

Time-gated fluorescence spectroscopy of porphyrin derivatives and aluminium phthalocyanine incorporated *in vivo* in a murine ascitic tumour model

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Abstract

The effect of systemic administration on drug uptake at cellular level was evaluated using time-gated fluorescence spectroscopy performed on a murine ascitic tumour model. Mice bearing L1210 leukaemia were injected intraperitoneally or intravenously with 25 mg per kg body weight hematoporphyrin derivative (HpD), 12.5 mg per kg body weight photofrin II (PII), 25 or 5 mg per kg body weight disulphonated aluminium phthalocyanine (AlS₂Pc). Every 2 h and for up to 22 or 30 h, mice were sacrificed, leukaemic cells extracted from the peritoneum, washed, and resuspended in buffer for fluorescence measurements. HpD and PII emission spectra were almost identical 12 h after intraperitoneal injection with main peaks at 630 nm and no appreciable changes afterwards. In the first 12 h, the PII fluorescence spectrum was constant, while in the case of HpD a shoulder at 615 nm was detectable. Similar fluorescence behaviour was observed after intravenous administration of porphyrin derivatives. These results seem to confirm that the tumour localizing fraction is the part actually retained by the cells. The AlS₂Pc spectrum peaked at 685 nm and did not change in any of our experiments. AlS₂Pc is incorporated more rapidly with respect to porphyrins, as was clearly observed in the case of intravenous administration, where the AlS₂Pc fluorescence was readily detectable after 2 h, whereas the PII emission became apparent only after 4–6 h.

1. Introduction

Hematoporphyrin derivative (HpD) and its tumour localizing fraction (TLF), contained at a high percentage in Photofrin II (PII), have been the most widely used photosensitizers in the photodynamic therapy of tumours in the last few years.

Phthalocyanines are, at present, being extensively studied as possible photosensitizers because of their interesting characteristics at long wavelength

(greater than 680 nm) absorption and chemical stability [1, 2]. In particular, disulphonated aluminium phthalocyanine (AlS₂Pc) showed promise in this field owing to its high efficiency as a photosensitizer [2], water solubility and phototoxicity [2–4], even though the data collected up to now relate to the use of broad band irradiation sources, and it is therefore difficult to evaluate the light dose actually employed.

The efficacy of photodynamic therapy of tumours is essentially based on drug preferential localization in neoplastic regions with respect to the surrounding tissue. The reasons for this behaviour and the elements that influence it have not yet been fully clarified.

Experiments performed on tumour cells incubated in the presence of different porphyrins [5] showed that the drug composition and the animal metabolism influence the pharmacokinetics. In fact, remarkable differences in the behaviour of the fluorescence characteristics as a function of the incubation time were observed, depending on whether the cells were stabilized *in vitro* or collected from mice at the time of the fluorescence measurements.

In this paper we present a study on the effect of systemic administration on drug uptake at cellular level. Time-resolved fluorescence spectroscopy was performed at different times on a murine ascitic tumour model after porphyrin intraperitoneal or intravenous injection into the mouse. Although intraperitoneal administration is not frequently utilized in clinical oncology, it is of interest since it realizes *in vivo* a situation similar to the usual incubation procedure of cell suspension where the drug is in direct contact with the cells.

Promising results recently published in the literature on the use of phthalocyanines suggest that a similar series of experiments should be done on AlS₂Pc, to study its fluorescence properties and uptake behaviour.

2. Materials and Methods

HpD was prepared following the procedure described by Lipson *et al.* [6] and stored as a lyophilized powder. PII was supplied by Quadra Logic Technologies (Vancouver, Canada) in solution at a concentration of 2.5 mg/ml. AlS₂Pc was kindly provided as a powder, together with HpD, by the Department of Chemistry, Paisley College of Technology (Paisley, U.K.).

All drugs were dissolved in physiological solution.

The chemically induced lymphoid leukaemia L1210 (H-2^d) [7] was maintained in hybrid (Balb/cxDBA/2 F₁) male mice by weekly intraperitoneal injection.

When ascitic tumours developed, the drug was injected intraperitoneally or intravenously into mice at a concentration of 25 mg per kg body weight (b.w.) for HpD, 12.5 mg per kg b.w. for PII, and 25 mg per kg b.w. for AlS₂Pc. A lower AlS₂Pc concentration (5 mg per kg b.w.) was also considered in the case of intravenous administration.

Every 2 h and up to 22 or 30 h after intraperitoneal or intravenous injection respectively, two mice were sacrificed, cells were drawn from the

peritoneum, washed, centrifuged, and resuspended in phosphate buffered solution at a concentration of $2 \times 10^6 \text{ cm}^{-3}$ for the fluorescence measurements. The cells were counted under an optical microscope and the living tumour cells were found to make up approximately 95% of the population.

At various times, in all of the experiments, control measurements were performed on peritoneal leukaemic cells extracted from untreated tumour-bearing mice.

A mode-locked argon ion laser tuned at 364 nm was used as the excitation source. The pulse repetition rate was reduced to approximately 750 kHz by an acousto-optic pulse-picker. The detection apparatus [8] consisted of a double microchannel-plate photomultiplier, whose output signal was sent to an electronic chain for time-correlated single photon counting, extended through a home-made acquisition unit. The whole instrumentation allowed simultaneous collection of the full-spectrum fluorescence decay curve, the time-integrated emission spectrum, and two time-gated spectra, with a temporal resolution of approximately 150 ps and a spectral resolution of approximately 2 nm. For the porphyrins the first gate was at zero delay and 500 ps wide, and the second gate was at 18 ns and 6 ns wide. For the AlS_2Pc the delay of the second gate was set at 8 ns while the first gate was left unchanged. The collected data were sent to a personal computer for analysis and storage. In particular, the decay curves were analysed using a non-linear least-squares fitting procedure. Different measurements provided variations in the time decays from 50 to 500 ps, depending on the decay value, and the fluctuations in the relative amplitudes were within a few per cent.

3. Results

Before describing the results obtained in the present experiment, it is worth recalling some general fluorescence properties of hematoporphyrin and its derivatives.

In addition to porphyrin monomers, HpD contains covalently linked porphyrins which constitute the tumour localizing (oligomer) fraction (the commercially available PII). Several time-resolved fluorescence studies have been reported in the literature on both HpD and PII in several solvents [9–12] and in biological systems [5, 11, 13]. The fluorescence decay curves can be fitted by two or three components whose relative amplitudes depend on the environment. The complexity of the oligomer fraction does not allow an unambiguous attribution of the molecular species responsible for this behaviour. On the basis of the experimental evidence, the short decay time (0.5–1 ns) is attributed to aggregates or oligomers in a folded configuration, and the long decay time (14–15 ns) to monomers and end rings of unfolded oligomers. The interpretation of the intermediate decay time (3–5 ns) is not yet quite clear. However, the results obtained in surfactants [10, 11] and in cells [13] seem to justify its relation with oligomers interacting with (or being encapsulated by) cellular structures or large aggregates. The monomeric

or end-ring porphyrins have the main emission peak at approximately 615 nm in water and at approximately 630 nm in hydrophobic environment. The fast and intermediate components contribute for the most part to the 630–680 nm region of the spectrum [11].

3.1. Intraperitoneal administration

Table 1 reports the fluorescence lifetime data for L1210 cells 4 h after intraperitoneal injection of HpD, PII and AlS_2Pc . Some fluctuations were observed, but neither the lifetime values nor their relative amplitudes varied meaningfully as the uptake time increased.

Fitting of the control decay curves, as a result of the natural fluorescence, leads to three lifetimes with the following typical values and relative amplitudes: 6–8 ns (about 10%–15%), 2–3 ns (about 30%–35%), and 0.7–1 ns (about 50%–60%). Thus, when porphyrin-treated cells are analysed, the natural fluorescence is superimposed on the drug emission signal, mainly contributing to the fast and intermediate components.

In agreement with results already observed in the case of cells incubated in suspension in the presence of porphyrins [5], in all of the measurements the slow component was predominant, as indicated by the high value (around 80%) of the product of its lifetime and its relative amplitude, and confirmed by the close similarity of the delayed spectrum with the time-integrated spectrum.

2 h after intraperitoneal injection, the HpD spectrum (Fig. 1(a)) peaked at 630 nm, with a marked shoulder at 615 nm. This shoulder disappeared after 12 h (Fig. 1(b)) and, in the following 10 h, the spectral lineshape did not show any significant variations.

The PII fluorescence spectra (Fig. 1(c)) peaked at 630 nm, but no shoulder was observed at shorter wavelengths, and they remained unchanged for further uptake times. Hence, about 12 h after injection, the HpD fluorescence characteristics tended to become very similar to those of PII.

For both porphyrin derivatives, the undelayed spectrum was characteristic of emission from a molecular species with low quantum yield. Accordingly, the fluorescence signal was increased in the 580–600 nm region (natural fluorescence) and in the 630–680 nm region (short and intermediate components). Its evolution as a function of the uptake time closely followed that of the time-integrated spectrum.

TABLE 1

Typical fluorescence decay times (τ) and relative amplitudes (A) 4 h after intraperitoneal injection

Drug	τ_1 (ns)	A_1 (%)	τ_2 (ns)	A_2 (%)	τ_3 (ns)	A_3 (%)
HpD	15.30	45.39	4.32	18.16	1.11	36.46
PII	14.90	40.09	4.91	18.32	1.18	41.58
AlS_2Pc	5.18	86.33	1.52	13.67	–	–

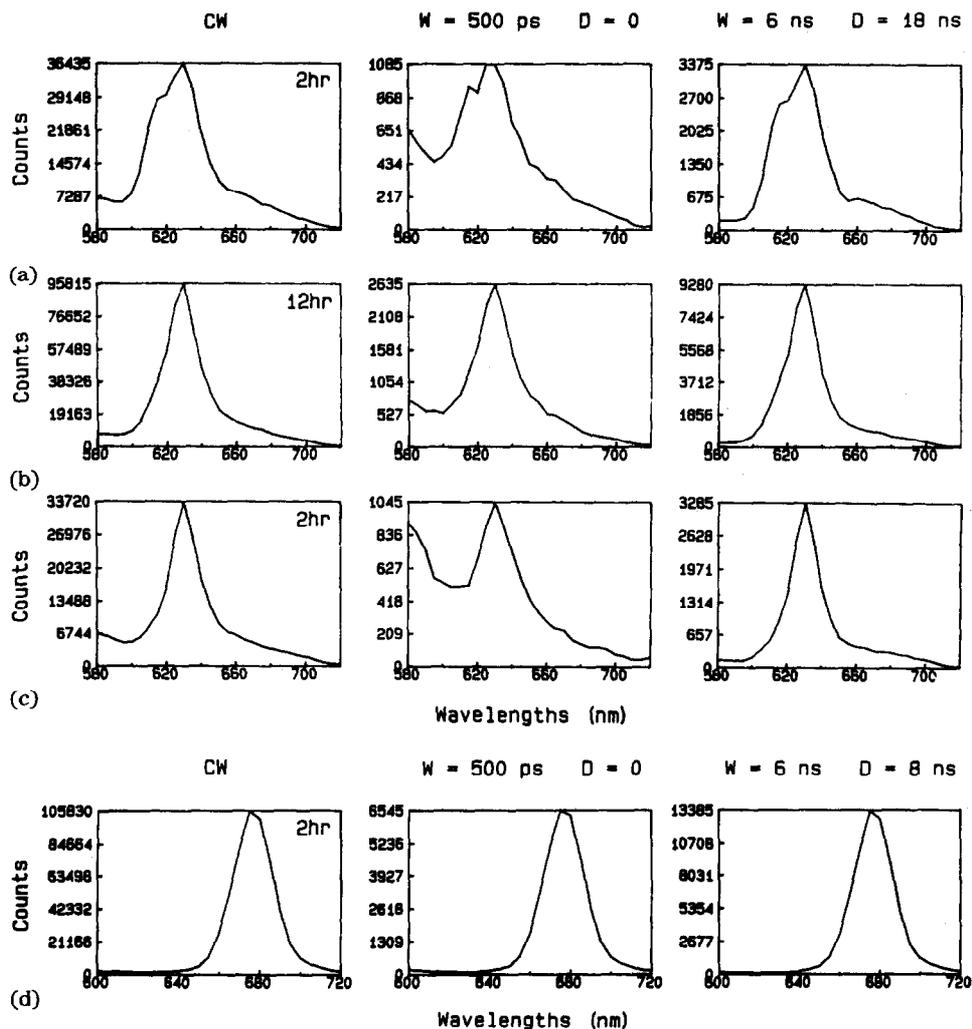


Fig. 1. Fluorescence spectra after intraperitoneal injection: HpD after (a) 2 h and (b) 12 h; (c) PII after 2 h, and (d) AlS₂Pc after 2 h. From left to right: time-integrated spectrum, time-gated spectrum (500 ps wide, undelayed), and time-gated spectrum (6 ns wide, 18 ns (a)–(c) or 8 ns (d) delayed).

The AlS₂Pc decay curves were best fitted using two exponential components, characterized by lifetimes of approximately 5 and 1.5 ns (Table 1). The short lifetime has been attributed to AlS₂Pc bound to intracellular sites [14]. However, the small amplitude of this component and its approximately constant value at different incubation times seem to indicate that in our cell system these possible binding sites are of limited relevance in the uptake process.

No significant variations were observed in any of the emission spectra, which presented the same shape with the peak at 685 nm (Fig. 1(d)). The

contribution of natural fluorescence was negligible owing to both its decrease in this spectral region and the high fluorescence signal of the phthalocyanine.

3.2. Intravenous administration

Analogous series of fluorescence measurements were repeated 2–30 h after intravenous administration of either porphyrins or phthalocyanine.

The HpD emission was not detectable after 2 h and was hardly visible at 630 nm after 4 h (Fig. 2(a)). The fluorescence intensity increased remarkably up to 14 h after injection, and remained approximately constant thereafter (Fig. 2(b)). A shoulder at 615 nm was barely observable for the first few hours and disappeared after 8–10 h. No significant spectral changes occurred subsequently as a function of the uptake time.

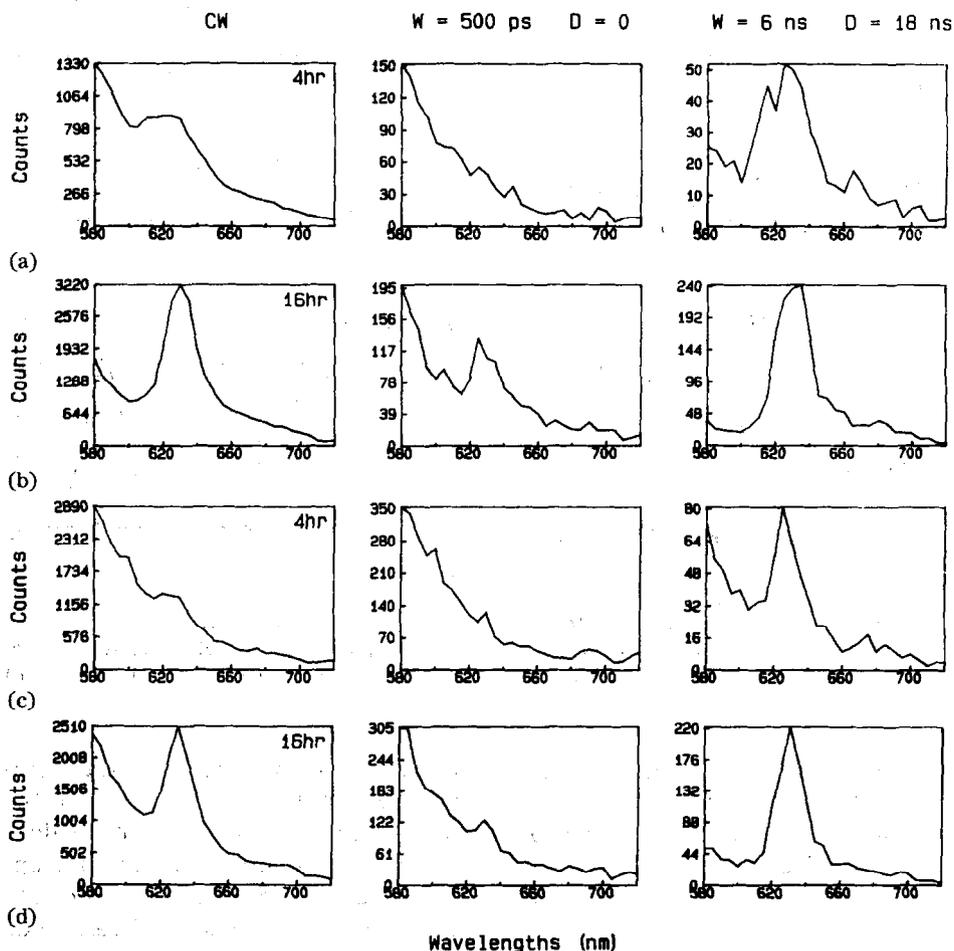


Fig. 2. Fluorescence spectra after intravenous injection: (a) HpD after 4 h and (b) 16 h; (c) PII after 4 h and (d) 16 h. From left to right: time-integrated spectrum, time-gated spectrum (500 ps wide, undelayed), and time-gated spectrum (6 ns wide, 18 ns delayed).

The PII spectra were characterized only by the emission at 630 nm and their lineshape did not show any variations at all with time. Similar to results observed in the case of HpD, the fluorescence intensity became measurable after 4–6 h, grew in the first 12–14 h, and remained unchanged afterwards (Fig. 2(c), (d)).

The porphyrin decay curves are still a result of three lifetimes with values (Table 2) similar to those obtained after intraperitoneal administration, but with a higher relative amplitude (around 65%–70%) of the short-lived species, which can be related to the stronger contribution of the natural fluorescence.

The AlS₂Pc at a concentration of 25 mg per kg b.w. gave rise to an intense emission at 685 nm after only 2 h (Fig. 3(a)) which remained constant up to 30 h. A lower AlS₂Pc concentration (5 mg per kg b.w.) was therefore investigated. In this case again, the drug fluorescence was clearly detectable after 2 h (Fig. 3(b)), but the maximum emission intensity was reached after 12 h (Fig. 3(c)). No changes were observed at subsequent times. For both concentrations, in agreement with results obtained after intraperitoneal injection, the time-gated spectra were very similar to the time-integrated spectra, consisting mainly in the emission at 685 nm.

Best fitting of the AlS₂Pc (5 mg per kg b.w.) decay curves led to three exponential components, with lifetimes of approximately 6 ns, 3 ns, and 1 ns (Table 2), and again no continuous trend of variation was observed with the uptake time. In this case, the contribution of the natural fluorescence was relevant and could account for the three-component fitting. This was also confirmed by the observation, for the higher AlS₂Pc dose, of only two components with fitting parameters similar to those reported in Table 1.

4. Discussion

To evaluate the results obtained, it is worth considering that the same cell line, when incubated in suspension with porphyrins, leads to a fluorescence spectrum where the emission at 615 nm is present at any time and is always dominant for HpD [5]. This emission, which is related to porphyrins in a water-like environment, is thus more relevant for the drug containing a larger proportion of monomers.

TABLE 2

Typical fluorescence decay times τ and relative amplitudes A 12 h after intravenous injection

Drug	τ_1 (ns)	A_1 (%)	τ_2 (ns)	A_2 (%)	τ_3 (ns)	A_3 (%)
HpD	14.36	10.83	3.12	20.07	0.61	69.10
PII	14.81	7.30	4.65	25.49	1.08	67.27
AlS ₂ Pc ^a	5.90	39.74	2.87	50.24	0.93	10.01

^a 5 mg per kg b.w.

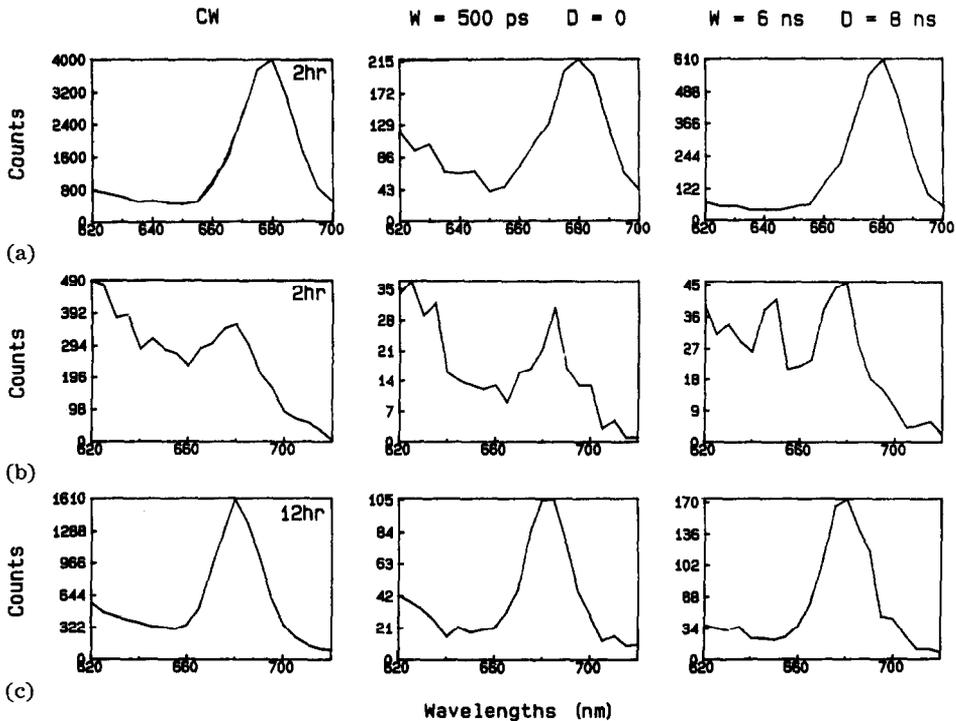


Fig. 3. Fluorescence spectra after intravenous injection: (a) AlS_2Pc (25 mg per kg b.w.) after 2 h, and (b) AlS_2Pc (5 mg per kg b.w.) after 2 h and (c) after 12 h. From left to right: time-integrated spectrum, time-gated spectrum (500 ps wide, undelayed), and time-gated spectrum (6 ns wide, 8 ns delayed).

Systemic administration seems to act as a means of selection of the drug components that can reach the cells. The 630 nm peak is constantly dominant and the 615 nm emission is visible just as a shoulder in the first hours and only after HpD injection. It is interesting to note that this is observed in the case of intraperitoneal administration where HpD monomers are injected in close contact with the cells. The peritoneum environment diminishes the amount of monomers available with time. Firstly they are weakly bound by the cells in hydrophilic binding sites (*e.g.* the outer cell membrane) and then they are progressively released as their concentration decreases in the peritoneum. The higher 630 nm to 615 nm emission intensity ratio for PII also confirms that the oligomers are actually the porphyrin fraction steadily retained by neoplastic cells and, therefore, those responsible for the therapeutic effect in the treatment of tumours. The selectivity seems to be even more marked for intravenous administration. In fact the difference in the emission spectra between HpD and PII is less relevant according to the hypothesis that it is the oligomeric fraction which mainly localizes and accumulates in neoplastic regions.

The AlS_2Pc was previously studied in solution at various concentrations either in aqueous or hydrophobic environments, and the data collected in that situation suggested the hypothesis that only monomers were present and that they did not tend to aggregate. The decay curves could always be fitted with a single exponential function, characterized by a lifetime of about 5 ns, and the spectra did not show any variation as the drug concentration or the solvent nature were changed. In the present measurements, the AlS_2Pc time-integrated and time-gated spectra are very similar to one another and all are unaltered 2–22 h or 30 h after administration. This behaviour of the fluorescence characteristics confirms that the drug is composed of monomers with no tendency to aggregation and suggests that the interaction mechanisms and/or binding sites are probably different from those observed in the case of porphyrins. This hypothesis is also supported by the observation that AlS_2Pc is incorporated by the cells in a shorter time with respect to porphyrins. Actually, a strong fluorescence signal is detected 2 h after intraperitoneal injection, and AlS_2Pc emission is observable 2 h after intravenous administration, even in the case of the lower drug dose. Moreover, while the cells release the monomeric porphyrins within the first 8–10 h, the phthalocyanine is retained in large amounts even after 30 h.

Recently the treatment of tumours in mice with a low dose (5 mg per kg b.w.) of AlS_2Pc produced curative effects, comparable with those obtained with a therapeutic dose (25 mg per kg b.w.) of HpD, even when the irradiation light dose in the case of AlS_2Pc was reduced by a factor of 4 [15]. Since the results obtained cannot simply be explained in terms of the larger light absorbance of AlS_2Pc , they seem to provide further evidence for the different action mechanisms of AlS_2Pc with respect to porphyrins.

A final comment should be made on the time-gated fluorescence spectroscopy. As shown in Fig. 3(c), the porphyrin emission is barely observable in the time-integrated spectrum, but is clearly visible in the delayed spectrum. This illustrates the diagnostic potentialities of the technique since it allows one to detect the presence of a fluorophore over an intense fluorescence background by the choice of a suitable observation time-window. Work is in progress to develop such a time-gated imaging system.

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