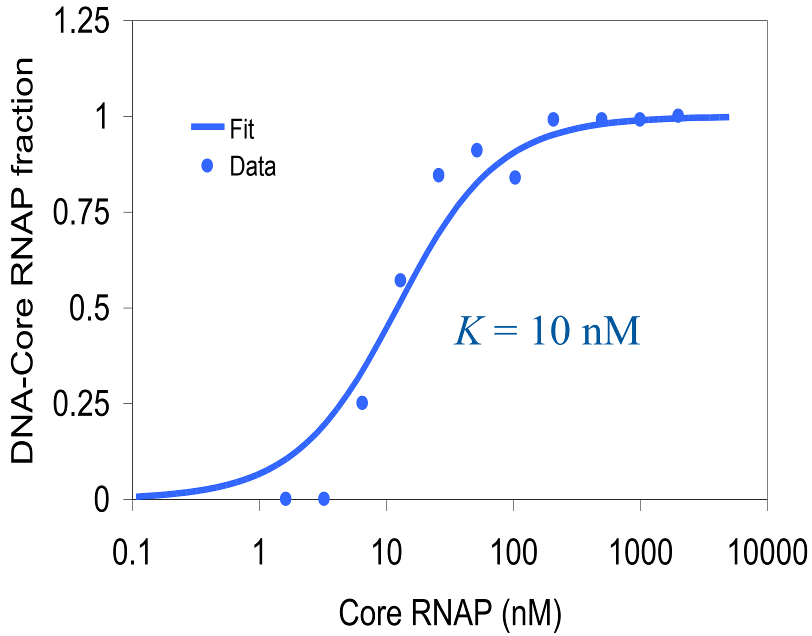
RNA Polymerase



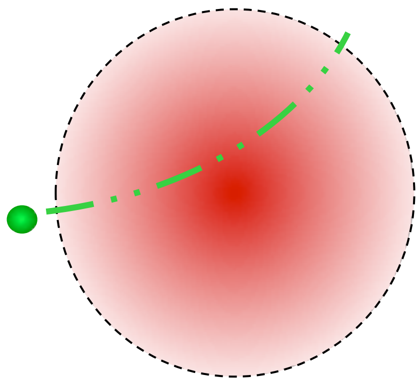
DNA Template

Titration of RNA Polymerase and DNA w/ 9 bp Artificial Bubble





Triplet State Blinking



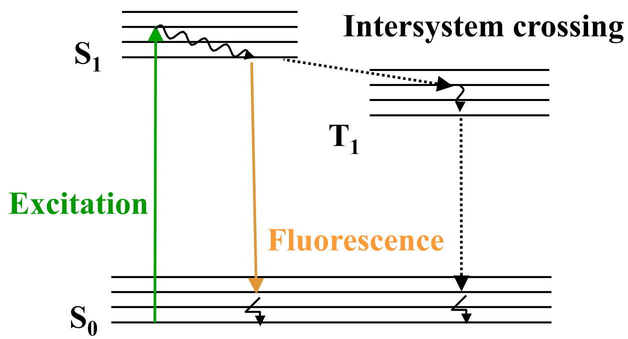
Freely diffusing, non-interacting particles in an open volume.

Photon are not detected stochastically, but in bursts when a molecule transverses the probe volume.

Several processes other than diffusion can lead to fluctuations in fluorescence intensity

e.g. Excitation into the Triplet State

If a particle blinks as it diffuses across the probe volume, an additional term appears in the fluctuation amplitude.

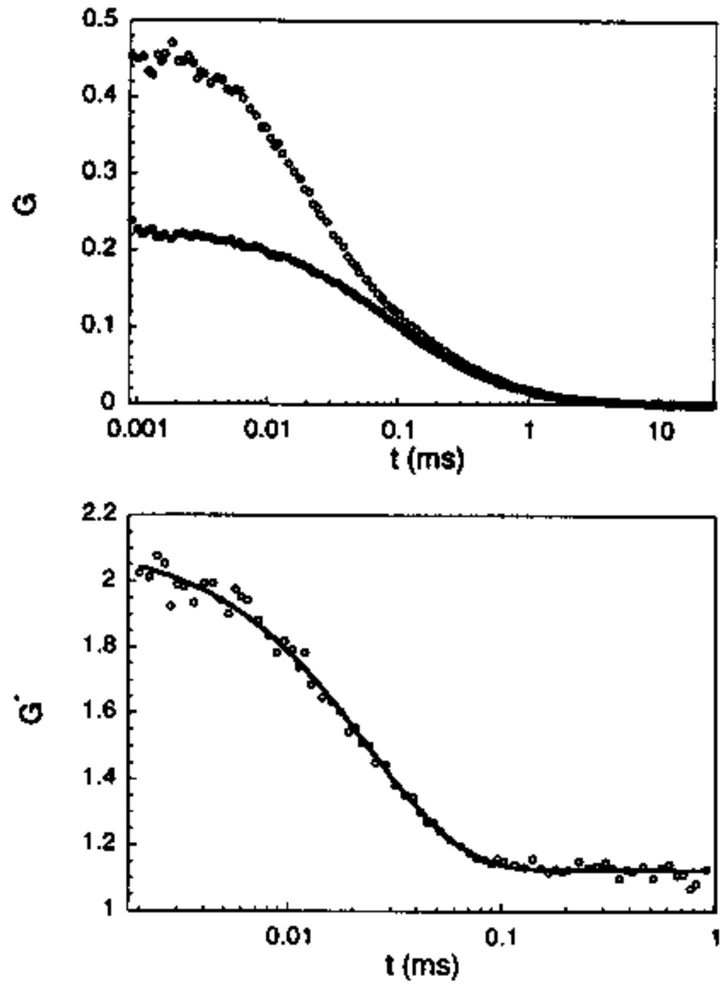


$$G(\tau) = \frac{\gamma}{\langle N \rangle}\left(\frac{1}{1+\tau/\tau_D}\right)\left(\frac{1}{1+\left(w_r/w_z\right)^2 \tau/\tau_D}\right)^{1/2}\left(1+\frac{T}{1-T}e^{\frac{t}{\tau_T}}\right)$$

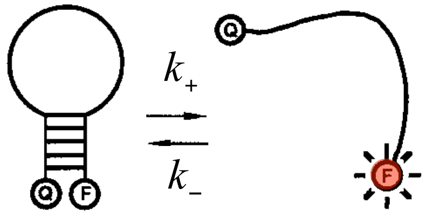
T is the fraction of molecules (on average) in the triplet state

τ_T is the triplet lifetime.

Dynamics of DNA Hairpin Formation



$$A \underset{k_-}{\overset{k_+}{\Leftrightarrow}} B$$



$$G(\tau) = G_D(\tau, N_A + N_B, \tau_D)\left[1 + K\left(\Im_A - \frac{\Im_B}{K}\right)^2 e^{-\lambda\tau}\right]$$

where $K = k_+ / k_-$, $\lambda = k_+ + k_-$, and $\Im$ is the fractional intensity of state A or B

$$G_c(\tau) = G_D(\tau, N_C, \tau_D)$$

Diffusion term drops out of the ratio

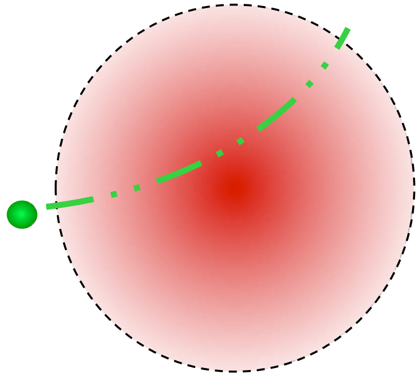
$$\frac{G_b(\tau)}{G_c(\tau)} = \frac{G_b(0)}{G_c(0)}\left(1 + \frac{1}{K}\exp(-\lambda\tau)\right)$$

$$\lambda = k_+ + k_-$$

$$1/\lambda = 24.2\ \mu s$$

Bonnet, Krichevsky, Libchaber *PNAS* (1998) 95:8602

Fluctuation Correlation Spectroscopy (FCS)



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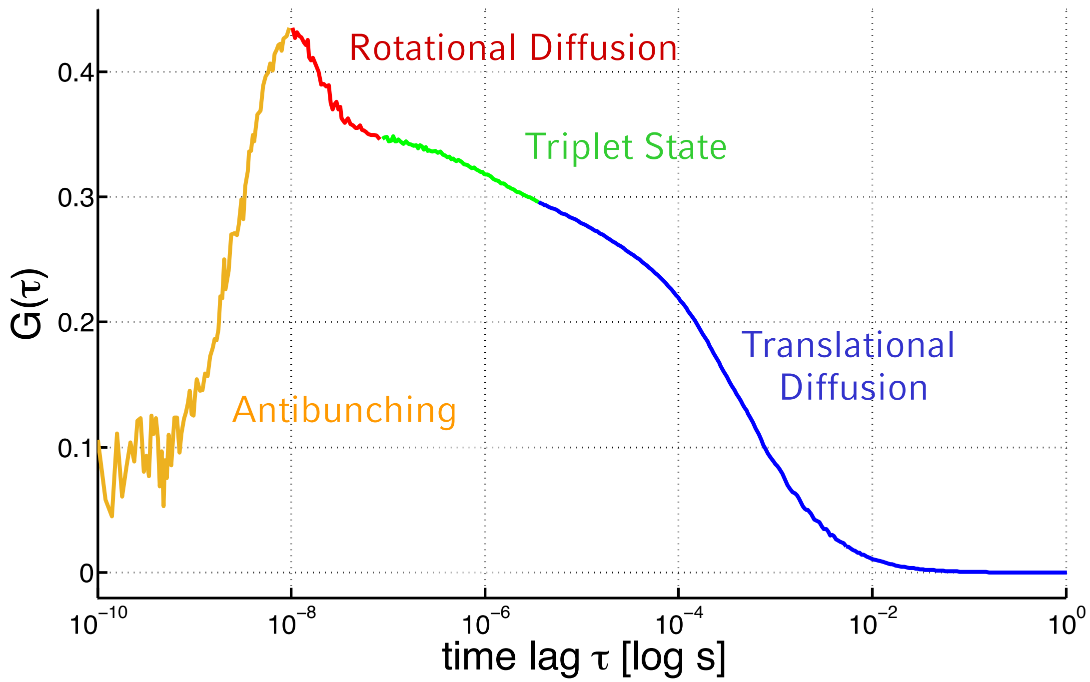


We can determine:

- Excited state lifetime
- Rotational Diffusion Constant
- Reaction Kinetics
- Triplet-State Lifetime
- Triplet-State Amplitude
- Translational Diffusion Constant
- Concentration

ACF with Multiple Species

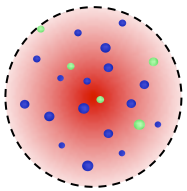
$$G(\tau) = \Im_1^2 \, G_{D1}\left(\tau, N_1, \tau_{D_1}\right) + \Im_2^2 \, G_{D2}\left(\tau, N_2, \tau_{D_2}\right)$$ 2 species

$$\Im_i = \varepsilon_i \langle N_i \rangle \Big/ \left(\varepsilon_1 \langle N_1 \rangle + \varepsilon_2 \langle N_2 \rangle \right)$$ Fractional Intensity

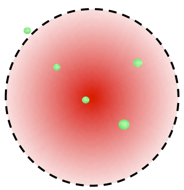
$$G(\tau) = \sum_{i=1}^{M} \Im_i^2 \, G_{Di}\left(\tau, N_i, \tau_{D_i}\right)$$ Multiple species

FCS measurements in a fluorescent background

With Background



Without Background



Situation: Large number of weakly fluorescing particles:

i.e. Although $Q_S >> Q_B$, $N_S << N_B \Rightarrow$

$$G_B(0) = \left(\frac{\gamma}{\langle N_B \rangle} \right) << \left(\frac{\gamma}{\langle N_S \rangle} \right) = G_S(0)$$

$$G(\tau)_{eff} = \Im_S^2 G_{Diff}\left(\tau, N_S, \tau_{D_S}\right) + \Im_B^2 G_{Diff}\left(\tau, N_B, \tau_{D_B}\right)$$

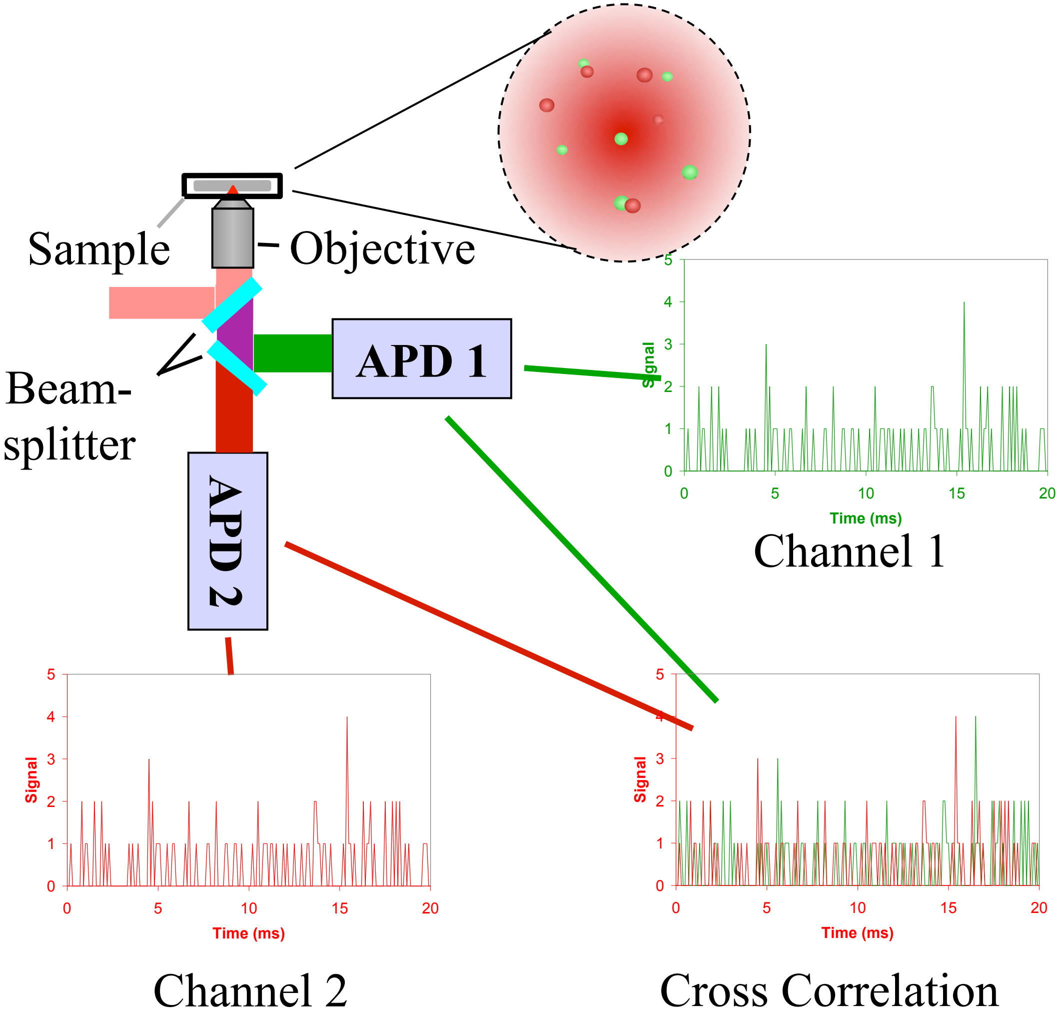
0

$$G(\tau)_{eff} = \Im_S^2 G_{Diff}\left(\tau, N_S, \tau_{D_S}\right)$$

The amplitude of the ACF is reduced by the *square* of the fractional intensity

Fluorescence Cross-Correlation Spectroscopy

Two-Channel Measurements

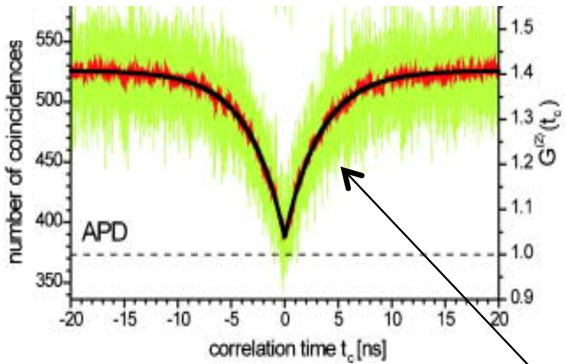


Type of Experiment	Optics and Channels
Fast Correlation	50/50 Beamsplitter
	Ch1: 50% of orignial Signal
	Ch2: 50% of original Signal
Two Color Cross-Correlation	Dichroic Mirror
	Ch1: Green
	Ch2: Red
Rotational Diffusion	Polarizing Beamsplitter
	Ch1: ⊥ Polarized light
	Ch2: || Polarized light

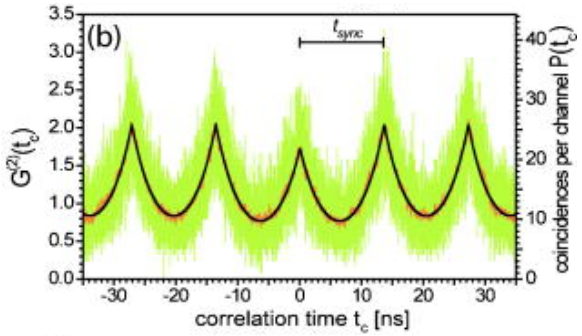
Fast correlation – correlation curves

Short-time correlation curves of Rhodamine 110 aqueous solutions measured with APD detectors:

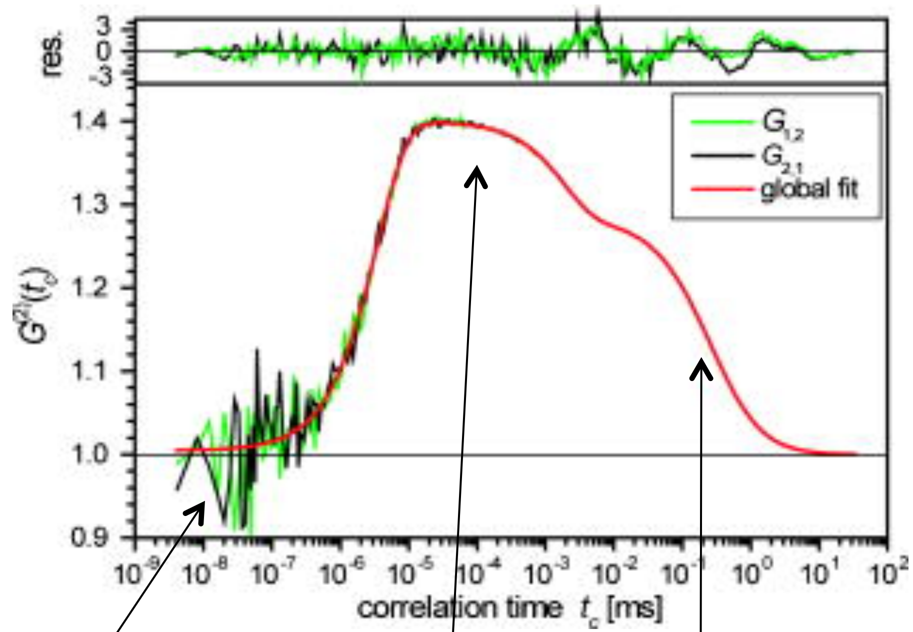
...using cw excitation:



...using pulsed excitation



Full ACF



Antibunching

(No Afterpulsing)

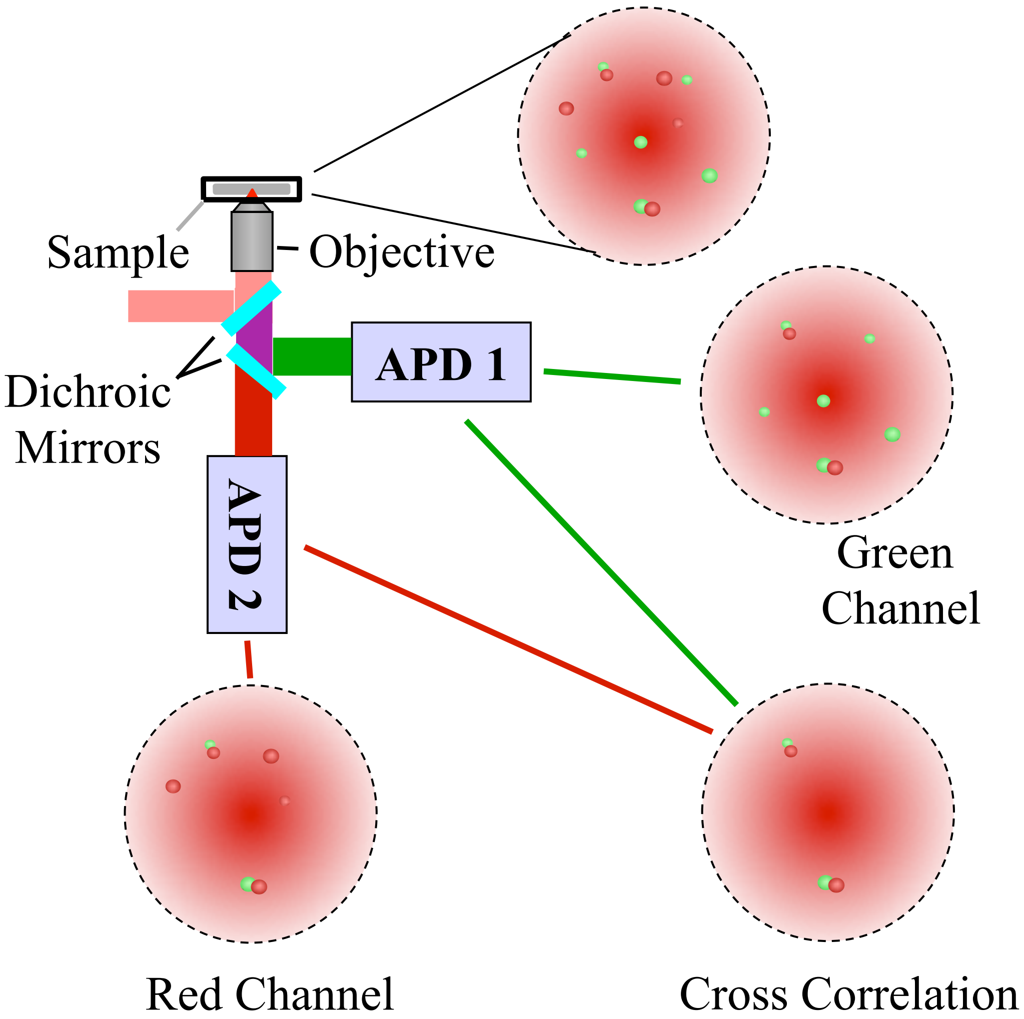
Triplet formation

Lateral diffusion

Rev. Sci. Instrum. 76, 083104 (2005)

Fluorescence Cross-Correlation Spectroscopy

Two-Channel Measurements



The sample consists of three species:

N_G Particles/complexes with a green label only

N_R Particles/complexes with a red label only

N_{GR} Particles/complexes containing both green and red labels

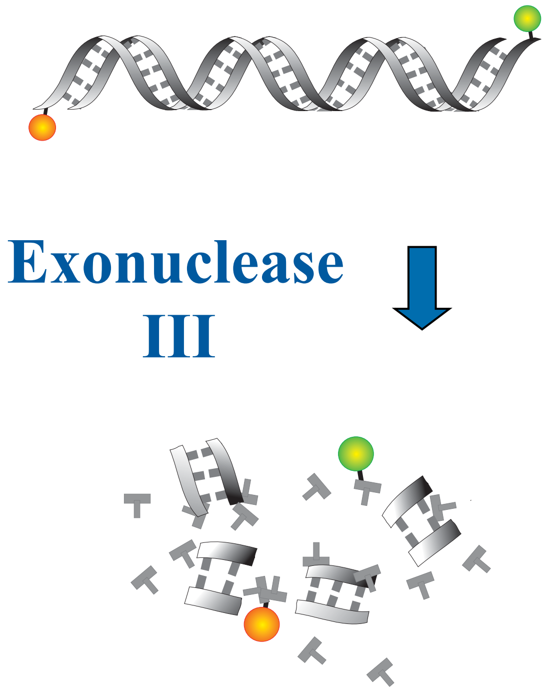
$$G(\tau) = \frac{\langle F_G(t)F_R(t+\tau)\rangle - \langle F_G(t)F_R(t)\rangle^2}{\langle F_G(t)F_R(t)\rangle^2}$$

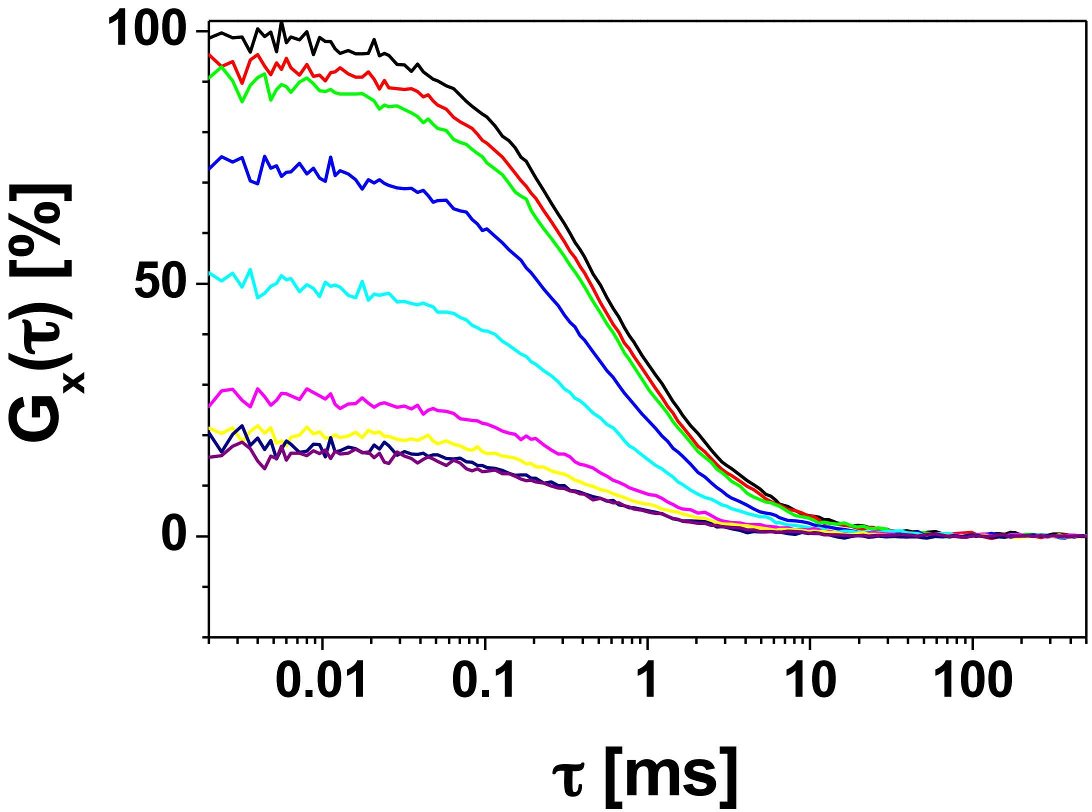
Ideally, only the N_{GR} particles cross correlate

$$G_{GR}(\tau) = \frac{\gamma \langle N_{GR}\rangle G_D(1, D_{GR}, \tau)}{\langle N_G + N_{GR}\rangle\langle N_R + N_{GR}\rangle}$$

Kinetics of DNA Degradation

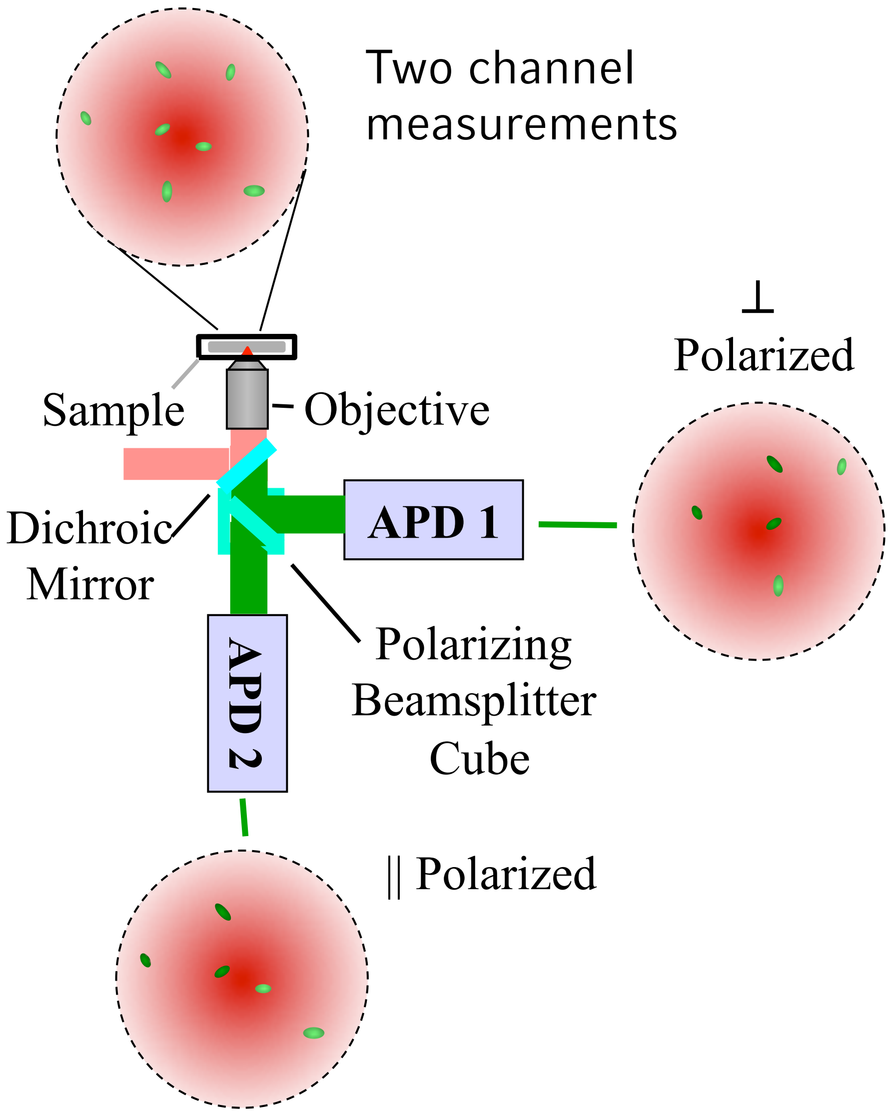
$$G_{GR}(0) = \frac{\gamma\langle N_{GR}\rangle}{\langle N_G + N_{GR}\rangle\langle N_R + N_{GR}\rangle}$$

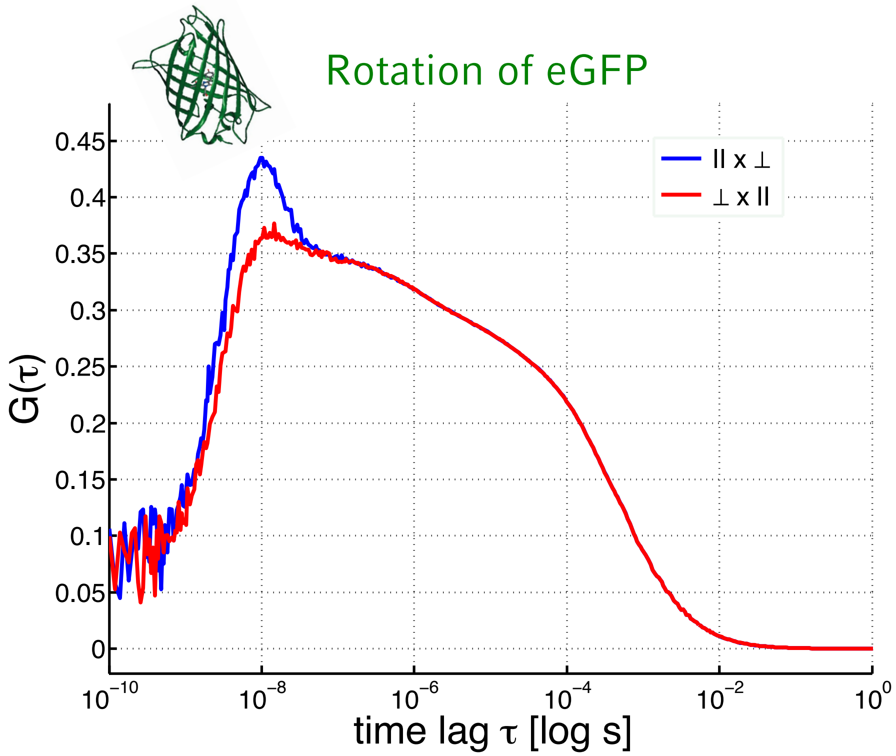


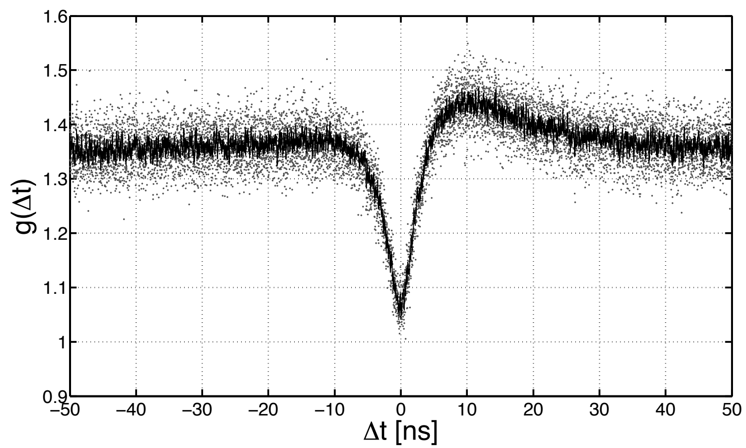


Kettling, Koltermann, Schwille, Eigen *PNAS* (1998) 95:1416

Rotational Diffusion







Fluctuations in Space

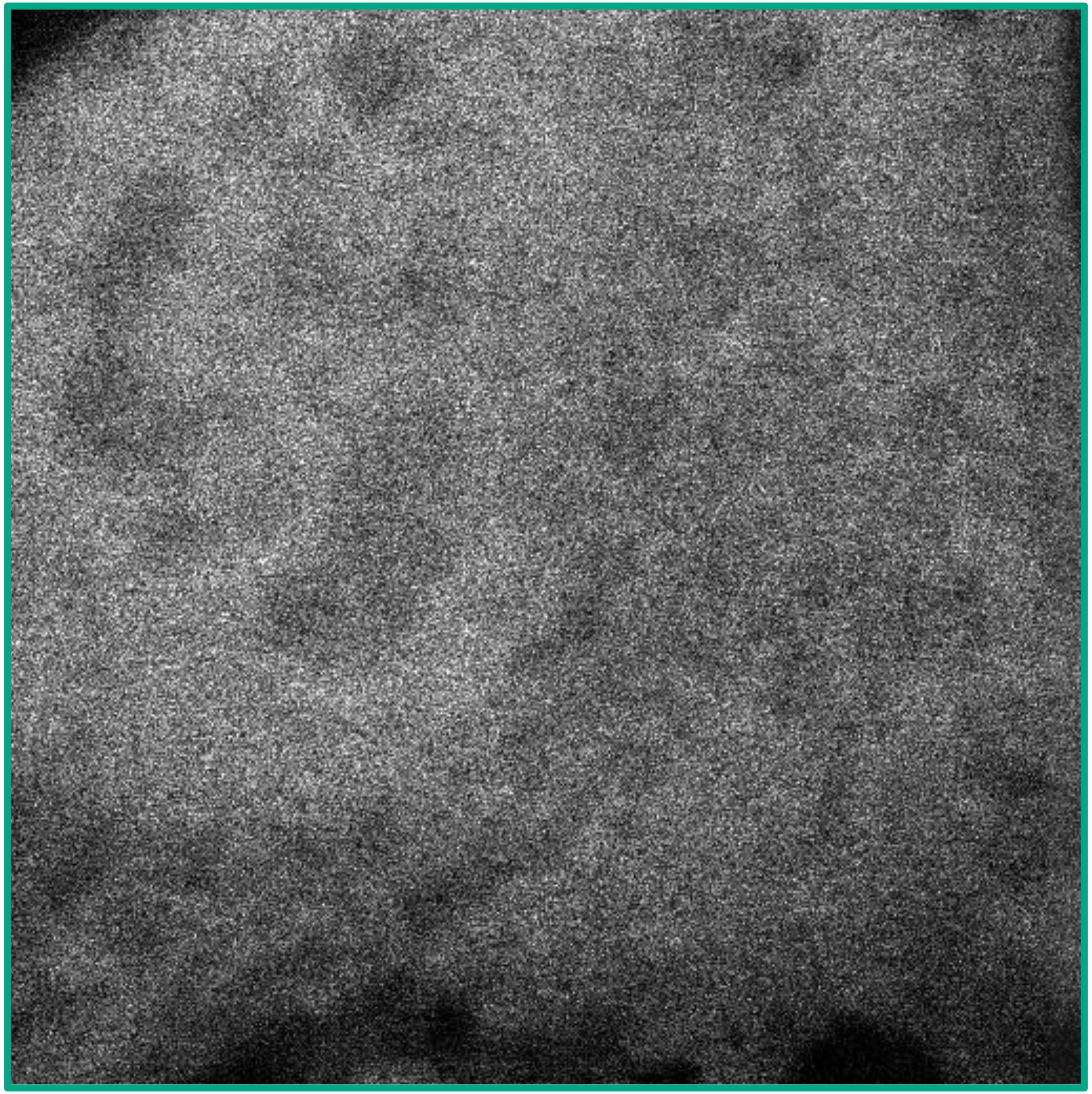
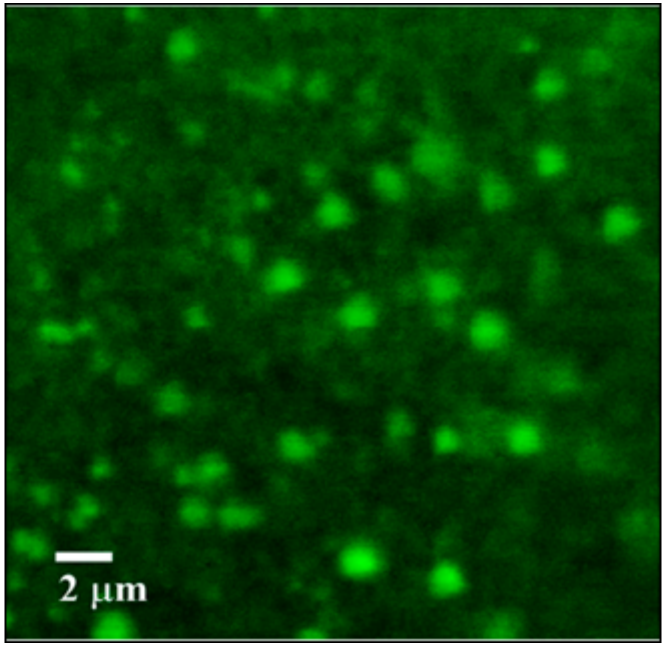


Image as a Record of Spatial Fluctuations



Ergodic: i.e. every sizeable sampling of the process is representative of the whole

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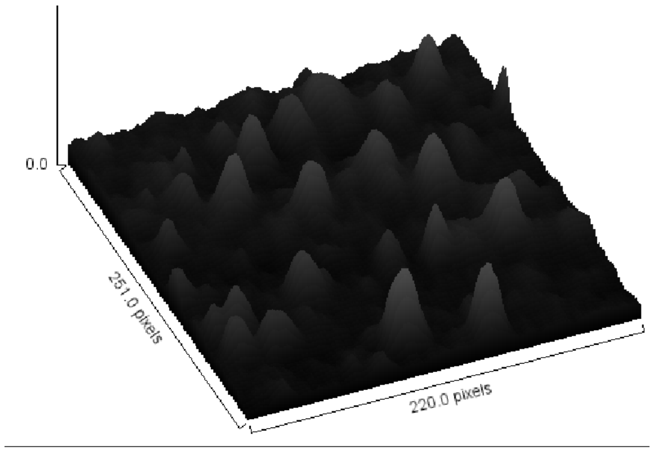
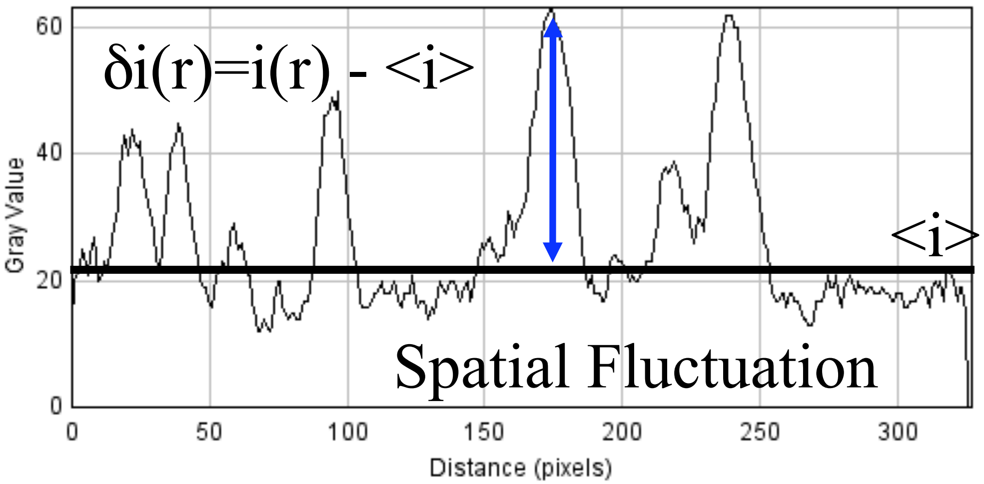
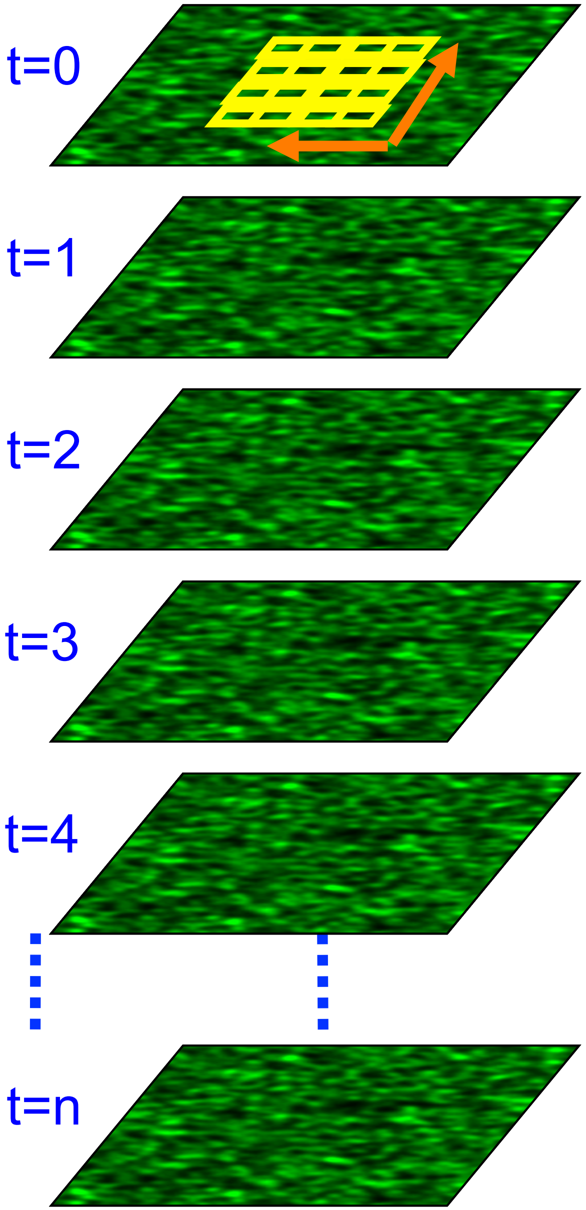




The Image records fluorescence fluctuations in space which reflect the distribution of labeled molecules

Image Correlation Spectroscopy (ICS)

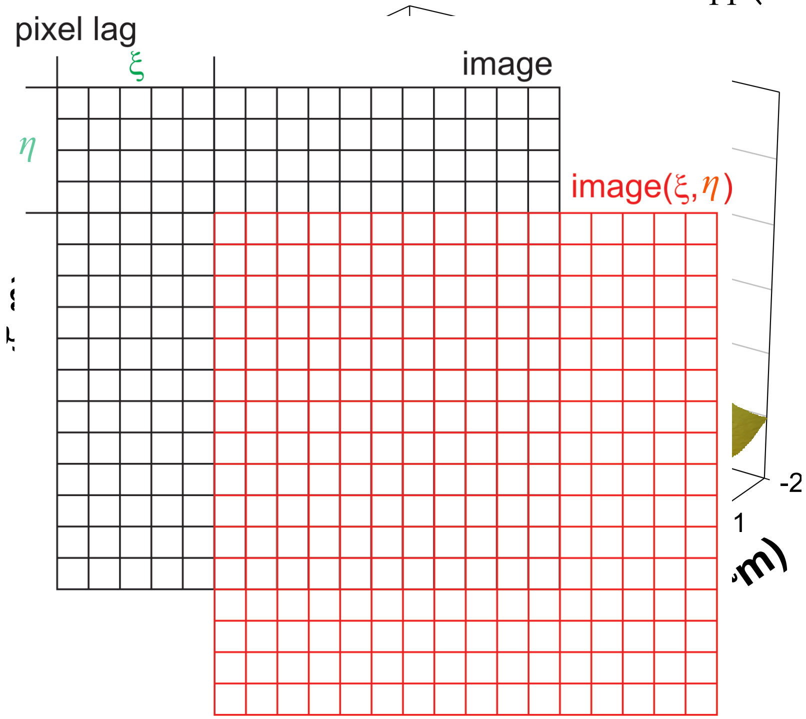


Spatial autocorrelation

of $\delta i(x,y,t) = i(x,y,t) - \langle i \rangle$

for each image in x, y

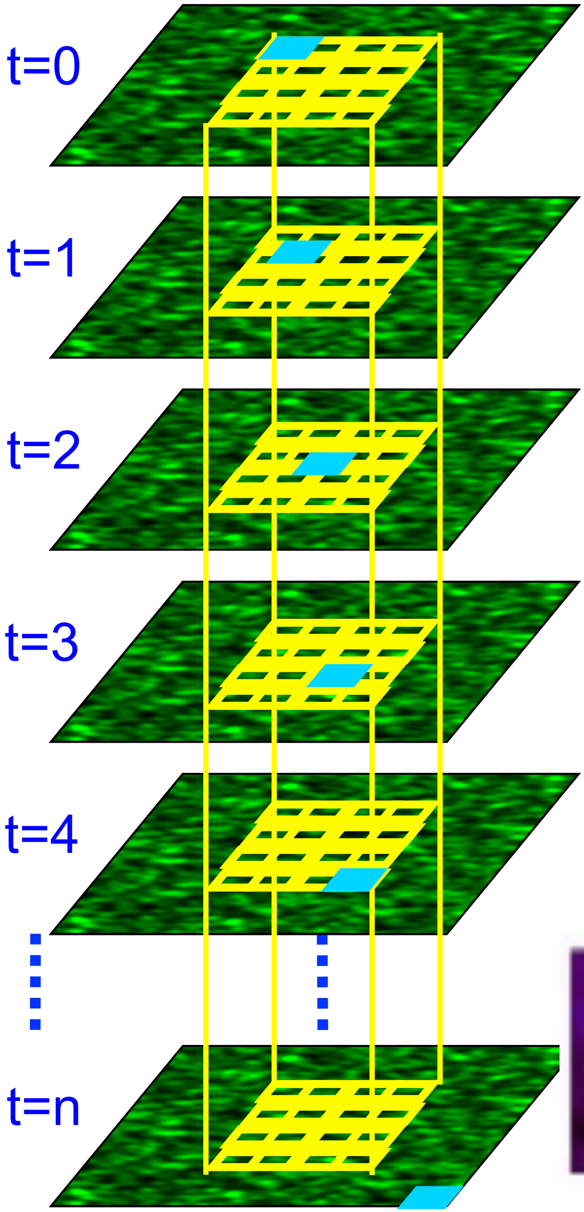
$$r_{11}(0,0)_j = \frac{1}{\langle N \rangle}$$



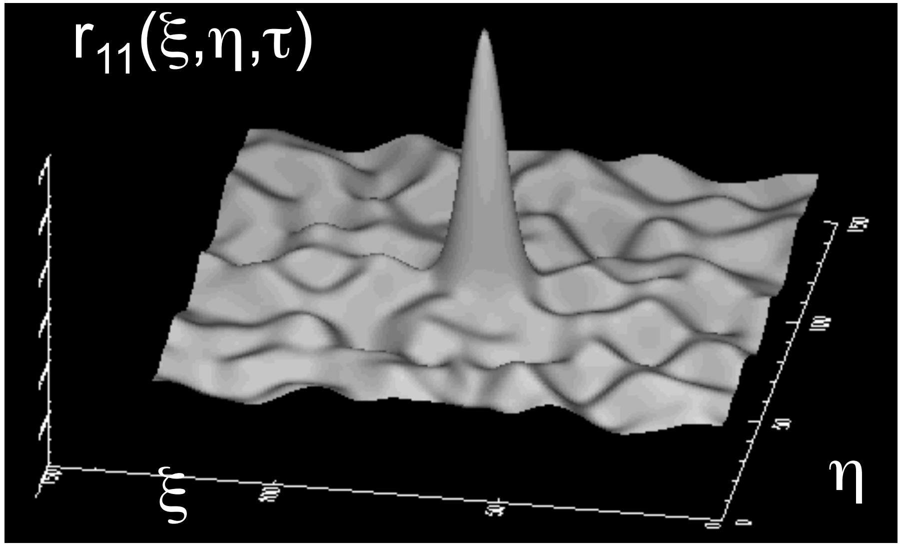
$$r_{11}(\xi,\eta)_j = \frac{\langle \delta i(x+\xi, y+\eta)\,\delta i(x,y) \rangle}{\langle i \rangle^2}$$

Petersen *et al. Biophys. J.* 65, 1135-1146 (1993);
Wiseman and Petersen, *Biophys. J.* 76, 963-977 (1999)

Spatio-Temporal Image Correlation Spectroscopy



Spatial correlation as a function of time



$$r_{11}(\xi,\eta,\tau) = \frac{\langle \delta i(x,y,t)\, \delta i(x+\xi, y+\eta, t+\tau) \rangle}{\langle i \rangle_t \langle i \rangle_{t+\tau}}$$

Hebert et al. Biophys. J. 88-3601 (2005)

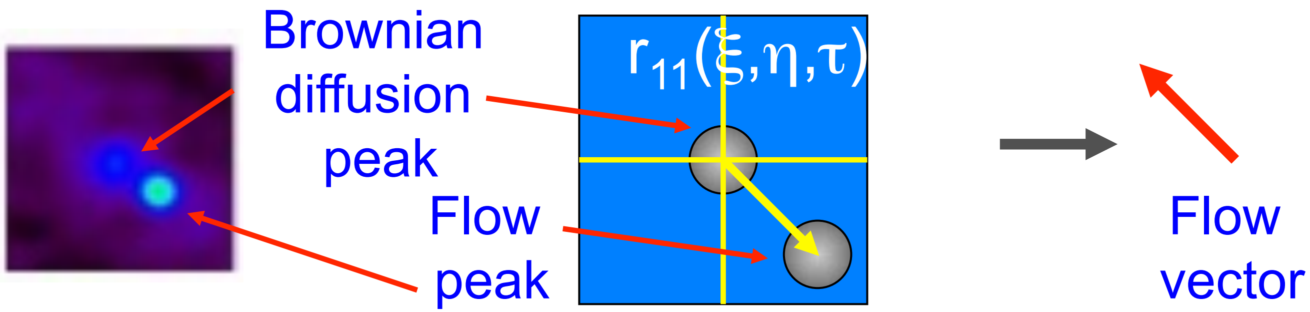
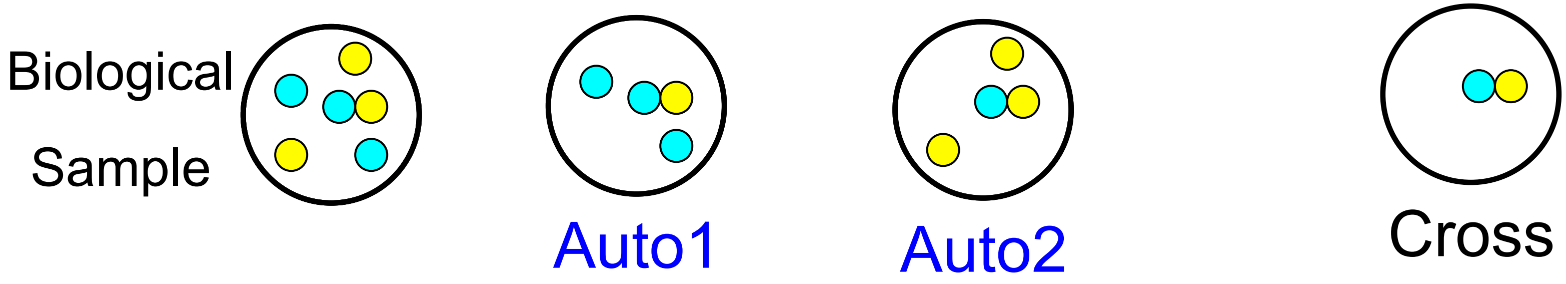
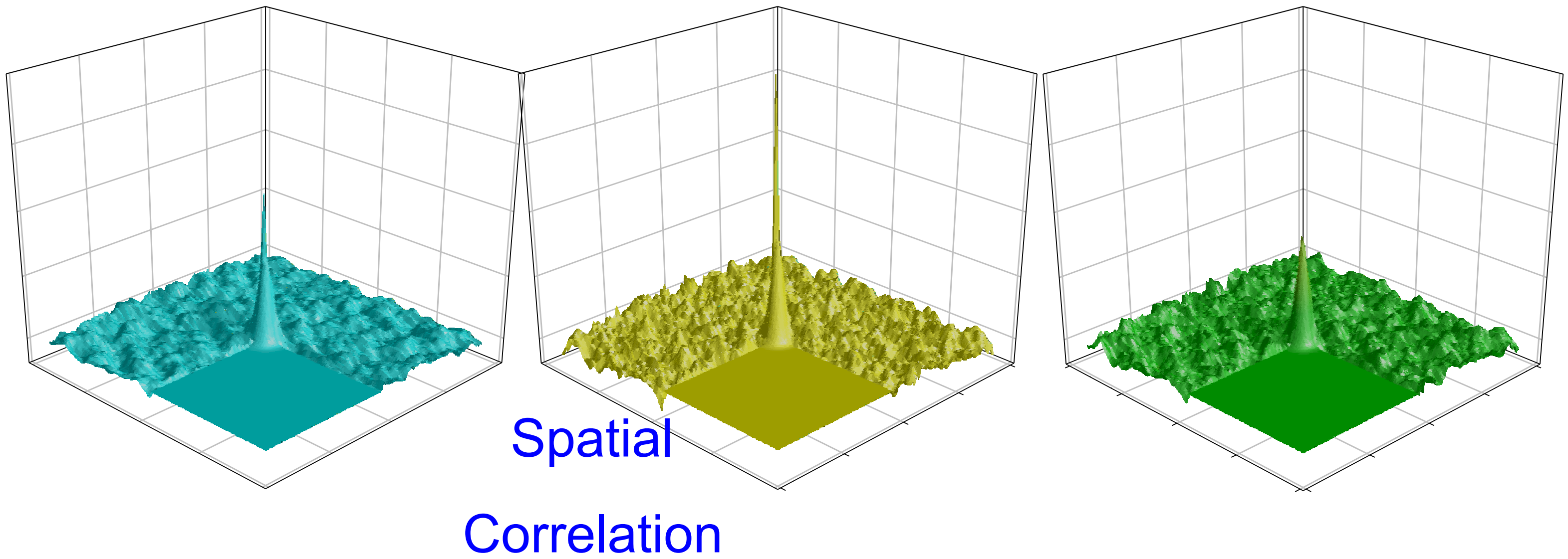


Image Cross-Correlation Spectroscopy (ICCS)

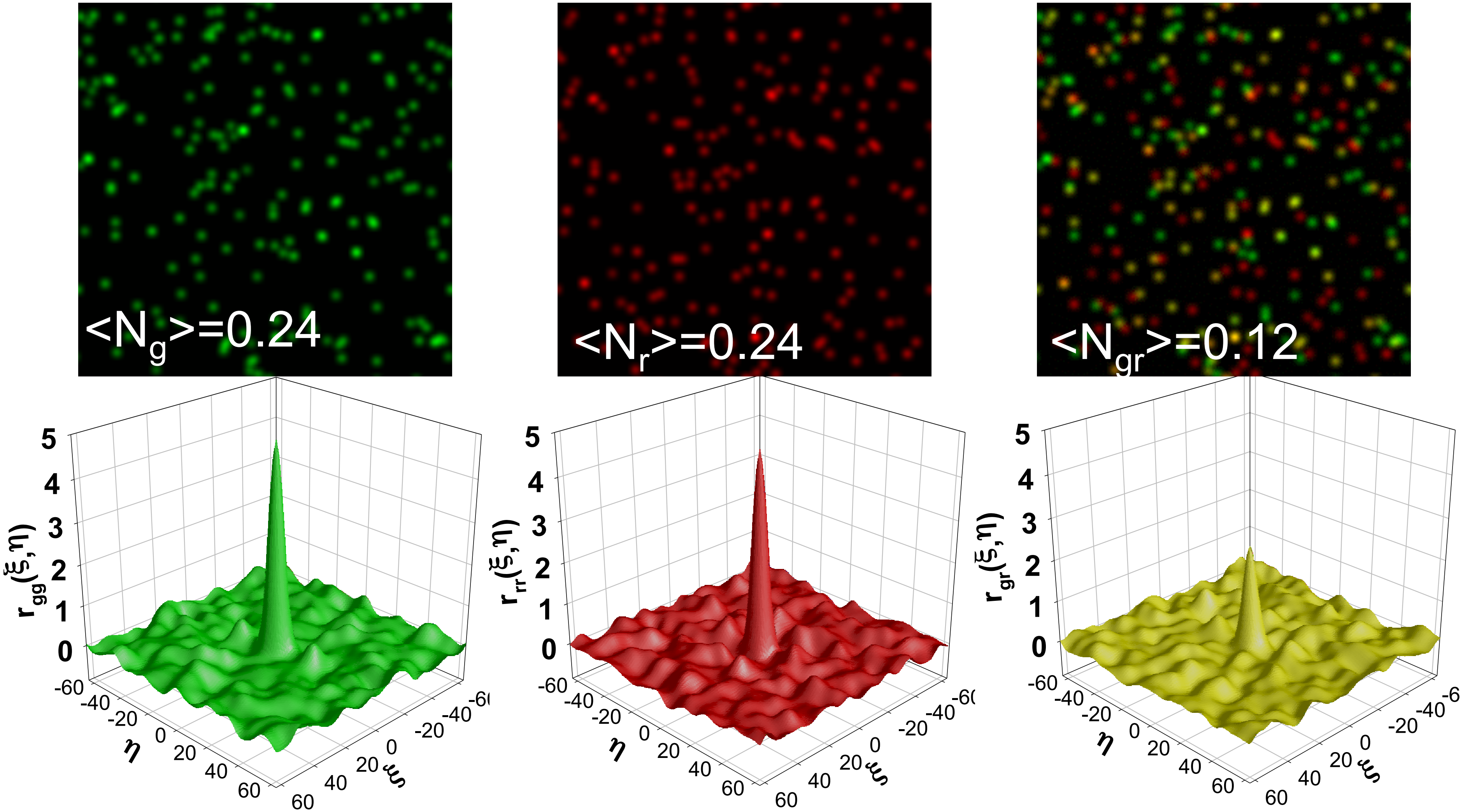
Wiseman et al., *J. Microscopy* 200, 14-25 (2000)





$$r_{ab}(\xi,\ \eta,\ 0)_j = \frac{\langle \delta i_a(x,y,t)\ \delta i_b(x+\xi,\ y+\eta,\ t)\rangle}{\langle i_a\rangle_t\ \langle i_b\rangle_t}$$

Spatial ICCS Simulations



$$\langle N_{gr} \rangle = \frac{g_{gr}(0,0)}{g_g(0,0)\, g_r(0,0)} = 0.10$$

Flavors of Image Correlation Spectroscopy

ICS (Image Correlation Spectroscopy) spatial autocorrelation of an image

Size and number of membrane aggregates (Paul Wiseman & Nils Petersen)

tICS (Temporal Image Correlation Spectroscopy)

time autocorrelation at one pixel (Mamta Srivastava Paul Wiseman, Nils Petersen)

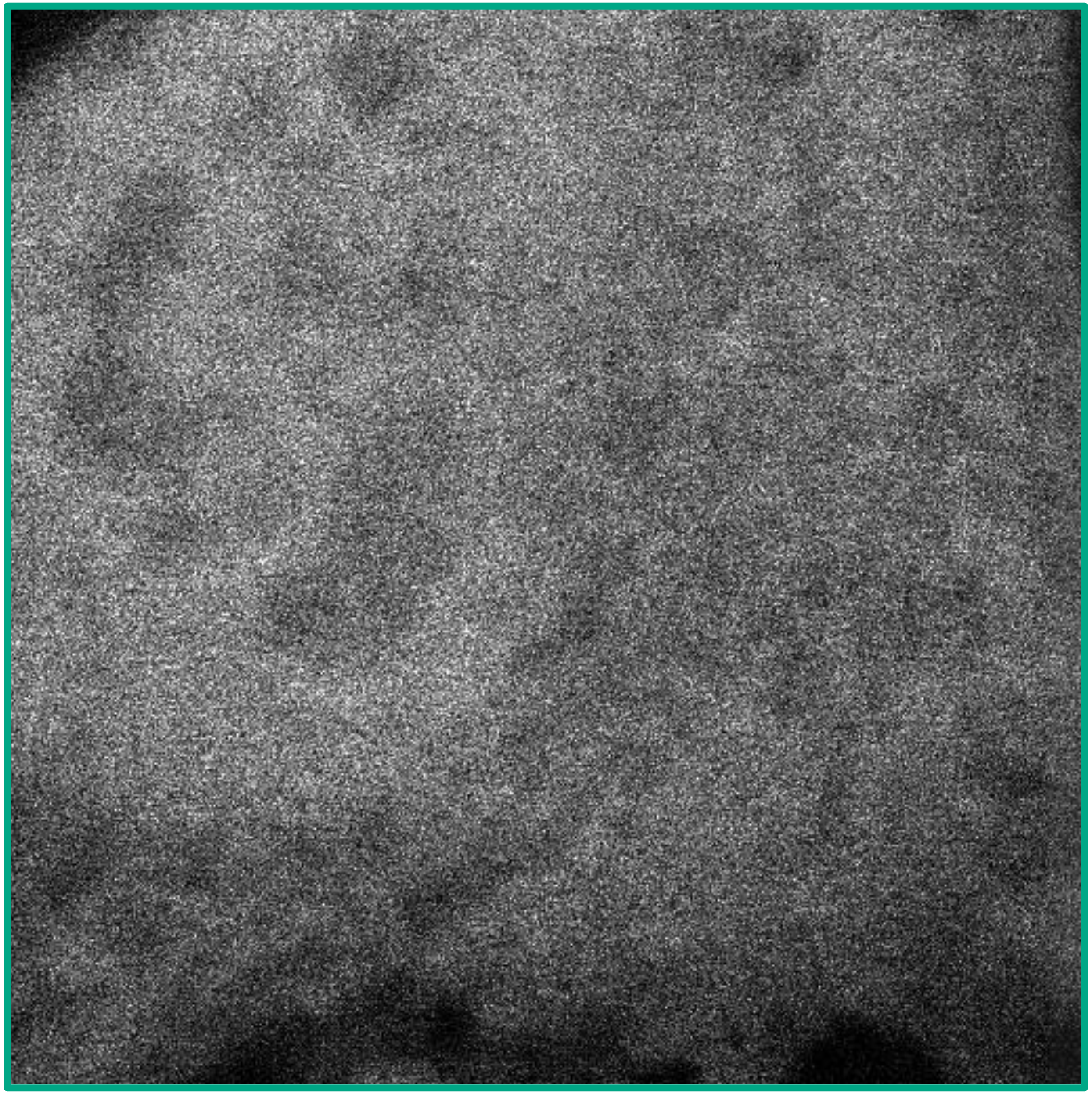
STICS (Spatio-Temporal Image Correlation Spectroscopy)

Diffusion and velocity in 2-D (Ben Herbert & Paul Wiseman)

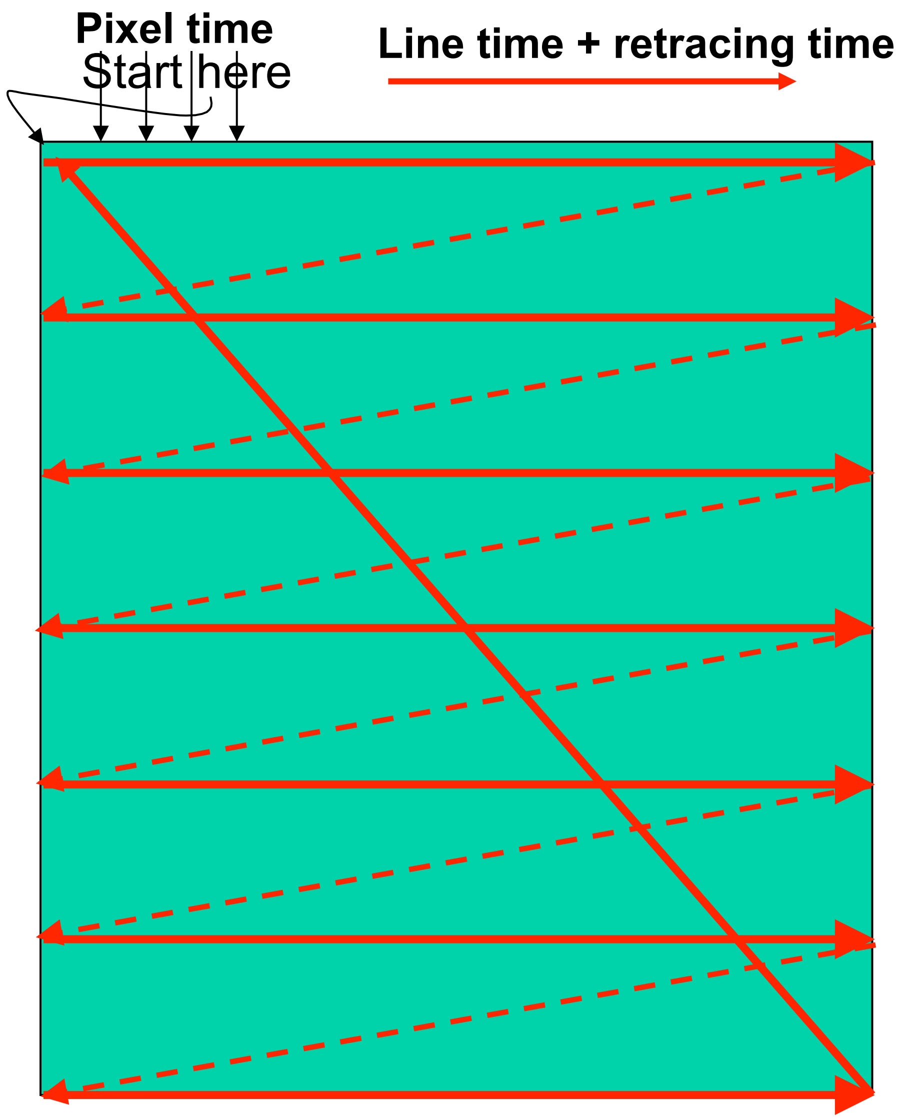
kICS (k-space Image Correlation Spectroscopy)

Distinguish between diffusion and binding on the basis of spatial correlations (Paul Wiseman, David Kolin, David Ronis)

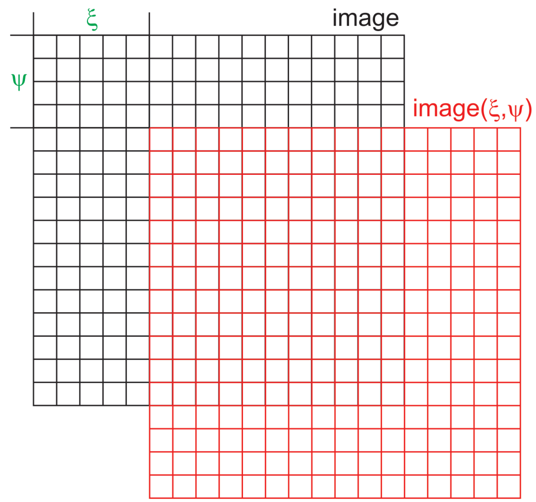
Fluctuations in Time and Space: RICS



Raster Image Correlation Spectroscopy



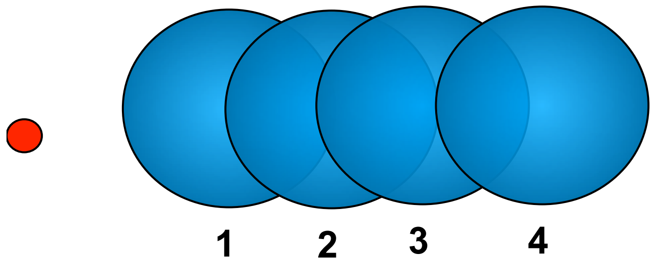
$$G_{ICS}(\xi,\psi) = \frac{\langle I(x,y) I(x+\xi, y+\psi)\rangle_{XY}}{\langle I(x,y)\rangle_{XY}^2}$$



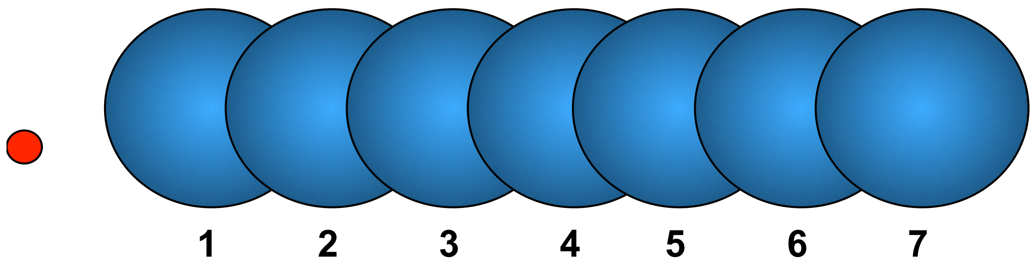
$$G_{RICS}(\xi,\psi) = \frac{\gamma}{N}\left(1 + \frac{4D(\tau_p\xi + \tau_l\psi)}{w_0^2}\right)^{-1} \left(1 + \frac{4D(\tau_p\xi + \tau_l\psi)}{w_z^2}\right)^{-1/2} exp\left(-\frac{\delta r^2(\xi^2+\psi^2)}{w_0^2 + 4D(\tau_p\xi + \tau_l\psi)}\right)$$

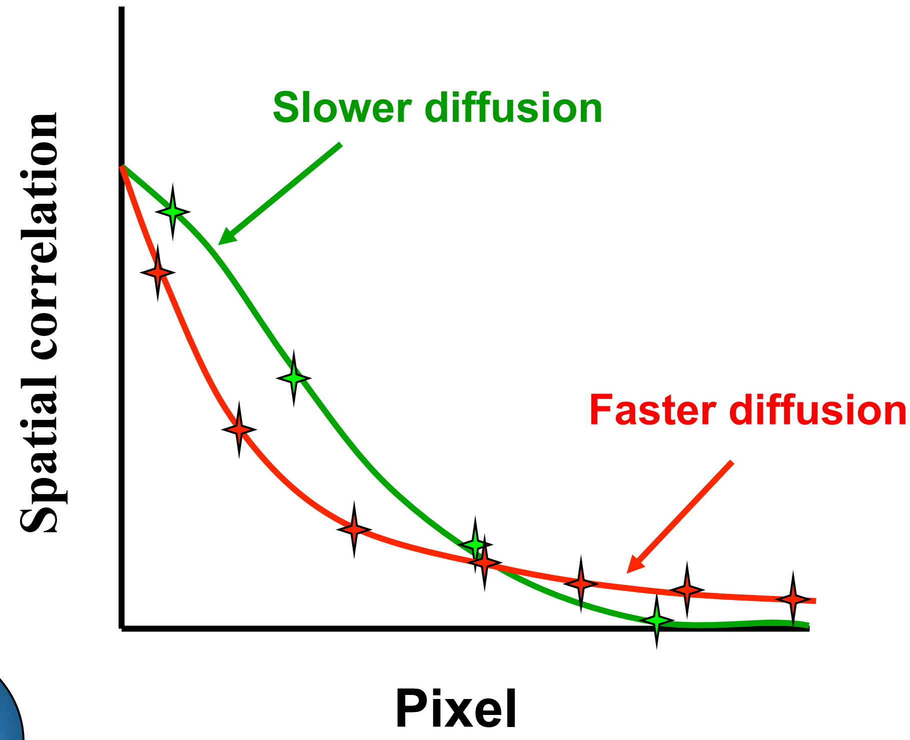
Temporal information hidden in the raster-scan image:

Situation 1:slow diffusion



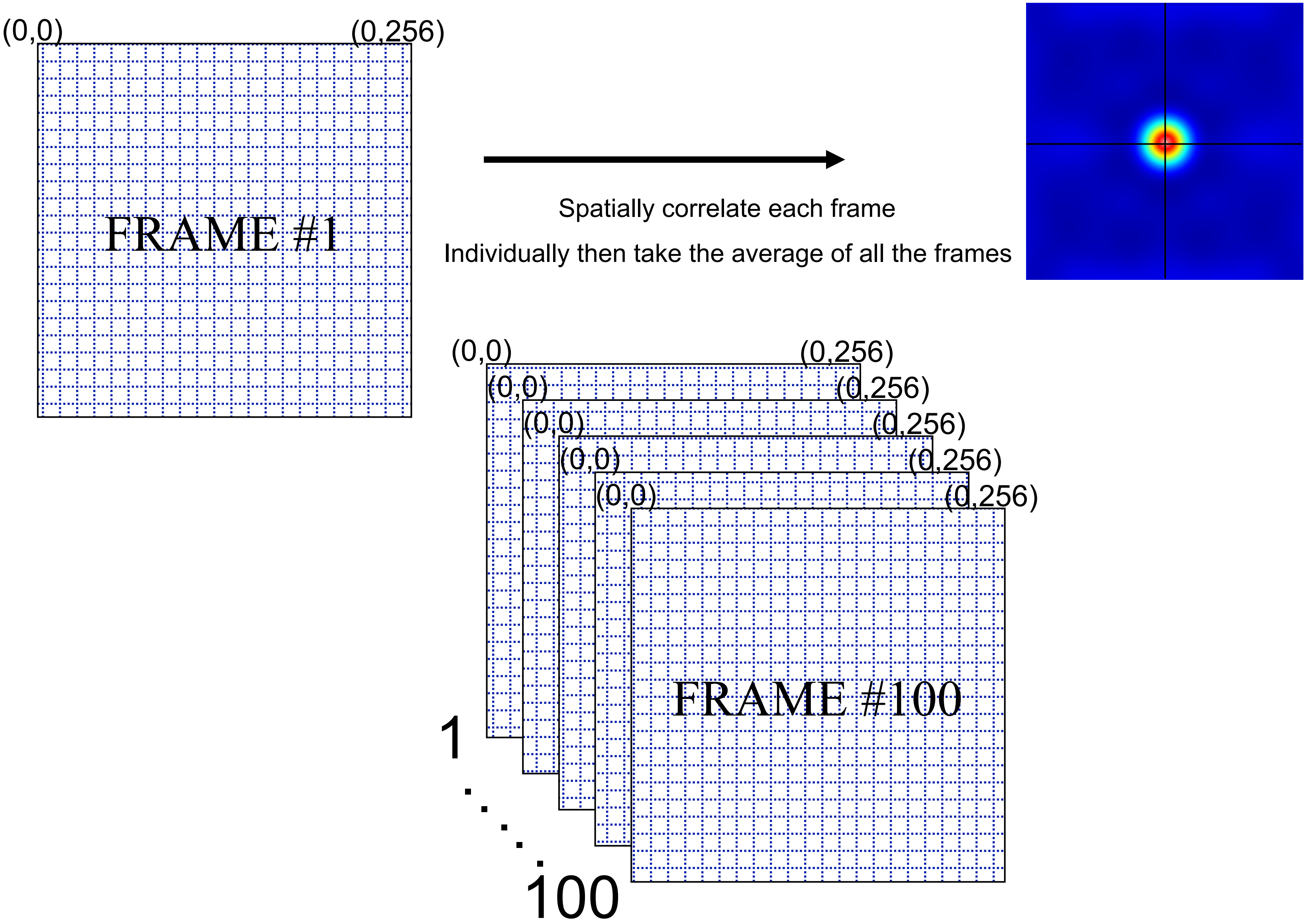
Situation 2: fast diffusion





Averaging in RICS

The image correlation is performed within each frame



RICS Simulations for Different Diffusion Coefficients

D = 0.1 μm²/s

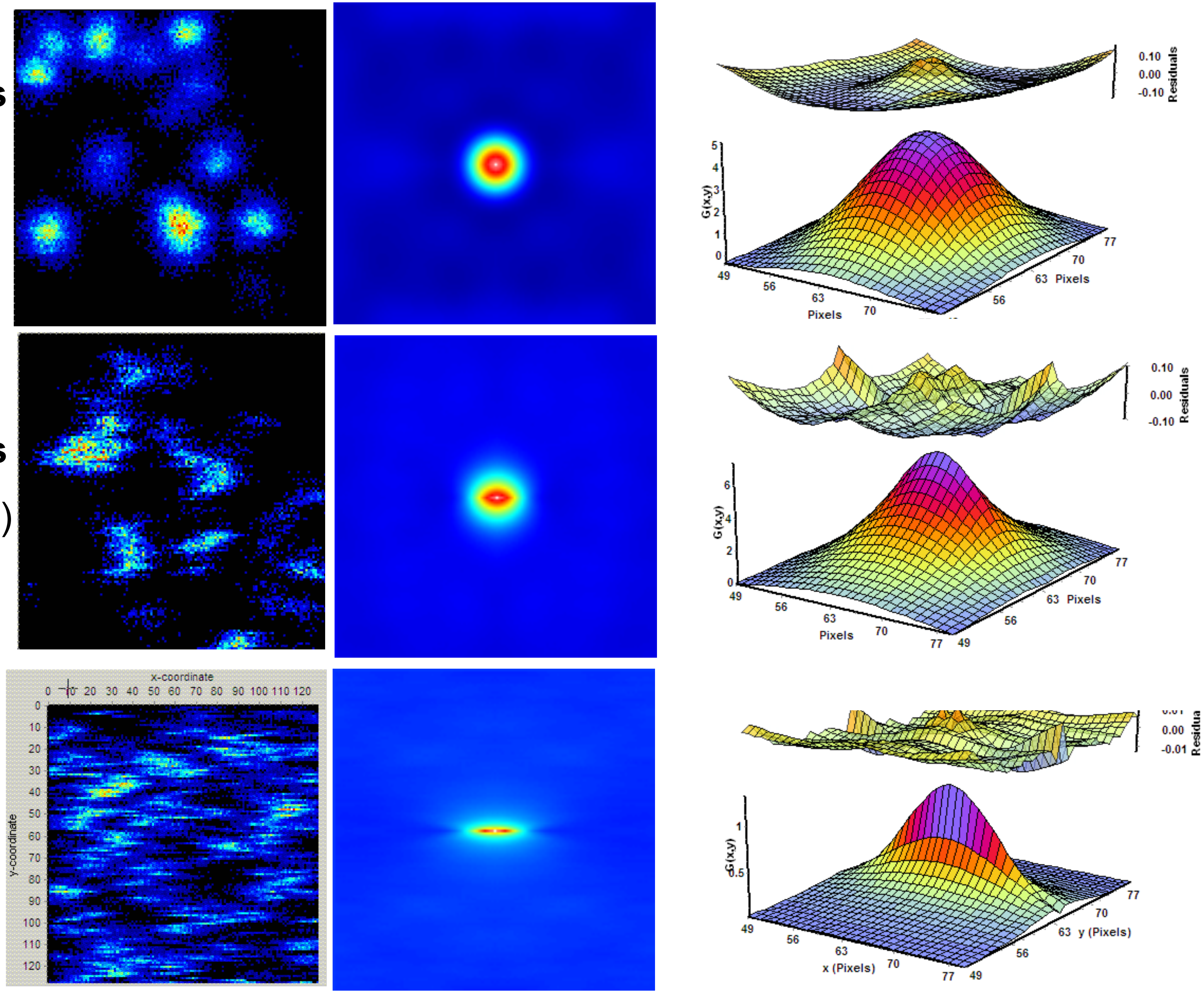
(membrane proteins)

D = 5.0 μm²/s

(40 nm beads)

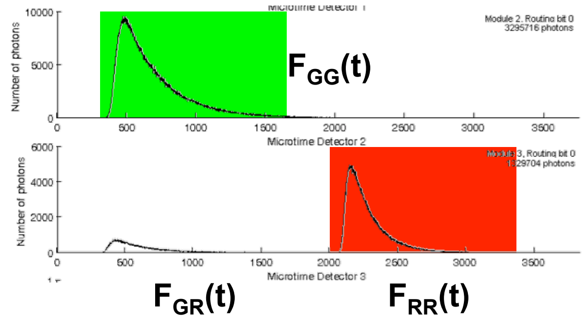
D = 90 μm²/s

(EGFP)



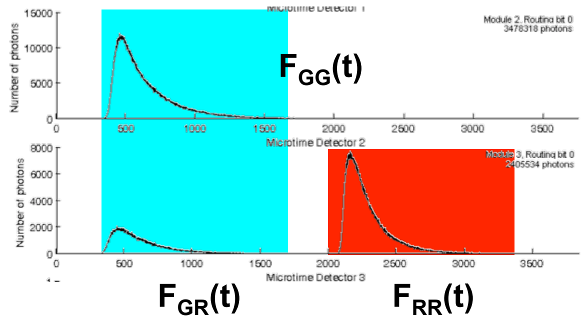
Cross-Correlation-Free RICS

eGFP + mCherry in HeLa cells | **eGFP-mCherry in HeLa cells**

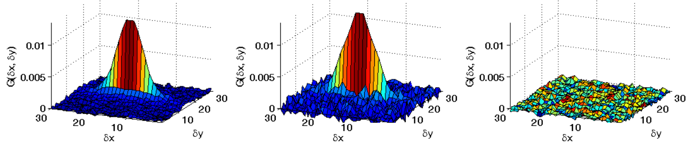


Green APD

Red APD

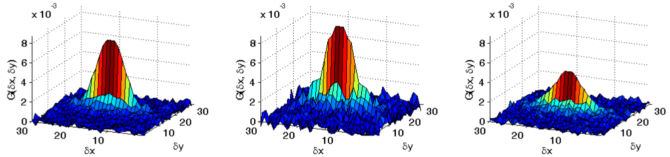


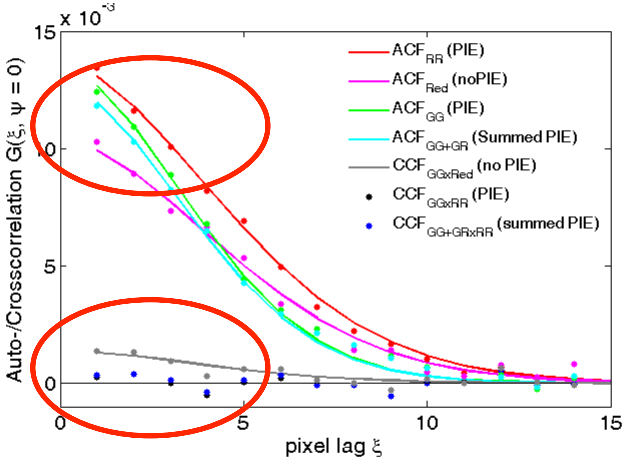
ACF eGFP ACF mCherry CCF



3D RICS

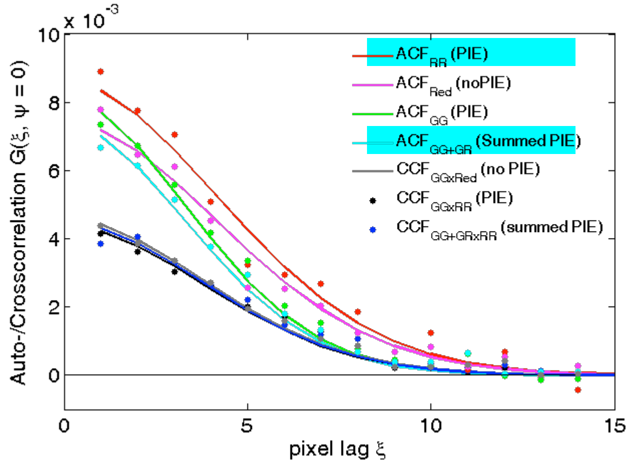
ACF eGFP ACF mCherry CCF



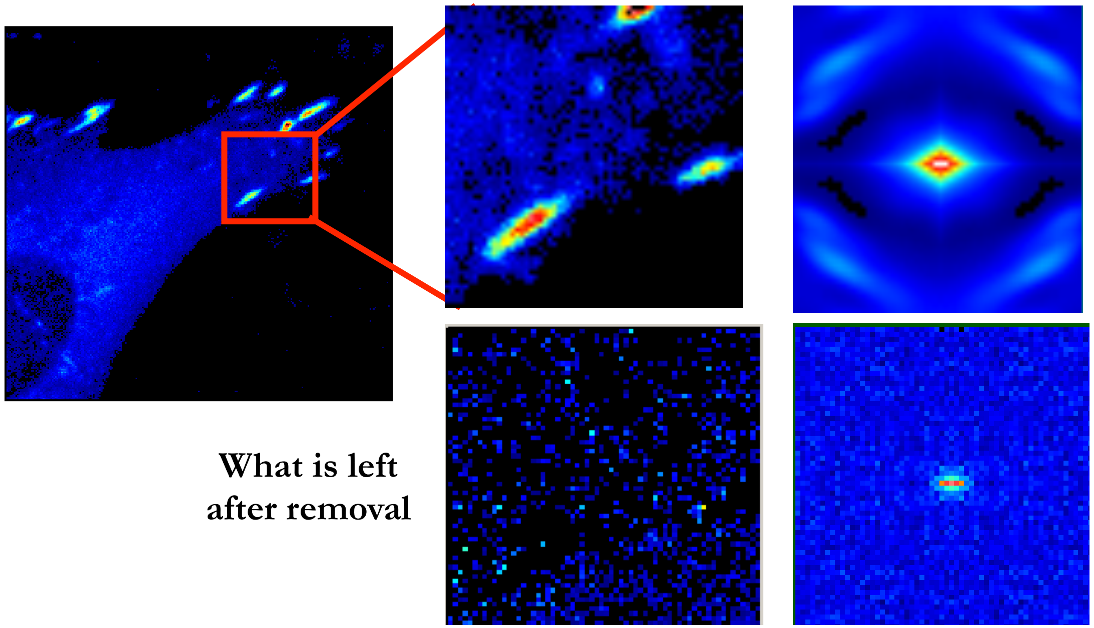


3D RICS

G(ξ,ψ=0)



Removal of Immobile Structures and Slow Moving Features



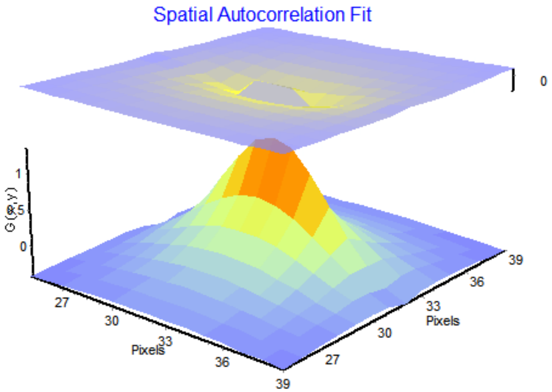
Spatial ACF

No removal

What is left
after removal

Spatial ACF

With removal



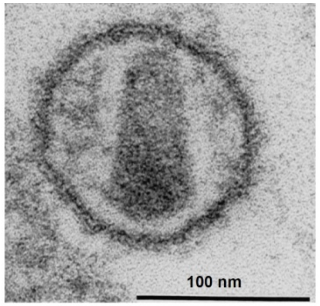
Fit using 3-D diffusion formula

Pixel size =	0.092 μm	G1(0) =	0.0062
Pixel time=	8 μs	D1 =	7.4 $\mu m^2/s$
Line time =	3.152 ms	G2(0) =	0.00023
Wo =	0.35 μm	D2 =	0.54 $\mu m^2/s$
		Bkgd =	-0.00115

HIV: membrane enveloped retrovirus with RNA genome

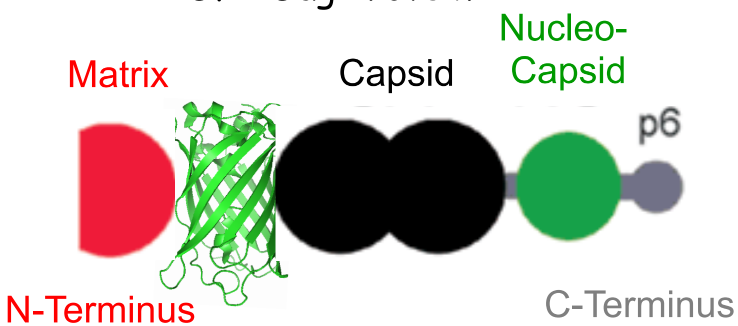
Electron Microscopy Images of HIV

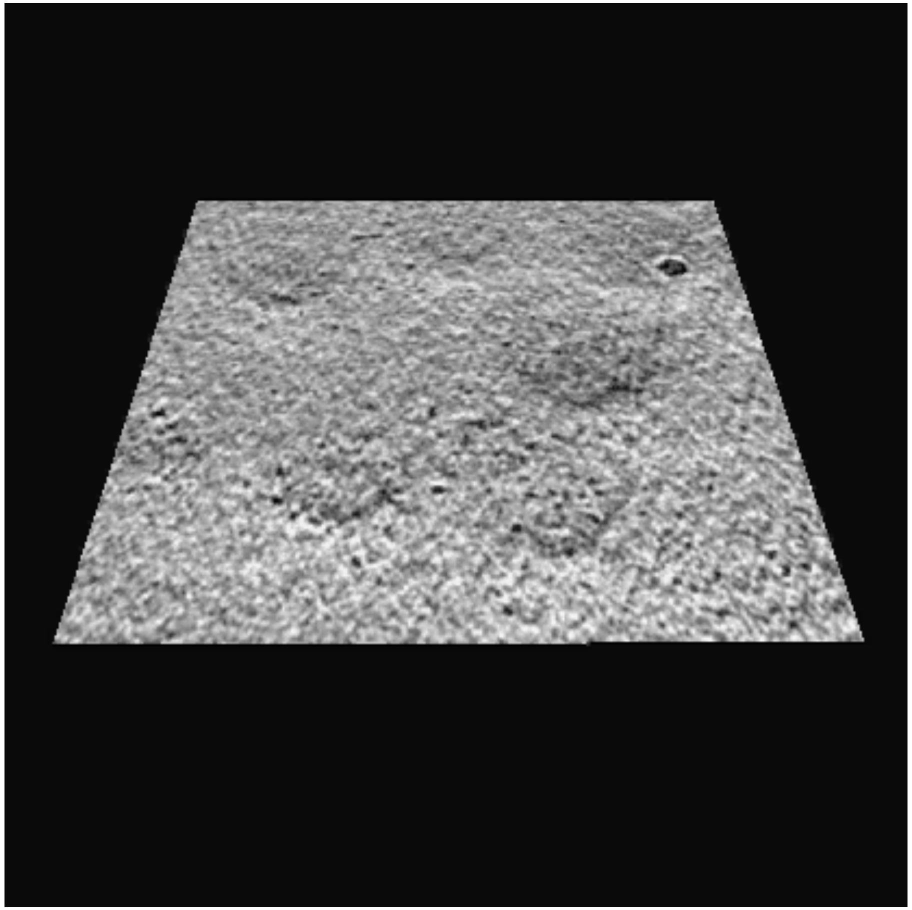
Welker, et al. 2000 J Virol **74**:1168.



Briggs et al. 2004 Nature Struct Mol Biol **11**:672

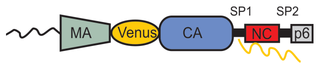
GFP-Gag Protein



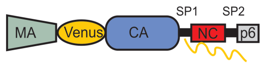


Briggs et al. 2006 Structure 14:15
Supplemental Movie

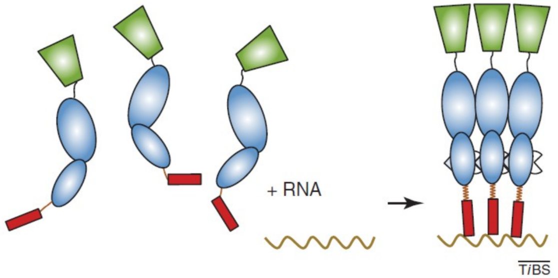
Contributions of Membrane, RNA and CA-CA Interactions



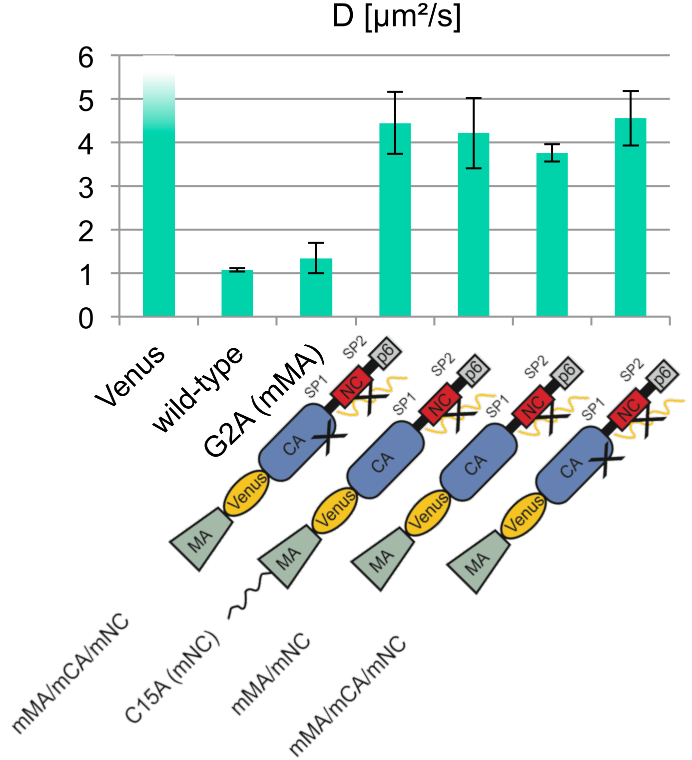
- $PI(4,5)P_2$ induces conformational switch in Gag, Myristate gets exposed and binds PM Ono et al.(2004), PNAS **101**, 14889-14894
- Matrix domain G2A mutant Göttlinger et al. (1989), PNAS **86**, 5781-5785
  - Defective for assembly and particle release
  - Interacts much less with membranes!



- In vitro: Gag assembles into VLPs upon addition of nucleic acid Campbell et al.(1995), J Virology **69**, 6487-6497
  - RNA primes Gag for interactions ?



from Rein et al. (2011), TIBS, 1-8



- Targeting Gag away from RNA or Gag
  - NC C15A inhibits Gag-RNA interaction Poon et al. (1996), J Virology **70**, 6607-6616
  - CA W184A/M185A inhibits CA-CA von Schwedler et al. (2003), J Virology **77**, 5439-5450