FCS
PCH analysis
Cross-correlation

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From cuvette to the microscope

1. Excitation & Emission Spectra
   • Local environment polarity, fluorophore concentration
2. Anisotropy & Polarization
   • Rotational diffusion
3. Quenching
   • Solvent accessibility
   • Character of the local environment
4. Fluorescence Lifetime
   • Dynamic processes (nanosecond timescale)
5. Resonance Energy Transfer
   • Probe-to-probe distance measurements
6. Fluorescence microscopy
   • Localization

7. Fluorescence Correlation Spectroscopy
   • Translational & rotational diffusion
   • Concentration
   • Dynamics

In the microscope, the spatial location matters: spatial correlations and distributions are a component of the experiment
Why we need FCS to measure the internal dynamics in cell??

Methods based on perturbation
Typically FRAP (fluorescence recovery after photobleaching)

Methods based on fluctuations
Typically FCS and dynamic ICS (Image Correlation Spectroscopy) methods

There is a fundamental difference between the two approaches, although they are related as to the physical phenomena they report on
Introduction to “number” fluctuations

In any open volume, the number of molecules or particles fluctuate according to a Poisson statistics (if the particles are not-interacting).

The average number depends on the concentration of the particles and the size of the volume.

The variance is equal to the number of particles in the volume.

This principle does not tell us anything about the time of the fluctuations which depends on transport phenomena.
If we perturb a system from equilibrium, it returns to the average value with a characteristic time that depends on the process responsible for returning the system to equilibrium.

**Spontaneous** energy fluctuations in a part of the system, can cause the system to locally go out of equilibrium. These spontaneous fluctuations **dissipate** with the same time constant as if we had externally perturbed the equilibrium of the system.
Experimental data on colloidal gold particles:

```
120002001324123102111131125111023313332211122422122612214
2345241141311423100100421123123201111000111_2110013200000
1001100010023221002110000201001_333122000231221024011102_
1222112231000110331110210110010103011312121010121111211_10
00322101230201212321110110023312242110001203010100221734
410101002112211444421211440132123314313011222123310121111
222412231113322132110000410432012120011322231200_253212033
2331111002100220130113211131200101314322112212232342230
321421532200202142123232043112312003314223452134110412322
220221
```

Collected data by counting (by visual inspection) the number of particles in the observation volume as a function of time using a “ultra microscope”
Particle Correlation

*Histogram of particle counts
*Poisson behavior
*Autocorrelation not available in the original paper. It can be easily calculated today.

Comments to this paper conclude that scattering will not be suitable to observe single molecules, but fluorescence could
What can cause a fluctuation in the fluorescence signal???

- Number of fluorescent molecules in the volume of observation, diffusion or binding
- Conformational Dynamics
- Rotational Motion if polarizers are used either in emission or excitation
- Protein Folding
- Blinking
- And many more

Example of processes that could generate fluctuations

Each of the above processes has its own dynamics. FCS can recover that dynamics
Generating Fluctuations By Motion

1. The Rate of Motion
2. The Concentration of Particles
3. Changes in the particle fluorescence while under observation, for example conformational transitions

We need a small volume!!

Observation Volume

Sample Space

What is Observed?

1. The Rate of Motion
2. The Concentration of Particles
3. Changes in the particle fluorescence while under observation, for example conformational transitions
Data presentation and Analysis

The time series

Detail of one time region

The autocorrelation function
N and relaxation time of the fluctuation

The histogram of the counts in a given time bin (PCH). N and brightness
How to extract the information on fluctuations and their characteristic time?

Distribution of the **amplitude** of the fluctuations

Distribution of the **duration** of the fluctuations

To extract the distribution of the duration of the fluctuations we use math based on calculation of the **correlation function**

To extract the distribution of the amplitude of the fluctuations, we use math based on the **PCH distribution**
The definition of the Autocorrelation Function

\[ \delta F(t) = F(t) - \langle F(t) \rangle \]

\[ G(\tau) = \frac{\langle \delta F(t) \delta F(t + \tau) \rangle}{\langle F(t) \rangle^2} \]
What determines the intensity of the fluorescence signal??

This is the fundamental equation in FCS

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

- \(kQ\) = quantum yield and detector sensitivity (how bright is our probe). This term could contain the fluctuation of the fluorescence intensity due to internal processes
- \(W(\mathbf{r})\) describes the profile of illumination
- \(C(\mathbf{r}, t)\) is a function of the fluorophore concentration over time. This is the term that contains the “physics” of the diffusion processes

The value of F(t) depends on the profile of illumination!
What about the excitation (or observation) volume shape?

\[ F(x, y, z) = I_0 I(z) e^{-\frac{2(x^2+y^2)}{w_0^2}} \]

\[ I(z) = \text{Exp}\left[-\frac{2z^2}{w_{0z}^2}\right] \quad \text{Gaussian z} \]

\[ I(z) = \frac{1}{1 + \left(\frac{z}{w_{0z}}\right)^2} \quad \text{Lorentzian z} \]

More on the PSF in PCH lecture

For the 2-photon case, these expression must be squared
In the simplest case, two parameters define the autocorrelation function: the amplitude of the fluctuation \( G(0) \) and the characteristic relaxation time of the fluctuation.

\[ G(0) \propto \frac{1}{N} \]

As time (tau) approaches 0.
The Effects of Particle Concentration on the Autocorrelation Curve

\[ \langle N \rangle = 2 \]

\[ \langle N \rangle = 4 \]

Observation volume

\[ G(t) \]

Time (s)
Why Is $G(0)$ Proportional to 1/Particle Number?

A Poisson distribution describes the statistics of particle occupancy fluctuations. For a Poisson distribution the variance is proportional to the average:

$$<N> = \langle \text{Particle\_Number} \rangle = \text{Variance}$$

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

Definition

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

$$G(0) = \frac{\text{Variance}}{\langle N \rangle^2} = \frac{\langle N \rangle}{\langle N \rangle^2} = \frac{1}{\langle N \rangle}$$
Effect of Shape on the (Two-Photon) Autocorrelation Functions:

For a 2-dimensional Gaussian excitation volume:

\[ G(\tau) = \frac{\gamma}{N} \left( 1 + \frac{4D\tau}{w_{2DG}^2} \right)^{-1} \]

For a 3-dimensional Gaussian excitation volume:

\[ G(\tau) = \frac{\gamma}{N} \left( 1 + \frac{4D\tau}{w_{3DG}^2} \right)^{-1} \left( 1 + \frac{4D\tau}{z_{3DG}^2} \right)^{-1/2} \]

2-photon equation contains a 8, instead of 4.
Blinking or other exponential processes:

If the particle blinks during the times it goes through the illumination volume, an additional term appears in the fluctuation amplitude.

How to account for this process??

**Reasoning:** let us assume that the particle is **not moving** and it is at the center of the PSF. The intensity will turn **ON** and **OFF**. The **OFF** time depends on the characteristic blinking time (triplet state lifetime). The **ON** time depends on the laser intensity. The larger the laser intensity, the lesser is the **ON** time.

$G(\tau) = (1 + \frac{T}{1-T} e^{-\frac{\tau}{\tau_T}})$

**Triplet state term:**

$T$ is the triplet state amplitude

$\tau_T$ is the triplet lifetime.
Blinking and binding processes

Until now, we assumed that the particle is not moving. If we assume that the blinking of the particle is independent on its movement, we can use a general principle that states that the correlation function splits in the product of the two independent processes.

\[ G_{Total}(\tau) = G_{Blinking}(\tau) \cdot G_{Diffusion}(\tau) \]

\[ G_{Binding}(\tau) = \left[ 1 + K \left( f_A - \frac{f_B}{K} \right)^2 e^{-\lambda \tau} \right] \]

\[ K = k_f / k_b \] is the equilibrium coefficient; \( \lambda = k_f + k_b \) is the apparent reaction rate coefficient; and \( f_j \) is the fractional intensity contribution of species \( j \)
How different is $G(\text{binding})$ from $G(\text{diffusion})$?

With good S/N it is possible to distinguish between the two processes. Most of the time diffusion and exponential processes are combined.
**Orders of magnitude** (for 1 μM solution, small molecule, water)

<table>
<thead>
<tr>
<th>Volume</th>
<th>Device</th>
<th>Size(μm)</th>
<th>Molecules</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>milliliter</td>
<td>cuvette</td>
<td>10000</td>
<td>6x10^{14}</td>
<td>10^4</td>
</tr>
<tr>
<td>microliter</td>
<td>plate well</td>
<td>1000</td>
<td>6x10^{11}</td>
<td>10^2</td>
</tr>
<tr>
<td>nanoliter</td>
<td>microfabrication</td>
<td>100</td>
<td>6x10^8</td>
<td>1</td>
</tr>
<tr>
<td>picoliter</td>
<td>typical cell</td>
<td>10</td>
<td>6x10^5</td>
<td>10^{-2}</td>
</tr>
<tr>
<td>femtoliter</td>
<td>confocal volume</td>
<td>1</td>
<td>6x10^2</td>
<td>10^{-4}</td>
</tr>
<tr>
<td>attoliter</td>
<td>nanofabrication</td>
<td>0.1</td>
<td>6x10^{-1}</td>
<td>10^{-6}</td>
</tr>
</tbody>
</table>

Table of characteristic times for diffusion
The Effects of Particle Size on the Autocorrelation Curve

**Diffusion Constants**

- 300 um²/s
- 90 um²/s
- 71 um²/s

**Stokes-Einstein Equation:**

\[ D = \frac{k \cdot T}{6 \cdot \pi \cdot \eta \cdot r} \]

and

\[ MW \propto Volume \propto r^3 \]

**Monomer --> Dimer**

Only a change in D by a factor of \(2^{1/3}\), or 1.26
Examples of different *Hela* cells transfected with AK1-EGFP

Examples of different *Hela* cells transfected with AK1β-EGFP

*Qiao Qiao Ruan, Y. Chen, M. Glaser & W. Mantulin Dept. Biochem & Dept Physics - LFD Univ II, USA*
Autocorrelation of EGFP & Adenylate Kinase -EGFP

Normalized autocorrelation curve of EGFP in solution (•), EGFP in the cell (●), AK1-EGFP in the cell (●), AK1β-EGFP in the cytoplasm of the cell (●).
A mixture of AK1b-EGFP in the cytoplasm and membrane of the cell.
Diffusion constants (um²/s) of AK EGFP-AKβ in the cytosol -EGFP in the cell (HeLa). At the membrane, a dual diffusion rate is calculated from FCS data. Away from the plasma membrane, single diffusion constants are found.
Two Channel Detection: Cross-correlation

1. Increases signal to noise by isolating correlated signals.
2. Corrects for PMT noise

Each detector observes the same particles.
Removal of Detector Noise by Cross-correlation

11.5 nM Fluorescein

Detector after-pulsing

Cross-correlation
Calculating the Cross-correlation Function

Detector 1: $F_i$

Detector 2: $F_j$

$t + t$

$G_{ij}(\tau) = \frac{\langle dF_i(t) \cdot dF_j(t + \tau) \rangle}{\langle F_i(t) \rangle \cdot \langle F_j(t) \rangle}$
Cross-correlation calculations

One uses the same fitting functions you would use for the standard autocorrelation curves.

Thus, for a 3-dimensional Gaussian excitation volume one uses:

\[ G_{12}(\tau) = \frac{\gamma}{N_{12}} \left(1 + \frac{4D_{12}\tau}{w^2}\right)^{-1} \left(1 + \frac{4D_{12}\tau}{z^2}\right)^{-1/2} \]

\( G_{12} \) is commonly used to denote the cross-correlation and \( G_1 \) and \( G_2 \) for the autocorrelation of the individual detectors. Sometimes you will see \( G_x(0) \) or \( C(0) \) used for the cross-correlation.
Two-Channel Cross-correlation

The cross-correlation
ONLY if particles are observed in both channels

Each detector observes particles with a particular color

The cross-correlation signal:

Only the green-red molecules are observed!!
Two-color Cross-correlation

Equations are similar to those for the cross correlation using a simple beam splitter:

$$G_{ij}(\tau) = \frac{\langle dF_i(t) \cdot dF_j(t + \tau) \rangle}{\langle F_i(t) \rangle \cdot \langle F_j(t) \rangle}$$

**Information Content**

Correlated signal from particles having **both colors**.

Autocorrelation from channel 1 on the **green particles**.

Autocorrelation from channel 2 on the **red particles**.

**Signal**

$$G_{12}(\tau)$$

$$G_1(\tau)$$

$$G_2(\tau)$$
Experimental Concerns: Excitation Focusing & Emission Collection

We assume exact match of the observation volumes in our calculations which is difficult to obtain experimentally.

Excitation side:
(1) Laser alignment
(2) Chromatic aberration
(3) Spherical aberration

Emission side:
(1) Chromatic aberrations
(2) Spherical aberrations
(3) Improper alignment of detectors or pinhole
(cropping of the beam and focal point position)
For two uncorrelated species, the amplitude of the cross-correlation is proportional to:

$$G_{12}(0) \propto \left[ \frac{f_{11}\bar{f}_{12}\langle N_1 \rangle + f_{21}\bar{f}_{22}\langle N_2 \rangle}{f_{11}f_{12}\langle N_1 \rangle^2 + (f_{11}f_{22} + f_{21}f_{12})\langle N_1 \rangle\langle N_2 \rangle + f_{21}f_{22}\langle N_2 \rangle^2} \right]$$
Discussion

1. The PSF: how much it affects our estimation of the processes?
2. Models for diffusion, anomalous?
3. Binding?
4. FRET (dynamic FRET)?
5. Bleaching?

6. ......and many more questions
Figure 4.2    Simulation of autocorrelation functions using equation (4.12). The diffusion coefficient used is $300 \mu m^2/\text{sec}$, $w_{3DG} = 0.3\mu m$, $z_{3DG} = 1.5\mu m$. 
100 red and 100 blue particles in the box. The detector is sensitive only to the blue particles. The particles perform a random motion in 3D. At random times after excitation, the blue particle (in the singlet state) can convert into the red particle (in the triplet state). After about $10^{-5}$s, the triplet state decays and the particle returns to be blue (singlet state). The particle is only detected when inside the illumination volume (in pink). The intensity is properly weighted according to a 3-D Gaussian intensity model.

Box size=6.4 μm
Diffusion coefficient $D=23 \, \mu m^2/s$
Periodic boundary conditions

$$t_D = \frac{w^2}{8D} = 2.6 \, ms$$
Correlation function for pure diffusion

Correlation function for diffusion and excited-state reaction (triplet state)

Panel 1: 100 particles in a box of approximately 6.4 μm side and a PSF of 0.5 μm waist and 1.5 μm axial waist.

Panel 2: 200 particles in a box. All particles undergo an excited state reaction with a decay rate of $10^{-5}$s. The system is at equilibrium with half the particles in the triplet excited state. What is the apparent $G(0)$ in panel 2? Why are the two correlation functions different?
Photon counting histogram for the sample with 100 particles in a box (panel 1) and with 200 particles (panel 2) undergoing an excited state reaction at a rate of $10^{-5}$s. The system is at equilibrium and half of the particles are in the triplet excited state. Why are the two histograms identical (within noise)?
A

Fluorescein at 4 nM

$G(0) = 0.11$

$w_{el} = 0.319 \mu m$

$D = 300 \mu m^2/sec$

---

B

Rhodamine 110 at 3 nM

$G(0) = 0.15$

$w_{el} = 0.319 \mu m$

$D = 272 \mu m^2/sec$
Case 1: Species vary by a difference in diffusion constant, $D$.

**Autocorrelation function can be used:**

$$G(\tau)_{sample} = \sum_{i=1}^{M} f_i^2 \cdot G(0)_i \cdot \left(1 + \frac{8D\tau}{w_{2DG}^2}\right)^{-1}$$

($2D$-Gaussian Shape)

$$G(0)_{sample} = \sum f_i^2 \cdot G(0)_i$$

$G(0)_{sample}$ is no longer $g/N$!
Case 2: Species vary by a difference in brightness assuming that \( D_1 \approx D_2 \)

The quantity \( G(0) \) becomes the only parameter to distinguish species, but we know that:

\[
G(0)_{sample} = \sum f_i^2 \cdot G(0)_i
\]

The autocorrelation function is not suitable for analysis of this kind of data without additional information.

We need a different type of analysis.
The Photon Counting Histogram: Statistical Analysis of Single Molecule Populations
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
Can we quantitate this?

What contributes to the distribution of intensities?
diffusing

Correlation plot (log averaged)

Immobile

Correlation plot (log averaged)

PCH average

G(t)

tau (s)

Counts
Same diffusion, same number of particles, brightness differs by 2

Same number of particles, monomer-dimer difference
If particles are not moving or otherwise fluctuating, the ratio between the variance and the average is 1

**Fixed Particle Noise (Shot Noise)**

Noise is Poisson

\[ Poi(k, \langle k \rangle) = \frac{\langle k \rangle^k}{k!} \exp\left(-\langle k \rangle\right) \]

Contribution from the detector noise
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:

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

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

\[ z_R = \frac{\pi \omega_0^2}{\lambda} \]
Extra fluctuation (variance) arises from particle motion

Single Particle PCH

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

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

This is not a poissonian!
• 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

Intensities just add

Distributions do not add!
Combining Distributions

Distributions convolute each other
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

Initial distribution

Sum over PSF

Convolve with Self

1 Particle PCH

Fixed Particle Shot Noise

2 Particle PCH

Conv. with 1 particle PCH

3 Particle PCH

Average weighted by number probability

Species 1 PCH

Species 2 PCH

... convolution

Final PCH

Total broadening
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

Fitting

\[ \chi^2 = \sum_k \left( M \frac{PCH_{\text{model}}(k) - PCH_{\text{observed}}(k)}{\sqrt{M \cdot PCH_{\text{observed}}(k) \cdot (1 - PCH_{\text{observed}}(k))}} \right)^2 \]

\[ k_{\text{max}} - d \]

M is number of observations

d is number of fitting parameters

Model Test

\[ \varepsilon = 9,030 \text{ cpsm} \]
\[ N = 1.28 \]

\[ \varepsilon = 91,330 \text{ cpsm} \]
\[ N = 0.12 \]
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

\[ \varepsilon = \varepsilon_{\text{monomer}} \]

\[ \varepsilon = 2 \times \varepsilon_{\text{monomer}} \]

All three species are present in equilibrium mixture

Typical one photon \( \varepsilon_{\text{monomer}} = 10,000 \text{ cpsm} \)
Photon Count Histogram (PCH)
Simulation Solution

$\varepsilon = 9,000$ cpm

$N = 1.3$

$\varepsilon = 16,000$ cpm

$N = 0.73$
Global Fitting: Fit Data Sets Simultaneously

\[ \varepsilon = 9,000 \text{ cpsm} \quad N = 1.3 \]

or

\[ \varepsilon_1 = 9,000 \text{ cpsm} \quad N_1 = 0.29 \quad \varepsilon_2 = 18,100 \text{ cpsm} \quad N_2 = 0.50 \]
What we measure is the number of particles in the PSF. How Do We Get Concentrations?

- N is defined relative to PSF volume \( V_{PSF} = \int \text{PSF}(\vec{r})d\vec{r} \)
- One photon:
  \[ V_{3DG} = w_0^2 z_0 (\pi / 2)^{3/2} \]
- 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 \( z_0 \))

\( w_0 = 0.21 \text{ um}, \ z_0 = 1.1 \text{ um}, \ V_{PSF} = 0.091 \text{ um}^3, \ C = 23 \text{ nM} \)
How to Improve Accuracy

• Minimize sources of instrument noise
  – PSF heterogeneity
  – Shot noise
• Maximize particle burst amplitudes
Effect of Brightness

$\varepsilon = 10,000$ cpsm

$\varepsilon = 100,000$ cpsm
Saturation Effect
Rhodamine 110 on the Zeiss Confocor 3

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

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

$V_{PSF} = 0.08 \text{ fL}$

$log(occurrences)$

$k (counts)$
Functional Form DOES Matter

- Functional Form
- 3DG
- GL
- Poisson

Graph showing the relationship between log(occurrences) and k (counts) with different functional forms.
Functional Form Matters for PCH

PSF z-Profile

PCH

3D Gaussian
GL Squared
Gauss-Lorentz Squared
Point Spread Function Effects

\[ p^{(1)}(k) = \frac{1}{V_0} \int_{V_0} Poi(k, \varepsilon PSF(\vec{r})) d\vec{r} \]

This equation will work for ANY PSF shape.
Alternative Methods

• Fluorescence Cumulant Analysis (FCA)
  – 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)
  – Fits PSF in fourier transformed space
  – Fits to non-physical parameterized PSF
2D PCH
Calculating the 2D PCH Function

\[
PCH(\varepsilon_A, \varepsilon_B, N; k_A, k_B) = \binom{k}{k_A} \left( \frac{\varepsilon_A}{\varepsilon} \right)^{k_A} \left( 1 - \frac{\varepsilon_A}{\varepsilon} \right)^{k-k_A} \cdot PCH(\varepsilon, N; k)
\]

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!

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