DCS-120 Confocal Scanning System: FLIM with NIR Dyes

Abstract: We used the DCS-120 confocal scanning FLIM system to test two typical near-infrared dyes, indocyanine green (ICG) and methylene blue, as markers in FLIM experiments. Using a 640 nm ps diode laser and near-infrared sensitive detectors, we were able to detect bright FLIM images from both dyes. Both dyes showed clearly detectable changes in their fluorescence decay parameters depending on the tissue structures they were bound to.

Motivation of using NIR Dyes

Near-infrared dyes are used as contrast agents and as fluorophores in diffuse optical imaging applications [5, 6]. Diffuse optical imaging (or diffuse optical tomography, DOT) reconstructs internal structures and tissue parameters from photons diffusing through thick biological tissue. It uses the fact that the scattering and absorption coefficients decrease with increasing wavelength. By using wavelengths between 650 and 900 nm information about the tissue condition can therefore be obtained up to a depths of about 5 cm. For these applications it is important to have information about binding of the dyes to proteins, DNA, collagen, and other cell constituents available. It is also important to know whether the dyes change their fluorescence lifetimes on binding, and whether these lifetime changes depend on the binding targets. Possible lifetime changes may interfere with the reconstruction of the tissue structure and tissue parameters from time-resolved data, but may also be exploitable to gain additional biological information.

Requirements to the FLIM system

FLIM measurements with NIR dyes require excitation in the 600 to 800 nm range, and detection in the range from 700 to 900 nm. These wavelengths are neither a problem on the excitation nor on the detection side. There is a wide range of suitable excitation sources: Picosecond diode lasers for the red and near-infrared range are available with 640 nm, 685 nm, and 785 nm. Also super-continuum lasers with acousto-optical filters or Ti:Sa lasers can be used. FLIM detection in the 700 to 900 nm range is easily achieved by bh HPM-100-50 hybrid detectors or by PMC-100-20 PMT modules.

Problems may, however, occur in the optics of the scanning system. The scan heads of laser scanning microscopes are not normally designed for excitation and detection in the NIR. The critical element is the main dichroic beamsplitter that separates the fluorescence from the excitation light. It must have a reflections-transmission curve that fits to the desired excitation and emission wavelengths. Replacing the main beamsplitter in the scan head of a laser scanning microscope is difficult or even impossible. The bh DCS-120 system is therefore available with a wideband (60/40%) beamsplitter [3, 4], and also the Zeiss LSM 710 has an 80/20 beam splitter in its beam splitter wheel. For the experiments described below we used a bh DCS-120WB (wideband) system with a HPM-100-50 hybrid detector.

ICG Experiments

For experiments with ICG (Indocyanin Green) we used samples of fresh pig skin. For staining the samples were immersed for 30 minutes in a 30 µm ICG solution. ICG has an absorption maximum around 870 nm. At concentrations higher than about 50 µm/l aggregates form which have an

absorption maximum at 690 nm. The fluorescence is emitted around 820 nm [7]. The small stokes shift between the absorption of the monomers and the emission makes it difficult to suppress scattered laser light and wideband spectral background of the laser [3, 4]. Fortunately, ICG has sufficient absorption down to 630 nm. We therefore used a 640 nm BDL-SMC ps diode laser for the experiments. This provides convenient spacing between the excitation and emission wavelengths, and avoides any filter leakage problems.

Results are shown in Fig. 1. The image on the left is from a focal plane about 20 µm below the top of the skin. The image on the right was taken from the back of the skin sample. Both images show the intensity-weighted lifetime of a double-exponential fit (t_i in SPCImage).

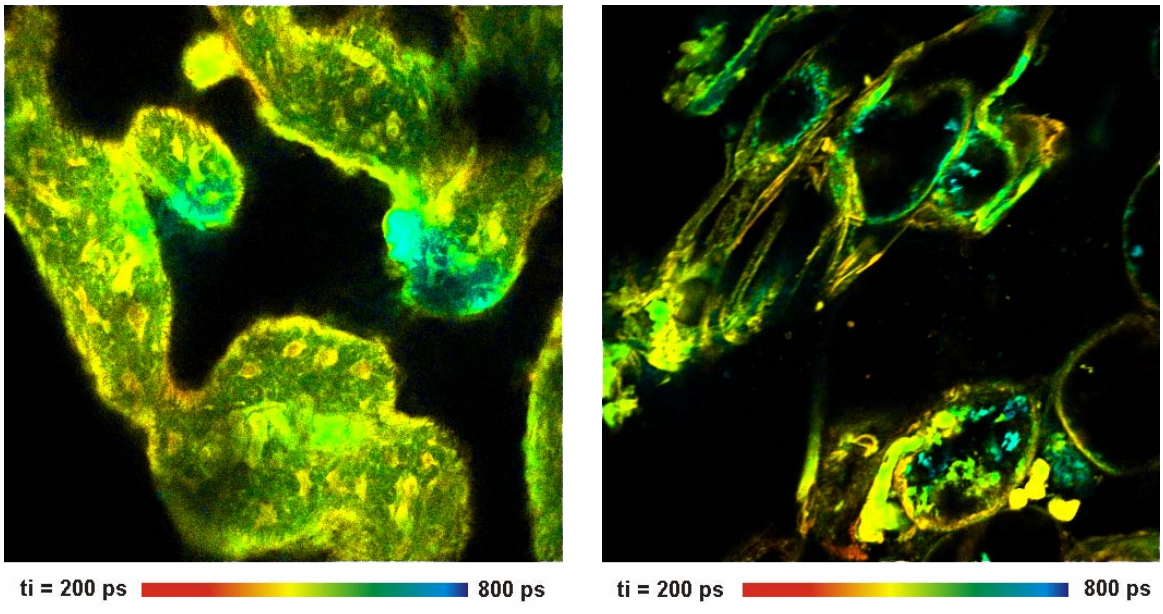

Fig. 1: Pig skin sample stained with ICG. Left: Image from the top of the skin, about 20 µm from the highest structures. Right: Image taken from the back of the skin sample. Fit with double-exponential model, intensity-weighted lifetime. FLIM data format 512 x 512 pixels, 256 time channels.

There are surprisingly large changes in the fluorescence lifetime throughout the sample. It is known that ICG has a lifetime of about 200 ps in water, and about 600 ps when bound to serum albumin. Typical fluorescence decay functions and the corresponding decay parameters are shown in Fig. 2. In the short-lifetime areas (yellow-orange) the data are marginally compatible with a mixture of bound and unbound ICG. In the long-lifetime areas (green) the decay functions are clearly incompatible with components of 200 ps and 600 ps. Two lifetimes, of about 470 ps and about 830 ps, were needed to fit the decay data, see Fig. 2, right.

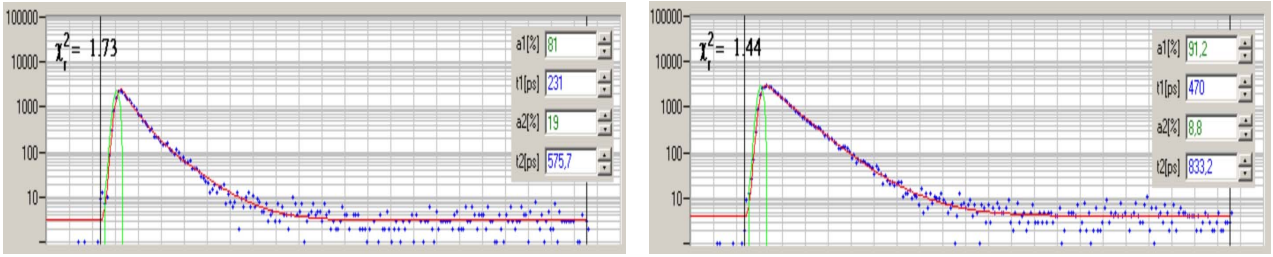

Fig. 2: Decay functions from selected 15x15 pixel areas of Fig. 1. Left: Short-lifetime areas (yellow orange). Right: Long-lifetime areas (green)

Methylene Blue

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. Methylene blue has been used as a drug against malaria and rheumatism. The use of methylen blue to treat Alzheimer disease is under clinical trial.

A lifetime image of pig skin stained with methylene blue is shown in Fig. 3, left. The decay data were fitted with a double-exponential model, the colour shows the amplitude-weighted average (t_m) of the decay components.

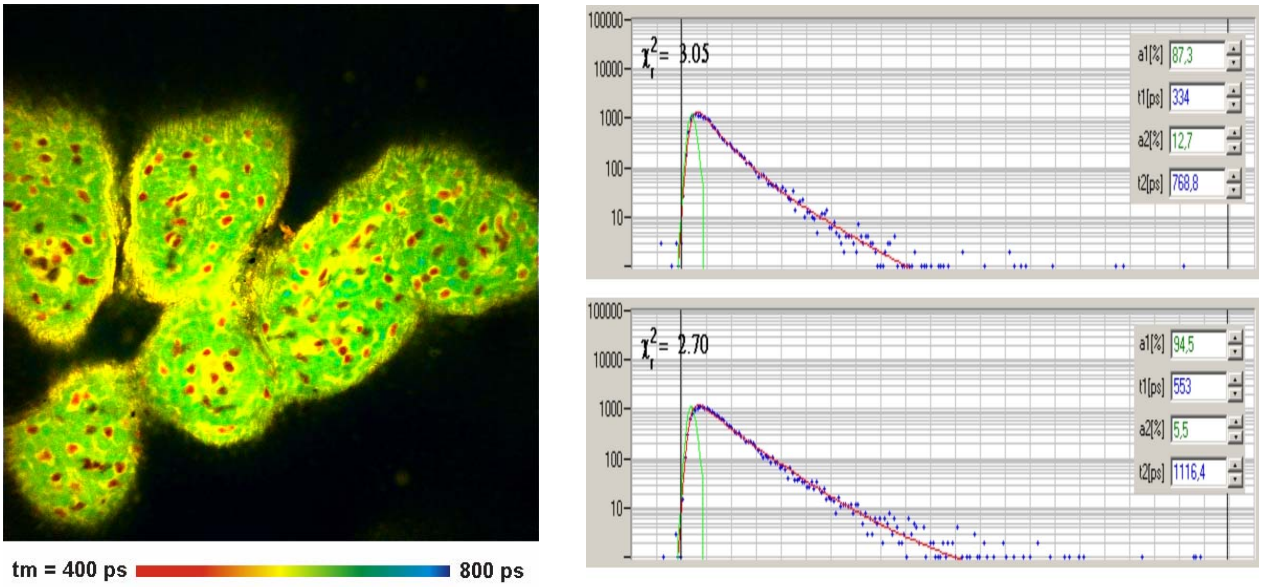

Fig. 3: Pig skin stained with methylen blue. Left: Lifetime image, double-exponential model, amplitude-weighted lifetime. Right: Decay curves in 3x3 pixel areas. Top: From red spots (fast decay). Bottom: From green areas (slow decay)

Also the methylen blue delivers different decay times depending on the local environment in the tissue. Both the lifetimes of the decay components and the amplitudes change. The exact mechanism of the lifetime changes is not known.

Summary

We have shown that FLIM with typical NIR dyes can be performed with the DCS-120WB confocal scanning FLIM system. Both ICG and methylene blue show clearly detectable changes in their decay behaviour depending on the tissue constituents they are bound to. The results show that even simple dyes may be probes for local environment parameters inside cells or tissues. Moreover, the results show that the assumption of an essentially invariable fluorescence lifetime of ICG in biological systems is not correct. The variability may have an impact on the reconstruction of tissue parameters in diffuse optical imaging experiments.

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