Fluorescence Correlation Spectroscopy of Living Cells

G. Vereb[*,1], **L. Ujlaky-Nagy**[1] **E. Friedländer**[1] **G. Vámosi**[2] and **J. Szöllősi**[1,2]

[1]Department of Biophysics and Cell Biology, Medical and Health Sicence Center, University of Debrecen, Nagyerdei krt 98, H-4032 Debrecen, Hungary
[2]Cell Biology and Signaling Research Group of the Hungarian Academy of Sciences, Medical and Health Science Center, University of Debrecen, Nagyerdei krt 98, H-4032 Debrecen, Hungary

Fluorescence correlation spectroscopy (FCS) is a fluctuation spectroscopic method established three decades ago. However, its application to cellular systems has only started to gain popularity recently. In FCS, fluctuations of fluorescence emission are observed from a small (fL to sub-fL, usually confocal) volume at high time resolution, and a time-dependent autocorrelation function is generated and evaluated to obtain time constants of photophysical and photochemical reactions, as well as of molecular diffusion in the observation volume. Molecules in various subcellular compartments of living cells – including the nucleus, cytoplasmic regions and the cell membrane – can be observed after labeling with fluorescent dyes (usually using antibodies) or upon generating their fusion constructs with fluorescent proteins. In addition to their diffusion constant, other parameters, such as the anomaly of diffusion, the absolute concentration in the observation volume, or the average fluorescence per diffusing entity can also be determined, which all can be characteristic of molecular interactions. A two-color version of FCS, fluorescence cross-correlation spectroscopy can also be applied to observe co-diffussion, that is, stable association of two distinct molecular species in their cellular environment. In this chapter, the theoretical background of FCS will be explained through an example of measuring membrane receptor diffusion, and some recent applications to cellular systems will be highlighted.

Keywords Fluorescence correlation spectroscopy; FCS; fluorescence cross-correlation spectroscopy (FCCS); single molecule sensitivity, visible fluorescent proteins; GFP; membrane receptor diffusion; co-diffusion; photochemical reactions

1. Introduction

While the detection of protein – protein interactions by fluorescence resonance energy transfer (FRET) has gained more and more space in the past years [1], another method revealing information on the interaction of molecules [2], established – at the level of principle – almost equally long time ago, is now making its way to cellular investigations. This method is termed FCS – fluorescence correlation spectroscopy [3-5].

Fluorescence correlation spectroscopy exploits the minute fluctuations of the fluorescence emitted by fluorescently tagged molecules from within a microscopic detection volume in order to evaluate the physical parameters contributing to these fluctuations. Using FCS, such physical parameters as local concentration, diffusion coefficients [6], chemical reaction rate constants as well as photophysical properties of fluorophores can be determined [7, 8]. The technique is characterized by high sensitivity to mobility, ability to distinguish between different diffusing species and diffusion mechanisms, while a high sampling rate also allows for separating other (physicochemical) mechanisms contributing to fluorescence fluctuations.

Modern FCS instruments possess single molecule sensitivity [9] made feasible by the advances in confocal microscopy. Using confocal principles sub-femtoliter observation volumes of FCS systems have been attained while the use of sensitive, low noise avalanche photodiodes has made the detection of photons from the few molecules observed at any given time possible. Present-day laser and detector technology allows stable excitation intensity void of noise that would otherwise interfere with the fluctuations originating form the biological system.

[*] Corresponding author: e-mail: vereb@dote.hu, Phone: +36 52 412623

2. Principles of fluorescence correlation spectroscopy

In an FCS experiment a focused laser beam continuously illuminates the specimen containing fluorescently tagged target molecules at a low concentration (see Fig. 1). Motion of a fluorescent molecule through the sensitive volume of the system results in a burst of fluorescence photons. The duration of a burst reflects the dwell time of the molecule within the observation volume and the intensity of the burst corresponds to the brightness of the molecule. The intensity time trace of the fluorescence signal is analyzed for its time-wise autocorrelation using

$$G(\tau) = \frac{\langle \delta F(t) \cdot \delta F(t+\tau) \rangle}{\langle F \rangle^2} \tag{1}$$

and

$$\delta F(t) = F(t) - \langle F \rangle \tag{2}$$

where $F(t)$ is the intensity of fluorescence at any time point, t, $\langle F \rangle$ is the average intensity obtained during the whole time of observation and is the deviation of fluorescence at this time point from, the average. The brackets $\langle\ \rangle$ in the formulas denote time averages of the appropriate terms.

The $\delta F(t)$ values are used to obtain the $G(\tau)$ autocorrelation function (see lower panel of Fig. 1 for a typical trace) by averaging the products of all possible ordered pairs of $\delta F(t)$ and $\delta F(t+\tau)$, where all possible τ time-differences are considered.

In Fig. 1, the autocorrelation function can be considered to originate primarily from the diffusion of labeled molecules, but other factors contribute as well. Since it is customary now to put in vivo visible fluorescent protein (VFP, for example eGFP – enhancend Green Fluorescent Protein) labels onto the molecules to be tracked, we shall consider such a situation [9].

Generally the data can be analysed using the Levenberg-Marquardt non-linear least-squares method to fit the data to a single- or multi-component, free or anomalous diffusion model. Additional terms often need to be included to take into consideration the fluctuations due to triplet state formation characteristic of many widely used fluorophores [10]; as well as dark state formation (blinking) exhibited by most VFPs [11]. This latter can result either from protonation or from light-induced conformational transition to a non-fluorescent state.

$$\mathrm{G}(\tau) = a_0 + \frac{1}{<\mathrm{N}>} \cdot G_{tr}.G_{bl}. \cdot \sum_i^n \left[w_i \left(1 + \left(\frac{\tau}{\tau_{d,i}} \right)^{2/d_{w,i}} \right)^{-1} \left(1 + \frac{1}{S^2} \left(\frac{\tau}{\tau_{d,i}} \right)^{2/d_{w,i}} \right)^{-1/2} \right] \tag{3}$$

where the triplet state correction is given by

$$G_{tr}(\tau) = \frac{\left(1 - T + Te^{-\tau/\tau_{tr}} \right)}{1 - T} \tag{4}$$

and the blinking correction by

$$G_{bl}(\tau) = \frac{\left(1 - \Theta_{bl} + \Theta_{bl}.e^{-\tau/\tau_{bl}} \right)}{1 - \Theta_{bl}} \tag{5}$$

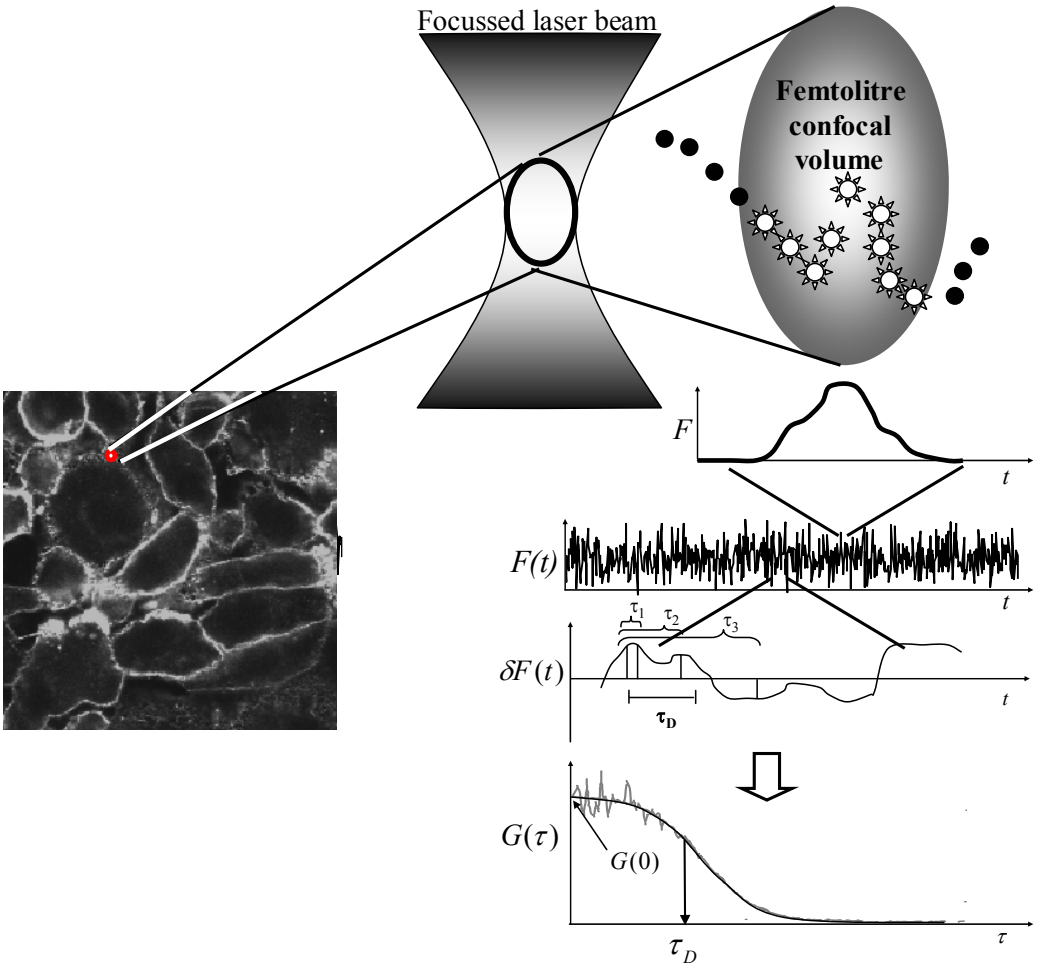

Fig. 1 Measuring the diffusion of GFP-fused flurescent receptors in the cell membrane using FCS. Fluorescent molecules entering and exiting the subfemtoliter confocal observation volume illuminated with a focussed laser beam generate temporary photon bursts that can be detected with quasi single-molecule sensitivity. The diffusion of molecules (in addition to other possible physical and photochemical processes) causes δ*F* fluctuation of the signal *F(t)*. The time course of fluctuations, δ*F(t)* can be used to generate a time-dependent autocorrelation function $G(\tau)$, which provides, by fitting, for the determination of the $N=1/G(0)$ number and $D = \omega^2/4\tau_D$ diffusion constant of the molecules in the observation volume from a single measurement.

In Eq. (3) $< N >$ is the average number of molecules in the detection volume, which is a great asset, since by fitting the autocorrelation function the concentration of labeled molecules can be obtained. The diffusional autocorrelation time, $\tau_{d,i}$ of the *i-th* diffusing species is also obtained from fitting; it represents the average time it takes for this species (of a weight fraction w_i) to traverse the detection volume. The detection volume is the space inside the surface where the detection efficiency, described by a 3D Gaussian function, falls to e^{-2} times the maximal value present at the center of this volume. This volume is a rotational ellipsoid, which is characterized by the structure parameter, $S = \omega_z/\omega_{xy}$ (ratio of axial radius ω_z to the lateral radius ω_{xy} of the ellipsoid, usually a value between 4-7). $d_{w,i}$ denotes the anomaly parameters of the diffusing components: a value of 2 is characteristic of free Brownian diffusion, while values larger than 2 hint at possible obstacles in the way of diffusing molecules – barriers, binding sites

temporarily slowing down diffusion, etc., and values below 2 result from facilitated or guided diffusion, which, obviously, is also a sign of molecular interactions. (Less importantly, a_0 is an offset to compensate for the nonzero baseline of the autocorrelation function arising from drifts in the fluorescence signal, such as photobleaching.)
In the triplet term, G_{tr}, T denotes the equilibrium molar fraction of fluorophores in the triplet state and τ_{tr} is the triplet lifetime. The "blinking term", G_{bl} accounts for the conformational fluctuations between fluorescent and dark state formation. Θ_{bl} is the fraction of fluorophores in the detection volume in the dark state and τ_{bl} is the corresponding relaxation time. Measurements can also be complicated by photobleaching, which, although it can be accounted for in the fitting algorithms, can bias the assessment of the more slowly or the faster diffusing molecular species [12].

The diffusional autocorrelation times, $\tau_{d,i}$ can be converted to a diffusion coefficient (D) using the equation:

$$D_i = \frac{\omega_{xy}^2}{4\tau_{\mathrm{d,i}}} \tag{6}$$

The radii ω_{xy} and ω_z are obtained by calibrating the system with a dilute solution of a dye of known concentration and diffusion coefficient. Rh6G of molecular mass 0.479 kDa is commonly used for its small size and photostability. In a dilute solution (~10 nM), for the Brownian diffusion of a single species that has a triplet state Eq. (3) then simplifies to

$$\mathrm{G}(\tau) = a_0 + \frac{1}{<\mathrm{N}>} \cdot G_{tr} \cdot \left(1 + \frac{\tau}{\tau_d}\right)^{-1} \left(1 + \frac{1}{S^2}\frac{\tau}{\tau_d}\right)^{-1/2} \tag{7}$$

The diffusion time, τ_d obtained from a Levenberg-Marquardt non-linear least-squares fit to Eq. (7) and the diffusion coefficient of a standard, e.g. Rh6G (280 $\mu m^2/s$ at 25°C) are used in Eq. (6) to obtain ω_{xy}. An estimate of ω_z can then be obtained from the fit result of S, or rigorously determined from measurements of a series of dilute concentrations of the dye. For a given concentration of dye, the average number of molecules in the detection volume can be expressed in terms of the dimensions of the ellipsoidal sensitive volume in the optical plane and the molar concentration c:

$$N = N_A c \pi^{3/2} \omega_{xy}^2 \omega_z \tag{8}$$

N_A is Avogadro's number. Thus, $\omega_{xy}^2 \omega_z$ can be derived from the slope of a plot of the apparent number of particles, N vs. dye concentration.

If the S factor is sufficiently large or in the case of diffusion of molecules in two dimensions, such as in the plane of the cell membrane, Eq. (7) simplifies to:

$$\mathrm{G}(\tau) = a_0 + \frac{1}{<\mathrm{N}>} \cdot G_{tr} \cdot \left(1 + \frac{\tau}{\tau_d}\right)^{-1} \tag{9}$$

3. Application to cellular systems

As for the biological significance, fluorescence correlation spectroscopy can yield information on molecule number and diffusion rates from temporal fluctuations arising from the passage of molecules through the confocal detection volume. Fluorescence correlation microscopy (FCM) integrates the FCS modality into a microscope system with high-sensitivity digital imaging and micro-positioning in order to perform FCS measurements in different locations or compartments of living cells [9].

The diffusion coefficient can be derived, and since it is inversely proportional to the square of the molecular weight, its change can hint at aggregation. In addition, changes in molecular aggregation can be detected from the fluorescence per molecule (fpm) calculated from dividing the time-averaged fluorescence by the average number of molecules in the observation volume.

As a most straightforward application to cellular systems, FCS was used to detect binding of ligands to membrane receptors, indicated by the marked decrease of their diffusion constants as compared to the initial values in solution. Such experiments can be can be used as a rapid technique for studying ligand-receptor interactions on living cells and represent a step forward toward large-scale drug screening in cell cultures [13]. Intracellular FCS measurements pose the problem of getting the fluorophor into the cell and attached to the appropriate target. The most wide-spread method currently is the generation of fusion constructs of the target protein and the green fluorescent protein (GFP) or one of its spectral variants. This approach was used to provide direct evidence for oligomer formation of polyglutamine-GFP fusion proteins expressed in cultured cells, based on the time-dependent increase in their particle size and diffusion time. These findings substantiated the *in vivo* significance of polyglutamine repeats in conformational neurodegenerative diseases such as Huntington's chorea [14]. Of course, it is also possible to introduce pre-labelled biomolecules into the cells, for example fluorescently labelled nucleic acids by transfection. For studying the diffusion mode of polyadenylated RNA, it was conjugated to fluorescein and after transfection into the cells it was allowed to form a complex with a splicing factor expressed as a chimera fused to red fluorescent protein (RFP). FCS, corroborated with photoactivation studies, has revealed that the movement of polyadenylated RNA in and out of nuclear speckles does not require metabolic energy, and therefore it cannot be considered as a stable structural component of these speckles [15]. FCS was also used to detect the diffusion characteristics of synaptic vesicles in hippocampal neurons. It was found that the stick-and-diffuse model where the vesicles bind and release from a cellular cytomatrix fits far better the observed data than the caged diffusion model, in which the vesicles diffuse in small circular cages located randomly in the synaptic bouton [16].

Analysing the anomaly of diffusion is also possible, and is relevant to revealing hindered vs. facilitated vs. free Brownian diffusion [17]. FCS was used to show that Golgi resident membrane proteins move subdiffusively in the endoplasmic reticulum and the Golgi apparatus in vivo. Based on Monte Carlo simulations for FCS on curved surfaces, it could also be ruled out that the observed anomalous diffusion was a result of the complex topology of the membrane [18]. FCS was also used to show that lipid raft-associated and cytoskeleton-linked transmembrane proteins exhibit distinctly different diffusion characteristics [19]. For analysing the movement of membrane proteins, the total internal reflection version of fluorescence correlation spectroscopy (TIR-FCS) [20] is suited ideally. Although presently it requires custom-built equipment, based on its ability to observe fluorescent molecules in a small area of an evanescent field it was successfully applied to study the reversible association and dissociation rates between fluorescent ligands and their receptors in supported phospholipid bilayers, as well as the lateral diffusion of a membrane-bound farnesylated eGFP [21].

Using two-photon excitation in FCS measurements of living systems can substantially improve signal quality in turbid preparations like plant cells and deep cell layers in tissue, and at comparable signal levels minimizes photobleaching in spatially restrictive cellular compartments, thereby facilitating long-term signal acquisition [22]. FCS can also provide a quick and straightforward method for determining interactions of biomolecules in cell lysates [23].

4. Fluorescence cross-correlation spectroscopy (FCCS)

A special version of FCS is fluorescence cross-correlation spectroscopy - FCCS - where two species are labeled using two spectrally distinct fluorophores (indexed a and b in the formula below) and their co-diffusion is examined [24, 25]. In this case, a cross-correlation function is calculated instead of the autocorrelation function:

$$G^{\times}(\tau) = \frac{\langle \delta F_a(t) \cdot \delta F_b(t+\tau) \rangle}{\langle F_a \rangle \langle F_b \rangle} \tag{10}$$

The difference is that here we relate the fluorescence of molecule *a* at time *t* to the fluorescence of molecule *b* at various times τ later. If the two fluorescence signals exhibit cross-correlation, a curve similar to the autocorrelation curve is obtained, and can be fitted to obtain, among others, a characteristic diffusion time. The fact that a nonzero $G^{x}(\tau)$ function is attainable is a definitive sign that in the population of the mixture of *a* and *b*, some *ab* complexes are present and are stable for at least the time they need to pass through the observation volume. This can be termed as co-mobility, which is an association relatively stable in time, and is revealed by a dynamic method. This is quite contrasting with the temporary associations revealed as momentarily being present using the FRET method. Incidentally, from the ratio of amplitudes of the individual autocorrelation functions (generated from the same data) and of the cross-correlation function, the proportion of di- (or oligo-)merized species can also be deduced.

FCCS was exploited to demonstrate that association of the alpha subunit of interleukin-15 receptor with both the IL-2 receptor alpha and MHC class I glycoproteins was stable in time, leading to co-diffusion of these molecular species [26]. FCCS has also revealed real-time interactions between Lyn and cross-linked FcεRI implicated in downstream signaling events of lymphocytes [27]. A pioneering application of cross-correlation spectroscopy of VFPs in living cells demonstrated that c-fos fused to eGFP and c-jun fused to monomeric red fluorescent protein 1 (mRFP1) expressed in HeLa cells co-diffuse and that mobility of the dimerized species is slow, indicating the stabilizing effect of DNA-binding on the dimer [28]. An interesting possibility of the application of cross-correlation to expressed VFP fusion products is the observation of protease activity in vivo [29].

Application of FCCS using VFPs appears to be a convenient way of screening molecular interactions, however, the spectral overlap of most VFPs can result in strong crosstalk between the two detection channels. A method applying interleaved pulsed excitation and detection of eCFP and eYFP on a shorter timescale compared to the average residence time of particles in the FCS volume element, and discriminating the fluorophores in excitation rather than in emission has been implemented successfully to overcome this difficulty [30].

Acknowledgements The authors are supported by the following grants: EU FP6 LSHB-CT-2004-503467, LSHC-CT-2005-018914, MCRTN-CT-035946-2, MRTN-CT-2005-019481; OTKA K62648, K68763, T48475, NK61412, ETT 066/2006, 070/2006. G. Vereb is recipient of an Öveges grant from the Hungarian National Office for Research and Technology.

References

[1] G. Vereb, J. Matko, and J. Szollosi, Methods Cell Biol **75**, 105 (2004)
[2] G. Vereb, J. Szollosi, J. Matko, P. Nagy, T. Farkas, L. Vigh, L. Matyus, T. A. Waldmann, and S. Damjanovich, Proc Natl Acad Sci U S A **100**, 8053 (2003)
[3] D. Magde, E. L. Elson, and W. W. Webb, Biopolymers **13**, 29 (1974)
[4] M. Ehrenberg, and R. Rigler, Q Rev Biophys **9**, 69 (1976)
[5] E. L. Elson, J Biomed Opt **9**, 857 (2004)
[6] P. Schwille, J. Bieschke, and F. Oehlenschlager, Biophys Chem **66**, 211 (1997)
[7] J. Widengren, and R. Rigler, Cell Mol Biol (Noisy-le-grand) **44**, 857 (1998)
[8] P. A. van den Berg, J. Widengren, M. A. Hink, R. Rigler, and A. J. Visser, Spectrochim Acta A Mol Biomol Spectrosc **57**, 2135 (2001)
[9] R. Brock, G. Vamosi, G. Vereb, and T. M. Jovin, Proc Natl Acad Sci U S A **96**, 10123 (1999)
[10] J. Widengren, Ü. Mets, and R. Rigler, J. Phys. Chem. **99**, 13368 (1995)
[11] U. Haupts, S. Maiti, P. Schwille, and W. W. Webb, Proc Natl Acad Sci U S A **95**, 13573 (1998)
[12] J. Widengren, and P. Thyberg, Cytometry A **68**, 101 (2005)
[13] A. Pramanik, and R. Rigler, Biol Chem **382**, 371 (2001)
[14] Y. Takahashi, Y. Okamoto, H. A. Popiel, N. Fujikake, T. Toda, M. Kinjo, and Y. Nagai, J Biol Chem2007)
[15] J. C. Politz, R. A. Tuft, K. V. Prasanth, N. Baudendistel, K. E. Fogarty, L. M. Lifshitz, J. Langowski, D. L. Spector, and T. Pederson, Mol Biol Cell **17**, 1239 (2006)
[16] C. Yeung, M. Shtrahman, and X. L. Wu, Biophys J **92**, 2271 (2007)
[17] M. Wachsmuth, W. Waldeck, and J. Langowski, J Mol Biol **298**, 677 (2000)
[18] M. Weiss, H. Hashimoto, and T. Nilsson, Biophys J **84**, 4043 (2003)
[19] L. Wawrezinieck, H. Rigneault, D. Marguet, and P. F. Lenne, Biophys J **89**, 4029 (2005)
[20] N. L. Thompson, and B. L. Steele, Nat Protoc **2**, 878 (2007)
[21] Y. Ohsugi, K. Saito, M. Tamura, and M. Kinjo, Biophys J **91**, 3456 (2006)
[22] P. Schwille, U. Haupts, S. Maiti, and W. W. Webb, Biophys J **77**, 2251 (1999)
[23] O. Stoevesandt, K. Kohler, R. Fischer, I. C. Johnston, and R. Brock, Nat Methods **2**, 833 (2005)
[24] P. Schwille, F. J. Meyer-Almes, and R. Rigler, Biophys J **72**, 1878 (1997)
[25] K. Bacia, S. A. Kim, and P. Schwille, Nat Methods **3**, 83 (2006)
[26] G. Vamosi, A. Bodnar, G. Vereb, A. Jenei, C. K. Goldman, J. Langowski, K. Toth, L. Matyus, J. Szollosi, T. A. Waldmann, and S. Damjanovich, Proc Natl Acad Sci U S A **101**, 11082 (2004)
[27] D. R. Larson, J. A. Gosse, D. A. Holowka, B. A. Baird, and W. W. Webb, J Cell Biol **171**, 527 (2005)
[28] N. Baudendistel, G. Muller, W. Waldeck, P. Angel, and J. Langowski, Chemphyschem **6**, 984 (2005)
[29] T. Kohl, E. Haustein, and P. Schwille, Biophys J **89**, 2770 (2005)
[30] E. Thews, M. Gerken, R. Eckert, J. Zapfel, C. Tietz, and J. Wrachtrup, Biophys J **89**, 2069 (2005)