The Photon Counting Histogram: Statistical Analysis of Single Molecule Populations

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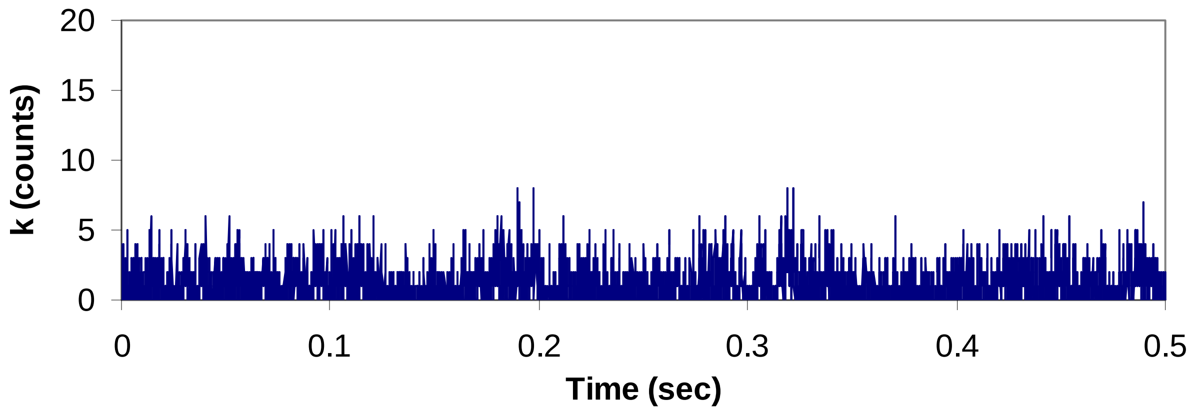
Transition from FCS

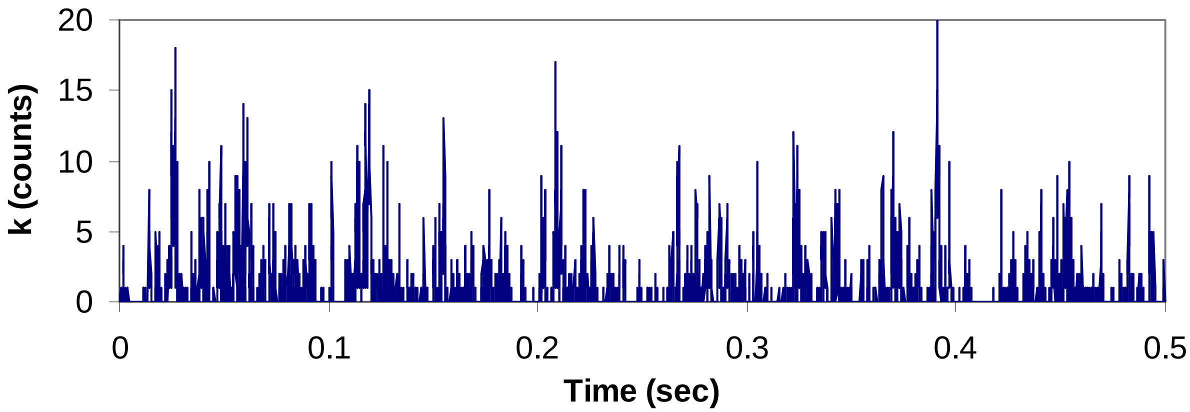
- The Autocorrelation function only depends on fluctuation duration and fluctuation density (independent of excitation power)
- PCH: distribution of intensities (independent of time)

Fluorescence Trajectories



Fluorescent Monomer:

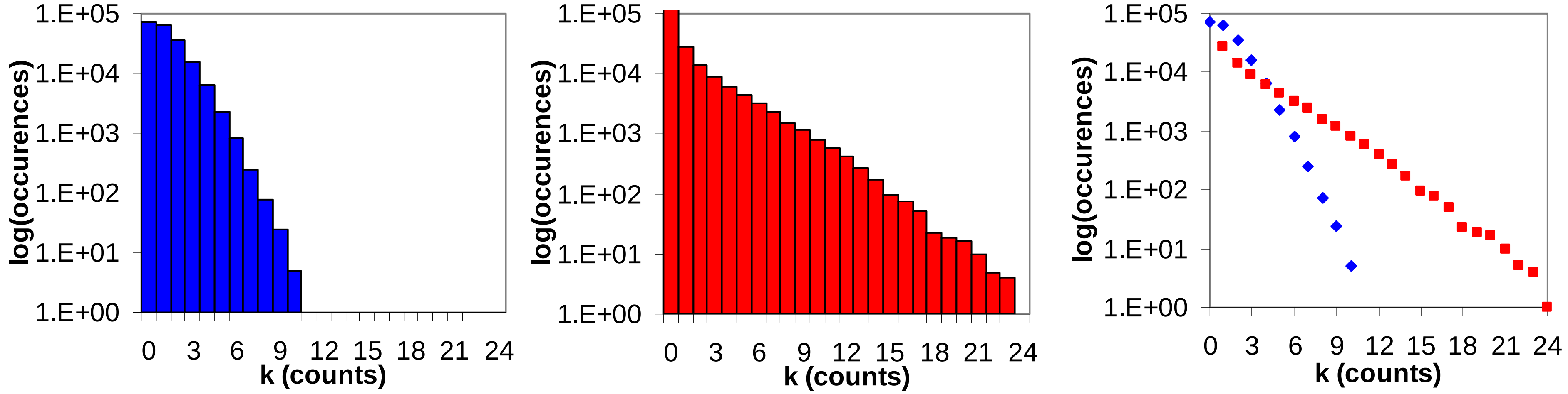
Intensity = 115,000 cps



Aggregate:

Intensity = 111,000 cps

Photon Count Histogram (PCH)

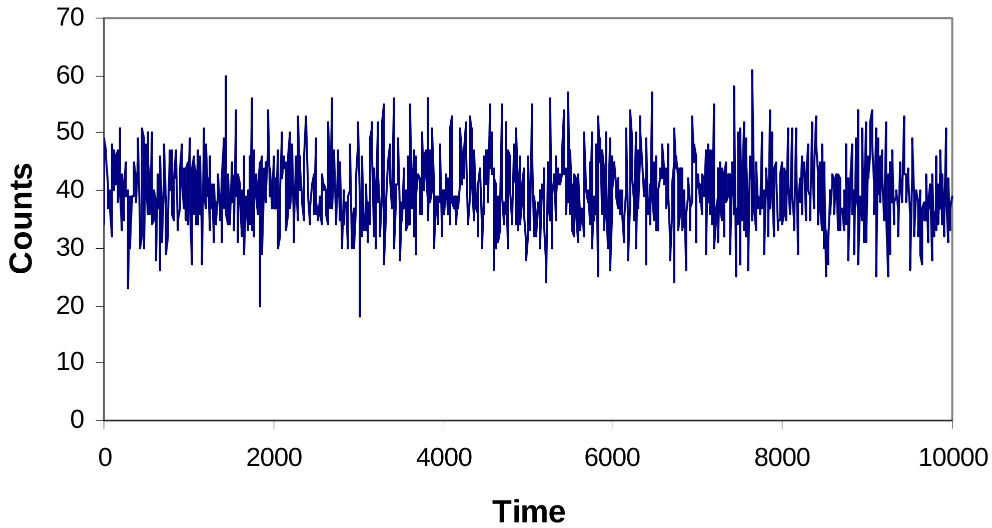


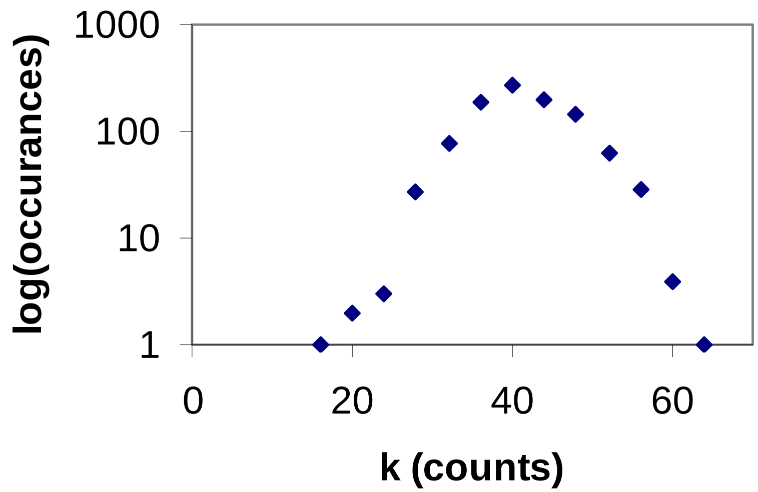
Can we quantitate this?

What contributes to the distribution of intensities?

Contribution from the detector noise

Fixed Particle Noise (Shot Noise)



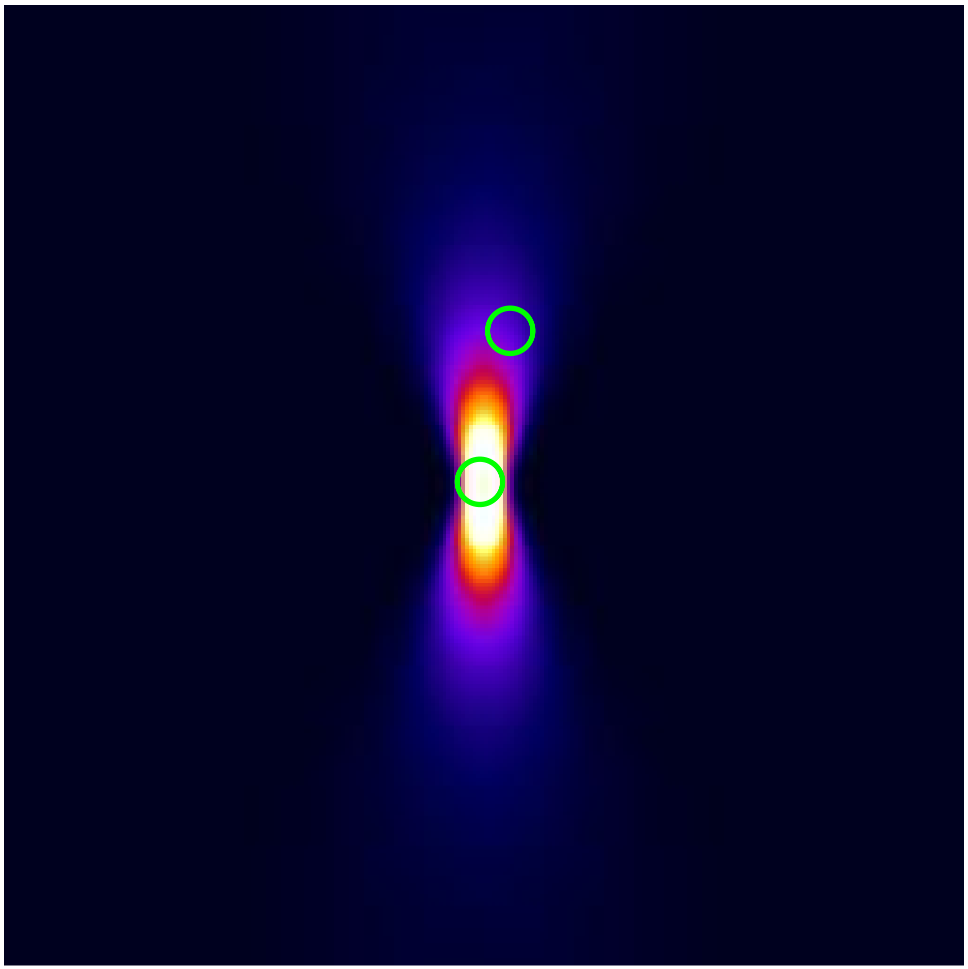


Noise is Poisson

$$Poi(k,\langle k\rangle) = \frac{\langle k\rangle^k}{k!}\exp(-\langle k\rangle)$$

Contribution from the profile of illumination

The Point Spread Function (PSF)



One Photon Confocal:

$$I_{3DG}(r,z) = \exp\left(-\frac{2r^2}{\omega_0^2} - \frac{2z^2}{z_0^2}\right)$$

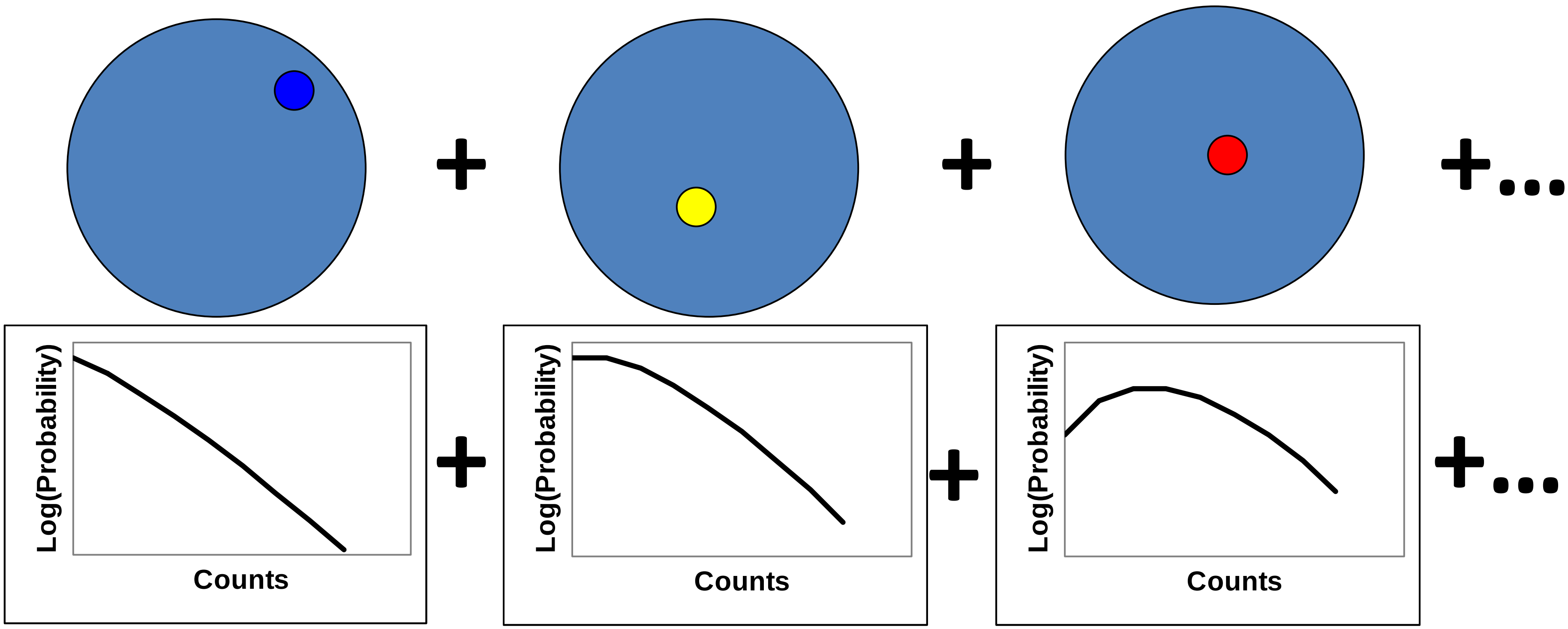
Two Photon:

$$I_{GL^2}(r,z) = \frac{4\omega_0^4}{\pi^2\omega^4(z)} \exp\left(-\frac{4r^2}{\omega^2(z)}\right)$$

$$\omega^2(z) = \omega_0^2\left(1+\left(\frac{z}{z_R}\right)^2\right)$$

$$z_R = \frac{\pi\omega_0^2}{\lambda}$$

Single Particle PCH



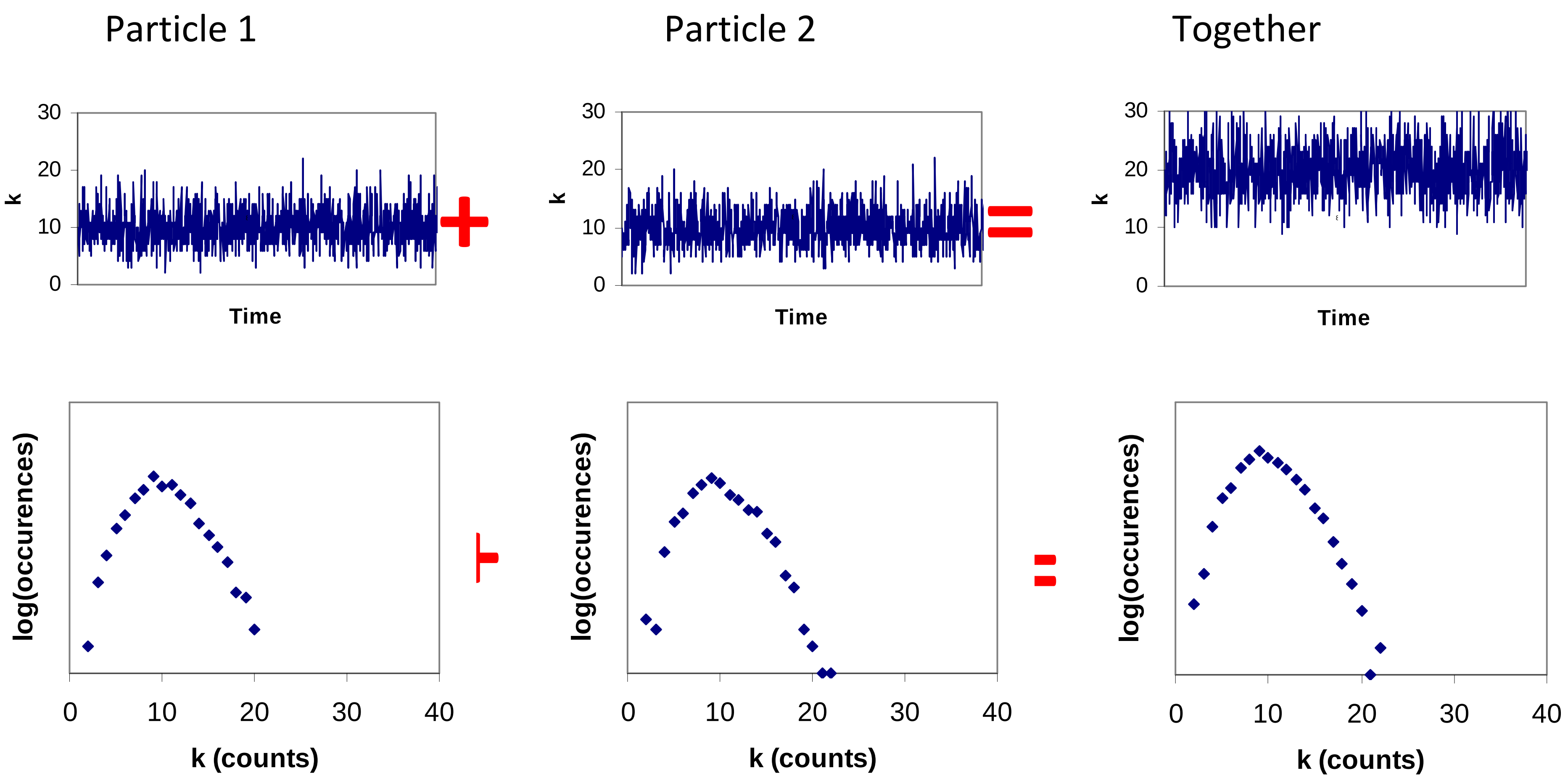
Have to sum up the poissonian distributions for all possible positions of the particle within the PSF

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

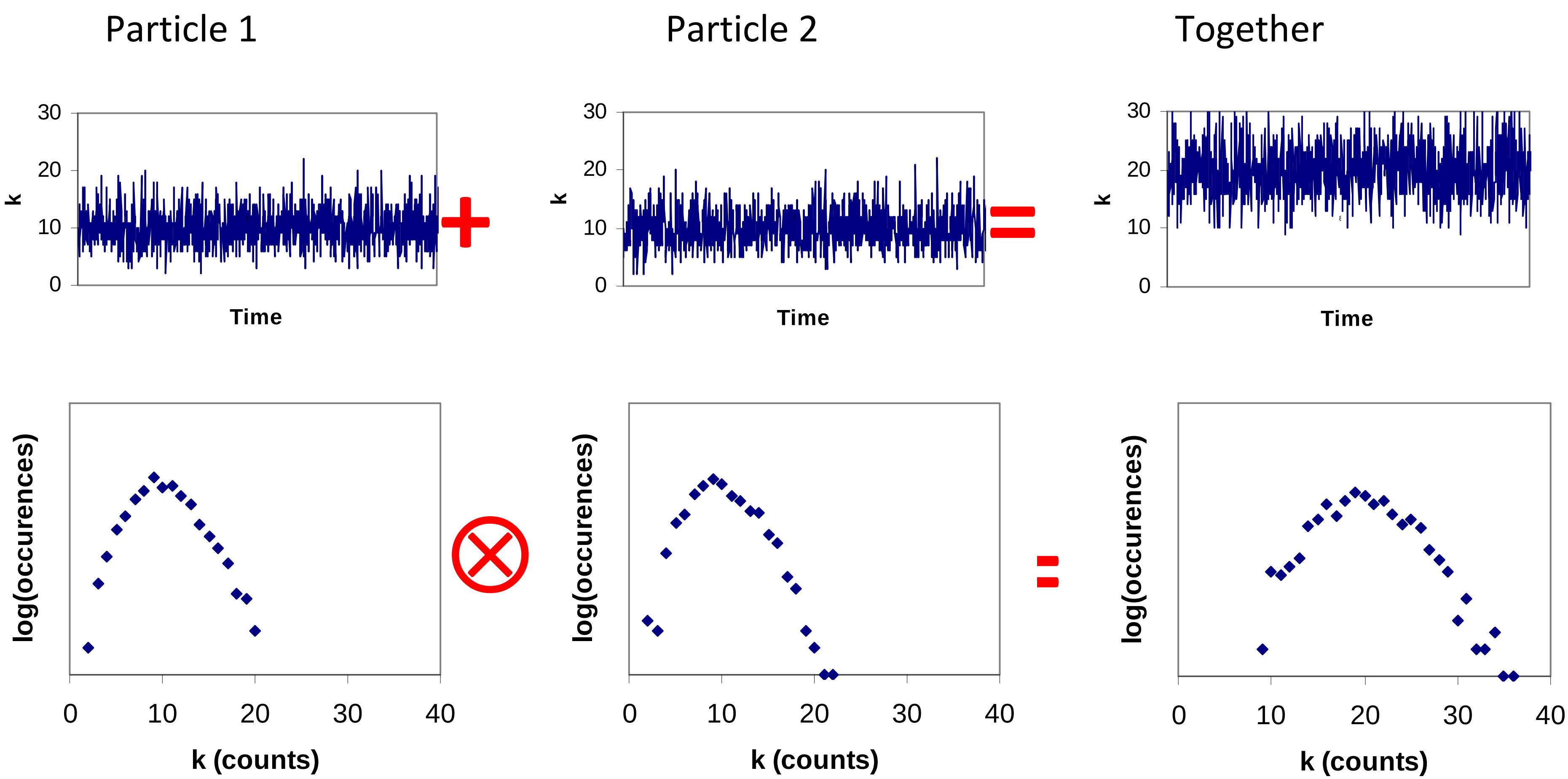
- What if I have two particles in the PSF?
- Have to calculate every possible position of the second particle for each possible position of the first!

Contribution from several particles of same brightness

Combining Distributions

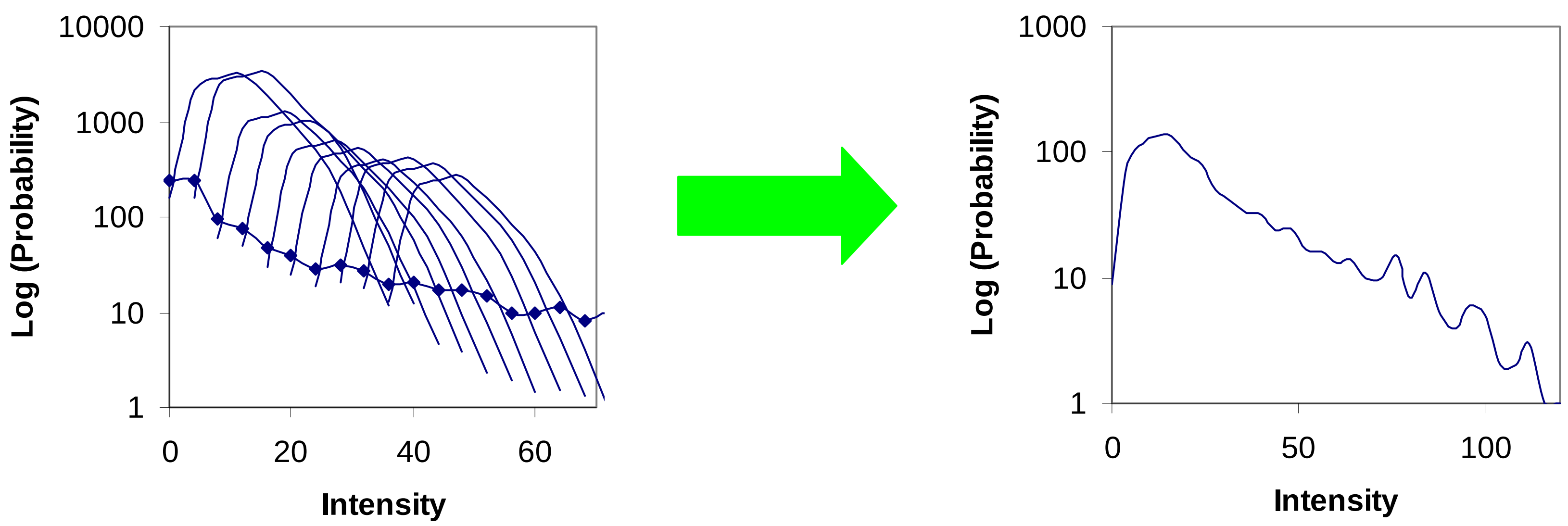


Combining Distributions



Convolution

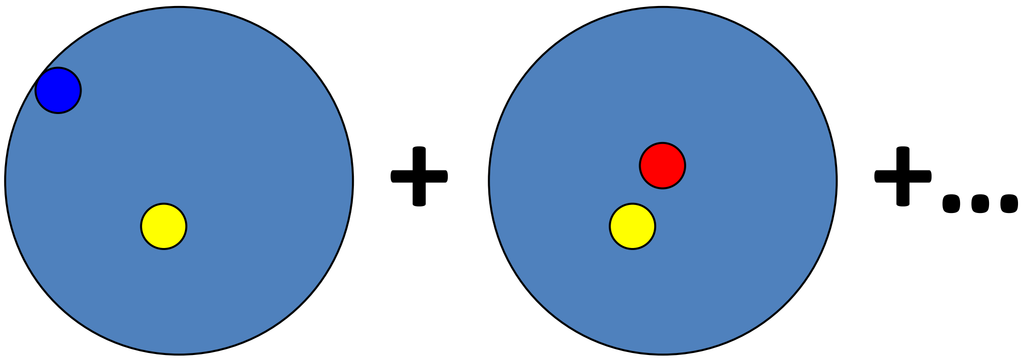
- Sum up all combinations of two probability distributions (joint probability distribution)
- Distributions (particles) must be independent



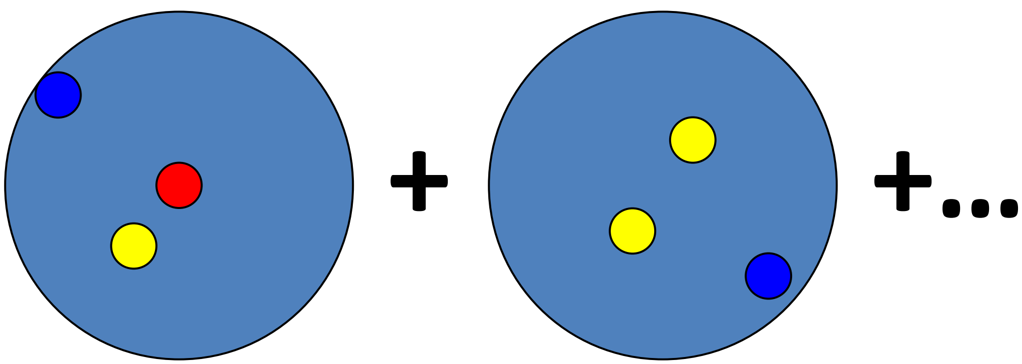
$$p^{(1+2)}(k) = \sum_{r=0}^{r=k} p^{(1)}(k-r) \cdot p^{(2)}(r)$$

Contribution from particles of different brightness

More Particles



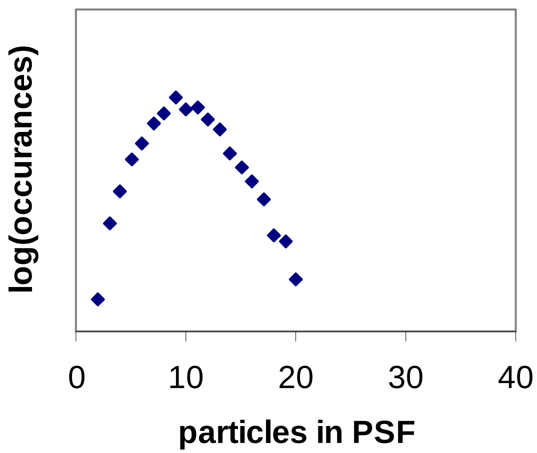
$$p^{(2)}(k) = p^{(1)}(k) \otimes p^{(1)}(k)$$



$$p^{(3)}(k) = p^{(1)}(k) \otimes p^{(2)}(k)$$

$$p^{(n)}(k) = p^{(1)}(k) \otimes p^{(n-1)}(k) = \sum_{r=0}^{r=k} p^{(1)}(k-r) \cdot p^{(n-1)}(r)$$

How Many Particles Do We Have in the PSF?



$$P(n,N) = Poi(n,N)$$

Particle occupation fluctuates around average, N with a poissonian distribution

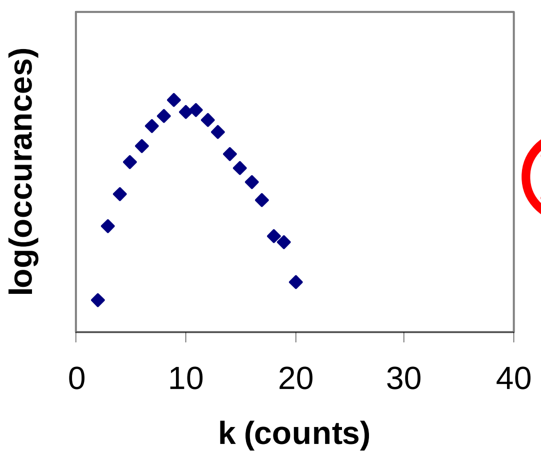
Calculate poisson weighted average of n particle distributions

$$PCH(k,N) = \sum_n p^{(n)}(k) \cdot P(n,N)$$

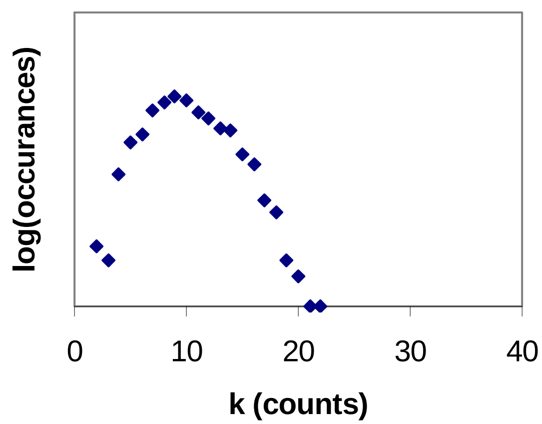
Multiple Species

- Species are independent so just convolute!

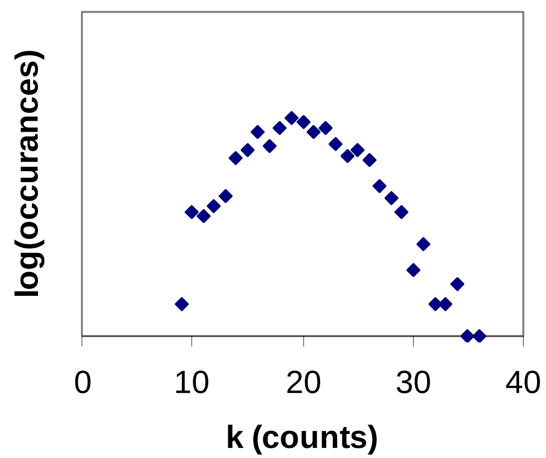
1 uM Fluorescein



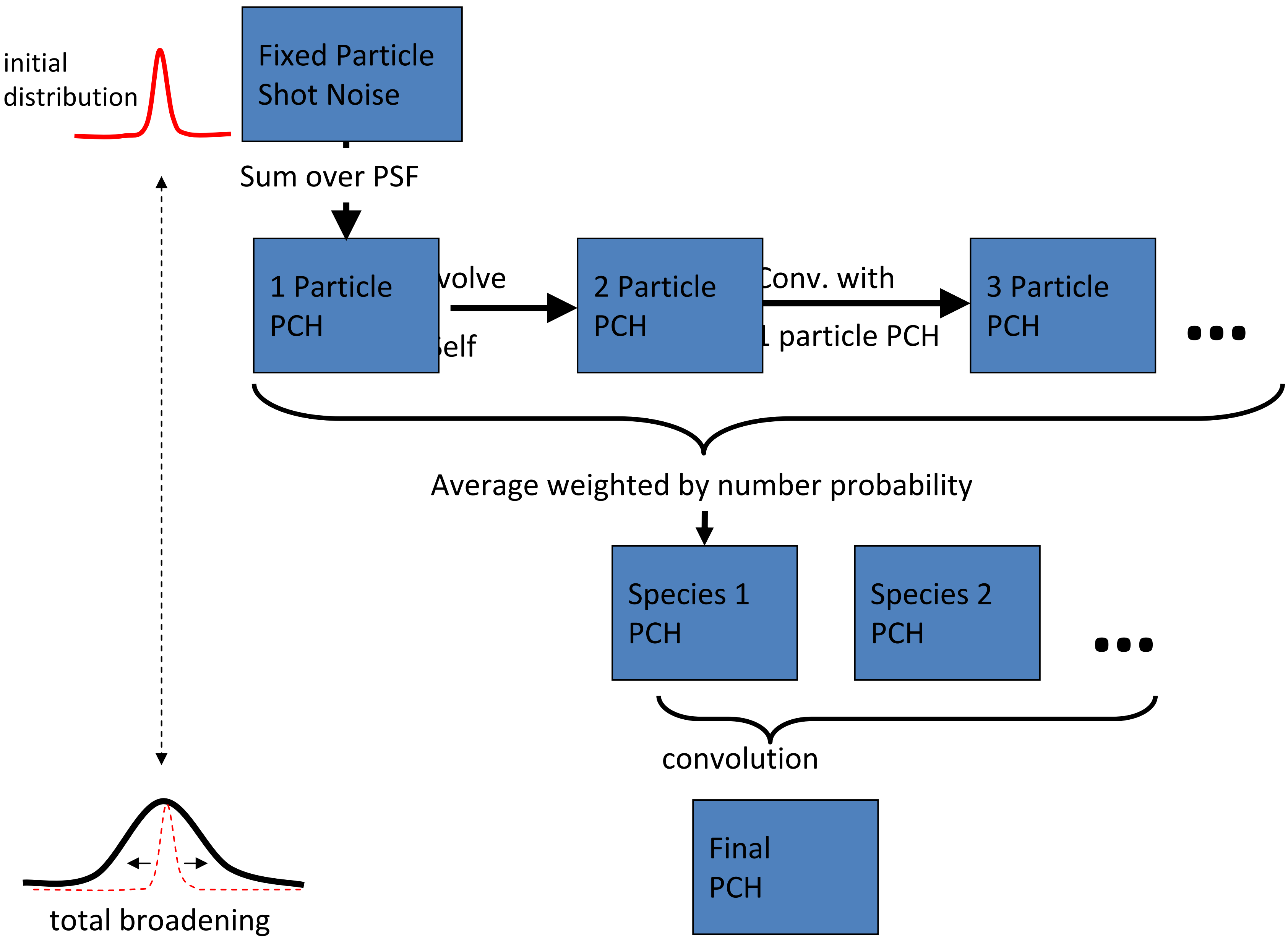
1 uM R110



1 uM Fl & 1uM R110



Recap: Factors that contribute to the final broadening of the PCH



Method

- Sum up Poisson distributions from all possible arrangements and number of fluorophores in excitation volume (PSF)
  - Intensity weighted sum of all possible single particle histograms (Poisson functions)
  - Convolution to get multiple particle histograms
  - Number probability weighted sum of multiple particle histograms
  - Convolution to get multi-species histograms

Chen et al., *Biophys. J.*, **1999,** *77,* 553.

Fitting

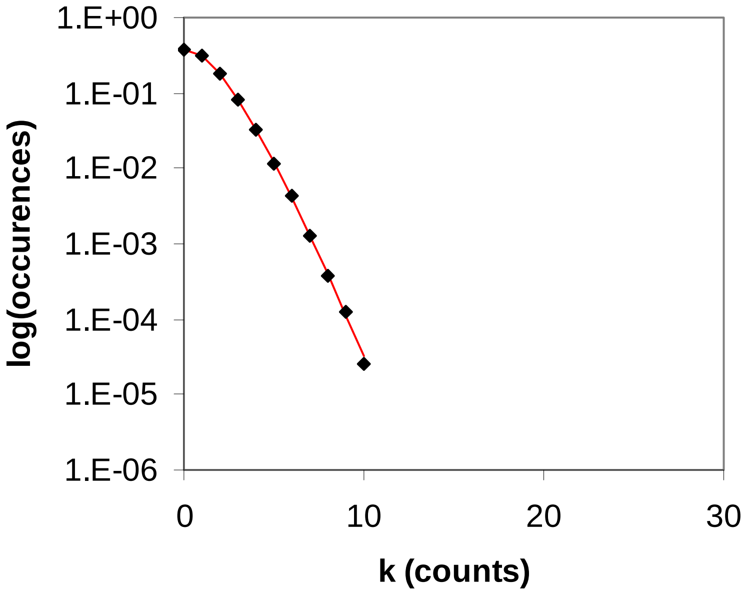
$$\chi^2 = \frac{\sum_k \left(M \frac{PCH_{model}(k) - PCH_{observed}(k)}{\sqrt{M \cdot PCH_{observed}(k) \cdot (1 - PCH_{observed}(k))}} \right)^2}{k_{\max} - d}$$

M is number of observations

d is number of fitting parameters

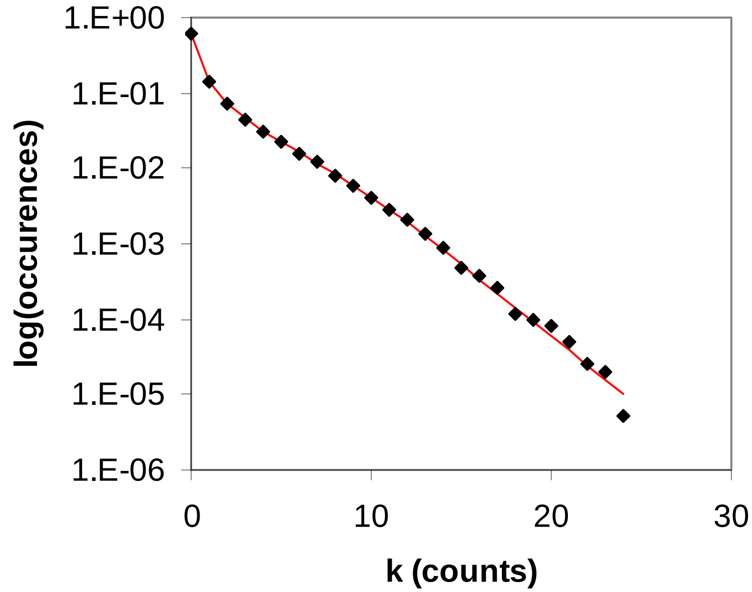
Chen et al., *Biophys. J.*, **1999,** *77,* 553.

Model Test



ε = 9,030 cpsm

N = 1.28



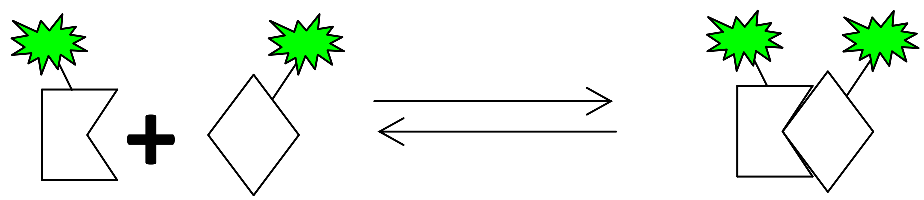
ε = 91,330 cpsm

N = 0.12

Hypothetical situation: Protein Interactions

- 2 proteins are labeled with a fluorophore
- Proteins are soluble
- How do we assess interactions between these proteins?

Dimer has double the brightness

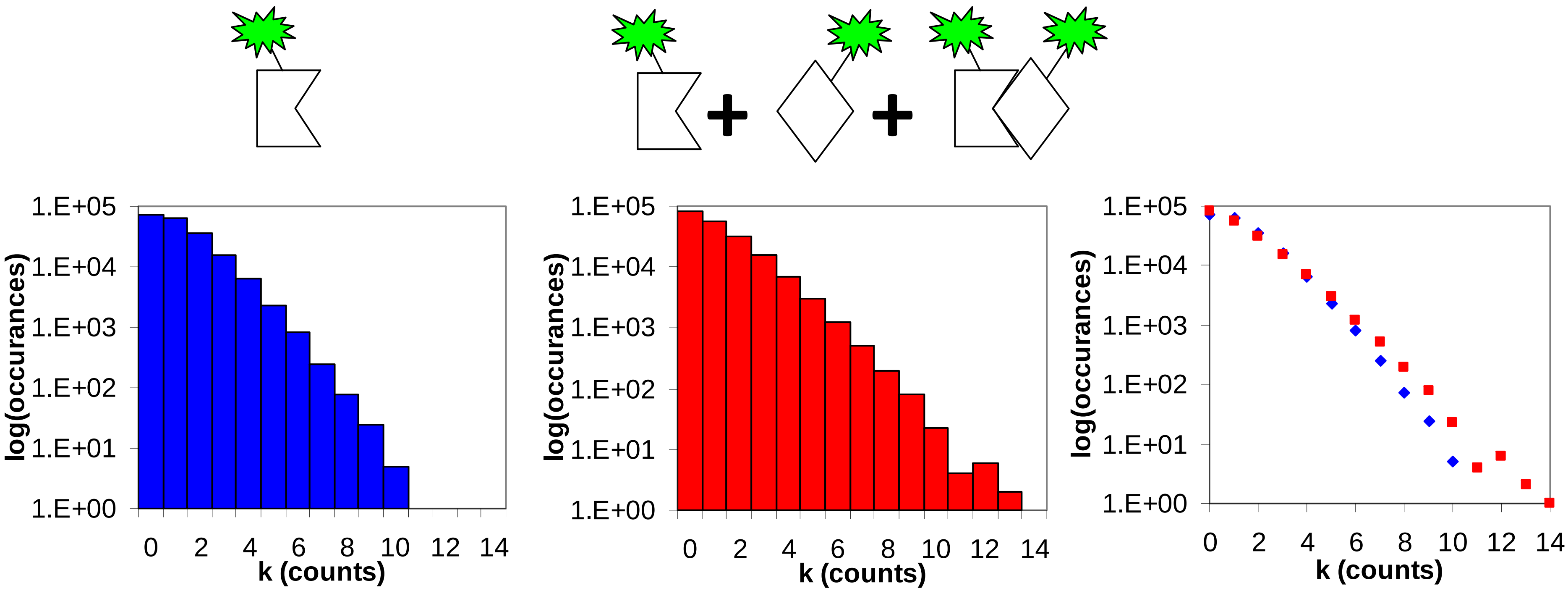


$\varepsilon = \varepsilon_{monomer}$ $\varepsilon = 2 \times \varepsilon_{monomer}$

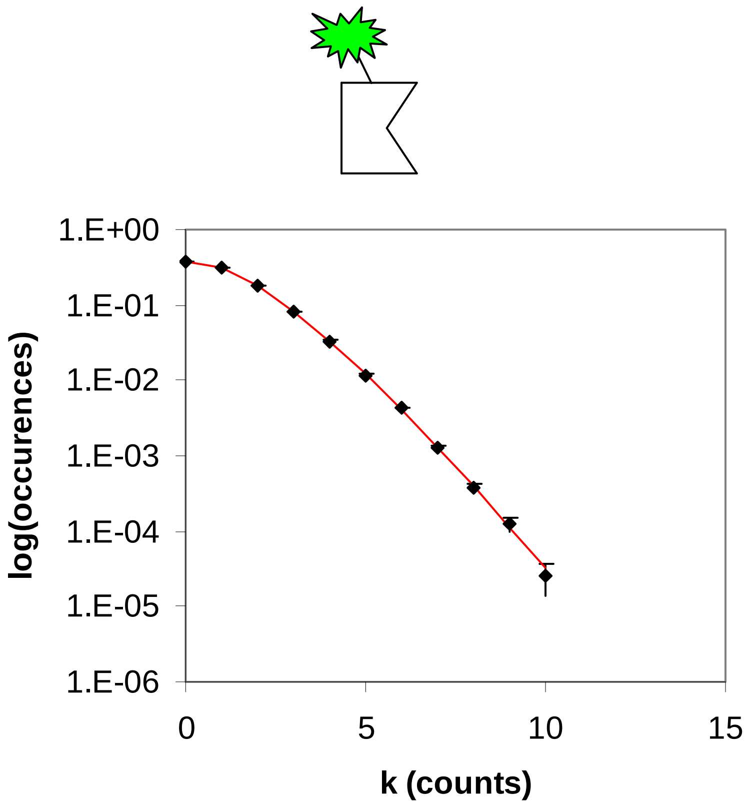
All three species are present in equilibrium mixture

Typical one photon $\varepsilon_{monomer}$ = 10,000 cpsm

Photon Count Histogram (PCH)

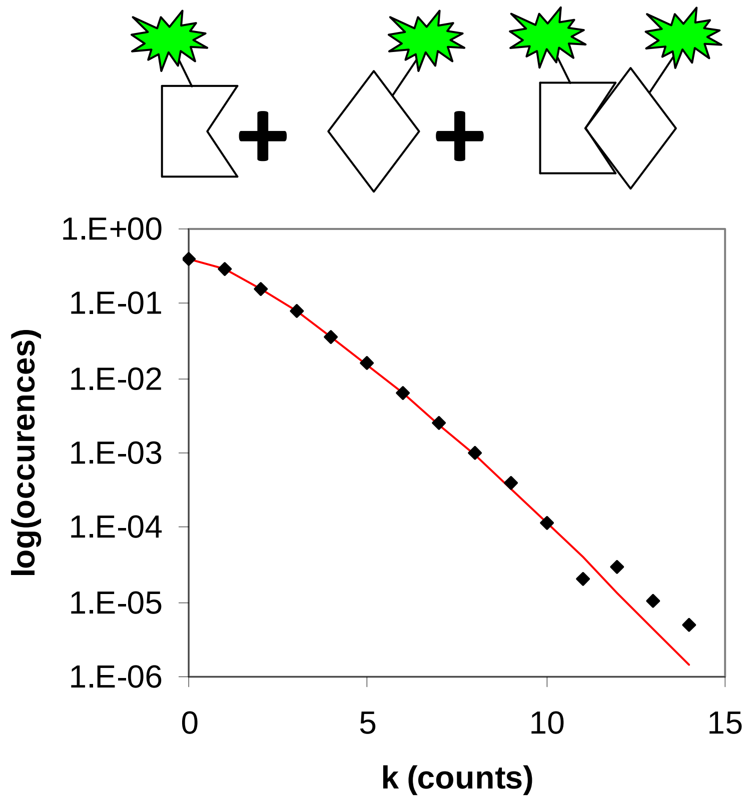


Simulation Solution



ε = 9,000 cpsm

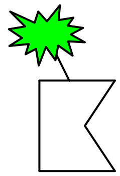
N = 1.3

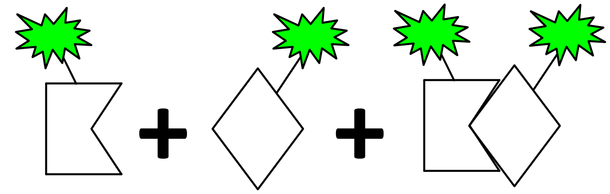


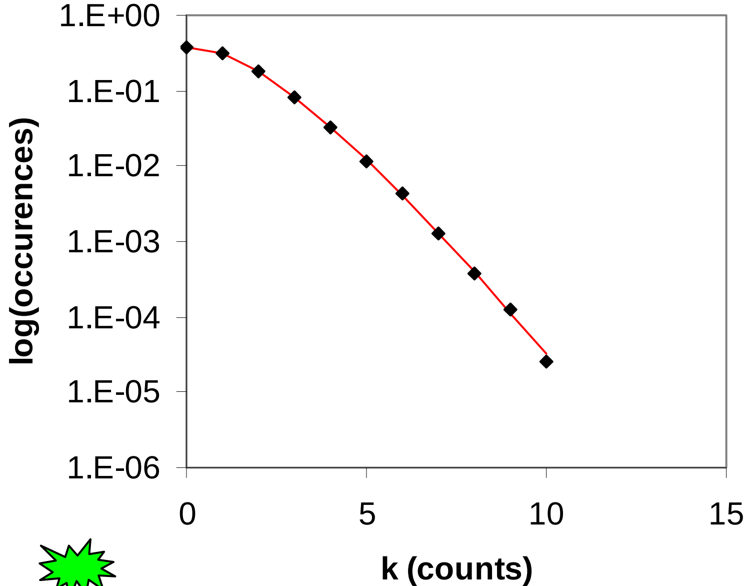
ε = 16,000 cpsm

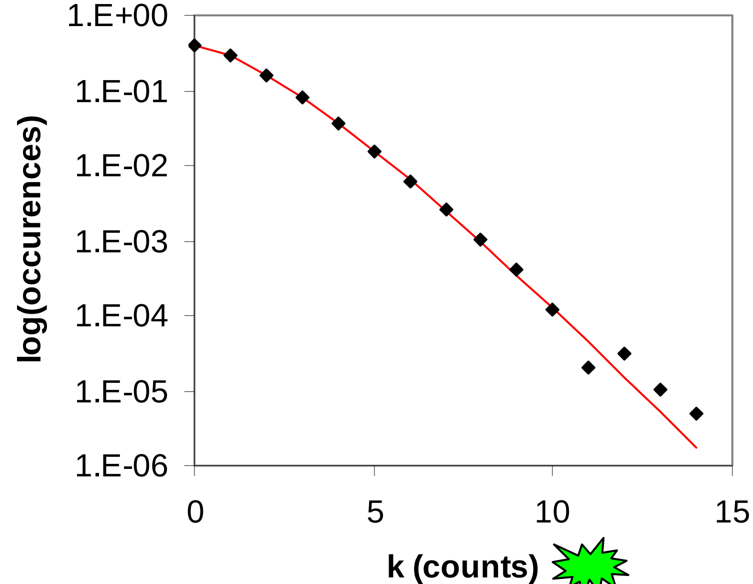
N = 0.73

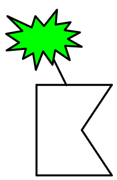
Global Fitting: Fit Data Sets Simultaneously







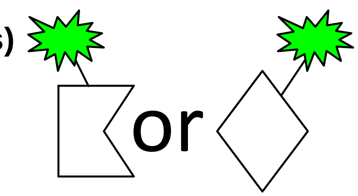


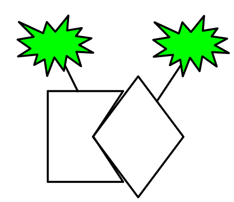


ε = 9,000 cpsm
N = 1.3

Link

ε_1 = 9,000 cpsm N_1 = 0.29 ε_2 = 18,100 cpsm N_2 = 0.50





What we measure is the number of particles in the PSF. How Do We Get Concentrations?

- N is defined relative to PSF volume
- One photon:

$$V_{3DG} = w_0^2 z_0 \left(\pi / 2\right)^{3/2} \qquad V_{PSF} = \int PSF(\vec{r})\, d\vec{r}$$

- Two photon:

$$V_{GL2} = \frac{\pi w_0^4}{\lambda}$$

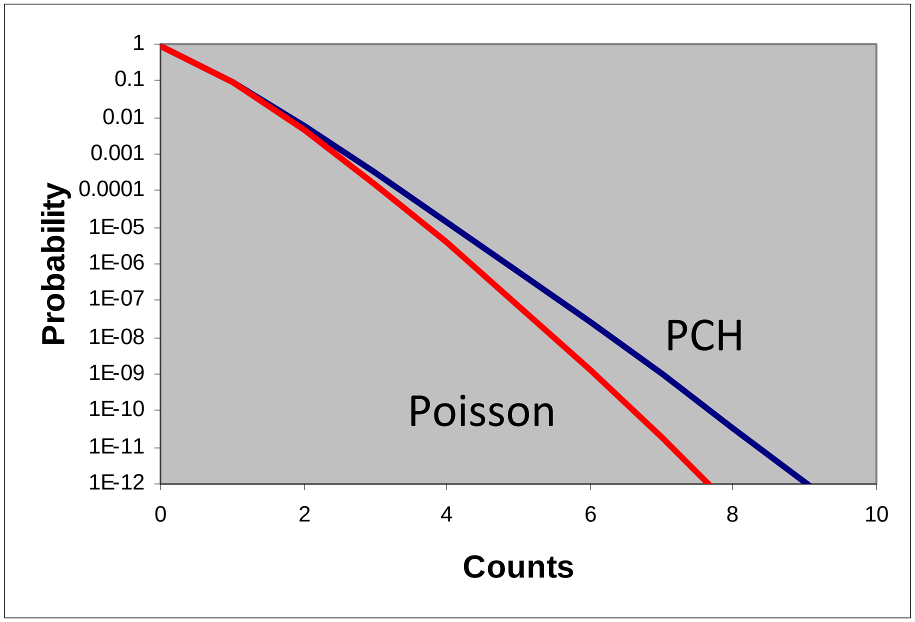
- Definition is same as for FCS
- Can use FCS to determine w_0 (and maybe z_0)

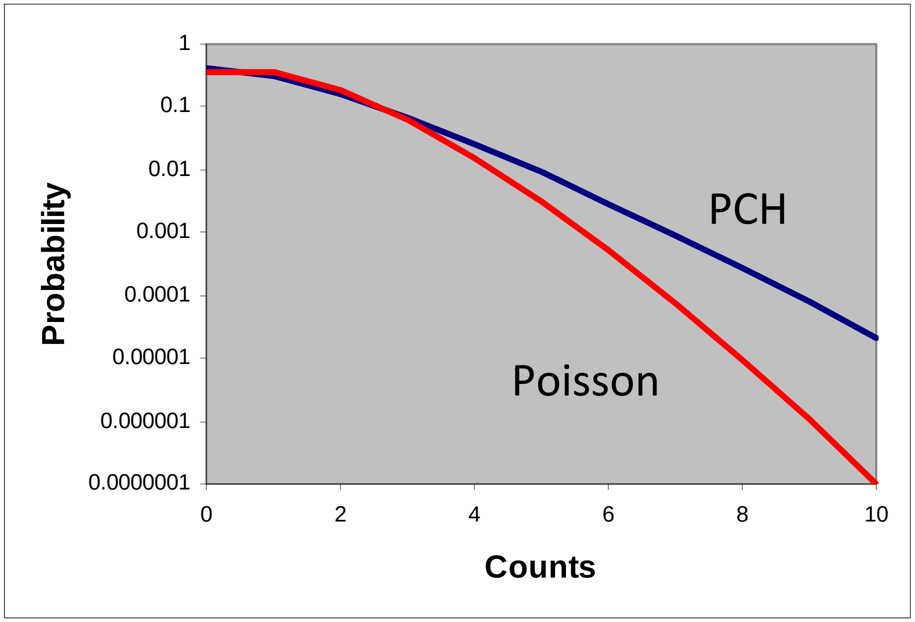
w_0 = 0.21 um, z_0 = 1.1 um, V_{PSF} = 0.091 um^3, C = 23 nM

How to Improve Accuracy

- Minimize sources of instrument noise
  - PSF heterogeneity
  - Shot noise
- Maximize particle burst amplitudes

Effect of Brightness





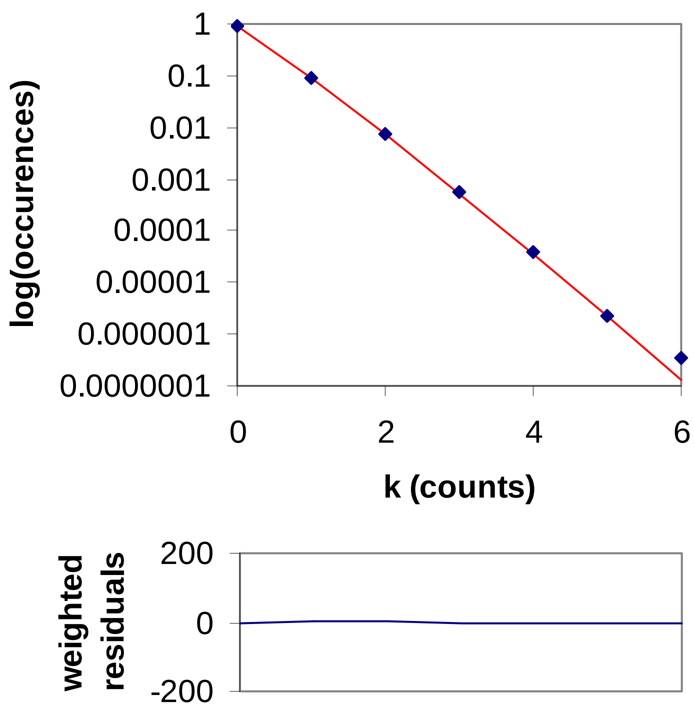
ε = 10,000 cpsm

ε = 100,000 cpsm

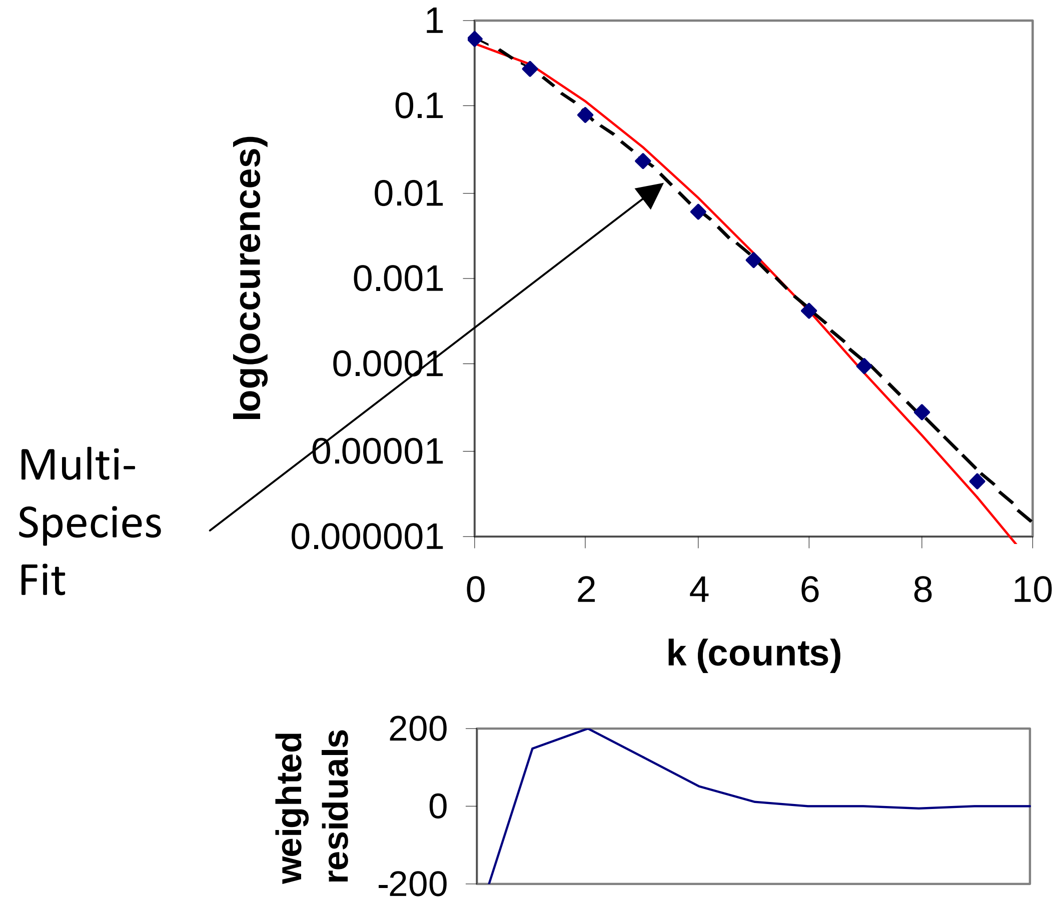
Saturation Effect

Rhodamine 110 on the Zeiss Confocor 3

10 uW laser

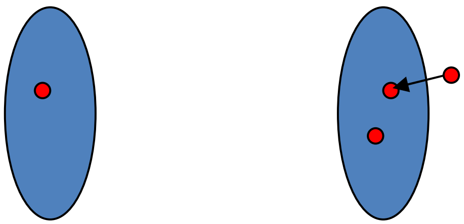


60 uW laser

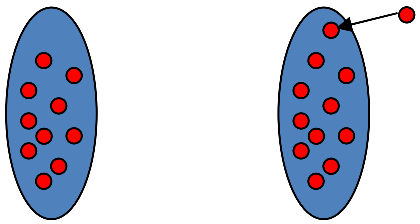


Laser power is not an infinite source of brightness!

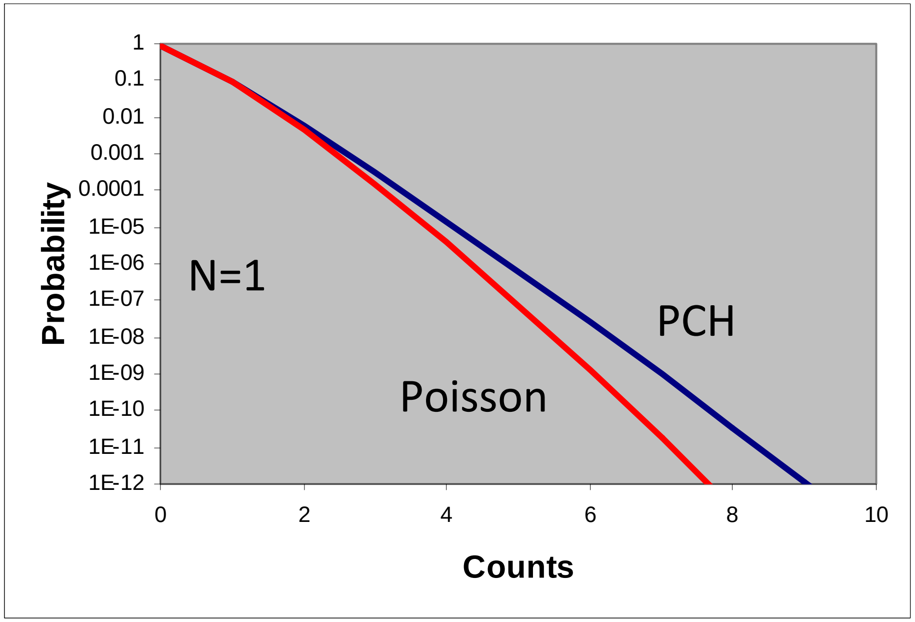
Concentration Effect

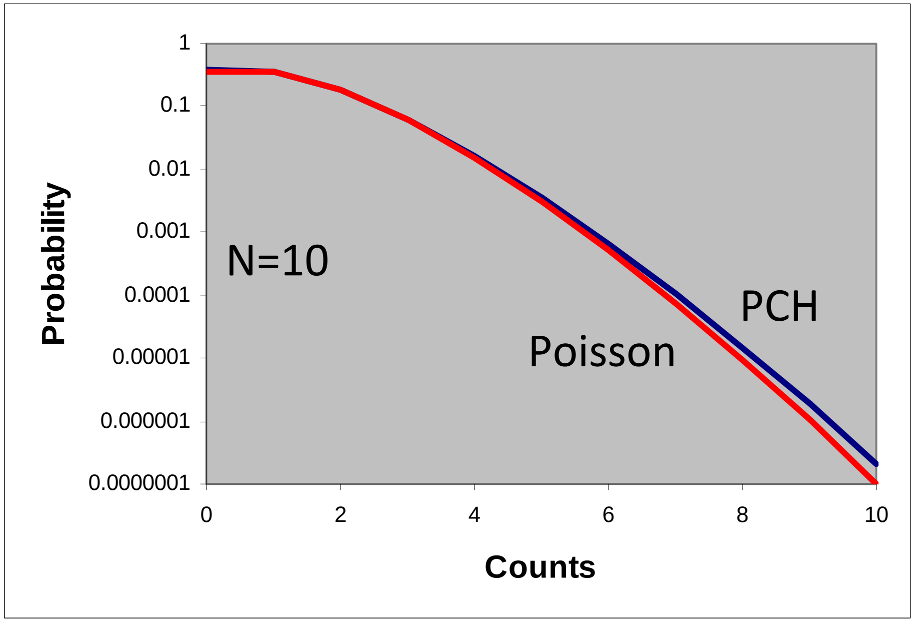


Brightness increases by 100%



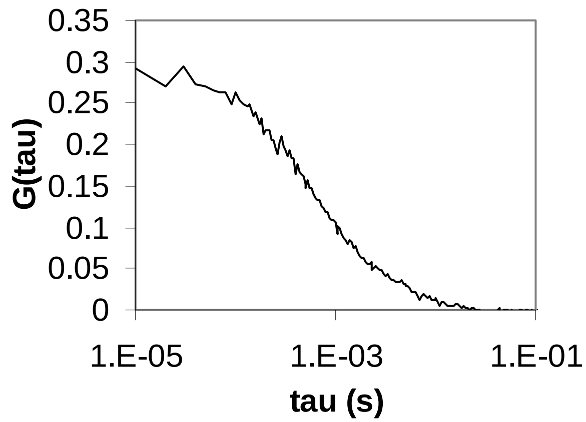
Brightness increases by 10%

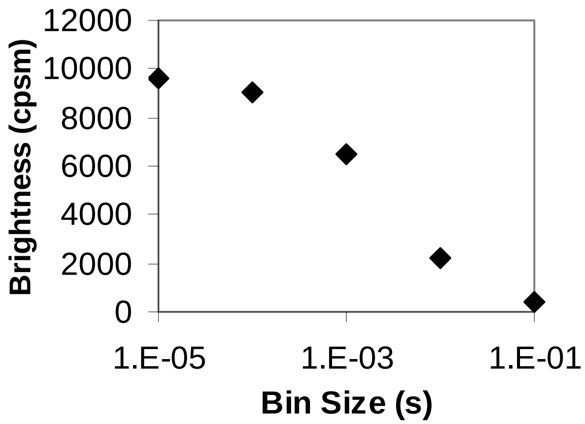


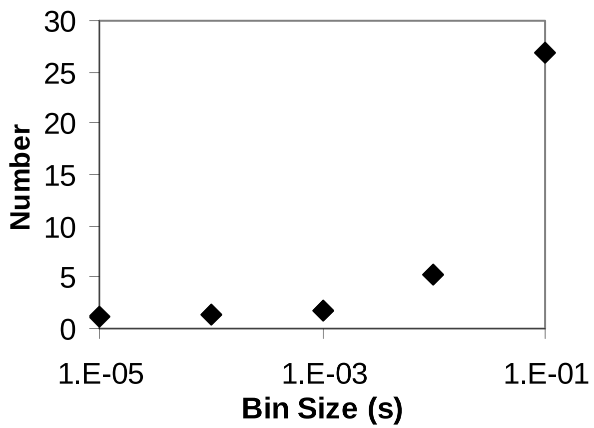


Note: if N is too low, experiment becomes photon limited

Sampling Time Effect





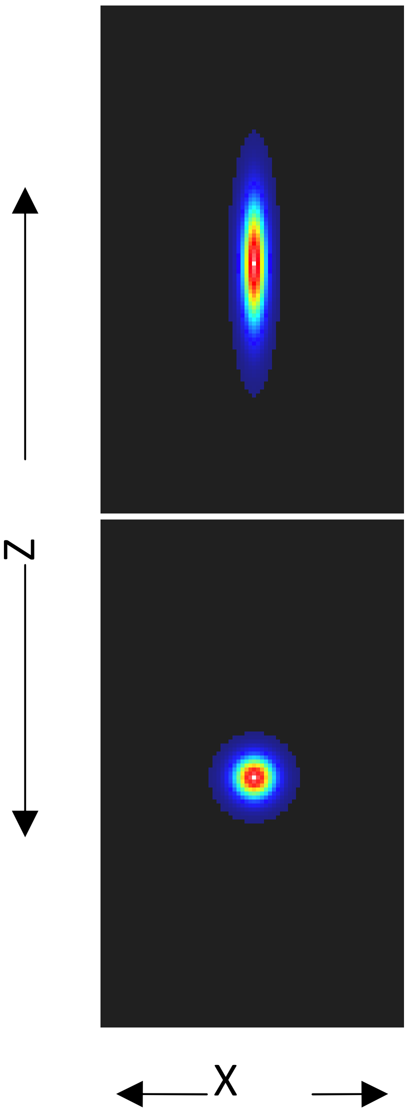


Again, shorter sampling leads to photon limited acquisition

In general sample as long as possible without diffusion averaging

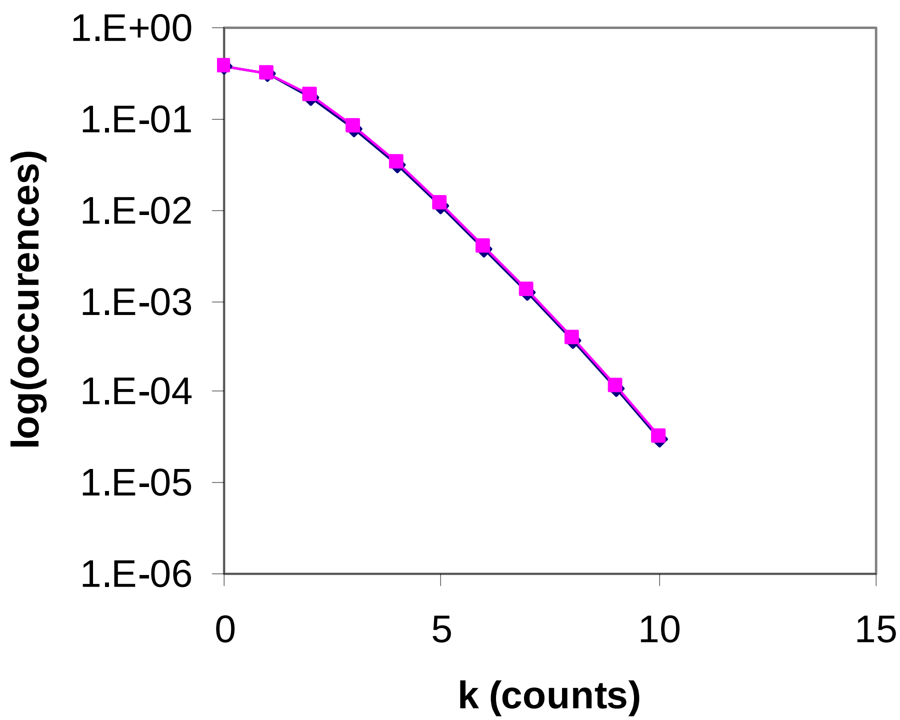
Wu and Mueller, *Biophys. J.*, **2005,** *89,* 2721.

PSF X,Y, and Z Dimensions Don't Matter

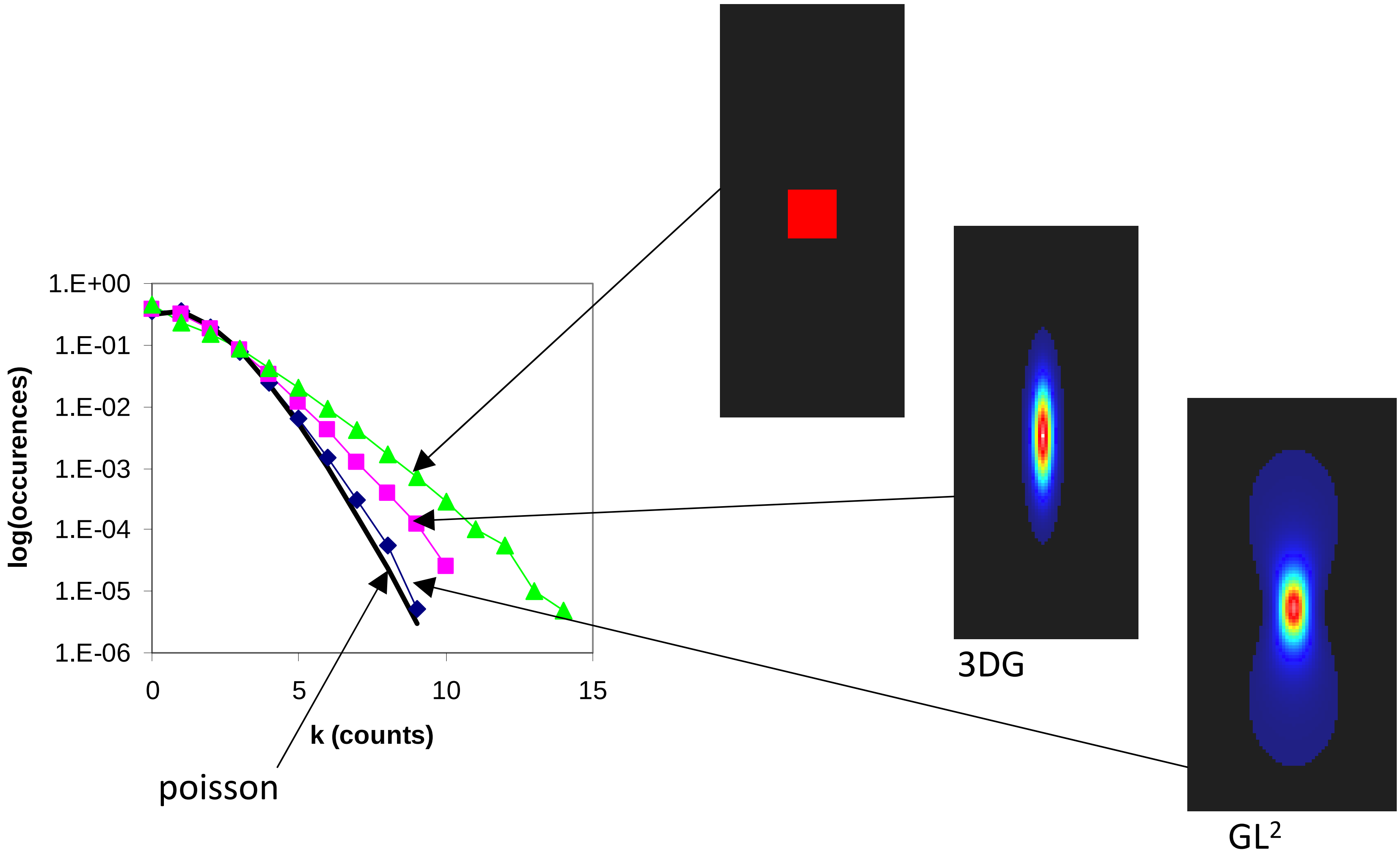


V_{PSF} = 0.08 fL

V_{PSF} = 0.08 fL

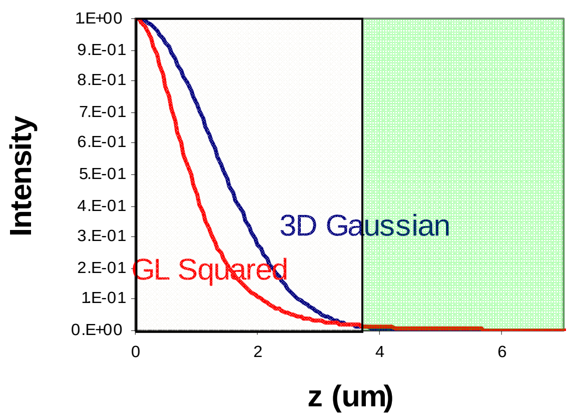


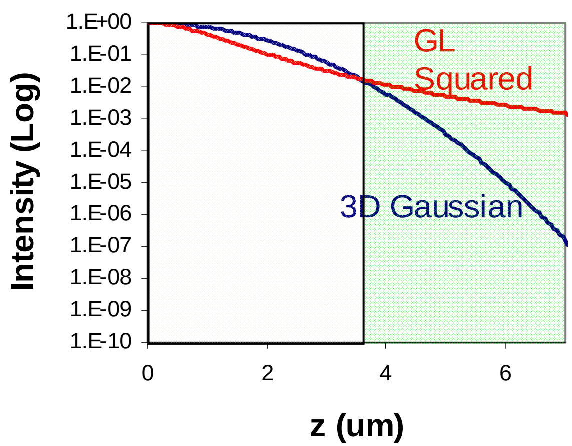
Functional Form DOES Matter



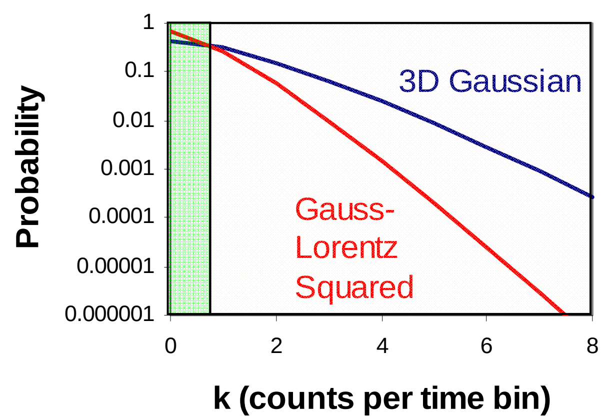
Functional Form Matters for PCH

PSF z-Profile





PCH



Point Spread Function Effects

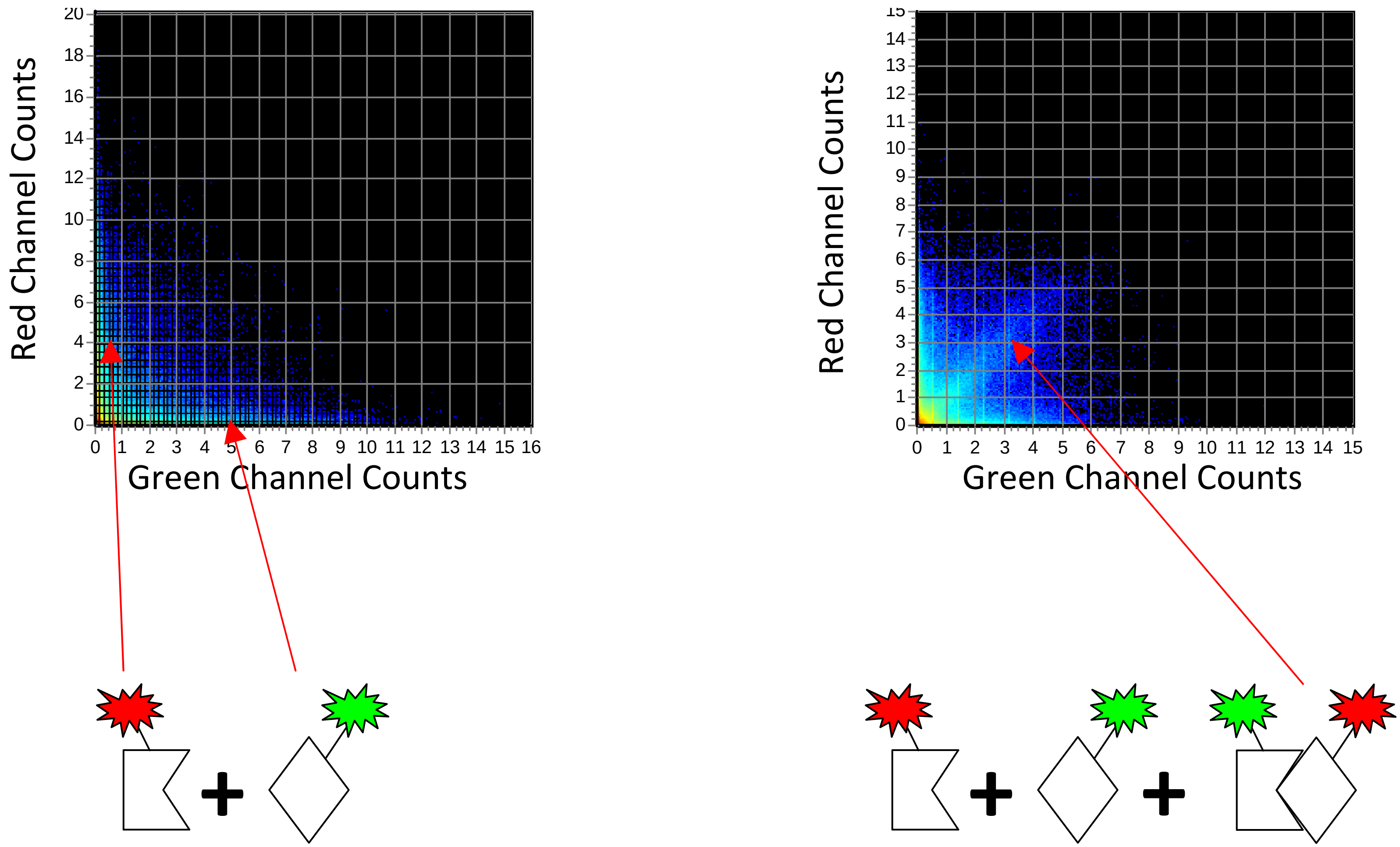
$$p^{(1)}(k) = \frac{1}{V_0} \int_{V_0} Poi\left(k, \varepsilon \overline{PSF}(\vec{r})\right) d\vec{r}$$

This equation will work for ANY PSF shape.

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



Red Channel Counts
Green Channel Counts
Red Channel Counts
Green Channel Counts

Calculating the 2D PCH Function

$$PCH(\varepsilon_A, \varepsilon_B, N; k_A, k_B) = \binom{k}{k_A} (\varepsilon_A / \varepsilon)^{k_A} (1 - \varepsilon_A / \varepsilon)^{k - k_A} \cdot PCH(\varepsilon, N; k)$$

the binomial distribution:

$$P(x, k, N) = \binom{N}{k} x^k (1 - x)^{N-k}$$

We can find the 2D PCH function from the single channel PCH function!

Chen et al., *Biophys. J.*, **2005,** *88,* 2177-2192.

Summary

- The photon count histogram can be modeled by integration of component noise sources
- Heterogeneous samples can be resolved through global analysis
- Accuracy is related to magnitude of particle fluctuations relative to instrument fluctuations