Supporting Methods

Theory of Spatio-Temporal ICS Analysis

Temporal correlation spectroscopy involves the calculation of the normalized autocorrelation function, $g(t)$, of a fluctuating fluorescence signal $i(t)$ at a given time t. A normalized temporal autocorrelation function can be expressed as:

$$g(\tau) = \frac{\langle \delta i(t) \delta i(t+\tau) \rangle}{\langle i \rangle^2}, \qquad \textbf{[S1]}$$

where $\delta i(t) = (i(t) - \langle i \rangle)$, $\langle i \rangle$ is the average intensity over all time points, and τ represents a temporal shift (measured as a change in time from the starting t).

An autocorrelation function can be similarly calculated in the spatial domain. Consider an image of M by N pixels, with the variables x and y representing pixel intensities as a function of discrete image space. x and y are spatial indices, measured as a function of distance (in μm) from the upper left corner of a given image; each point (x,y) has an associated pixel intensity $i(x,y)$. The spatial autocorrelation function is defined as:

$$g(\xi,\eta) = \frac{\langle \delta i(x,y) \delta i(x+\xi, y+\eta) \rangle}{\langle i \rangle_{x,y}^2}, \qquad \textbf{[S2a]}$$

where $\delta i(x,y) = (i(x,y) - \langle i \rangle_{x,y})$ and the spatial mean intensity is defined as

$$\langle i \rangle_{x,y} = \frac{1}{MN} \sum_{x=1}^{M} \sum_{y=1}^{N} i(x,y) \qquad \textbf{[S2b]}$$

and where ξ and η represent spatial lag variables measured as a change in position from the starting x and y values. Alternatively, the spatial autocorrelation function can be calculated by taking the Fourier transform of the power spectrum of the data, as described by Petersen *et al*. (1).

If the two autocorrelation functions are used simultaneously, the result is the combined spatio-temporal autocorrelation function for spatio-temporal ICS, and is defined as:

$$g(\xi,\eta,\tau) = \frac{\langle \delta i(x,y,t)\delta i(x+\xi,y+\eta,t+\tau)\rangle}{\langle i(t)\rangle_{x,y}\langle i(t+\tau)\rangle_{x,y}}, \quad \textbf{[S3]}$$

where $\langle i \rangle_{x,y}$ denotes a spatial average (but not a temporal average) over x and y at time point t. The spatial intensity fluctuations are defined as $\delta i(x,y,t) = (i(x,y,t) - \langle i(t) \rangle_{x,y})$ and τ is the correlation time.

Once the spatio-temporal autocorrelation function has been calculated, the resulting curves must be fit to obtain physical information about the systems. The temporal and spatial domains of Eq. **S3** may be treated separately by calculating both $g(\xi,\eta,0)$ and $g(0,0,\tau)$. The two can be separated because the $g(0,0,0)$ term needed to fit $g(0,0,\tau)$ (the temporal autocorrelation portion, Eq. **S6**) is obtained from the spatial autocorrelation equation for all $\tau = 0$. Separating the two terms reduces the computational power required. The spatial autocorrelation portion was fit by nonlinear least squares with a 2D Gaussian function, as described by Petersen (1):

$$g(\xi,\eta,0) = g(0,0,0)e^{-(\xi^2+\eta^2)/w^2} + g_0, \quad \textbf{[S4]}$$

where $g(0,0,0)$ is the zero spatial lag value, w is the calculated e^{-2} beam radius in the focal plane, and g_0 is a component residual resulting because the autocorrelation does not decay to zero due to limited sampling size; all three variables are fit parameters for this equation.

The number of particles per image (also called number density) can be obtained from the zero lag value $g(0,0,0)$ from the following equation, where '*pixelsize*' is the edge length of each square pixel in the image (1):

$$n = \frac{MN * (pixelsize)^2}{g(0,0,0)\pi w^2}. \quad \textbf{[S5]}$$

By calculating the spatial correlation per image, the number density for each image in the series can be determined.

The temporal autocorrelation portion of Eq. **S3** can now be fitted. For ease of computation, the following equation was used to evaluate $g(0,0,\tau)$ for each value of τ (2):

$$g(0,0,\tau) = \frac{1}{T-\tau}\sum_{t=1}^{T-\tau}\frac{1}{MN} * \sum_{x=1}^{M}\sum_{y=1}^{N}\frac{\langle \delta i(x,y,t)\delta i(x,y,t+\tau)\rangle}{\langle i(t)\rangle_{x,y}\langle i(t+\tau)\rangle_{x,y}}, \quad \textbf{[S6]}$$

where T is the total number of time points and τ ranges from 0 to T - 1.

The temporal autocorrelation equation can provide information about the dynamics of the system. Past experiments on intracellular behaviors involving microtubules have indicated the presence of a diffusive-like term and often a directed velocity term for short periods of time. In this case, the effective diffusion term represents Brownian processes that follow a random walk model. Using the value of $g(0,0,\tau)$ from above, the following equation (with zero velocity) can be fit by least squares to obtain an effective diffusion term:

$$g(0,0,\tau) = \frac{g(0,0,0)}{\left(1+\frac{\tau}{\tau_d}\right)\left[1+\left(\frac{\langle w\rangle}{z}\right)^2\left(\frac{\tau}{\tau_d}\right)\right]^{1/2}} + h_0, \quad \textbf{[S7]}$$

where $\langle w \rangle$ is the average beam radius, as determined from the spatial autocorrelation, τ_d is the best fit characteristic diffusion time, h_0 is a fitting offset parameter accounting for cases when the correlation function does not decay to zero at long lag times, and z is the beam radius in the axial direction (determined from the point spread function) (3). The effective diffusion term D then can be calculated from τ_d using the following relation: $D = \langle w \rangle^2/4\tau_d$.

Eq. **S7** can be expanded to allow for a directed velocity term in addition to the effective diffusion term for a single population. This velocity term corresponds to motion superimposed upon that expected by a random walk process.

$$g(0,0,\tau) = \frac{g(0,0,0)}{\left(1+\frac{\tau}{\tau_d}\right)\left[1+\left(\frac{\langle w\rangle}{z}\right)^2\left(\frac{\tau}{\tau_d}\right)\right]^{1/2}} \exp\left(-\left(\frac{\tau}{\tau_f}\right)^2 \frac{1}{\left(1+\frac{\tau}{\tau_d}\right)\left[1+\left(\frac{\langle w\rangle}{z}\right)^2\left(\frac{\tau}{\tau_d}\right)\right]^{1/2}}\right) + h_0, \quad \textbf{[S8]}$$

where v is the calculated velocity, τ_f is the characteristic velocity time ($\tau_f = w/v$), and the other constants are as above (3).

Alternatively, Eq. **S7** can be expanded to allow for directed velocity and diffusion for two different populations. In this case, objects alternate between diffusive motion and directed transport. An example of such motion would be directional velocity caused by the concerted action of microtubule motors on a vesicle

$$g(0,0,\tau) = \frac{g(0,0,0)}{\left(1+\frac{\tau}{\tau_d}\right)\left[1+\left(\frac{\langle w\rangle}{z}\right)^2\left(\frac{\tau}{\tau_d}\right)\right]^{1/2}} + g(0,0,0)\ \exp\left(-\left(\frac{\tau}{\tau_f}\right)^2\right) + h_0, \qquad \textbf{[S9]}$$

where the variables are as described (4).

Eqs. **S7**, **S8**, and **S9** are the standard equations used in fluorescence correlation spectroscopy and ICS for fitting autocorrelation functions to find diffusion and velocity terms. The fit functions for diffusion (Eq. **S7**), diffusion plus velocity for one population (Eq. **S8**), and diffusion plus velocity for two populations (Eq. **S9**) were calculated for each temporal correlation function and the appropriate fit was selected based on the minimum residual value (maximum R^2 value) for the fits.

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