Characterisation of cell-free protein production using FCS

Application note

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In this application note, we demonstrate the use of Fluorescence Correlation Spectroscopy (FCS) on the EI-FLEX to characterise cell-free protein synthesis of YFP-CopB, a fusion of Chlamydia Outer Protein (Cop) B and Yellow Fluorescent Protein (YFP)[1]. CopB is used here as a model protein, while YFP fusion is necessary for fluorescence detection of the expressed fusion protein. FCS has previously been used to monitor protein expression in cell-free protein synthesis lysates in real time[2–5].

Overview of this application note:

- Cell-free synthesis of the YFP-CopB fusion protein was measured using FCS on the EI-FLEX over a 10-hour period
- Calculation of the confocal volume was performed using Cy3B, a common dye with a known diffusion coefficient
- Autofluorescence of the cell-free lysate can be distinguished from the photons emitted by the YFP-CopB fusion protein under 520 nm laser excitation

Glossary of terms used in this application note

Confocal volume: The confocal volume (often called the effective volume) is the small, 3D ellipsoidal region from which the fluorescence signal is detected. It is defined by the intersection of the laser excitation beam and the detection optics (the pinhole).

Hydrodynamic radius: The radius of a theoretical hard sphere that diffuses at the same rate as the particle being measured. It includes the molecule itself plus any hydration shell (water molecules) and ions that move along with it through the solvent.

Experimental Set-up

To begin expression, 10 ng/µL YFP-CopB plasmid was added to ClearColi (CC) lysate, a cell-free protein synthesis lysate derived from *E. coli*. Plasmid concentration can be tuned to achieve the desired rate of production and final protein concentration[2]. After the plasmid was mixed with CC lysate, 8 µL of this mixture was pipetted into a well in a 1.8 mm spacer on a glass coverslip. 8 µL of CC lysate alone was pipetted into a separate well as a negative control, and the wells were sealed with an additional coverslip. A uniform layer of air was left at the top of the well, as this is essential for fluorescent protein maturation and cell-free protein production[2,3]. A diagram and photo of this setup are shown in Figure 1. The slide was placed in the EI-FLEX, and one minute of data was acquired for each of the two samples, approximately once every 20-30 minutes for 10 hours.

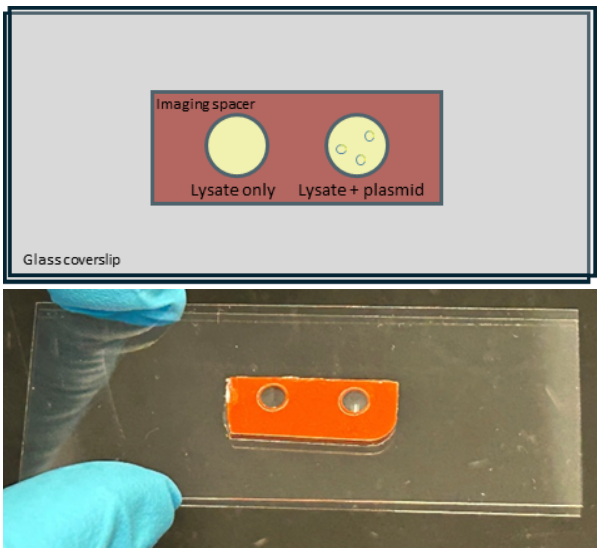

Figure 1 – Experimental set-up on the EI-FLEX
Top: Diagram showing slide format. An imaging spacer (red) is placed on top of a glass coverslip to form wells. Solution is pipetted into the wells, leaving some space at the top of the well. When a second glass coverslip is placed on top of the spacer, the wells are sealed with some air left above the solution. The left well contains lysate alone; the right contains lysate and the plasmid for YFP-CopB expression. The slide is placed on a stage on the EI-FLEX over the objective for data collection.
Bottom: Photo showing the actual slide with two wells.

Calibration of the confocal volume of the 520 nm laser

Firstly, in order to calculate YFP-CopB concentration in the time series experiments, it was necessary to calibrate the confocal volume of the 520 nm laser. The calibration was performed using a series of dilutions of free Cy3B, which have known concentrations as measured by a spectrophotometer. Six concentrations of Cy3B were measured in triplicate using the EI-FLEX 520 nm laser.

Data were analysed to calculate $g^{(2)}$ correlations as a function of time delay (Figure 2a). The simple diffusion model below was fit to the correlations, which gives the amplitude f_0 and the average diffusion time of a fluorescent particle through the laser focus, τ_D.

$$g^{(2)}(\tau) = \frac{f_0}{1+\frac{\tau}{\tau_D}} + 1$$

Lower amplitudes represent higher concentrations of fluorescent dye. The diffusion time depends on the hydrodynamic radius of the particle; a small dye molecule has a faster diffusion through the focus of the laser than a larger protein. Normalised correlations fall on top of each other with little noise (Figure 2b). The fitted diffusion times remain constant over concentrations of Cy3B used. Fluorescent counts are expected and observed to be linear with concentration (Figure 2c).

Plotting inverse amplitude (from the correlations) versus concentration of Cy3B allows calculation of the confocal volume of the 520 nm laser. The confocal volume is given by the slope of the line in units of 1/nM. To convert to femtoliters (fL), the value is multiplied by 10^{24} and then divided by Avogadro's number. The confocal volume of the 520 nm laser was calculated to be 2.54 ± 0.09 fL.

Calibration of the confocal volume of the 520 nm laser

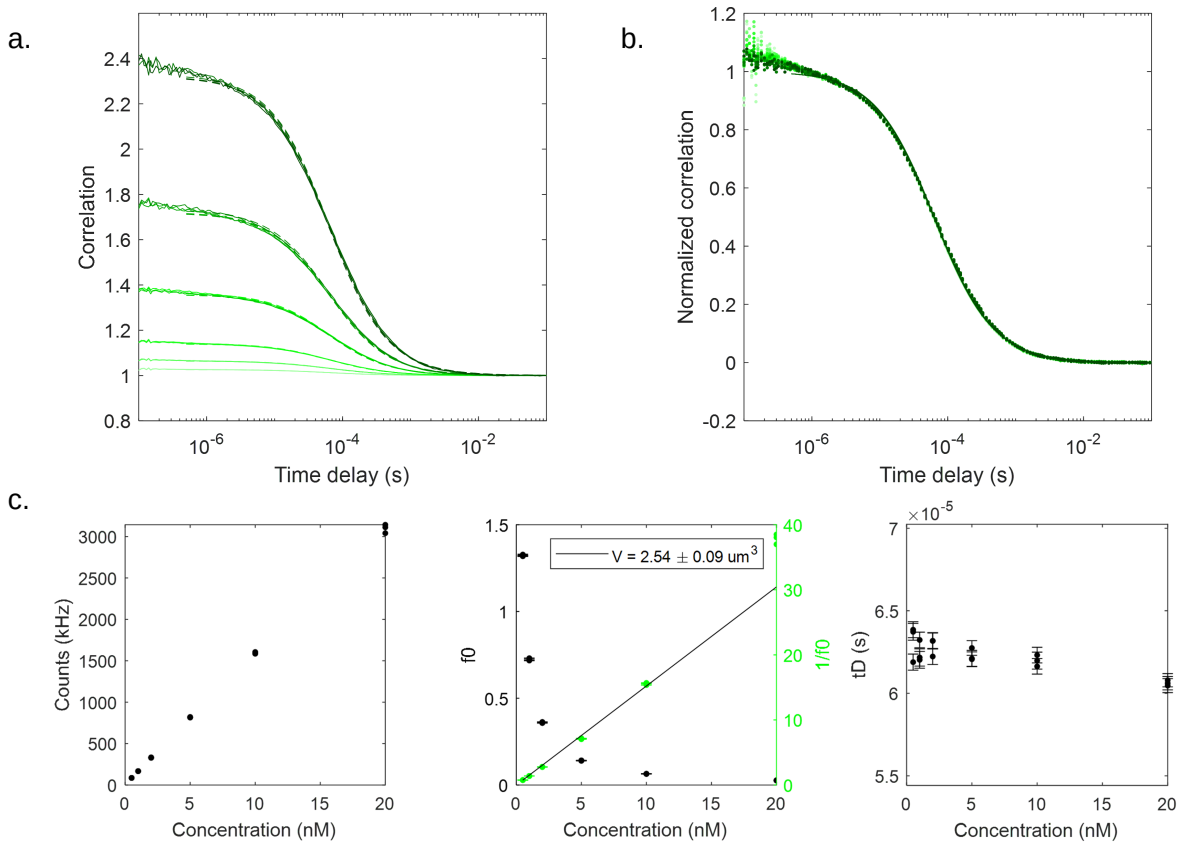

Figure 2 –Calibration of the confocal volume of the EI-FLEX 520 nm laser using Cy3B dye

a) Correlation versus time delay from FCS measurements of serially diluted Cy3B dye.
b) Correlations normalised to have an amplitude of 1.
c) Left: fluorescent counts versus concentration of Cy3B. Centre: amplitude (black) and inverse amplitude (green) versus concentration of Cy3B. A line is fit to the inverse amplitude versus concentration. The slope of the line is used to calculate the confocal volume of the 520 nm laser in femtolitres. The confocal volume calculated (V) is given in the legend in μm^3, equivalent to fL. Right: Fitted diffusion time τ_D in seconds versus concentration of Cy3B. Diffusion times are constant over replicate measurements and concentrations of dye.

Establishing the fluorescence background of cell-free lysate

The CC lysate alone was found to have some autofluorescence following exposure to the 520 nm laser that decreased over the ten hours of measurement. It also showed correlations, although these were found to be noisy (Figures 3a and 3b). After an initial decrease in fluorescence, counts remained relatively constant over the time series (Figure 3c). Equivalently, after an initial increase, amplitudes remained relatively constant over the time series. Diffusion times also remained relatively constant over the time course and likely represent some small autofluorescent component of the CC lysate. The decrease in fluorescence observed over time could have several explanations, including photobleaching, reaction of the autofluorescent molecules with other lysate components, exposure to oxygen, or temperature.

Liu et al. reported time series FCS measurements of GFP expression in cell-free lysate using a home-built system with a 488 nm laser[2]. They used CC lysate prepared by the same method reported here, also observing autofluorescence of the lysate that decreased over time.

Time series measurements of YFP-CopB expression

Once YFP-CopB plasmid is added to CC lysate, expression begins. An increase in YFP concentration can be monitored by the EI-FLEX by measuring fluorescence following excitation by the 520 nm laser (Figure 4).

Early data (light green correlations) are dominated by lysate background and have low amplitude. As the concentration of YFP-CopB increases over this background, the amplitude increases and then falls again at higher concentrations of YFP-CopB (later time points, dark green correlations).

Establishing the fluorescence background of cell-free lysate

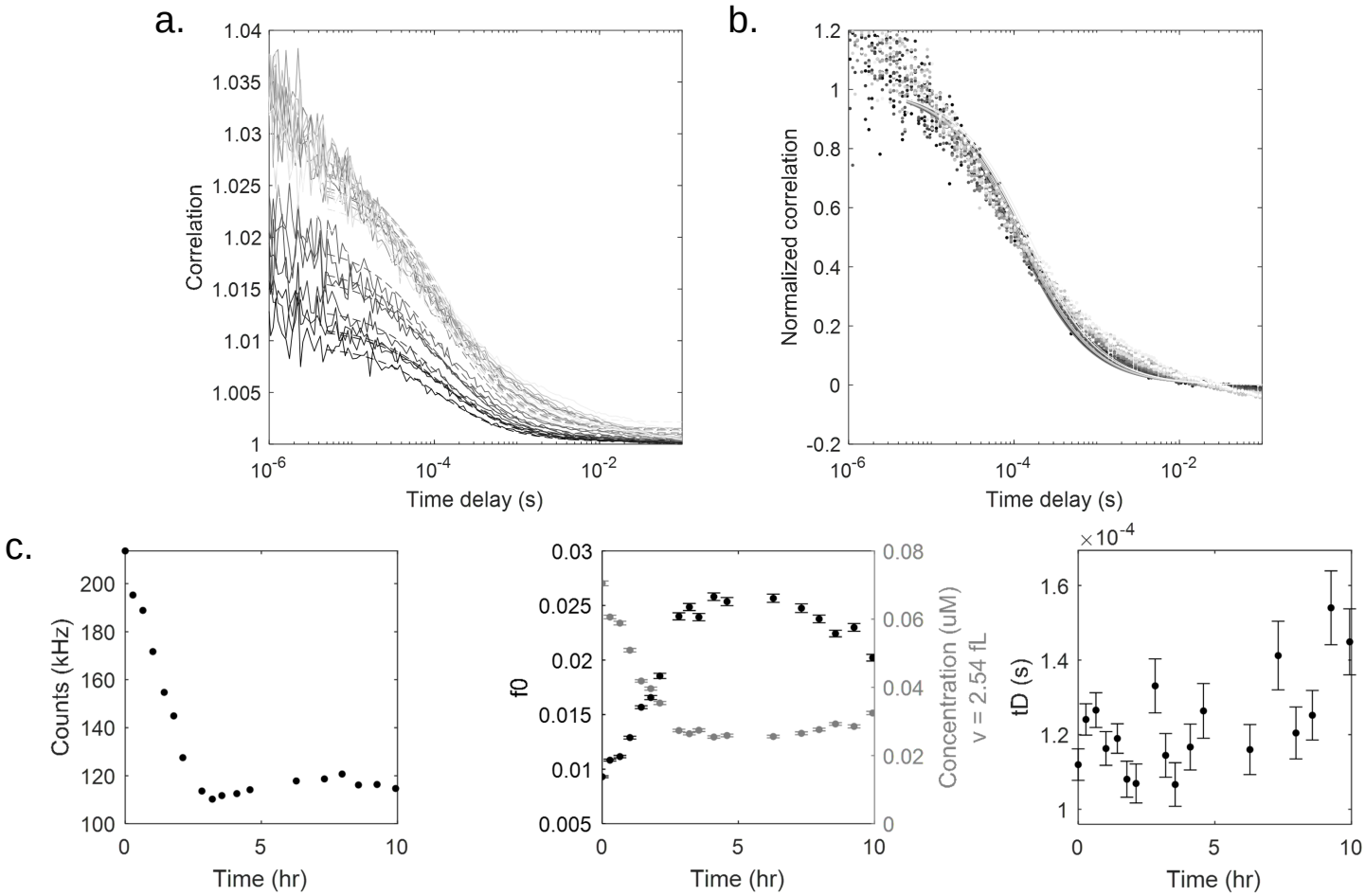

Figure 3 – ClearColi cell-free lysate monitored over time using the EI-FLEX
a) Correlation versus time delay from FCS measurements of CC lysate. Correlations (grey) represent separate measurements taken at intervals over ten hours. Light grey correlations represent early time points. Dark grey correlations represent data taken at a later point in time. A fit to the correlations gives amplitude f_0 and diffusion time τ_D.
b) Correlations normalised to have an amplitude of 1.
c) Left: fluorescent counts of the CC lysate versus time since the first measurement. The first measurement is taken to be Time = 0. One minute of data (one time point on the graph) was collected every 20-30 minutes over ten hours. Centre: amplitude (black) and concentration (grey) versus time. Concentrations were calculated from the measured amplitude and previously calibrated confocal volume of 2.54 fL. Right: Fitted diffusion time in seconds versus experimental time.

Time series measurements of YFP-CopB expression

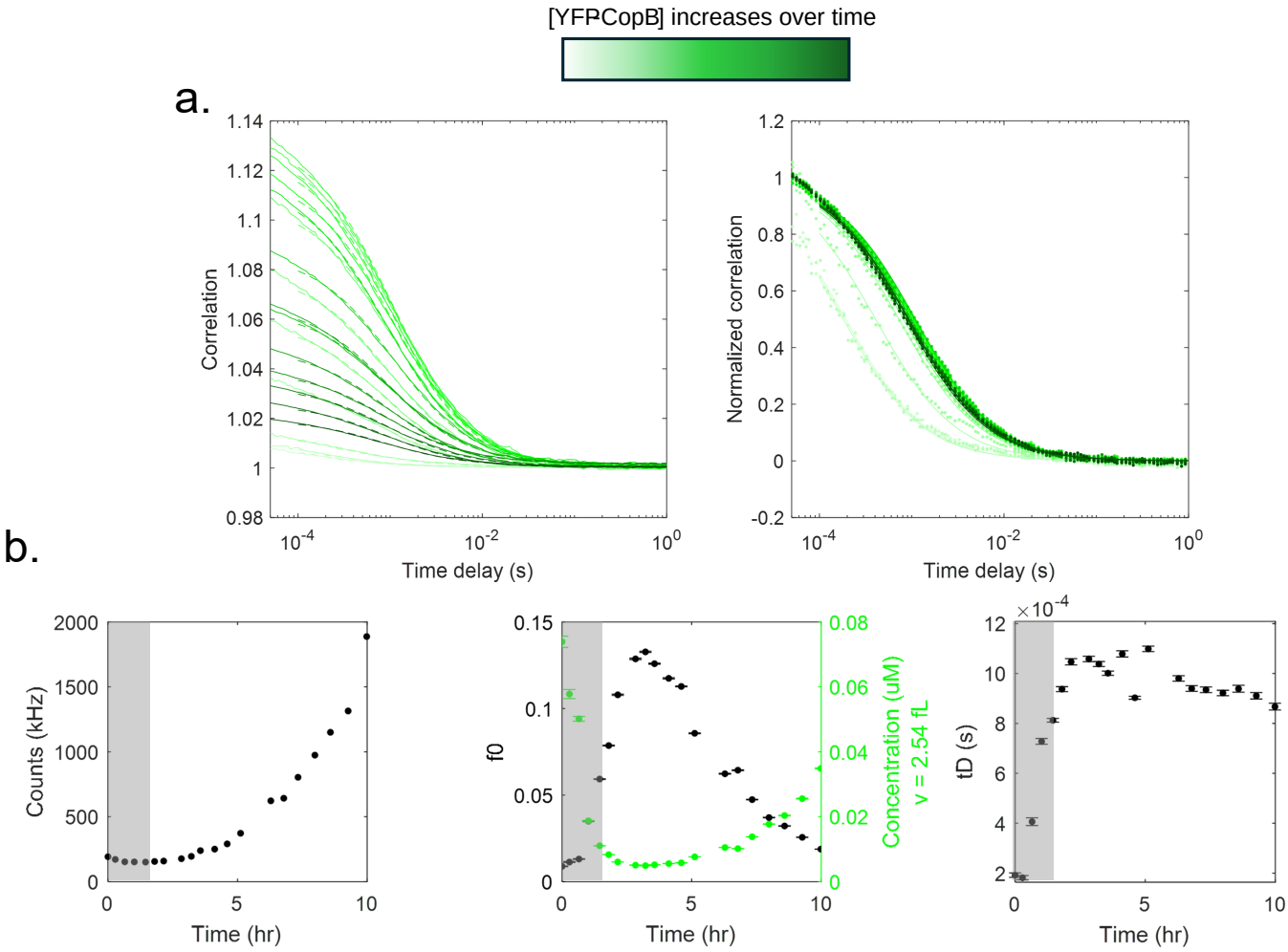

Figure 4 –YFP-CopB protein expression in ClearColi cell-free lysate monitored over time using the EI-FLEX

a) Top: Colour gradient used in plots shifts from light green to dark green with time, representing an increase in concentration of YFP-CopB.
Left: Correlation versus time delay from FCS measurements of YFP-CopB expressed in CC lysate. Correlations (green) represent separate measurements taken at intervals over ten hours. Light green correlations represent early time points. Dark green correlations represent data taken at a later point in time. A fit to the correlations gives amplitude f_0 and diffusion time τ_D.
Right: Correlations normalised to have an amplitude of 1.
b) Left: fluorescent counts versus time since the first measurement. The first measurement is taken to be Time = 0. One minute of data (one time point on the graph) was collected every 20-30 minutes over ten hours.
Centre: amplitude (black) and concentration (green) versus time. Grey shading indicates where early background fluorescence dominated. Concentrations were calculated from the measured amplitude and previously calibrated confocal volume of 2.54 fL. Right: Fitted diffusion time in seconds versus experimental time.

Time series measurements of YFP-CopB expression

As shown in Figure 4b, fitted diffusion times are initially small due to low YFP-CopB concentration and high contribution of CC lysate background. As YFP-CopB concentration becomes significant over this background, the diffusion time increases to ~1 ms. The uniformity of the normalised correlations suggests that no obvious protein aggregation occurred during expression; the population of cell-free expressed YFP-CopB appears as a relatively constant size.

However, this observed diffusion time was higher than expected for monomeric YFP-CopB (~0.3 ms). The expected diffusion time for YFP-CopB was estimated using the diffusion time of Cy3B dye; the ratio of the diffusion times of YFP-CopB and Cy3B was set equal to the cube root of the ratio of their masses. The combination of high diffusion time with uniform correlations suggests that YFP-CopB formed oligomers or possibly complexed with lysate proteins. This is consistent with the character and function of CopB; it is hydrophobic, has two predicted transmembrane domains, and participates in protein-protein interactions to perform its suggested role of forming a membrane-bound pore[6].

Measured concentrations C were calculated using fitted amplitudes f_0, calibrated confocal volume V, and Avogadro's number N_A.

$$C = \frac{1}{f_0 N_A V}$$

The final concentration of YFP-CopB at hour 10 was determined to be ~40 nM (Figure 4b). Since the counts derived from the CC lysate-only condition remained low and constant after the initial few hours (~120 kHz) compared to the YFP-CopB- derived counts that increased to over 1,500 kHz, the influence of the CC lysate is likely small on the reported concentration. If only relative changes of YFP-CopB expression are required, then background subtraction is not necessary. For highly accurate, absolute concentrations, the autofluorescence of the CC-lysate should be taken into account to avoid overestimation of protein concentration.

Summary

Here, we have demonstrated the use of the EI-FLEX for FCS measurements of fluorescently labelled protein expression using a cell-free protein synthesis system. The expression of YFP-CopB was tracked in solution over ten hours, permitting the calculation of increasing protein concentration. Information about protein size and oligomerisation was also interpreted from EI-FLEX data.

References

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