The Photon Counting Histogram (PCH)

While Fluorescence Correlation Spectroscopy (FCS) can measure the diffusion coefficient and concentration of fluorophores, a complementary method, a photon counting histogram (PCH) analysis, can be used to differentiate between species of similar diffusion coefficient through their molecular brightness (Chen et al, 1999, Kask et al, 1999, Thompson et al, 2002). In this manner, the PCH can also resolve aggregation through specie brightness levels. An attractive feature of this method is that the same data obtained to calculate the autocorrelation function can also be used to generate a histogram, although a time series is not required for the PCH.

The PCH is based on the probability distribution of photon counts in a small volume (~one femtoliter); these data can be used to measure:

1. The average **photon counts ‹*k*›***,* i.e. **concentration**, of fluorescence species (where a monomer or oligomer is counted as a single species).
2. The **molecular brightness (ε)** : The average number of photons per sampling time per molecule. The molecular brightness is proportional to the product of the quantum yield, the excitation of the fluorophore (extinction coefficient), and the instrument efficiency in measuring photons.

THE CONCEPT OF THE PCH:

A constant source of light produces a Poisson distribution of detected counts (Eq.1).

$$p(N) = \frac{\langle N \rangle^{N} \cdot e^{-\langle N \rangle}}{N!} (Eq.1)$$

$$\langle k \rangle = \frac{\varepsilon}{V_0} \int_{V_0} \overline{\mathrm{PSF}(\vec{r})} d\vec{r} = \varepsilon \frac{V_{PSF}}{V_0} (Eq.2)$$

Where:
p(N) = probability of N events
N = number of events

Where:
<k> = average photon counts
ε = the molecular brightness
V_{PSF} = the illumination volume
V_o = total sample volume

The molecular brightness is defined as:

$$\varepsilon = I_0^N \beta \eta_w T (Eq.3)$$

Where:

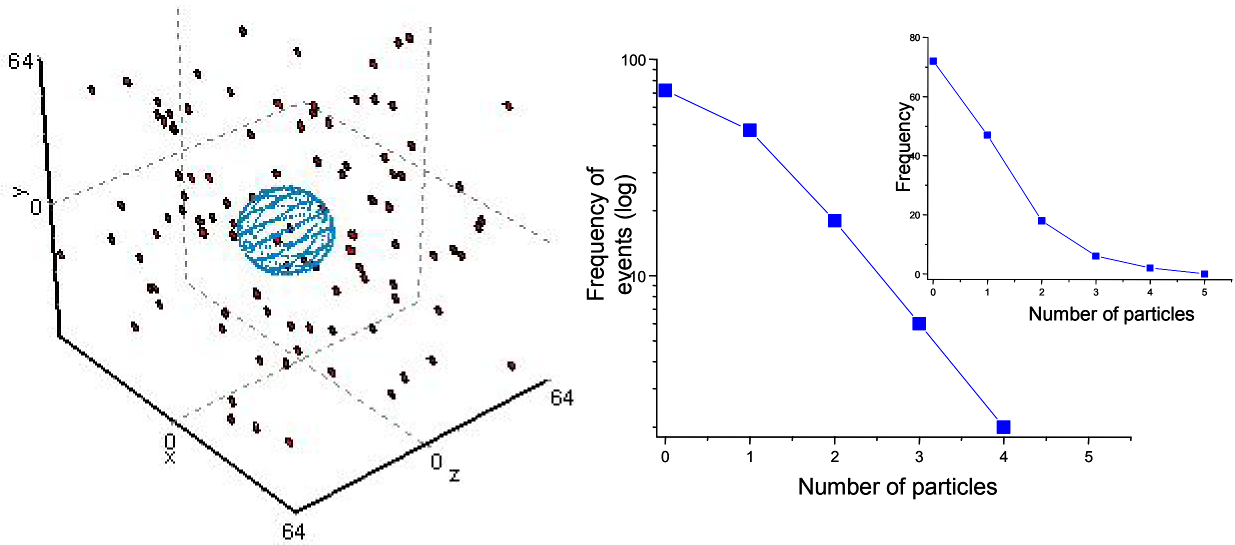
T = the integration time increment
η_w = the detection efficiency
I_o = maximum excitation when the fluorophore at center of V_{PSF}
$I_o = I_o \cdot \beta \cdot \mathrm{PSF}(r_o)$
N = 2, for 2-photon, and 1, for single photon excitation
β = excitation probability; quantum yield, and instrument bias

- In PCH the photon count distribution originates from a convolution of two primary sources: the particle number fluctuations and the photon detection statistics. Both of these sources are described by Poisson statistics while their convolution displays a broader distribution known as super-Poisson. There is a further complication due to the non-uniform nature of the PSF that also contributes to the broadening. The photon distribution from a given fluorophore will depend on its position within the beam, and thus the PCH for a fluorophore will be a convolution of the photon distributions from the various possible positions. The final super-Poisson shape is fit to a theoretical model that accounts for the PSF shape and then resolves the average number of fluorescence particles within the beam and their brightness (Eq.1 & 2) (Qian and Elson, 1989,1990;Chen et al, 1999, Muller et al, 2000).

- In more complex mixtures the super Poisson histogram can be fit to give the number of species of different brightness, with their respective brightness and concentration. This allows the determination of the nature of complex mixtures consisting of either molecules with different fluorophores or of different brightness due to aggregation into monomers, dimers, and higher aggregates (Chen et al, 2002; Sanchez et al, 2002; Ruan et al,2002).

Basic Poisson Statistics

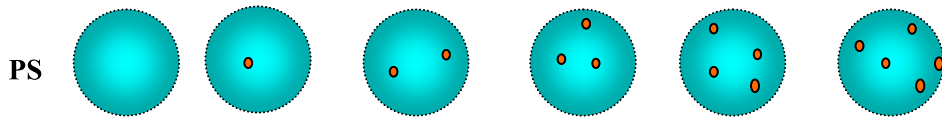
The occupation number within the laser beam volume follows Poisson statistics. Thus, the probability of observing a large number of particles in a small volume (V_{PSF}) at the same time is very low.



Total number of particles in the sample volume (V_o) is 100

Histogram of the number of particles in the focal volume (V_{PSF})

$$p(N) = \frac{\langle N \rangle^{N} \cdot e^{-\langle N \rangle}}{N!} (Eq.1)$$

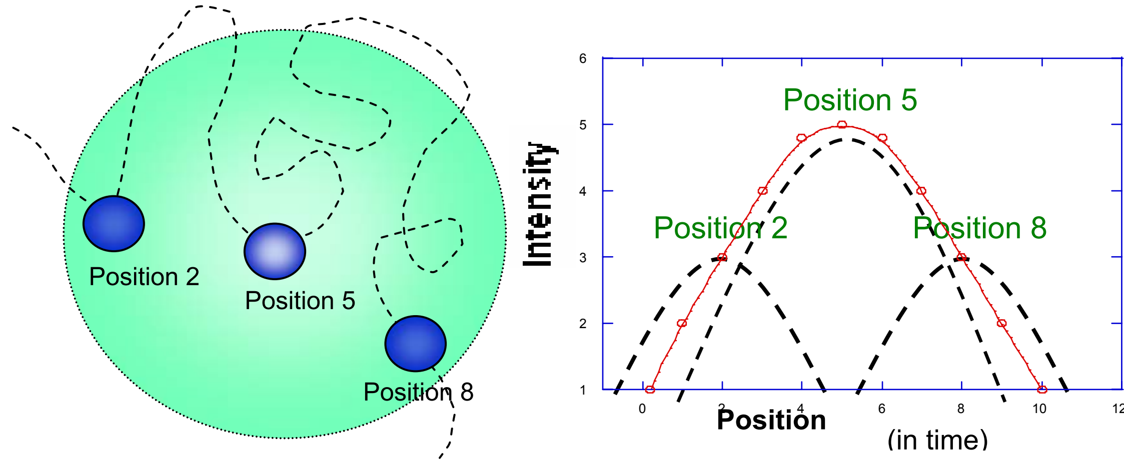


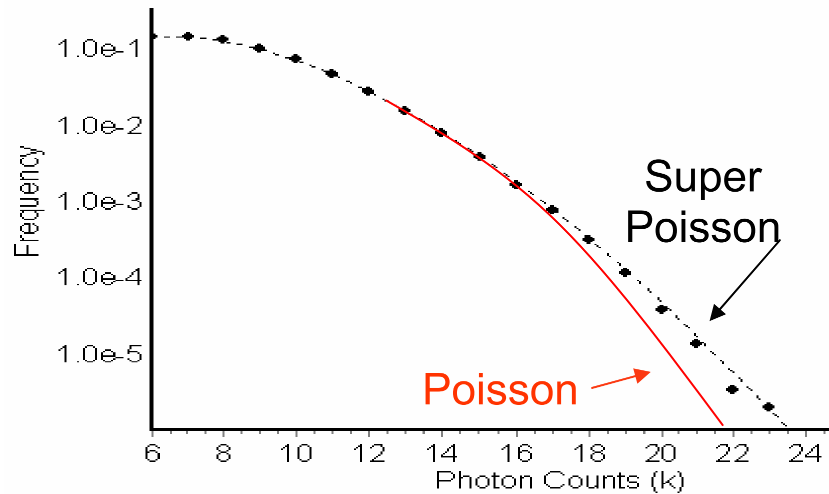
Number of particles in PSF	0	1	2	3	4	>5
Frequency of events (F_t=145)	72	47	18	6	2	0
Proportion	0.497	0.324	0.124	0.041	0.014	0

Figure 1: Simple Poisson statistics using [Eq.1]

Mathematical formula for PCH analysis

The volume of the PSF is not illuminated uniformly for a single particle so the intensity at each position will differ.





Each position has a probability distribution of photon counts resulting in a super Poisson distribution. The Poisson distribution is a convolution of all the "histograms" for the particle at different positions within the focal volume (V_{PSF}):

$$p_{3DG}^{(1)}(k;V_0,\varepsilon)=\frac{1}{V_0}\frac{\pi\omega_0^2 z_0}{2k!}\int_0^\infty \gamma(k,\varepsilon e^{-4x^2})dx \qquad \text{For } k>0$$

Where we consider a three dimensional Gaussian-Lorentzian to describe the V_{PSF} with a beam waist in the x-y of ω_o and in z of z_o using the incomplete γ function.

Mathematical formula for PCH analysis

If there are a number of fluorescent particles each with a fluorescence intensity, I, a standard deviation, σ, and a detection efficiency of η_w, the total measured intensity within the focal volume for a given time interval is the sum of all the contributions from each particle within the volume during that time. The resulting intensity distribution is characterized by Poisson statistics.

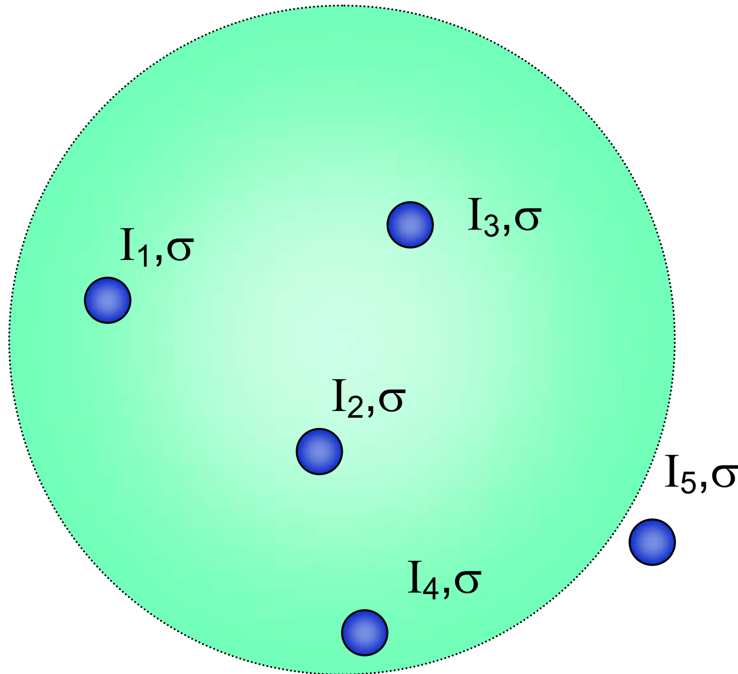


Figure 2: Each fluorescent particle at a given position will have an intensity (I) distribution and a standard deviation (σ).

- The probability of observing *k* photoelectron events at time t is given by Mandel's formula and is directly related to the sum of the intensity contribution from each particle over the integration time, T. The integral indicates the sum of all the particles in a given volume:

$$\mathrm{p}(k,t,T)=\int_0^\infty \frac{(\eta_W W(t))^k e^{-\eta_W W(t)}}{k!}\mathrm{p}(W(t))dW(t)$$

- Where W(t) is the energy distribution and is related to the light intensity (I), detector area and the integration time. The particles can have different brightness, and the volume can be occupied by a different number of particles.

Mathematical formula for PCH analysis with multiple populations

A distribution of different brightness can arise from either different fluorophores with different brightness or from different aggregation states of a single fluorophore.

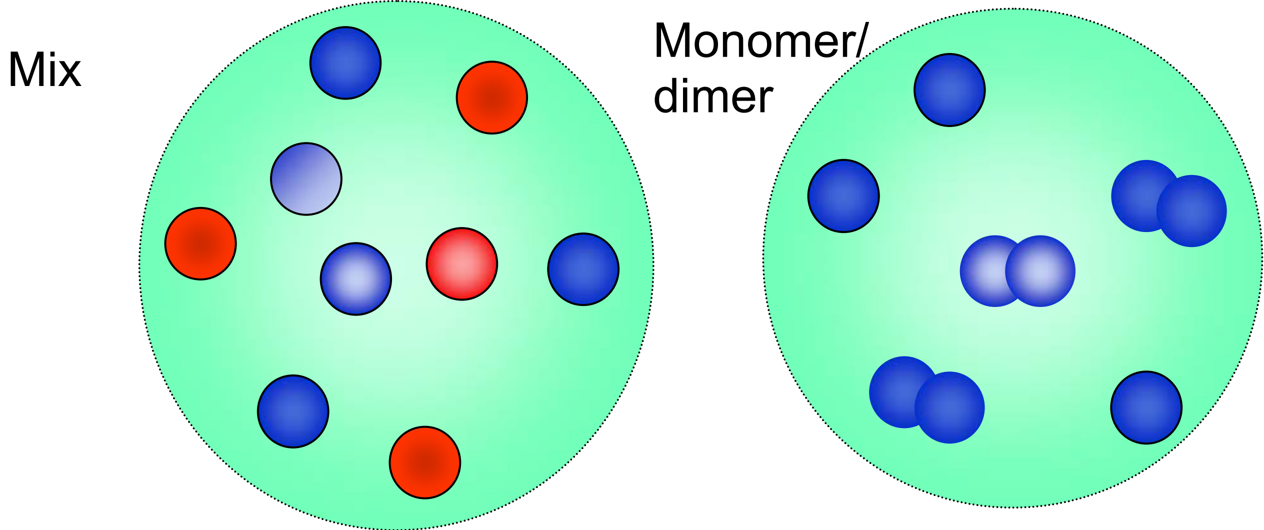


Figure 4: Multiple species with distinct brightness (ε).

- The PCH distribution for several particles is defined by the probability of observing *k* photon counts in a focal volume, V_{PSF} (See Chen, Thesis 1999 for more detail):

$$p^{(1)}(k;V_o,\varepsilon) = \int\int \text{Poi}\,(k, \varepsilon\overline{\text{PSF}(\vec{r}_1)} + \varepsilon\overline{\text{PSF}}(\vec{r}_2))\text{p}(\vec{r}_1)\,p(\vec{r}_2)\,d\vec{r}_1 d\vec{r}_2$$

Where:

- $\vec{r}_1$ and $\vec{r}_2$ are two position coordinates for each particle.

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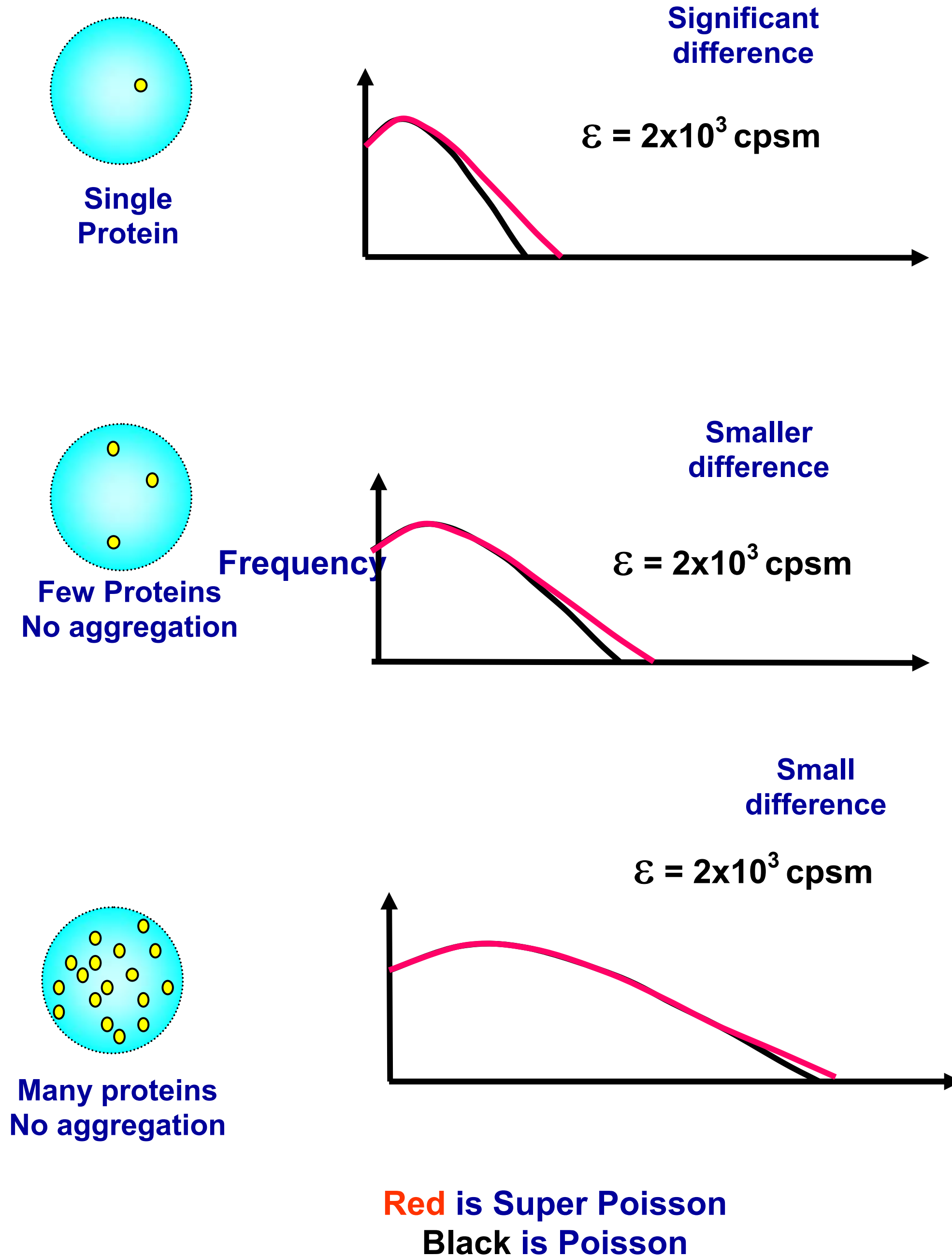
For two particles of different brightness the PCH is the convolution of the one particle PCH with itself:

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

Brightness will increase by 2 fold for dimers, three fold for trimers, and so on.

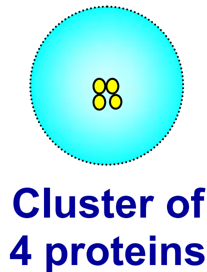
Detecting protein concentration

Protein concentrations can be determined from the shape of the Poisson distribution with broadening occurring as photon counts go up. Deviations from Poisson statistics also lessen as concentrations increase. (cpsm = counts per second per molecule)

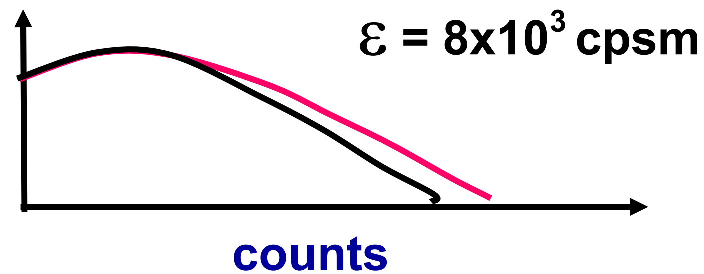


Detecting protein aggregation

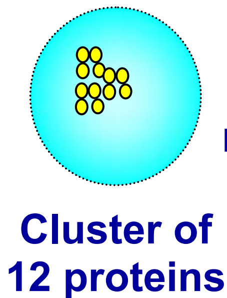
Protein aggregation can be determined from the shape of the Poisson distribution with the molecular brightness increasing with aggregation (here ε is the brightness per cluster not per molecule per say). Deviations from Poisson statistics also lessen as cluster size increases. (cpsm = counts per second per protein cluster)

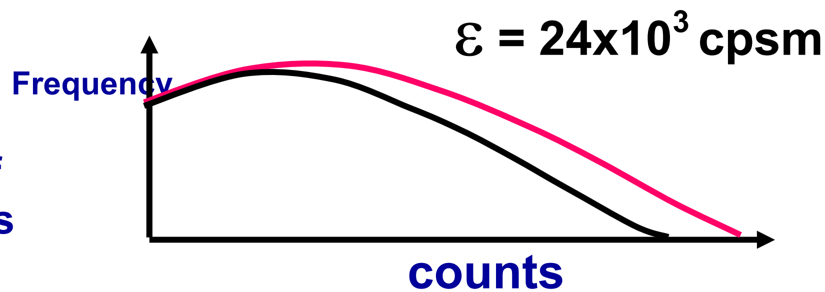


Significant difference

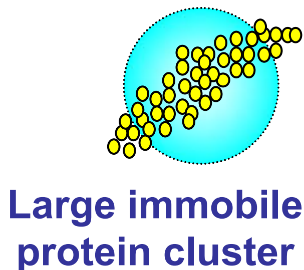


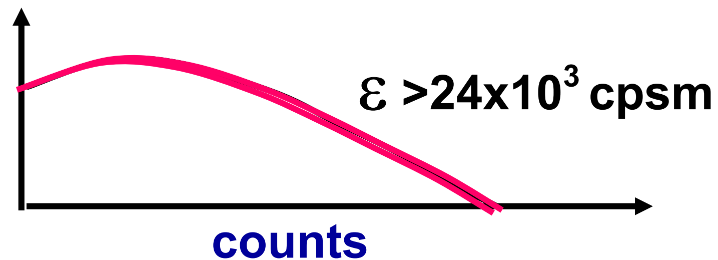
Largest difference





No difference

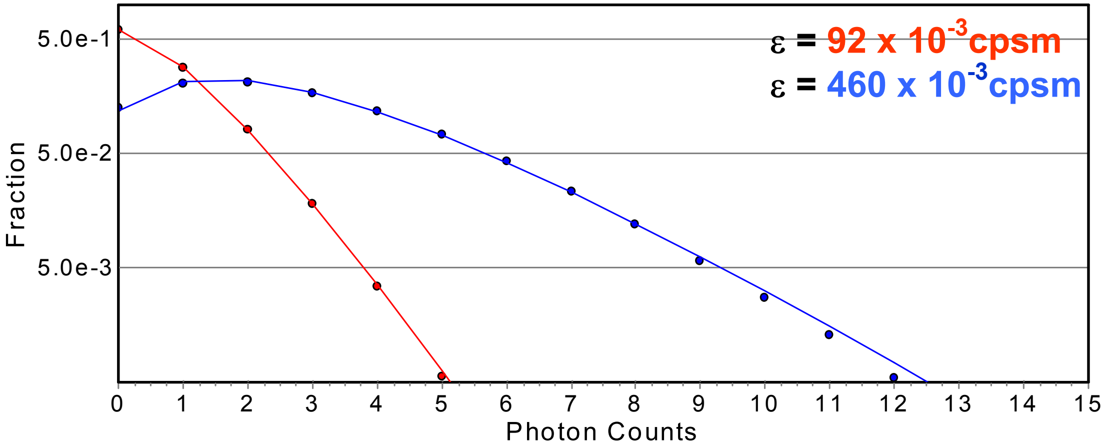




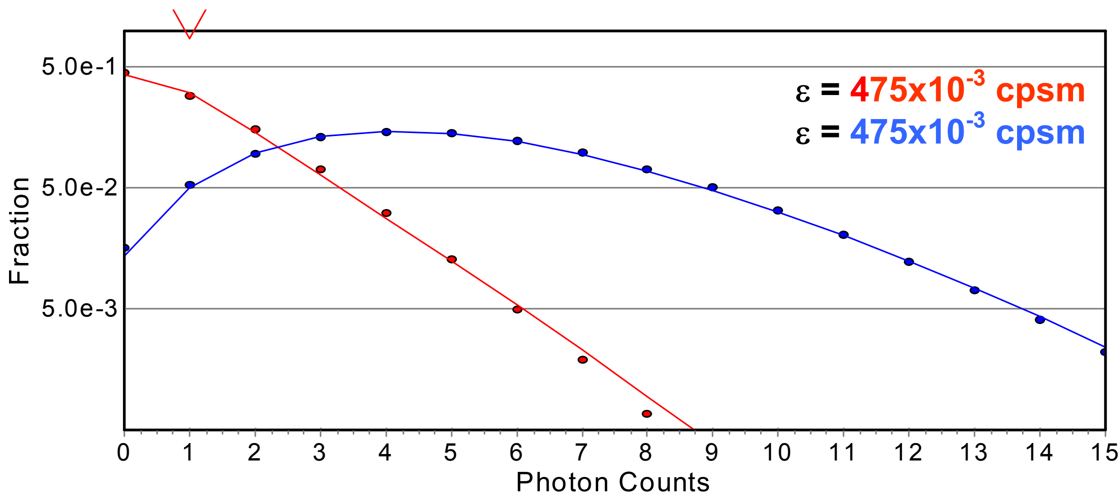
Red is Super Poisson
Black is Poisson

Simulations of PCH Data

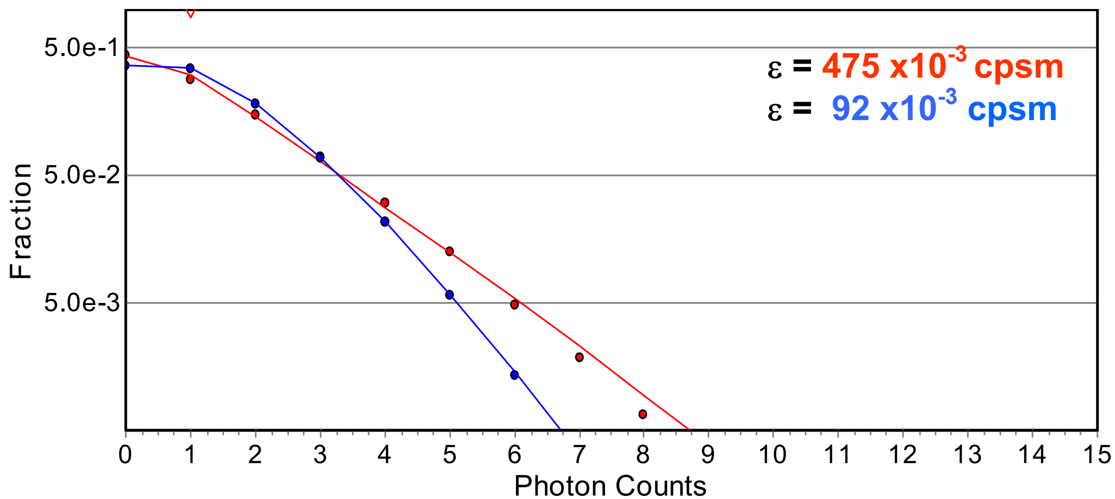
Equal concentration: 50 nM, **Different brightness (ε):** 20,000 vs 100,000 counts



Different concentration: 20 nM vs 100 nM, **Equal brightness (ε):** 100,000 counts



Different concentration: 20 nM vs 100 nM
Different brightness: 20,000 vs 100,000 counts



Example of PCH analysis on EGFP in solution and in the cell

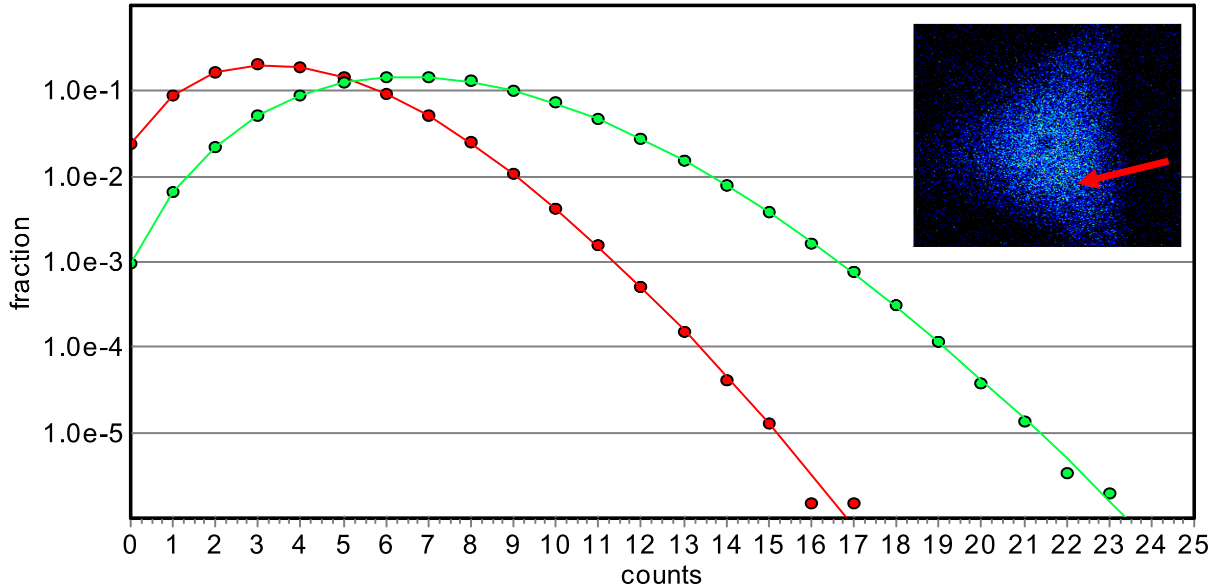


Figure 5: PCH analysis of EGFP in solution vs EGFP in the cytosol. The Fitted PCH curves for EGFP (red) in solution and EGFP inside CHO K1 cells (green) have similar molecular brightness. The red arrow points to the spot where the data acquisition occurred. There is a slight deviation from the curve due to the higher concentration which yields higher photon counts

Proteins	Molecular Brightness ε ($x10^3$ cpsm)	Concentration of protein (nM)
EGFP in solution	2.68	15.0
EGFP inside CHO cells	2.06	36.1

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