High-Resolution Measurement of NADH and FAD Fluorescence Decay with the DCS-120 MP

Wolfgang Becker, Lukas Braun, Axel Bergmann

Becker & Hickl GmbH, Berlin, Germany

Abstract: The bh DCS-120 MP system is able to record single decay functions at extremely high precision and time resolution. We used the system to record decay functions of NADH and FAD with an IRF width of 19 ps. Fluorescence decay functions were obtained for NADH and FAD in aqueous solution, and in a diluted solution of citric acid at pH = 4. The decay curves are multi-exponential, with decay components as fast as 115 ps for NADH and 59 ps for FAD. The curves measured at pH = 7 and pH = 4 are significantly different, and they are different from decay curves recorded in cells. The procedures described can be used to supplement FLIM experiments with precision decay parameters of the fluorophores involved. Similar measurement can be performed with other bh FLIM systems, especially if these are equipped with bh's ultra-fast HPM-100-06 detectors.

Precision Recording of Decay Functions with bh FLIM Systems

FLIM experiments give direct insight into molecular processes in live cells and tissues [1, 2]. The experiments often have to be supplemented by precision measurements of the decay functions of the fluorophores involved. FLIM users then usually resort to an additional fluorescence lifetime spectrometer for cuvette-based measurements of decay functions. In many cases, however, such fluorescence decay data can be favourably be recorded with the FLIM system itself [2, 5]. Recording the decay functions with the FLIM system has several advantages. The obvious one is that the data are recorded under exactly the same conditions as with the FLIM system. The excitation and detection wavelengths are the same, the system IRF is the same, and the geometric configuration is the same. The influence of the anisotropy decay cancels by the high NA of the objective lens, transit time-effects in a cuvette are avoided, and reabsorption effects are negligible due to the small size of the observation volume [2]. Most importantly, however, measurements with bh's FLIM systems provide superior time resolution. With femtosecond lasers and bh's HPM-100-06 detectors an IRF width of <20 ps FWHM is obtained [4], see Fig. 1, left. Even for diode-laser excitation the IRF width stays below 50 ps FWHM [7], see see Fig. 1, right.

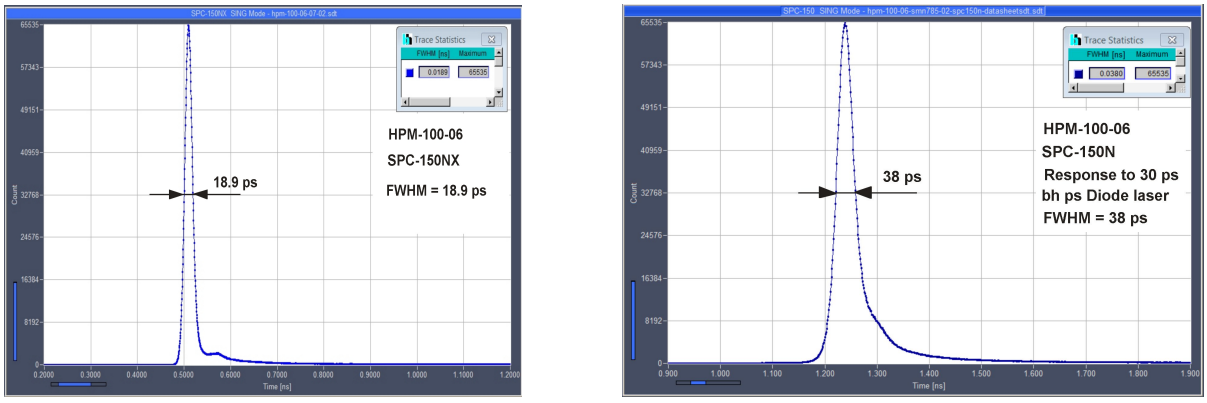

Fig. 1: Left: IRF with femtosecond laser. Right: IRF with bh BDS-405nm diode laser

For the experiments described below we used a DCS-120 MP multiphoton system with a Toptica 785 nm Femto Fibre Pro femtosecond laser, non-descanned detection, and HPM-100-06 hybrid detectors [5]. The IRF width of this system is about 19 ps, FWHM. The DCS scan head was attached to a NIKON TE 2000 inverted microscope.

The dye solution to be investigated was put in a cell dish, placed under the microscope and scanned the same way as a normal cell or tissue sample. The laser power was adjusted to obtain a count rate of about 10^6 photons per second. The entire 'image' was sent to the SPCImage NG data analysis software [2, 5] and [6]. Data of a large ROI or of the entire image were combined by the 'lock' function of SPCImage. The resulting decay curve contains several million photons and can thus be precisely analysed by fitting with double- or triple-exponential decay models.

NADH and FAD Decay Curves

NADH decay curves are shown in Fig. 2 and Fig. 3. Fig. 2 is in pure water, Fig. 3 is in dilute citric acid, pH = 4.

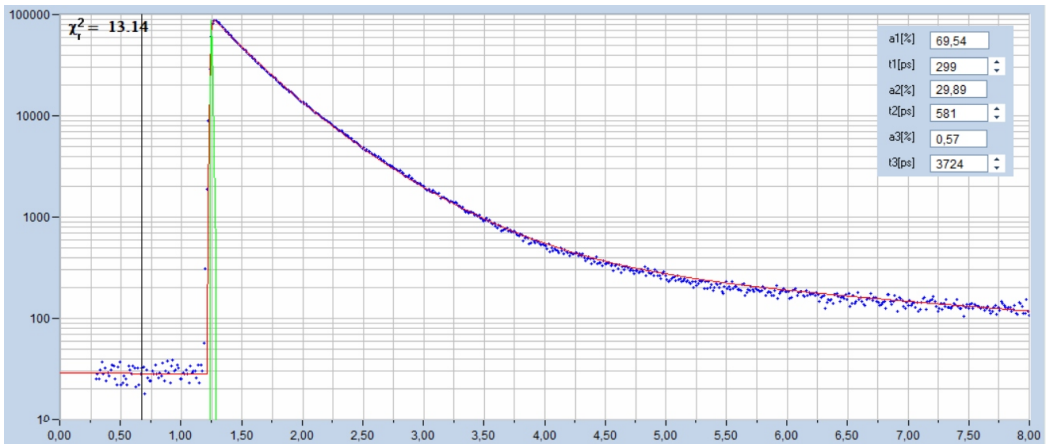

Fig. 2: NADH dissolved in pure water. Lifetimes and amplitudes of decay components shown upper right.

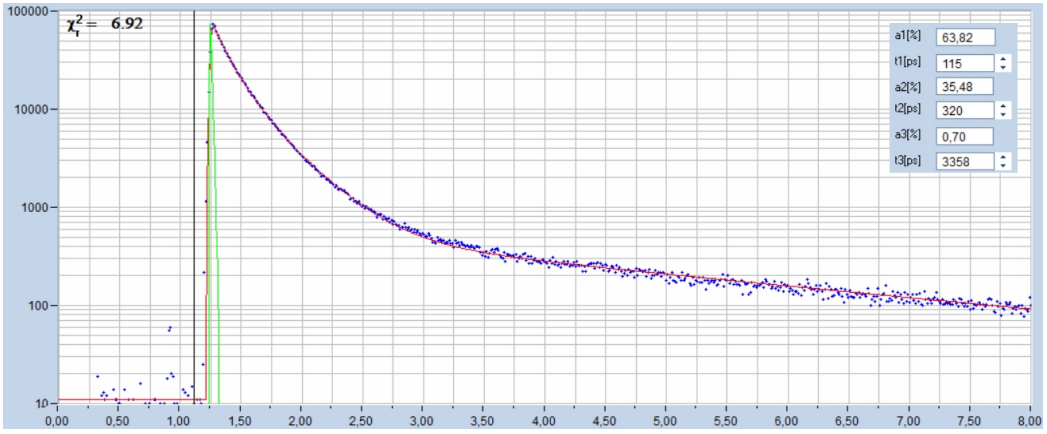

Fig. 3: NADH in water with citric acid, pH = 4. Lifetimes and amplitudes of decay components shown upper right.

Already at first glance, it can be seen that the decay functions are multi-exponential. That means NAD(P)H exist in different conformations or modifications. The decay times and the amplitudes of the components are shown in the inserts in the upper right of the figures. The fastest components are

299 ps and 115 ps. This is shorter than normally found in cells. Nevertheless, short components sometimes show up also in cells. The results show that these components may indeed by real.

A comparison of Fig. 2 and Fig. 3 further shows that there is a significant change in the decay parameters with the molecular environment. It is possible that the change is mostly induced by the change in pH, which would be in agreement with [12]. However, it is possible that also the redox potential of the molecular environment of the NADH molecules has an influence. It is known that it has a dramatic influence on the fluorescence intensity of NADH and FAD [9], and it may have an influence on the lifetimes as well.

Fig. 4 and Fig. 5 show similar decay curves for FAD. Fig. 4 was recorded in purely aqueous solution, Fig. 5 in diluted citric acid, with pH = 4. Interestingly, the data recorded in pure water show an extremely fast decay component of 59 ps. It is present with an amplitude of about 30%. The existence of a fast components has already been reported in [8], where it was extracted from the data by multi-exponential fit procedures. Fig. 4 shows it directly, for the first time, indicating that the component is real. The decay data recorded with citric acid (Fig. 5) do not show the fast component. Moreover, the decay function at pH = 4 is visually undistinguishable from a single-exponential decay. (A single-exponential fit delivers τ = 3.1 ns.) Also here, we cannot tell whether the change in the decay curve is induced by a change in pH or by a change in the redox potential.

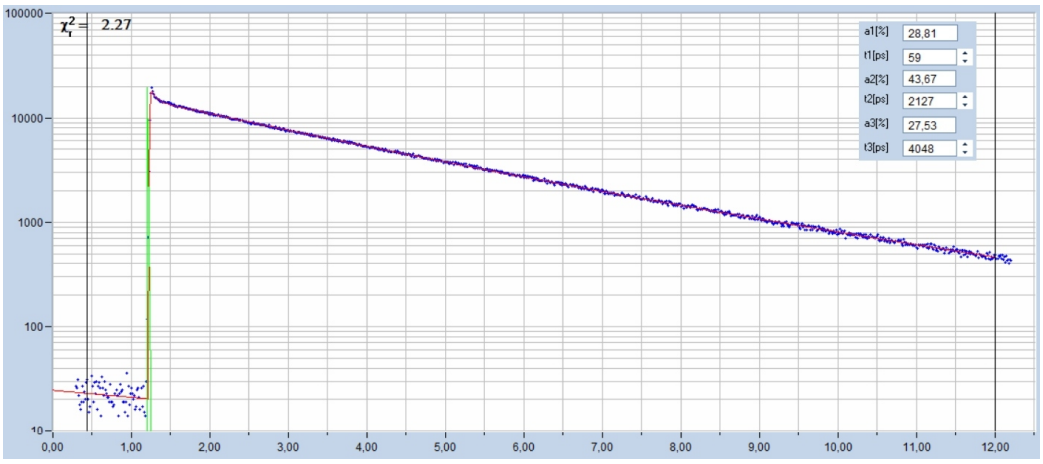

Fig. 4: FAD in water. Lifetimes and amplitudes of decay components shown upper right.

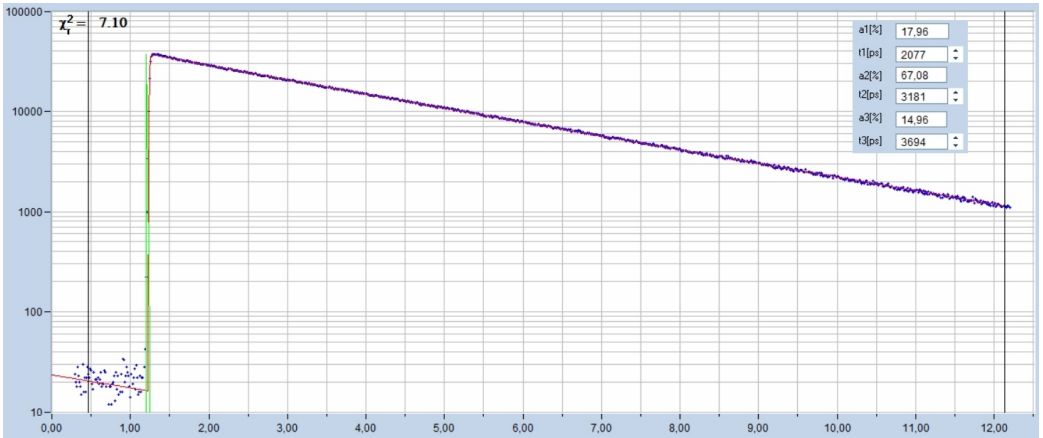

Fig. 5: FAD in water with citric acid, pH = 4. Lifetimes and amplitudes of decay components shown upper right.

Comparison with Decay Curves in Cells

Fig. 6 and Fig. 7 are showing NADH and FAD decay functions measured in cells. The curves were extracted from FLIM images of excised human epithelial bladder tissue. Excitation for NADH and FAD was performed quasi-simultaneously by ps diode lasers of 375 nm and 410 nm. Details of the experiments are described in [3]. The decay curves are totally different from the curves obtained in solution. In particular, there are no traces of the ultra-fast decay components. However, the difference in the decay profiles is not surprising. In solution the decay components originate from intrinsically different modifications or conformations of NADH and FAD. In cells, the decay functions are dominated by the lifetimes of bound and unbound NAD(P)H and bound an unbound FAD [2, 10, 11]. It should also be noted that there may be additional fluorophores present in the cells. The FAD signals from cells usually contain a small amount of fluorescence of FMN. The lifetime of the FMN fluorescence is 4 ... 5 ns. The slow decay component in Fig. 7 may therefore originate from FMN.

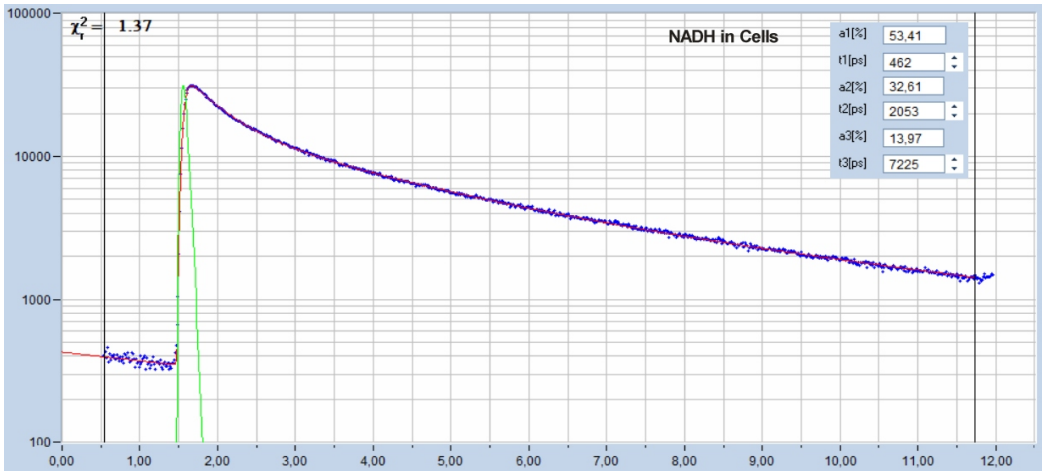

Fig. 6: NADH decay function in cells

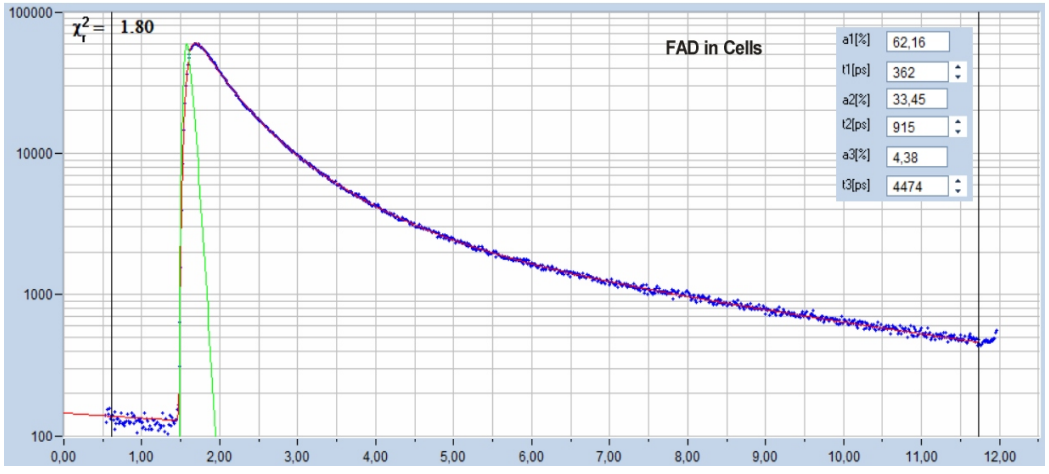

Fig. 7: FAD decay function in cells

Summary

The bh DCS-120 MP system is able to record single fluorescence decay curves at extraordinarily high time resolution. Here, we have demonstrated the use of the system for recording precision

decay functions of NADH and FAD in solution. The data show fast decay components on the order of 115 ps for NADH and 59 ps for FAD. The presence of a fast FAD component has been suspected earlier, but the DCS-120 MP measurement shows it directly for the first time. Similar measurements can be performed with other bh FLIM systems, especially if these are equipped with the ultra-fast HPM-100-06 hybrid detectors.

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Contact:

Wolfgang Becker
Becker & Hickl GmbH
Berlin, Germany
Email: becker@becker-hickl.com
info@becker-hickl.com