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Review

Recent applications of fluorescence correlation spectroscopy in live systems

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ABSTRACT

Fluorescence correlation spectroscopy (FCS) is a widely used technique in biophysics and has helped address many questions in the life sciences. It provides important advantages compared to other fluorescence and biophysical methods. Its single molecule sensitivity allows measuring proteins within biological samples at physiological concentrations without the need of overexpression. It provides quantitative data on concentrations, diffusion coefficients, molecular transport and interactions even in live organisms. And its reliance on simple fluorescence intensity and its fluctuations makes it widely applicable. In this review we focus on applications of FCS in live samples, with an emphasis on work in the last 5 years, in the hope to provide an overview of the present capabilities of FCS to address biologically relevant questions.

1. Introduction

It is now more than 40 years ago that the first paper on fluorescence correlation spectroscopy (FCS) was published [1]. Invented to measure chemical reaction rates and diffusion coefficients by analysing thermodynamic fluctuations in the fluorescence intensity of a system, it is now a well-established biophysical tool, which is used routinely in live cells and organisms to obtain quantitative data on the molecular level. Although it developed rather slowly in the beginning, mainly due to technical limitations, it received a boost by its combination with confocal microscopy [2] and its extension to dual-colour fluorescence cross-correlation spectroscopy (FCCS) [3]. This made FCS in live cells possible, in particular with fluorescent proteins [4], allowed characterization of biomedically important samples [5], and opened the way to measure biomolecular interactions at physiological concentrations [6]. Since then FCS has been applied to a wide range of biological problems from bacteria, yeast, and cells to live organisms and has been used to characterize most parts of a cell from nucleus over cytoplasm and organelles to the plasma membrane. Fig. 1 shows schematically some of the topics addressed by FCS in cells and embryos. In this review we will focus mainly on FCS and those of its modalities which provide directly temporal information and we direct the interested reader to recent reviews of other fluorescence fluctuation spectroscopy modalities [7–10]; Table 1 gives an overview of FCS modalities included in our review. Since the methodology of all those FCS modalities has been explained repeatedly in literature (e.g. references in Table 1), we only schematically illustrate their principles here (Figs. 2 and 3). We restrict ourselves to work within the last 5 years. Earlier work has been extensively reviewed [11–14]. We thus hope to give the reader an up to date view of FCS as applied within the life sciences and provide a perspective of FCS as a tool for quantitative bioimaging either in combination with other imaging tools or as an imaging tool in itself.

2. Structural information

Although FCS does not provide directly any structural information, the indirect information derived from properties of molecular diffusion can be exploited to characterise structures which are not easily accessible to more direct methods due to their small dimensions and dynamic nature. FCS has been used in numerous studies to elucidate the organization of molecules in the plasma membrane as well as in several works on chromatin structure.

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2.1. Membrane organization

The lateral organization of lipids and proteins in cell membranes is believed to play an important role in processes such as endocytic or signalling pathways by dynamically sequestering molecules involved in those processes. Several FCS studies of diffusion of membrane-associated proteins or peptides identified slowly diffusing populations of those molecules, which are indicative of binding to more ordered domains in the plasma membrane [15–17] (number 2 in Fig. 1). Comparison of diffusion of several proteins can show the selectivity of proteins for certain domains [16,17], while the increase in diffusion coefficient (D) upon cholesterol depletion indicates affinity of the concerned proteins for cholesterol enriched domains [16]. Diffusion of whole domains containing different adhesion molecules was measured by temporal ICS (TICS) and found to correlate with the function of the respective molecules [18]. The selectivity of proteins for certain domains was also demonstrated by Lillemeier et al. [19]. They showed by FCCS that T cell antigen receptor (TCR) and linker for activation of T cells (Lat) are segregated to separate domains in quiescent cells and that those domains concatenate upon activation.

A widely tested hypothesis of membrane lateral organization is the raft hypothesis of Simons and Ikonen according to which domains of more tightly packed lipids enriched in sterols and sphingolipids are present among a more fluid surrounding [20]. The rafts are expected to be small and highly dynamic, which renders their observation by traditional FCS and microscopic techniques practically impossible. FCS with stimulated emission depletion (STED) excitation has been recently employed to study membrane rafts [21–25]. Thanks to the reduction of the lateral diameter of the detection area achieved by STED illumination, STED–FCS can provide more accurate information on nanoscale domains and their influence on diffusion in membranes. According to the law of free diffusion, the diffusion time τ_D (the average time molecules spend diffusing through the observation area) is proportional to the observation area. Any deviation of the diffusion law from proportionality serves as a clear indication of hindrance of diffusion either by barriers or by transient binding to immobile structures. By measuring FCS with observation areas ranging from diffraction limited down to approximately 40 nm diameter, Eggeling et al. [21] and Mueller et al. [22] found that while diffusion of glycerophospholipids is very close to free diffusion, diffusion of sphingomyelin, ganglioside GM1 and other putative raft markers was strongly influenced by transient binding to domains of diameter estimated not to be larger than 20 nm. Depletion of cholesterol as well as disruption of the cytoskeleton reduced the transient binding of sphingomyelin; the effect was less pronounced in the case of other raft markers [22]. Analysis of autocorrelation functions allowed determining of kinetic rates of binding of the raft markers to the domains [23]. The diffusion patterns of the individual raft markers were independent of the fluorophores by which they were labelled; that is in contradiction to partitioning studies performed on phase-separated artificial lipid bilayers, which show strong influence of the fluorophore moieties on the partitioning. It suggests that native sphingolipids and gangliosides may be involved in yet other types of domains that are inaccessible to their fluorescently labelled analogues [24]. Further evidence for more complex patterns of lipid segregation in plasma membranes was provided by Triffo et al. who studied colocalization of three types of lipid anchors by FCCS with pulsed interleaved excitation (PIE–FCCS). Two of the anchors (from lymphocyte cell kinase and RhoA) were found to localize to different clusters although neither of them is expected to exhibit preference for rafts while the K-Ras anchor did not partition into any clusters [26].

Although the sub-diffraction observation areas are beneficial for elucidating membrane nanostructures, they are not strictly required to obtain information on nanoscale features by FCS. The diffusion law, used in the above mentioned studies [21,22], can be also obtained by FCS measurements with observation areas equal to or larger than the diffraction limit and then extrapolating the dependence of τ_D on the effective observation area A_{eff} to zero ($\tau_D(t) = \tau_0 + A_{eff} / D$). Free diffusion results in a straight line passing through $\tau_0 = 0$, but as was shown previously, transient binding to domains (number 2 in Fig. 1) results in a positive intercept $\tau_0 > 0$ and diffusion in a meshwork of semi-permeable barriers (number 1 in Fig. 1) in a negative one $\tau_0 < 0$ [27,28] as schematically illustrated in Fig. 3A.

This approach has been utilized to study hindered diffusion of proteins in plasma membranes [29–31]. For example Ganguly and Chattopadhyay found diffusion of serotonin receptor 5-HT_{1A}R to be hindered by a meshwork. Free diffusion was recovered by actin disruption as well as by cholesterol depletion; the involvement of cholesterol in the interaction between membrane proteins and cytoskeleton is a possible explanation of the result [31]. Other evidence for the role of cholesterol in mediating of diffusion hindering by actin comes from Sankaran et al. who studied the influence of cholesterol depletion and actin disruption on diffusion of a sphingolipid binding domain (SBD). By using imaging FCS with total internal reflection (TIR) excitation (ITIR–FCS) they observed temporarily decreased membrane heterogeneity and increased probe mobility after either cholesterol depletion or cytoskeleton disruption. However, even under cholesterol depletion, the mem-

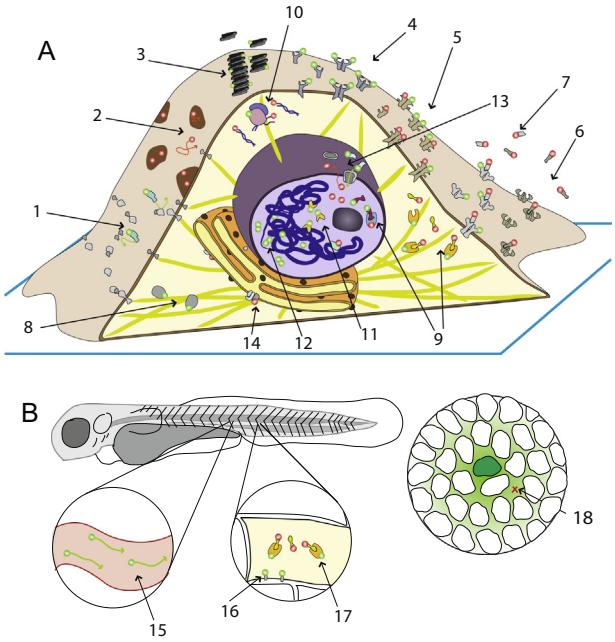

Fig. 1. Illustration of the selected topics addressed by FCS in living cells (A) and zebrafish embryos (B): diffusion of proteins and lipids in plasma membrane and its hindrance by cytoskeletal meshwork and/or tracer partitioning to domains (1, 2); aggregation of amyloid peptides on plasma membrane (3); oligomerization state of membrane receptors (4, 5); binding of ligands to membrane receptors (6, 7); diffusion of proteins in cytoplasm (8); protein–protein interactions in cytosol and nucleus (9); interaction of proteins with small nucleic acid molecules (10); binding of proteins to chromatin and nuclear DNA (11); influence of chromatin architecture on diffusion of inert tracers (12); passive or active transport of molecules between nucleus and cytoplasm (13); anomalous diffusion of protein complexes in endoplasmic reticulum (14); in embryos 3 days post fertilization: blood flow (15) and protein diffusion in plasma membrane (16) [135] and protein–protein interactions in cytosol (17) of muscle cells; morphogen gradients originating from a single source region (depicted as the green cell) in gastrula stage embryos (18).