Biophysical Journal, Volume *111*

Supplemental Information

Diffusion Tensor Analysis by Two-Dimensional Pair Correlation of Fluorescence Fluctuations in Cells

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Supplementary information

The 2D-pCF provides information on the directionality of molecular motion

As recently demonstrated by Hofling et al. [56], the single-point fluorescence autocorrelation function can be directly linked to the molecule displacement with no a priori assumption on the motion of the fluorescent molecule. Here, we extend this result to the 2D-pCF and show that this function can provide information on the directionality of molecular displacement. We define each image as: $I(\mathbf{r},t)=\varepsilon\int d\mathbf{r}'W(\mathbf{r}-\mathbf{r}')c(\mathbf{r}',t)$, where $c(\mathbf{r},t)$ labels the concentration of the molecules at position **r** at time t, $W(\mathbf{r})$ is the probability density function of measurement uncertainty (the so-called point spread function, PSF), and ε is the total efficiency in photon production, collection and detection. Considering that $W(\mathbf{r})=W(-\mathbf{r})$ and admitting the dilution condition [56], we obtain for Eq. 1 in reciprocal space **k**:

$$G(\mathbf{r}_0;\boldsymbol{\rho},\tau)=\alpha\int d\mathbf{k}e^{-i\boldsymbol{\rho}\mathbf{k}}W(\mathbf{k})^2P(\mathbf{k},\tau), \qquad \text{S1}$$

where $\alpha=\left[N\int d\mathbf{k}W(\mathbf{k})^2\right]^{-1}$ is a normalization factor, $W(\mathbf{k})$ and $P(\mathbf{k},\tau)$ represent the (spatial) Fourier transform of instrumental PSF and diffusion propagator, respectively. In order to simplify Eq. S1 we can use the fact that in most acquisition systems the PSF is well approximated by a Gaussian function defined as:

$$W(\boldsymbol{\rho})=\frac{1}{2\pi\sigma_0^2}\exp\left(-2\frac{\boldsymbol{\rho}^2}{\sigma_0^2}\right), \qquad \text{S2}$$

where σ_0^2 is called radial waist. In light of this, Eq. S1 can be recast into:

$$G(\mathbf{r}_0;\boldsymbol{\rho},\tau)=\frac{1}{N}\left\langle\exp\left(-\frac{\left|\Delta\mathbf{R}_i(\mathbf{r}_0;\tau)-\boldsymbol{\rho}\right|^2}{\sigma_0^2}\right)\right\rangle_i, \qquad \text{S3}$$

where $\left|\Delta\mathbf{R}_i(\mathbf{r}_0;\tau)-\boldsymbol{\rho}\right|$ is the distance between the new i-th molecule position after a delay τ and the point under consideration (**ρ**), $\langle...\rangle_i$ represents the ensemble average over all molecules observed in $\mathbf{r}_0$ at τ=0. According to Eq. S3, each observed molecular displacement $(\Delta\mathbf{R}_i(\mathbf{r}_0;\tau))$ contributes to $G(\mathbf{r}_0;\boldsymbol{\rho},\tau)$ with a gaussian function centered in $\Delta\mathbf{R}_i(\mathbf{r}_0;\tau)$. As a result, summing together all these contributions for each time delay, the 2D-pCF will picture the spatial distribution of molecular displacements, independently on the nature of particle motion. Consequently, in presence of obstacles or barriers able to constrain the motion of molecules along a certain direction, $G(\mathbf{r}_0;\boldsymbol{\rho},\tau)$ becomes direction-dependent and represents a tool to characterize such directionality.

The 2D-pCF in simple case of particle motion

As already shown by Elson and Magde [57], in the case of active transport (flux) characterized by a velocity $\mathbf{v}(\mathbf{r_0})$ at point $\mathbf{r_0}$, the correlation function is:

$$G(\mathbf{r}_0;\boldsymbol{\rho},\tau)=\frac{1}{2\pi N}\exp\left(-\frac{\left|\mathbf{v}(\mathbf{r}_0)\tau-\boldsymbol{\rho}\right|^2}{\sigma_0^2}\right), \qquad \text{S4}$$

similarly to the result obtained for STICS [19]. If we now consider a 2D diffusive motion characterized by a Gaussian propagator of molecular displacement [57], we can define:

$$P(\mathbf{r}_0;\boldsymbol{\rho},\tau)=\frac{1}{2\pi\left\langle\Delta\mathbf{R}_i(\mathbf{r}_0;\tau)^2\right\rangle_i}\exp\left(-\frac{\boldsymbol{\rho}^2}{\left\langle\Delta\mathbf{R}_i(\mathbf{r}_0;\tau)^2\right\rangle_i}\right), \qquad \text{S5}$$

where $\left\langle\Delta\mathbf{R}_i(\mathbf{r}_0;\tau)^2\right\rangle_i$ is the molecule mean square displacement for the molecule observed in $\mathbf{r}_0$. By considering Eq. S5 we can rewrite Eq. S3 as:

$$G(\mathbf{r}_0;\boldsymbol{\rho},\tau)=\frac{1}{2\pi N\sigma_r^2(\tau)}\exp\left(-\frac{\boldsymbol{\rho}^2}{\sigma_r^2(\mathbf{r}_0;\tau)}\right), \qquad \text{S6}$$

where $\sigma_r^2(\mathbf{r}_0,\tau)=\left\langle \Delta\mathbf{R}_i(\mathbf{r}_0,\tau)^2 \right\rangle_i+\sigma_0^2$ is called image-derived Mean Square Displacement or *i*MSD in keeping with the nomenclature already adopted for the analogous quantity derived by STICS[20]. This definition reflects the contribution to the correlation function of the PSF as well as that of the molecular MSD.

Differences between 2D-pCF and STICS

It is probably useful to discuss the relationship between 2D-pCF and STICS. For the latter the correlation function is defined as [19]:

$$G_{STICS}(\xi,\psi,\tau)=\frac{\left\langle \delta I(x,y,t)\delta I(x+\xi,y+\psi,t+\tau)\right\rangle_{x,y}}{\left\langle I(x,y,t)\right\rangle_{x,y}\left\langle I(x,y,t+\tau)\right\rangle_{x,y}}, \qquad \text{S7}$$

where $\langle ... \rangle_{x,y}$ indicates spatial averaging. As a consequence, the displacement of all molecules in the selected region of interest (ROI) enters only as an average. Therefore, although the STICS correlation function can be measured for several small ROIs in the acquired image series, only one STICS correlation function exists for each ROI. On the other hand, as clearly visible in Eq. 1, in order to measure a 2D-pCF, a single starting point $\mathbf{r_0}$ must be selected. As a consequence, once a small ROI is chosen (e.g. 16x16 pixels) a different 2D-pCF must be considered at each pixel (i.e. 256 different 2D-pCFs). Moreover, it should be noted that a ROI significantly larger than the optical resolution of the measurement is typically required to fit the STICS function with the commonly-used Gaussian approximation. As a matter of fact, with a measurement resolution of about 250-300 nm, an at least micrometer-sized region of the sample must be analyzed to properly measure the tail of the STICS function. Similarly, since the STICS function is actually a probability distribution function, proper spatial sampling is required to fit to any model. While here we measure the 2D-pCFs including all the points which are closer than 1.6 µm from the starting point (32x32 pixels wide). STICS would lead to averaging the molecular dynamics over a region that is approximately 9 µm^2 wide.

Supplementary Figures

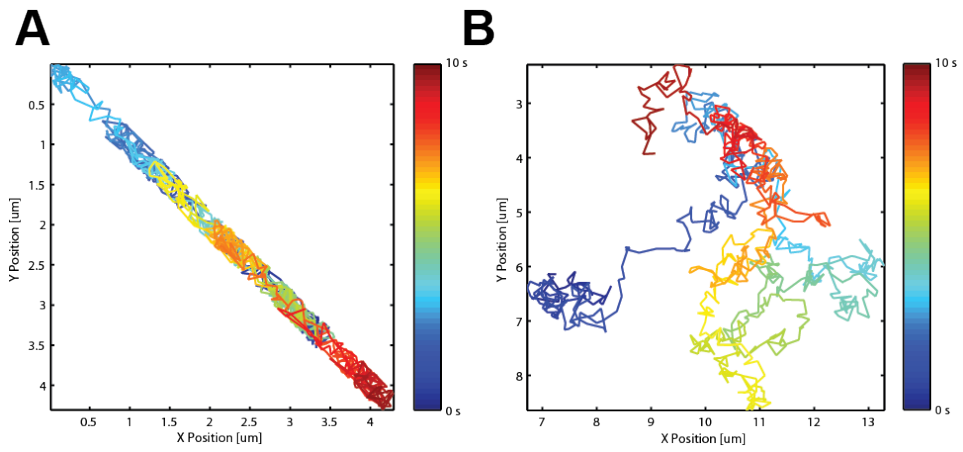

Figure S1. Representative simulated trajectories of molecules diffusing inside the channel (*A*) and far from it (*B*)

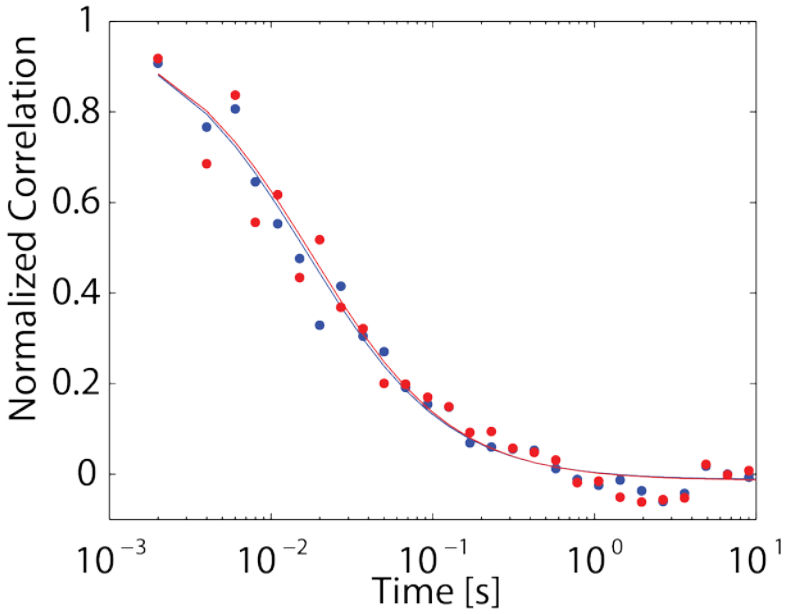

Figure S2 Comparison of between the FCS curves measured in r_1 and r_2 selected in Fig. 7.

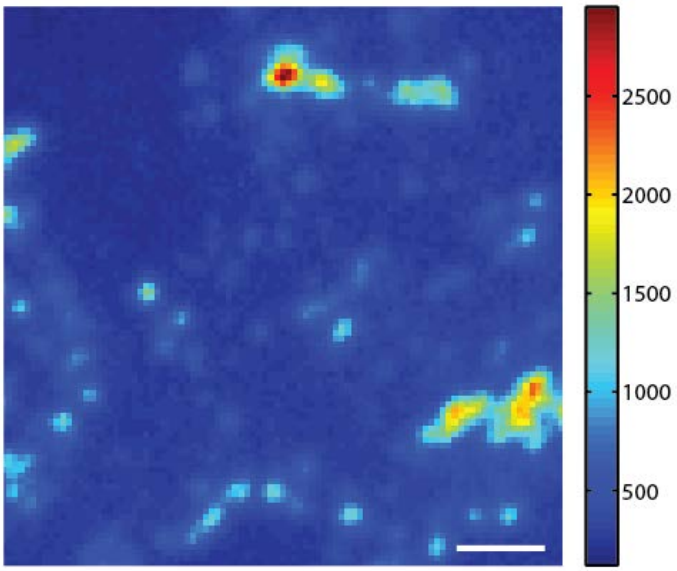

Figure S3 False color image of the Vin-RFP fluorescence collected in TIRF and shown as red in Fig. 9. Scale bar: 2 µm.

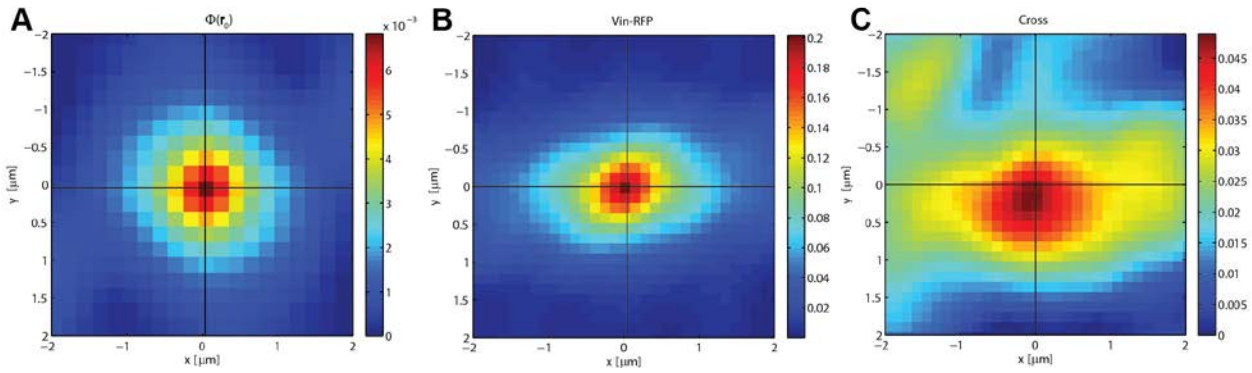

Figure S4 Correlation of diffusion anisotropy measured on H-Ras-GFP fluorescence (*A*) and Vin-RFP (*B*) with the relative cross correlation (*C*) for the region shown in Fig. 9**.**