FLUORESCENCE AND BIOMEDICAL INSTRUMENTATION

Fluorescence Measurements on an ISS PC1 Spectrofluorimeter Using a Stopped-Flow Accessory

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Introduction

When combined with an entry-level stopped-flow device spectrofluorimeters can provide a very usable platform to study transient kinetics. We demonstrate the use of a Hi-Tech Scientific SFA 20 (TgK Scientific Ltd, UK) stopped-flow accessory with PC1, the spectrofluorimeter from ISS. When interfaced with the ISS instrument the acquisition of kinetic data with this accessory is straightforward and is capable of producing good quality, reproducible results.

Methodology

The dead time of a mixing device for transient kinetics can be determined experimentally using a well-characterized reaction where both the starting amplitude and the subsequent kinetic change can be measured. Extrapolating it back to the starting amplitude allows the determination of the dead time, the time between the start of the reaction and the first observable point. We used N-acetyltryptophanamide (NATA) and N-bromosuccinimide (NBS) according to Peterman [1] for this experiment.

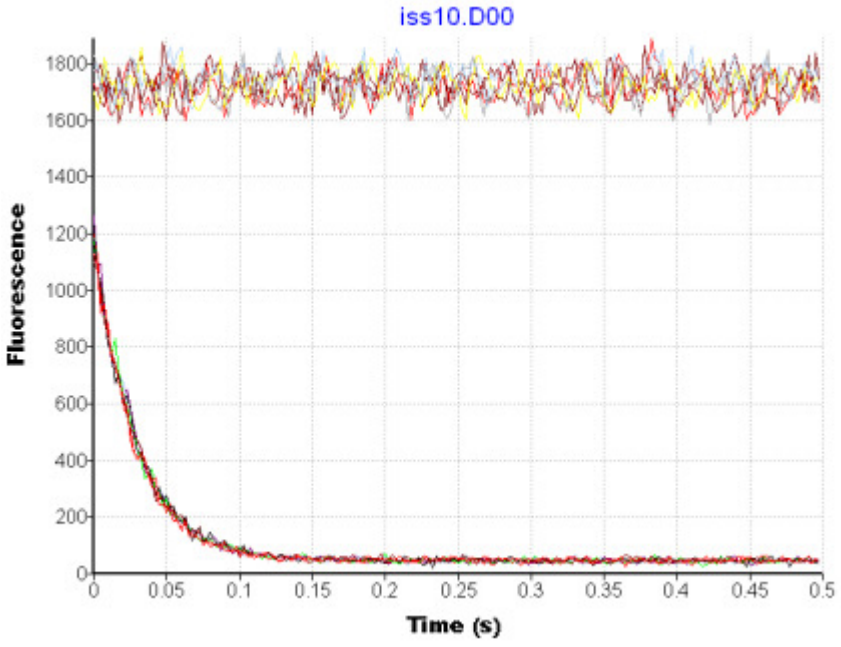

Figure 1. Overlay of multiple experiments using the stopped-flow device.

The instrument set up described below was then used to study the displacement of calcium from *Drosophila* calmodulin. This was a more challenging test of the set up since the signal comes from a single tyrosine in the calmodulin.

Experimental Data

The syringes of the SFA 20 stopped-flow accessory were loaded with the two reactants; 5 µM NATA was mixed with 50 µM NBS (syringe concentrations) both in 0.1 M sodium phosphate (pH 7.5) at 20ºC. Excitation was at 280 nm and emission was through a WG 320 filter. This is a second order reaction in which the NBS reacts with the NATA to produce a non-fluorescent product. The reaction was done under pseudo-first order conditions. The individual shots were pushed by hand. Data acquisition was initialised with a TTL trigger unit supplied with the SFA-20 (OPT-20L), which connects with the ISS PC1 external triggering facility.

The use of a trigger at stopping to start data collection automatically allows data to be averaged since all traces are referenced to a common time zero.

Figure 1 shows two series of traces overlaid. At the top are shown seven traces of NATA pushed against buffer, which give the starting amplitude; below are seven traces of the NATA/NBS reaction showing good reproducibility between pushes.

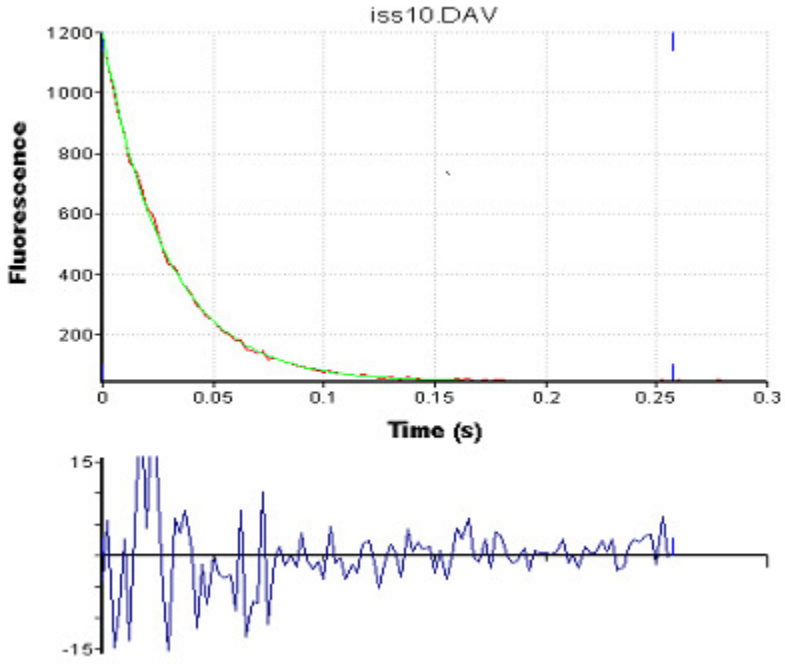

Figure 2. Average of seven shots fitted with a 1EXP+C model; residuals are given in the sub-plot.

In a further set of measurements to explore the use of the set up under more demanding conditions, 11 µM calmodulin plus 200 µM Ca^{2+} was mixed with 2.5 mM EGTA (syringe concentrations). Both solutions also contained 25 mM Tris, 50 mM KCl (pH 7.5). In this experiment, the emission was observed through the right emission monochromator instead through a cut-off filter. The excitation wavelength was 280 nm and emission control wavelength was 315 nm.

Results

The NATA/NBS reaction traces were averaged and fitted to a single exponential. A good fit was obtained with a rate constant of 35 s^{-1} (see Figure 2).

The dead time, t_d was calculated from the relationship:

$$k_{obs} \times t_d = \ln\left(\frac{F_{obs}}{F_{total}}\right)$$

where F_{obs} is the observed amplitude of the signal in the reaction and F_{total} is the amplitude between the start of the reaction (i.e. NATA alone) and the end of the reaction and k_{obs} is the fitted rate constant. Hence the dead time was 10.8 ms.

Figure 3 shows the average of 4 traces where the calmodulin/Ca^{2+} was mixed with buffer including 6 traces where it was mixed with EGTA. The reaction traces were averaged and the data were fitted to a single exponential. This was a good fit giving a rate constant of 4.9 s^{-1}.

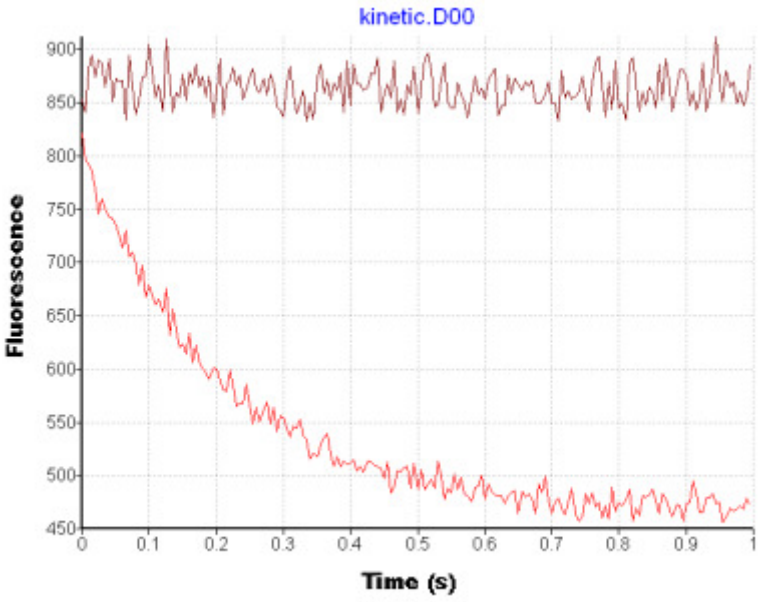

Figure 3. Average of shots – upper against buffer; lower against EGTA.

Calmodulin contains two low affinity calcium binding-sites in the N-terminal domain (k_{off} > 700 s^{-1}) and two high affinity sites in the C-terminal domain. Only the dissociation from the C-terminal sites is monitored by tyrosine fluorescence because the single tyrosine in calmodulin is located in the C-terminal domain. The result obtained here is consistent with published values for somewhat different buffer conditions (7.3 s^{-1} in 25 mM Tris, 100 mM KCl, pH 8 [2]).

Conclusion

From these results it can be seen that the combination of the SFA-20 stopped-flow accessory and the ISS PC1 Spectrofluorimeter can produce good quality results. The method is quite economical in terms of sample consumption; a total shot volume of 300 µl was used in these tests. Thus the set up is quite acceptable for experiments with precious biological reagents.

Acknowledgement

TgK Scientific thank Drs J F Eccleston and S R Martin (NIMR, Mill Hill, London, UK) for their help in conducting these tests.

References:

1. Peterman B. F., *Anal. Biochem.* 93, 442–444 (1978).
2. Brown et al., *J. Biol. Chem.* 272, 3389-3397 (1997).

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