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Recent applications of fluorescence correlation spectroscopy in live cells

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As a time-domain analogue of fluorescence imaging, FCS offers valuable insights into molecular dynamics, interactions, and concentrations within living cells. The primary insight generated by FCS is molecular mobility and concentration, which makes it useful for investigating molecular-scale details without the need for enrichment or separation. A specific strength of FCS is the ability to probe protein–protein interactions in live cells and several recent applications in this area are summarized. FCS is also used to investigate plasma membrane protein organization, with many applications to cell surface receptors and the mechanisms of drug binding. Finally, FCS is undergoing continual methodological innovations, such as imaging FCS, SPIM-FCS PIE-FCCS, STED-FCS, three-color FCS, and massively parallel FCS, which extend the capabilities to investigate molecular dynamics at different spatial and temporal scales. These innovations enable detailed examinations of cellular processes, including cellular transport and the spatial organization of membrane proteins.

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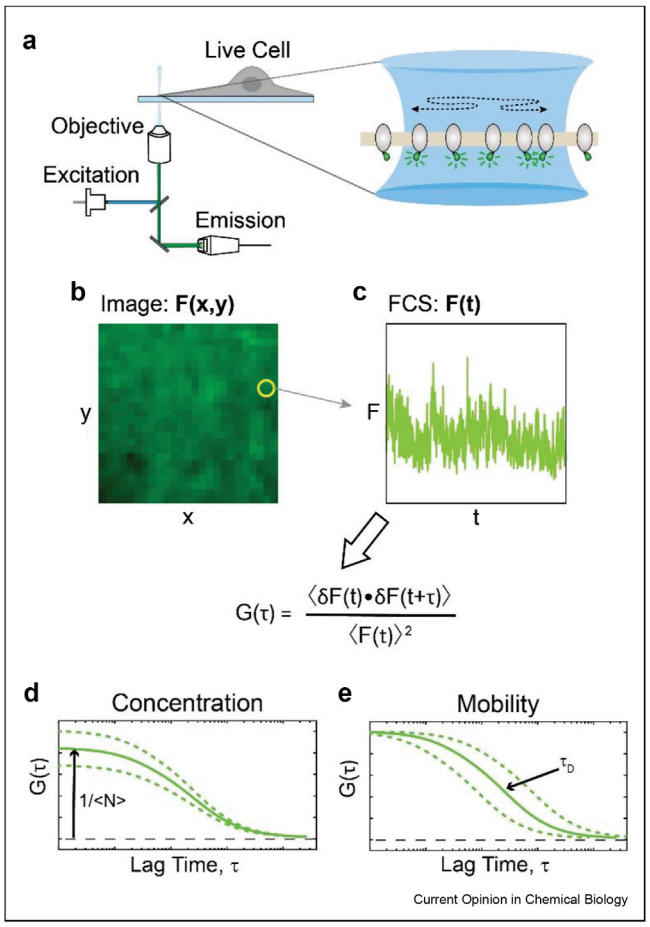
Introduction

Quantitative imaging and spectroscopy methods are highly valued in cell biology and molecular biophysics because they can reveal important mechanisms in the complex environment of living cells. Fluorescence methods in particular are flourishing because they provide a combination of sensitivity, selectivity, versatility, and accessibility. At a basic level, traditional live cell imaging can report on the subcellular localization of large ensembles of labeled molecules. While useful for many problems, the traditional imaging approach does not typically reveal molecular scale information in the sense of molecular diffusion, assembly, or binding because the optical diffraction limit (~200 nm) is much larger than many biomolecules of interest. Methods like single-molecule tracking (SMT) can circumvent the diffraction limit by lowering the molecular density and recording single trajectories using successive localization events. Fluorescence correlation spectroscopy (FCS) spans these two concentration regimes: it is sensitive to single-molecule events while being able to record data and much higher molecular densities and higher temporal resolution [1–3]. These advantages make FCS a powerful quantitative method for in situ studies of protein diffusion, protein–protein interactions, and protein expression levels [4].

This review will focus on several recent applications of FCS measurements in live cells. There are many examples to choose from, so I focused on representative studies that highlight the strengths and advantages of FCS. These application areas include the determination of local concentrations of biomolecular species, the quantification of protein–protein interactions, the investigation of protein-nucleic acid interactions (e.g. transcription factors), and the organization and dynamics of proteins in the plasma membrane. I will also summarize some recent and ongoing technological innovations that are creating unique opportunities in cell biology research. There are already several excellent reviews that summarize the FCS method and more broad-ranging applications in and outside of chemical biology fields [2,5,6]. I will, therefore, only briefly summarize the method and its interpretation in the remaining paragraphs of this introduction. For a new user of FCS, I would recommend an excellent recent book that contains detailed information about FCS hardware, data analysis, and specialized applications [7].

FCS can be thought of as a time-domain cousin to fluorescence imaging. Rather than recording intensity as a function of spatial coordinates to generate an image, FCS collects intensity as a function of time from the same position in the sample (Figure 1). The timescale of data collection varies depending on the sample, but generally ranges from seconds to minutes with sampling rates ranging from microseconds to picoseconds [2]. The time-dependent fluorescence intensity is sensitive to any time-varying property like structural fluctuations, molecular rotations, triplet relaxation, photobleaching,

Figure 1



FCS Overview. (**a**) Diagram of an inverted microscope-based FCS instrument. An objective focuses the excitation laser beam to a diffraction-limited spot in a live cell sample. In the zoom illustration, GFP-labeled membrane proteins are shown diffusing in the plasma membrane. Fluorescence emission is collected by a single-photon avalanche photodiodes (SPAD) detector and recorded for analysis. (**b**) A fluorescence image is created by measuring the signal intensity at a series of x and y positions, F(x,y), at a fixed time. (**c**) FCS data is collected from one (x,y) position as a function of time to generate a fluctuating signal, F(t). The time-dependent signal is analyzed by a time−time ACF to generate, G(τ), shown in panels **d** and **e**. (**d**) The amplitude of the ACF at G(τ = 0) is quantitatively related to concentration, and (**e**) the decay time, τ_D, is quantitatively related to the mobility of the diffusing species.

etc. [2]. For most live-cell studies, the fluctuations of primary interest are from individual fluorophores entering and leaving the observation volume (Figure 1a). In a typical experiment, data is collected for a set amount of time, during which a fluctuating signal is observed. These fluctuations are largely a result of single-molecule diffusion events, and are analyzed with an autocorrelation function (ACF) approach. The ACF is characterized by the average time scale of the fluctuations, τ_D, and the average amplitude of the fluctuations, $G(\tau = 0)$ (Figure 1c–e). A model fit is used to convert these values into a diffusion coefficient (D) and the average number of particles, $\langle N \rangle$ or local concentration, C. These parameters are hugely valuable in cell biology because they allow one to determine the expression level or local concentration of a molecule of interest as well as its mobility (Figure 1d and e). Choosing the right model fit for the data can be challenging, but is essential for proper interpretation of the correlation functions. A Gaussian volume model is typically used, but other models may be selected depending on the details of the system [7].

Because mobility is directly related to the size of the molecule and its local environment, it is possible to extract molecular properties like multimerization, binding events, and transport between heterogeneous regions. As an example of FCS to resolve translational mobilities in living cells, a recent paper used polarization-dependent FCS to characterize green fluorescent protein (GFP) in several cell lines [8]. The translational mobility of GFP was heterogeneous within each cell line and across the four different cell lines, as reported in the distributions of single-cell diffusion coefficients. Treatment with nocodazole, a microtubule inhibitor, was used to disrupt the cytoskeleton, which significantly reduced the diffusion heterogeneity. The study is an example of how FCS can reveal differences in cellular nano-environments between cell lines and how they change during cell cycle phases.

Concentration is another key output of FCS measurements, and is often used in the applications discussed below. However, the sensitivity of FCS to concentration can be counterintuitive to a newcomer, so it may be useful to explain how it is observed in the raw data. At very low concentrations (e.g. less than one particle in the observation area at any given time), the signal will consist of short bursts of intensity when the occasional fluorophore diffuses into the observation volume. As the concentration increases, the bursts will become more and more frequent, eventually overlapping in time and leading to a fluctuating signal around a mean value. At very high concentrations, the signal intensity is also high, so that the fluctuations are quite small relative to the signal strength. Another way of thinking about this is that with many fluorophores in the observation volume, the signal is less sensitive to single molecules entering or leaving the area. Imagine a dance party observed from a mezzanine above the floor. For a small party of 10 or so people, it will be very obvious to the observer when someone enters or leaves the dance floor. For a large party of hundreds of people, the observer is much less likely to notice someone ducking out. Practically, this sensitivity to signal *fluctuations* means there is an upper limit to the concentrations that can be assayed with FCS. The exact upper limit depends on a variety of instrument details, but is generally at $\langle N \rangle > 200$ or $\sim$1 μM in an aqueous solution. In 2-dimensional

systems, the limit is also around $\langle N \rangle > 200$, or 10^3 molecules/μm^2. There are ways around these limits, like shrinking the observation volume using stimulated emission depletion (STED). Fortunately, however, these concentration limits fall into an ideal range for many important proteins of interest as will be shown below.

Recent applications of FCS in live cells

Concentration of specific molecular species

Concentration and mobility are the two primary outputs of FCS measurements. This section will focus on recent studies that use FCS to quantify the concentration of specific biomolecules in a live cell context. In the first example, FCS was used to measure the concentration of a secreted protein, Fam20C, from a live cell culture in situ [9]. The protein was expressed via transfection of a SNAP-tag fusion and labeled in situ. SNAP tags are derived from a mutant version of the DNA repair protein O6-alkylguanine-DNA alkyltransferase, which has been engineered to accept small molecules as substrates. SNAP fusions are very common in quantitative fluorescence imaging because they are genetically encodable like fluorescent proteins and yet can be conjugated with small molecule fluorescent probes that are much brighter than fluorescent proteins. The FAM20C kinase phosphorylates secreted proteins and knowing the functions of FAM20C kinase is important for understanding glycosaminoglycan biosynthesis, biomineralization, and the regulation of secreted proteins in various biological processes. Using FCS ACF amplitudes, the authors investigated the concentration of FAM20C secreted into the media and measured the effects of the FAM20C signal peptide, RNA interference, and myriocin, a drug that modifies sphingosine, an activator of FAM20C. This FCS approach allows for real-time, in situ measurement of secreted proteins without the need for enrichment or separation, and could be a useful analytical strategy for detecting other extracellular secretions.

While fluorescence correlation spectroscopy (FCS) is minimally invasive method for live cell studies, practical issues can arise, such as low signal/noise ratios and long-term photobleaching. One recent study proposed a new data analysis approach using the artificial slow variation in fluorophore concentration due to photobleaching to create an in situ FCS calibration curve, enabling precise FCS-calibrated concentration maps in complex and dynamic environments [10]. The model developed for ACF fitting incorporates long-term photobleaching and background noise, allowing for precise determination of key parameters, even in high excitation intensity and extended measurement times. This eliminates the need to avoid photobleaching entirely, potentially reshaping the approach to single-point FCS experiments in cellular studies. For the in vivo application, the authors investigated drosophila embryos expressing different fluorescent protein fusions. Utilizing single-point FCS measurements and the new model, the researchers analyzed the changes in concentration. The results demonstrate the method's effectiveness in accounting for molecular brightness and background noise to determine concentration in a dynamic cellular context.

Finally, FCS was recently employed to investigate nitric oxide levels (NO) in cells using a fluorescent sensor diaminofluorescein 2 (DAF-2 and DAF-2T) [11]. FCS data was first collected in a series of standard calibration samples to characterize the fluorescence characteristics of the sensor in the presence and absence of NO. FCS data was then taken in live HeLa cells to measure subnanomolar to micromolar concentrations of NO. Some consideration of the observation volume in the cytoplasm vs a dilute aqueous buffer was taken into consideration. The authors also characterized the sensor in two different cell lines and at different spatial positions within the same cell. This approach revealed a significant degree of intracellular spatial heterogeneity in the NO concentrations.

Protein–protein interactions

Protein interactions are nodes of communication in many cellular processes, and so assessing protein interaction partners and characterizing the dynamic landscape of protein interactions is fundamental to cell biology. One recent study employed FCS to quantitatively analyze the uptake and intracellular interactions of a fluorescent derivative of olaparib (PARPi-FL), a drug used in the treatment of ovarian cancer and breast cancer associated with BRCA mutations [12]. Olaparib inhibits DNA repair by binding to the PARP1 protein, leading to DNA damage accumulation and potential cell death. Using the decay times from FCS data, this study identified three mobile components in the nucleus of TNBC cells: freely diffusing olaparib analog, PARP1-olaparib analog complex, and interactions with RNA. The study explored the different cytoplasmic interactions and compared the findings across cell lines, revealing that the therapeutic effect may depend more on the expression level of BRCA1 protein than PARP1. The study emphasized that drug penetration and accumulation in the nucleus do not guarantee therapeutic success, as sensitivity depends on factors such as the presence of BRCA1 protein [12].

Another recent study focused on regulatory protein complexes in the cell cycle: the assembly and disassembly of Cyclin B1—Cdk1-Cks complexes [13]. Cyclin-dependent kinases (Cdks) play a crucial role in cell cycle control, with Cyclin B1 binding and activating Cdk1 to form the major mitotic kinase for mitosis entry. The study employs FCS and fluorescence cross-correlation spectroscopy (FCCS) combined with CRISPR/Cas9-mediated gene editing to tag Cyclin B1

biallelically with a fluorescent protein. In FCCS, two different fluorophores with distinct emission wavelengths are used, and a cross-correlation function between the two emission channels is calculated. Cross-correlation can reveal information about stable molecular interactions between the two labeled species. The FCS decay times reveal two Cyclin B1 species, suggesting free and Cdk1-bound populations. Together, FCS and FCCS were used to characterize the cell cycle-regulated binding dynamics between Cyclin B1 and Cdk1. This approach provides high temporal and spatial resolution for studying protein complex dynamics in living cells [13].

FCS has also been used recently to quantify the interactions between the chaperone trigger factor (TF) and nascent protein chains during translation [14]. Chaperones, such as TF, assist in the folding process. Previous research on TF-nascent chain interactions in *Escherichia coli* indicated dynamic and length-dependent associations. FCS was employed in both in vitro and in vivo settings to characterize the length-dependent interactions between TF and nascent chains. Measurements were conducted on free enhanced GFP (EGFP) and TF-EGFP. Results showed that EGFP behaved similarly in *E. coli* and *S. cerevisiae* cells. TF-EGFP exhibited significantly slower diffusion than EGFP, indicating interaction with nascent chains. Inhibition of protein synthesis partially eliminated this effect, while a TF mutant showed a further decrease in diffusion. One remarkable aspect of this work was that they were able to record FCS data from live *E. Coli*. The diameter of *E. Coli* is comparable to the beam waist of the FCS excitation area, and so it can be challenging to collect data from these cells because of photobleaching and cell movement. To solve this problem, researchers elongated the cells with an antibiotic. The study demonstrates FCS's applicability for investigating translation-reaction-dependent interactions within living cells [14].

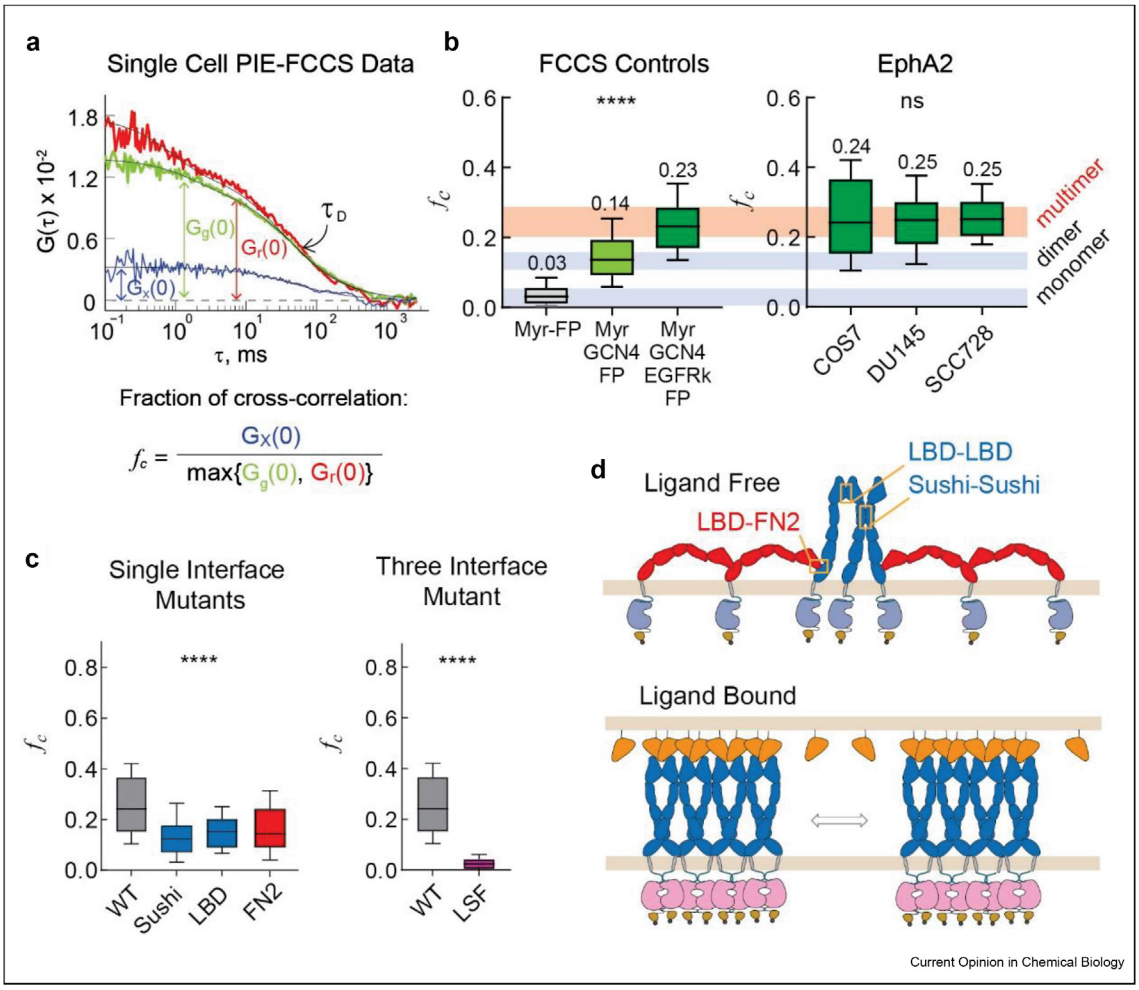
Membrane protein organization

One important application of FCS is to measure membrane protein dynamics and interactions. Membrane proteins constitution a large portion of the human proteome and yet are underrepresented in biophysical studies because of the difficulties expressing and purifying membrane proteins for high resolution structural methods. Furthermore, the plasma membrane is composed of many different lipid species and a large diversity of proteins. This chemical complexity is difficult to reproduce in model systems, so live cell studies are critical for many questions about membrane protein−protein interactions. In one recent study, FCS was used to investigate the behavior of the β2 adrenergic receptor (β2AR) in various cell types [15]. β2AR is a G protein-coupled receptor (GPCR) that responds to the hormone adrenaline (epinephrine) and undergoes ligand-directed internalization. In the study, researchers generated haplotype-specific SNAP-tagged β2AR human embryonic stem (ES) cell lines. This method enables the comparison of receptor expression and internalization in ES progenitor cells, fibroblasts, and cardiomyocytes. FCS was selected to monitor the surface expression of β2AR across the cell lines. The amplitudes of the ACFs were used to determine the surface density of the receptors in live cells. The study revealed differences in receptor behavior related to cell phenotype, suggesting potential implications for drugs targeting β2-agonists [15].

Work in my own lab is focused on membrane protein organization using specialized FCS method called pulsed interleaved excitation−fluorescence cross-correlation spectroscopy (PIE-FCCS) [16]. This method is a two-color, time-correlated single photon counting technique that resolves fluorescence lifetimes, FCS, and FCCS (Figure 2a). The pulse arrival times are interleaved so that spectral cross-talk can be removed from the cross-correlation data [17]. PIE-FCCS reduces the likelihood of false positive cross-correlation, especially for heterogeneous systems like the plasma membrane. In one recent application, we resolved the multimerization state of Ephrin type-A receptor 2 (EphA2) in cancer cells [18]. EphA2, a receptor for membrane-bound ephrin ligands, exhibits a unique duality in signaling—its bound state induces tumor-suppressive effects, while the unbound state promotes oncogenesis. The study focused on deciphering the molecular assembly of ligand-free EphA2, which initiates oncogenic signaling through phosphorylation of S897. With PIE-FCCS we measured EphA2 diffusion, oligomerization, and conformational changes, by which we discovered that ligand-free EphA2 forms multimers through symmetric and asymmetric interactions (Figure 2). The symmetric interactions drive ligand-induced receptor clustering and tumor-suppressive signaling, while the asymmetric interactions support oncogenic signaling, emphasizing the pivotal role of EphA2 assembly in dictating its dual functions in oncogenesis (Figure 2) [18].

Other recent studies with PIE-FCCS have investigated the role of dimerization and multimerization of EGFR, a membrane protein targeted in lung cancer therapy. In one study, we investigated structure-function relationships for exon 19 deletion (ex19del) variants of EGFR, one of the most common variants in non-small cell lung cancer [19]. Using a computational approach, the ex19dels were sorted and classified, and representative members of each group were identified for experimental work: E746_A750, E746_S752 > V, and L747_A750 > P. These variants were studied with biochemical analyses, revealing distinct activation profiles, enzyme kinetics, and TKI sensitivities. Using PIE-FCCS, one of the variants, L747_A750 > P, exhibited ligand-independent dimerization, in contrast to wild-type (WT),

Figure 2



PIE-FCCS reveals the multimerization state of EphA2. (**a**) Sample PIE-FCCS data from a single cell includes two ACF and one CCF. The fraction of cross-correlation is calculated from the amplitudes of the correlation functions. (**b**) Many single-cell measurements are collected to assess the distribution of fc values. Control membrane proteins are used to characterize a protein of interest based on the degree of cross-correlation. EphA2 is a multimer in the absence of a ligand in three different cell lines. (**c**) Mutagenesis experiments were used to determine the interfaces that mediate the multimeric state of EGF. Single interface mutants reduced the multimerization state. (**d**) model resulting from the data in the manuscript. EphA2 is a multimer with three key interfaces, when ligand binds, the two interfaces mediate assembly into large polymeric complexes. Figure adapted from Ref. [18].

E746_A750, and E746_S752 > V, which were predominantly monomeric. In the presence of EGF ligand, all variants, including WT, formed a mixture of dimers and multimers, suggesting that ex19del mutations may inhibit ligand-dependent multimeric assemblies. These results emphasize the need for personalized treatment strategies based on the properties of specific EGFR-mutant NSCLC [19]. In another recent PIE-FCCS study, we investigated the mechanism and prevalence of EGFR-KDD, a kinase domain duplication mutant of EGFR [20]. Utilizing clinical genomics, computational structural biology, and biochemical analyses, the researchers find that EGFR-KDD forms a catalytically active asymmetric intra-molecular dimer independent of EGF-ligand stimulation. With PIE-FCCS we found that EGF-ligand stimulation induced the formation of catalytically active homo- and hetero-dimers and multimers, highlighting the ability of EGFR-KDD to activate other HER/ERBB family members. The findings provide insights into the oncogenicity of EGFR-KDD and suggest therapeutic strategies for patients with EGFR-KDD-mediated cancers [20].

Transcription factors

Another common use of FCS is to resolve protein-nucleic acid interactions [21]. One interesting recent example is an investigation of the impact of doxorubicin (Dox), an anticancer drug, on chromatin binding to proteins, including high mobility group B1 (HMGB1), linker histone H1, and retinoic acid receptor (RARα), in

live cells [22]. The researchers employed fluorescence recovery after photobleaching (FRAP) and fluorescence correlation spectroscopy (FCS) to analyze the mobility of these proteins. At lower Dox doses, HMGB1 binding to DNA increased, while linker histone H1 binding decreased. Conversely, at higher doses corresponding to peak plasma concentrations during chemotherapy, Dox reduced HMGB1 binding. The researchers propose that Dox induces a biphasic effect, involving competition between ligands and local conformational changes in nucleosome-free DNA. The study also suggests a decrease in overall RARα binding to DNA upon Dox treatment, potentially contributing to Dox-induced cardiotoxicity. HMGB1 promotes nucleosome sliding and decompacts chromatin, while histone H1 stabilizes nucleosome structure. The study indicates that Dox's interference with H1 binding facilitates HMGB1 association with DNA. Additionally, the reduction in RARα binding to DNA, observed under Dox treatment, introduces a potential link between Dox-induced cardiotoxicity and the retinoic acid signaling pathway. These findings provide insights into the interactions of Dox with chromatin components and highlight its multifaceted effects on DNA-binding proteins, shedding light on both the therapeutic efficacy and side effects of this widely used anticancer drug [22].

Methodological developments

Like many fluorescence methods, FCS is undergoing continuous development [23]. Some examples, like PIE-FCCS, were referred to in the previous sections. In this section, I will highlight new areas of innovation, while also presenting examples of their application to live cell biophysical problems.

Imaging FCS

Imaging Fluorescence Correlation Spectroscopy (imFCS) is an extension of FCS that incorporates spatial information into the traditional temporal measurements [24,25]. Traditional FCS measures the fluctuations in fluorescence intensity over time within a small observation volume, providing insights into molecular mobility and interactions. ImFCS extends this concept to image sequences, allowing for the spatiotemporal analysis of fluorescence fluctuations in two-dimensional or three-dimensional space. The development of imFCS involves capturing spatially resolved fluorescence intensity data across an image and correlating these fluctuations over time to extract valuable information about molecular dynamics within cellular or subcellular structures. Data collection is usually performed with emCCD cameras, but new scientific cMOS cameras are also capable of collecting imFCS data. This technique is particularly useful for investigating heterogeneous or dynamic environments like live cells because it has the inherent ability to study multiple regions simultaneously. ImFCS has been applied to study the dynamics of molecules within cellular membranes, providing insights into lipid diffusion, protein mobility, and interactions within the lipid bilayer [26].

One recent study using imFCS focuses on the signaling stimulated by antigen (Ag) through cell surface immunoreceptors, particularly the high-affinity receptor for immunoglobulin E (IgE), FcεRI, in rat basophilic leukemia (RBL) mast cells [27]. The signaling involves lipid-based interactions in the plasma membrane, specifically the organization of liquid-ordered (Lo)-like nanodomains around clustered IgE-FcεRI. Lyn, a tyrosine kinase, is a key player and is known to preferentially partition into these Lo-like nanodomains. The study utilizes imFCS to analyze the diffusion properties of various probes, including lipid probes with Lo-preference, Lyn variants with modified interactions, and tyrosine phosphatase (PTPα). The results demonstrate that Lyn's lipid-based partitioning in the Lo-like nanodomains enhances its coupling with Ag-clustered FcεRI, leading to efficient kinase/receptor coupling and stimulated phosphorylation. The study provides valuable experimental evidence on how subtle changes in lipid organization contribute to protein interactions essential for TM signaling initiation.

Single plane illumination FCS (SPIM-FCS)

First reported in 2010, SPIM-FCS is a technique that merges light sheet microscopy with fluorescence correlation spectroscopy (FCS) [28]. It is related to imFCS, but differs in that SPIM employs a thin sheet of light to illuminate a single plane of the sample at a time, minimizing photobleaching and phototoxicity [29]. This selective illumination, combined with orthogonal detection, enhances optical sectioning and image contrast. As described in the previous section, imFCS analyzes fluorescence intensity fluctuations within a small observation volume to extract information about the number and dynamics of fluorescent molecules using autocorrelation analysis. The integration of these techniques allows for precise measurement of molecular dynamics within a defined plane of the sample. The combination of SPIM and FCS offers significant advantages, including reduced photobleaching, improved spatial resolution, and the ability to perform FCS measurements in thick and live samples. This makes SPIM-FCS particularly useful for studying live cells and organisms, enabling long-term observation of dynamic processes with minimal damage. The technique is valuable for investigating membrane dynamics, such as the behavior of membrane proteins and lipids, and for studying developmental biology by tracking molecular movements in embryos and tissues.

One recent application used SPIM-FCS to investigate the formation of spatiotemporal gradients in early *Caenorhabditis elegans* embryos [30]. The researchers

focus on the dynamics of PIE-1 and MEX-5 proteins, which are crucial for body axis formation and asymmetric cell division. Using three-dimensional time-resolved SPIM-FCS, they monitored the emergence of protein concentration gradients and observed a previously unreported depletion zone of PIE-1 near the cell cortex, not seen in MEX-5. The SPIM-FCS data revealed the successive formation of a mobility gradient for PIE-1 along the anterior–posterior axis, highlighting a linear decrease in the fraction of fast-diffusing PIE-1 proteins from the anterior to the posterior end. This detailed spatiotemporal mapping suggests that the current one-dimensional models of gradient formation might be oversimplified and that a three-dimensional approach could provide a more accurate representation of the underlying biological processes. This study demonstrates the versatility of SPIM-FCS in capturing the three-dimensional nature of embryonic self-organization events, providing new insights into the complexity of developmental processes in *C. elegans* [30].

STED FCS

Stimulated Emission Depletion (STED) FCS is an advanced fluorescence microscopy technique that combines the principles of FCS with STED microscopy, a super-resolution technique [31]. As discussed in this review, FCS measures the fluctuations in fluorescence intensity over time as fluorescent molecules move through a small detection volume, providing information about molecular mobility and interactions. STED microscopy, on the other hand, achieves super-resolution by using a second-shaped laser beam to deplete fluorescence from the outer regions of the excitation spot, resulting in a smaller effective detection volume [32]. This spatial confinement provided by STED enhances the resolution of FCS measurements, allowing for the investigation of molecular dynamics at a finer scale. This technique is particularly advantageous for studying processes within subcellular structures or regions with dimensions below the diffraction limit of conventional microscopy [32]. For example, STED-FCS has been used to measure the diffusion of plasma membrane species to identify mechanisms like confined diffusion and association with lipid raft-like assemblies [33,34]. One recent application of STED FCS was to the diffusion and interaction dynamics of the peroxisomal cargo receptor PEX5 in the cytosol, which is crucial for the import of peroxisomal matrix proteins [32]. PEX5 plays a key role in transporting proteins into peroxisomes by recognizing peroxisomal targeting signals. The study utilized advanced microscopy and spectroscopy techniques, including FCS, FCCS, and STED-FCS. The results showed that PEX5 exhibited slow diffusion in the cytosol, independent of cargo binding, and this slow diffusion is linked to its N-terminal region. The study investigated several mechanisms, such as PEX5 oligomerization and interactions with other proteins, and concluded that the slow diffusion of PEX5 is associated with cytosolic factors yet to be identified [32]. The research provides insights into the dynamic nature of cellular interactions involved in peroxisomal protein import.

Three-color FCS

A natural extension of FCS is into multiple detection channels. As discussed above, FCCS is a two-color version of FCS. Some work has been done to perform simultaneous three-color FCS [35], and recently a multi-color FCS approach was extended to live cells to monitor the diffusion and concentration of three proteins in the MAPK pathway [34]. The MAPK pathway, crucial in eukaryotic cell signaling, regulates cell processes such as proliferation, differentiation, migration, and apoptosis. Dysregulation is linked to various diseases. Traditional methods (MS, WB, ELISA, DIGE) study kinase activity offline, lacking spatial–temporal data in living cells. The researchers designed three probes to bind kinases in the MAPK pathway including BRAF, MEK1/2, and ERK1/2. Three spectrally distinct probes were designed by conjugating inhibitors for these proteins (vemurafenib, selumetinib, and ulixertinib) with fluorescent dyes (BODIPY TR, BODIPY 493, and ATTO 647N). This approach enabled simultaneous monitoring of the kinases in single living cells with multi-channel FCS. The system utilized a laser scanning confocal configuration with three lasers and three single photon counting modules for high-resolution scanning confocal imaging and in situ multi-channel FCS measurements of single living cells.

The multi-channel FCS system enabled the acquisition of three-color cell images and real-time recording of spatiotemporal information for labeled endogenous kinases [34]. Fluorescence fluctuations of multiple labeled kinases were simultaneously measured in three channels, and data correlation analysis was performed using different diffusion models. For FCS measurements in solution, a single component free diffusion model was employed to characterize the diffusion of fluorescent molecules. In cellular FCS measurements, the ACFs of fluorescent molecules in each channel were fitted with an anomalous diffusion model. The binding ratio of the probe to the target kinase was a crucial factor for evaluating kinase activity, and it was determined by fitting the data with the FCS two-component model. The increase in the binding ratio represents an increase in the expression level of the kinase, indirectly reflecting an increase in kinase activity under certain conditions, with the total concentration of the fluorescent probe held constant. The method was applied to study kinase responses to external stimuli, drugs, oxidative stress, and MAPK pathway activation through PI3K-mTOR modulation [34]. This strategy provides a new

approach for comprehensive in situ monitoring of multiple MAPK kinases in single living cells.

Massively parallel FCS

FCS relies on the ability to detect small fluctuations in fluorescence signal typically at a single photon level. This requires high-sensitivity detectors, most commonly SPAD detectors. Technological advances have led to the increased availability and affordability of SPAD arrays [36], that enable massively parallel (mp)-FCS [37]. A recent study utilized a diffractive optical element for simultaneous excitation of fluorescent molecules in multiple spots, generating 1024 illuminated spots. Detection was carried out in a confocal arrangement using a matching matrix detector with a 32×32 SPAD array. The software enabled fast auto- and cross-correlation analysis through parallel signal processing using a graphic processing unit (GPU). mpFCS exhibits single-molecule sensitivity and is demonstrated to map location-specific differences in the concentration and translational diffusion of molecules in various subcellular compartments of live cells. The technique is particularly valuable for studying molecules with specific biological functions and their interactions in complex cellular environments [37].

In the most recent application, mpFCS is introduced as a promising modality to map the spatial organization of nucleocytoplasmic transport [38]. The study focuses on understanding the spatial organization of nucleocytoplasmic transport in live cells, specifically the directionality of transport. While various fluorescence-based techniques have been valuable in studying nucleocytoplasmic shuttling dynamics, existing methods have limitations, such as dependence on image settings and pixel averaging. The model system was live HEK cells expressing the glucocorticoid receptor (GR) fused with enhanced green fluorescent protein (eGFP-GR). By calculating two-foci cross-correlation coefficients, the direction of particle motion and 2D velocity can be mapped. The study reveals the simultaneous occurrence of ligand-induced eGFP-GR nuclear import and export at different locations along the nuclear envelope. The mpFCCS method proves versatile for understanding active transport in live cells, local heterogeneities in fluid flow, and the role of the fluid phase in long-range material transport and biological system synergy.

Outlook and conclusions

This review highlights recent applications of FCS in live cell studies. The sensitivity of FCS to single-molecule diffusion events allows for the quantification of various parameters, including mobility, local concentration, protein–protein interactions, protein-nucleic acid interactions, and membrane protein organization. As the time-domain analogue of fluorescence imaging, FCS provides valuable insights into the dynamic behavior of molecules within living cells by analyzing the fluctuations in fluorescence intensity over time. The concentration and mobility outputs of FCS measurements serve as metrics to characterize the expression levels, local concentrations, and mobility of biomolecules of interest. The technique has been employed to investigate a wide array of biological phenomena, ranging from protein secretion, metabolite concentrations, diffusion phenomena, and protein–protein interactions.

Recent methodological advances such as imFCS, SPIM-FCS, STED FCS, three-color FCS, and mpFCS have extended the range of potential applications of FCS for biochemical and cell biological studies. These advancements enable the incorporation of spatial information, super-resolution imaging, and parallelized detection, significantly enhancing the capabilities of FCS in mapping molecular dynamics within cellular and subcellular structures. The examples discussed here illustrate the power of FCS in providing detailed insights into cellular processes. For instance, studies on the β2AR shed light on receptor behavior across different cell types, emphasizing the potential implications for drug therapy. Investigations into protein-nucleic acid interactions under the influence of anticancer drugs like doxorubicin contribute to our understanding of drug effects on chromatin components. Methods like PIE-FCCS, which are optimized for the study of membrane protein organization, have revealed the multimerization states and dynamic behavior of RTKs such as EGFR and EphA2 within live cells.

While FCS and related methods have many advantages, there are several limitations to consider. Low biomolecule concentrations and high autofluorescence backgrounds in cells can result in a low signal-to-noise ratio (SNR), affecting the accuracy of concentration measurements and the interpretation of FCS data. Developing more sensitive detection systems, advanced background subtraction techniques, and utilizing brighter and more stable fluorophores can enhance the SNR. Photobleaching of fluorescent proteins during data collection leads to a decrease in signal over time, complicating the analysis and potentially introducing artifacts. Techniques to minimize photobleaching, such as using lower excitation intensities, photostable fluorophores, or real-time photobleaching correction algorithms, can mitigate this issue. Advanced modeling and simulation techniques that account for cellular heterogeneity and dynamics can improve data interpretation. At high molecular concentrations, the signal fluctuations become small relative to the signal strength, reducing the sensitivity of FCS to individual molecular events. Methods like STED-FCS can enhance the dynamic range by reducing the observation volume, but the development of new analysis techniques that can handle higher concentrations effectively should also be pursued to address this limitation.

Overall, FCS provides quantitative insights into biochemical processes that occur in complex cellular environments. The wide range of applications and continuous methodological developments discussed in this review position FCS as an adaptable tool for generating new insight into cell biochemistry. The ability to probe molecular dynamics, interactions, and spatial organization with high sensitivity and precision makes FCS an essential component of the still-growing field of quantitative imaging and spectroscopy methods in cell biology and molecular biophysics.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Adam W. Smith reports financial support was provided by American Lung Association. Adam W. Smith reports financial support was provided by National Science Foundation.

Data availability

No data was used for the research described in the article.

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