Fluorescence Fluctuation Spectroscopy

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7h Annual Course of Principles of Fluorescence Techniques
Genova, Italy
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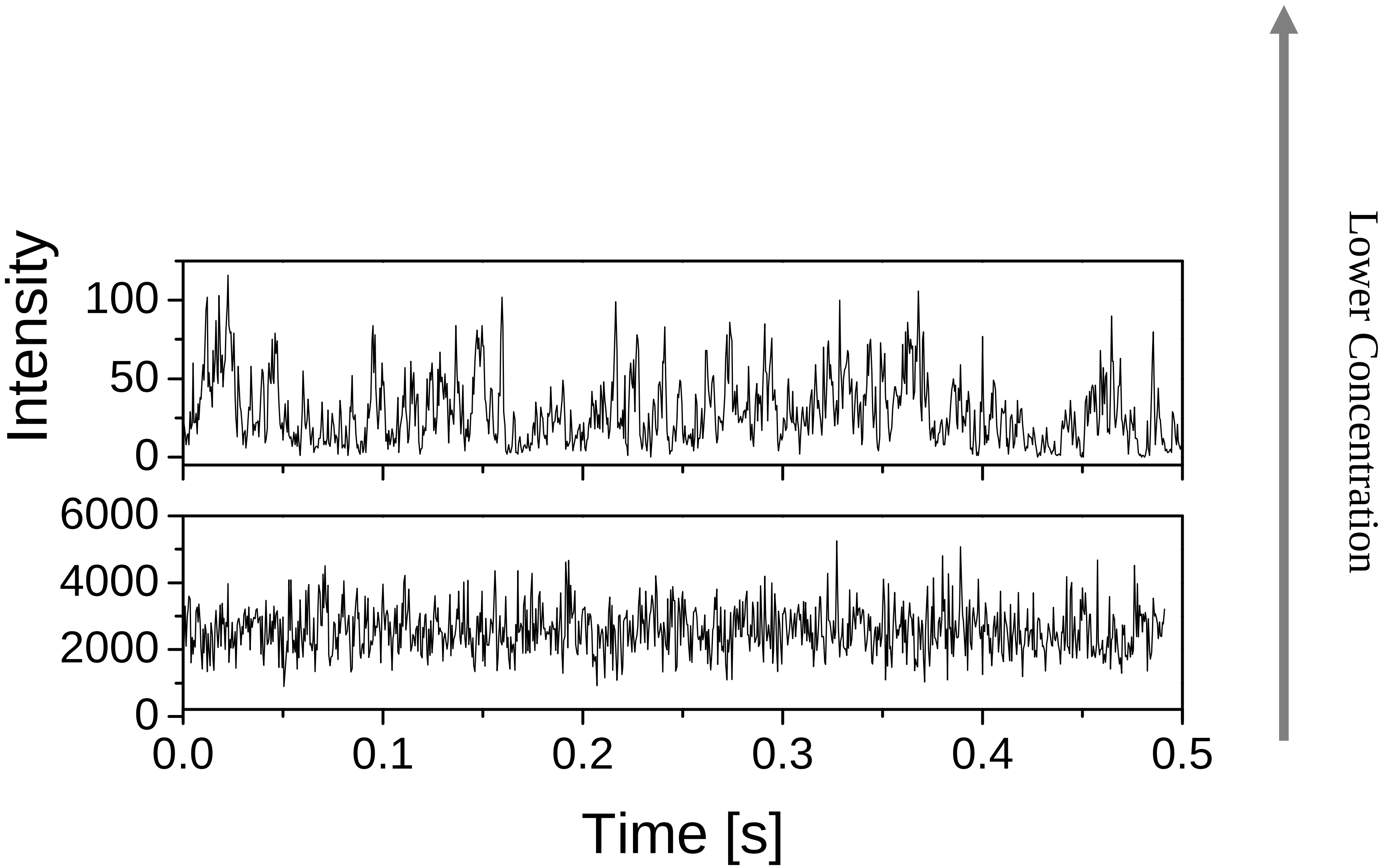
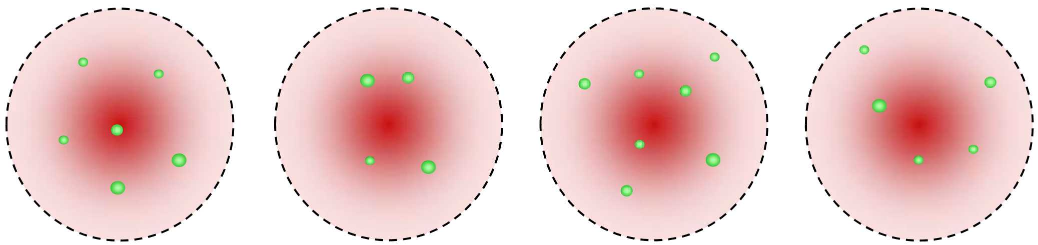
Fluctuation Measurements



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.

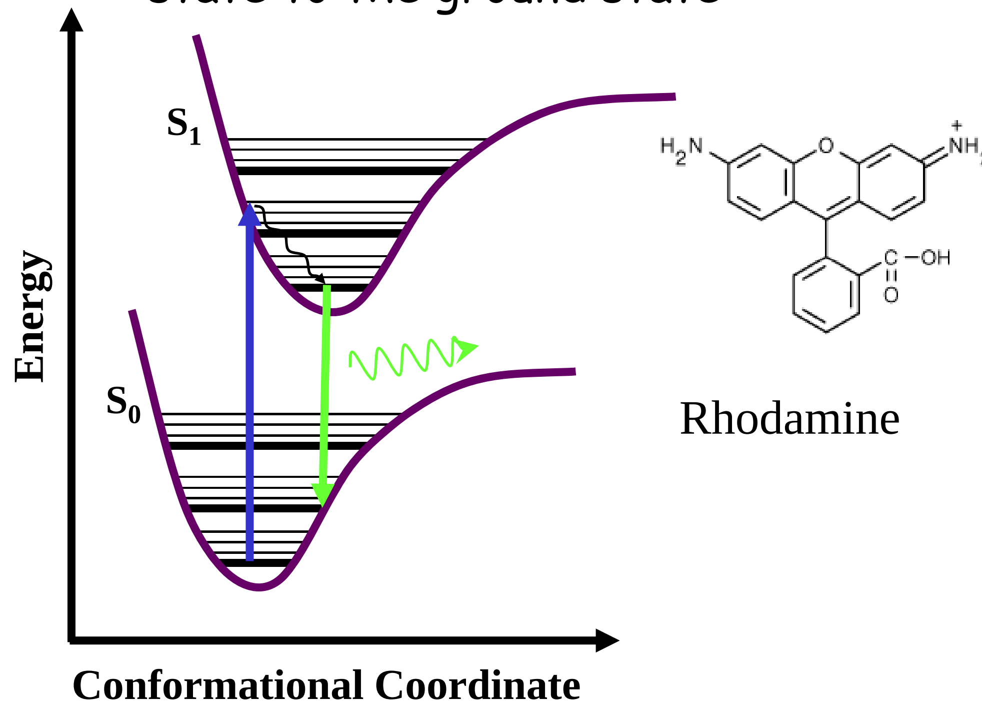
b) The probability of exactly one change occurring in a sufficiently short interval of length h is approximately λh

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

What is FCS?

What are its capabilities and limitations?

Fluorescence: The property of a molecule to emit light upon the transition from the lowest excited electronic state to the ground state

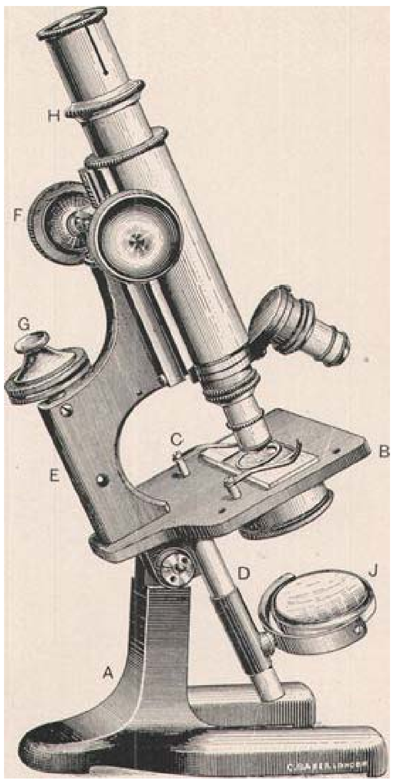


Rhodamine

Correlation

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



12000200132412310211113112511102331333221112242212261222
14234524114131142310010042112312320111100011-21100
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21024011102-12221123100011033111021010010103011312121
010121111211-1000322101230201212132111011002331224211
0001203010100221734410101002112211444421211440132123314
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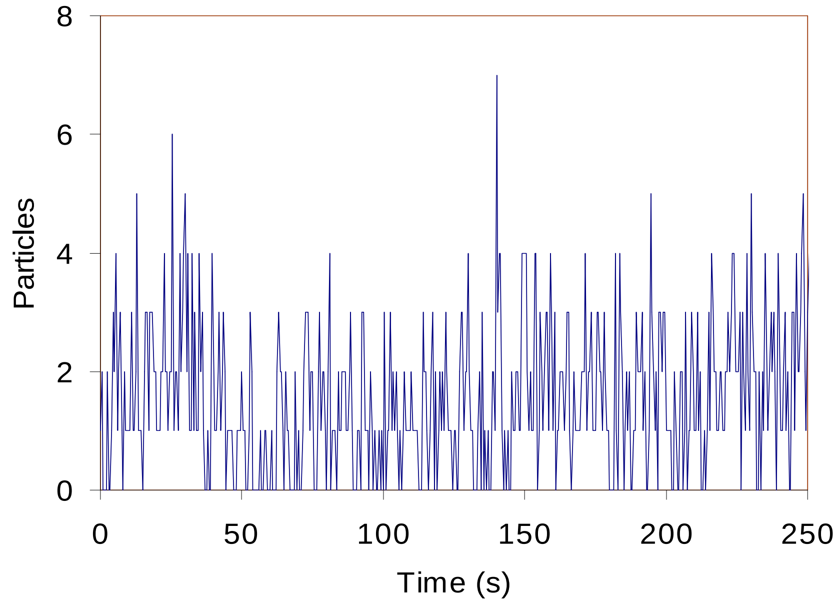
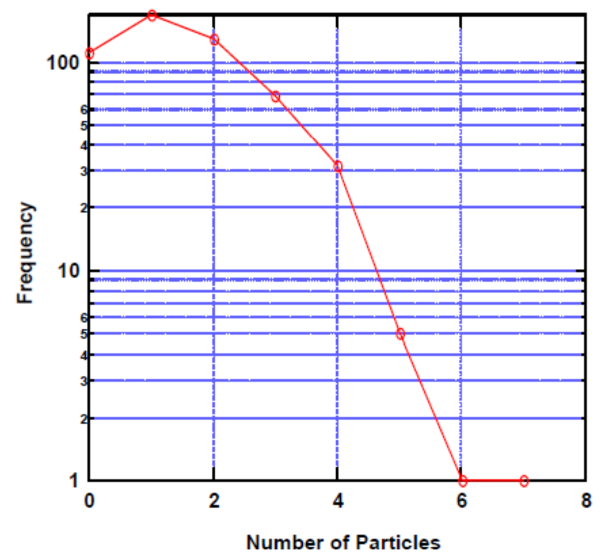
20011322231200-2532120332331111002100220130113211
3120010131432211221122323442223032142153220020214212323
2043112312003314223452134110412322220221

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

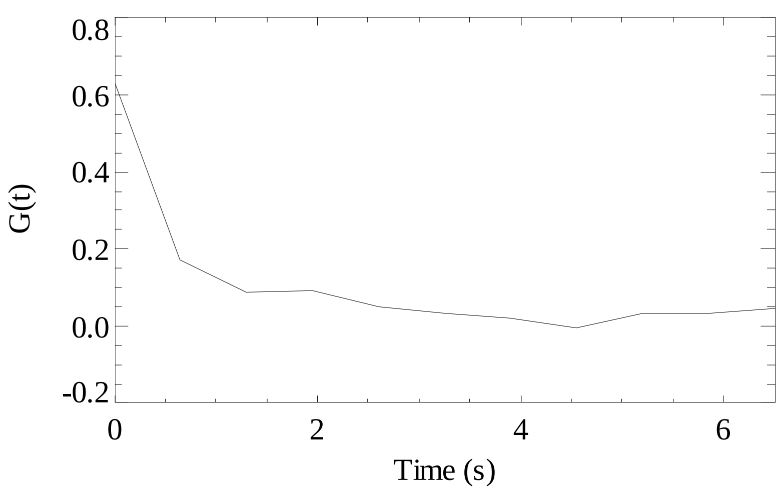
Autocorrelation Analysis



PCH



Autocorrelation Analysis



⟨N⟩ = 1.55 particles

τ ~ 1.5 s

Autocorrelation Analysis

The normalized autocorrelation function (ACF) is given by:

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

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

where

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

For processes that are:

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

the ACF is independent of the absolute time

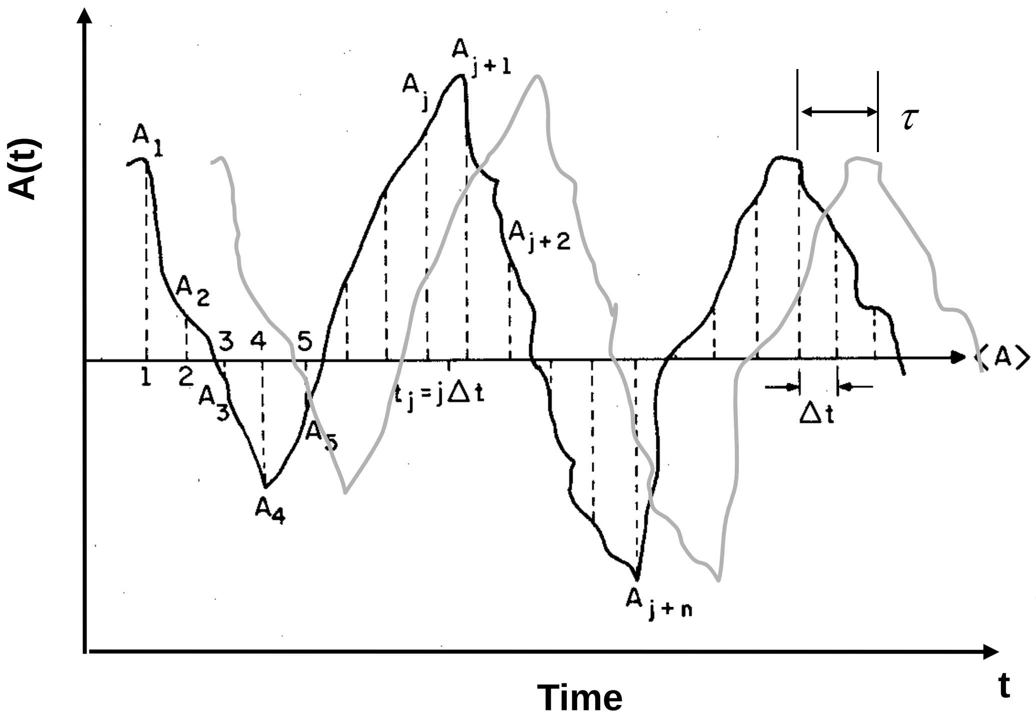
Ergodic: i.e. every sizeable sampling of the process is representative of the whole

the time average is equal to the ensemble average

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

Properties of the Autocorrelation Function

The autocorrelation function (ACF) measures the self similarity of the observable A as a function of τ



$$G(\tau) = \frac{\langle \delta A(0)\,\delta A(\tau)\rangle}{\langle A\rangle^2}$$

$$= \frac{\sum_{i=1}^{\ell} \delta A_i \delta A_{i+\tau} \Big/ \ell}{\left(\sum_{i=1}^{\ell} A_i \Big/ \ell\right)^2}$$

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

$\delta A_i \delta A_{i+\tau}$ 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

$$G(0) = \frac{\langle \delta A(0) \delta A(0) \rangle}{\langle A \rangle^2} = \frac{\sum_{i=1}^{\ell} \left(A_i - \langle A \rangle\right)^2 \Big/ \ell}{\left(\sum_{i=1}^{\ell} A_i \Big/ \ell \right)^2}$$

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

For a Poissonian process

For non-conserved, non-periodic signals

$G(\tau) \to 0$ as $\tau \to \infty$

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

ACF of Freely Diffusing Molecules

The fluorescence intensity is given by:

$$F(t) = \kappa Q \int d\mathbf{r}\, W(\mathbf{r}) C(\mathbf{r},t)$$

Where κ is the detection efficiency

$Q = \sigma\phi$; Effective Quantum Yield

$W(\mathbf{r}) = I^n(\mathbf{r}) S(\mathbf{r}) X(\mathbf{r})$; Probe Volume

$C(\mathbf{r},t)$ = Number Density

$I^n(\mathbf{r})$ = laser intensity profile for *n*-photon excitation

$S(\mathbf{r})$ = Sample extent

$X(\mathbf{r})$ = Detection efficiency

The ACF is given by:

$$G(\tau) = \frac{\langle \delta F(0) \delta F(\tau) \rangle}{\langle F \rangle^2} = \frac{\iint d\mathbf{r}\, d\mathbf{r}'\, W(\mathbf{r}) W(\mathbf{r}') \langle \delta C(\mathbf{r},\tau) \delta C(\mathbf{r}',0) \rangle}{\left[\langle C \rangle \int d\mathbf{r}\, W(\mathbf{r}) \right]^2}$$

Point-Spread-Function

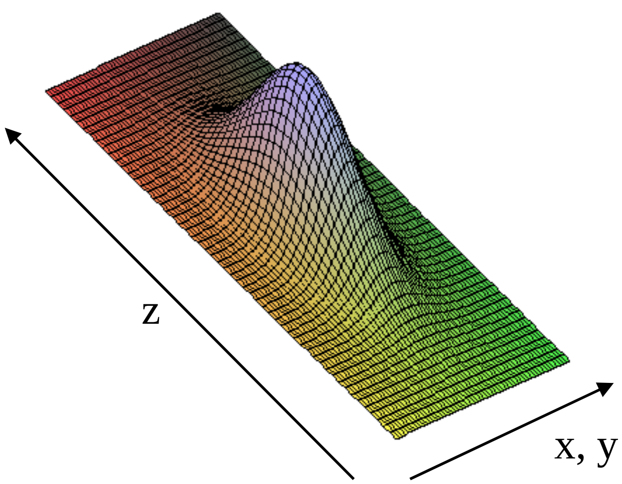
The PSF is the measured fluorescence intensity of a point particle at the position **r** within the excitation volume

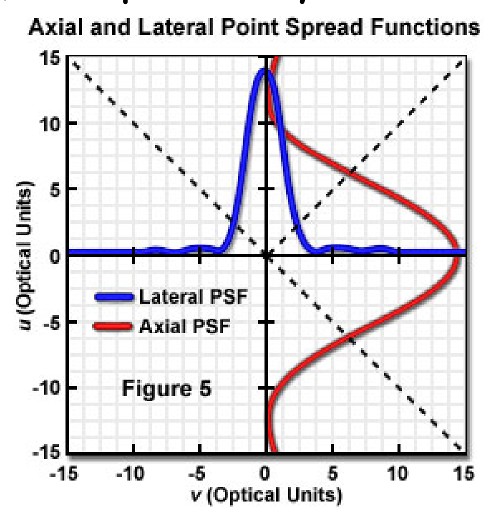
1-photon excitation, confocal detection:

Approximate the PSF by a 3 dimensional Gaussian

$$W(x,y,z) = I_0(0,0,0)\exp\left[-\frac{2\left(x^2+y^2\right)}{w_r^2}-\frac{2z^2}{w_z^2}\right]$$

where w_r and w_z the radial and axial distance from the center to where the intensity has decayed by $(1/e)^2$ respectively





Point-Spread-Function

The PSF is the measured fluorescence intensity of a point particle at the position **r** within the excitation volume

2-photon excitation:

Approximate the PSF by the expression for the Gaussian Beam Waist of a focused Laser beam: Gaussian Lorentzian

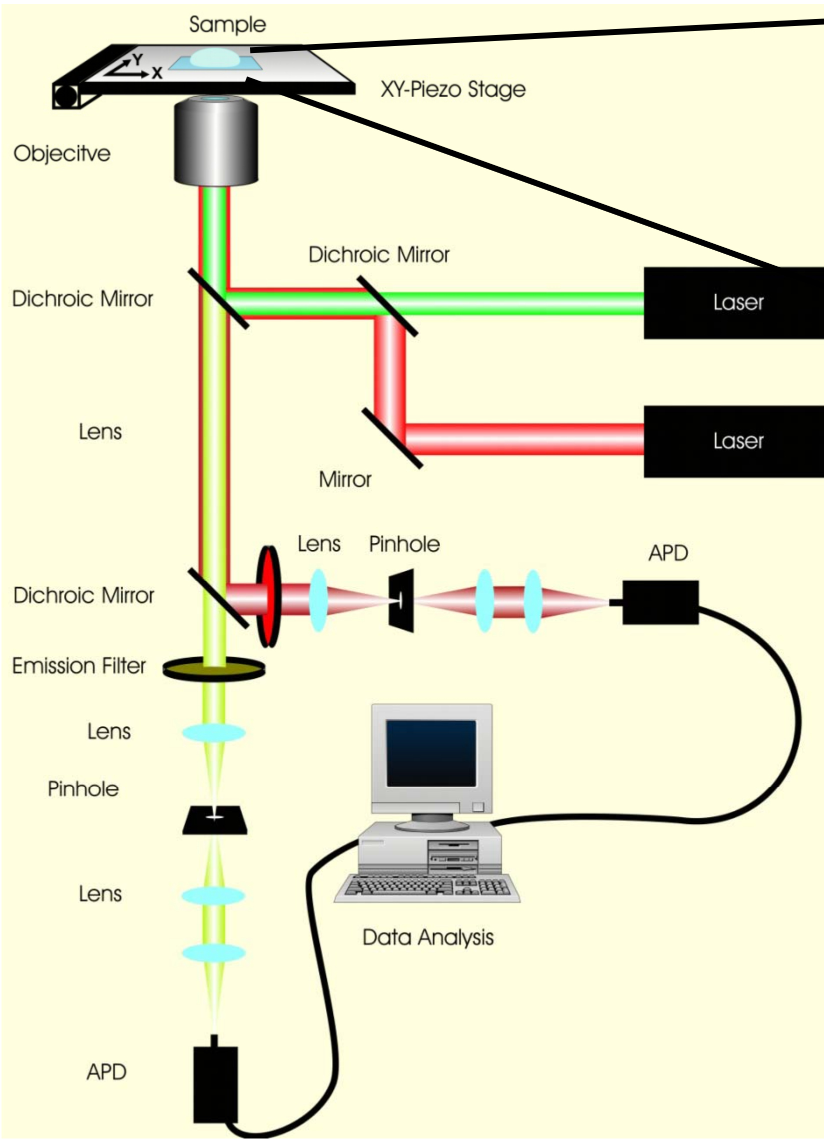
$$I(x,y,z) = I_0(0,0,0)\frac{w_0^2}{w(z)^2}\exp\left[-\frac{2\left(x^2+y^2\right)}{w(z)^2}\right]$$

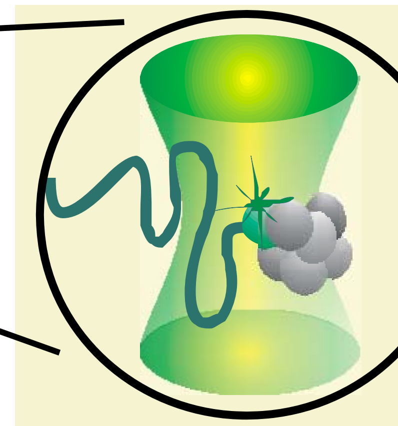
$$\text{where} \qquad w(z)^2 = w_0^2\left[1+\frac{z}{\left(\pi w_0^2/\lambda\right)}\right]$$

w_0 is the beam waist and λ is the excitation laser wavelength

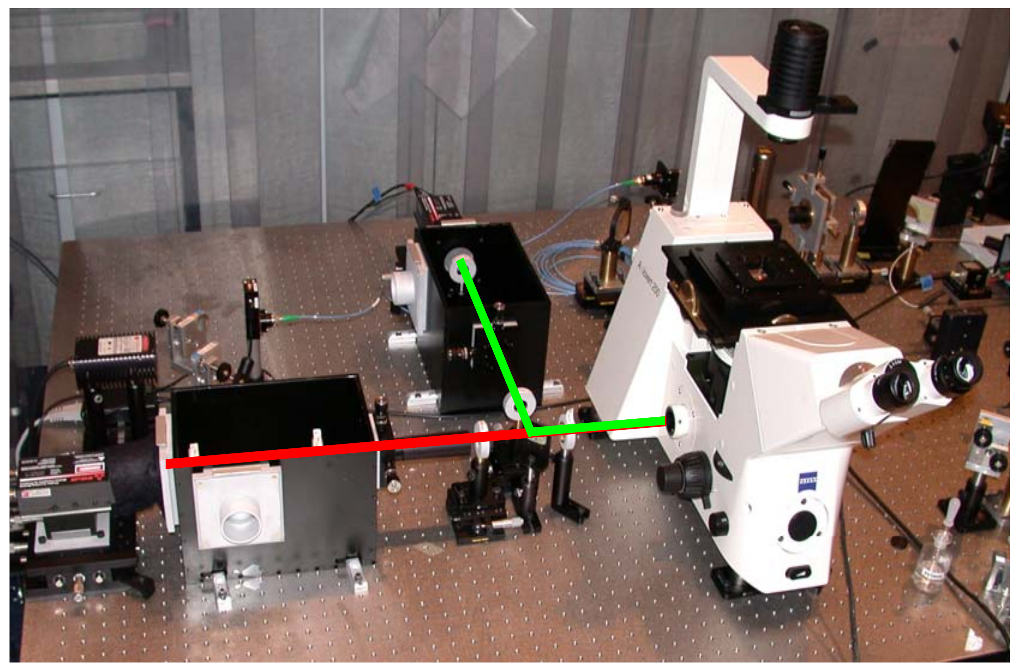
$$W(x,y,z) = I_0^2(0,0,0)\frac{w_0^4}{w(z)^4}\exp\left[-\frac{4\left(x^2+y^2\right)}{w(z)^2}\right]$$

Experimental Setup



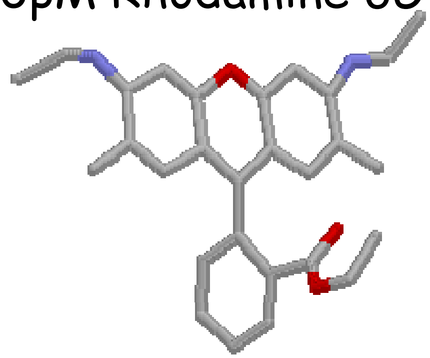


Two Channel Confocal Microscope

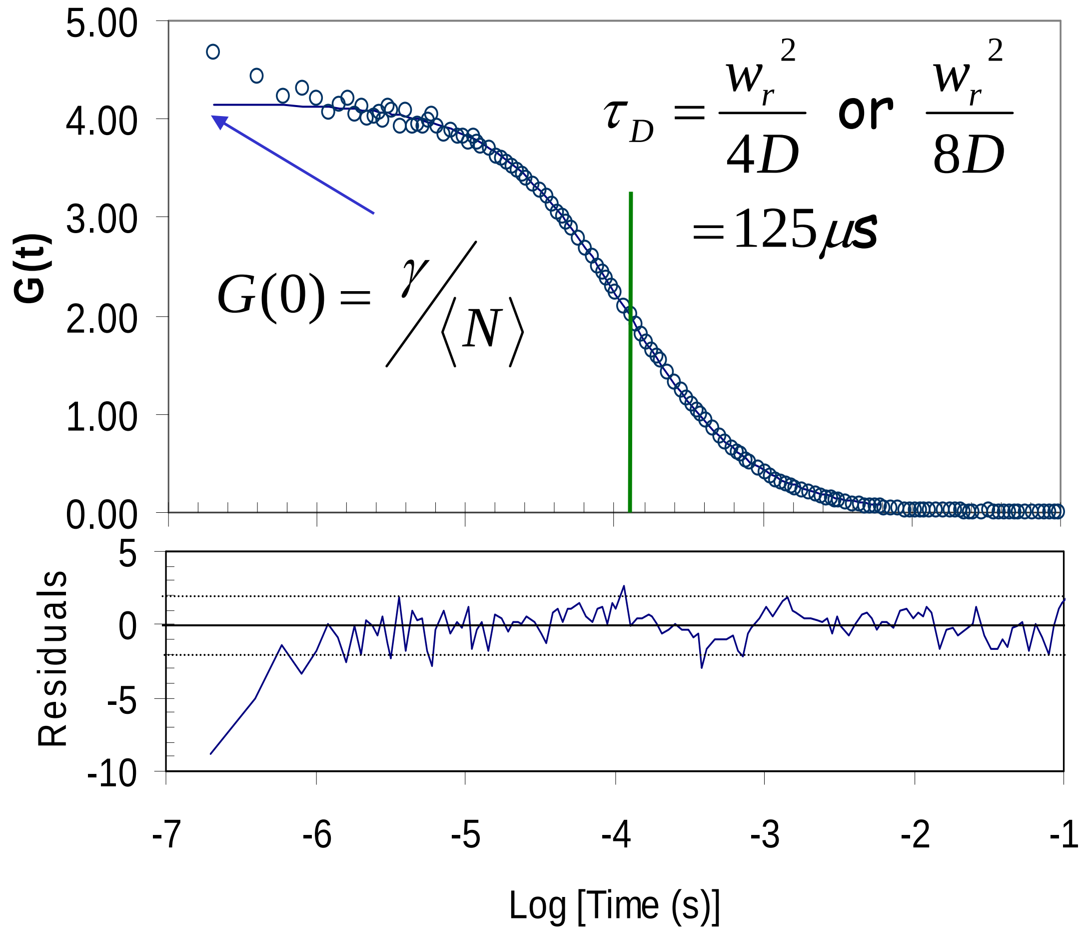


ACF of Freely Diffusing Molecules

230pM Rhodamine 6G in buffer



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



Amplitude ACF ⇒

Concentration

$\langle N \rangle$ = 0.085 molecules

Decay ACF ⇒

Diffusion Constant

D = 280 $\mu m^2/s$ (w_r = 374 nm)

The Gamma Factor

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

For a single particle fixed at the center of the PSF: $C(\mathbf{r}, t) = \delta(0)$

The fluorescent intensity is given by: $F(t) \equiv \varepsilon = \kappa Q W(0)$

For molecules freely diffusing in solution:

$$\begin{aligned}\langle F(t) \rangle &= \varepsilon \langle N \rangle \\ &= \varepsilon \langle C \rangle V_{eff}\end{aligned}$$

$$V_{eff} = \int d\mathbf{r} \frac{W(\mathbf{r})}{W(0)} = \left(\frac{\pi}{2}\right)^{\frac{3}{2}} w_r^2 w_z \text{ for a 3D Gaussian}$$

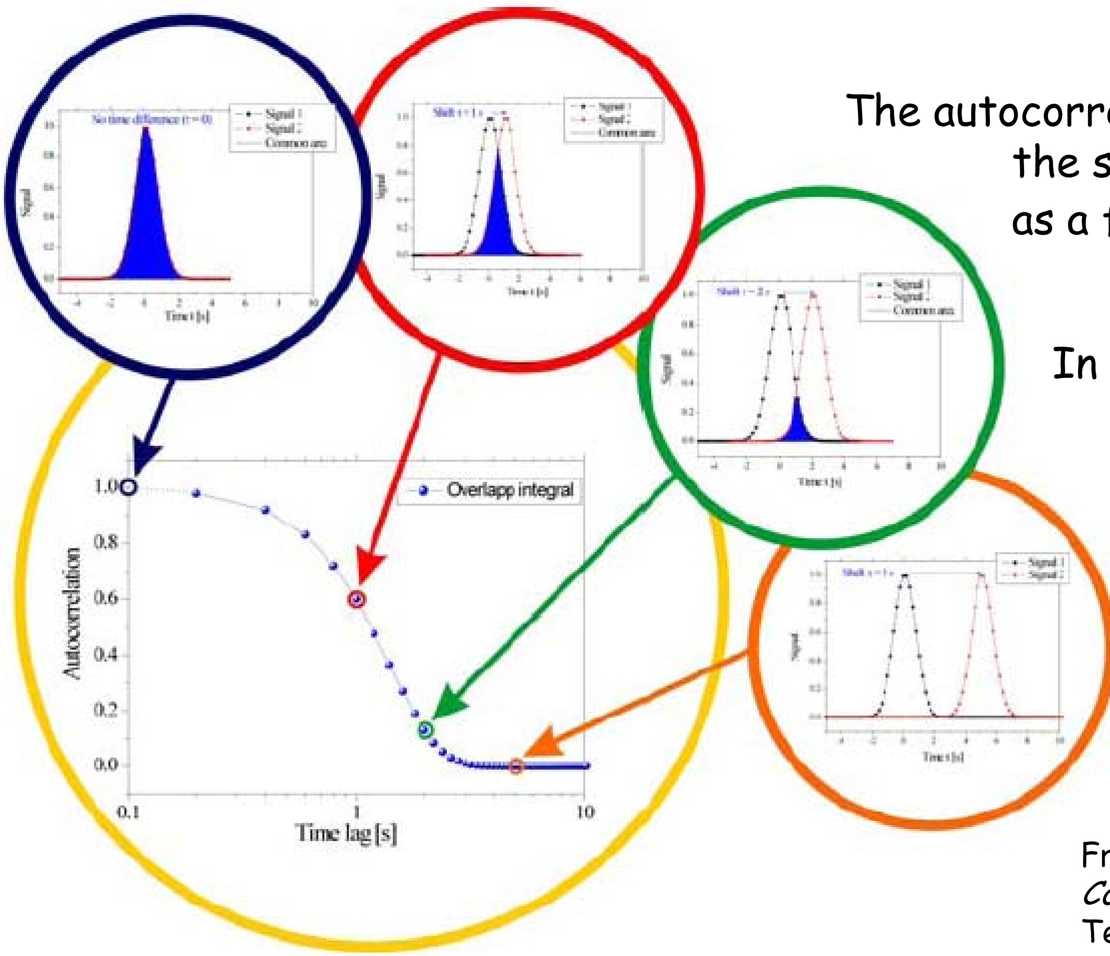
V_{eff} is the volume of uniform illumination at maximum intensity that yields the same overall detection rate

$$\varepsilon = \langle F \rangle / \langle N \rangle$$

$$\gamma = \frac{\int d\mathbf{r} \left(W(\mathbf{r})/W(0)\right)^2}{\left[\int d\mathbf{r} \left(W(\mathbf{r})/W(0)\right)\right]}$$

Autocorrelation Function

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



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?

?

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

ACF with Multiple Species

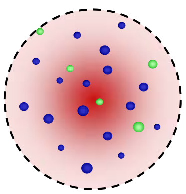
$$G(\tau) = \Im_1^2 G_{D1}\left(\tau, N_1, \tau_{D_1}\right) + \Im_2^2 G_{D2}\left(\tau, N_2, \tau_{D_2}\right)$$ 2 species

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

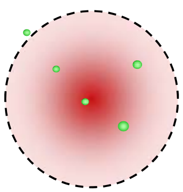
$$G(\tau) = \sum_{i=1}^{M} \Im_i^2 G_{Di}\left(\tau, N_i, \tau_{D_i}\right)$$ Multiple species

FCS measurements in a fluorescent background

With Background



Without Background



Situation: Large number of weakly fluorescing particles:

i.e. Although $Q_S \gg Q_B$, $N_S \ll N_B \Rightarrow$

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

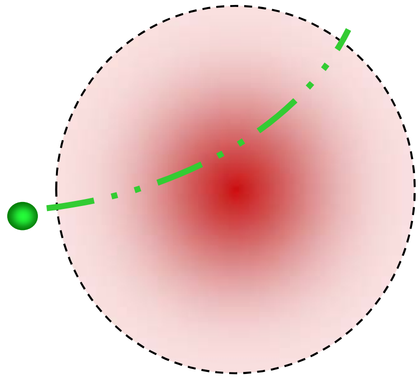
$$G(\tau)_{eff} = \Im_S^2 G_{Diff}\left(\tau, N_S, \tau_{D_S}\right) + \Im_B^2 G_{Diff}\left(\tau, N_B, \tau_{D_B}\right)$$

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

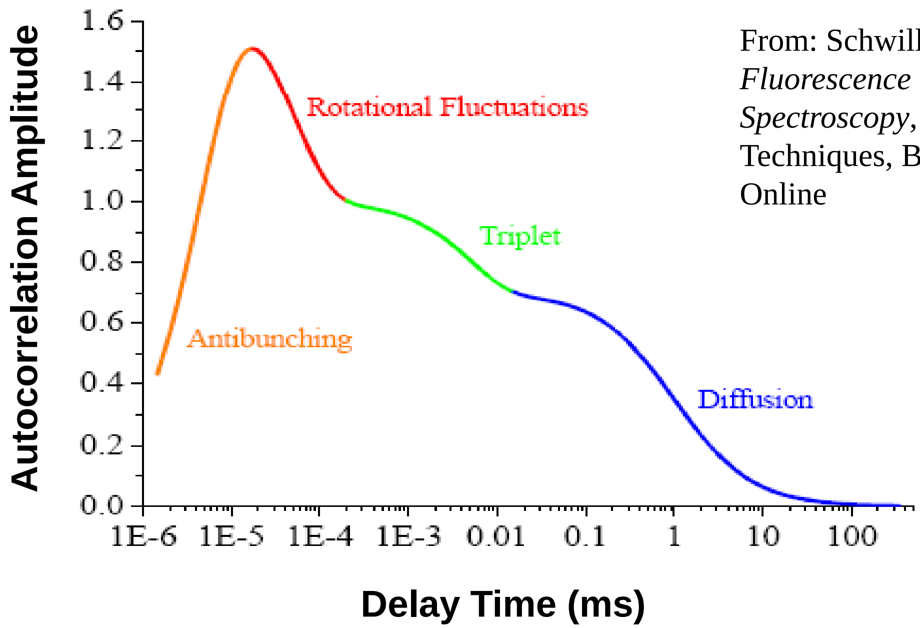
Fluctuation Correlation Spectroscopy (FCS)



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

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

Schematic Autotcorrelation Function:



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

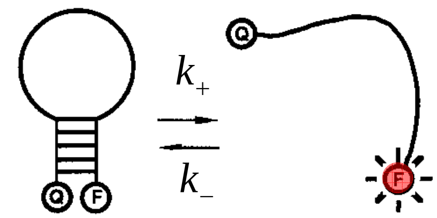
We can determine:

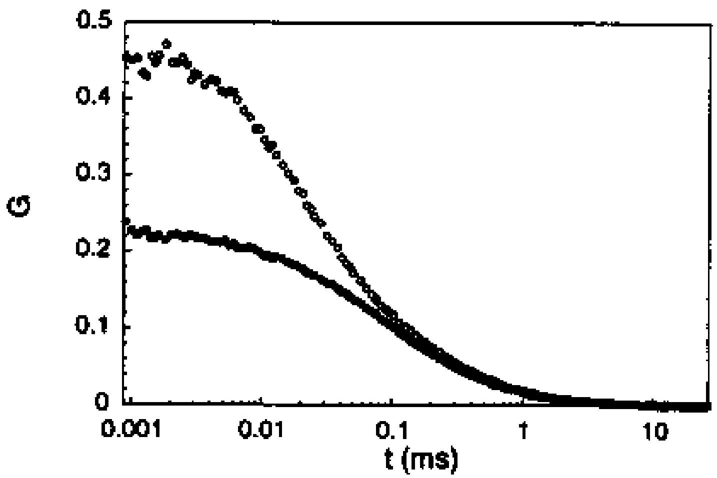
- Excited state lifetime
- Rotational Diffusion Constant
- Reaction Kinetics
- Triplet-State Lifetime
- Triplet-State Amplitude
- Translational Diffusion Constant
- Concentration

Unimolecular Reaction

Dynamics of DNA Hairpin Formation

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

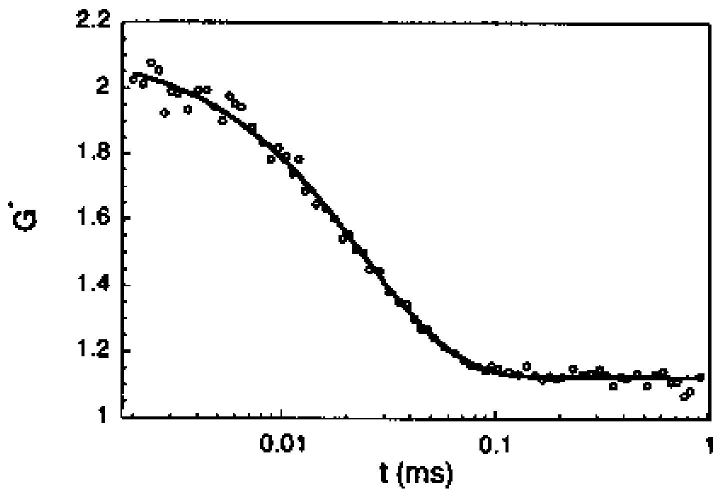




$$G(\tau) = G_D\left(\tau, N_A + N_B, \tau_D\right)\left[1 + K\left(\Im_A - \frac{\Im_B}{K}\right)^2 e^{-\lambda\tau}\right]$$

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

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



Diffusion term drops out of the ratio

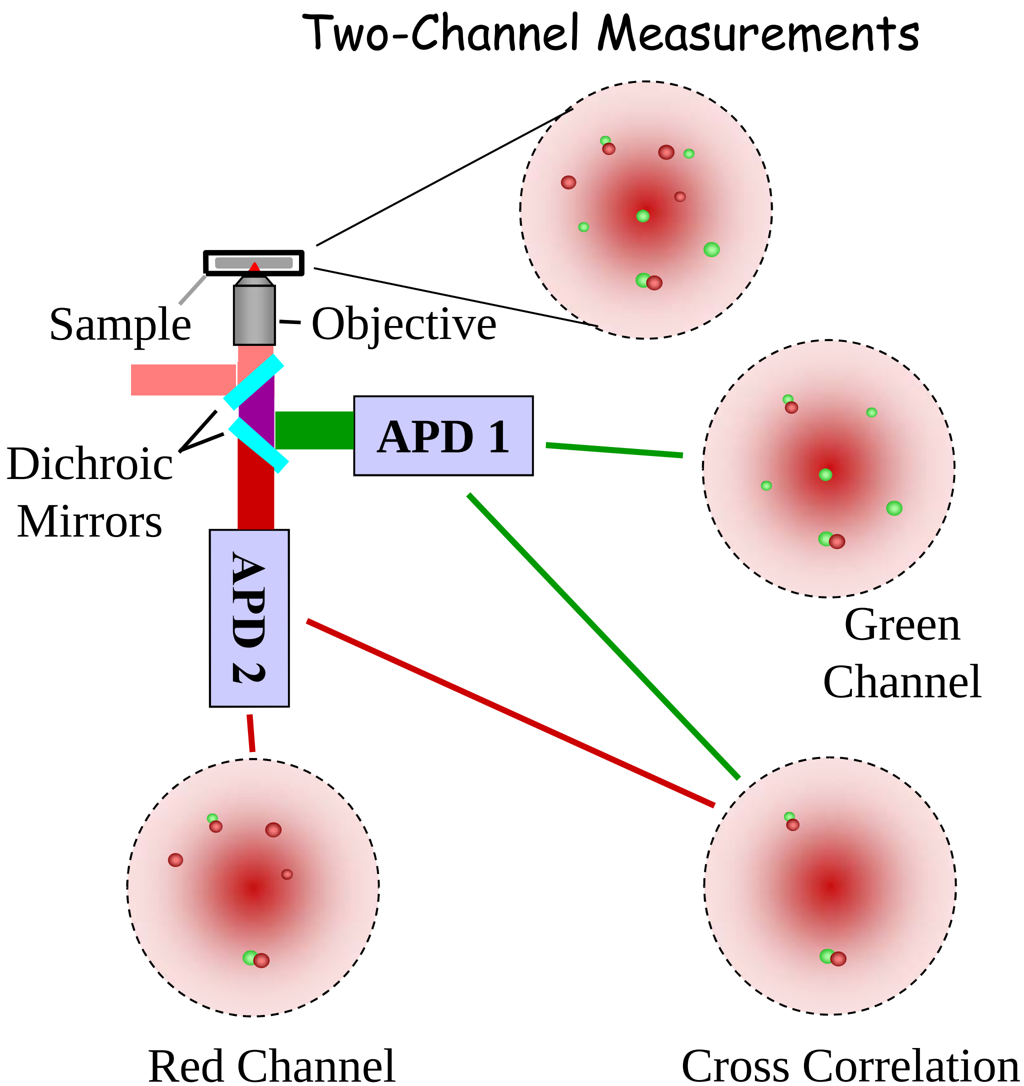
$$\frac{G_b(\tau)}{G_c(\tau)} = \frac{G_b(0)}{G_c(0)}\left(1 + \frac{1}{K}\exp(-\lambda\tau)\right)$$

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

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

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

Fluorescence Cross-Correlation Spectroscopy



The sample consists of three species:

N_G Particles/complexes with a green label only

N_R Particles/complexes with a red label only

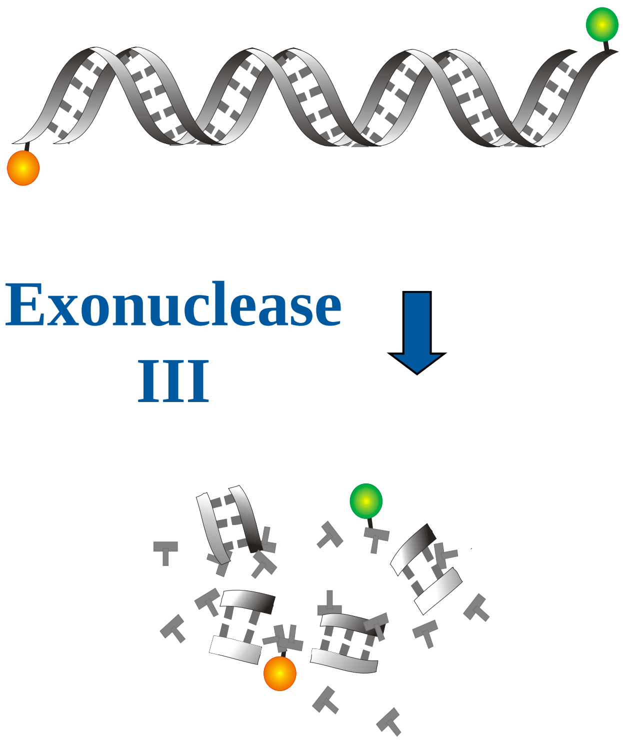
N_{GR} Particles/complexes containing both green and red labels

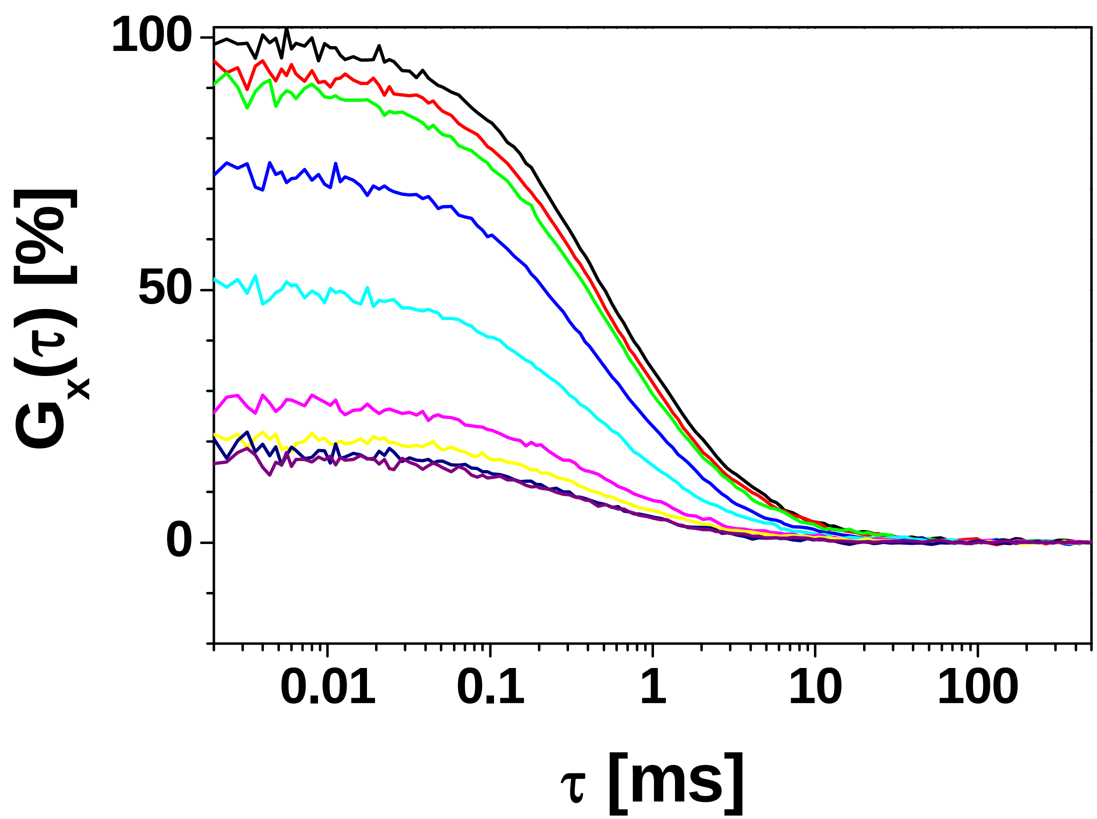
$$G(\tau) = \frac{\langle F_G(t)F_R(t+\tau)\rangle - \langle F_G(t)F_R(t)\rangle^2}{\langle F_G(t)F_R(t)\rangle^2}$$

Ideally, only the N_{GR} particles cross correlate

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

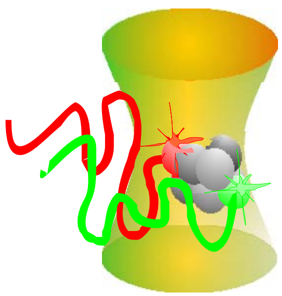
Kinetics of DNA Degradation





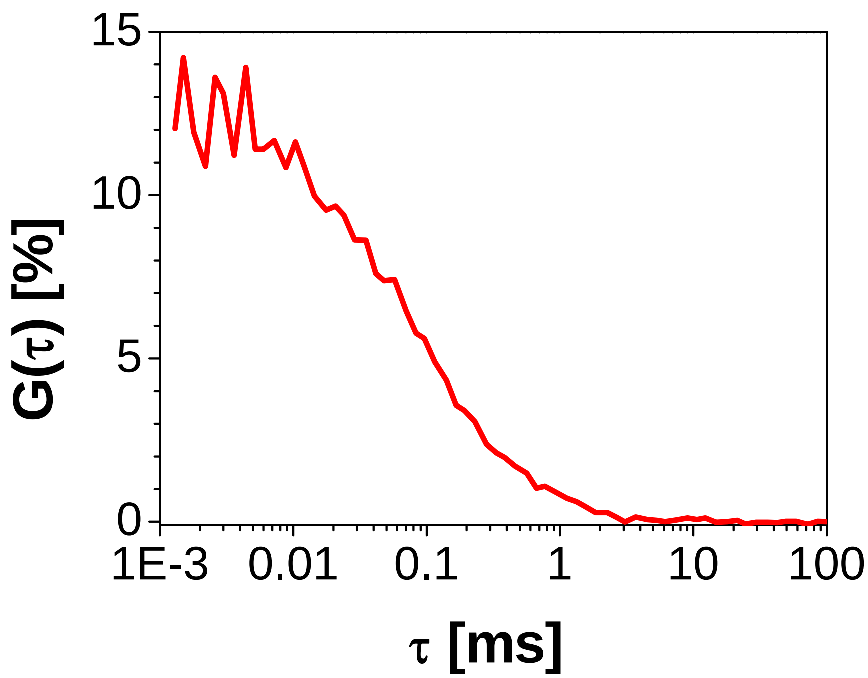
Kettling, Koltermann, Schwille, Eigen *PNAS* (1998) 95:1416

Effect of Cross Talk on Amplitude of the CCF



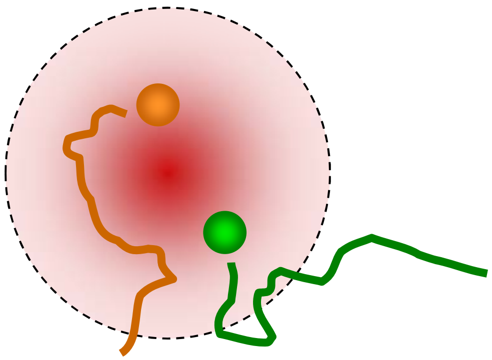
$$G_{G\times R}(\tau)=\frac{\gamma\,\Im^{R}_{G,R}}{\left\langle N_G+\frac{\varepsilon_{GR,G}}{\varepsilon_{G,G}}N_{GR}\right\rangle}\left(\frac{1}{1+4D_G\tau/\omega_r^2}\right)\left(\frac{1}{1+4D_G\tau/\omega_z^2}\right)^{1/2}$$

$$+\frac{\gamma\,\Im^{R}_{GR,R}}{\left\langle \frac{\varepsilon_{G,G}}{\varepsilon_{GR,G}}N_G+N_{GR}\right\rangle}\left(\frac{1}{1+4D_{GR}\tau/\omega_r^2}\right)\left(\frac{1}{1+4D_{GR}\tau/\omega_z^2}\right)^{1/2}$$



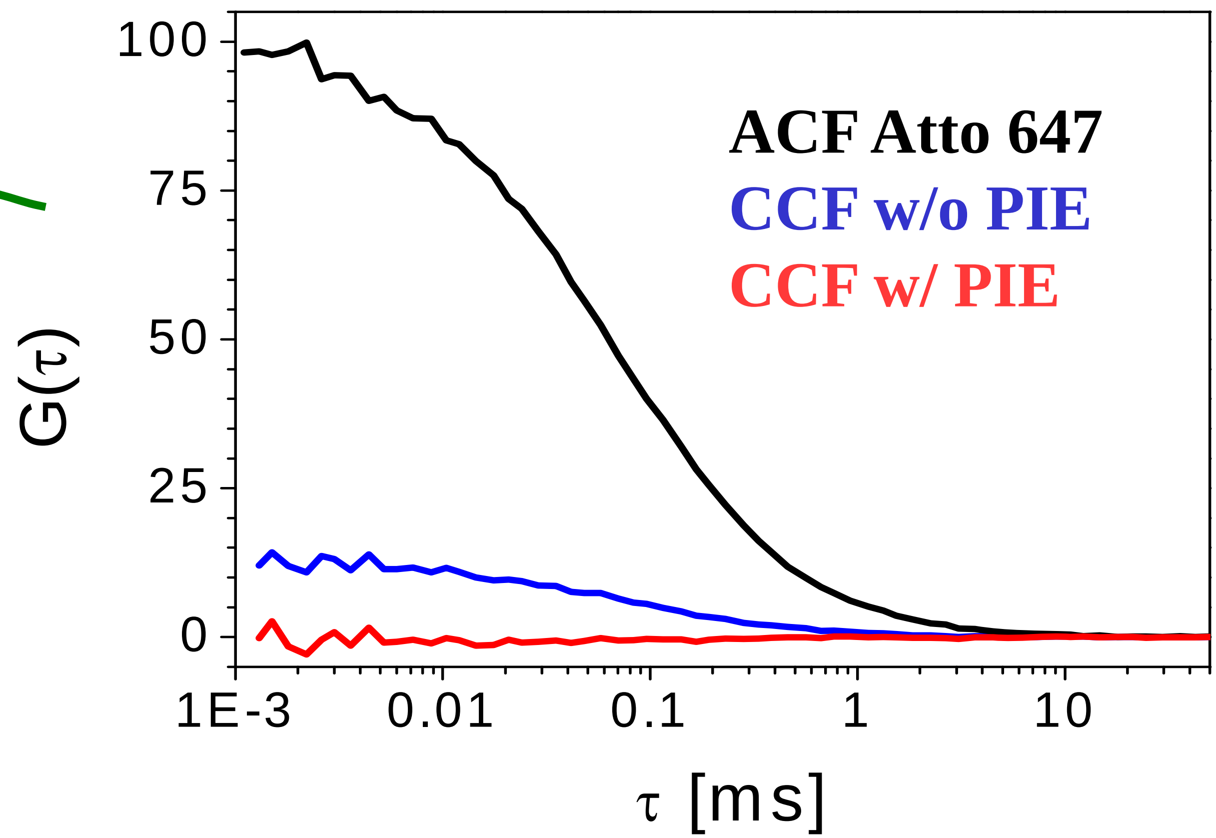
Cross Correlation of Independent Dyes

Cross-correlation of Atto 532 and Atto 647 freely diffusing in solution



Atto 532

Atto 647



Photon Counting Histogram

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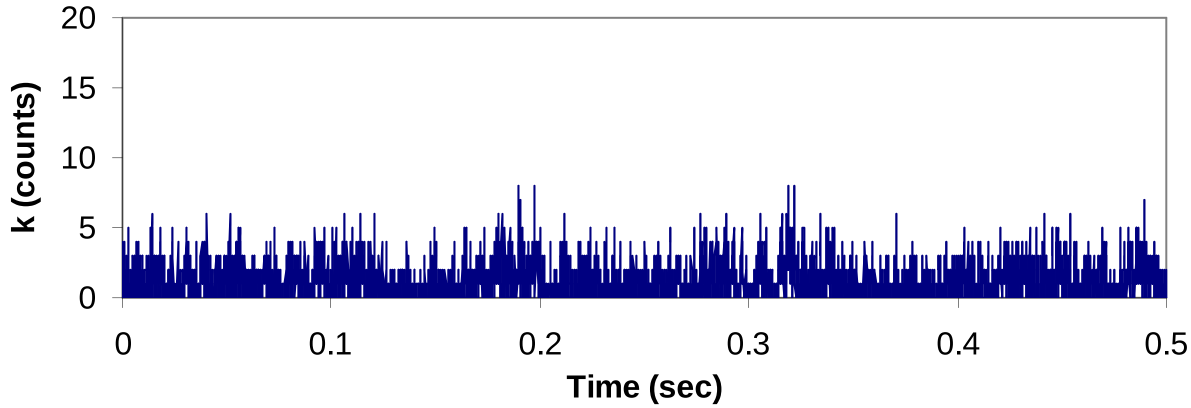
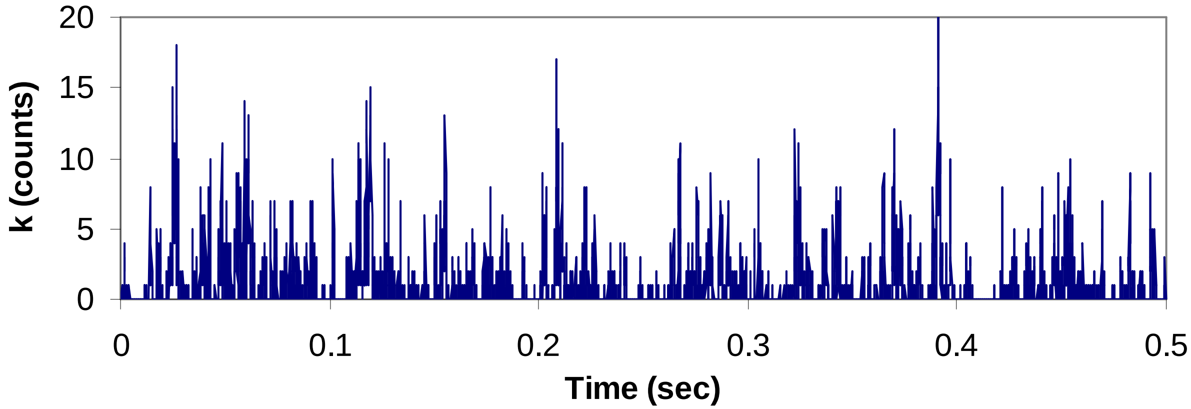
Effect of Brightness on Measured Fluorescence Intensity



Fluorescent Monomer:

Intensity = 115,000 cps

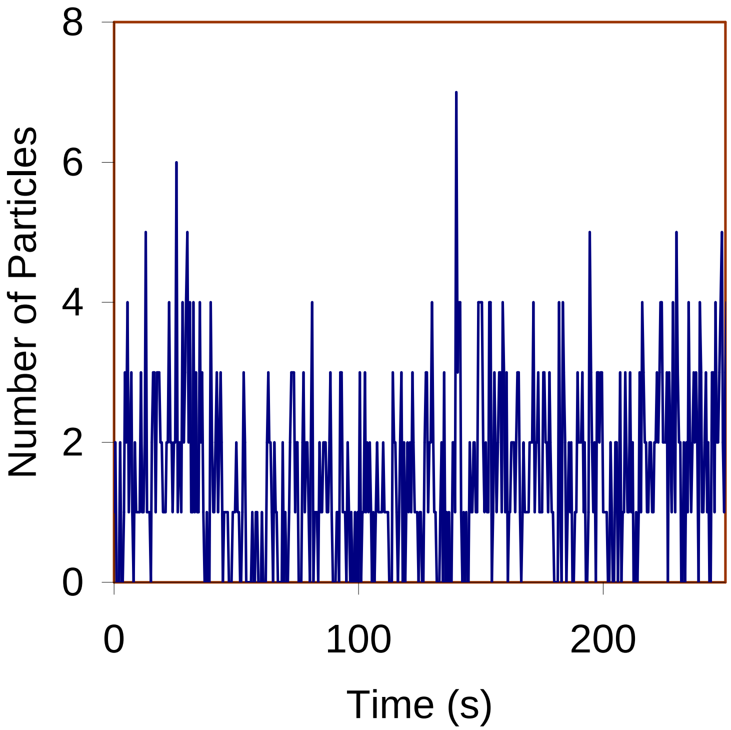


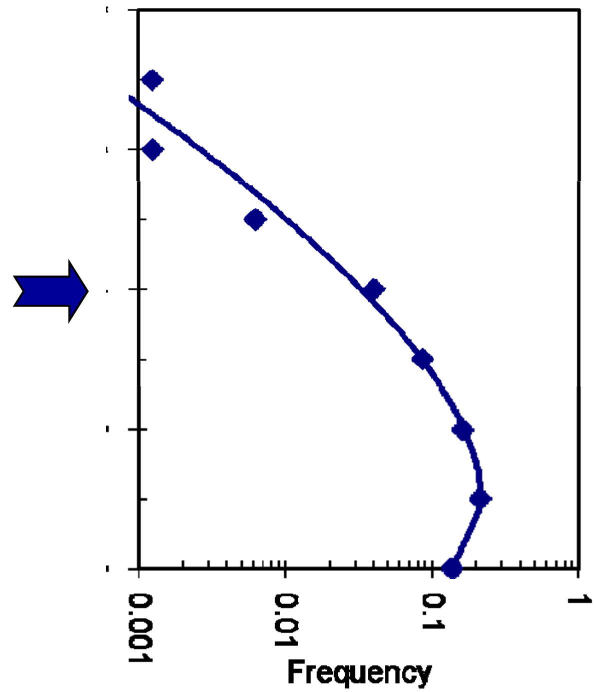
Aggregate:

Intensity = 111,000 cps

Amplitude Fluctuations

A photon counting histogram analysis investigates the amplitude of the fluctuations





Svedberg and Inouye, *Zeitschr f. Physik Chemie* 1911, 77:145-119

For a Poisson Distribution:

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

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

$\langle \Delta N^2 \rangle < \langle N \rangle$ super-Poissonian Statistics

$\langle \Delta N^2 \rangle > \langle N \rangle$ sub-Poissonian Statistics

The measured probability function for detecting *N* photons in a time bin is a renormalization of the histogram of the photon counting data.

$$P_{\exp}(N) = \frac{freq(N)}{N_{Total\ photons}}$$

Photon Counting Statistics

The number of detected photons from a constant intensity light source is governed by Poisson statistics

$$p\left(k,\langle k\rangle\right)=\frac{\left(\eta_E E\right)^k e^{-\eta_E E}}{k!}\equiv \mathrm{Poi}\left(k,\langle k\rangle\right)$$

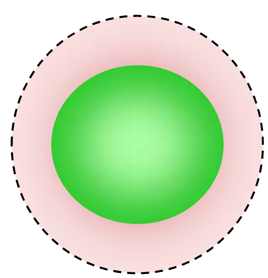
where: k is the number of detected photons

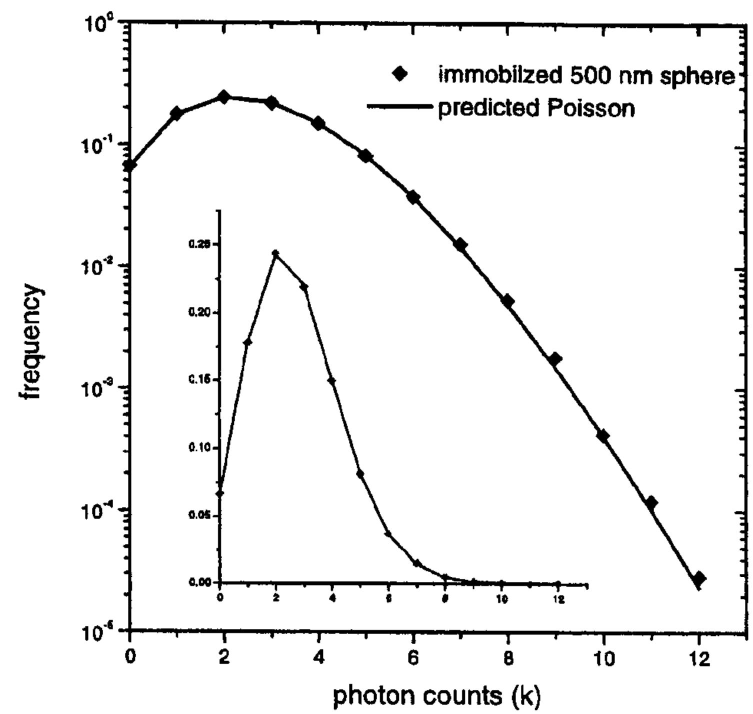
$\langle k\rangle = \eta_E E$ is the average number of detected photons

η_E is the detection efficiency

E is the energy impinging on the detector

e.g. A non-diffusing 500 nm fluorescent bead in the excitation volume:





Mandel's Formula

For a fluctuating intensity source, the photon counting distribution is given by Mandel's formula: Mandel, *Proc. Phys. Soc.* (1958) 72:1037-1048

$$p(k,t,T)=\int_0^\infty \frac{(\eta_E E(t,T))^k e^{-\eta_E E}}{k!} P(E(t,T))dE$$

where: $P(E(t,T))$ is the energy probability distribution

T is the integration time of the measurement

$$E(t,T)=\int_t^{t+T} I_D(t)dt$$

where: I_D is the intensity reaching the detector

Effect of binning:

For $T \rightarrow 0$: power fluctuations tract intensity fluctuations

For $T \rightarrow \infty$: intensity fluctuations average out, $p(E) \rightarrow \delta(E - \langle E\rangle)$

Choose T small enough to tract intensity fluctuations:

$$E(t) = I_D(t)T$$

$$p(k,t,T)=\int_0^\infty \frac{(\eta_I I_D(t))^k e^{-\eta_I I_D(t)}}{k!} P(I_D(t))\, dI_D \qquad \text{where: } \eta_I = \eta_E T$$

Diffusing Particle in a Confocal Volume

I_D depends upon the position of the particle

The *PSF* gives the *measured fluorescence intensity* of a point particle at the position r within the probe volume

The intensity at the detector from a fluorophore at position r is given by:

$$I_D(\mathbf{r}) = \frac{I_{ex}^n \beta\, \overline{PSF}^n(\mathbf{r})}{n}$$

where n = number of absorbed photons per excitation

β includes corresponding scale factors between excitation and detection intensity

We define the Molecular Brightness to be the measured intensity of a molecule at the center of the *PSF*:

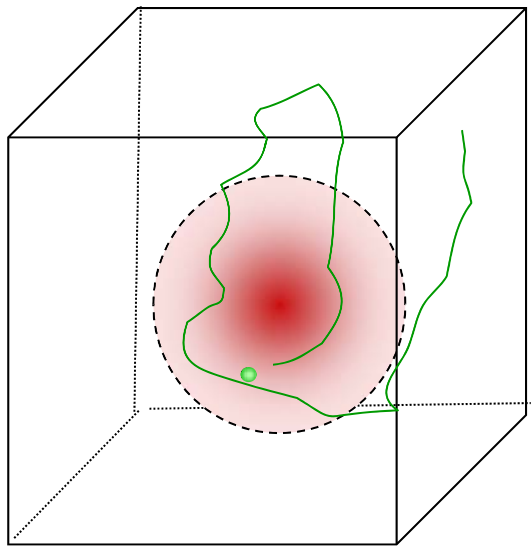
$$\varepsilon = \frac{I_0^n \beta \eta_I}{n} = \frac{kQW^n(0)}{n} \quad ; \quad \eta_I I_D(\mathbf{r}) = \varepsilon\, \overline{PSF}^n(\mathbf{r})$$

$$p^{(1)}(k;\varepsilon) = \int \frac{\left[\varepsilon\, \overline{PSF}^n(\mathbf{r})\right]^k \exp\left(-\varepsilon\, \overline{PSF}^n(\mathbf{r})\right)}{k!} P(\mathbf{r})\, d\mathbf{r}$$

$$p^{(1)}(k;\varepsilon) = \int \mathrm{Poi}\left(k, \varepsilon\, \overline{PSF}^n(\mathbf{r})\right) P(\mathbf{r})\, d\mathbf{r}$$

PCH for Particles in a Box

The probability of detecting k photons from a single molecule in a box of volume V_0 is given by:



$$p^{(1)}(k;V_0,\varepsilon) = \int \mathrm{Poi}\left(k, \varepsilon \overline{PSF}^n(\mathbf{r})\right) P(\mathbf{r})\, d\mathbf{r}$$

$$= \frac{1}{V_0} \int_{V_0} \mathrm{Poi}\left(k, \varepsilon \overline{PSF}^n(\mathbf{r})\right) d\mathbf{r}$$

The average count rate is:

$$\langle k \rangle = \frac{1}{V_0} \int_{V_0} \varepsilon \overline{PSF}^n(\mathbf{r})\, d\mathbf{r} = \frac{\varepsilon V_{PSF}}{V_0}$$

For multiple particles in a box:

$$p^{(2)}(k;V_0,\varepsilon) = \iint \mathrm{Poi}\left(k, \varepsilon \overline{PSF}^n(\mathbf{r}_1) + \varepsilon \overline{PSF}^n(\mathbf{r}_2)\right) P(\mathbf{r}_1) P(\mathbf{r}_2) d\mathbf{r}_1 d\mathbf{r}_2$$

$$p^{(N)}(k;V_0,\varepsilon) = \int_1 \ldots \int_N \mathrm{Poi}\left(k, \varepsilon \sum_i^N \overline{PSF}^n(\mathbf{r}_i)\right) \prod_i^N P(\mathbf{r}_i) d\mathbf{r}_i$$

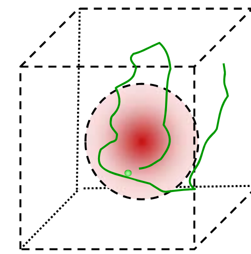
The expression can also be written as a convolution:

$$p^{(2)}(k;V_0,\varepsilon) = \left(p^{(1)} \otimes p^{(1)}\right)(k,V_0,\varepsilon) = \sum_{j=0}^{k} p^{(1)}(j;V_0,\varepsilon)\, p^{(1)}(k-j;V_0,\varepsilon)$$

$$p^{(N)}(k;V_0,\varepsilon) = \left(\overbrace{p^{(1)} \otimes \cdots \otimes p^{(1)}}^{N \text{ times}}\right)(k,V_0,\varepsilon)$$

PCH in an Open Volume

Particles can enter and leave the subvolume V_0



The probability of having N particles in the subvolume V_0 is given by:

$$p^{(1)}(N) = \mathrm{Poi}(N, \langle N \rangle)$$

The probability of observing k photons is given by the product of the probability of observing k photons with N particles in the volume multiplied by the probability of having N particles in the volume:

$$\Pi(k; \langle N_{PSF} \rangle, \varepsilon) = \sum_{N=0}^{\infty} p^{(N)}(k; V_0, \varepsilon) \mathrm{Poi}(N, \langle N_{PSF} \rangle) \qquad \text{where} \qquad p^{(0)}(k; V_0, \varepsilon) = \delta(k)$$

The average count rate is given by: $\langle k \rangle = \varepsilon \langle N_{PSF} \rangle$

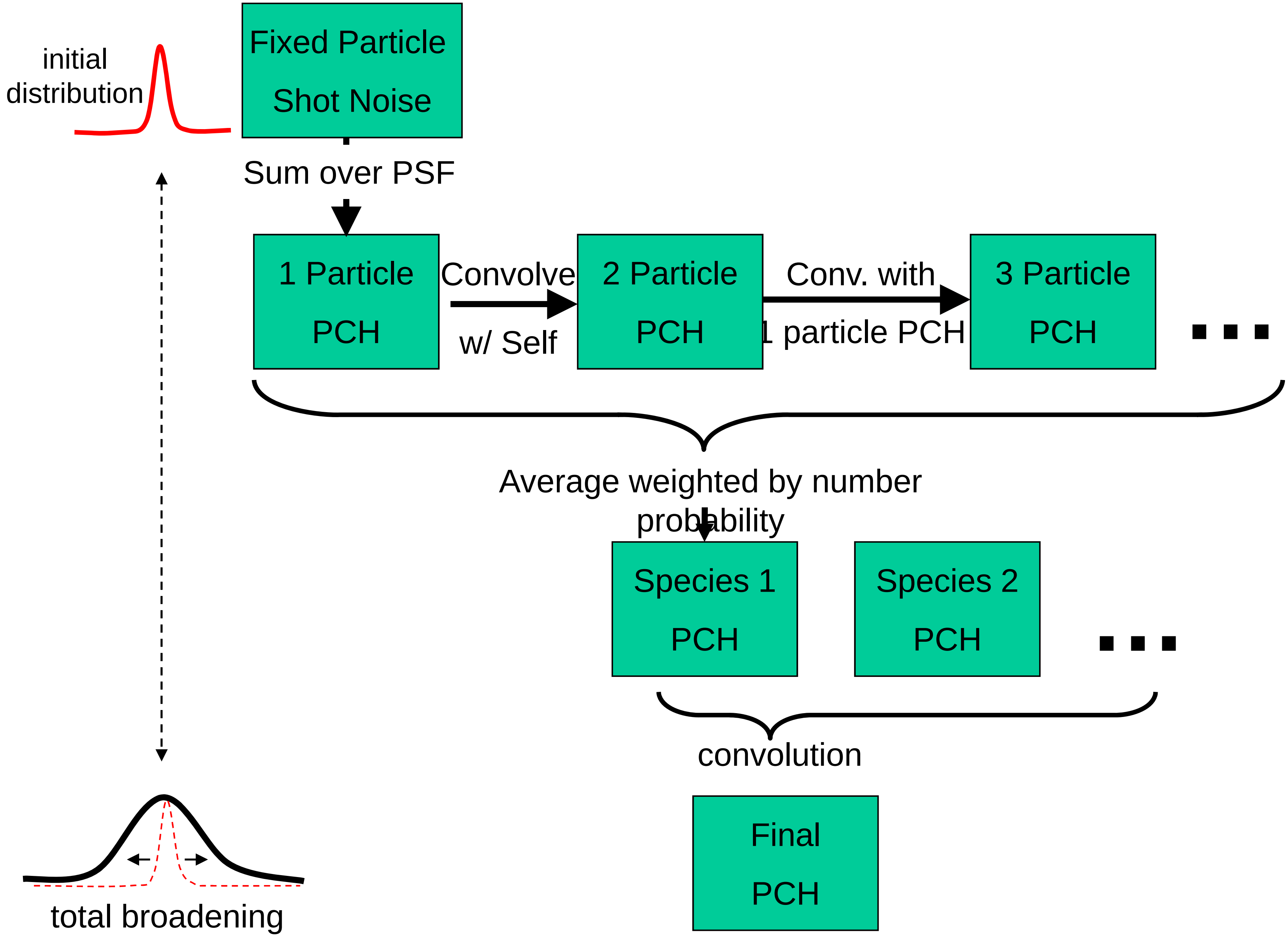
Information available from analysis: $\varepsilon, \langle N_{PSF} \rangle$

Two key assumptions for PCH:

1) The molecule does not move significantly during a time bin

2) The molecular brightness is constant in time and follows the spatial profile of the excitation volume (no reactions, photophysics, etc . . .)

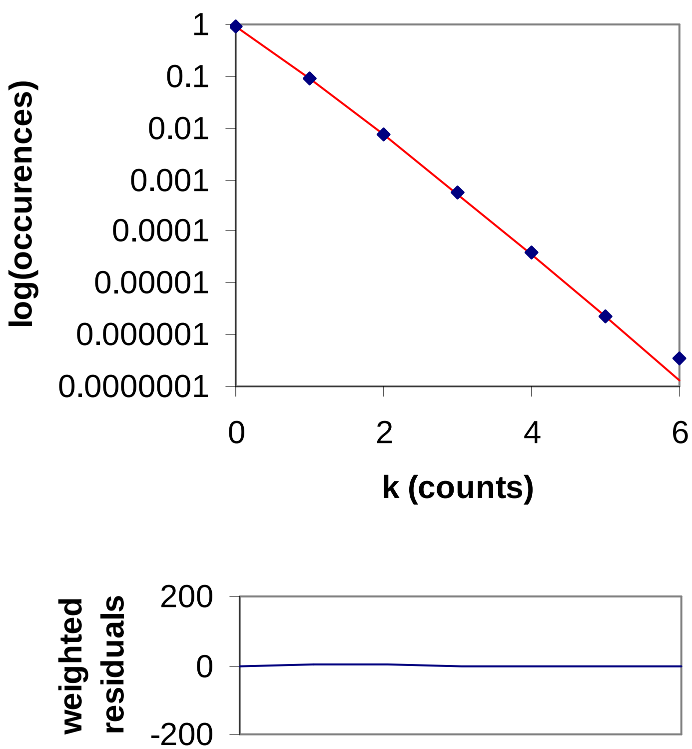
Factors Effecting PCH (Recap)



Saturation Effects

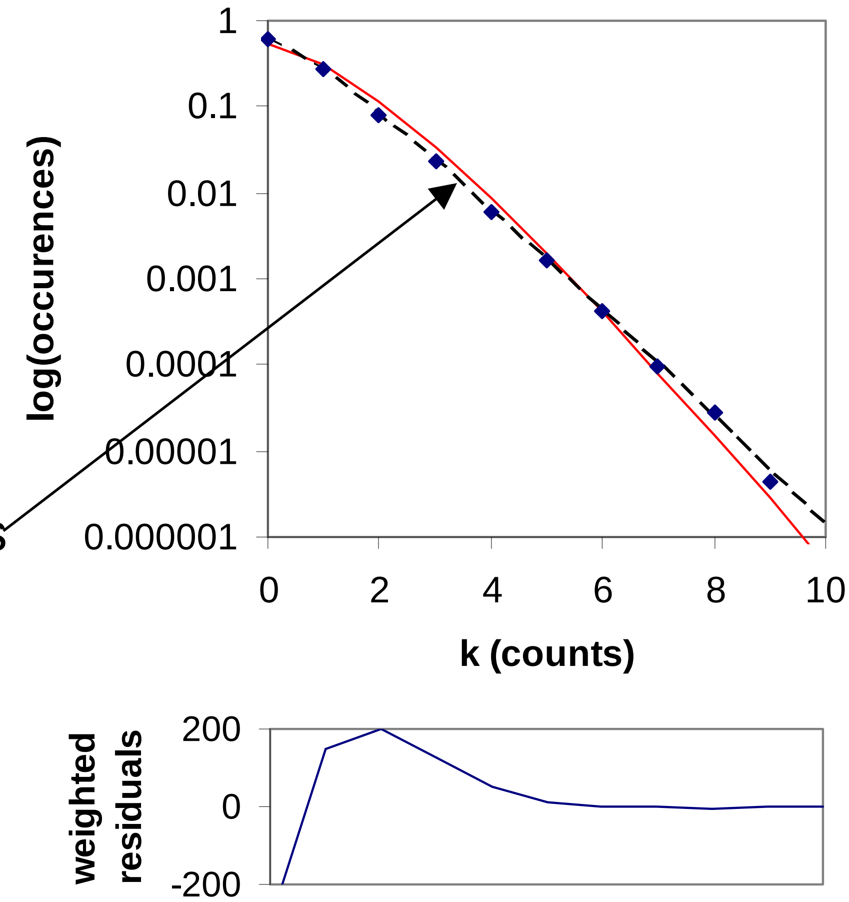
Rhodamine 110 on the Zeiss Confocor 3

10 uW laser



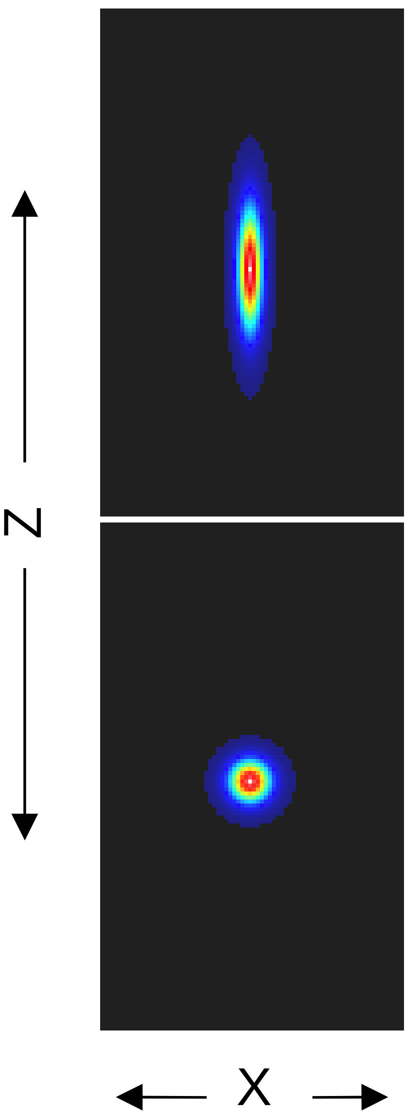
60 uW laser

Multi-Species Fit



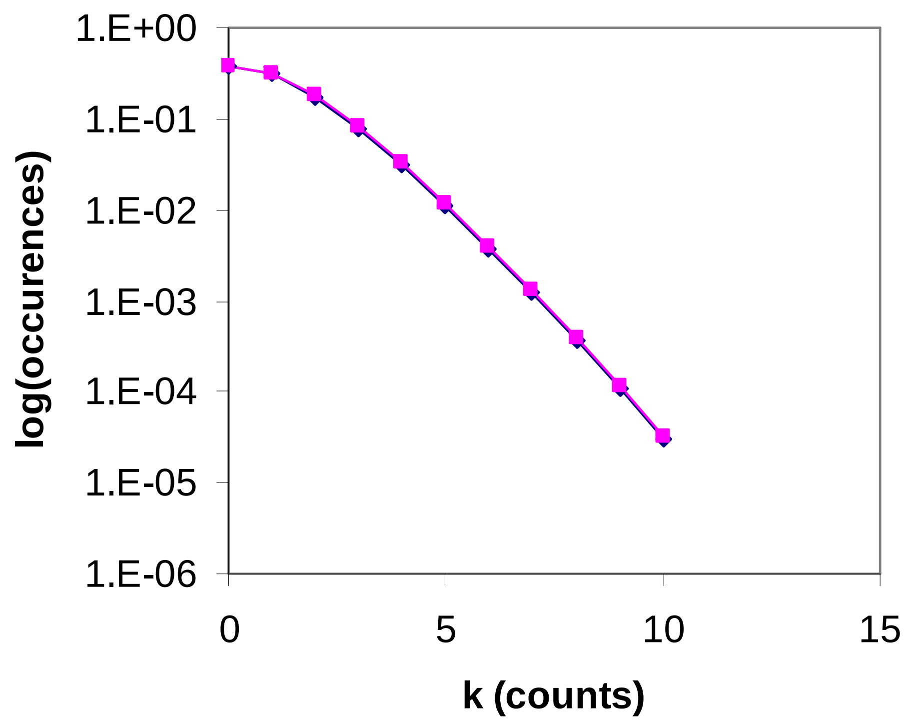
Laser power is not an infinite source of brightness!

Size of the PSF does not influence the PCH

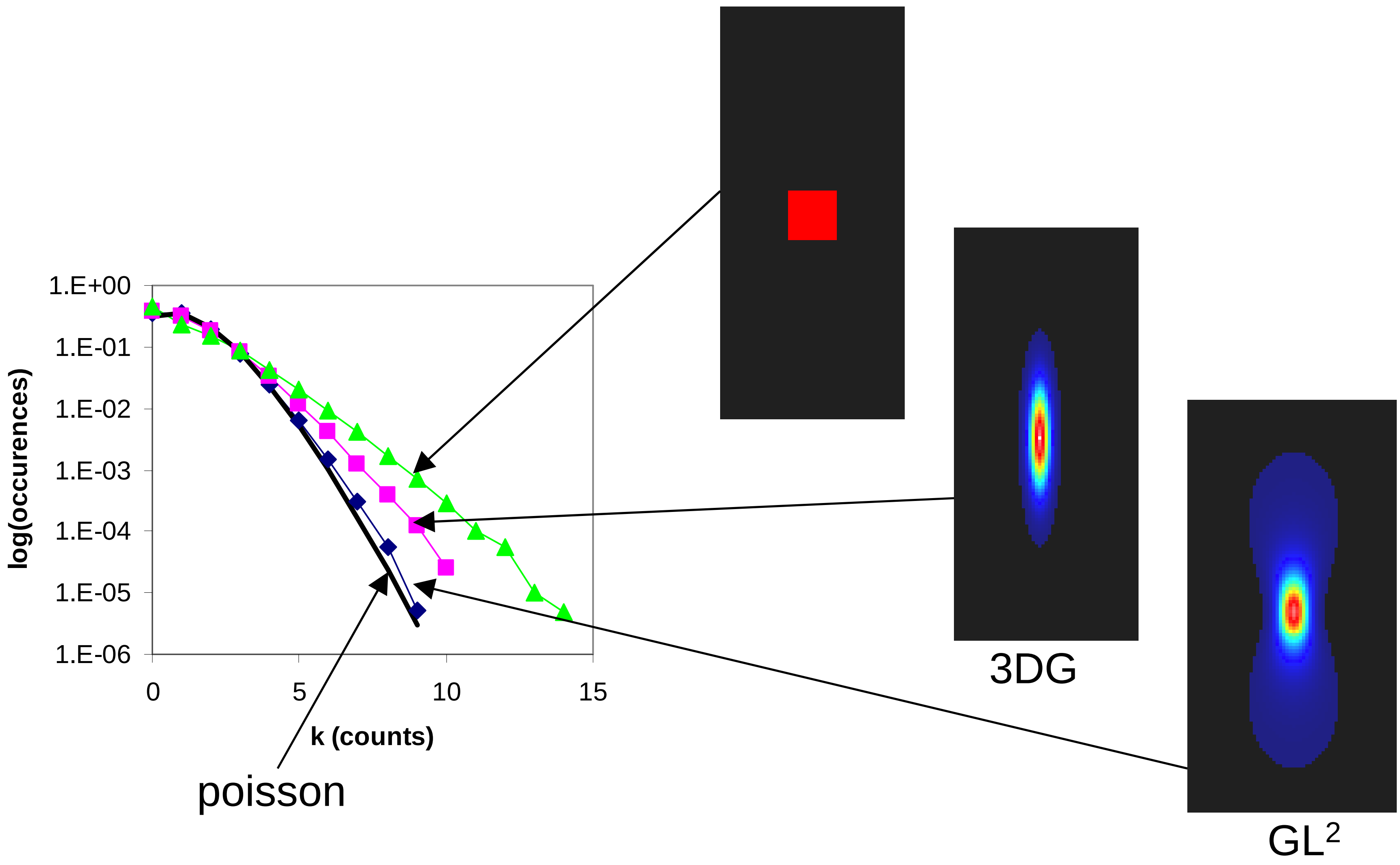


$V_{PSF} = 0.08$ fL

$V_{PSF} = 0.08$ fL

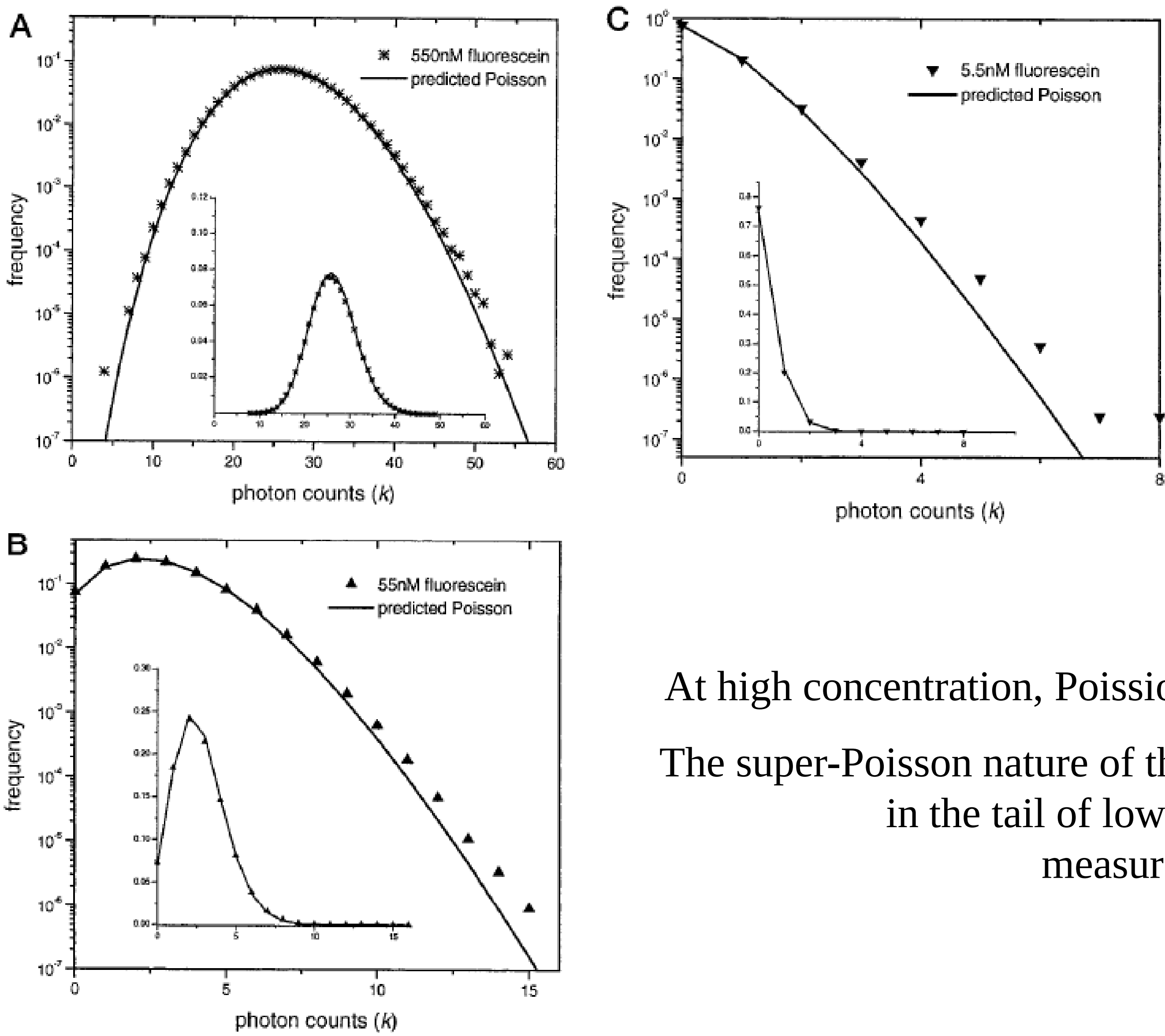


Functional Form Influences the Distribution



PCH versus Concentration

PCH measurements and Fits to Poisson distribution



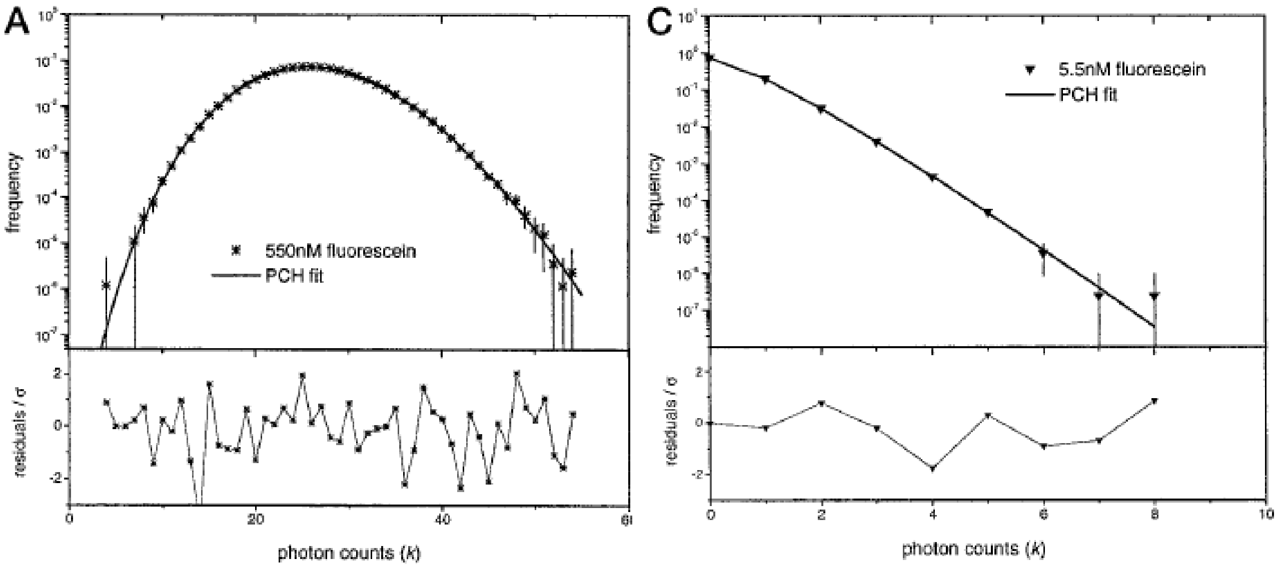
From: Chen *et al*. 1999
Biophys J 77:553

At high concentration, Poission statistics dominate

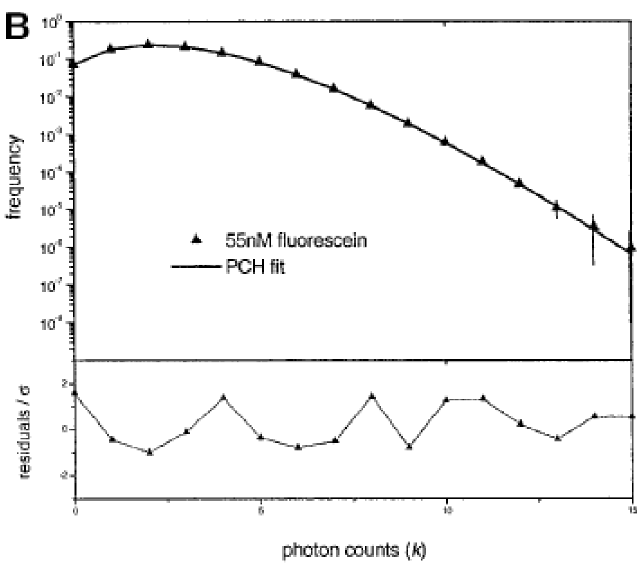
The super-Poisson nature of the distribution is seen in the tail of lower concentration measurements

PCH versus Concentration

Determination of $\langle N_{PSF}\rangle$ and ε by fitting to the probability function



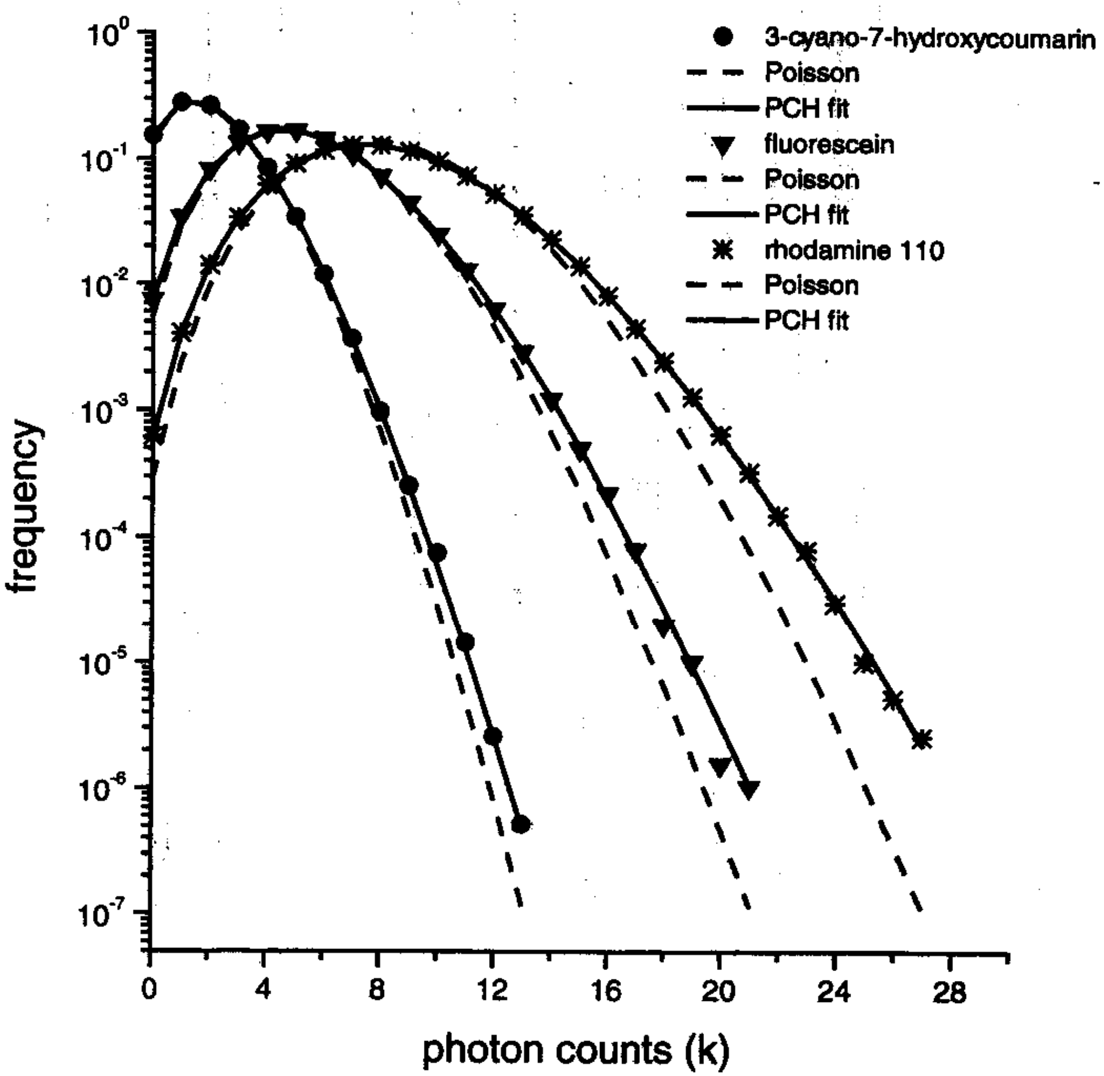
From: Chen *et al.* 1999
Biophys J 77:553



$$\Pi\left(k;\langle N_{PSF}\rangle,\varepsilon\right)=\sum_{N=0}^{\infty}p^{(N)}\left(k;V_0,\varepsilon\right)\mathrm{Poi}\left(N,\langle N_{PSF}\rangle\right)$$

c (nM)	*c* (nM)/ 5.5 nM	⟨k⟩	⟨k⟩/ 0.28	ε	⟨N⟩	⟨N⟩/ 0.347	χ^2
550	100	26.25	93.8	0.807	32.53	93.7	1.14
55	10	2.71	9.7	0.807	3.36	9.7	0.98
5.5	1	0.28	1	0.807	0.347	1	0.84

PCH versus ε

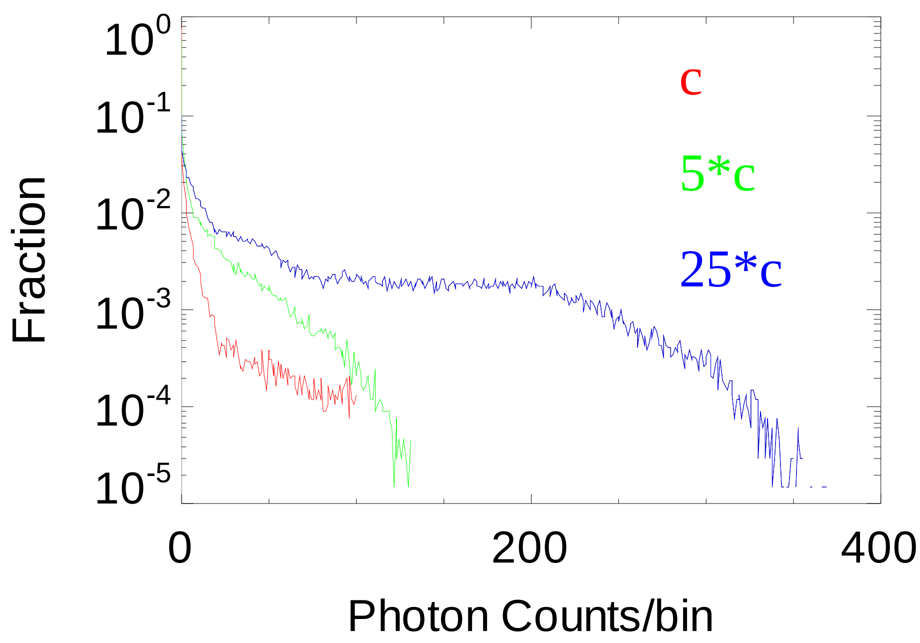


The brighter the molecule, the more clearly the non-Poissonian statistics are observable

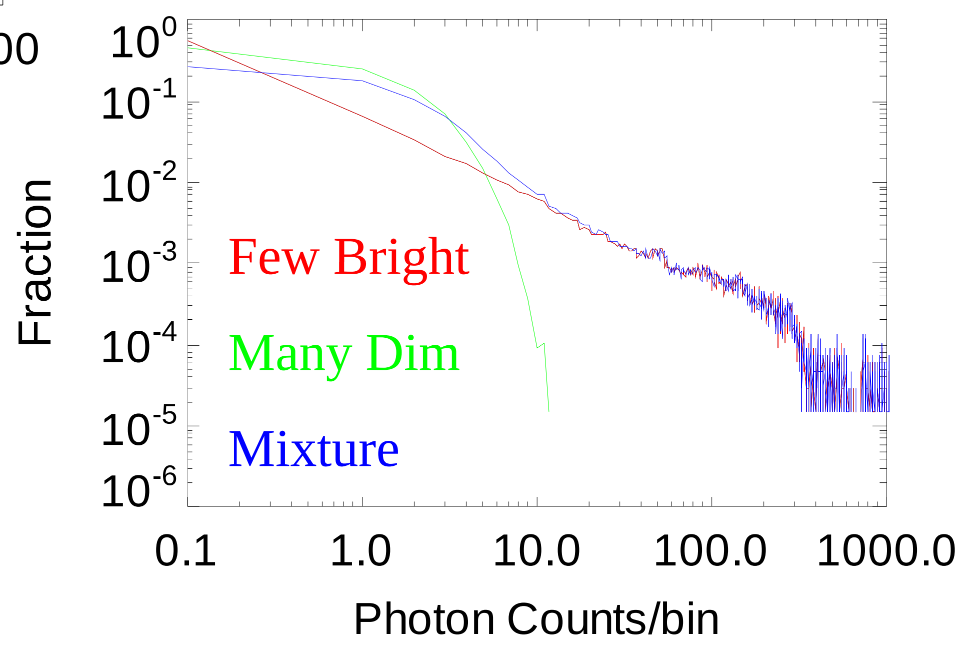
From: Chen *et al*. 1999
Biophys J 77:553

Bright, Slow Molecules

PCH vs Concentration



PCH with and without many dim molecules



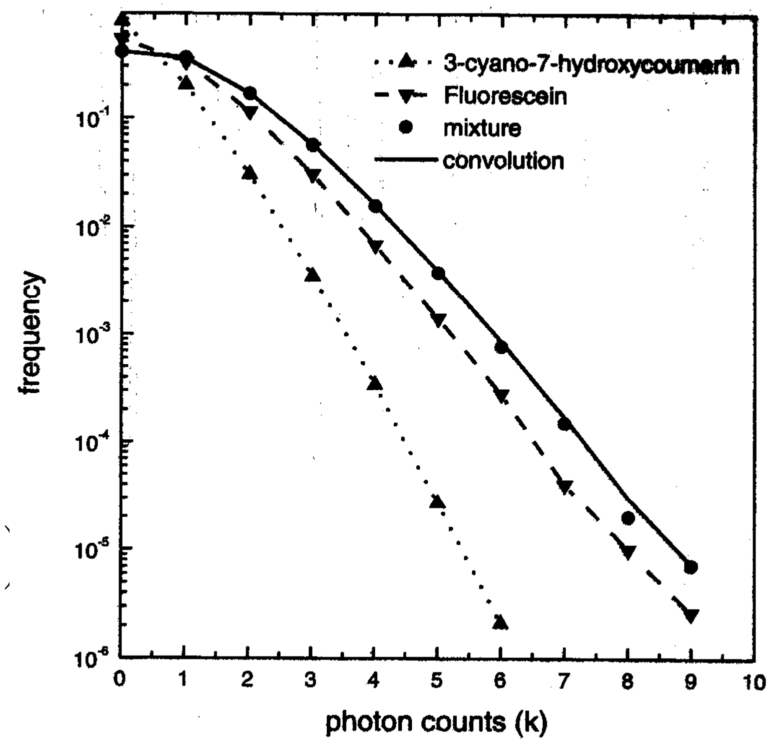
Multiple Species

PCH distinguishes between different species via the molecular brightness, independent of the diffusion time.

$$p^{(N_1,N_2)}(k;V_0,\varepsilon_1,\varepsilon_2)=\int_1 \dots \int_{N_1+N_2} \prod_i^{N_1+N_2} P(\mathbf{r}_i)\,d\mathbf{r}_i$$

$$\mathrm{Poi}\left(k,\varepsilon_1\sum_i^{N_1} PSF(\mathbf{r}_i)+\varepsilon_2\sum_j^{N_2} PSF(\mathbf{r}_j)\right)$$

$$\Pi\left(k;\langle N_1\rangle,\varepsilon_1,\langle N_2\rangle,\varepsilon_2\right)=\Pi\left(k;\langle N_1\rangle,\varepsilon_1\right)\otimes\Pi\left(k;\langle N_2\rangle,\varepsilon_2\right)$$

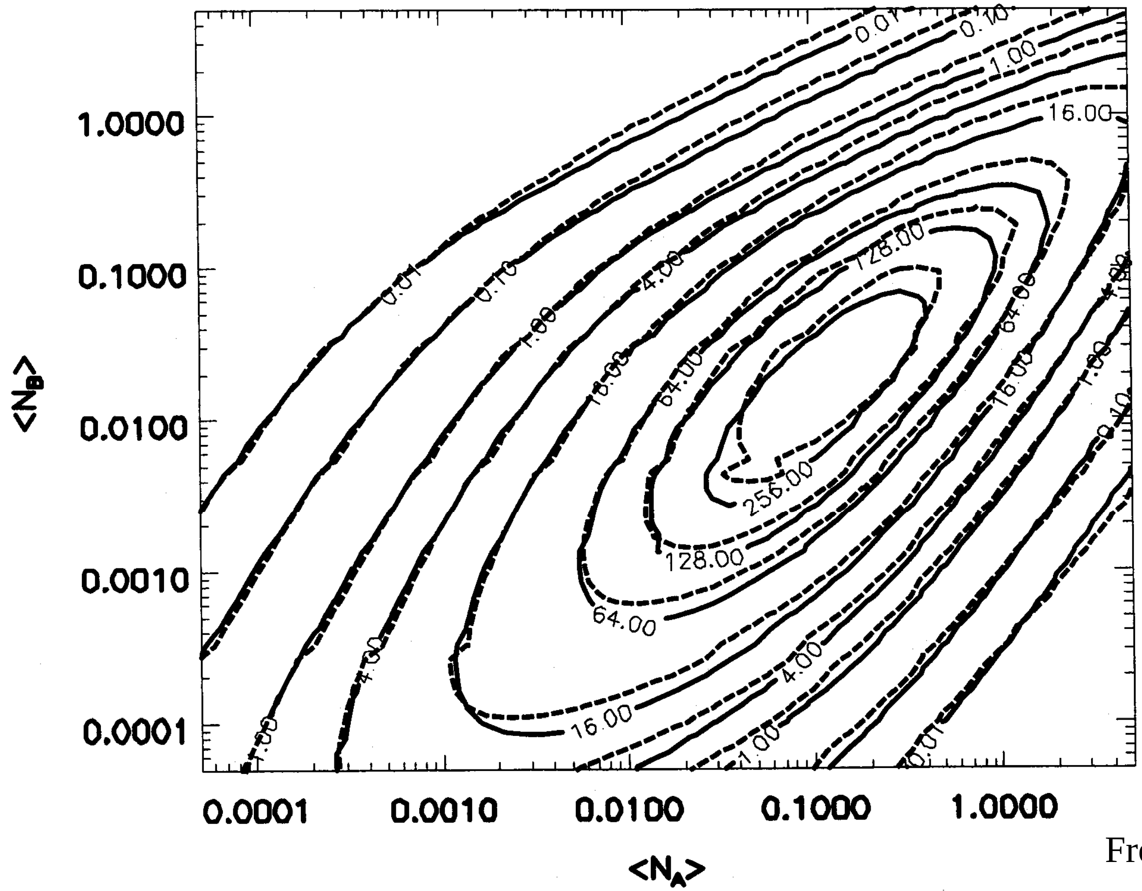


Background, Dark counts, scattered laser light, etc. can be treated as an additional species

From: Chen *et al*. 1999 *Biophys J* 77:553

Resolvability

χ^2 misfit contour map for 1.6×10^7 photons with $\varepsilon_A = 1.5$ and $\varepsilon_B = 6.0$ (solid lines) and $\varepsilon_A = 0.25$ and $\varepsilon_B = 1.0$ scaled by 61.6 (dashed lines)



From: Müller, Chen, Gratton 2000 *Biophys J* 78:474

The small number of data points in the fit limitations the number of parameters one can reliably fit to.

Alternative Methods

- Fluorescence Cumulant Analysis (FCA)
  - Mueller *Biophys. J.* **2004**, *86,* 3981.
  - Similar to method of moments
  - Any distribution can be described by a sum of moments
  - Simple algebraic formulas for cumulants

- Fluorescence Intensity Distribution Analysis (FIDA)
  - Kask et al. *PNAS* **1999,** *96,* 13756.
  - Fits PSF in fourier transformed space
  - Fits to non-physical parameterized PSF

2D PCH

With two channel detection, 2D PCH can be analyzed

Chen et al., *Biophys. J.*, **2005,** *88*, 2177.