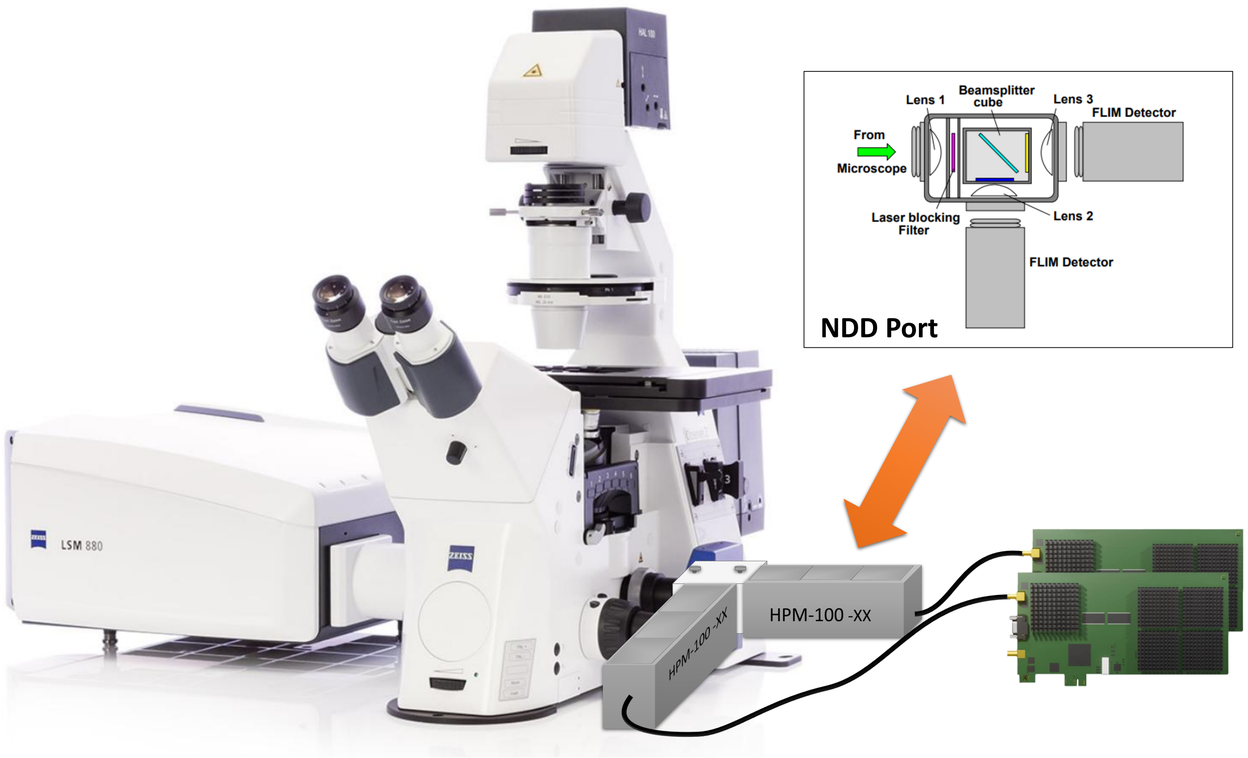
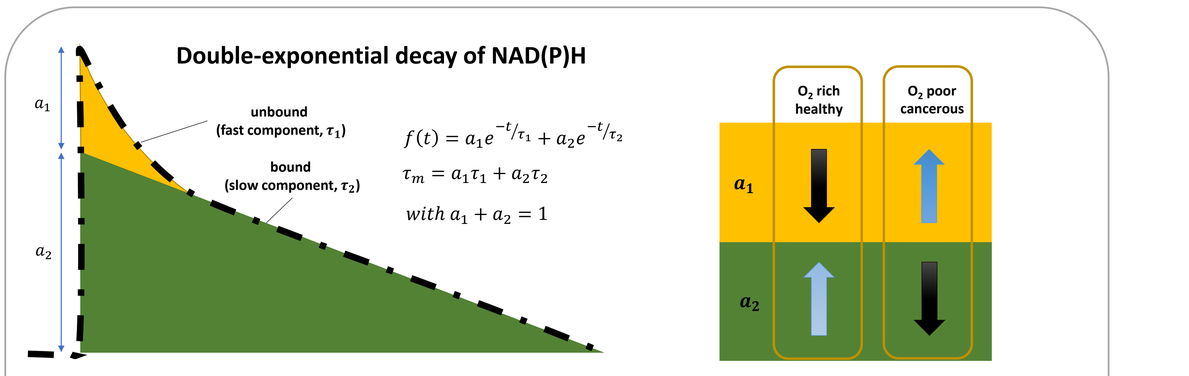
Multiphoton FLIM for ZEISS LSM

Becker & Hickl GmbH



A multiphoton laser scanning microscope uses a femtosecond-pulsed near-infrared (NIR) laser to excite fluorescence in a sample through the simultaneous non-linear absorption of multiple photons. NIR wavelengths have lower absorption and scattering than visible or UV light, allowing for deeper tissue penetration. The nonlinear excitation process confines fluorescence generation to the focal point, improving depth resolution and reducing photobleaching of out-of-focus layers. Fluorescence is collected from the sample and sent to the detector at the non-descanned detection port (NDD).

The TCSPC module records the time and position of each photon to create a pixel array, where each pixel contains a fluorescence decay curve. The fluorescence lifetime and other FLIM parameter are then calculated in each of those pixels, and given a corresponding pseudo-color-code to be represented on the FLIM image.

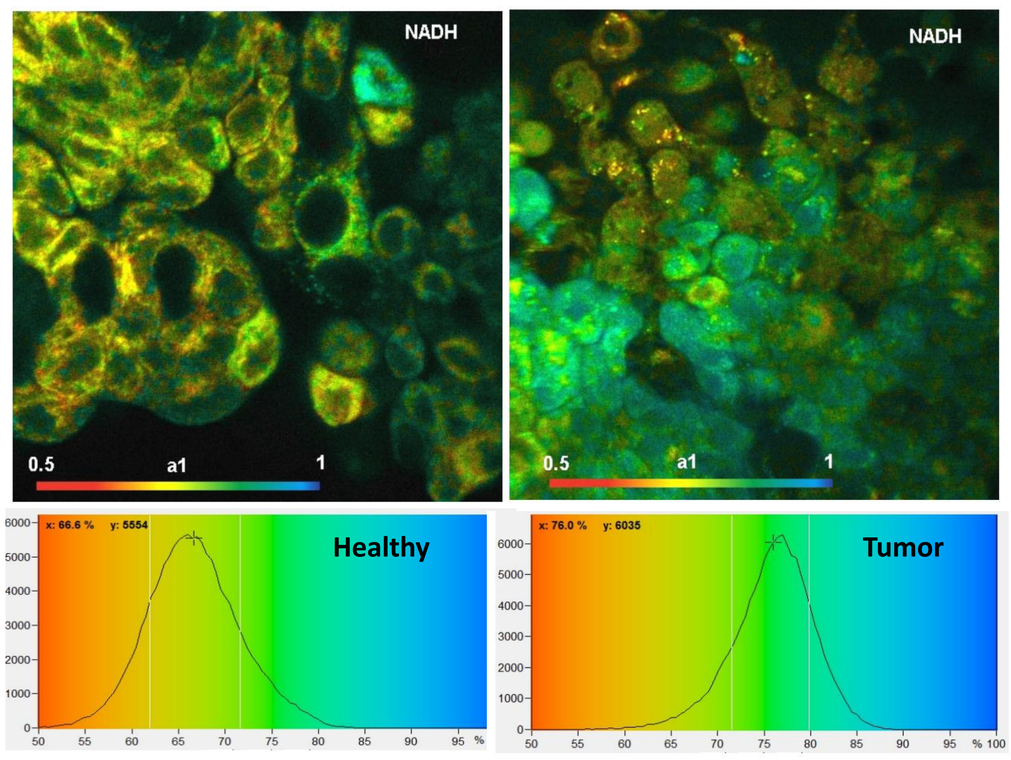


Autofluorescence metabolic FLIM approach

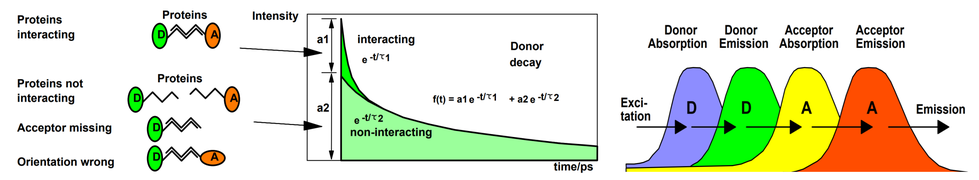
With 785 nm excitation wavelength, the system is ideally suited for NAD(P)H FLIM. The fluorescence decay of NAD(P)H provides key insights into the metabolic state of the cells, with primary information found in the amplitudes of the fast and slow decay components, a1 and a2.

The ratios of unbound (a1) to bound (a2) fraction of NAD(P)H is influenced by the glycolysis and oxidative phosphorylation in the cells. In cancer cells reductive glycolysis dominates resulting in increased value of a1 shortening the mean lifetime.

NAD(P)H FLIM requires efficient photon detection, high time-resolution, and advanced data analysis to perform double-exponential fitting of the measured data, all of which are provided by bh FLIM-systems.



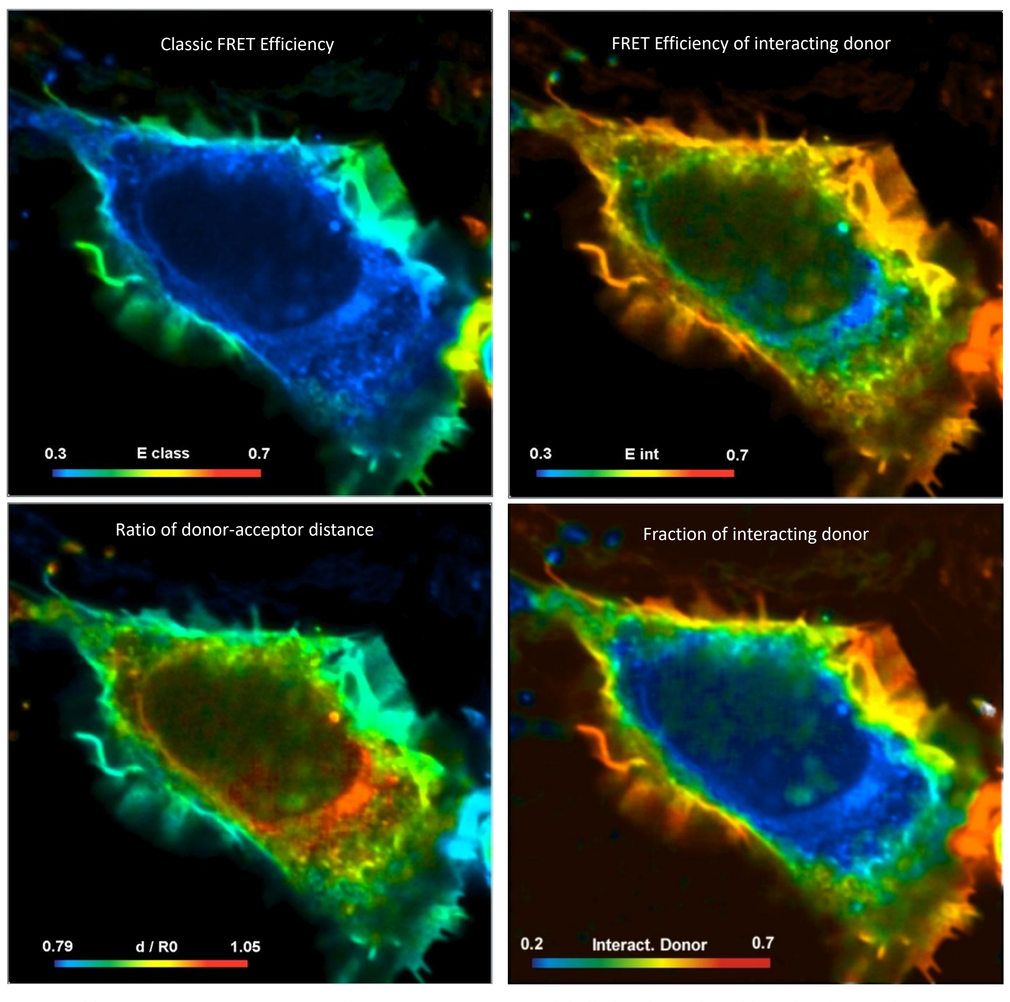
NAD(P)H images of a1, the amplitude of the fast decay component.
The left panel depicts healthy human bladder cells. The right panel shows tumor cells [1].



Principle of FLIM-FRET [2]

Förster Resonance Energy Transfer (FRET) is an interaction between two fluorophore molecules that are positioned in close proximity to each other. When the donor emission spectrum overlaps with the acceptor absorption spectrum, energy from the donor can be transferred to the acceptor resulting in a shorter donor fluorescence lifetime.

The donor decay can be fitted to a double-exponential function, which consists of a fast component (τ1) corresponding to the interacting donor fraction, and a slower component (τ2) representing the not interacting donor fraction.



Calibration Free FLIM-FRET: HEK cell expressing two proteins labelled with Cerulean (donor) and Venus (acceptor). Interaction occurs preferentially in the cell membrane [2].

A key advantage of the double-exponential FLIM-FRET approach is that it is calibration free. Unlike traditional methods that need reference measurements from a donor-only sample, the reference lifetime is the τ2 value, which can be directly obtained from the same measurement.

The process involves recording a FLIM dataset at the donor's emission wavelength, performing FLIM analysis using SPCImage NG software to calculate the desired FRET parameters just in few seconds.

	Tryptophan [3][4]	Keratin [5][6]	Collagen [7][4]	NAD(P)H [1]	ATTO 488 [8]	EGFP [9]	Alexa Fluor 488 [10][11]	Venus [9][12]	FAD [1][3]	Melanin [5]	mOrange2 [9]	ATTO 565 [13]	Alexa Fluor 568 [10][11]	mCherry [9]	mKate 2 [9]	mGarnet [9]
Possible λex [nm]	280	350	375	375	488	473/488	488	515	445	375	515/532	515/532	532	515/532	515/532	532/594
Max. λex [nm]	220	277	335	350	495	488	500	515	350 /450	335	549	564	579	587	588	598
Recom. 2p λex [nm]	550 780 (3p)	760	720-880	720-780		800-980	720-830	940	920	760-820	760 900-1100		750-850	750 – 800	720-800	
Max. λem [nm]	350	382	400	470	500	507	520	528	535	550	565	590	603	610	633	670
Lifetime [ns] [*]	0.5 – 2.5 ^{bound} 3.1 ^{free}	1.4	2.85	0.3 – 0.4 ^{free} 1.2 ^{bound}	3.2	2.6	4.1	3	0.12 ^{bound} 3.38 ^{free}	0.1-1.8	2.7	4	3.6	1.4	2.5	0.8

(*) The fluorescence lifetimes may vary depending on the environment parameters, e.g. temperature, pH-value or other parameters. For detailed information, please check the TCSPC Handbook.

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