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
Can we quantitate this?

What contributes to the distribution of intensities?

Photon Count Histogram (PCH)
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:

\[
I_{3DG}(r, z) = \exp\left(-\frac{2r^2}{\omega^2} - \frac{2z^2}{z^2_0}\right)
\]

Two Photon:

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

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

\[\quad z_R = \frac{\pi \omega_0^2}{\lambda}\]
Single Particle PCH

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

\[ p^{(1)}(k) = \frac{1}{V_0} \int_{V_0} Poi(k, e^{PSF(\vec{r})}) d\vec{r} \]
• 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!
Combining Distributions

Contribution from several particles of same brightness

Particle 1

Particle 2

Together
Combining Distributions

Particle 1

Particle 2

Together

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?

\[ P(n, N) = \text{Poi}(n, N) \]

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

1. Fixed Particle Shot Noise
2. 1 Particle PCH
3. 2 Particle PCH
4. 3 Particle PCH
5. ... (continuing)

Sum over PSF

Convolve with self

Conv. with 1 particle PCH

Average weighted by number probability

Species 1 PCH
Species 2 PCH
... (continuing)

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

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

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

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

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

All three species are present in equilibrium mixture

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

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

\[ N = 1.3 \]

\[ \varepsilon = 16,000 \text{ cpsm} \]

\[ N = 0.73 \]
Global Fitting: Fit Data Sets Simultaneously

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

\[ \varepsilon_1 = 9,000 \text{ cpsm} \]
\[ N_1 = 0.29 \]
\[ \varepsilon_2 = 18,100 \text{ cpsm} \]
\[ N_2 = 0.50 \]
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:
  \[ V_{3DG} = w_0^2 z_0 \left( \frac{\pi}{2} \right)^{3/2} \]
  \[ V_{PSF} = \int PSF(\vec{r})d\vec{r} \]
- 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 \( z_0 \))

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

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

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

$\log(\text{occurrences})$

$k (\text{counts})$
Functional Form DOES Matter

- Poisson Distribution
- 3DG
- $GL^2$
Functional Form Matters for PCH
Point Spread Function Effects

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

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

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!

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