Critical Review

Dynamics of Intracellular Processes in Live-Cell Systems Unveiled by Fluorescence Correlation Microscopy

Nicolás González Bardeci[1]
Juan Francisco Angiolini[1]
María Cecilia De Rossi[1]
Luciana Bruno[2]
Valeria Levi[1]*

[1]Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina, IQUIBICEN, UBA-CONICET
[2]IFIBA, UBA-CONICET, Argentina

Abstract

Fluorescence fluctuation-based methods are non-invasive microscopy tools especially suited for the study of dynamical aspects of biological processes. These methods examine spontaneous intensity fluctuations produced by fluorescent molecules moving through the small, femtoliter-sized observation volume defined in confocal and multiphoton microscopes. The quantitative analysis of the intensity trace provides information on the processes producing the fluctuations that include diffusion, binding interactions, chemical reactions and photophysical phenomena. In this review, we present the basic principles of the most widespread fluctuation-based methods, discuss their implementation in standard confocal microscopes and briefly revise some examples of their applications to address relevant questions in living cells. The ultimate goal of these methods in the Cell Biology field is to observe biomolecules as they move, interact with targets and perform their biological action in the natural context. © 2016 IUBMB Life, 69(1):8–15, 2017

Keywords: *fluorescence microscopy; fluorescence correlation spectroscopy; intracellular dynamics; diffusion; autocorrelation; cross-correlation*

Introduction: A Long Trip From Qualitative To Quantitative Microscopy

Fluorescence microscopy has evolved during the last years changing the way we explore and understand cell function. Initially used to observe the distribution of cell components in fixed specimens, microscopy now provides quantitative information on biological processes with minimal perturbation.

Microscopy methodologies such as those based on fluorescence recovery after photobleaching (1,2), photoactivation (3), single molecule/particle tracking (4,5) and fluorescence correlation spectroscopy (FCS) constituted major innovations in the Cell Biology field since they allowed exploring the motion of biomolecules in systems of increasing complexity ranging from solutions (6,7), artificial and natural biomembranes (8,9), live cells (2,10,11) and organisms (12,13).

FCS was introduced more than 40 years ago (14) but it was not until the 1990s with the advent of confocal microscopy, photon-counting detection and improved computational power (15) that FCS could spread to various scientific fields. The development of new technologies to label biomolecules *in situ* (reviewed in Refs. 16 and 17) promoted the uses of FCS in biological studies. FCS is especially well suited for these studies since it requires a low concentration of fluorescent molecules and does not need synchronizing or perturbing the system. This versatile method also provides high spatiotemporal resolution. The spatial resolution typically reaches the optical diffraction limit (~200 nm), although FCS with sub-diffraction resolution has also been achieved (18,19). For processes in live-cell systems the temporal resolution usually lies in the microsecond to millisecond range, but faster, photophysical phenomena taking place up to the nanosecond scale can be studied by FCS (20).

Volume 69, Number 1, January 2017, Pages 8–15
Address correspondence to: Valeria Levi, Departamento de Química Biológica, Universidad de Buenos Aires Facultad de Ciencias Exactas y Naturales, Buenos Aires, Argentina.
E-mail: vlevi12@gmail.com
Received 30 August 2016; Accepted 7 November 2016
DOI 10.1002/iub.1589
Published online 28 November 2016 in Wiley Online Library (wileyonlinelibrary.com)

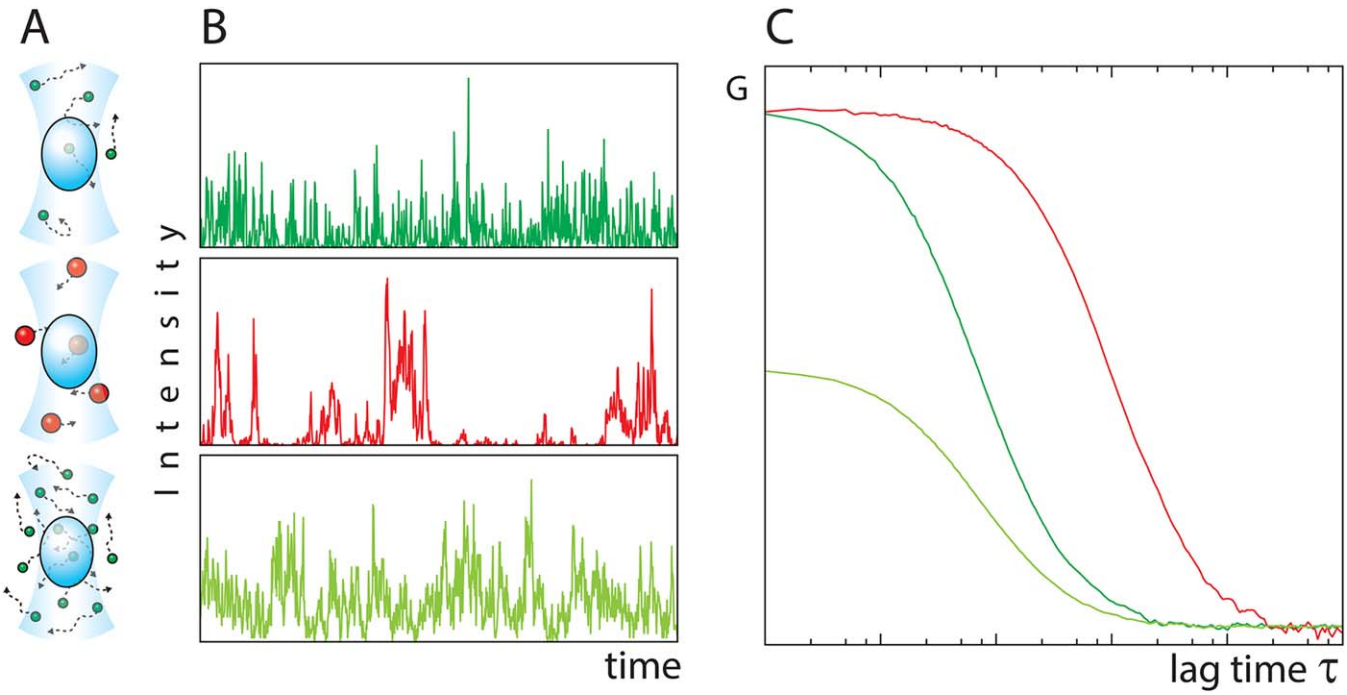

FIG 1

Single-point FCS. (A) Schematic representation of the observation volume (light blue) and slow (red) or fast (green) moving fluorescent molecules. (B) The movement of the molecules through the observation volume generates fluctuations in the intensity trace. (C) Autocorrelation analysis of the intensity traces obtained for the slow and fast moving molecules. Slow-moving molecules introduce longer-lived fluctuations and thus the autocorrelation function slowly decreases with τ in comparison to fast molecules. On the other hand, the amplitude of the autocorrelation function is inversely proportional to the number of fluorescent molecules in the confocal volume, and therefore to the local concentration.

FCS has continually expanded, evolved and diversified into numerous and different modalities (21); it is nowadays a powerful and well-established biophysical tool which is routinely used to obtain quantitative data on dynamical processes.

In this review, we describe the basics of FCS methods, provide general guidelines for setting up measurements in commercial confocal microscopes and survey new FCS approaches. Finally, we selected two major life sciences topics to illustrate how this methodology provides fundamental information to comprehend biological processes *in vivo*.

Theoretical Background: Detecting Fluctuations From Single Molecules

In a classical single-point FCS experiment, the laser beam of a confocal or multiphoton excitation microscope focuses to a diffraction-limited volume within the sample. The fluorescence intensity in the femtoliter-sized observation volume optically defined in these microscopes (15,22,23) is collected as a function of time with high temporal resolution.

Let us consider a very simple system such as a solution of fluorescently-labeled molecules (Fig. 1). The spontaneous motion of the molecules through the observation volume introduces fluctuations in the intensity trace. Qualitatively, the duration of the fluctuations depends on how molecules move, e.g. slow-moving molecules produce long-lasting fluctuations while fast molecules introduce short fluctuations.

To extract quantitative information on both the mobility and local concentration of fluorescent molecules, the intensity trace should be examined with an adequate statistical analysis. In the simplest case, the trace is evaluated calculating the temporal autocorrelation function that quantifies the self-similarity of the intensity trace at time t and after a lag time τ:

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

where $I(t)$ represents the fluorescence intensity at time t, the brackets indicate average values over the time-course of the experiment, and $\delta I(t) = I(t) - <I(t)>$ represents the fluorescence fluctuation at a given time.

Then, the autocorrelation data can be analyzed using functional forms for $G(\tau)$ derived from the theoretical analysis of the molecular mechanism assumed to be responsible for the intensity fluctuations. This analysis also requires modeling the observation volume defined by the microscope setup. We will not describe the theory behind FCS modeling; excellent reviews on this topic can be found elsewhere (23–25).

In the simple example showed in Figure 1 the data can be fitted with the following equation that considers a confocal setup and Brownian diffusion of the molecules (15):

$$G(\tau)=\frac{1}{\langle N\rangle}\left(1+\frac{\tau}{\tau_D}\right)^{-1}\left(1+\frac{\tau}{\omega^2\tau_D}\right)^{-1/2} \quad (2)$$

where $\tau_D = \omega_{xy}{}^2/4D$ is the characteristic diffusion time and $\omega = \omega_z/\omega_{xy}$ is the ratio of axial to radial e^{-2} radii of the confocal observation volume ($V_{obs} = \pi^{3/2}\omega_{xy}{}^2\omega_z$ (23)), and $<N>$ is the mean number of fluorescent particles in the observation volume.

This procedure allows determining the diffusion coefficient (D) of the molecules and the mean concentration of molecules in the observation volume.

Intensity fluctuations arise from a wide variety of dynamical processes including binding interactions, chemical reactions and photophysical phenomena and thus FCS could potentially provide phenomenological parameters characterizing these processes (24,26). The analysis can also be complemented with Monte Carlo simulations to explore the dynamics of even more complex processes as those observed in living cells (27,28). Moreover, FCS resolves populations of molecules following fluctuation-generating processes in different temporal windows (29).

We should emphasize that FCS provides dynamical information of either equilibrium or steady-state systems. In addition, the low concentration of fluorescent molecules (nM–μM range, (30)) minimally perturbs the system. Both properties make FCS an appealing technique for live cells studies.

A Toolkit For Single-Point FCS Measurements

We will now provide general and very basic guidelines for the experimental realization of single-point FCS in living cells. Further details on the step-by-step procedures and instrumentation required for FCS measurements can be found elsewhere (31,32). Here, we focus on FCS measurements run in commercial confocal microscopes equipped with photon-counting detectors since this setup is nowadays a standard equipment in microscopy facilities of many research institutes and universities.

The first step in every FCS experiment is calibrating the observation volume. One of the simplest calibration protocols consists on running a single-point FCS experiment in a sample with known mobility properties such as a solution of a fluorescent probe of known diffusion coefficient (33). Equation (2) is then fitted to the experimental autocorrelation curve holding constant D to the known value to obtain ω_{xy} and ω (31). An alternative and independent calibration of the observation volume is to measure the intensity distribution of subresolution-sized fluorescent particles (for details, Ref. (34)). More sophisticated, calibration-free FCS methods based on optical setups that produce two laser foci laterally shifted in a known distance were also used to measure diffusion coefficients (35,36).

FCS experiments in living cells/organisms require maintaining the environmental properties that keep the sample in good conditions during the measurements. It is highly recommendable to use bright and photostable fluorescent probes to improve the signal-to-noise ratio (37,38) and to check that the background and cellular autofluorescence represent less than 10% of the total fluorescence (31) and do not present significant correlation in the explored time-window. This statement should not prevent users that might not have the choice to the probe from using FCS; measurements with ordinary fluorophores have also shown to provide valuable dynamical information although with higher error.

The laser power should also be set as low as possible to avoid photodamage and photobleaching but high enough to register fluctuations with adequate signal-to-noise ratio (S/N). Photobleaching may cause an overall reduction of the intensity and introduce artifacts in the measurements since molecules turn off before leaving the confocal volume. In this condition, fluctuations become shorter (see for example, Ref. (39)) and the correlation curve shifts to lower τ values. To establish the range of operational laser powers that preserves the time scale of the correlation with high S/N, it is recommendable to run calibration experiments varying the laser power (32).

Finally, the data acquisition frequency and total time of the experiment are set using an initial estimation for the characteristic time of the intensity fluctuations (t_c). As a general rule, the total duration of the FCS experiment should be 10^4 t_c to obtain a ~1% precision on this parameter (23) whereas the sampling time should be $\sim 2/3 t_c$ (40).

The autocorrelation curves obtained in single-point FCS measurements in cells usually present dispersion due to inter and/or intracellular variability (31). Thus, FCS normally requires the analysis of numerous data sets before reaching to a biological conclusion with statistical validity.

An Introduction To Advanced FCS Modes

Initial single-point FCS experiments showed the great potential of this relatively simple method to reveal hidden dynamical information of living specimens. This enthusiasm triggered the idea of extracting even more complex aspects of cellular organization through the study of intensity fluctuations and led to major developments in the field (revised in Refs. (26), (41), and (42)).

One of the most interesting expansions of the original point-FCS analysis consisted on measuring the correlation between intensity traces registered simultaneously in two detectors of the microscope set to collect fluorescence in different spectral windows (43). Figure 2A considers the case of molecules labeled with spectrally different fluorescent probes that associate forming a complex. These associated molecules move together within the confocal volume introducing simultaneous fluctuations in both channels. In contrast, the intensity traces will not show any correlation when molecules move independently from each other. The amount and dynamics of the associated molecules can be extracted through the analysis of the cross-correlation function defined as (32,44):

$$G_{ab} = \frac{\langle \delta I_a(t) \cdot \delta I_b(t+\tau) \rangle}{\langle I_a(t) \rangle \langle I_b(t) \rangle} \quad (3)$$

where a and b refer to the different channels of the microscope.

Generalizing, dual-color fluorescence cross-correlation spectroscopy (FCCS) measures synchronicity between processes that introduce intensity fluctuations in different spectral ranges. It allows recovering information regarding interactions between molecules, kinetics of chemical reactions and colocalization in sub-resolution compartments (45).

One of the main drawbacks of single-point FCS measurements is that it only provides information of a diffraction-limited volume of the sample. Several methods were developed to gain spatial resolution including sampling multiple confocal spots simultaneously (35,46) and scanning the laser in circular orbits and lines (see for example, Refs. 47–49). These last methods have the advantage of their easy implementation in standard confocal or multiphoton setups. In addition, the combination of microscopy methods that confines the excitation to a thin z-section of the sample such as total internal reflection fluorescence microscopy, TIRF (50) or single plane illumination microscopy, SPIM (51), with fast and highly sensitive cameras allowed the simultaneous and parallel collection of intensity traces at every pixel of the image.

These developments constitute the basis of temporal imaging-FCS methods (see for example, Refs. 52–55). The calculation of the temporal autocorrelation (Equation (1)) at every pixel of the scanned region creates a map of dynamical information (Fig. 2B). Temporal imaging-FCS is frequently used to

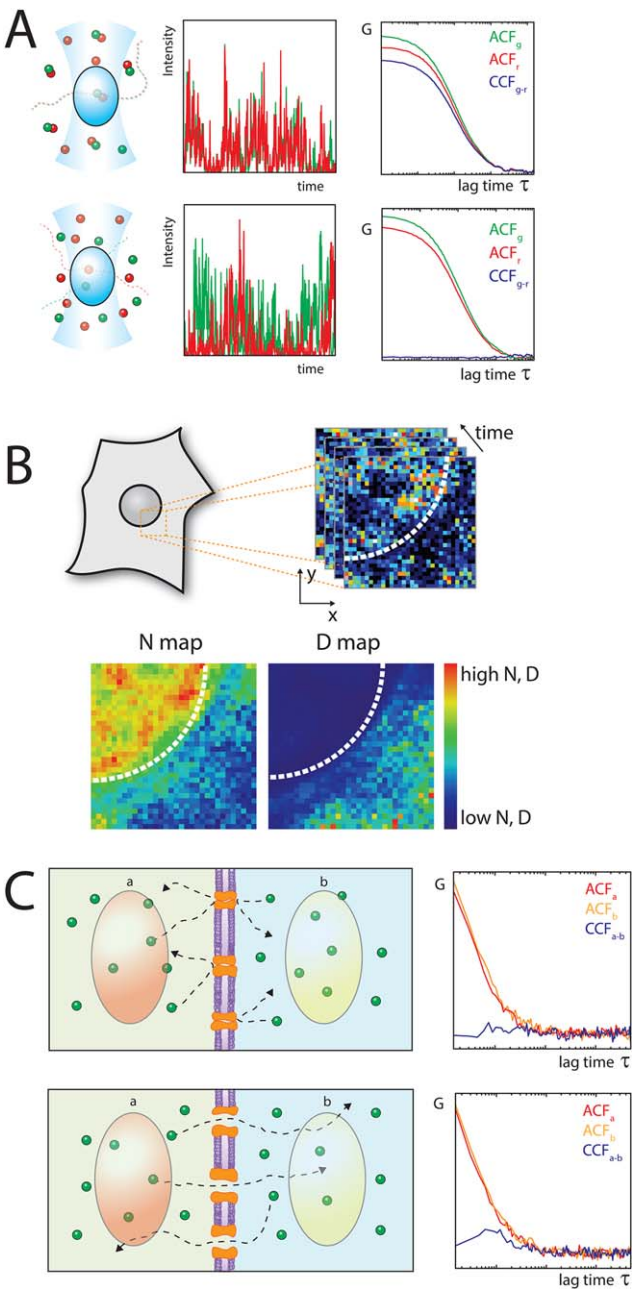

FIG 2

Advanced FCS modalities. (A) Dual-color fluorescence cross-correlation spectroscopy. Schematic representation of the observation volume (light blue) and molecules labeled with different-color fluorescent probes (red and green). Molecules forming a complex move together through the observation volume generating simultaneous fluctuations in both intensity traces and resulting in a positive cross-correlation ($CCF_{g\text{-}r}$). The time-decay of this function provides information on the complex dynamics whereas the autocorrelation function of each intensity trace (ACF) depends on the dynamics of the whole population of either green or red-labeled molecules. Fluctuations in both channels are independent from each other when molecules do not interact resulting in the absence of cross-correlation. (B) Imaging-FCS. Schematic representation of a region of a cell imaged in an optical z-sectioning microscopy setup. The fluorescence intensity at every pixel within the region of interest is collected as a function of time and the autocorrelation function is calculated pixel-by-pixel. This analysis generates maps of number of molecules (N) and diffusion coefficients (D). In the scheme, a high concentration of slower-migrating molecules is observed in the nucleoplasm compared to the cytoplasm. (C) Pair-correlation function analysis. Schematic representation of cellular compartments separated by impermeable (top panel) or permeable (bottom panel) barriers. The confocal microscope is set to repetitively scan the laser along a line crossing both compartments. The intensity at every pixel of the line is collected as a function of time. In this particular example, the cross-correlation ($CCF_{a\text{-}b}$) is calculated between the positions schematized in the figure, each one located in a different compartment. The absence of cross-correlation indicates that molecules cannot move between compartments (top panel) whereas a positive cross-correlation indicates communication between compartments and allows quantifying the characteristic transit time from the left to the right compartment. The ACF analysis at the selected positions provides the dynamics and concentrations of molecules in the compartments.

study relatively slow-processes since the time resolution is limited by the acquisition frequency of the orbit, line or frame depending on the method.

The capability of scanning the laser through the sample opened the possibility of analyzing the correlation between intensity signals measured at different pixels, and thus at different times (reviewed in Ref. (41)). The fundamental idea behind spatiotemporal correlation methods is that molecules located at a position p_i can be observed at a position $p_i + dp_i$ after a lag time τ. The probability of this event depends on how molecules move and on the local environment.

Recently, Gratton et al. (56–58) described the pair correlation function analysis (pCF). This method is based on repetitively scanning the laser along a line and calculating the temporal cross-correlation of fluorescence fluctuations between pairs of pixels apart from each other in a distance selected by the user (Fig. 2C). The amplitude and characteristic time of this cross-correlation depends on the distance between pixels, the motion properties of the molecules and the presence of channels, obstacles, barriers and other architectural accidents that may affect the molecule paths. pCF analysis can detect sub-diffraction anisotropies in the microenvironment that affect the molecules motion but are not necessary observable in the image.

The raster image correlation spectroscopy (RICS) approach (59) makes use of the temporal information hidden in images acquired in laser-scanning microscopes. These images are in fact generated by raster-scanning the laser through the sample and collecting the intensity pixel-by-pixel. The pixel acquisition time is normally in the microsecond time-scale whereas the line is collected in milliseconds. RICS calculates a correlation matrix that contains the information of the correlation of intensities obtained between every pair of pixels, as a function of the distance between the pixels. The shape of the correlation matrix depends on the molecules mobility in the observed area. This methodology was used, for example, to measure the mobility of a specific transcription factor in *Arabidopsis* roots (12).

A related method, spatiotemporal image correlation spectroscopy (STICS) (60), analyzes raster image series and correlates intensity fluctuations observed at pixels of different images as a function of the lag time between images. This methodology can be used to detect and measure the magnitude and direction of macromolecular flow in subregions of the sample. Wiseman et al. exploited this method to map α5-integrin or α-actinin diffusion and flow during cell migration (61).

Fluctuation-based methods can also be used to evaluate formation of oligomers through the analysis of the intensity distribution instead of the duration of the fluctuations. This distribution depends on the number of fluctuating particles and their molecular brightness (i.e. photons emitted/time). Brightness is a key parameter to determine self-associated species since it is expected to increase linearly with the number of fluorescently-tagged subunits. The number and brightness (N&B) method developed by Gratton et al. (62) allows recovering the brightness at every pixel of an image by the simple calculation of the average intensity of a pixel and its variance across an image stack. We have recently used this method to map the oligomerization state of the glucocorticoid receptor in living cells (63). The main drawback of N&B is its inability to resolve different self-associated species within the observation volume and thus it only provides a weighted mean brightness of the species. Methods based on the analysis of the whole intensity distribution (PCH and FIDA methods (64,65)), and on the calculation of high-order moments of fluorescence fluctuations (66,67) have been proposed to resolve oligomeric species. N&B and PCH two-color versions were also developed for the analysis of heterocomplexes (68,69).

A Dynamic View of Biological Membranes

FCS in combination with advanced fluorescence microscopy methods provided fundamental evidences that enriched the original fluid-mosaic model proposed for the organization of biological membranes (reviewed in Ref. 70). We now know that interactions between membrane components segregate relatively transient or more stable structures that play relevant roles in signaling (71,72), membrane trafficking (73,74), and viral infection cycles (75–77). Here we selected some foundational FCS works that contributed to set the basis for modern concepts on membrane organization (reviewed in Ref. 78).

Initial works in the area applied single-point FCS methodologies to quantify the mobility of fluorescent lipids or proteins in synthetic systems such as giant unilamellar vesicles (GUVs) or supported lipid bilayers (reviewed in Ref. (79)). These works showed that lipid bilayers may present domains with different dynamical organization, depending on their composition (80). Further works focused on analyzing heterogeneities on natural membranes. Ruan et al. (49) made use of scanning-FCS to quantify the mobility of a membrane protein in GUVs assembled from natural membranes; the scanning approach allowed locating the membrane and correct from its natural motion. The development of FCS methods specifically designed for measuring dynamics in membranes (46) extended these studies to living cells (see, for example Ref. 81). One of the aims in the area was to observe lipid rafts since these small and highly dynamic structures were proposed to be fundamental for several membrane-related biological processes (70) and their observation remained elusive due to their small size in relation to the optical resolution (82).

To overcome this limitation, Sanchez et al. (9) used the fluorescent probe Laurdan that senses the polarity of its microenvironment through spectral shifts in the emission and thus it reports on the local packing of the membrane. By studying simultaneous intensity fluctuations of Laurdan in two different spectral windows, these authors detected membrane microdomains sizing 20 to 300 nm with the characteristic high lipid packing proposed for lipid rafts.

More recently, single-point and scanning FCS methodologies were combined with STED (stimulated emission depletion) microscopy to obtain subdiffraction information on membrane heterogeneities (83–85). These works revealed that fluorescent raft markers present transient interactions with regions smaller than 80 nm, sizes compatible with those proposed for lipid rafts.

Dynamical Organization of The Nucleus Through Fluctuations Techniques

The puzzling organization of the nucleus into functional compartments with no physical barriers separating them from the nucleoplasm raised key questions regarding how molecules move and reach their specific nuclear targets during relevant processes such as transcription, replication and repair (86–88).

Fluorescence fluctuation approaches permitted exploring how biomolecules move within this intricate compartment with unprecedented resolution. These methods showed that the nuclear milieu behaves as a heterogeneous multiscale porous medium with randomly distributed obstacles that constrain the diffusion of molecules (58,89,90) reinforcing the idea that nuclear crowding induces volume exclusion and affects the diffusion and speed of chemical reactions (91).

FCS was also well-suited to study protein-chromatin interactions *in situ* since binding to relatively immobile targets introduces a delay on the protein diffusion detectable through fluctuations techniques (89,92–94). In simple cases, the FCS analysis provides quantitative information regarding the fractions of molecules engaged in interactions and the dwell time on the targets (95). This methodology was employed to explore the dynamics of transcription-related proteins in cultured cells and allowed determining interactions in the millisecond to second time scale (for example, Refs. 92,94,95). We have recently combined the use of photoactivatable fluorescent probes with single-point FCS to quantify the dynamics of transcription factors in developing mouse early embryos (13). This methodology showed that transcription factors relevant to early-development are engaged in short- and long-lived interactions with DNA. Moreover, we found variations on DNA-Sox2 interactions among blastomeres of the four-cell embryo that are modulated by DNA accessibility and correlate with the cell fate of the progeny.

Spatiotemporal correlation methods are powerful tools to investigate exchange of biomolecules between cellular compartments and were then used to explore the receptor-mediated bidirectional transport of proteins through nuclear pores (56,96,97). One of the problems in these studies is the natural motion of the pore complex within the nuclear membrane. Cardarelli et al. (56) combined pCF analysis with a tracking technique that allows the real time positioning of the center of the scanned orbit on top of a single nuclear pore complex (98). Based on their pCF analysis at the pore, these authors proposed that molecular transport through the nuclear pore complex can be powered, at least in part, by the directed motion of specific nucleoporins.

Conclusions

Years of research and applications of fluorescence fluctuations methodologies in chemistry, physics, biology, biophysics, and biotechnology have turned FCS into a well-established and almost routine microscopy method. The combination of advances in imaging techniques, detectors technology and computational power are pushing the limits on what we can see and study through FCS. The observation of molecules as they move in whole organisms such as plants (12), mouse (13), and zebrafish (99,100) embryos are just some examples on the exquisite dynamical information that FCS provides and on the future direction of this technology in the Cell Biology field.

The aim of this review was to present the recent advances in FCS and related techniques to a broad biological community, focusing on questions of general interest. We also wanted to provide a guide to these techniques to make them more accessible to a large number of researchers. In this direction, we have provided two examples in different fields to illustrate the potentiality of this methodology.

We envision a promising future where a growing number of novel fluctuation-based tools in combination with new microscopy methods will help us to achieve the ultimate goal of mapping the dynamics and interactions of biomolecules *in situ*.

Acknowledgements

The work was supported by ANPCyT (PICT 2012-0899), UBACyT (20020110100074). L.B. and V.L. are CONICET members.

References

[1] Axelrod, D., Koppel, D. E., Schlessinger, J., Elson, E., and Webb, W. W. (1976) Mobility measurement by analysis of fluorescence photobleaching recovery kinetics. Biophys. J. 16, 1055–1069.

[2] Reits, E. A., and Neefjes, J. J. (2001) From fixed to FRAP: measuring protein mobility and activity in living cells. Nat. Cell. Biol. 3, E145–E147.

[3] Lippincott-Schwartz, J., Altan-Bonnet, N., and Patterson, G. H. (2003) Photobleaching and photoactivation: following protein dynamics in living cells. Nat. Cell. Biol. Suppl, S7–S14.

[4] Cognet, L., Leduc, C., and Lounis, B. (2014) Advances in live-cell single-particle tracking and dynamic super-resolution imaging. Curr. Opin. Chem. Biol. 20, 78–85.

[5] Kusumi, A., Tsunoyama, T. A., Hirosawa, K. M., Kasai, R. S., and Fujiwara, T. K. (2014) Tracking single molecules at work in living cells. Nat. Chem. Biol. 10, 524–532.

[6] Bismuto, E., Gratton, E., and Lamb, D. C. (2001) Dynamics of ANS binding to tuna apomyoglobin measured with fluorescence correlation spectroscopy. Biophys. J. 81, 3510–3521.

[7] Klingler, J., and Friedrich, T. (1997) Site-specific interaction of thrombin and inhibitors observed by fluorescence correlation spectroscopy. Biophys. J. 73, 2195–2200.

[8] Sanchez, M. F., Dodes Traian, M. M., Levi, V., and Carrer, D. C. (2015) One-photon lithography for high-quality lipid bilayer micropatterns. Langmuir 31, 11943–11950.
[9] Sanchez, S. A., Tricerri, M. A., and Gratton, E. (2012) Laurdan generalized polarization fluctuations measures membrane packing micro-heterogeneity in vivo. Proc Natl Acad Sci USA 109, 7314–7319.
[10] Izeddin, I., Recamier, V., Bosanac, L., Cisse, I. I., Boudarene, L., et al. (2014) Single-molecule tracking in live cells reveals distinct target-search strategies of transcription factors in the nucleus. eLife 3, e02230.
[11] Larson, D. R., Zenklusen, D., Wu, B., Chao, J. A., and Singer, R. H. (2011) Real-time observation of transcription initiation and elongation on an endogenous yeast gene. Science 332, 475–478.
[12] Clark, N. M., Hinde, E., Winter, C. M., Fisher, A. P., Crosti, G., et al. (2016) Tracking transcription factor mobility and interaction in Arabidopsis roots with fluorescence correlation spectroscopy. eLife 5, e14770.
[13] White, M. D., Angiolini, J. F., Alvarez, Y. D., Kaur, G., Zhao, Z. W., et al. (2016) Long-lived binding of Sox2 to DNA predicts cell fate in the four-cell mouse embryo. Cell 165, 75–87.
[14] Magde, D., Elson, E., and Webb, W. W. (1972) Thermodynamic fluctuations in a reacting system-measurement by fluorescence correlation spectroscopy. Phys. Rev. Lett. 29, 705–708.
[15] Rigler, R., Mets, U., Widengren, J., and Kask, P. (1993) Fluorescence correlation spectroscopy with high count rate and low background: analysis of translational diffusion. Eur. Biophys. J. 22, 169–175.
[16] Dean, K. M., and Palmer, A. E. (2014) Advances in fluorescence labeling strategies for dynamic cellular imaging. Nat. Chem. Biol. 10, 512–523.
[17] Zhang, J., Campbell, R. E., Ting, A. Y., and Tsien, R. Y. (2002) Creating new fluorescent probes for cell biology. Nat. Rev. Mol. Cell Biol. 3, 906–918.
[18] Kastrup, L., Blom, H., Eggeling, C., and Hell, S. W. (2005) Fluorescence fluctuation spectroscopy in subdiffraction focal volumes. Phys. Rev. Lett. 94, 178104.
[19] Levene, M. J., Korlach, J., Turner, S. W., Foquet, M., Craighead, H. G., et al. (2003) Zero-mode waveguides for single-molecule analysis at high concentrations. Science 299, 682–686.
[20] Kask, P., Piksarv, P., and Mets, Ü. (1985) Fluorescence correlation spectroscopy in the nanosecond time range: photon antibunching in dye fluorescence. Eur. Biophys. J. 12, 163–166.
[21] Elson, E. L. (2013) 40 years of FCS: how it all began. Methods Enzymol. 518, 1–10.
[22] Berland, K. M., So, P. T., and Gratton, E. (1995) Two-photon fluorescence correlation spectroscopy: method and application to the intracellular environment. Biophys. J. 68, 694–701.
[23] Elson, E. L. (2013) Brief introduction to fluorescence correlation spectroscopy. Methods Enzymol. 518, 11–41.
[24] Elson, E. L. (2011) Fluorescence correlation spectroscopy: past, present, future. Biophys J. 101, 2855–2870.
[25] Krichevsky, O., and Bonnet, G. (2002) Fluorescence correlation spectroscopy: the technique and its applications. Rep. Prog. Phys. 65, 251–297.
[26] Macháň, R., and Wohland, T. (2014) Recent applications of fluorescence correlation spectroscopy in live systems. FEBS Lett. 588, 3571–3584.
[27] Angiolini, J., Plachta, N., Mocskos, E., and Levi, V. (2015) Exploring the dynamics of cell processes through simulations of fluorescence microscopy experiments. Biophys. J. 108, 2613–2618.
[28] Dix, J. A., Hom, E. F. Y., and Verkman, A. S. (2006) Fluorescence correlation spectroscopy simulations of photophysical phenomena and molecular interactions: a molecular dynamics/Monte Carlo approach. J. Phys. Chem. B 110, 1896–1906.
[29] Meseth, U., Wohland, T., Rigler, R., and Vogel, H. (1999) Resolution of fluorescence correlation measurements. Biophys. J. 76, 1619–1631.
[30] Fitzpatrick, J. A., and Lillemeier, B. F. (2011) Fluorescence correlation spectroscopy: linking molecular dynamics to biological function in vitro and in situ. Curr. Opin. Struct. Biol. 21, 650–660.
[31] Bulseco, D. A., and Wolf, D. E. (2007) Fluorescence correlation spectroscopy: molecular complexing in solution and in living cells. Methods Cell Biol. 81, 525–559.
[32] Kim, S. A., Heinze, K. G., and Schwille, P. (2007) Fluorescence correlation spectroscopy in living cells. Nat. Methods 4, 963–973.
[33] Slaughter, B. D., and Li, R. (2010) Toward quantitative "in vivo biochemistry" with fluorescence fluctuation spectroscopy. Mol. Biol. Cell 21, 4306–4311.
[34] Di Rienzo, C., Gratton, E., Beltram, F., and Cardarelli, F. (2014) From fast fluorescence imaging to molecular diffusion law on live cell membranes in a commercial microscope. J. Vis. Exp. (92), e51994.
[35] Dertinger, T., Pacheco, V., von der Hocht, I., Hartmann, R., Gregor, I., et al. (2007) Two-focus fluorescence correlation spectroscopy: A new tool for accurate and absolute diffusion measurements. Chemphyschem 8, 433–443.
[36] Ries, J., and Schwille, P. (2006) Studying slow membrane dynamics with continuous wave scanning fluorescence correlation spectroscopy. Biophys. J. 91, 1915–1924.
[37] Chen, H., Farkas, E. R., and Webb, W. W. (2008) Chapter 1: In vivo applications of fluorescence correlation spectroscopy. Methods Cell Biol. 89, 3–35.
[38] Kohl, T., and Schwille, P. (2005) Fluorescence correlation spectroscopy with autofluorescent proteins. Adv. Biochem. Eng. Biotechnol. 95, 107–142.
[39] Stasevich, T. J., Mueller, F., Michelman-Ribeiro, A., Rosales, T., Knutson, J. R., et al. (2010) Cross-validating FRAP and FCS to quantify the impact of photobleaching on in vivo binding estimates. Biophys. J. 99, 3093–3101.
[40] Tcherniak, A., Reznik, C., Link, S., and Landes, C. F. (2009) Fluorescence correlation spectroscopy: criteria for analysis in complex systems. Anal. Chem. 81, 746–754.
[41] Digman, M. A., and Gratton, E. (2011) Lessons in fluctuation correlation spectroscopy. Annu. Rev. Phys. Chem. 62, 645–668.
[42] Kolin, D. L., and Wiseman, P. W. (2007) Advances in image correlation spectroscopy: measuring number densities, aggregation states, and dynamics of fluorescently labeled macromolecules in cells. Cell Biochem. Biophys. 49, 141–164.
[43] Schwille, P., Meyer-Almes, F. J., and Rigler, R. (1997) Dual-color fluorescence cross-correlation spectroscopy for multicomponent diffusional analysis in solution. Biophys. J. 72, 1878–1886.
[44] Ries, J., and Schwille, P. (2012) Fluorescence correlation spectroscopy. Bioessays 34, 361–368.
[45] Bacia, K., Kim, S. A., and Schwille, P. (2006) Fluorescence cross-correlation spectroscopy in living cells. Nat. Methods 3, 83–89.
[46] Ries, J., and Schwille, P. (2008) New concepts for fluorescence correlation spectroscopy on membranes. Phys. Chem. Chem. Phys. 10, 3487–3497.
[47] Inoue, M., Digman, M. A., Cheng, M., Breusegem, S. Y., Halaihel, N., et al. (2004) Partitioning of NaPi cotransporter in cholesterol-, sphingomyelin-, and glycosphingolipid-enriched membrane domains modulates NaPi protein diffusion, clustering, and activity. J. Biol. Chem. 279, 49160–49171.
[48] Ries, J., Chiantia, S., and Schwille, P. (2009) Accurate determination of membrane dynamics with line-scan FCS. Biophys. J. 96, 1999–2008.
[49] Ruan, Q., Cheng, M. A., Levi, M., Gratton, E., and Mantulin, W. W. (2004) Spatial-temporal studies of membrane dynamics: scanning fluorescence correlation spectroscopy (SFCS). Biophys. J. 87, 1260–1267.
[50] Schneckenburger, H. (2005) Total internal reflection fluorescence microscopy: technical innovations and novel applications. Curr. Opin. Biotechnol. 16, 13–18.
[51] Huisken, J., Swoger, J., Del Bene, F., Wittbrodt, J., and Stelzer, E. H. (2004) Optical sectioning deep inside live embryos by selective plane illumination microscopy. Science 305, 1007–1009.
[52] Guo, S. M., Bag, N., Mishra, A., Wohland, T., and Bathe, M. (2014) Bayesian total internal reflection fluorescence correlation spectroscopy reveals hIAPP-induced plasma membrane domain organization in live cells. Biophys. J. 106, 190–200.
[53] Guo, S. M., He, J., Monnier, N., Sun, G., Wohland, T., et al. (2012) Bayesian approach to the analysis of fluorescence correlation spectroscopy data II: application to simulated and in vitro data. Anal. Chem. 84, 3880–3888.
[54] Hassler, K., Leutenegger, M., Rigler, P., Rao, R., Rigler, R., et al. (2005) Total internal reflection fluorescence correlation spectroscopy (TIR-FCS) with low background and high count-rate per molecule. Opt. Express 13, 7415–7423.
[55] Wohland, T., Shi, X., Sankaran, J., and Stelzer, E. H. (2010) Single plane illumination fluorescence correlation spectroscopy (SPIM-FCS) probes inhomogeneous three-dimensional environments. Opt. Express 18, 10627–10641.
[56] Cardarelli, F., Lanzano, L., and Gratton, E. (2012) Capturing directed molecular motion in the nuclear pore complex of live cells. Proc. Natl. Acad. Sci. USA 109, 9863–9868.

[57] Digman, M. A., and Gratton, E. (2009) Imaging barriers to diffusion by pair correlation functions. Biophys. J. 97, 665–673.
[58] Hinde, E., Cardarelli, F., Digman, M. A., and Gratton, E. (2010) In vivo pair correlation analysis of EGFP intranuclear diffusion reveals DNA-dependent molecular flow. Proc. Natl. Acad. Sci. USA 107, 16560–16565.
[59] Digman, M. A., Brown, C. M., Sengupta, P., Wiseman, P. W., Horwitz, A. R., et al. (2005) Measuring fast dynamics in solutions and cells with a laser scanning microscope. Biophys. J. 89, 1317–1327.
[60] Hebert, B., Costantino, S., and Wiseman, P. W. (2005) Spatiotemporal image correlation spectroscopy (STICS) theory, verification, and application to protein velocity mapping in living CHO cells. Biophys. J. 88, 3601–3614.
[61] Wiseman, P. W., Brown, C. M., Webb, D. J., Hebert, B., Johnson, N. L., et al. (2004) Spatial mapping of integrin interactions and dynamics during cell migration by image correlation microscopy. J. Cell Sci. 117, 5521–5534.
[62] Digman, M. A., Dalal, R., Horwitz, A. F., and Gratton, E. (2008) Mapping the number of molecules and brightness in the laser scanning microscope. Biophys. J. 94, 2320–2332.
[63] Presman, D. M., Ogara, M. F., Stortz, M., Alvarez, L. D., Pooley, J. R., et al. (2014) Live cell imaging unveils multiple domain requirements for in vivo dimerization of the glucocorticoid receptor. PLoS Biol. 12, e1001813.
[64] Chen, Y., Muller, J. D., So, P. T., and Gratton, E. (1999) The photon counting histogram in fluorescence fluctuation spectroscopy. Biophys. J. 77, 553–567.
[65] Kask, P., Palo, K., Ullmann, D., and Gall, K. (1999) Fluorescence-intensity distribution analysis and its application in biomolecular detection technology. Proc. Natl. Acad. Sci. USA 96, 13756–13761.
[66] Palmer, A. G., III, and Thompson, N. L. (1989) High-order fluorescence fluctuation analysis of model protein clusters. Proc. Natl. Acad. Sci. USA 86, 6148–6152.
[67] Qian, H., and Elson, E. L. (1990) Distribution of molecular aggregation by analysis of fluctuation moments. Proc. Natl. Acad. Sci. USA 87, 5479–5483.
[68] Chen, Y., and Muller, J. D. (2007) Determining the stoichiometry of protein heterocomplexes in living cells with fluorescence fluctuation spectroscopy. Proc. Natl. Acad. Sci. USA 104, 3147–3152.
[69] Digman, M. A., Wiseman, P. W., Choi, C., Horwitz, A. R., and Gratton, E. (2009) Stoichiometry of molecular complexes at adhesions in living cells. Proc. Natl. Acad. Sci. USA 106, 2170–2175.
[70] Jacobson, K., Mouritsen, O. G., and Anderson, R. G. (2007) Lipid rafts: at a crossroad between cell biology and physics. Nat. Cell Biol. 9, 7–14.
[71] Miguel, L., Owen, D. M., Lim, C., Liebig, C., Evans, J., et al. (2011) Primary human CD4+ T cells have diverse levels of membrane lipid order that correlate with their function. J. Immunol. 186, 3505–3516.
[72] Zech, T., Ejsing, C. S., Gaus, K., de Wet, B., Shevchenko, A., et al. (2009) Accumulation of raft lipids in T-cell plasma membrane domains engaged in TCR signalling. EMBO J. 28, 466–476.
[73] Klemm, R. W., Ejsing, C. S., Surma, M. A., Kaiser, H. J., Gerl, M. J., et al. (2009) Segregation of sphingolipids and sterols during formation of secretory vesicles at the trans-Golgi network. J. Cell. Biol. 185, 601–612.
[74] Schuck, S., and Simons, K. (2004) Polarized sorting in epithelial cells: raft clustering and the biogenesis of the apical membrane. J. Cell Sci. 117, 5955–5964.
[75] Brügger, B., Glass, B., Haberkant, P., Leibrecht, I., Wieland, F. T., et al. (2006) The HIV lipidome: a raft with an unusual composition. Proc. Natl. Acad. Sci. USA 103, 2641–2646.
[76] Saad, J. S., Miller, J., Tai, J., Kim, A., Ghanam, R. H., et al. (2006) Structural basis for targeting HIV-1 Gag proteins to the plasma membrane for virus assembly. Proc. Natl. Acad. Sci. USA 103, 11364–11369.
[77] Takeda, M., Leser, G. P., Russell, C. J., and Lamb, R. A. (2003) Influenza virus hemagglutinin concentrates in lipid raft microdomains for efficient viral fusion. Proc. Natl. Acad. Sci. 100, 14610–14617.
[78] He, H. T., and Marguet, D. (2011) Detecting nanodomains in living cell membrane by fluorescence correlation spectroscopy. Annu. Rev. Phys. Chem. 62, 417–436.
[79] Machan, R., and Hof, M. (2010) Lipid diffusion in planar membranes investigated by fluorescence correlation spectroscopy. Biochim. Biophys. Acta 1798, 1377–1391.
[80] Korlach, J., Schwille, P., Webb, W. W., and Feigenson, G. W. (1999) Characterization of lipid bilayer phases by confocal microscopy and fluorescence correlation spectroscopy. Proc. Natl. Acad. Sci. USA 96, 8461–8466.
[81] Lenne, P. F., Wawrezinieck, L., Conchonaud, F., Wurtz, O., Boned, A., et al. (2006) Dynamic molecular confinement in the plasma membrane by microdomains and the cytoskeleton meshwork. EMBO J. 25, 3245–3256.
[82] Bacia, K., Scherfeld, D., Kahya, N., and Schwille, P. (2004) Fluorescence correlation spectroscopy relates rafts in model and native membranes. Biophys. J. 87, 1034–1043.
[83] Eggeling, C., Ringemann, C., Medda, R., Schwarzmann, G., Sandhoff, K., et al. (2009) Direct observation of the nanoscale dynamics of membrane lipids in a living cell. Nature 457, 1159–1162.
[84] Honigmann, A., Mueller, V., Ta, H., Schoenle, A., Sezgin, E., et al. (2014) Scanning STED-FCS reveals spatiotemporal heterogeneity of lipid interaction in the plasma membrane of living cells. Nat. Commun. 5, 5412.
[85] Mueller, V., Ringemann, C., Honigmann, A., Schwarzmann, G., Medda, R., et al. (2011) STED nanoscopy reveals molecular details of cholesterol- and cytoskeleton-modulated lipid interactions in living cells. Biophys. J. 101, 1651–1660.
[86] Lanctot, C., Cheutin, T., Cremer, M., Cavalli, G., and Cremer, T. (2007) Dynamic genome architecture in the nuclear space: regulation of gene expression in three dimensions. Nat. Rev. Genet. 8, 104–115.
[87] Misteli, T. (2007) Beyond the sequence: cellular organization of genome function. Cell 128, 787–800.
[88] Weidtkamp-Peters, S., Weisshart, K., Schmiedeberg, L., and Hemmerich, P. (2009) Fluorescence correlation spectroscopy to assess the mobility of nuclear proteins. Methods Mol. Biol. 464, 321–341.
[89] Baum, M., Erdel, F., Wachsmuth, M., and Rippe, K. (2014) Retrieving the intracellular topology from multi-scale protein mobility mapping in living cells. Nat. Commun. 5, 4494.
[90] Di Rienzo, C., Piazza, V., Gratton, E., Beltram, F., and Cardarelli, F. (2014) Probing short-range protein Brownian motion in the cytoplasm of living cells. Nat. Commun. 5, 5891.
[91] Bancaud, A., Huet, S., Daigle, N., Mozziconacci, J., Beaudouin, J., et al. (2009) Molecular crowding affects diffusion and binding of nuclear proteins in heterochromatin and reveals the fractal organization of chromatin. EMBO J. 28, 3785–3798.
[92] Brazda, P., Szekeres, T., Bravics, B., Toth, K., Vamosi, G., et al. (2011) Live-cell fluorescence correlation spectroscopy dissects the role of coregulator exchange and chromatin binding in retinoic acid receptor mobility. J. Cell Sci. 124, 3631–3642.
[93] Kaur, G., Costa, M. W., Nefzger, C. M., Silva, J., Fierro-Gonzalez, J. C., et al. (2013) Probing transcription factor diffusion dynamics in the living mammalian embryo with photoactivatable fluorescence correlation spectroscopy. Nat. Commun. 4, 1637.
[94] Mikuni, S., Tamura, M., and Kinjo, M. (2007) Analysis of intranuclear binding process of glucocorticoid receptor using fluorescence correlation spectroscopy. FEBS Lett. 581, 389–393.
[95] Michelman-Ribeiro, A., Mazza, D., Rosales, T., Stasevich, T. J., Boukari, H., et al. (2009) Direct measurement of association and dissociation rates of DNA binding in live cells by fluorescence correlation spectroscopy. Biophys. J. 97, 337–346.
[96] Cardarelli, F., and Gratton, E. (2010) In vivo imaging of single-molecule translocation through nuclear pore complexes by pair correlation functions. PLoS One 5, e10475.
[97] Cardarelli, F., Lanzano, L., and Gratton, E. (2011) Fluorescence correlation spectroscopy of intact nuclear pore complexes. Biophys. J. 101, L27–L29.
[98] Levi, V., Ruan, Q., and Gratton, E. (2005) 3-D particle tracking in a two photon microscope. Application to the study of molecular dynamics in cells. Biophys. J. 88, 2919–2928.
[99] Wang, Y., Wang, X., Wohland, T., and Sampath, K. (2016) Extracellular interactions and ligand degradation shape the nodal morphogen gradient. eLife 5, e13879.
[100] Yu, S. R., Burkhardt, M., Nowak, M., Ries, J., Petrasek, Z., et al. (2009) Fgf8 morphogen gradient forms by a source-sink mechanism with freely diffusing molecules. Nature 461, 533–536.