High-Resolution Multiphoton FLIM Reveals Ultra-Fast Fluorescence Decay in Human Hair

Wolfgang Becker, Cornelia Junghans, Becker & Hickl GmbH, Berlin
Vladislav Shcheslavskiy, Privolzhskiy Research Medical University, Nizhni Novgorod

Abstract: We used the bh DCS-120 MP multiphoton FLIM system to record fluorescence lifetime images (FLIM) of human hair. The system employs a femtosecond fibre laser for excitation, a galvanometer scanner, a non-descanned detection beam path, ultra-fast hybrid detectors and ultra-fast SPC-180NX TCSPC FLIM modules. The instrument-response function of the system has a width of <19 ps, FWHM. FLIM results obtained from human hair showed a dominating fluorescence-decay component of 8.5 ps lifetime in black and 14 ps in brown hair, but more or less 'normal' decay profiles in blond and grey hair. Decay data obtained from melanin solutions confirmed melanin as the source of the fast decay.

Ultra-Fast Fluorescence Decay in Biological Material

Using high-resolution FLIM with femtosecond excitation, fast hybrid detectors, and fast TCSPC / FLIM [1, 2] modules we have found ultra-fast fluorescence decay in a wide variety of biological materials. Examples are mushroom spores [4], pollen grains [5] and other plant tissue, carotenoids [6], melanoma tissue [7], and even Scottish whiskey [8]. It seems that almost any material with strong optical absorption and low or virtually no fluorescence emits such fast fluorescence components. The explanation is straightforward: If a compound absorbs light but does not emit fluorescence it must dissipate the energy via an extremely fast radiationless deactivation path. However, it can do so only at finite speed. That means the excited state is not instantly depleted - the radiationless decay just reduces the time the molecules stay in the excited state. That means there is fluorescence, but it decays at a very fast rate. Interestingly, the peak amplitude of the fluorescence intensity remains the same. With a detection system that has a sufficiently fast IRF this fluorescence can be detected. The situation is illustrated in Fig. 1.

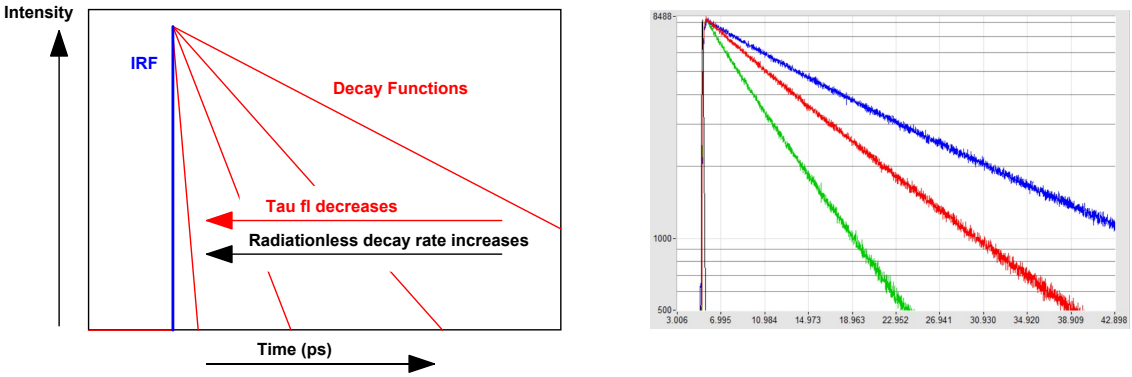

Fig. 1: An increasing radiationless decay rate decreases the fluorescence lifetime but does not change the peak intensity of the decay curve. With a sufficiently fast detection system the decay curve can be detected even in presence of extremely fast radiationless decay. Right: Practical example.

Here, we use our high-resolution DCS-120MP FLIM system to record extremely fast fluorescence decay in human hair. Fast fluorescence decay in human hair has been reported earlier. Ehlers et al.

found fast decay components of 0.4 ns and 0.2 ns in FLIM data of blond and black hair [10]. The authors further report on measurements with a fast MCP PMT. The measurements delivered lifetimes of 30 ps for eumelanin in black hair and 40 ps for DOPA melanin. All these lifetimes were in the range of the IRF widths of the instruments used. It could therefore not be told how short the lifetimes really were. With our fast TCSPC / FLIM systems there is chance that fluorescence lifetimes can be determined at reasonable accuracy even in the range below 10 ps.

High-Resolution FLIM System

The system we are using is a bh DCS-120 MP multiphoton FLIM system [2] with a femtosecond fibre laser [3] for excitation, a fast galvanometer scanner, a Nikon TE2000 inverted microscope, a non-descanned detection beam path, ultra-fast hybrid detectors and ultra-fast SPC-150NX or SPC-180NX TCSPC FLIM modules [1]. The principle is shown in Fig. 2.

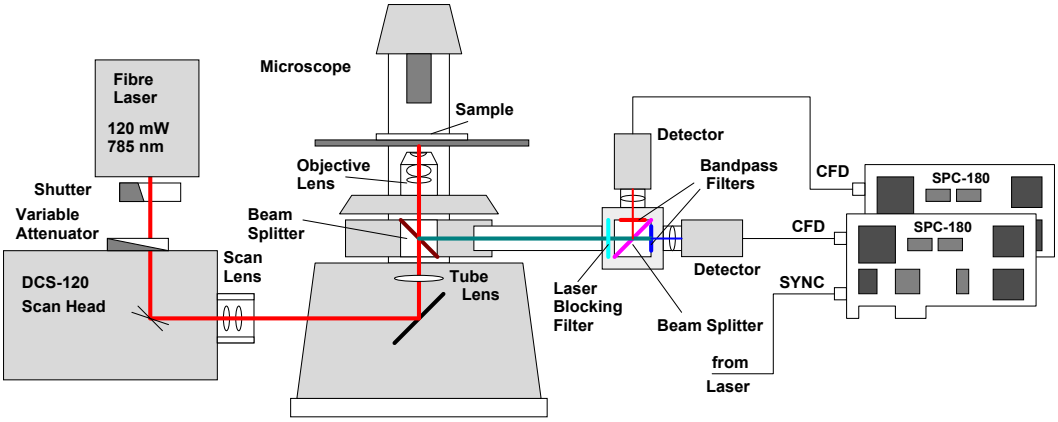

Fig. 2: Principle of the DCS-120 MP FLIM system

The optical system does not contain any elements that introduce pulse dispersion on a time scale longer than a few 100 femtoseconds. There are no optical fibres, no large lenses with large wavefront aberrations, and no Fresnel lenses. The timing jitter in the TCSPC modules is <3.5 ps, FWHM [1]. Therefore, the IRF width is almost entirely determined by the transit-time spread in the detectors, which is about 16 to 18 ps. A typical IRF of the system is shown in Fig. 3.

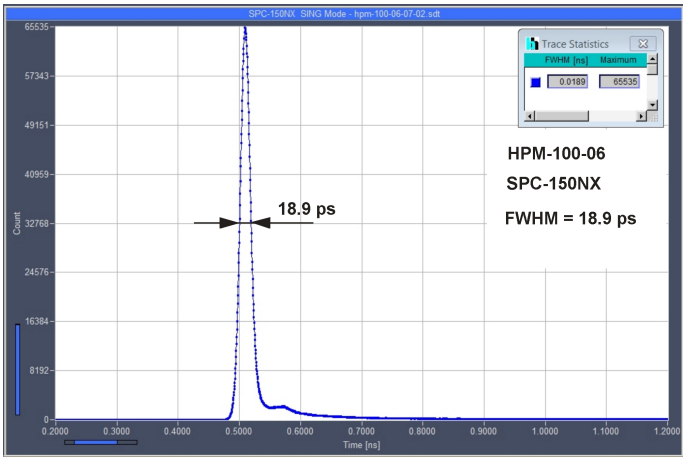

Fig. 3: IRF of the DCS-120 MP system with the ultra-fast hybrid detector. The FWHM of the IRF is 18.9 ps

With an IRF width this short, fluorescence decay times of less than 10 ps can be detected, even when these are components of a multi-exponential decay [9].

The entire DCS-120 system is controlled by bh SPCM data acquisition software. Operation mode is 'FIFO Imaging'. We used a FLIM format of 512 x 512 pixels and 1024 time channels.

FLIM of Human Hair

Imaging Protocol

A Nikon TE 2000 inverted microscope with an NA=1.3, 63x, oil immersion microscope lens was used. The hair samples were placed on microscopy slides, immersed in oil, and covered with full-size 0.18 mm cover slips. The full-size cover slips adhere well to the slides, and the entire stack can easily be placed on the microscope stage. After being placed on the stage, the samples were brought into focus visually via the eyepieces. After visual focusing, the beam path of the microscope was switched to the side port and the filter carousel rotated as to place the NDD beamsplitter in the beam path [2]. Then the 'Preview' function of the scanner panel in the SPCM software was started, and the focus fine-tuned by inspecting the two-photon fluorescence image on the computer screen. The laser intensity was adjusted to obtain no more than 100,000 photons / second on the detector. This count rate may appear ridiculously low, and it is indeed much lower than the FLIM system can record. It should be noted, however, that the fluorescence is strongly quenched. That means, the largest part of the absorbed light is converted into heat. Moreover, the samples, especially the darker ones, have noticeable absorption at the fundamental wavelength of the laser. Sample damage by thermal effects is therefore a real possibility. After focusing, the recording was started by pushing the 'Start' button of the SPCM scanner panel. The recording was continued until about 1000 photons in the brightest pixels were recorded [2]. The data were then saved and analysed by bh SPCImage NG data analysis software. All data were analysed by maximum-likelihood estimation (MLE) and with a triple-exponential incomplete-decay model.

Results

The images shown in Fig. 4 to Fig. 6 were recorded by the protocol described above. Only one channel of the system was used, with the entire light being fed to the detector in the 0° beam path. An SP700 short pass filter was used to block scattered laser light, a 420 nm long pass filter to block SHG signals. A FLIM image of black hair is shown in Fig. 4. A t_m (amplitude-weighted lifetime) image is shown on the left. The colour scale is from 20 ps (red) to 500 ps (blue). A decay curve at the cursor position is shown on the right, the insert shows the amplitudes and lifetimes of the decay components. Already at first glance, it is evident that the decay is very fast. SPCImage data analysis shows that the dominating decay component has a lifetime of 8.5 ps and an amplitude of almost 96%. The amplitude-weighted average decay time, t_m, is 19.3 ps.

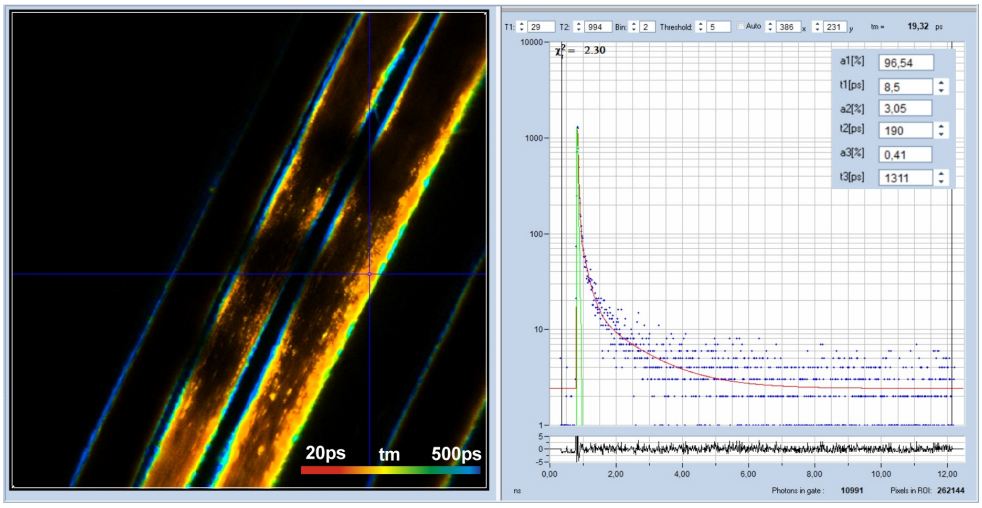

Fig. 4: Black hair, lifetime image (left) and decay function at cursor position (right). Decay components shown in the insert upper right. Triple-exponential analysis by SPCImage NG. The fastest component, t_1, has a lifetime of 8.5 ps and an amplitude of 96.5%.

Fig. 5 shows brown hair (bottom) and grey hair (top) from the same donor. Decay curves and decay components are shown upper and lower right. The brown hair has a fast decay component, t_1, of 14.2 ps lifetime and 96.5% amplitude, which is only slightly slower than for the black hair in the figure above. The fast component is not present in the grey hair, see decay curve upper right. Here, the fast component, t_1, has a lifetime of 180 ps, with an amplitude of only 45.5%. The amplitude-weighted mean lifetimes, t_m, of the brown and the black hair are 68 ps and 1300 ps, respectively.

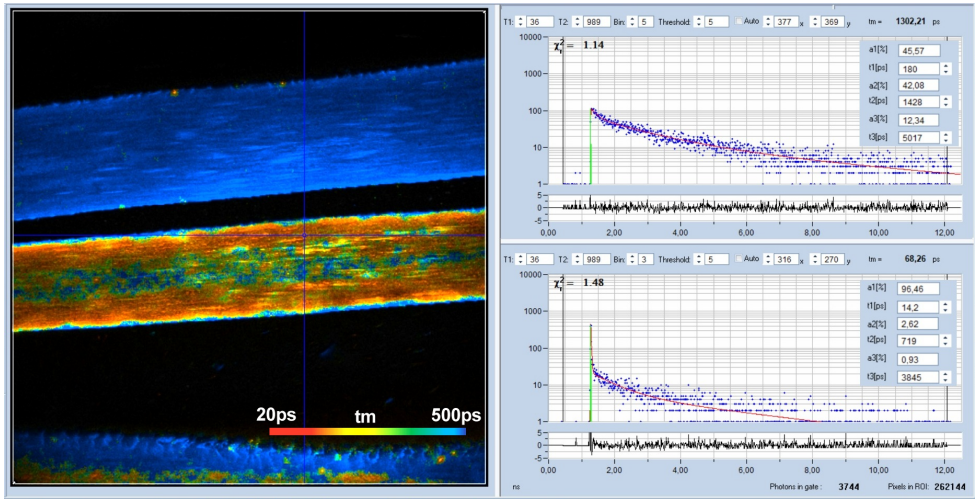

Fig. 5: Brown hair (bottom) and grey hair (top) from the same donor. Decay curves and decay components are shown upper and lower right.

Blond hair is shown in Fig. 6. The entire decay is much slower than for the black and brown hair. The lifetime of the fast component, t_1, is 54 ps, and the amplitude 58%. This is fast for average FLIM standard, but by far not as fast as for the black and brown hair. The amplitude-weighted mean lifetime, t_m, at the cursor position is 782 ps. Also this is much longer than in the figures above. Please note the different colour scale of the image.

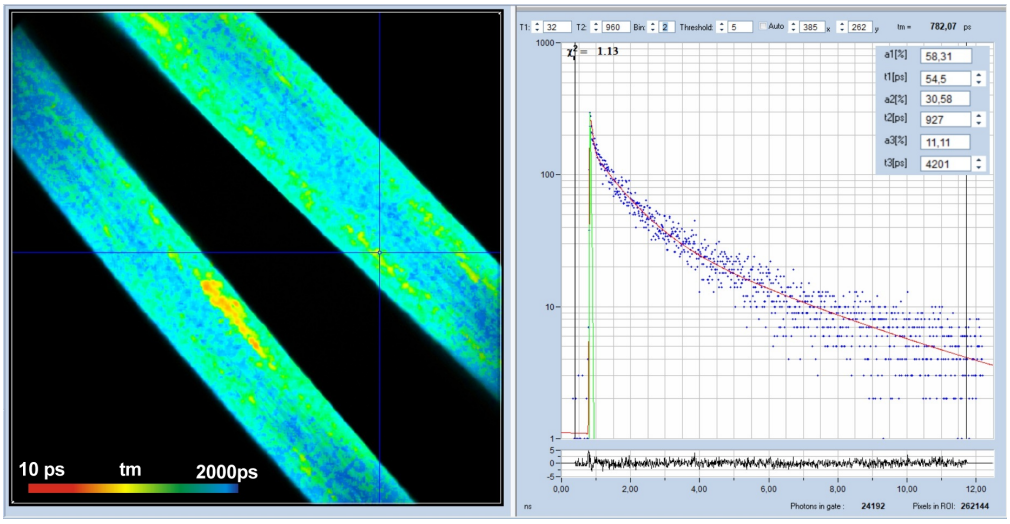

Fig. 6: Blond hair. Left: Lifetime image, tm = 10 ps to 2000 ps. Right: Decay curve at cursor position.

The results shown in Fig. 4 to Fig. 6 do, of course, suggest that the ultra-fast decay component comes from melanin. We therefore recorded fluorescence decay data from synthetic melanin. The melanin was purchased from Thermo Fisher. It was dissolved in 1 n NH_4OH, and the solution was scanned with the DCS-120 MP system. The measurement was performed at 408 femtoseconds per time channel, which is the highest time resolution available for the SPC-180NX. The data were combined into a single decay curve. The result is shown in Fig. 7. The curve has an FWHM of 17.9 ps, which is even a bit less than the system IRF, see Fig. 3. That means the dominating fluorescence decay time must be significantly shorter than 5 ps. This makes it plausible that the fast decay component in the hair indeed comes from melanin.

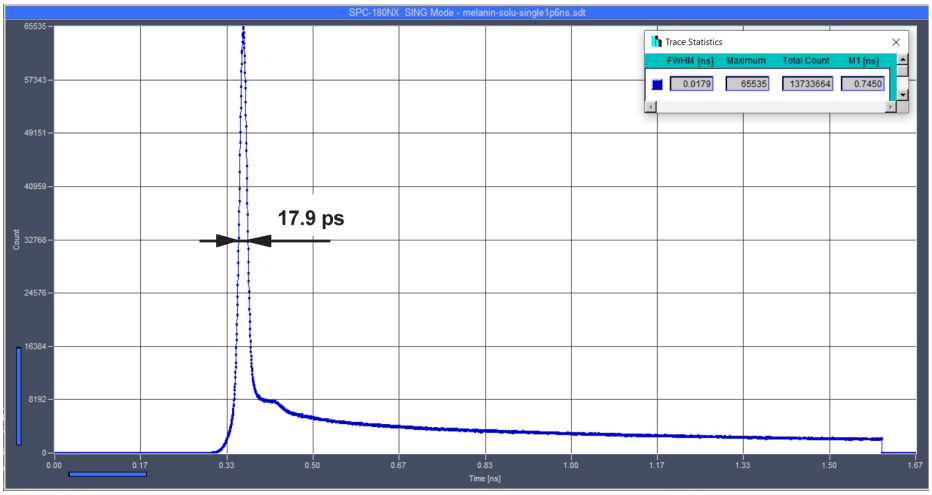

Fig. 7: Fluorescence decay curve of a melanin solution. Time-channel width 408 femtoseconds. The fast peak at the beginning is undistinguishable from the system IRF.

Conclusions

Using a DCS-120 MP FLIM system with ultra-fast detectors, we found fluorescence decay components of less than 10 ps lifetime in human hair. The lifetimes and the amplitudes of the fast component vary for different hair colour. Lifetimes of less than 10 ps and amplitudes of 95 % were

found in black hair. For brown hair the lifetimes were slightly longer but still remained in the sub-20 ps range. In grey hair and blond hair we did not find a similarly fast decay component. The findings are in agreement with [10]. The fast decay components are attributed to melanin (eumelanin). Grey and blond hair contains very little eumelanin, which explains that the ultra-fast components are not prominent or even missing. The fluorescence is then expected to come from keratin, with a lifetime on the order of 1 ns. We further support the melanin hypothesis by measurements of melanin solutions. In solution, the fluorescence decay was so fast that it could not be resolved even by the DCS-120 MP system. That means the dominating decay component must have a lifetime of less than 5 ps. The differences between the decay parameters in hair and in solution are not surprising. First, melanin does not have a very rigid molecular structure. The fluorescence lifetime may therefore change with the molecular environment. Moreover, the molecular structure of melanin can vary by binding different end groups which also can influence the decay time.

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