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?
Contribution from the detector noise

Fixed Particle Noise (Shot Noise)

Noise follows the Poisson distribution $\rightarrow$ average=variance

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

\[ p^{(1)}(k) = \frac{1}{V_0} \int_{V_0} \text{Poi}(k, \varepsilon \text{PSF}(\vec{r})) \, d\vec{r} \]
• 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!
Contribution from several particles of same brightness

Combining Distributions
Combining Distributions

Particle 1

Particle 2

Together

Combining Distributions

Particle 1

Particle 2

Together

Combining Distributions

Particle 1

Particle 2

Together

Combining Distributions

Particle 1

Particle 2

Together

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Combining Distributions

Particle 1

Particle 2

Together
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

Initial distribution

Sum over PSF

1 Particle PCH

Convolve with Self

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

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

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

M is number of observations

d is number of fitting parameters

Model Test

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

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

[Diagram showing the reaction]

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

All three species are present in equilibrium mixture

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

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

\[ \varepsilon = 16,000 \text{ cpsm} \]
\[ N = 0.73 \]
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:
  \[ V_{3DG} = w_0^2 z_0 \left( \pi / 2 \right)^{3/2} \]
  \[ V_{PSF} = \int \text{PSF}(\vec{r}) d\vec{r} \]
• 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

\[ \varepsilon = 10,000 \text{ cpsm} \]

\[ \varepsilon = 100,000 \text{ cpsm} \]
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

\[ V_{PSF} = 0.08 \text{ fL} \]

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\[ 1 \times 10^{-06} \]

\[ 1 \times 10^{-05} \]

\[ 1 \times 10^{-04} \]

\[ 1 \times 10^{-03} \]

\[ 1 \times 10^{-02} \]

\[ 1 \times 10^{-01} \]

\[ 1 \times 10^{00} \]

\[ \log(\text{occurences}) \]

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

log(occurences) vs k (counts)

- Poisson
- 3DG
- GL²
Functional Form Matters for PCH
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

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