Multiphoton NDD FLIM at NIR Detection Wavelengths with the Zeiss LSM 7MP and OPO Excitation

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Abstract: We demonstrate multiphoton NDD FLIM of tissue samples stained with near-infrared dyes. For the experiments we used a Zeiss LSM 7MP multiphoton microscope with a Coherent Chameleon OPO (optical parametric oscillator) as an excitation source. The excitation wavelengths range from 1000 nm to 1300 nm. The fluorescence was detected by an HPM-100-50 NIR hybrid detector attached to the NDD (non-descanned detection) port of the microscope; the FLIM data were recorded by a standard bh TCSPC FLIM system. We demonstrate the performance of the system for tissue samples stained with Methylene Blue, Indocyanin Green (ICG), and 3,3'-Diethylthiatricarbocyanine (DTTCC). All three dyes could be efficiently excited at wavelengths from 1200 nm to 1300 nm. The dyes showed remarkable variability in their fluorescence lifetimes. The lifetimes clearly depended on the tissue structures the dyes were located in.

Keywords: FLIM, TCSPC, Multiphoton Excitation, OPO, NIR Dyes, Methylene Blue, Indocyanin Green, Fluorescence Lifetime

FLIM with NIR Fluorophores

Fluorophores emitting in the near-infrared region are used as markers in diffuse optical tomography and small-animal imaging. For these applications, it is important to know how the fluorescence lifetime depends on the binding target and on the local molecular environment on the cell level. Moreover, lifetime variation due to variable local molecular environment may be exploitable as a probe function [7, 8]. Another advantage of NIR probes is that there is no contamination from autoflourescence. Moreover, light propagation through biological tissue at infrared wavelengths is less impaired by scattering and absorption than in the visible range. We have recently shown that even one-photon excitation and confocal detection in the NIR yields surprisingly clear images from tissue layers up to 50 µm deep [2, 3, 5]. It can be expected that the penetration depth is further enhanced by two-photon excitation and non-descanned detection.

For the experiments described here we used a Zeiss LSM 7MP with OPO excitation [9] and a bh Simple Tau 150 TCSPC FLIM system [1, 2, 4]. To obtain sensitivity in a wavelength range up to 900 nm the HPM-100-40 GaAsP hybrid detector of the FLIM system was replaced with an HPM-100-50 GaAs hybrid detector. The detector was attached to the NDD port of the microscope via a Zeiss T adapter [4]. To be able to detect fluorescence up to 900 nm we replaced the standard two-photon beamsplitter in the beam path at the back of the objective lens with a 980 nm dichroic mirror. The beamsplitter is not critical, an 80/20 wideband beamsplitter can be used as well. The standard 700 nm short pass (laser blocking) filter in the T adapter was replaced with a 980 nm short pass filter. The microscope objective lens was an x20/1.0 DIC VIS-IR Plan-Apochromate for water immersion.

FLIM Images

Methylene Blue

Methylene Blue is a biomedically interesting compound. It has anti-viral and anti-bacterial effects, and it has been evaluated as a drug against malaria [12]. It has been applied to induce apoptosis in

cancer cells [13], and to treat psoriatic skin lesions by photodynamic therapy [11]. The use of methylene blue to treat Alzheimer disease has been under clinical trial [10].

Methylene Blue has an absorption band from 550 to 690 nm, with a maximum at 660 nm. Fluorescence is emitted from 650 nm to 750 nm, with a maximum at 680 nm. It can thus be expected that two-photon excitation works well in the range from 1000 nm to 1200 nm.

A FLIM image obtained from a pig skin sample stained with Methylene Blue is shown in Fig. 1, left. The sample was excited at 1200 nm, the fluorescence was detected from 680 nm to 780 nm. Excitation was surprisingly efficient. No more than 4 percent of the available OPO power were needed to obtain a count rate on the order of 1 MHz. Decay curves from characteristic spots are shown in Fig. 1, right. The decay profiles are multi-exponential but can be reasonably fitted by a double-exponential decay model. The amplitude-weighted lifetime in different parts of the tissue varies by almost a factor of two.

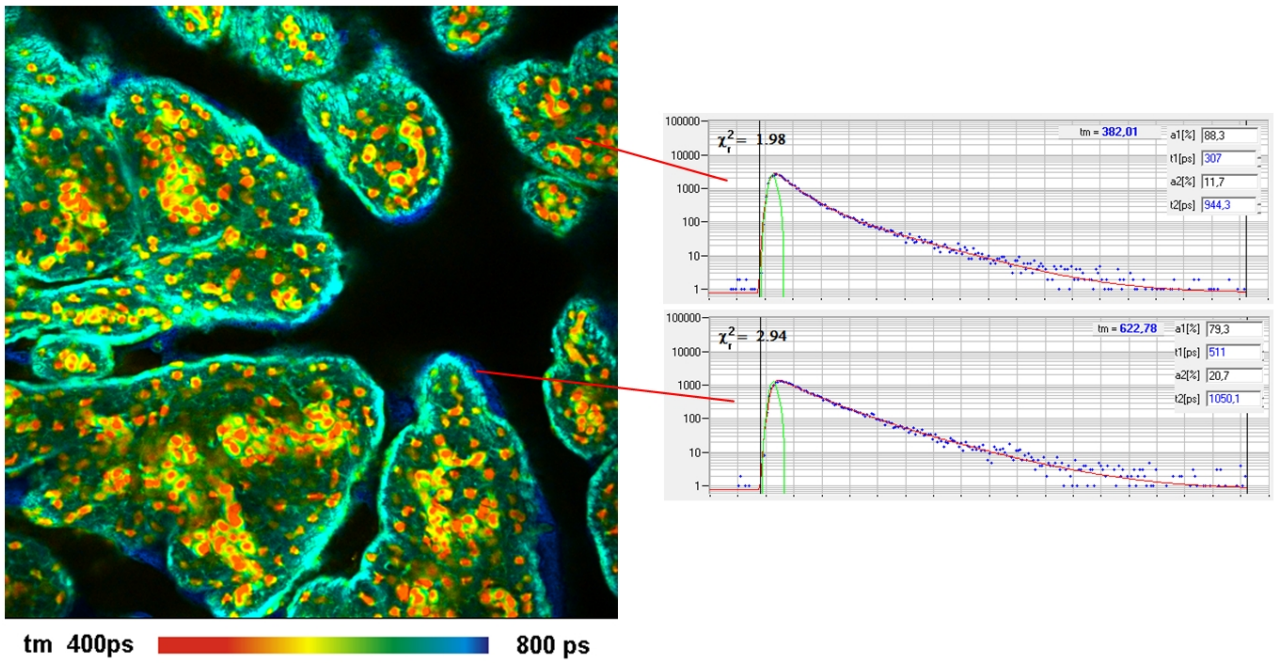

Fig. 1: Pig skin stained with methylene Blue. Left: Lifetime image, double-exponential decay model, amplitude-weighted lifetime. Two-photon excitation at 1200 nm, 512 x 512 pixels, 256 time channels. Right: Decay curves in characteristic spots of the image.

Indocyanine Green (ICG)

FLIM Images of a similar sample stained with ICG are shown in Fig. 2. The data were analysed by a double-exponential decay model. The lifetime shown is the amplitude-weighted average of the decay components. The fluorescence was excited at 1200 nm, fluorescence was detected from 780 nm to 850 nm. As for the Methylene Blue, about 3 % of the available excitation power was sufficient to obtain a count rate on the order of 1 MHz.

The range of the lifetime and its variability is in agreement with FLIM data obtained by one-photon excitation [3, 5]. The results show that the common assumption of an essentially invariable fluorescence lifetime (and thus quantum efficiency) of ICG in biological systems is not correct.

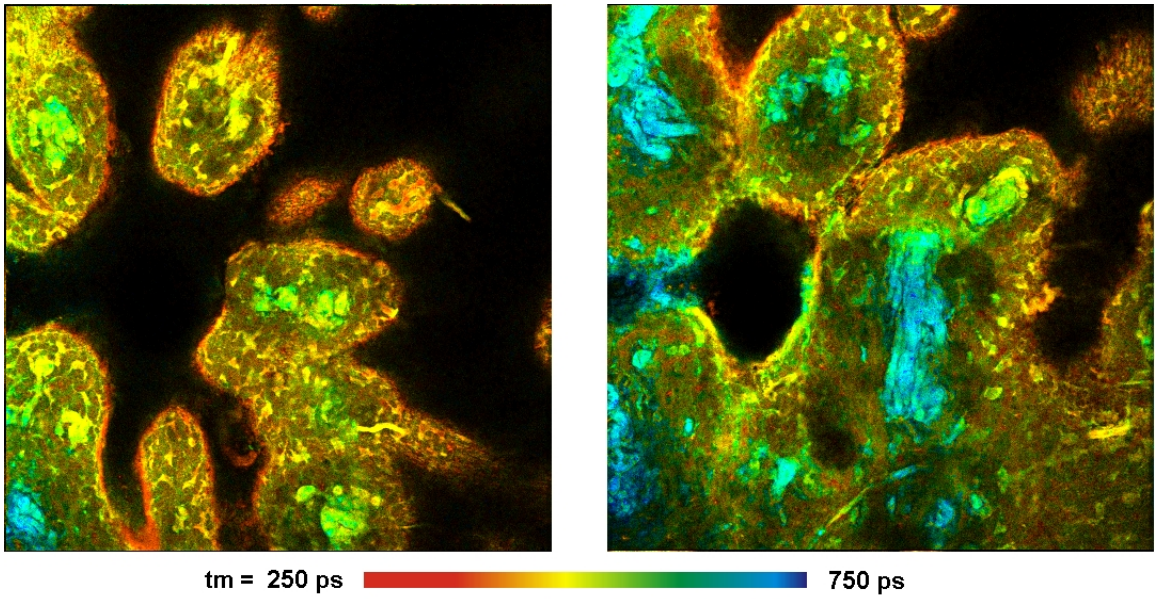

Fig. 2: Pig skin stained with Indocyanin Green. Two-photon excitation at 1200 nm, detection from 780 to 850 nm. Amplitude-weighted lifetime of double-exponential decay. Depth from top of tissue 10 µm (left) and 40 µm (right). 512x512 pixels, 256 time channels.

3,3'-Diethylthiatricarbocyanine (DTTCC)

A FLIM image of a pig skin sample stained with DTTCC is shown in Fig. 3. The excitation wavelength was 1200 nm, the fluorescence was detected from 780 nm to 850 nm. The excitation power was 2% of the available OPO power. The fluorescence lifetime is generally longer than for ICG. The general lifetime behaviour is the same as found in [3, 5]. The lifetime is short where the tissue was exposed to high dye concentration. However, there is also variability inside the tissue.

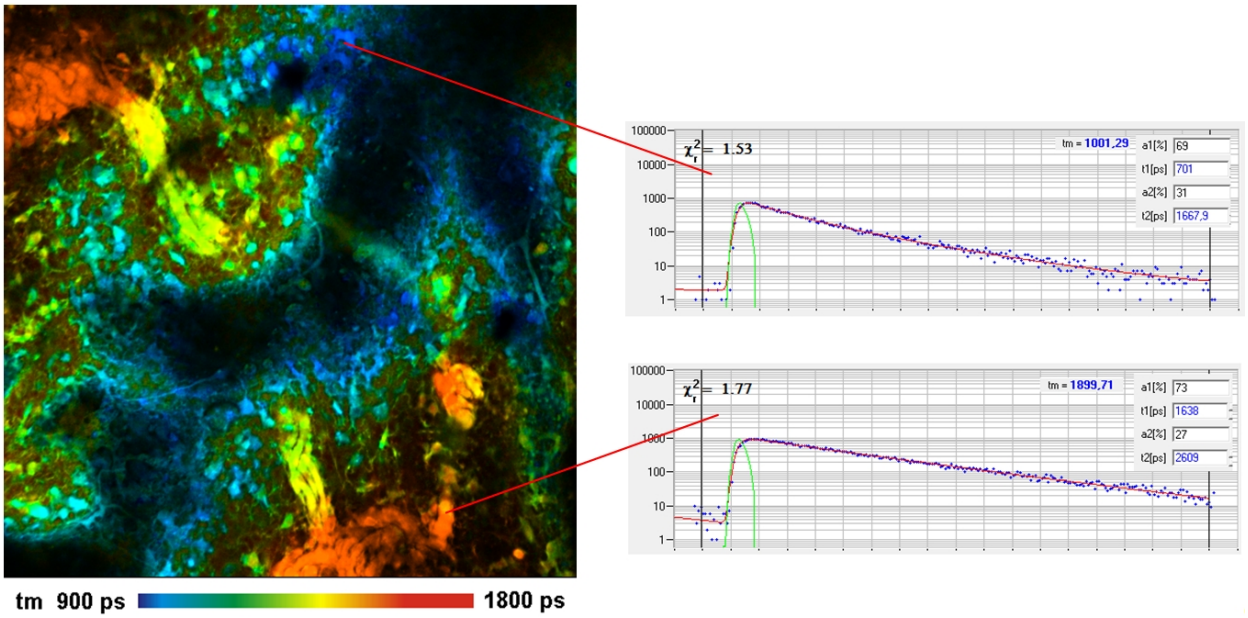

Fig. 3: Pig skin stained with DTTCC. Left: Lifetime image, amplitude-weighted lifetime of double-exponential decay model, 512 x 512 pixels, 256 time channels. Right: Decay curves in selected spots of the image.

FLIM Z Stacks

To explore the dependence of the fluorescence decay parameters on the staining conditions we recorded FLIM Z stacks for the samples shown above. The Z stacks were recorded by the Mosaic Imaging function of the SPCM 64-bit software [6]. Each Z stack contains 16 images from the surface of the tissue down to about 60 µm. The results are shown in Fig. 4 through Fig. 6.

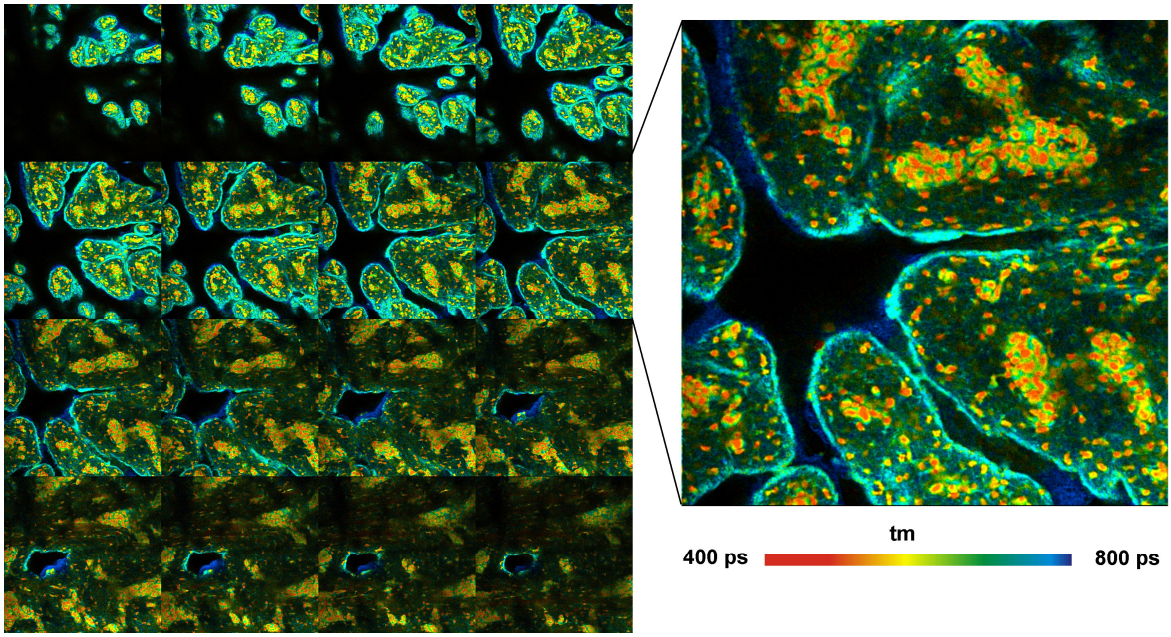

Fig. 4: Pig skin stained with Methylene Blue. Z-stack recorded by Mosaic FLIM, 16 planes from 0 to 60 µm from top of sample, each plane 512x512 pixels, 256 time channels. Amplitude-weighted lifetime of double-exponential decay. Plane 8 shown magnified on the right.

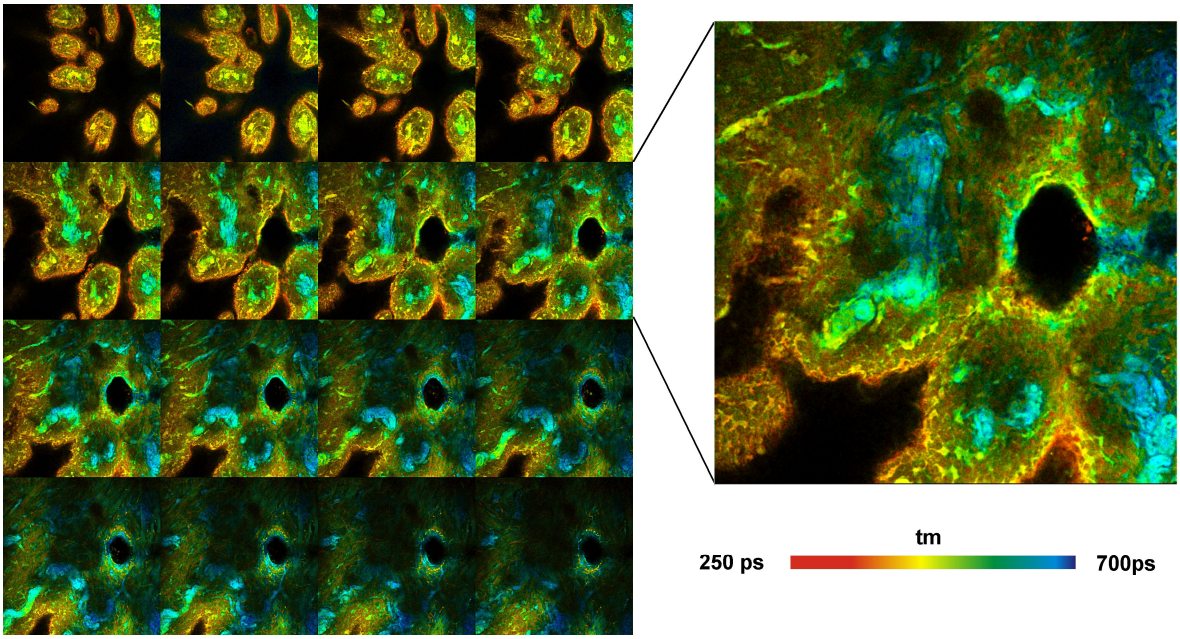

Fig. 5: Pig skin stained with Indocyanin Green. Z-stack recorded by Mosaic FLIM, 16 planes from 0 to 60 µm from top of sample, each plane 512x512 pixels, 256 time channels. Amplitude-weighted lifetime of double-exponential decay. Plane 8 shown magnified on the right.

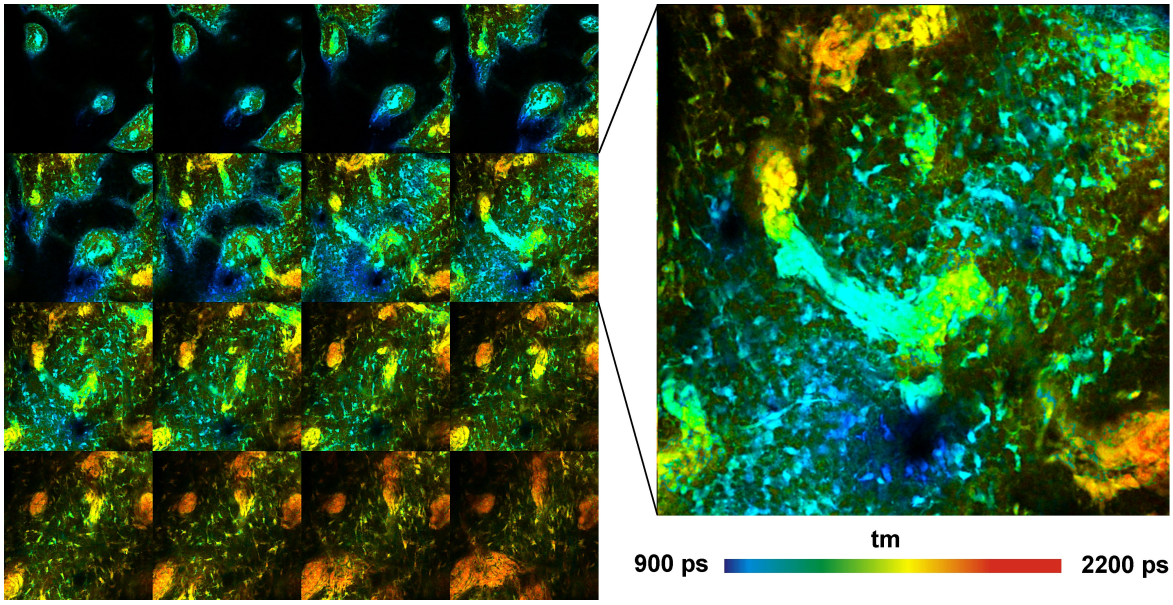

Fig. 6: Pig skin stained with DTTCC. FLIM Z-stack, recorded by Mosaic FLIM, 16 planes, 0 to 60 um from top of tissue. Each element has 512x512 pixels and 256 time channels per pixel. Amplitude-weighted lifetime of double-exponential decay. Plane 8 shown magnified on the right.

The Z stacks show that, in the samples investigated, the lifetime changes with the depth in the tissue. We used an incubation time of only 15 minutes. It is therefore likely that the change is caused by concentration changes or different binding efficiency due to incomplete incubation. We did not explore the dependence on incubation time and concentration. It appears, however, indicated to keep both under close control to obtain reproducible lifetimes.

Summary

We presented a multiphoton NDD FLIM system for deep-tissue lifetime imaging with near-infrared dyes. The system is based an a Zeiss LSM 7MP laser scanning microscope with OPO excitation, and a bh Simple-Tau 150 FLIM system with a HPM-100-50 near-infrared hybrid detector. Methylene Blue, Indocyanin Green (ICG) and 3,3'-Diethylthiatricarbocyanine (DTTCC) could easily excited with the OPO system. No more than a few percent of the available OPO power were needed. Lifetime images and FLIM Z stacks obtained from stained pig skin showed remarkable resolution of the tissue structure and surprisingly large variability in the fluorescence lifetime.

References

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