**iMSD calculation and interpretation**

In brief, the iMSD analysis on the acquired image-stack were carried out using a custom script working on MATLAB (script provided here as **Supplementary File 2**) or alternatively, using the dedicated routine in the SimFCS Software . In detail, first, the spatiotemporal correlation function of the fluorescence intensity fluctuations *g* (Eq. 1) is computed by Fourier methods:

$g\left(ξ,η,τ\right)=\frac{\left〈i\left(x,y,t\right)∙i\left(x+ξ,y+η,t+τ\right)\right〉}{\left〈i\left(x,y,t\right)\right〉^{2}}-1$ (1)

where ξ and η are the distance between correlated pixels in the x and y directions, respectively, τ is the time lag, i(x, y, t) is the fluorescence intensity at point (x, y) and time t, and 〈...〉 indicates the average over spatial and time variables x, y, and t. g(ξ, η, τ) is then fitted by a standard 2D Gaussian function, i.e., (Eq. 2).

$g\left(ξ,η,τ\right)=g\_{0}+g\_{1}\left(τ\right)exp\left\{-\frac{(ξ-v\_{ξ}τ)^{2}+(η-v\_{η}τ)^{2}}{σ^{2}(τ)}\right\}$ (2)

Where the numerator of the exponential term describes the net flux of particles along a specific direction in terms of average velocity, i.e., $\left〈\vec{v}\right〉=\vec{v}\_{φ}=(v\_{ξ},v\_{η})$ and the variance σ2(τ)represents the mean square displacement of the ensemble as a function of the time lag. Categorization of motion can be achieved by fitting σ2(τ) to a power-law equation, i.e. (Eq. 3).

$σ^{2}\left(τ\right)=σ\_{0}^{2}+κ τ^{α}$ (3)

Where σ02 is a y-axis intercept value and affords a quantitative view on the average size of the diffusing object (see below), and α discriminates the dynamics as i) Brownian diffusion (α=1), ii) super-diffusive motion (α>1) and iii) sub-diffusion (α<1). An anomalous diffusion with α < 1 can be regarded as a confined motion and the trend of σ2(τ)can be fitted to the following relationship described by Eq. 4.

$σ^{2}\left(τ\right)=σ\_{0}^{2}+4D\_{M}τ+\frac{L^{2}}{3}\left(1- exp\left\{-\frac{τ}{τ\_{c}}\right\}\right) $ (4)

Where L defines the linear size of the confinement area, τc is an index of how fast confinement occurs, DM is the particle diffusivity on a large time scale and represents 1/4th of the derivative of σ2 for τ→∞. Similarly, the short-term diffusivity Dm is measured by the slope of σ2 for τ → 0 and reads $D\_{m}=D\_{M}+{L^{2}}/{(12τ\_{c})}$. However, if α >1, the trend of σ2(τ) is ascribable to the sum of a linear contribution due to Brownian diffusion and a parabolic term that describes a component of active transport along different directions on the focal plane. In other words:

$σ^{2}\left(τ\right)=σ\_{0}^{2}+4Dτ+v\_{σ}^{2}τ^{2}$ (5)

Where vσ2 represents the variance of particle velocity (i.e. $v\_{σ}^{2}=\left〈\left(\vec{v}-\left〈\vec{v}\right〉\right)^{2}\right〉$) and D is the diffusion coefficient (which is the same both for short and long time scales). Thus, we characterized the intracellular dynamics through Eq. 3–5. Finally, to describe more complex dynamics, as for instance, that of particles undergoing super-diffusive motion on a short time scale and confined diffusion over a larger time range, the following generalization of the aforementioned models can be introduced:

$σ^{2}\left(τ\right)=σ\_{0}^{2}+4D\_{M}τ+\frac{L^{2}}{3}\left(1- exp\left\{-\frac{τ}{τ\_{c}}\right\}\right)+v\_{σ}^{2}τ^{2} exp\left\{-\frac{τ}{τ\_{v}}\right\}$ (6)

where τv (τv < τc) represents a characteristic time below which the super-diffusive trend is dominant. As the parabolic contribution decreases exponentially, it becomes negligible at larger time lags and the *i*MSD trend is driven by the confinement term. This "global" model describes hybrid super/sub-diffusive behaviors and preserves the physical meaning and the corresponding derivation of all the parameters. In Eq. (3), (4), and (5), σ02 is defined as the y-axis intercept value. As already demonstrated for diffusing molecules1 and for cytoplasmic organelles2,3, this value is related to the average particle size (for organelles) or to the PSF (for molecules). In particular, for objects bigger than confocal PSF, such as organelles, the apparent particle size could be calculated using:

$Size\_{app}=\sqrt{σ\_{0}^{2}}$ (7)

In this case, Sizeapp (apparent) represents the average diameter of imaged organelles, i.e., the real size of the organelles convolved with instrument’s PSF.

(1) Di Rienzo, C., Gratton, E., Beltram, F., Cardarelli, F. Fast spatiotemporal correlation spectroscopy to determine protein lateral diffusion laws in live cell membranes. *Proceedings of the National Academy of Sciences of the United States of America.* **110** (30), 12307–12312 (2013).

(2) Digiacomo, L. et al. Dynamic fingerprinting of sub-cellular nanostructures by image mean square displacement analysis. *Scientific Reports.* **7** (1), 14836 (2017).

(3) Ferri, G. et al. Insulin secretory granules labelled with phogrin-fluorescent proteins show alterations in size, mobility and responsiveness to glucose stimulation in living β-cells. *Scientific Reports.* **9** (1), 2890 (2019).