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Accounting for misalignments and thermal fluctuations in Fluorescence Correlation Spectroscopy experiments on membranes

Supplementary Information

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Here, it is detailed the methodology used to account for misalignments and thermal fluctuations in fluorescence correlation spectroscopy (FCS) experiment on membranes. The proposed model is based on the mathematical properties of the prolate ellipsoid which describes the energy spatial distribution generated by a laser, focalized through an adequate optics in a given region of the sample. A cut-off is considered in order to delimit the detection volume (DV). The cut-off is the isosurface corresponding to the lowest detectable intensity value. This isosurface is reasonably assumed being an ellipsoid. When FCS measurements are carried out on membranes, the detection region is defined through the cross-section of the membrane and the ellipsoid. Since, any planar cross-section of an ellipsoid forms an ellipse on its surface, given the membrane flat, the detection area (DA) results elliptical and it degenerates into a circle when aligned to the optical axes.

The circular DA is usually assumed during the experiments, but in certain cases this assumption could be weak leading to apparent anomalous behaviors. Given an elliptical DA, we calculate the its semi-axes and then the fluorophores residence time inside it. Given the detection eccentricity, we define two characteristic times, one for each semi-axis of the detection area. Therefore, the temporal correlation of the collected intensity fluctuations (ACF) is generally 2D asymmetrical. Finally, we removed the membrane motionless assumption. The membrane is now considered fluctuating around its equilibrium position. The characteristic relaxation time of these fluctuations is related to the ratio of the surface tension and the viscosity of the surrounding solvent which acts as a damper. The thermal fluctuation amplitude depends instead on the thickness and the bending modulus of the membrane. This additional degree of freedom, even if limited, affects the 2D ACF which becomes similar to a 3D decorrelation.

1 FCS on arbitrary oriented membranes

Before getting into the issues concerning the analysis of fluorescence correlation spectroscopy measurements, we report few mathematical definitions, Eqs. 1-4, needed to understand the main passages of the model proposed in the manuscript. The general quadratic curve

$$au^2 + 2buv + cu^2 + 2dv + 2fu + g = 0 \tag{1}$$

is an ellipse, if after defining,

$$\Delta = \begin{vmatrix} a & b & d \\ b & c & f \\ d & f & g \end{vmatrix} \quad , \quad J = \begin{vmatrix} a & b \\ b & c \end{vmatrix} \quad \text{and} \quad I = a + c \,, \tag{2}$$

Δ is not equal to zero, J is greater then zero and Δ/I is lower then zero. In that case, the center of the ellipse, $\{c_u, c_v\}$, is given by

$$c_u \to \frac{cd-bf}{b^2-ac} \quad \text{and} \quad c_v \to \frac{af-bd}{b^2-ac}\,, \tag{3}$$

while the expression of the length of the semi-axes, a_u and a_v, are

$$a_u^2 \to \frac{2\left(acg-af^2+b^2(-g)+2bdf-cd^2\right)}{\left(\sqrt{(a-c)^2+4b^2}-a-c\right)(ac-b^2)} \quad \text{and} \quad a_v^2 \to \frac{2\left(acg-af^2+b^2(-g)+2bdf-cd^2\right)}{\left(-\sqrt{(a-c)^2+4b^2}-a-c\right)(ac-b^2)}\,. \tag{4}$$

Thus a generic quadratic curve describes an ellipse only if certain mathematical conditions are fulfilled, we assumed these conditions satisfied in our case, since any planar section of an ellipsoid is an ellipse by definition. In order to determine the extent of the elliptical detection area traced by the membrane, we perform a change of reference. In particular, we define a reference aligned to the membrane, hereinafter referred to as the local reference $\{u,v,k\}$, and an other one oriented according to the confocal optical axes, from now defined as the absolute reference $\{x,y,z\}$.

In the local reference, the u and v coordinates represent the fluorophore location on the membrane while k represents the membrane displacement from the laser focus, thus associated with the unit vector normal to the planar membrane. The membrane is passing through the laser focus when the k coordinate is equal to 0. The two references are related through a rotation matrix R which describes the affine transformation from the absolute to the local reference. The matrix R is a rotation wide θ around the $\hat{y}$ axis. Due to the symmetry of the ellipsoid, this is sufficient to describe all possible inclinations of the membrane with the respect to the focal plane.

$$\begin{pmatrix} x \\ y \\ z \end{pmatrix} = R^T \begin{pmatrix} u \\ v \\ k \end{pmatrix} \quad \text{where} \quad R = \begin{pmatrix} \cos(\theta) & 0 & -\sin(\theta) \\ 0 & 1 & 0 \\ \sin(\theta) & 0 & \cos(\theta) \end{pmatrix} \tag{5}$$

Based on the assumption so far, the relations between the coordinates of the two references are the following

$$x \to u\cos(\theta) - k\sin(\theta) \quad , \quad y \to v \quad \text{and} \quad z \to k\cos(\theta) + u\sin(\theta) \tag{6}$$

Using these relations, the expression of the ellipsoid, $x^2 + y^2 + (z/s)^2 - r_{xy}^2 = 0$, associated with the intensity value I_o/e is reformulated as follows

$$-\frac{k^2\cos^2(\theta)}{s^2} - k^2\sin^2(\theta) + \frac{k\left(s^2-1\right)u\sin(2\theta)}{s^2} + r_{xy}^2 + u^2\left(-\frac{\sin^2(\theta)}{s^2} - \cos^2(\theta)\right) - v^2 = 0 \tag{7}$$

where I_o, r_{xy} and s and are the intensity at the laser focus, the characteristic size and shape factor of the detection volume defined by the confocal optics. By comparing of this last result with the general quadratic curve, Eq. 1, we obtain,

$$\begin{aligned} g &\to \tfrac{-k^2s^2\sin^2(\theta)-k^2\cos^2(\theta)+s^2r_{xy}^2}{s^2} \\ f &\to 0 \\ d &\to \tfrac{k\left(s^2-1\right)\sin(2\theta)}{2s^2} \\ c &\to -1 \\ b &\to 0 \\ a &\to \tfrac{-s^2\cos^2(\theta)-\sin^2(\theta)}{s^2}, \end{aligned} \tag{8}$$

and by replacing the identities so determined in the equation Eqs. 3-4, the expression of the position and length semi-axes of the ellipse that represents the detection area,

$$c_u \to -\tfrac{k\left(s^2-1\right)\sin(2\theta)}{\left(s^2-1\right)\cos(2\theta)+s^2+1} \quad , \quad c_v \to 0 \tag{9}$$

$$a_u^2 \to r_{xy}^2 - \tfrac{2k^2}{\left(s^2-1\right)\cos(2\theta)+s^2+1} \quad \text{and} \quad a_v^2 \to \tfrac{2s^2\left(r_{xy}^2\left(\left(s^2-1\right)\cos(2\theta)+s^2+1\right)-2k^2\right)}{\left(\left(s^2-1\right)\cos(2\theta)+s^2+1\right)^2}. \tag{10}$$

The assumptions made in order to obtain these last results are met up to the condition of tangency between the membrane and the ellipsoid, $|k| < k_o$, where k_o is obtained by determining at which membrane displacement, k_o value, the detection area degenerates into a point, $a_u(k_o) = 0$ or $a_v(k_o) = 0$,

$$k_o \to \frac{r_{xy}\sqrt{\left(s^2-1\right)\cos(2\theta)+s^2+1}}{\sqrt{2}}. \tag{11}$$

Once determined the extents of the detection area, Eq. 10, two characteristic times can be defined, $t_u = a_u^2/(4D)$ and $t_v = a_v^2/(4D)$, leading to a 2D ACF asymmetrical model as the most general for the analysis of fluorescence correlation experiments on membranes. 2D ACF symmetrical models, both ideal and anomalous, are also adopted for those analysis. Indeed, the detection area becomes circular in perfect alignment conditions and thus the symmetrical 2D models are reasonable, if the membrane inclination is tolerable. Therefore, several authors reported their results on membrane by the mean of a single characteristic diffusion time. To esteem a single equivalent characteristic time given the DA eccentricity, we first solve, with the respect of the variable c, the following expression

$$\sqrt{\tfrac{t}{a}+1}\sqrt{\tfrac{t}{b}+1}-(\tfrac{t}{c}+1)=0 \quad , \quad c \rightarrow \frac{t}{\sqrt{\frac{(a+t)(b+t)}{ab}}-1} \tag{12}$$

and then we approximate at both short and long lag times,

$$\begin{aligned} &\lim_{t\rightarrow 0}\; c \rightarrow \frac{t}{\sqrt{\frac{(a+t)(b+t)}{ab}}-1} \rightarrow \frac{2ab}{a+b} \\ &\lim_{t\rightarrow \infty}\; c \rightarrow \frac{t}{\sqrt{\frac{(a+t)(b+t)}{ab}}-1} \rightarrow \sqrt{ab}, \end{aligned} \tag{13}$$

obtaining the following inequality,

$$\frac{1}{\frac{t(a+b)}{2ab}+1} < \frac{1}{\sqrt{\frac{t}{a}+1}\sqrt{\frac{t}{b}+1}} < \frac{1}{\frac{t}{\sqrt{ab}}+1}. \tag{14}$$

2 Accounting for the thermal fluctuations of the membrane

Here, we remove the assumption of the motionless membrane, and now, the membrane can move up-and-down inside the detection volume. This less restrictive assumption is consistent with what experimentalists already assume by neglecting the membrane curvature. The up-and-down motion is described through the relaxation of the low-frequency harmonics of the membrane shape. The low-frequency harmonics are characterized by long relaxation times that are comparable with the characteristic diffusion times measured on membranes. To include their effect into the temporal correlation of the intensity signal (ACF), we consider the Smoluchoski's equation,

$$\frac{\partial p(z,t)}{\partial t} = -\frac{1}{t_m}\frac{\partial (zp(z,t))}{\partial z} + \frac{(mr_z)^2}{4t_m}\frac{\partial^2 p(z,t)}{\partial^2 z}, \tag{15}$$

which leads to the following probability density distribution,

$$p(z;z',t) = \frac{\sqrt{2}}{\sqrt{\pi (mr_z)^2(1-e^{-2t/t_m})}} e^{-\frac{2(z-z'e^{-\frac{t}{t_m}})^2}{(1-e^{-\frac{t}{t_m}})(mr_z)^2}}, \tag{16}$$

where t_m and m are the relaxation time of the thermal fluctuations and the ratio between the characteristic fluctuation amplitude and the characteristic size of the detection volume along the membrane motion direction. Assuming the membrane aligned but moving, the ACF contribution of the thermal fluctuations is formulated through the standard expression of the correlation along the membrane motion direction,

$$\iint_{-\infty}^{+\infty} e^{-2\frac{z}{r_z}} e^{-2\frac{z'}{r_z}} p(z;z',\tau)dzdz' = \frac{\sqrt{\pi} r_z e^{\tau/(2t_m)}}{2\sqrt{cosh(\tau/t_m)+sinh(\tau/t_m)m^2}}. \tag{17}$$

Below, we treat the simultaneous effect of the misalignments and the thermal fluctuations of the membrane. When the membrane is misaligned, the intensity fluctuations due to the membrane motion and those generated by the fluorophores diffusion are strongly coupled. This strong coupling is due to the displacement and distortion of the DA caused by the up-and-down movements of the membrane. The small misalignments could cause the fluctuation of the DA center position, transferring part of the membrane motion to the diffusing fluorophores and making the removal of artifacts even more challenging. Indeed, to take into account all effects, we had to integrate along all possible directions, simultaneously. This makes the mathematical treatment more complex, however, the results reported below can be derived using standard integration methods. In the general case of a moving membrane arbitrarily oriented, the ACF is generalized as follows

$$\begin{aligned} G(\tau) = &\frac{1}{\langle N\rangle}\frac{1}{\sqrt{1+\frac{\tau}{t_d}}}\frac{1}{\sqrt{p_2(1+p_2\frac{\tau}{t_d})}}\frac{1}{\sqrt{1-e^{-\frac{2\tau}{t_m}}}\sqrt{-\frac{e^{\frac{2\tau}{t_m}}}{m^2}+\frac{e^{\frac{2\tau}{t_m}}\coth(\frac{\tau}{t_m})}{m^2}+p_4\sqrt{\frac{m^2(4p_4^2-p_5^2)\sinh(\frac{\tau}{t_m})+8p_4\cosh(\frac{\tau}{t_m})+4p_5}{(m^2p_4+1)\sinh(\frac{\tau}{t_m})+\cosh(\frac{\tau}{t_m})}}}} \\ &p_1 = \frac{\cos^2\theta}{S^2}+\sin^2\theta, \qquad p_2 = \frac{\sin^2\theta}{S^2}+\cos^2\theta, \quad p_3 = \frac{(S^2-1)\sin 2\theta}{S^2}, \\ &p_4 = -\frac{p_3^2(2p_2\tau/t_d+1)-8p_1p_2(p_2\tau/t_d+1)}{4p_2(p_2\tau/t_d+1)}, \quad p_5 = -\frac{p_3^2}{2p_2^2\tau/t_d+2p_2}. \end{aligned} \tag{18}$$