DCS-120 FLIM System Detects FMN in Live Cells

Wolfgang Becker, Lukas Braun, Becker & Hickl GmbH

Abstract: It has long been suspected that FAD metabolic FLIM data are biased by a fluorescence decay component from FMN. We therefore checked for possible traces of FMN fluorescence in FLIM data from our DCS-120 metabolic FLIM system. Indeed, we found a decay component with a lifetime around 4.8 ns which cannot be attributed to FAD but very well to FMN. The amplitude of the FMN component was 3.9% in normal cells and 8.8% in cancer cells.

The fluorescence decay functions of NAD(P)H (nicotinamide adenine (pyridine) dinucleotide) and FAD (flavin adenine dinucleotide) contain information on the metabolic state of the cells. Bound and unbound NAD(P)H and FAD have different lifetimes, and the relative amount of bound and unbound forms varies with the metabolic state. Therefore the apparent lifetime or, better, the ratio of the amplitudes of the decay components, a1/a2, can be used as an indicator of the metabolic state [1].

Although FLIM results are largely conclusive for NADH [2,3,5,6], this is not the case for FAD. In contrast to NADH, where the fast decay component comes from the free form, for FAD the slow component is associated to the free form. Therefore, the lifetime effect of a change in the free/bound ratio for FAD and NADH goes in opposite directions. Often in FAD FLIM data the signal from FAD is not cleanly separated from that of NADH. The result then depends on the relative amounts of FAD and NADH. This can modify or even reverse the observed lifetime effect. However, also for perfectly recorded data the FAD results are often inconclusive. It has long been suspected that the FAD signal contains fluorescence from FMN (flavin mononucleotide) [4]. We therefore looked for possible traces of FMN fluorescence in NADH/FAD data recorded with our DCS-120 metabolic FLIM system [1, 2].

Fig. 2 shows NAD(P)H and FAD FLIM images of freshly excised human epithelial bladder cells [6]. The images were recorded by multiplexed excitation at 375 nm (NAD(PH) and 410 nm (FAD). NAD(P)H was detected from 420 to 470 nm, FAD from 490 to 580 nm. Please see [1,2] for technical details. Both images show the amplitude of the fast decay component, a_1 [1]. The sample contains mostly normal cells (area A), but there are also a few tumorous cells with an enhanced fraction of free NADH (area B).

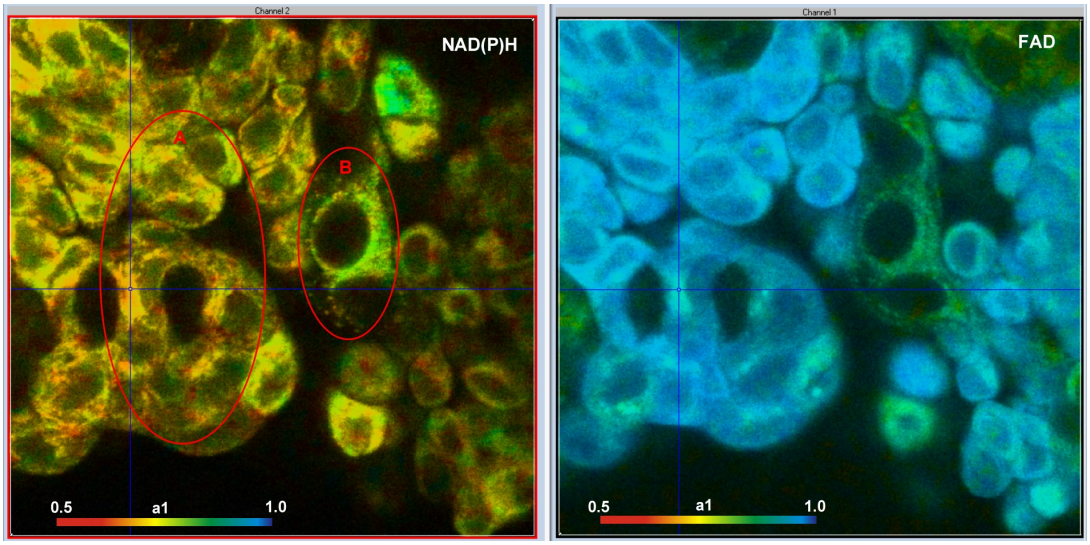

Fig. 1: FLIM images of human epithelium bladder cells. NAD(P)H (left) and FAD (right). Amplitude, a1, of fast decay component

To check for possible FMN contribution in the FAD data, an ROI was chosen which contains only cells that qualify 'normal' in the NADH channel. Please see Fig. 2. The decay curve of the combined pixels within the ROI is shown on the right. Triple-exponential analysis clearly delivers a decay component of 4.83 ns, present with an amplitude of 3.9 %. A lifetime of 4.83 ns cannot be attributed to FAD, but very well to FMN.

Fig. 3 shows the same data, but the ROI was set on cells which are classified as 'cancer' in the NADH image. The lifetime of the third component is 4.38 ns, not much different than in Fig. 2. However, the amplitude has increased to 8.84 %.

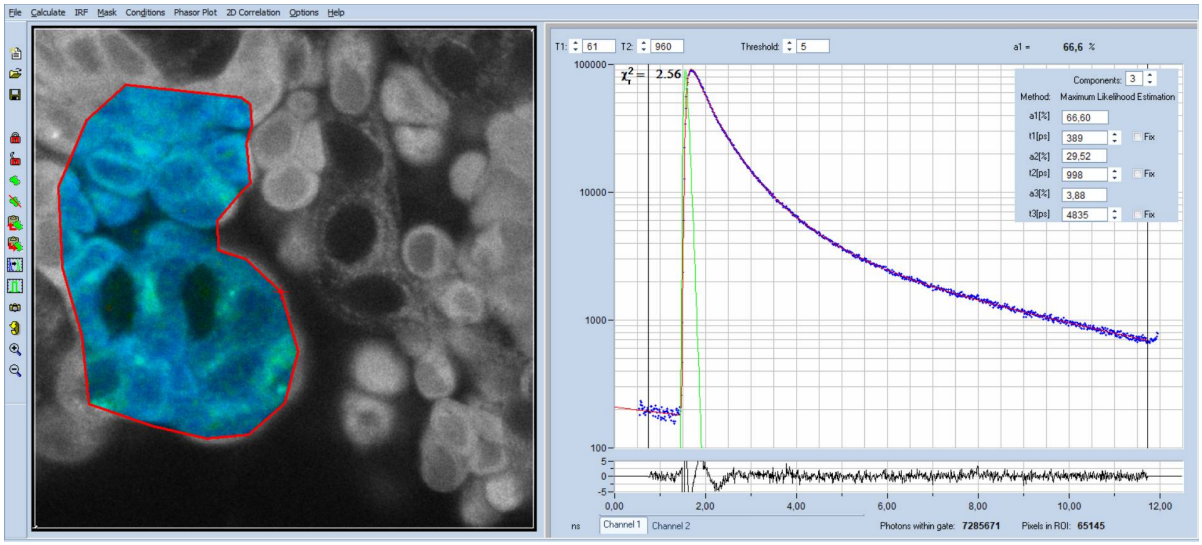

Fig. 2: Left: FAD Image of human epithelial bladder cells. The region of interest includes healthy cells only. Right: Decay curve of combined pixels in ROI, and triple-exponential fit. The third component, with t3 = 4.835 ns and a3 = 3.88%, is compatible with FMN.

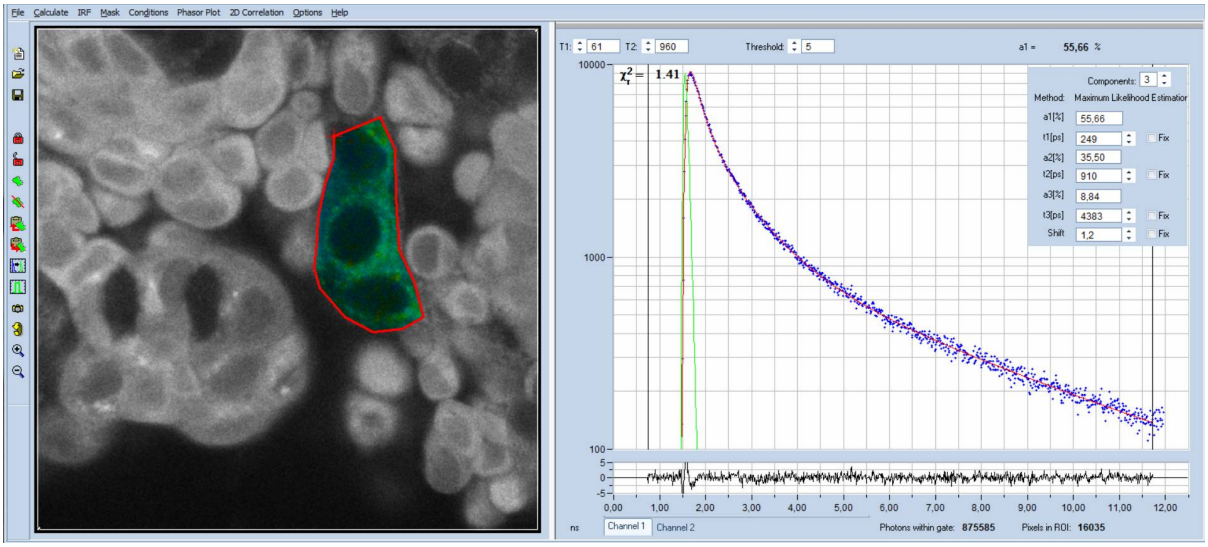

Fig. 3: Same data as in Fig. 2, but ROI on cells which are probably tumor ells. The third component is t3 = 4.383 ns and a3 = 8.84%.

Fig. 4 shows an attempt to extract the amplitude of the FMN component on a pixel-by-pixel basis. Of course, this poses a challenge to the data analysis. Triple-exponential analysis has to be used, the

number of photons is lower than in a region of interest, and the slow component of FAD, t_2, and the FMN component, t_3, differ only by a factor of two in lifetime. Moreover, the amplitude of the FMN component is low compared to the amplitudes of the FAD components. Nevertheless, with a binning factor of 8 in SPCImage NG (17x17 pixels, the entire image has 512x512 pixels) and MLE analysis we obtained a very satisfactory result. As expected, the amplitude of the FMN component, a_3, is about 4% in the normal cells, and about 8% in the tumor cells. This does not mean that the absolute amount of FMN in the tumor cell is higher. The higher a_1 can also come from the lower intensity of the tumor cell. Whether the lower intensity is a result of lower FAD concentration or lower redox ratio in the tumor cell is not known. In the second case, a_3 may be another expression of the redox ratio. Whether it can be used to distinguish cells of different metabolic state should be subject of further investigation.

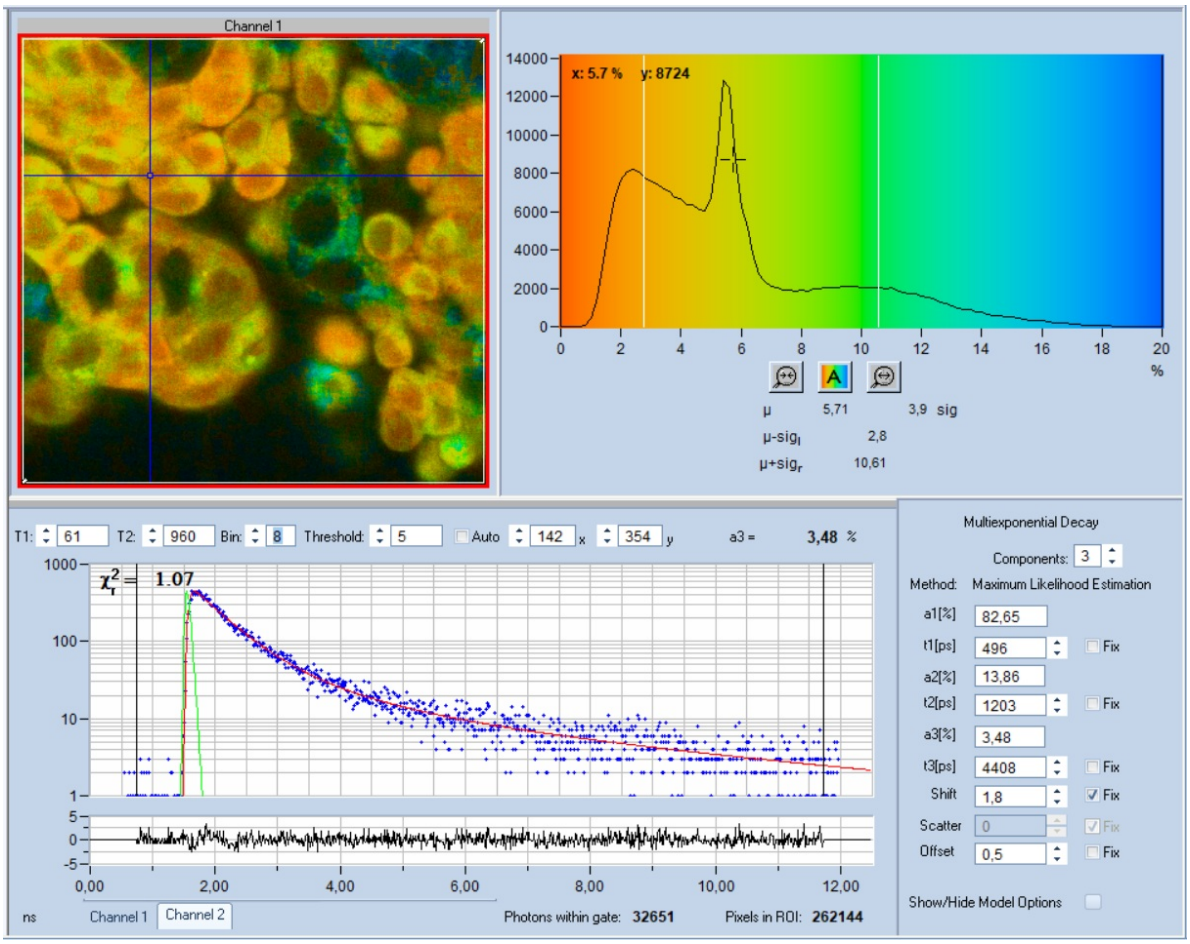

Fig. 4: Triple-exponential analysis of the image in the FAD channel. Colour shows amplitude, a3, of FMN component.

The presence of a decay component from FMN may have implications to the use of FAD data for cancer diagnosis, especially if the amplitude, a1, the amplitude ratio, a1/a2 or the FLIRR ratio are used as cancer indicators. Certainly, an amplitude contribution of 3.8 % or even 8.84 % does not render a_1 or a_1/a_2 of FAD or the FLIRR useless. It can, however, introduce unpredictable shifts in these parameters. The results then can no longer be considered quantitative. Kalinina et al. therefore suggested new definitions of the FLIRR based on triple-exponential analysis of the FAD [4].

References

1. W. Becker, The bh TCSPC Handbook, 9th edition (2021)
2. W. Becker, A. Bergmann, L. Braun, Metabolic Imaging with the DCS-120 Confocal FLIM System: Simultaneous FLIM of NAD(P)H and FAD, Application note, available on www.becker-hickl.com (2018)
3. Becker Wolfgang, Suarez-Ibarrola Rodrigo, Miernik Arkadiusz, Braun Lukas, Metabolic Imaging by Simultaneous FLIM of NAD(P)H and FAD. Current Directions in Biomedical Engineering 5(1), 1-3 (2019)
4. S. Kalinina, C. Freymueller, N. Naskar, B. von Einem, K. Reess, R. Sroka, A. Rueck, Bioenergetic Alterations of Metabolic Redox Coenzymes as NADH, FAD and FMN by Means of Fluorescence Lifetime Imaging Techniques. Int. J. Mol. Sci. 22, 5952, 1-15 (2021)
5. M. M. Lukina, V. V. Dudenkova, N. I. Ignatovaa, I. N. Druzhkova, L. E. Shimolina, E. V. Zagaynovaa, M. V. Shirmanova, Metabolic cofactors NAD(P)H and FAD as potential indicators of cancer cell response to chemotherapy with paclitaxel. BBA – General Subjects 1862, 1693-1700 (2018)
6. Rodrigo Suarez-Ibarrola, Lukas Braun, Philippe Fabian Pohlmann, Wolfgang Becker, Axel Bergmann, Christian Gratzke, Arkadiusz Miernik, Konrad Wilhelm, Metabolic Imaging of Urothelial Carcinoma by Simultaneous Autofluorescence Lifetime Imaging (FLIM) of NAD(P)H and FAD. Clinical Genitourinary Cancer (2020)

Contact:

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