Two-Photon FLIM of Mushroom Spores Reveals Ultra-Fast Decay Component

Wolfgang Becker, Cornelia Junghans, Axel Bergmann, Becker & Hickl GmbH

Abstract: We performed FLIM on the spores of a variety of mushrooms that are commonly found in the middle-European and north-American area. Using our DCS-120 MP FLIM system with ultra-fast detectors, we found extremely fast components in the decay functions. The decay times ranged from about τ_1 = 8 ps to 80 ps, with amplitudes a_1 up to 99.5%. The decay times and the amplitudes correlate with the colour of the spores. The darker the spores are the more pronounced the fast component is. We attribute the mechanism to an extremely efficient energy-transfer process, without being able to tell what exactly the mechanism might be.

Motivation

Mushrooms belong to the oldest organisms on earth. By recycling organic material, they are an integral part of the ecological system. Moreover, many mushrooms live in symbiosis with trees. This symbiosis is of vital importance not only for the mushrooms but also for the trees, which do not grow well without the mushrooms. The spores of mushrooms remain viable under harshest conditions. They can rise up to the stratosphere and literally travel around the planet. Doing so, they survive heat, extreme cold, extreme exsiccation, and UV radiation. The resistance of the spores to UV radiation is particularly interesting. Considering the small size there is essentially no conceivable way to efficiently block UV light from the inner part of a spore. This raises the question whether there possibly is another protection mechanism at work. A possible hint to that mechanism could be turned up by fluorescence-lifetime measurements. We therefore performed FLIM on the spores of a variety of mushrooms commonly found in Europe and North America. A few examples are shown in Fig. 1.

Fig. 1: A selection of mushrooms the spores of which were investigated by FLIM. Left to right: Amanita muscarina, Agaricus campestris, Coprinus comatus, Boletus edulis, Hypholoma fasciculare.

Experiment Setup

From earlier experiments we knew that fluorescence lifetimes of mushroom spores can be in the sub-100-ps range. Typical one-photon confocal systems with diode-laser excitation then show an apparent lifetime but do not reliably resolve the decay functions into individual decay components. Therefore, we used our DCS-120 MP multiphoton system. The instrument is based on fast beam scanning, two-photon excitation with a femtosecond fibre laser (Toptica Femto Fibre Pro) and detection by ultra-fast hybrid detectors (bh HPM-100-06) [2, 4]. The data were recorded with by bh's multi-dimensional TCSPC technique (bh SPC-150NX TCSPC FLIM modules). Please see [1, 2] for details. The instrument response of the DCS-120 MP system is about 18 ps, full width at half

maximum. This is about 5 times faster than for a typical diode-laser based system with GaAsP hybrid detectors, and 10 times faster than for FLIM systems with conventional PMT detectors.

Sample Preparation and FLIM Procedure

Pieces of fresh mushroom caps were placed over 180 µm thick microscope slides to collect the spores. For FLIM measurement, the slides were placed on the sample stage of an inverted microscope. To obtain maximum resolution and photon collection efficiency we used a microscope lens with oil immersion, NA = 1.3 numerical aperture, and 40x magnification. The emitted photons were recorded via the non-descanned detection path of the DCS-120 MP system. A Chroma SP 700 short-pass filter was used to reject scattered laser light, and a 400-nm long pass filter to suppress possible SHG light. Consequently, fluorescence was detected from about 400 nm up to the upper detection limit of the detector, which is about 650 nm.

We admit that, from the point of optics, the configuration of the sample is not entirely correct. For best resolution, the spores should be embedded in a medium with a refractive index close to 1.3. However, attempts to image the spores in a solid environment failed because of fluorescence of the embedding medium. Attempts to image the spores in water or immersion oil failed because the laser beam induced motion in the sample. The best and only way to run the experiments was to image the bare spores on the glass from the back of the slide, as described above.

Another problem was that the spores, especially those of brown or black colour, absorb at the fundamental wavelength of the excitation laser. They are therefore easily destroyed by the laser. To avoid any scanning artefacts, we kept the laser power below 2 mW, in some cases even below 1.5 mW. Due to the low laser power the photon detection rate was no higher than 200,000 counts per second for white spores and no higher than 20,000 counts per second for dark spores. Consequently, the data acquisition times were relatively long, typically in the range from one to ten minutes. The long acquisition time was no problem, however, because our system has excellent timing stability and a detector background count rate of no more than 60 counts per second [3].

Results

Fig. 2 shows FLIM data of spores of Amanita Muscarina. Amanita muscarina has white spores. A colour-coded image of the mean lifetime (amplitude-weighted lifetime of triple-exponential model) is shown left, a decay curve integrated over the pixels of a single spore is shown right. The data do not show any surprise. The mean lifetime is in the range of 1 ns to 2 ns. The lifetimes and amplitudes of the components are compatible with NAD(P)H or FAD, or a mixture of both. No matter whether or not the fluorescence comes from these compounds, the decay function is similar to the decay functions found in other live matter.

FLIM data of spores of Agaricus campestris are shown in Fig. 3. In contrast to Amanita muscarina, the spores of Agaricus campestris are dark red-brown. The mean lifetime is in the sub-20 ps range. The real surprise comes with the decay function. It is dominated by an ultra-fast component, with a lifetime on the order of 10 ps, and an amplitude of 99.1%. The other two components are almost entirely suppressed in favour of the fast component.

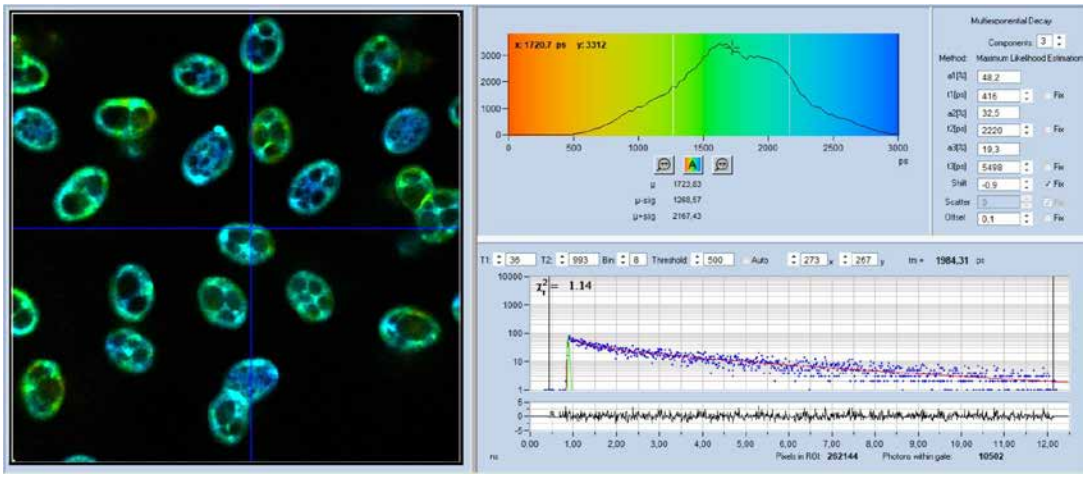

Fig. 2: Decay data of Amanita muscarina. Lifetime image of mean decay time shown left, lifetime range 0 to 3000 ps. Decay function shown lower right. lifetime histogram and decay parameters upper right. Field size is approximately 80 x 80 µm.

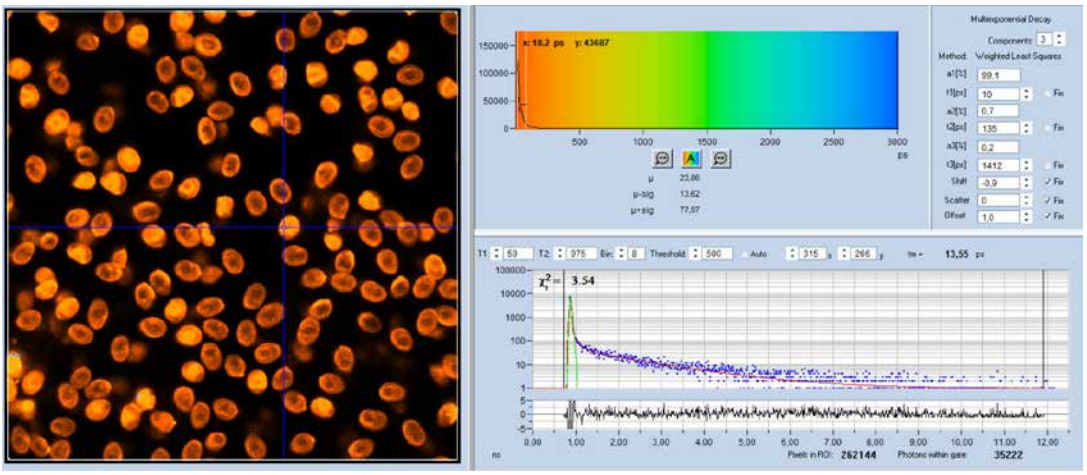

Fig. 3: Decay data of Agaricus campestris. Lifetime image of mean decay time shown left, lifetime range 0 to 3000 ps. Decay function shown lower right. lifetime histogram and decay parameters upper right. Field size is approximately 125 x 125 µm.

It could be argued that the fast component may be SHG or insufficiently blocked laser light. However, an additional laser blocking filter in front of the detector neither changed the signal intensity nor the shape of the decay curves. An additional long-pass filter with 450 nm cutoff wavelength reduced the photon rate but did not change the decay curves noticeably. Therefore leakage of laser light or SHG can be excluded as a source of the effect. The ultra-fast component is real.

To get a clue on the mechanism of the fast emission we did FLIM on spores of a variety of other mushrooms. The results revealed two interesting facts. First, there is a continuous transition from the 'normal' decays to the decays with an ultra-fast component. Second, the lifetime of the fast component is the lower and the amplitude the higher the darker the spores are. Simultaneously, the intensity of the slow components decreases. The results are listed in Table 1 and Table 2.

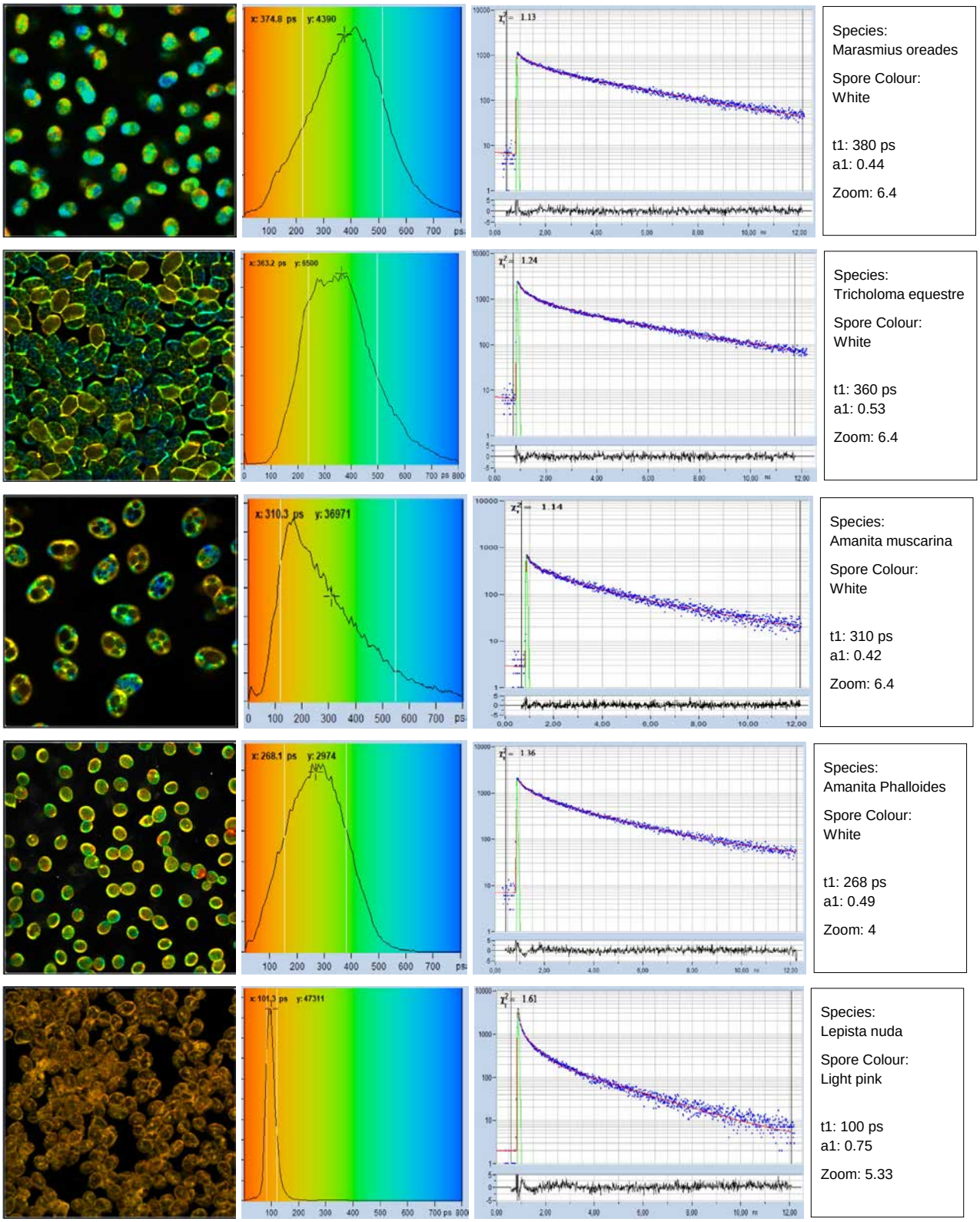

Table 1: Spores with lifetimes, t1, of the fast component from 360 ps to 100 ps, in order of decreasing t1. Left to right: t1 lifetime images (red to blue = 0 to 800 ps), distribution of t1 over pixels, decay curve in selected spot, and text field describing species, spore colour, t1 an a1, and zoom factor used for the recording. Field size is 500 µm / Zoom.

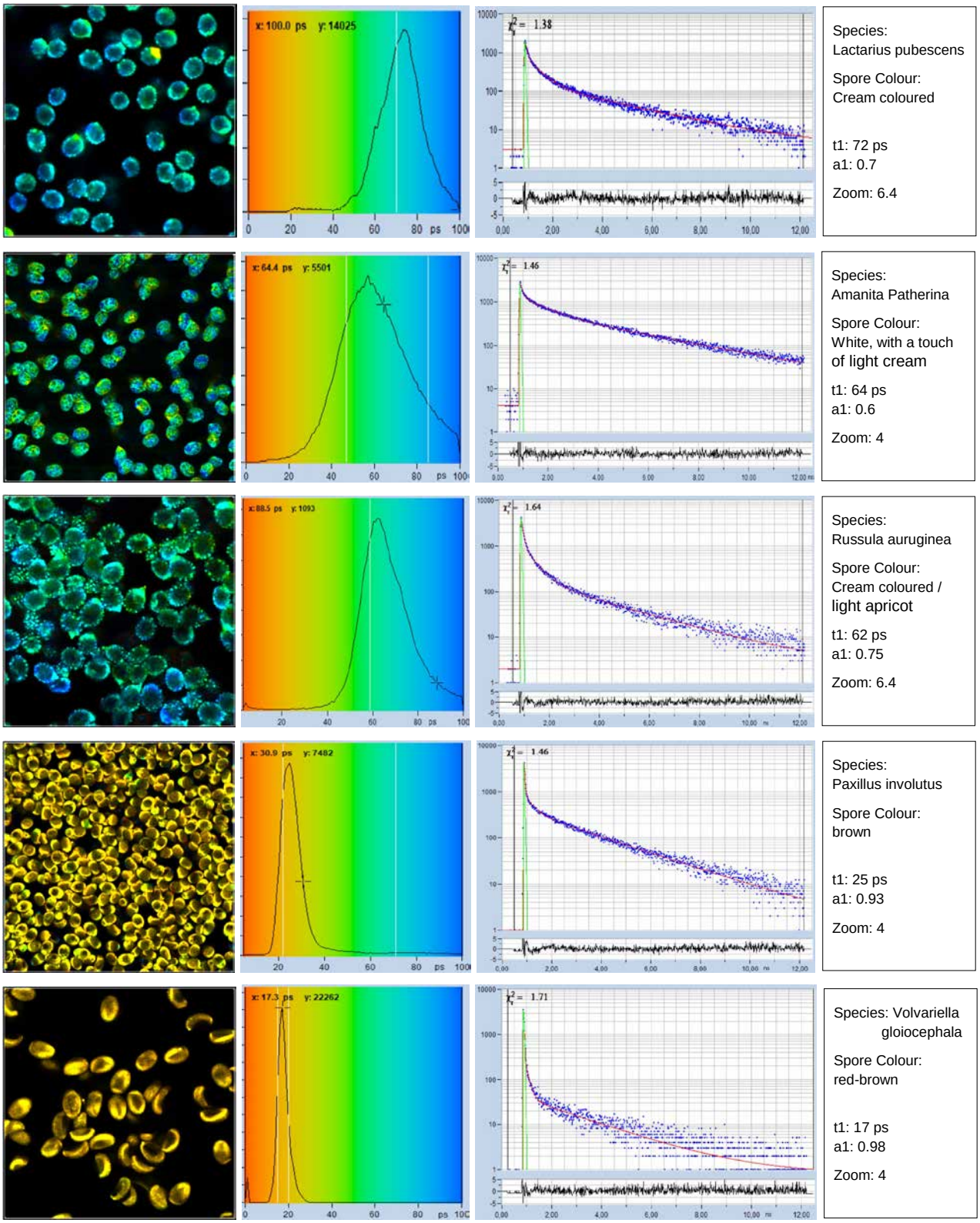

Table 2: Spores with lifetimes, t1, of the fast component below 100 ps, in the order of decreasing t1. Left to right: t1 lifetime images (red to blue = 0 to 100 ps), distribution of t1 over pixels, decay curve in selected spot, and text field describing species, spore colour, t1 an a1, and zoom factor used for the recording. Field size is 500 µm / Zoom.

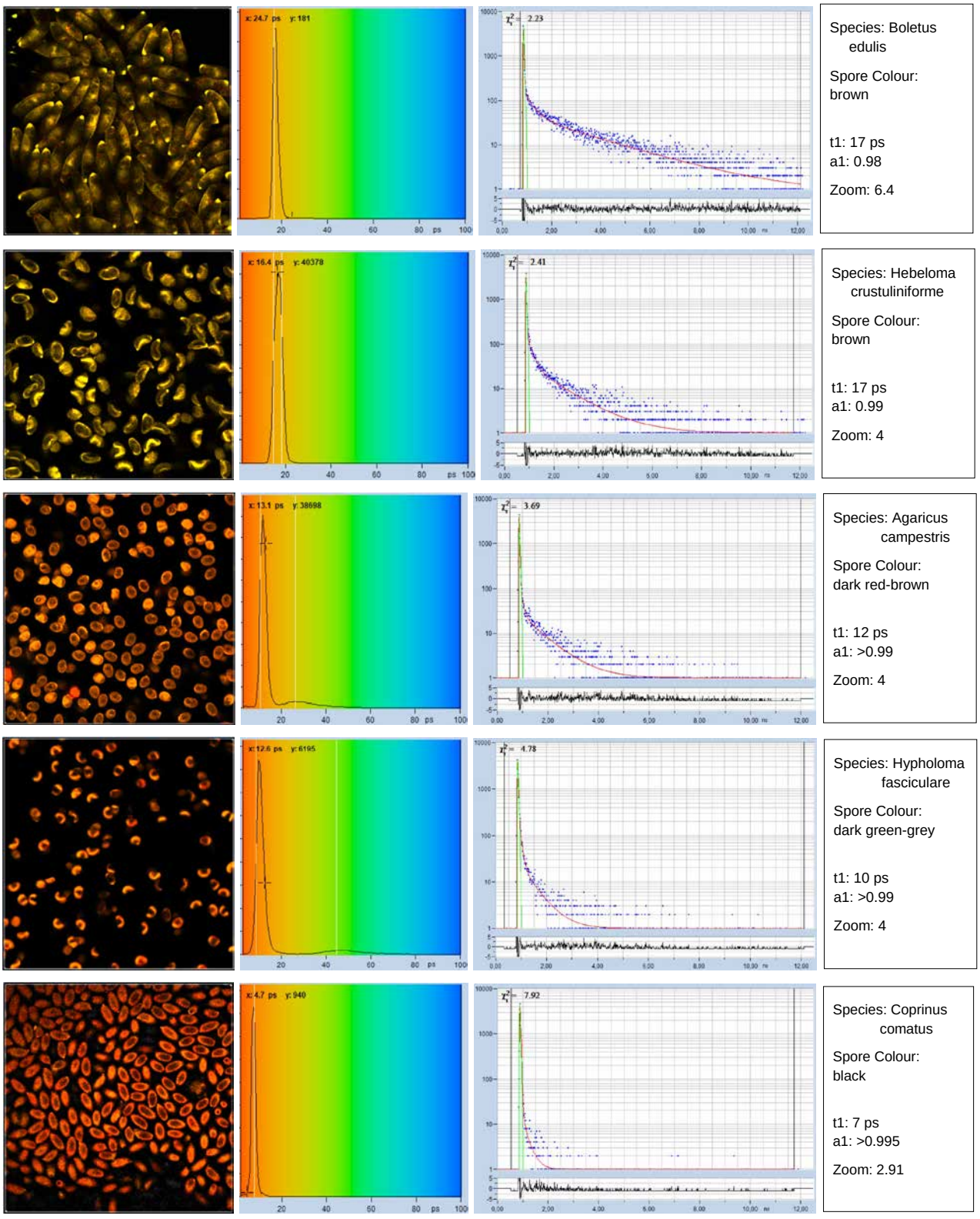

Table 2, continued: Spores with lifetimes, t1, of the fast component below 100 ps, in the order of decreasing t1. Left to right: t1 lifetime images (red to blue = 0 to 100 ps), distribution of t1 over pixels, decay curve in selected spot, and text field describing species, spore colour, t1 an a1, and zoom factor used for the recording. Field size is 500 µm / Zoom.

Interpretation of the Results

The decay functions found in these experiments are unique and absolutely unusual. There is no known fluorophore which has a fluorescence lifetime in the range of 10 to 20 ps. Such short lifetimes can occur only if an extremely strong quenching process is at work. This raises the question of what this process may be. In principle, one could presume that the absorber present in the darker spores is fluorescent but strongly quenched by some intramolecular deactivation process. The fact that the fluorescence lifetime is related to the concentration of the absorber could be explained by aggregation. The size of the aggregates certainly would depend on the concentration of the absorber, and short lifetimes of aggregates are not unusual. However, this model does not describe the observed decrease in intensity of the slow lifetime components with increasing absorber concentration. Of course, there should be an intensity drop simply due to increasing absorption both for the excitation light and the fluorescence. However, the intensity of the slow components decreases by several orders of magnitude even for moderately coloured spores. Absorption is thus unlikely to account for the full amount of intensity decrease.

An alternative model that describes the results without these problems is inter-molecular energy transfer. Energy would be transferred from the 'normal' fluorophores (acting as a donor) into a non-fluorescent absorber (acting as an acceptor). The result would be a quenching of the 'normal' fluorescence, causing a decrease in fluorescence lifetime, and a decrease in intensity. The lifetime of the remaining fluorescence would depend on the coupling efficiency. If not all of the fluorophore molecules interact with the absorber (which is not unusual) some of the original fluorescence would remain. The fraction of non-interacting molecules would depend on the absorber concentration. This explains the decrease in the amount of remaining 'normal' fluorescence with increasing absorber concentration. Problems remaining are the extremely high energy transfer rate needed to explain the short lifetime, and the gradual change in the decay time of the fast component with the spore colour. If FRET is assumed to be the source of the massive lifetime decrease the FRET efficiency must be on the order of 0.99 and more. Such high FRET efficiencies have not yet been seen. The gradual change in the lifetime of the fast component with the spore colour requires that also the FRET efficiency changes gradually. This is no problem if it is assumed that the absorbers in different species have different absorption coefficients. Moreover, it is not unusual that the coupling efficiency gets higher for higher acceptor concentration. The reason is that simultaneous interaction with several acceptor molecules becomes more likely. Finally, there is possibly another, even more elegant explanation. If the absorber increasingly forms aggregates with increasing concentration the energy transfer efficiency could increase dramatically, causing a similarly dramatic decrease in lifetime.

Altogether, this makes the energy transfer mechanism more likely than the pure absorption mechanism. And, finally, if nature has implemented a protection mechanism in mushroom spores it would probably have developed one that not only blocks the light from the vital constituents but also pulls out the energy from them.

A final decision between the two models could be facilitated by spectral measurements. The problem of such measurements is that there is no spectral FLIM detector which comes anywhere near to the time resolution of the HPM-100-06 hybrid detector. Therefore, spectral experiments have to be performed by subsequent measurements through different narrow-band filters. These experiments will probably have to wait until the next mushroom season.

References

1. W. Becker, The bh TCSPC handbook. 8th edition (2019), available on www.becker-hickl.com
2. Becker & Hickl GmbH, DCS-120 Confocal and Multiphoton Scanning FLIM Systems, user handbook 8th ed. (2019). Available on www.becker-hickl.com
3. Becker & Hickl GmbH, The bh TCSPC Technique. Principles and Applications. Available on www.becker-hickl.com.
4. Becker & Hickl GmbH, Two-Photon FLIM with a femtosecond fibre laser. Application note, available on www.becker-hickl.com

Contact:

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