$$= \frac{2Q^2\sigma^2}{\pi\sqrt{\pi}\omega^2}\int_{-\infty}^{\infty}\int_{-\infty}^{\infty}\exp\left(-\frac{2\left(x^2+\left(x+\sqrt{4D\tau}\eta\right)^2\right)+\omega^2\eta^2}{\omega^2}\right)d\eta dy \quad (23)$$
$$= \frac{2Q^2\sigma^2}{\pi\sqrt{\pi}\omega^2}\int_{-\infty}^{\infty}\left\{\int_{-\infty}^{\infty}\exp\left(-\frac{4\left(x+\sqrt{D\tau\eta}\right)^2}{\omega^2}\right)dx\right\}\exp\left(-\frac{(4D\tau+\omega^2)\eta^2}{\omega^2}\right)d\eta$$

where we used the fact,

$$\int_{-\infty}^{\infty}\int_{-\infty}^{\infty}\exp\left(-\frac{2\left(x^2+\left(x+\sqrt{4D\tau}\eta\right)^2\right)}{\omega^2}-\eta^2\right)d\eta dy$$
$$= \int_{-\infty}^{\infty}\int_{-\infty}^{\infty}\exp\left(-\frac{4x^2+4\sqrt{4D\tau}x\eta+2(4D\tau)\eta^2+\omega^2\eta^2}{\omega^2}\right)d\eta dy$$
$$= \int_{-\infty}^{\infty}\int_{-\infty}^{\infty}\exp\left(-\frac{4\left(x^2+2\sqrt{D\tau}x\eta+\left[\sqrt{D\tau}\eta\right]^2\right)+\left[4D\tau+\omega^2\right]\eta^2}{\omega^2}\right)d\eta dy$$
$$= \int_{-\infty}^{\infty}\int_{-\infty}^{\infty}\exp\left(-\frac{4\left(x+\sqrt{D\tau\eta}\right)^2+\left[4D\tau+\omega^2\right]\eta^2}{\omega^2}\right)d\eta dy$$

Now, we can evaluate the inner integral in Eq. 23 using a substitution $z = x + \sqrt{D\tau}\eta$

$$\int_{-\infty}^{\infty}\exp\left(-\frac{4\left(x+\sqrt{D\tau\eta}\right)^2}{\omega^2}\right)dx = \int_{-\infty}^{\infty}\exp\left(-\frac{4z^2}{\omega^2}\right)dz = \frac{\omega\sqrt{\pi}}{2}$$

where we used Eq 14. Back to Eq. 23,

$$G(\tau) = \frac{2Q^2\sigma^2}{\pi\sqrt{\pi}\omega^2}\int_{-\infty}^{\infty}\left\{\int_{-\infty}^{\infty}\exp\left(-\frac{4\left(x+\sqrt{D\tau\eta}\right)^2}{\omega^2}\right)dx\right\}\exp\left(-\frac{(4D\tau+\omega^2)\eta^2}{\omega^2}\right)d\eta$$
$$= \frac{Q^2\sigma^2}{\omega\pi}\int_{-\infty}^{\infty}\exp\left(-\frac{(4D\tau+\omega^2)\eta^2}{\omega^2}\right)d\eta$$
$$= \frac{Q^2\sigma^2}{\omega\pi}\sqrt{\frac{\omega^2\pi}{4D\tau+\omega^2}} \quad (24)$$
$$= \frac{Q^2\sigma^2}{\omega\sqrt{\pi}}\frac{1}{\sqrt{1+\tau/\tau\_D}}$$

by Eq. 14 where $\tau_D = \omega^2/(4D)$, which is often referred to a diffusion time.
Since we assumed that $f(t)$ follows the Poisson statistics (Eq. 16) that has equal variance and mean,

$$G(0) = \langle\delta f(t)\delta f(t+0)\rangle_t = \langle\left(\delta f(t)\right)^2\rangle_t = \bar{f}$$

by Eq. 20. On the other hand, by Eq. 24,

$$G(0) = \frac{Q^2\sigma^2}{\omega\sqrt{\pi}}$$

Which indicates that

$$\bar{f} = \frac{Q^2\sigma^2}{\omega\sqrt{\pi}} \quad (25)$$

By replacing the bulk parameters in Eq. 24 with $\bar{f}$,

$$G(\tau) = \frac{\bar{f}}{\sqrt{1+\tau/\tau_D}}$$

In many applications, the normalized autocorrelation function is convenient to compare different FCS data. The normalized autocorrelation function can be derived as

$$\begin{aligned} g(\tau) &= \langle \frac{\delta f(t)}{\bar{f}} \cdot \frac{\delta f(t+\tau)}{\bar{f}} \rangle_t \\ &= \frac{1}{\bar{f}^2} \langle f(t) \delta f(t+\tau) \rangle_t \\ &= \frac{1}{\bar{f}^2}\, G(\tau) \\ &= \frac{1}{\bar{f}} \frac{1}{\sqrt{1+\tau/\tau_D}} \end{aligned}$$

FCS equation for normal diffusion in $\mathbb{R}^m$

For high dimensional cases in $\mathbb{R}^m$ ($m \geq 2$), consider a laser profile

$$I_{\mathbf{c}}(\mathbf{x}) = \prod_{i=1}^{m} \sqrt{\frac{2}{\pi \omega_i^2}} \exp\left(-2 \frac{(x_i - c_i)^2}{\omega_i^2}\right)$$

where $\mathbf{x} = (x_1, x_2, \cdots, x_m)$, $\mathbf{c} = (c_1, c_2, \cdots, c_m)$ is the location of the laser, and $\boldsymbol{\omega} = (\omega_1, \omega_2, \cdots, \omega_n)$ where ω_i is the half width of laser at the e^{-2} of the maximum in ith direction. Similarly, the solution of diffusion equation is given by

$$\begin{aligned} \mathbb{P}(\mathbf{x}, t | \mathbf{y}, t) &= \prod_{i=1}^{m} \frac{1}{\sqrt{4\pi D(t-t_0)}} \exp\left(-\frac{(x_i - y_i)^2}{4D(t-t_0)}\right) \\ &= \frac{1}{\sqrt{4\pi D(t-t_0)}} \exp\left(-\frac{|\mathbf{x}-\mathbf{y}|^2}{4D(t-t_0)}\right) \\ &= \Phi_{D(t-t_0)}(\mathbf{x}-\mathbf{y}) \end{aligned}$$

where $\mathbf{x} = (x_1, x_2, \cdots, x_m)$, and $\mathbf{y} = (y_1, y_2, \cdots, y_m)$. The same computation leads

$$\begin{aligned} G(\tau) &= \bar{f} \prod_{i=1}^{m} \sqrt{\frac{1}{1+\tau/\,\tau_D^{(i)}}} \\ g(\tau) &= \frac{1}{\bar{f}} \prod_{i=1}^{m} \sqrt{\frac{1}{1+\tau/\,\tau_D^{(i)}}} \end{aligned} \qquad (11)$$

where $\tau_D^{(i)} = \omega_i^2/(4D)$ and $N = \frac{Q^2\sigma^2}{(\omega\sqrt{\pi})^m}$. As the most familiar form in $\mathbb{R}^3$,

$$g(\tau) = \frac{1}{\bar{f}} \cdot \frac{1}{\left(1+\tau/\tau_{D_{x,y}}\right)\sqrt{1+\tau/\tau_{D_z}}}$$

where $\tau_{D_{x,y}} = \omega_x^2/(4D)$ with $\omega_x = \omega_y$ and $\tau_{D_z} = \omega_z^2/(4D)$.

Discussion

We have derived FCS equations for free diffusion using basic mathematical tools based on two statistical assumptions: (1) the number of fluorescence molecules (or photons) from confocal volume follows Poisson distribution and (2) fluorescence fluctuation at different locations are statistically independent without stationarity and ergodicity of Brownian motion (or diffusion). Since the first assumption (Poisson distribution) was used to represent a bulk parameter $Q^2\sigma^2/\left(\omega\sqrt{\pi}\right)^m$ as $\bar{f}$ in $\mathbb{R}^m$ (Eq. 25), it is not a crucial assumption to derive the FCS equation. On the other hand, independence of fluorescence fluctuations at different locations was used to simplify the integral, which is one of the most critical steps.

If stationarity and ergodicity assumption was used to derive the FCS equation, then this will raise a question of what types of kinetic process are stationary and ergodic so that they can be interpreted by FCS equation. For an instant, some lines of evidence strongly indicated the breakdown of ergodicity in anomalous diffusion processes (Lubelski 2009, Krichevsky 2002) in which the current FCS equation derivation is not applicable.
Once the prerequisites for FCS equations are met, FCS equations can serve as an elegant and powerful tool for probing underlying kinetics occurring in living cells. We hope this tutorial is understandable as well as give readers a solid theoretical foundation for FCS.

References

Magde, D., Elson, L. E., Webb, W. W.: "Fluorescence correlation spectroscopy. II. An experimental realization", *Biopolymers*, pp. 29—61, 1974.

Elson, E. L., Magde, D.: "Fluorescence correlation spectroscopy I. Conceptual basis and theory", Biopolymers. 13: 1–27, 1974.

Magde, D., Elson, L. E., Webb, W. W.: "Thermodynamic Fluctuations in a Reacting System - Measurement by Fluorescence Correlation Spectroscopy", *Phys. Rev. Lett.*, pp. 705—708, 1972.

Krichevsky, O and Bonnet, G: "Fluorescence correlation spectroscopy: the technique and its applications", Rep. Prog. Phys. 65:251–297, 2002.

Brown, R.: "On the particles contained in the pollen of plants; and on the general existence of active molecules in organic and inorganic bodies", *Phil. Mag.*, pp. 161—173, 1828.

Bachelier, J. L.: "Theorie de la speculation", *Annales scientifiques de l'Ecole Normale Superieure, Serie*, pp. 21-86, 1900.

Einstein, A.: "On the movement of small particles suspended in a stationary liquid demanded by the molecular kinetic theory of heat", *Annalen der Physik*, pp. 549—560, 1905.

Derman, C.: "Ergodic Property of the Brownian Motion Process", *Proc. Natl. Acad. Sci. U.S.A.*, pp. 1155—1158, 1954.

Nadler, B. and Schuss, Z.: "The Stationary Arrival Process of Independent Diffusers from a Continuum to an Absorbing Boundary Is Poissonian", *SIAM J. APPL. MATH.*, pp. 433—447, 2001.

Lubelski, A. and Klafter, J.: "Fluorescence correlation spectroscopy: the case of subdiffusion", *Biophys. J.*, pp. 2055—2063, 2009.

Krichevsky, O. and Bonnet, G.: "Fluorescence correlation spectroscopy: the technique and its applications", *Reports on Progress in Physics*, pp. 251, 2002.

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