Supporting Information

Accompanying: *The spectroscopic basis of Fluorescence Triple Correlation Spectroscopy*

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Details on the solution of the molecular correlation function

The solution proceeds by writing $\phi_{jjj}(\mathbf{r}_1, \mathbf{r}_2, \mathbf{r}_3, \tau_1, \tau_2)$ in terms of Fourier substitutions:

$$F_{\mathbf{k}}\{\delta C(\mathbf{r},\tau)\} = \frac{1}{(2\pi)^{3/2}} \int e^{i\mathbf{k}\cdot\mathbf{r}} \delta C(\mathbf{r},\tau) d\mathbf{r} = \delta \widetilde{C}(\mathbf{k},\tau)$$
$$F_{\mathbf{r}}^{-1}\{\delta \widetilde{C}(\mathbf{k},\tau)\} = \frac{1}{(2\pi)^{3/2}} \int e^{-i\mathbf{k}\cdot\mathbf{r}} \delta \widetilde{C}(\mathbf{k},\tau) d\mathbf{k} = \delta C(\mathbf{r},\tau)$$

$$\begin{aligned}\phi_{jjj}(\mathbf{r}_1, \mathbf{r}_2, \mathbf{r}_3, \tau_1, \tau_2) &= \langle \delta C(\mathbf{r}_1, 0) \delta C(\mathbf{r}_2, \tau_1) \delta C(\mathbf{r}_3, \tau_2) \rangle \\ &= \left\langle \delta C(\mathbf{r}_1, 0) F_{\mathbf{r}_2}^{-1} \left\{ F_{\mathbf{k}_2} \left\{ \delta C(\mathbf{r}_2, \tau_1) \right\} \right\} F_{\mathbf{r}_3}^{-1} \left\{ F_{\mathbf{k}_3} \left\{ \delta C(\mathbf{r}_3, \tau_2) \right\} \right\} \right\rangle \\ &= F_{\mathbf{r}_2}^{-1} \left\{ F_{\mathbf{r}_3}^{-1} \left\{ \left\langle \delta C(\mathbf{r}_1, 0) F_{\mathbf{k}_2} \left\{ \delta C(\mathbf{r}_2, \tau_1) \right\} F_{\mathbf{k}_3} \left\{ \delta C(\mathbf{r}_3, \tau_2) \right\} \right\rangle \right\} \right\} \\ &= F_{\mathbf{r}_2}^{-1} \left\{ F_{\mathbf{r}_3}^{-1} \left\{ \left\langle \delta C(\mathbf{r}_1, 0) \delta \widetilde{C}(\mathbf{k}_2, \tau_1) \delta \widetilde{C}(\mathbf{k}_3, \tau_2) \right\rangle \right\} \right\} \end{aligned} \quad \text{(S2)}$$

The expectation value is reached if $\delta \widetilde{C}(\mathbf{k}_2, \tau_1)$ and $\delta \widetilde{C}(\mathbf{k}_3, \tau_2)$ are solved in time using the diffusion equation

$$\frac{\partial \delta C(\mathbf{r},\tau)}{\partial \tau} = D \nabla^2 \delta C(\mathbf{r},\tau) \quad \text{(S3)}$$

$$
\begin{aligned}
F_{\mathbf{k}}\left\{\frac{\delta C(\mathbf{r},\tau)}{\partial\tau}\right\} &= F_{\mathbf{k}}\{D\nabla^2\delta C(\mathbf{r},\tau)\} \\
\frac{\delta\widetilde{C}(\mathbf{k},\tau)}{\partial\tau} &= \frac{D}{(2\pi)^{3/2}}\nabla^2\int e^{i\mathbf{k}\cdot\mathbf{r}}\delta C(\mathbf{r},\tau)d\mathbf{r} \\
\frac{\delta\widetilde{C}(\mathbf{k},\tau)}{\partial\tau} &= \frac{-\mathbf{k}^2 D}{(2\pi)^{3/2}}\int e^{i\mathbf{k}\cdot\mathbf{r}}\delta C(\mathbf{r},\tau)d\mathbf{r} \\
\frac{\delta\widetilde{C}(\mathbf{k},\tau)}{\partial\tau} &= -\mathbf{k}^2 D\delta\widetilde{C}(\mathbf{k},\tau) \\
\Rightarrow \delta\widetilde{C}(\mathbf{k},\tau) &= \delta\widetilde{C}(\mathbf{k},0)e^{-\mathbf{k}^2 D(\tau)}
\end{aligned}
$$

Where the last step is the solution to the differential equation. To apply this result to the expectation value, delay times τ_1 and $\tau_2-\tau_1$ are substituted for τ in a manner consistent with Eq. (14):

$$
\delta C(\mathbf{r}_1,0)\delta\widetilde{C}(\mathbf{k}_2,\tau_1)\delta\widetilde{C}(\mathbf{k}_3,\tau_2) = \delta C(\mathbf{r}_1,0)\delta\widetilde{C}(\mathbf{k}_2,0)\delta\widetilde{C}(\mathbf{k}_3,0)e^{-\mathbf{k}_2^2 D\tau_1}e^{-\mathbf{k}_3^2 D(\tau_2-\tau_1)} \tag{S4}
$$

Combining Eq. (S2) and Eq. (S4),

$$
\begin{aligned}
&\phi_{jjj}(\mathbf{r}_1,\mathbf{r}_2,\mathbf{r}_3,\tau_1,\tau_2) \qquad\qquad (\mathrm{S5})\\
&\quad= F_{\mathbf{r}_2}^{-1}\left\{F_{\mathbf{r}_3}^{-1}\left\{\left\langle\delta C(\mathbf{r}_1,0)\delta\widetilde{C}(\mathbf{k}_2,0)\delta\widetilde{C}\mathbf{k}_3,0)\right\rangle e^{-\mathbf{k}_2^2 D_j\tau_1}e^{-\mathbf{k}_3^2 D_j(\tau_2-\tau_1)}\right\}\right\} \\
&\quad= F_{\mathbf{r}_2}^{-1}\left\{F_{\mathbf{r}_3}^{-1}\left\{\left\langle\delta C(\mathbf{r}_1,0)F_{\mathbf{k}_2}\{\delta C(\mathbf{r}_2,0)\}F_{\mathbf{k}_3}\{\delta C(\mathbf{r}_3,0)\}\right\rangle e^{-\mathbf{k}_2^2 D_j\tau_1}e^{-\mathbf{k}_3^2 D_j(\tau_2-\tau_1)}\right\}\right\} \\
&\quad= F_{\mathbf{r}_2}^{-1}\left\{F_{\mathbf{r}_3}^{-1}\left\{F_{\mathbf{k}_2}\left\{F_{\mathbf{k}_3}\left\{\left\langle\delta C(\mathbf{r}_1,0)\delta C(\mathbf{r}_2,0)\delta C(\mathbf{r}_3,0)\right\rangle e^{-\mathbf{k}_2^2 D_j\tau_1}e^{-\mathbf{k}_3^2 D_j(\tau_2-\tau_1)}\right\}\right\}\right\}\right\}
\end{aligned}
$$

Applying the boundary condition in Eq. (16):

$$\begin{aligned}
&\phi_{jjj}(\mathbf{r}_1,\mathbf{r}_2,\mathbf{r}_3,\tau_1,\tau_2)\\
&=F^{-1}_{\mathbf{r}_2}\left\{F^{-1}_{\mathbf{r}_3}\left\{F_{\mathbf{k}_2}\left\{F_{\mathbf{k}_3}\left\{\overline{C}_j\delta(\mathbf{r}_2-\mathbf{r}_1)\delta(\mathbf{r}_3-\mathbf{r}_2)e^{-\mathbf{k}_2^2D_j\tau_1}e^{-\mathbf{k}_3^2D_j(\tau_2-\tau_1)}\right\}\right\}\right\}\right\}\\
&=\frac{\overline{C}_j}{(2\pi)^3}F^{-1}_{\mathbf{r}_2}\left\{F^{-1}_{\mathbf{r}_3}\left\{e^{i\mathbf{k}_2\cdot\mathbf{r}_1}e^{-\mathbf{k}_2^2D_j\tau_1}e^{i\mathbf{k}_3\cdot\mathbf{r}_2}e^{-\mathbf{k}_3^2D_j(\tau_2-\tau_1)}\right\}\right\}
\end{aligned}\tag{S6}$$

The final form of ϕ_{jjj} is calculated using a Fourier relation:

$$\int e^{i\mathbf{x}\cdot\xi}e^{-t\mathbf{x}^2}d\mathbf{x}=\left(\frac{\pi}{t}\right)^{3/2}e^{-\xi^2/4t}\tag{S7}$$

$$\phi_{jjj}(\mathbf{r}_1,\mathbf{r}_2,\mathbf{r}_3,\tau_1,\tau_2)=\frac{\overline{C}_j\,e^{-(\mathbf{r}_2-\mathbf{r}_1)^2/4D_j\tau_1}e^{-(\mathbf{r}_3-\mathbf{r}_2)^2/4D_j(\tau_2-\tau_1)}}{64\pi^3D_j^3\tau_1^{3/2}(\tau_2-\tau_1)^{3/2}}\tag{S8}$$

Details on the solution of the single-species FCS function $G_{jjj}(\tau_1,\tau_2)$

$G_{jjj}(\tau_1,\tau_2)$ is written explicitly with the definitions of $I(\mathbf{r})$ and $\phi_{jjj}(\mathbf{r}_1,\mathbf{r}_2,\mathbf{r}_3,\tau_1,\tau_2)$:

$$\begin{aligned}
G_{jjj}(\tau_1,\tau_2)&=\iiint I(\mathbf{r}_1)I(\mathbf{r}_2)I(\mathbf{r}_3)\phi_j(\mathbf{r}_1,\mathbf{r}_2,\mathbf{r}_3,\tau_1,\tau_2)d\mathbf{r}_1d\mathbf{r}_2d\mathbf{r}_3\\
&=\frac{\overline{C}_j}{2^{3/2}\pi^{15/2}D^3\tau_1^{3/2}(\tau_2-\tau_1)^{3/2}r_0^6z_0^3}\int e^{-2(r_{1,x}^2+r_{1,y}^2)/r_0^2}e^{-2r_{1,z}^2/z_0^2}\\
&\quad\times e^{-2(r_{2,x}^2+r_{2,y}^2)/r_0^2}e^{-2r_{2,z}^2/z_0^2}e^{-2(r_{3,x}^2+r_{3,y}^2)/r_0^2}e^{-2r_{3,z}^2/z_0^2}\\
&\quad\times e^{-(\mathbf{r}_2-\mathbf{r}_1)^2/4D\tau_1}e^{-(\mathbf{r}_3-\mathbf{r}_2)^2/4D(\tau_2-\tau_1)}d\mathbf{r}_1d\mathbf{r}_2d\mathbf{r}_3
\end{aligned}\tag{S9}$$

This integral is solved by repeated use of Fourier convolution relations that progressively collapse the function down to a single three-dimensional integral, the solution of which is written in terms of $\tau_D = \frac{r_0^2}{4D_j}$ and $\omega = z_0/r_0$,

$$G_{jjj}(\tau_1,\tau_2) = \frac{\overline{C}_j}{\pi^3 r_0^4 z_0^2}\left(\frac{4}{3}\right)^{3/2}\left(1+4\tau_1(\tau_2-\tau_1)/3\tau_D^2+4\tau_2/3\tau_D\right)^{-1}$$
$$\times\left(1+4\tau_1(\tau_2-\tau_1)/3\omega^4\tau_D^2+4\tau_2/3\omega^2\tau_D\right)^{-1/2}$$

Numerical solution of the triple-correlation function

Analytical solutions to Eq. (9) rely on analytical forms of both point-spread functions $I(\mathbf{r})$ and molecular correlation functions $\phi(\mathbf{r}_1,\mathbf{r}_2,\mathbf{r}_3,\tau_1,\tau_2)$, which might not exist. In particular, point-spread functions for high-N.A. objectives are best described by diffraction-based numerical models.[35]

A direct numerical solution to Eq. (9) involves a computationally-expensive double convolution integral that can be avoided for very simple systems for which it is possible to write $\phi_{jkl}(\mathbf{r}_1,\mathbf{r}_2,\mathbf{r}_3,\tau_1,\tau_2)$ as the product of two double correlation functions:

$$\frac{\phi_{jkl}(\mathbf{r}_1,\mathbf{r}_2,\mathbf{r}_3,\tau_1,\tau_2)}{\phi_{jkl}(\mathbf{r}_1,\mathbf{r}_2,\mathbf{r}_3,0,0)} = \frac{\phi_{jk}(\mathbf{r}_2-\mathbf{r}_1,\tau_1)}{\phi_{jk}(\mathbf{r}_2-\mathbf{r}_1,0)}\frac{\phi_{kl}(\mathbf{r}_3-\mathbf{r}_2,\tau_2-\tau_1)}{\phi_{kl}(\mathbf{r}_3-\mathbf{r}_2,0)} \qquad \text{(S10)}$$

Diffusion of a single species j is an example of such a process:

$$\begin{aligned}\frac{\phi_{jjj}(\mathbf{r}_1,\mathbf{r}_2,\mathbf{r}_3,\tau_1,\tau_2)}{\phi_{jjj}(\mathbf{r}_1,\mathbf{r}_2,\mathbf{r}_3,0,0)} &= \frac{e^{-(\mathbf{r}_2-\mathbf{r}_1)^2/4D_j\tau_1}e^{-(\mathbf{r}_3-\mathbf{r}_2)^2/4D_j(\tau_2-\tau_1)}}{64\pi^3 D_j^3\tau_1^{3/2}(\tau_2-\tau_1)^{3/2}}\\ &= \left[\frac{e^{-(\mathbf{r}_2-\mathbf{r}_1)^2/4D_j\tau_1}}{8(\pi D_j\tau_1)^{3/2}}\right]\left[\frac{e^{-(\mathbf{r}_3-\mathbf{r}_2)^2/4D_j(\tau_2-\tau_1)}}{8(\pi D_j(\tau_2-\tau_1))^{3/2}}\right]\\ &= \frac{\phi_{jj}(\mathbf{r}_2-\mathbf{r}_1,\tau_1)}{\phi_{jj}(\mathbf{r}_2-\mathbf{r}_1,0)}\frac{\phi_{jj}(\mathbf{r}_3-\mathbf{r}_2,\tau_2-\tau_1)}{\phi_{jj}(\mathbf{r}_3-\mathbf{r}_2,0)}\end{aligned}$$

Numerical solutions can then use the same Fourier relations that also simplify numerical solutions to double correlation integrals[34]

$$G(\tau_1,\tau_2) = \iiint I(\mathbf{r}_1)I(\mathbf{r}_2)I(\mathbf{r}_3)\phi_{jkl}(\mathbf{r}_1,\mathbf{r}_2,\mathbf{r}_3,\tau_1,\tau_2)d\mathbf{r}_1 d\mathbf{r}_2 d\mathbf{r}_3$$

$$G(\tau_1,\tau_2) = \frac{\phi_{jkl}(\mathbf{r}_1,\mathbf{r}_2,\mathbf{r}_3,0,0)}{\phi_{jk}(\mathbf{r}_2-\mathbf{r}_1,0)\phi_{kl}(\mathbf{r}_3-\mathbf{r}_2,0)}\iiint I(\mathbf{r}_1)I(\mathbf{r}_2)I(\mathbf{r}_3)$$

$$\times\,\phi_{jk}(\mathbf{r}_2-\mathbf{r}_1,\tau_1)\phi_{kl}(\mathbf{r}_3-\mathbf{r}_2,\tau_2-\tau_1)\,d\mathbf{r}_1 d\mathbf{r}_2 d\mathbf{r}_3$$

$$G(\tau_1,\tau_2) = \frac{\phi_{jkl}(\mathbf{r}_1,\mathbf{r}_2,\mathbf{r}_3,0,0)}{\phi_{jk}(\mathbf{r}_2-\mathbf{r}_1,0)\phi_{kl}(\mathbf{r}_3-\mathbf{r}_2,0)}\int B(\mathbf{r}_3)I(\mathbf{r}_3)d\mathbf{r}_3$$

$$B(\mathbf{r}_3) = F_{\mathbf{r}_2}^{-1}\left\{F_{\mathbf{k}_2}\{A(\mathbf{r}_2)I(\mathbf{r}_2)\}F_{\mathbf{k}_2}\{\phi_{kl}(\mathbf{r}_3-\mathbf{r}_2,\tau_2-\tau_1)\}\right\}$$

$$A(\mathbf{r}_2) = F_{\mathbf{r}_1}^{-1}\left\{F_{\mathbf{k}_1}\{I(\mathbf{r}_1)\}F_{\mathbf{k}_1}\{\phi_{jk}(\mathbf{r}_2-\mathbf{r}_1,\tau_1)\}\right\}$$

Calculation of effective volumes for double and triple correlations

Effective volumes[22] for double and triple correlations utilizing two-photon excitation were calculated using numerical models of point-spread functions[34,35] that were scaled to match FCS data by reducing the laser excitation wavelength λ to 85% of the actual value (Table S1). The exact values of γ_2 and γ_3 are uncertain ($\pm$40%) because the calculation is sensitive to both size and spacing of the grid used to calculate the point-spread functions (which corresponds experimentally to the the size of the APD detector head), but the ratio of contrast ratios consistently falls in the range $1.5 < \gamma_3/\gamma_2^2 < 1.9$. Using the notation of Hess *et al.*,[34] the overfilling factor β is the ratio of objective back aperture radius to the laser $1/e^2$ radius.

Table S1: Contrast ratios of various point-spread functions

PSF model	β	γ_2^{-1} (aL)	$\gamma_3^{-1/2}$ (aL)	γ_3/γ_2^2
Gaussian	N/A	93	75	$\left(\frac{4}{3}\right)^{3/2} = 1.54$
	1/4	80	60	1.65
(Wolf-Richards)^2	1/2	80	60	1.66
	1	100	80	1.69

Adjustments for multiphoton spectroscopy

Two- and three-photon fluorescence excitation can be achieved with $\sim$ 150fs laser pulses focussed to a lateral radius r_0 and longitudinal radius z_0, as with single-photon excitation before. The effective point-spread function for n-photon excitation is proportional to the single-photon point-spread-function raised to the n power and renormalized,

$$I^n(\mathbf{r}) = \left(\frac{2}{\pi}\right)^{3/2} \frac{n^{3/2}}{r_0^2 z_0} e^{-2n(r_x^2+r_y^2)/r_0^2} e^{-2nr_z^2/z_0^2} \tag{S11}$$

Using $I^n(\mathbf{r})$, expressions for FCS decays obtained using n-photon excitation is obtained by substituting $r_0^2 \to r_0^2/n$ into the fit functions and using n-photon variants $V_{\text{eff}}^{(n)}$ and $\tau_D^{(n)}$:

$$\begin{aligned} G(\tau_1,\tau_2) &= \left(\overline{C}V_{\text{eff}}^{(n)}\right)^{-2}\left(\frac{4}{3}\right)^{3/2}\left(1+4\tau_1(\tau_2-\tau_1)/3\left(\tau_D^{(n)}\right)^2+4\tau_2/3\tau_D^{(n)}\right)^{-1} \\ &\times\left(1+4\tau_1(\tau_2-\tau_1)/3\omega^4\left(\tau_D^{(n)}\right)^2+4\tau_2/3\omega^2\tau_D^{(n)}\right)^{-1/2} \\ V_{\text{eff}}^{(n)} &= \left(\frac{\pi}{n}\right)^{3/2} r_0^2 z_0; \quad \tau_D^{(n)} = \frac{r_0^2}{4nD} \end{aligned} \tag{S12}$$

Weight functions relating exact and multiple-tau triple correlations

The multiple-tau method calculates $G_{\alpha\times\beta\times\gamma}^{\text{sm}\tau}(\tau_1,\tau_2)$, which is a weighted average of exact correlations $G_{\alpha\times\beta\times\gamma}(\tau_1,\tau_2)$,

$$G_{\alpha\times\beta\times\gamma}^{\text{sm}\tau}(\tau_1,\tau_2) = \sum_{a=-2^j}^{2^j}\sum_{b=-2^\omega}^{2^\omega} W(a,b)G_{\alpha\times\beta\times\gamma}(\tau_1+a,\tau_2+b) \tag{S13}$$

The weight function is an irregular prism with a hexagonal or octagonal base, depending on the relative sizes of binning values j,ω corresponding to each pair of delay times τ_1,τ_2. When $2^j = 2^\omega$, the weighted average and weight function are calculated as:

$$W(a,b) = \begin{cases} A\left[N^{\star} - \max[|a|,|b|]\right] & ab > 0 \\ A\left[N^{\star} - |a-b|\right] & ab < 0 \end{cases} \tag{S14}$$
$$N^{\star} = 2^{j}, \quad A^{-1} = 2^{j}2^{\omega}N^{\star}$$

When $2^j \neq 2^{\omega}$, $W'(a,b)$ is used instead:

$$W'(a,b) = \begin{cases} W(a,b') & |b| \geq (2^{\omega}-2^{j})/2 \\ N^{\star} - |a| & |b| < (2^{\omega}-2^{j})/2 \end{cases}$$
$$b' = \begin{cases} b-(2^{\omega}-2^{j})/2 & b > 0 \\ b+(2^{\omega}-2^{j})/2 & b < 0 \end{cases} \tag{S15}$$
$$N^{\star} = \min[2^{j}, 2^{\omega}]$$

Averaging is a consequence of using binned data. When data from two bins are multiplied together, the time delays between each original (un-binned) piece of data are not all uniform, e.g. the time delay between the earliest datum in one bin and the latest datum in another is greater than the average time delay between the bins.

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