Ultra-Fast Fluorescence Decay in Malignant Melanoma

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Abstract: Using a multiphoton TCSPC-FLIM system with ultra-fast detectors, we found extremely fast fluorescence-decay components in a wide variety of biological material. Here, we report on FLIM of malignant melanoma. We found decay components with lifetimes, τ_1, from 10 ps to 20 ps, and with amplitudes, a_1, as large as 98%. The lifetimes and amplitudes are in sharp contrast to the decay parameters in healthy tissue (τ_1 = 185 ps, a_1 = 55%) and in material from benign pigmented lesions (τ_1 = 96 ps, a_1 = 45%).

Introduction

It is commonly believed that autofluorescence lifetimes of biological material are in the range from a few 100 ps to about 5 ns. This is supported by fluorescence-decay data of NADH, which exhibits a lifetime of about 400 ps for the free form and 3 ns for the protein-bound form, and of FAD, with about 250 ps for the bound form and 2 ns for the free form [6, 14]. Lifetimes of other endogenous fluorophores are in the same range, with fast decay components down to about 200 ps [16]. The fact that there are no faster decay times known may in part be due to the limited time resolution of the instruments. Commonly used FLIM systems have instrument response functions with a full width at half-maximum (fwhm) of about 250 ps (when using PMT detectors) and 100 ps (when using hybrid detectors with GaAsP cathodes) [1]. Faster fluorescence decay times may therefore have eluded attention, especially when they appeared as components of a multi-exponential decay.

Recently, Becker & Hickl GmbH Berlin, have introduced ultra-fast hybrid detectors and ultra-fast TCSPC-FLIM modules to their FLIM systems [1, 11]. In combination with femtosecond-lasers and two-photon excitation the systems deliver an IRF of <20 ps fwhm [1, 9, 10]. We have used the systems to look for extremely fast fluorescence decay processes in a variety of biological samples. The result was a surprise. Ultra-fast decay components were found in mushroom spores [2], pollen grains [3], in carotenoids [4], and even in Scottish whiskey [5]. Lifetimes were found as short as 10 ps, and amplitudes as large as 0.99. In mushroom spores, the amplitude and the lifetimes were strictly related to the colour [2]. An equivalent, yet not similarly strict relationship was found for pollen and plant tissue [3].

There have been earlier indications that short fluorescence lifetimes occur also in tissue of malignant melanoma. Decreased fluorescence lifetimes have been found by Dimitrow et al. [12, 13] and by Seidenari et al. [17]. With the limited time resolution of the instruments used, it was not possible to decide whether the reported lifetimes were the true lifetime of the melanoma tissue, whether the resulting decay functions were single- or multiexponential, and which of the decay components were the source of the change. It also remained an open question whether the lifetime changes were a result of a change in the bound / unbound ratio of NADH, as found by Pastore et al. [15], or a result of the presence of fluorophores with extremely short decay times. Having ultra-fast FLIM systems at hand, it was therefore a logical step to look for fast decay in tissue samples from malignant melanoma.

Experiment

For melanoma imaging we used a bh FLIM system attached to a Zeiss LSM 880 NLO multiphoton microscope in the inverted (Axio Observer) version [10]. HPM-100-06 hybrid detector modules (Becker & Hickl GmbH) were attached to the NDD port of the LSM 880 NLO via a Zeiss NDD beamsplitter module. The recording electronics consisted of two parallel SPC-150 N modules and a DCC-100 detector controller (all Becker & Hickl GmbH). Fluorescence was excited by two-photon absorption of the near-infrared femtosecond laser of the LSM 880 NLO, FLIM data were recorded by bh's multi-dimensional TCSPC technique [1]. The instrument response of the FLIM system is about 18 ps, full width at half maximum [7]. This is about 5 times faster than for systems with GaAsP hybrid detectors, and 10 times faster than for FLIM systems with conventional PMT detectors. Fast decay components thus become directly visible in the decay curves, without indirect evidence by deconvolution from an IRF wider than the decay time.

For imaging of benign skin lesions we used a similar system based on the bh DCS-120 scanner and a femtosecond fibre laser [11].

Fresh tumor samples were obtained from tumor surgery at the Institute of Transplantology, Nizhi Novgorod, and imaged within 1 hour after being excised. A 40x NA = 1.2 water immersion lens was used. To obtain a good match of the refractive index on the way to the sample and back the samples were placed in cell dishes and the space between the glass and the sample surface filled with buffer solution. The excitation wavelength was 750 nm, the detection wavelength interval from 435 nm 485 nm. A 690 nm short-pass filter of Chroma was used to block scattered excitation light. The back side of the sample was protected by a black cover to avoid daylight pickup and to avoid fluorescence light to be reflected back into the beam path from the condensor lens or the lamp reflector. Such reflections show up as nasty distortions in the decay data, especially when the decay functions contain fast decay components of large amplitude.

Data analysis was performed by bh SPCImage NG, using an MLE fit and triple-exponential decay models [8].

Results

FLIM results obtained from the melanoma are shown in Fig. 1 and Fig. 2. Fig. 1 shows a lifetime image of a vertical section through the tissue. Superficial layers are shown on the right in the image, deeper layers on the left. Colour coding shows the amplitude-weighted lifetime, tm, obtained by fitting the decay data by a triple-exponential model [1, 8]. As can be seen from the image, the lifetime is extraordinarily short in a layer close to the surface of the tissue. The lifetime is about 20 ps in the superficial layers (orange areas), and about 1200 ps in deeper layers (green areas). A closer inspection of the data shows that the short lifetime is caused by the presence of an extremely fast decay component. An image displaying the lifetime of the fastest component, t1, of the triple-exponential decay is shown in Fig. 2. Decay curves from selected spots of the image are shown on the right. The short value of t1 shows up clearly in the t1 image. It is visible as a sharp peak in the decay curve from the superficial tissue layer, see bottom, right. The fit delivers a lifetime, t1, of 13 ps and an amplitude, a1, of 98% for the fast component. The amplitude ratio, a1/(a2+a3), is about 57, which is unusually high for biological material. The peak is not present in the decay curve from deep tissue layers, see top right. The component lifetimes in these areas are in a more or less 'normal' range, and compatible with a mixture of NADH, FAD, and possibly FMN [6]. The parameters are t1 = 185 ps, a1 = 23.8%, a1/(a2+a3) = 0.31.

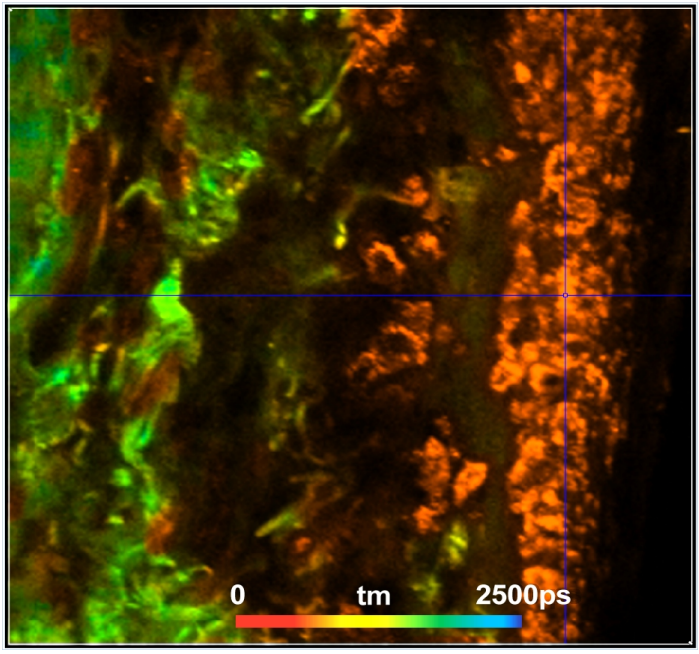

Fig. 1: Vertical section through melanoma sample, colour-coded image of the amplitude-weighted lifetime, tm, of a triple-exponential fit of the decay data. Red to blue corresponds to 0 ps to 2500 ps.

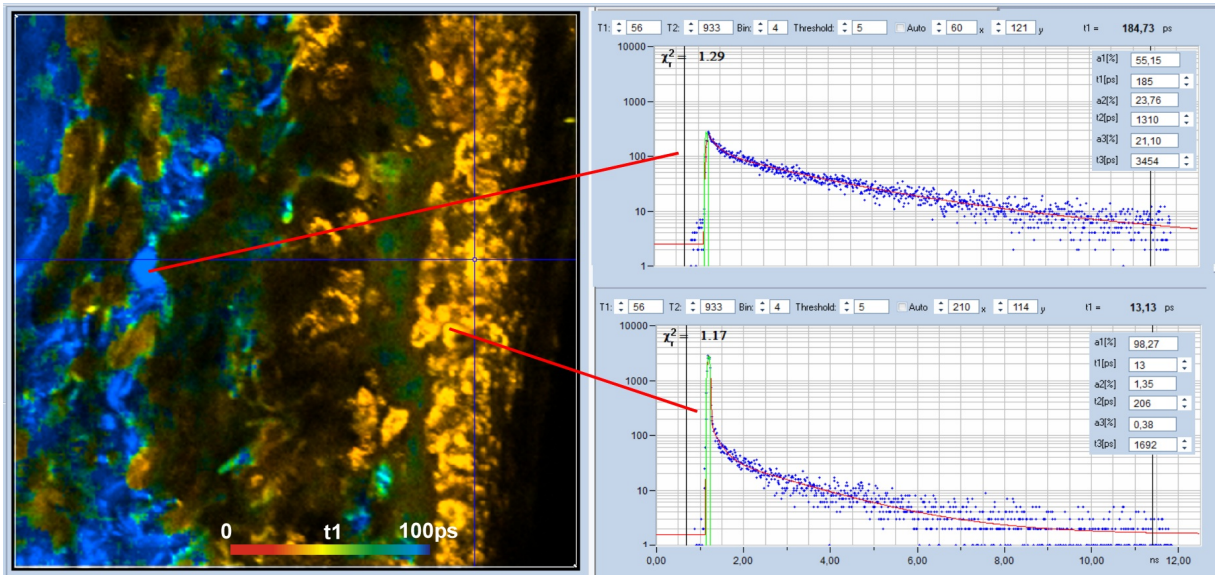

Fig. 2: Colour-coded image of the lifetime of the fast component, t1, of a triple-exponential fit of the data. Red to blue corresponds to 0 to 100 ps. Decay curves in characteristic spots of the image are shown on the right.

For comparison, Fig. 3 shows a FLIM image of a sample from a benign pigmented lesion recorded under similar conditions. A tm image is shown on the left, a decay curve from a selected spot on the right. As can be seen from the figure there is no ultra-fast component of high amplitude, as in the melanoma data. The fast decay component has a lifetime of 96 ps, and an amplitude of 45%. The amplitude ratio, a1/(a2+a3), is about 0.8, i.e. 70 times smaller than for the malignant melanoma.

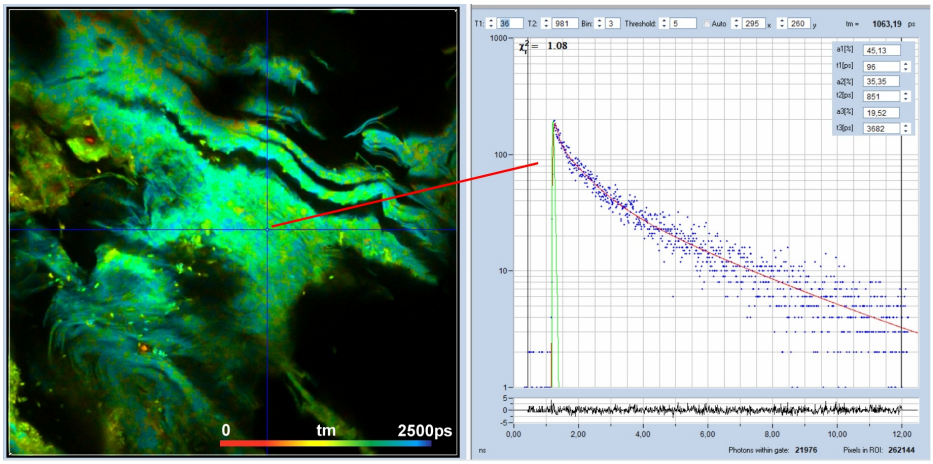

Fig. 3: Left: tm image of a sample from a benign pigments skin lesion. Red to blue corresponds to tm = 0 ps to 2500 ps. Right: decay curve in a selected spot of the image. There is no high-amplitude ultra-fast decay component.

Discussion of the Results

Fluorescence decay functions measured in malignant-melanoma tissue differ significantly from that in normal skin tissue, and from the tissue of benign pigmented lesions. The most striking difference is in the lifetime of the fast decay component, t1, and in the amplitude ratio of the fast component, a1/(a2+a3). The decay parameters can potentially be used to identify malignant melanoma, and to investigate their development mechanisms. Ultra-fast decay effects should therefore no longer be put aside as a peculiarity but considered as a potential source of biological information.

As for the origin of the fast decay component we can only speculate. It is reasonable to assume that it comes from special forms of melanin. A likely source are aggregates, forming by the high concentration of melanin in the melanoma. It is not unusual that aggregates exhibit extremely short fluorescence lifetimes, and it would not be surprising if the same effect occurred for melanin. However, why other material with high melanin content does not show the fast decay component remains an open question.

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