Fundamentals of Fluctuation Spectroscopy I: The basics of Fluorescence Correlation Spectroscopy

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A Poisson Process

Experiments that result in counting the number of events in a given time or in a given object can be described by a Poisson process provided:

a) Number changes on nonoverlapping intervals are independent.

b) The probability of exactly one change occurring in a sufficiently short interval of length *h* is approximately λh

c) The probability of two or more changes in a sufficiently short interval is essentially zero.

e.g. Fluctuations in molecular number in a small volume.



The First FCS Measurement

Observation of gold colloids using an ultra-microscope (Svedberg and Inouye, *Zeitschr f. Physik Chemie* **1911**, 77:145-119)

Measurement of the Equilibrium Thermodynamic Fluctuations in Molecular Number

1200020013241231021111311251110233133 3221112242212261221423452411413114231 00100421123123201111000111-2110013200 0001001100010002322102110000201001-33 3122000231221024011102-12221123100011 0331110210100101030113121210101211112 11-1000322101230201212132111011002331 2242110001203010100221734410101002112 2114444212114401321233143130112221233 1012111122241223111332213211000041043 2012120011322231200-25321203323311110 0210022013011321131200101314322112211 2232344222303214215322002021421232320 4311231200331422345213411041232222022 1

The First FCS/PCH Measurement



Experimental Setup





count rates *higher* than 1 count per bin

count per bin

Autocorrelation Analysis



Freely diffusing, noninteracting particles in an open volume.

Photon are not detected stochastically, but in bursts when a molecule transverses the probe volume.

Schematic Autotcorrelation Function:



Delay Time (ms)

We can determine:

Excited state lifetime Rotational Diffusion Constant Triplet-State Lifetime Triplet-State Amplitude Translational Diffusion Constant Concentration Molecular Brightness

Analysis Techniques

The autocorrelation function is one of many techniques to analyze the data



Other Analysis Approaches Include:

Histogram Analysis



Multidimensional Analysis



Autocorrelation Analysis

The normalized autocorrelation function (ACF) is given by:

$$G(\tau) = \frac{\left\langle A(t) A(t+\tau) \right\rangle - \left\langle A(t) \right\rangle^{2}}{\left\langle A(t) \right\rangle^{2}}$$
$$= \frac{\left\langle \delta A(t) \delta A(t+\tau) \right\rangle}{\left\langle A(t) \right\rangle^{2}}$$

 $\delta A\left(t\right) = A\left(t\right) - \left\langle A\left(t\right) \right\rangle$

For processes that are:

where

Stationary: i.e. the average parameters do not change with time

the ACF is independent of the absolute time

Ergodic: i.e. every sizeable sampling of the process is representative of the whole

the time average is equal to the ensemble average

$$\frac{\left\langle \delta A\left(t\right)\delta A\left(t+\tau\right)\right\rangle}{\left\langle A\left(t\right)\right\rangle^{2}}=\frac{\left\langle \delta A\left(0\right)\delta A\left(\tau\right)\right\rangle}{\left\langle A\right\rangle^{2}}$$

Occasionally in the literature, the ACF is defined as:

$$g(\tau) = \langle A(t) A(t + \tau) \rangle$$

Properties of the Autocorrelation Function

The autocorrelation function (ACF) measures the self similarity of the observable A as a function of τ



 $G(\tau) = \frac{\langle \delta A(0) \delta A(\tau) \rangle}{\langle A \rangle^2} = \frac{\sum_{i=1}^{\ell} \delta A_i \delta A_{i+\tau}}{\left(\sum_{i=1}^{\ell} A_i / \ell\right)^2}$ $(\delta A_i)^2 \ge 0$

 $\delta A_i \delta A_{i+\tau}$ can be < 0

Properties of the Autocorrelation Function

The amplitude is proportional to the size of the fluctuations

$$G(0) = \frac{\langle \delta A(0) \delta A(0) \rangle}{\langle A \rangle^2} = \frac{\sum_{i=1}^{\ell} (A_i - \langle A \rangle)^2 / \ell}{\left(\sum_{i=1}^{\ell} A_i / \ell\right)^2}$$
$$G(0) = \frac{\sigma^2}{\mu^2}$$

For non-conserved, non-periodic signals

 $G(\tau) \rightarrow 0$ as $\tau \rightarrow \infty$

 $G(\tau)$ is the probability distribution of detecting a photon at delay τ when a photon was detected at $\tau = 0$



Autocorrelation Function, Single Species

The fluorescence intensity is given by:

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

Where κ is the detection efficiency

 $Q = \sigma \phi ; \qquad \text{Effective Quantum Yield}$ $W(\mathbf{r}) = I^n(\mathbf{r})S(\mathbf{r})X(\mathbf{r}) ; \text{ Probe Volume}$ $I^n(\mathbf{r}) = \text{laser intensity profile} \\ \text{for } n\text{-photon excitation}$ $S(\mathbf{r}) = \text{Sample extent}$ $X(\mathbf{r}) = \text{Detection efficiency}$ $C(\mathbf{r}, \mathbf{t}) = \text{Number Density}$

The ACF is given by:

$$G(\tau) = \frac{\left\langle \delta F(0) \delta F(\tau) \right\rangle}{\left\langle F \right\rangle^2}$$

$$G(\tau) = \frac{\iint d\mathbf{r} d\mathbf{r}' W(\mathbf{r}) W(\mathbf{r'}) \langle \delta C(\mathbf{r}, \tau) \delta C(\mathbf{r'}, 0) \rangle}{\left[\langle C \rangle \int d\mathbf{r} W(\mathbf{r}) \right]^2}$$

Point-Spread Functions (PSF)

The PSF is the measured fluorescence intensity of a point particle at the position **r** within the excitation volume

1-photon excitation, confocal detection:

Approximate the PSF by a 3 dimensional Gaussian

$$W(x, y, z) = I_0(0, 0, 0) \exp \left[-\frac{2(x^2 + y^2)}{w_r^2} - \frac{2z^2}{w_z^2}\right]$$

where w_r and w_z the radial and axial distance from the center to where the intensity has decayed by $(1/e)^2$ respectively

2-photon excitation:

Approximate the PSF by the expression for the Gaussian Beam Waist of the laser:

$$I(x, y, z) = I_0(0, 0, 0) \frac{w_0^2}{w(z)^2} \exp\left[-\frac{2(x^2 + y^2)}{w(z)^2}\right]$$

where $w(z)^2 = w_0^2 \left[1 + \left(\frac{z}{(\pi w_0^2/\lambda)}\right)^2\right]$

 w_0 is the beam waist and λ is the excitation laser wavelength

$$W(x, y, z) = I_0^2(0, 0, 0) \frac{w_0^4}{w(z)^4} \exp\left[-\frac{4(x^2 + y^2)}{w(z)^2}\right]$$

Autocorrelation Function, Single Species

For a freely diffusing species: $\frac{\partial \delta C(\mathbf{r},t)}{\partial t} = D\nabla^2 \delta C(\mathbf{r},t)$

$$\begin{split} \delta C(\mathbf{r},t) &= \int d\mathbf{\rho} \ e^{i\mathbf{\rho}\cdot\mathbf{r}} \delta C_{f}(\mathbf{\rho},t) \\ \frac{\partial \delta C(\mathbf{r},t)}{\partial t} &= \int d\mathbf{\rho} \ e^{i\mathbf{\rho}\cdot\mathbf{r}} \left\{ -D\rho^{2} \delta C_{f}(\mathbf{\rho},t) \right\} \\ \delta C(\mathbf{r},t) &= \int d\mathbf{\rho} \ e^{i\mathbf{\rho}\cdot\mathbf{r}} \left\{ \delta C_{f}(\mathbf{\rho},0) e^{-D\rho^{2}t} \right\} \\ \left\langle \delta C(\mathbf{r},t) \delta C(\mathbf{r}',0) \right\rangle &= \int d\mathbf{\rho} \ e^{i\mathbf{\rho}\cdot\mathbf{r}} \left\{ \left\langle \delta C_{f}(\mathbf{\rho},0) \delta C(\mathbf{r}',0) \right\rangle e^{-D\rho^{2}t} \right\} \\ &= \int d\mathbf{\rho} \ e^{i\mathbf{\rho}\cdot\mathbf{r}} \left\{ \frac{1}{2\pi} \int d\mathbf{r}'' \ e^{-i\mathbf{\rho}\cdot\mathbf{r}''} \left\langle \delta C(\mathbf{r}'',0) \delta C(\mathbf{r}',0) \right\rangle e^{-D\rho^{2}t} \right\} \\ &= \frac{\langle C \rangle}{2\pi} \int d\mathbf{\rho} \ e^{i\mathbf{\rho}\cdot|\mathbf{r}-\mathbf{r}'|-D\rho^{2}t} \\ \left\langle \delta C(\mathbf{r},t) \delta C(\mathbf{r}',0) \right\rangle &= \frac{\langle C \rangle}{(4\pi Dt)^{\frac{n}{2}}} e^{-\left(\frac{|\mathbf{r}-\mathbf{r}'|^{2}}{4Dt}\right)} \\ G(\tau) &= \frac{\iint d\mathbf{r} d\mathbf{r}' W(\mathbf{r}) W(\mathbf{r}') \left\langle \delta C(\mathbf{r},\tau) \delta C(\mathbf{r}',0) \right\rangle}{\left[\langle C \rangle \int d\mathbf{r} W(\mathbf{r}) \right]^{2}} \end{split}$$

For a 3-Dimensional Gaussian Probe Volume:

$$G_D(\tau, N, \tau_D) = \frac{\gamma}{\langle N \rangle} \left(\frac{1}{1 + \tau / \tau_D} \right) \left(\frac{1}{1 + (w_r / w_z)^2 \tau / \tau_D} \right)^{1/2}$$

where $\tau_D = \frac{w_r^2}{4D}$

Autocorrelation Function, Single Species

$$G_D(\tau, N, \tau_D) = \frac{\gamma}{\langle N \rangle} \left(\frac{1}{1 + \tau / \tau_D}\right) \left(\frac{1}{1 + \left(w_r / w_z\right)^2 \tau / \tau_D}\right)^{\frac{1}{2}}$$



Freely Diffusing Particles with Flow

For flow (or scanning) in the y direction in a 3-Dimensional Gaussian Probe Volume :



$$\frac{\partial \delta C(\mathbf{r},t)}{\partial t} = D\nabla^2 \delta C(\mathbf{r},t) - v \frac{\partial \delta C(\mathbf{r},t)}{\partial y}$$
$$\left\langle \delta C(\mathbf{r},t) \delta C(\mathbf{r}',0) \right\rangle = \frac{\left\langle C \right\rangle}{\left(4\pi Dt\right)^{\frac{3}{2}}} e^{-\left(\frac{|\mathbf{r}-\mathbf{r}'|^2 + 2|y-y'|^2 + v^2 t^2}{4Dt}\right)}$$

$$G_D(\tau, N, \tau_D) = \frac{\gamma}{\langle N \rangle} \left(\frac{1}{1 + \tau/\tau_D}\right) \left(\frac{1}{1 + \left(w_r/w_z\right)^2 \tau/\tau_D}\right)^{1/2} \left(e^{-\frac{v^2 \tau^2}{w_r^2 (1 + \tau/\tau_D)}}\right)$$

TMR Labeled Endothelin



Gamma Factor

For a single particle fixed at the center of the PSF:

 $C(\mathbf{r},t) = \delta(0)$

The fluorescent intensity is given by:

$$F(t) = \kappa Q \int d\mathbf{r} W(\mathbf{r}) C(\mathbf{r}, t)$$
$$= \kappa Q \int d\mathbf{r} W(\mathbf{r}) \delta(0)$$
$$= \kappa Q W(0)$$

We define the molecular brightness as $\varepsilon = \kappa QW(0)$ For molecules freely diffusing in solution:

$$\langle F(t) \rangle = \varepsilon \langle N \rangle$$

$$= \left\langle \varepsilon \int d\mathbf{r} \, \frac{W(\mathbf{r})}{W(0)} \, C(\mathbf{r}, t) \right\rangle$$

$$= \varepsilon \langle C \rangle \int d\mathbf{r} \, \frac{W(\mathbf{r})}{W(0)}$$

$$= \varepsilon \langle C \rangle V_{eff}$$

$$\langle N \rangle = \langle C \rangle V_{eff}; \quad V_{eff} = \int d\mathbf{r} \, \frac{W(\mathbf{r})}{W(0)}$$

 V_{eff} is the volume of uniform illumination at maximum intensity that yields the same overall detection rate

Hence the molecular brightness can be determined from the ACF and the average intensity

$$\varepsilon = \langle F \rangle / \langle N \rangle$$

Gamma Factor

$$G(0) = \frac{\iint d\mathbf{r} d\mathbf{r}' W(\mathbf{r}) W(\mathbf{r}') \langle \delta C(\mathbf{r}, 0) \delta C(\mathbf{r}', 0) \rangle}{\left[\langle C \rangle \int d\mathbf{r} W(\mathbf{r}) \right]^2} \\ \langle \delta C(\mathbf{r}, 0) \delta C(\mathbf{r}', 0) \rangle = \langle C \rangle \delta(\mathbf{r} - \mathbf{r}') \\ G(0) = \frac{\iint d\mathbf{r} d\mathbf{r}' W(\mathbf{r}) W(\mathbf{r}') \langle C \rangle \delta(\mathbf{r} - \mathbf{r}')}{\left[\langle C \rangle \int d\mathbf{r} W(\mathbf{r}) \right]^2} \\ = \frac{\langle C \rangle \int d\mathbf{r} W^2(\mathbf{r})}{\langle C \rangle^2 \left[\int d\mathbf{r} W(\mathbf{r}) \right]^2} \\ = \frac{\int d\mathbf{r} (W(\mathbf{r}) / W(0))^2}{\langle C \rangle \left[\int d\mathbf{r} (W(\mathbf{r}) / W(0)) \right]^2} = \frac{\gamma}{\langle N \rangle} \\ \gamma = \frac{\langle N \rangle}{\langle C \rangle} \frac{\int d\mathbf{r} (W(\mathbf{r}) / W(0))^2}{\left[\int d\mathbf{r} (W(\mathbf{r}) / W(0)) \right]^2} \\ = \frac{\langle N \rangle V_{eff}}{\langle N \rangle} \frac{\int d\mathbf{r} (W(\mathbf{r}) / W(0))^2}{\left[\int d\mathbf{r} (W(\mathbf{r}) / W(0)) \right]^2} \\ = \frac{\int d\mathbf{r} (W(\mathbf{r}) / W(0))^2}{\left[\int d\mathbf{r} (W(\mathbf{r}) / W(0)) \right]^2}$$

Afterpulsing

Autocorrelation from Reflected Laser Light



The amplitude of the Autocorrelation Function is inversely proportional to the intensity

A Power Law is used to empirically fit the afterpulsing



Signal-to-Noise Considerations

High Intensity Limit:

Uncertainty dominated by number of fluctuations:

$$\frac{S}{N} \approx \left(\frac{t_{\exp}}{\tau_{C}}\right)^{\frac{1}{2}}$$

where t_{exp} is the measurement time of the experiment and τ_C is the correlation time of the fluctuations

Low Intensity Limit:

Uncertainty dominated by number of photons:

$$\frac{S}{N} \approx \left(t_{\exp}\right)^{\frac{1}{2}} I_T \frac{\gamma}{\langle N \rangle}$$
$$I_T = \varepsilon \langle N \rangle$$
$$\frac{S}{N} \approx \left(t_{\exp}\right)^{\frac{1}{2}} \varepsilon \gamma$$

Only possibilities to improve the S/N ratio are: extend the measurement time increase the counts per molecule second change the geometry

S/N is independent of sample concentration!!!

Limitations

Time Scale: $ns/\mu s \rightarrow ms/s/hrs$

Early time limit: Detector afterpulsing (100 ns - 5 μs) Detector deadtime: (2 ns - 30 ns) Numbers of available photons: (10 ns - 100 ns)

Long time limit:

Time molecule remains in the excitation volume (Typically ~ 1 ms)

Increase the long time limit by: Increasing the excitation volume: (10 ms) Place sample in viscous solvents or gels: (s)

Slow reactions can be measured by changes in the autocorrelation function with time. (hrs)

Concentration Limits: $\sim 1 \ \mu M \rightarrow 10 \ pM$

Maximum Concentration: (1 µM) The change in signal from thermodynamic fluctuations becomes comparable to other sources of noise in the system

Minimum Concentration: (10 pM) Limit statistics Impurities