# Stopped-flow in the mid-IR using dual comb spectroscopy

## I. Introduction

Protein chemists have always wanted to follow kinetics in the mid infra-red region (mid-IR) in order to better understand how proteins fold, and to obtain information about secondary structure changes involved in the folding process. Traditionnally, the stopped-flow is coupled to a Fourier Transform Infra-Red (FT-IR) spectrometer. Despite the improvement in FT-IR technology and use of step scan acquisition, the main limitation of such set-ups for rapid kinetics is often the time resolution of the FT-IR spectrometer. The fastest acquisition speed is often in the 30-50 ms range (or even longer) so it does not ideally match the few milliseconds dead time (with FTIR cell) of the Biologic SFM stopped-flow. Indeed, these spectrometers have been designed for steady-state study applications and the use of interferometers limits data acquisition speed. So rapid kinetics shorter than 100-200 ms are just impossible to observe with such technology.

Some home-made IR detection systems have been developed in laboratories[1] to reach such acquisition speeds and obtain millisecond resolution but none have, as of yet, been commercialised.

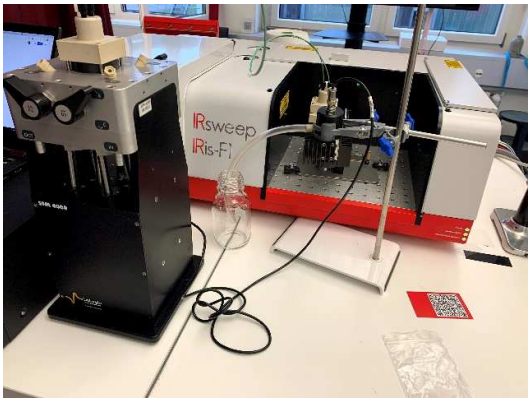


**Figure 1** **SFM-4000 coupled to IRis-F1 from IRsweep.**

**At a glance: Performance data**

- 5 ms dead time
- 75-100 µl of sample per shot
- Umbilical volume = 200 µl
- Single, double and triple mixing
- 100 µm to 500 µm light path
- IR-spectra with 4µs acquisition time
- 1000 spectra/second continuous reaction monitoring at 0.3 $cm^{-1}$ resolution
- $10^{-3}$ AU noise floor in single shot

Another limitation is the signal to noise ratio of the spectrometer as it is directly linked to the acquisition speed. Commercial FT-IR spectrometers with faster acquisition speeds often require averaging over many stopped-flow shots to improve signal to noise ratio which could prove problematic when working with the precious or concentrated samples required in the IR region.

To overcome the acquisition speed and sensitivity of FT-IR spectrometers a new type of IR spectrometer based on dual comb spectroscopy has been developed by IRsweep[2]. The IRis-F1 spectrometer from IRsweep is a dual comb spectrometer especially designed for rapid kinetics measurements. The aim of this application note is to show how the BioLogic SFM can be connected to the IRis-F1 from IRsweep, to follow reactions in the millisecond time scale, and to observe never-seen-before mid-IR reactions.

## II. What is dual comb spectroscopy?[3]

Dual-comb spectroscopy relies on fundamentally different principles to conventional Fourier transform - or dispersive spectrometers. Broadband infrared beams (frequency combs) emitted by two closely

matched laser sources are superimposed on a single detector element after passing through the sample. The wavelengths of the detected light are resolved based on the frequency of the radio-frequency signal observed at the detector. Since no moving parts are needed, a full infrared spectrum can be recorded in only four microseconds in a single experiment. Millisecond reaction dynamics as studied by the stopped-flow technique can hence be easily followed based on infrared spectral signatures at superior signal to noise ratios, as is demonstrated in this application note. Being based on high power laser sources, the dual-comb spectrometer also features high brightness, allowing for measurements in strongly absorbing solvents with high spectral and time resolution.

## III. Experimental set-up

An SFM-4000 was used in this application note together with an IRis-F1 spectrometer (see Figure 1). The SFM-4000 has 3 mixers and 4 syringes driven by independent stepping motors to give the user full control of volume, injection speed and mixing ratio. The SFM's FT-IR accessory is fully compatible with the Iris-F1 sample compartment.

The flow cell uses CaF2 windows and the user can freely select light paths from 100 µm to 500 µm by changing a spacer between windows. For the purposes of this application note a 100µm spacer was installed which corresponds to a dead volume (from last mixer to the center of the cell), of 15µl, so to a dead time of 5 ms using a 3ml/s flow rate. The last mixer is directly integrated into the FT-IR accessory to minimize the dead volume.

The SFM-4000 main body is connected to the FT-IR flow cell using an umbilical connector, this umbilical brings the solutions coming from exit of mixer 2 and from syringe 4 to the last mixer for the final mixing stage. The instantaneous stop of the flow is ensured by the synchronization of the hard-stop valve with the motors stop . The synchronization between the stopped-flow push and the IRis-F1 is made using a 5V TTL trigger.

## IV. β-Lactoglobulin structural change

The fast transition from β-sheet to α-helix in β-lactoglobulin secondary structure has been described by Gerwert *et al.*[4] using trifluoroethanol as denaturant agent. This fast reaction is used here to demonstrate the performance of the SFM-4000 and IRis-F1 coupling.

The transition in secondary structure is initiated by mixing a solution of 25 mg/ml β-lactoglobulin in 10 %$_V$ trifluoroethanol/20 mM DCl in $D_2O$ with 60 %$_V$ trifluoroethanol/20 mM DCl in $D_2O$ at a ratio of 1:1 (see Figure 2), resulting in a concentration step of trifluoroethanol from 10 %$_V$ to 35 %$_V$.

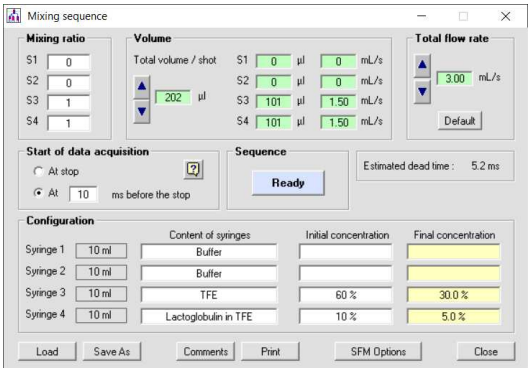


**Figure 2 Mixing sequence used for the β-lactoglobulin reaction**

Deuterated water and hydrochloric acid were used to avoid the strong background absorbance of $H_2O$ at 1650 $cm^{-1}$. For the experiments, 100 µl of each solution were injected at 3 ml/s resulting in a 5 ms dead time.

Spectra were continuously acquired every 4 µs for 130 ms. Pre-trigger spectra, corresponding to the initial state of the reaction, were used as the background for absorbance spectra. In post-processing, spectra were co-averaged over 1 ms and spectral averaging over 3 $cm^{-1}$ was performed.

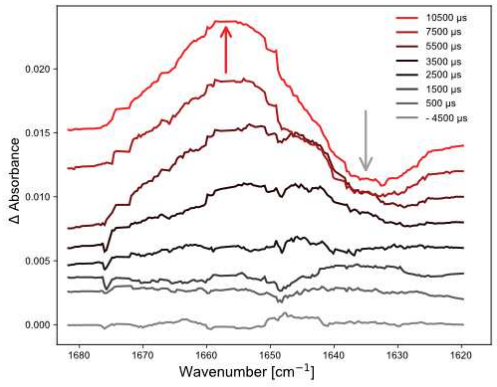


**Figure 3** **IR spectra obtained during the first 10ms of the reaction. Consecutive offset for visualization purposes.**

Figure 3 shows the evolution of the IR spectra measured during the first 10 ms and the bands at 1635 cm-1 and 1660 cm-1 confirm the presence of a fast intermediate as observed by Gerwert et al.

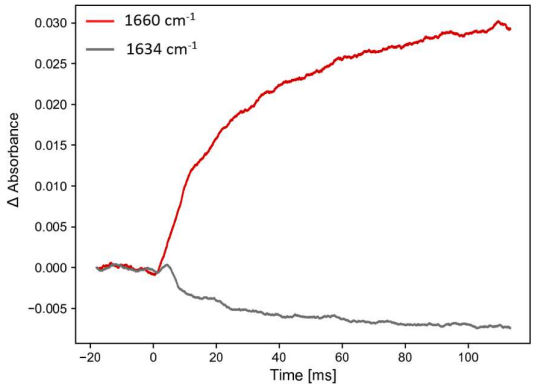


**Figure 4** **β-lactoglobulin structural change: time-traces of ΔA, average of 4 stopped-flow shots**

Figure 4 shows an average of 4 stopped-flow experiments after correction of the baseline drift by subtracting the absorbance at 1623 $cm^{-1}$. The reaction is finished in about 100 ms but a very defined signal can be obtained in its early time below 10 ms.

# V. Conclusion

The BioLogic SFM can be coupled easily to the Iris-F1 dual comb spectrometer using the standard FT-IR and an umbilical connector accessory. Sub-10 ms bio-chemical reaction kinetics can be studied in the mid-IR region where traditional FT-IR experiments are limited.

With the IRis-F1, absorbance spectra from a single stopped-flow shot are acquired at $10^{-3}$ AU (absorbance units) with 1 ms time resolution. Sensitivity can be further increased by co-averaging multiple shots or reducing the time resolution.

Only two SFM-4000 syringes were used for the described experiments, but the two additional syringes can be useful if users wish to program automatic concentration studies or double mixing experiments, in such cases one umbilical flow line is used as a delay line to age the first mixing. SFM-2000 and SFM-3000 can also be coupled to the IRis-F1 spectrometer.

## REFERENCES


1) **J. Tang, F. Gai, A Millisecond Infrared Stopped-Flow Apparatus. *Applied Spectroscopy*. 2006; 60(12) ; 1477-1481.**
2) **A. Hugi, G. Villares, S. Blaser, H.C. Liu, J. Faist, Mid-infrared frequency comb based on a quantum cascade laser. Nature 492, 229–233 (2012).**
3) **https://irsweep.com/technology/**
4) **Kauffmann, E., Gerwert, K. et al. (2001). *PNAS*, *98*(12), 6646–6649**


***Revised in 10/2020***

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