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Supplemental Information

Spatiotemporal Fluctuation Analysis: A Powerful Tool for the Future Nanoscopy of Molecular Processes

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Supporting Materials and Methods

Estimation of temporal resolution in SML experiments

In SML experiments, the localization precision (σ_{SML}) depends on the number of collected photons (N_{phs}) and on the imaging resolution (i.e. the single-molecule spot size, σ_{PSF}) according to (see also Ref. [43] in the main text):

$$\sigma_{SML}^2 = \frac{\sigma_{PSF}^2}{N_{phs}}. \quad \textbf{(S1)}$$

At this point, if we define the molecular brightness as the rate of photons collection form each single molecule, B= N_{phs} /t, then Eq. S1 can be rewritten as:

$$\sigma_{SML}^2 = \frac{\sigma_{PSF}^2}{Bt}, \quad \textbf{(S2)}$$

indicating that, given a certain brightness of the label, the achievable resolution (σ_{SML}) is set by the acquisition time *t*. Equation S2 is represented in Fig. 1A for the 3 representative brightness of 10, 100 and 1000 kPhs/s.

Estimation of precision in fitting the width of the correlation function

Following previous results, we define the STICS correlation function as (see also Ref. [54] in the main text):

$$G(\xi, \psi, \tau) = \frac{\langle I(\mathrm{x,y,t}) \cdot I(\mathrm{x}+\xi,\mathrm{y}+\psi,\mathrm{t}+\tau) \rangle}{\langle I(\mathrm{x,y,t}) \rangle^2} - 1, \quad \textbf{(S3)}$$

where $I(\mathrm{x,y,t})$ is the fluorescence intensity measured in the position *x*,*y* at time *t* and ξ, ψ and τ are the spatial and temporal lags, respectively. In the *i*MSD approach, the STICS correlation function is approximated to a gaussian function defined as (see also Ref. [44] in the main text):

$$G(\xi, \psi, \tau) = g(\tau) \exp\left(-\frac{\xi^2+\psi^2}{\sigma_f^2(\tau)}\right) + g_\infty(\tau), \quad \textbf{(S4)}$$

where $g(\tau)$ and $g_\infty(\tau)$ represent the amplitude and the offset of the correlation function, respectively, and

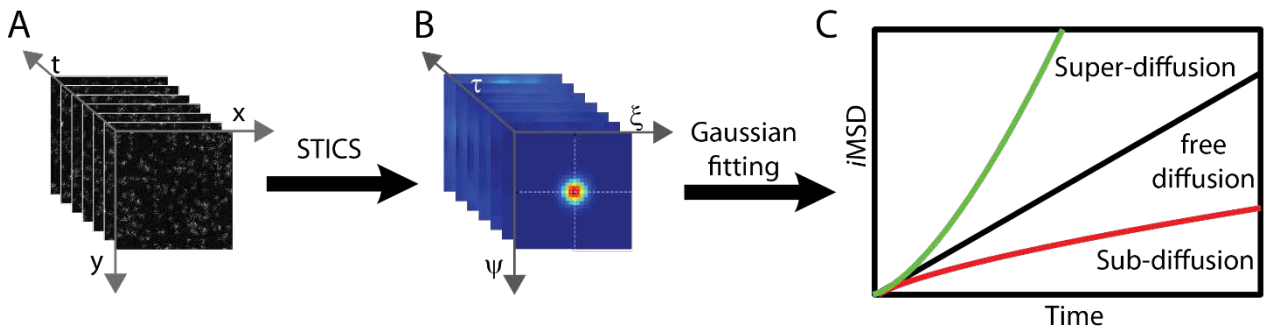
$$\sigma_r^2(\tau) = MSD(\tau) + \sigma_0^2. \quad \textbf{(S5)}$$

In particular, in the case of immobile molecules $MSD(\tau) = 0$. In this case, $\sigma_r^2(\tau)$ is expected to be constant and the residual variance can be used to estimate the precision of the fitting procedure. Thus, we simulated the STICS correlation function of immobile molecules by varying the number of molecules and the number of photons collected from each molecule. In brief, a time-series of 1000 frames, 256x256 pixels wide is simulated. First, a variable number of molecules are randomly seeded in the simulated space. Then, for each simulated frame, a random number of photons is associated with each molecule according to the chosen molecular brightness by using a Poissonian distributed random number generator. Finally, the actual position at which each photon is collected can be obtained by adding a random number drawn from a Gaussian distribution to the molecular position in both the spatial directions, with a full-width-at-half-maximum (FWHM) equal to the imposed imaging resolution (that in turn corresponds to 3 simulated pixels). The obtained image series is used to calculate the STICS correlation function according to Eq. S3. Then, fitting to Eq. S4 enables to obtain $\sigma_r^2(\tau)$. Finally, we estimate σ_{iMSD}^2 as the standard deviation of $\sigma_r^2(\tau)$.

Simulation of particles diffusing within moving vesicles

A time-series of 8000 frames, 256x256 pixels wide is simulated. In order to simulate the diffusion of particles trapped inside a vescicle, we simulated an empty sphere containing 10 point-like objects. The radius of the sphere is set equal to the imaging resolution in the radial plane (σ_{PSF}), that in turn corresponds to 3 simulated pixels. 150 spheres were randomly seeded in the 3D simulated box (256,256,64 pixels in x,y and z direction, respectively, where the focal plane is set in the middle of the simulated volume). 3D Brownian motion is simulated both for the trapped point-like objects and for the whole sphere (the vesicle). In particular, the point-like objects are let free to diffuse inside the sphere with a diffusivity $D_1=10^{-3}$ σ^2_{PSF}/frame, while the sphere is let free to diffuse inside the simulated box with $D_2=D_1/10$. The motion of the point-like objects is limited inside the sphere by applying reflective boundaries at the sphere surface. Moreover, circular boundaries are applied to the motion of the spheres. Simulated frames are then obtained, as described above, by setting the brightness of each point-like object to 0.1 photons per frame (when objects are in the focal plane).

Supporting Figure 1



Supporting Figure 1. Measuring molecular displacement by the *i*MSD approach. (A) First, an image series of the sample is collected, in order to map the spatial and temporal fluctuations of the fluorescence intensity. (B) By calculating the STICS correlation function, all the measured fluorescence fluctuations are averaged together before measuring the molecular displacement. (C) Through the Gaussian fitting of the STICS correlation function at different time delays the average molecular displacement is directly measured in the form of an image-derived Mean Square Displacement or *i*MSD. By plotting the recovered *i*MSD vs time, free diffusion can be easily distinguished from both sub-diffusive and super-diffusive behavior. Further details can be found in Refs. [54,55] of the main text.