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Potentials and pitfalls of inverse fluorescence correlation spectroscopy

Stefan Wennmalm

SciLifeLab, Royal Institute of Technology, Department of Applied Physics, Experimental Biomolecular Physics Group, Solna 171 65, Sweden

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ABSTRACT

Inverse Fluorescence Correlation Spectroscopy (iFCS) is a variant of FCS where unlabeled particles in solution, or domains in membranes, displace their surrounding, signal-generating molecules and thereby generate fluctuations. iFCS has to date been applied to unlabeled as well as labeled particles and protein molecules, using fluorescence as well as Raman scattering as a signal source, in diffraction-limited detection volumes as well as in nano-wells, and on fixed surfaces as well as in lipid bilayers. This review describes these applications and discusses the potentials and pitfalls when using iFCS.

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1. Introduction

Fluorescence Correlation Spectroscopy (FCS) was introduced in the early 1970's as a technique to measure concentrations, diffusion and chemical reactions of fluorescent molecules at nanomolar concentrations [1,2]. It was developed further throughout the 70's and the 80's but it was not until the 1990's that it became widespread, as a result of an improved signal to noise [3] as well as the production of commercial instruments by Zeiss and Evotec Biosystems. Analysis of molecular interactions in solution [4,5] as well as in living cells [6] followed, and groups demonstrated that fluorescence fluctuations of disparate origin can be analyzed by FCS and give information on photophysical processes [7] as well as molecular dynamics [8–14]. In 1997 Schwille et al. introduced dual-color Fluorescence Cross-Correlation Spectroscopy (FCCS) which has become widely used for detection of interactions between binding partners labeled with fluorophores of different colors [15]. A number of other variants of FCS have also been developed, using high order correlations [16], high moment analysis [17,18], image correlation [19,20], intensity distribution analysis in the form of PCH [21] and FIDA [22], and 2-focus-FCS [23] and scanning-FCS [24], to mention a few.

FCS is often used to determine the size of particles via the diffusion coefficient D, in solution and on membranes. This works well

E-mail address: stewen@kth.se

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in solution for diffusing proteins and particles where D scales with the inverse radius of spherical particles. The same relationship has recently been shown to hold on model lipid bilayers for single protein molecules of various sizes [25,26]. For protein oligomers and clusters however the dependence is likely weaker [27,28]. For micrometer sized membrane domains, models exist for predicting domain size from the diffusion time [29,30], however these models have been shown to work [30] as well as not to work [31]. On cell membranes, diffusion is an even less reliable size-indicator due to factors such as possible interactions with the cytoskeleton, interactions with other membrane molecules, and the presence of cellular compartments.

PCH is closely related to FCS but analyses molecular brightness instead of diffusion coefficients and should in principle be applicable for analysis of nanodomains on membranes. For estimation of the size of nanodomains PCH requires however very long data collection times and has therefore been reported as being unpractical [31,32].

From the above discussion it is clear that fluctuation techniques have difficulties in determining sizes of molecules whose motion is not governed by diffusion alone. The ability to assist in such situations is one of the main strengths of Inverse FCS (iFCS). iFCS has since its introduction in 2009 gone through a slow but steady development. When first introduced the label-free aspect of iFCS was emphasized [33], but soon its ability to determine the particle- or domain-size, independently from the diffusion coefficient, emerged as a main strength [31,34–38]. The sizing ability of iFCS makes it related to the super-resolution techniques STED- and PALM/STORM-imaging whose strengths are the enhanced resolution in fluorescence images and the ability to determine sizes down to 20–30 nm. iFCS should in combination with the STED technique allow sizing of membrane domains well below 10 nm, and in contrast to the super-resolution techniques, in highly dynamic living cells.

This review describes the developments and applications of Inverse Fluorescence Correlation Spectroscopy (iFCS) to date, and discusses potential applications as well as pitfalls.

2. Theory

In iFCS, the diffusion part of the autocorrelation function (ACF) curve is the same as in standard FCS [33]. The amplitude of the ACF however relates differently to the particle concentration compared to the case in standard FCS. In standard FCS the amplitude is given as G(0)-1 = 1/N in the case of negligible background signal, where N is the number of fluorescent particles in the detection volume. In iFCS the amplitude of the ACF curve is instead given by

$$G(0) - 1 = \frac{N}{\left(1/V_q - N\right)^2} \tag{1}$$

where $V_q = V_p/V_{DV}$ where V_p is the particle volume and V_{DV} is the detection volume [33]. Thus the amplitude depends on the particle concentration as well as the particle volume, and either one of them cannot be derived from Eq. (1) alone without knowing the other. In most cases $1/V_q >> N$ which implies that

$$G(0) - 1 \approx V_q^2 \cdot N \tag{2}$$

Thus G(0)-1 is proportional to N, as opposed to the case in standard FCS where G(0)-1 = 1/N at negligible background signals. However, in standard FCS in the case of high background signals a background-corrected amplitude has to be used. The amplitude is then $\beta = N/(N + N')^2$ where N is the particle number and N′ is the number of particles that the background signal corresponds to [39]. By comparing the expression for β and Eq. (1), one sees that N′ in standard FCS relates to $1/V_q$ in iFCS, though the expressions differ because the particle fluctuations are positive in standard FCS and negative in iFCS. The Koppel correction could become useful also in iFCS, if for example some domains in a membrane unexpectedly accumulate the dyes intended as background marker. Such domains would then result in positive fluorescence signals on top of that of the background.

Eq. (1) is valid when the background molecules themselves do not give rise to an autocorrelation curve. This is the desired situation in iFCS, and therefore a high concentration of background molecules should be used (see Section 4.4 for details on concentrations to be used), combined with a low excitation intensity, which reduces the chance that the ACF picks up the diffusion of the background molecules. When sub-diffraction limited volumes are used, however, the number of background dye molecules will be reduced. In such cases a fast component in the iFCS curve originating from the background dyes may have to be accepted. It will then be useful to consider the model derived in the supplemental information of Jiang et al. [31], which takes into account the diffusion of the background dyes as well as the dark domains in the membrane.

While the ACF amplitude in iFCS does not give the concentration and particle number separately, the two can be deduced by two alternative approaches. The first approach can be used when a single color iFCS measurement is recorded at low particle concentrations, such that $N \approx 0.1$ or lower. Then the data trace can be analyzed by creating an intensity histogram (Fig. 4d). The histogram will display a main distribution corresponding to the baseline, i.e. the mean intensity when the detection volume is void of particles, while a smaller distribution will correspond to the average intensity when a particle is present in the detection volume. The difference between the centers of the two distributions will thus give the mean particle volume, given that the size of the detection volume has been estimated [40].

The second approach was recently presented by Jiang et al., who show that by calculating the Scewness of the fluorescence intensity the concentration of objects in the detection area, $m_d(t)$, can be obtained from a single iFCS measurement by

$$Scewness(F(t)) = \frac{\left\langle (\delta F(t))^3 \right\rangle}{\left\langle (\delta F(t))^2 \right\rangle^{3/2}} = \frac{1}{\sqrt{m_d(t)}} \tag{3}$$

Once the concentration of objects has been obtained, the mean size of objects is given from Eq. (1) above [31].

As will be described below, also labeled particles can be analyzed by iFCS if they are emitting at a different wavelength than the surrounding, signal-generating molecules (the surrounding, signal-generating molecules will below be referred to as background in iFCS). In this approach, called iFCCS, the cross-correlation between the signals from the studied particles and the background fluorophores is calculated. The cross-correlation amplitude in iFCCS was derived as being

$$G_{CC}(0) - 1 \approx \frac{-V_p}{\sqrt{V_g V_r}} \tag{4}$$

in the case of negligible cross-talk and when $1/V_{qg} \gg N_g$ [36]. Here V_p is the particle volume and V_g and V_r are volume of the green and the red detection volumes respectively. V_{qg} is the ratio between the particle volume and the green detection volume, and N_g is the particle number for the green detection volume. V_g and V_r can be estimated accurately from standard FCS measurements on dyes with known diffusion coefficients. Thus Eq. (4) gives a direct measure of the volume of the analyzed particles, a measure that is not based