Density filtered Fluorescence Correlation Spectroscopy for highly concentrated solutions

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Fluorescence Correlation Spectroscopy (FCS) is widely used to detect and quantify diffusion processes at the molecular level. The molecules of which diffusion is studied are marked with fluorescent dyes. It is commonly maintained that this technique only applies to systems where the concentration of fluorescent molecules is low. Even if this is the optimal operational condition, we show that FCS can be used also at high concentrations of fluorescent molecules: the initial condition of very bad signal to noise ratio (SNR) can be hampered by suitable statistical averaging, as usual in other contexts of signal analysis.

Keywords: Fluorescence Correlation Spectroscopy; Molecular diffusion

I. INTRODUCTION

Fluorescence Correlation Spectroscopy (FCS) has been developed in the ’70s [1] and rapidly became a useful technique in various fields from biology to chemistry. But FCS users get in troubles when dealing with highly concentrated solutions. First of all, the autocorrelation functions (ACFs) tend to be squeezed as the number of fluorescent molecules in solution is increased, this fact leads to the common belief that these curves cannot be fitted any longer. Then, a second problem is introduced by the detector, because at increasing dye-concentration it can quickly attain saturation. Moreover, some authors have drawn attention to another problem: fluorescence fluctuations stemming from laser emission variations, become comparable to the fluorescence fluctuation level coming from the dyes. To fix these problems, some authors resorted to techniques conceived to reduce the observable volume with plasmonic nanoantennas [4] or plasmonic gold bowtie nanoantennas [3]. Laurence et al. [2] also show that the mentioned difficulties can be overcome by using several connected detectors, each one receiving part of the fluorescent beam - coming from the sample - after having separated it through beamsplitters. Thus, this setup needs many detectors and sometimes cannot be the optimal choice. An alternative, that we are putting forward here, is based on the use of absorptive filters to attenuate fluorescent light, and long time averaging in order to overcome the bad Signal to Noise Ratio (SNR).

II. RESULTS

II.1. FCS with density filters

Experiments have been performed using watery solutions of the Alexa Fluor 488 dye (AF488), at different concentrations of the dye, that is 1 nM, 10 nM and 500 nM respectively, and with different optical density filters of density 2.0 (OD2, 1% transmission), 1.3 (OD1.3, 5% transmission) and 1.0 (OD1, 10% transmission) on a spot-variation FCS setup (svFCS), built on a confocal microscope. The watery solutions of the AF488-dye were put in 8 wells Labtek supports.

Our results, reported in Figure 1, show that for short lag times the combined effect of a bad SNR and of the afterpulsing [5] tend to deform the ACFs. On the other hand, the ACFs recorded with different filters are found to be overlapping for a lag time interval ranging from several microseconds to several seconds, as shown in Figure 1. These results were found to agree independently of the density of the filter used, whereas, for the same acquisition duration, the higher the OD value the noisier the ACF, as shown by Figure 1. For graphical reasons and limited space we report only the most unfavourable SNR condition obtained with the OD2 filter.

II.2. Improvement of SNR through statistics

Following a standard practice, in order to improve the SNR for data records obtained with OD filters, we resort to an increase of statistics by performing longer data recording. Figure 2 shows two ACFs obtained with the OD2 filter but with different acquisition durations: the green curve being recorded for a time interval 80 times longer than that corresponding to the black curve (4000...
Figure 1. Comparison of ACFs recorded without filter in red, and with a OD2 in black. On the left panel there are the ACFs from a 10nM AF488 solution. On the right panel, the x-axis has been truncated, starting from 10µs, to highlight the overlapping parts of the ACFs.

Figure 2. Comparison of ACFs recorded with OD2 and for different durations. In black it is shown the ACF recorded for 50 seconds (1 acquisition of 50 seconds) for a 10nM AF488 solution. In green the ACF has been recorded from the same solution but for 4000 seconds (80 acquisitions of 50 seconds). More precisely, the green curve corresponds to the average of 80 different ACFs, each one worked out for a 50 seconds acquisition. As expected, the SNR has been increased, obtaining less noisy average ACFs, and better global fitting results for the multiple runs.

II.3. FCS measurements at high concentration

What we learn from the results reported in Figure 2 is that the standard smooth pattern of the ACF, which is obtained without filter and displayed in Figure 1 (red line), is recovered also in presence of an OD2 filter provided that the acquisition duration is sufficiently long: the ACF reported in the right panel of Figure 2 has been worked out with an overall acquisition time of 4000 seconds. Thus we observe that a good statistics can convert a very noisy pattern into a definitely smoother one, not surprisingly indeed. The further and main step now consists in comparing the ACFs obtained with two samples at different concentrations of 1nM and 500nM without filter and with OD2 filter respectively. Both ACFs refer to 20 acquisitions of a duration of 50 seconds each. By fitting the ACFs on a time interval ranging from 20µs to 4 seconds, their characteristic parameters have been estimated by skipping the triplet state and the afterpulsing peak; this led to a diffusion time of 96.2±1.3µs and 2.0 molecules in the confocal volume for the 1nM solution, and a diffusion time of 94.4±1.3µs and 963.0 molecules in the confocal volume for the 500nM solution. And, in fact, after a suitable normalisation of the two ACFs, the right panel of Figure 3 clearly shows a very good superposition of the two curves.

III. CONCLUSION

In the present note we have put forward the possibility of using the FCS technique to investigate molecular diffusion processes also at large concentrations of fluorescently labeled molecules. This can be achieved by the joint use of optical density filters and a sufficient statistics to suitably raise the SNR.

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