Fast-Gated Intensified Charge-Coupled Device Camera to Record Time-Resolved Fluorescence Spectra of Tryptophan

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The possibilities of a 200 ps gated intensified charge-coupled device (CCD) camera to record time-resolved fluorescence were explored using the fluorescing amino acid tryptophan and its derivative N-acetyl-tryptophan amide (NATA) as model compounds. The results were compared to complementary data from time-correlated single-photon counting (TCSPC) experiments. If a spectral resolution of 1–2 nm is desired, the fast-gated intensified CCD (ICCD) camera is the method of choice. For a 10⁻⁴ M tryptophan solution, time-resolved emission spectra and intensity decays (measured over 12 ns at 25 ps resolution) could be obtained in typically 10 minutes, giving the well-known lifetimes of 0.5 and 3 ns. In addition, a longer lifetime of 7 ns was found at the red edge of the spectrum. The very short gate time of the ICCD camera allowed us to observe a shift in the emission maximum of tryptophan even within the first nanosecond of decay of the fluorescence emission. As expected from the tryptophan rotamer model, such a shift is not observed in NATA. Using amplitudes obtained by global analysis, decay-associated spectra of these lifetimes were constructed.

Index Headings: Time-gated charge-coupled device; CCD; Time-resolved fluorescence; Tryptophan.

INTRODUCTION

Intensified charge-coupled device (CCD) cameras are used for many types of spectroscopy (fluorescence,¹² phosphorescence,¹ Raman,⁴ imaging¹⁵¹⁶) in fields such as biology, chemistry, and physics. In fluorescence experiments, CCD cameras are mainly applied to record steady-state emission spectra, but can be useful for time-resolved experiments as well. The most commonly used gated intensified CCD cameras have gating times ranging from 2 to 100 ns. To obtain time-resolved information, these cameras are usually combined with ns-pulsed light sources, such as Nd: YAG or excimer lasers with pulse widths of typically 3 and 15 ns, respectively. The main disadvantage of the long gating times of ICCD cameras is that time-resolved fluorescence experiments are not possible if lifetimes are below or around 3 ns. This is, for instance, a hindrance to the full exploration of the compound tryptophan, an important intrinsic fluorescent probe in proteins, which has lifetimes as short as 0.5 and 3.1 ns.⁷ To overcome this problem both faster laser sources and faster detection systems are required.

Recently, robust picosecond-range lasers have become available. These Ti:sapphire lasers have a pulse width of about 2 ps, which makes them very useful for time-resolved experiments on a sub-nanosecond level. Because of their high repetition rate of 76 MHz, many measurements can be done in a short time, so they are very appropriate for combining with time-correlated single-photon counting (TCSPC) detection.⁸

In this explorative study we combined a Ti:sapphire laser with a recently commercialized ultrafast-gated intensified CCD camera to record time-resolved emission spectra and fluorescence decay curves. To evaluate the performance of this combination, the results will be compared to those obtained by means of TCSPC experiments and with data from the literature.

Tryptophan was chosen for our explorative study because of its interesting fluorescence properties. It exhibits two dominant fluorescence lifetimes of about 0.5 and 3 ns, which are associated with different emission spectra.⁷ A shorter lifetime (about 13 ps)⁷ and a much longer lifetime of 7–9 ns have also been observed under particular conditions.¹⁰ Tryptophan fluorescence emission is very dependent on its microenvironment.¹¹ Temperature, pH, and polarity are, among others, factors that influence both spectral shapes and lifetimes. For this reason, tryptophan is often used as a probe for observing structural changes in proteins.

In the experiments discussed below, only the unbound tryptophan molecule is used, as well as its derivative N-acetyl-tryptophan amide (NATA), frequently presented as a model for tryptophan in a protein because of the extra groups simulating peptide bonds, as shown in Fig. 1.

The multiple lifetimes found for tryptophan are rather unexpected and complicating since a single lifetime is anticipated for a single fluorophore. Generally, this is attributed to different rotameric structures of tryptophan,⁷¹² in which fluorescence is quenched by an electron transfer mechanism due to the positively charged amino group positioned over the indole ring.¹³ In NATA this is not possible: it behaves as expected for a single compound and shows only one lifetime (3.0 ns). Time-resolved emission spectra of tryptophan show a red shift of the emission spectrum in the first five nanoseconds of emission, as observed by Szabo and Rayner.⁷ Decay-associated spectra of the two shortest lifetimes have been constructed. It was shown that the spectrum of the 3.0 ns component is red-shifted compared to the 0.5 ns component. These results should be reproduced by our experiments using the fast-gated intensified CCD camera.

EXPERIMENTAL

Laser System. The same laser system was used for both the TCSPC and the ICCD camera experiments. The system consists of a Mira 900-P (Coherent, Santa Clara, CA), which emits 3 ps pulses at a repetition rate of 76 MHz, many...
MHz, pumped by an Innova-300 argon-ion laser (Coherent, Santa Clara, CA). The Mira 900-P is a mode-locked titanium–sapphire laser, tunable from approximately 700 to 1000 nm. The laser emission is led through a pulse picker (APE PulseSelect, Berlin, Germany), which reduces the repetition rate to 3.8 MHz, to avoid double excitation of molecules. A frequency tripler (Oplaz Technologies fs-tripler, Chatsworth, CA) is used to provide the used output wavelength of 290 nm. This light is used to excite the sample, and fluorescence emission is collected at 90° and dispersed by a spectrograph (TVC JarrellAsh Monospec 18, Grand Junction, CO) and then detected by either the ICCD camera or the TCSPC system. Scattered laser light was filtered out using a WG-320 filter (Schott, Mainz, Germany).

**Intensified Charge-Coupled Device Camera.** Time-resolved emission spectra were recorded from 330 to 440 nm with an intensified CCD camera with ultrafast gate times (LaVision, Göttingen, Germany). This system contains an image intensifier that provides very good sensitivity and acts as an extremely fast optical shutter with a shortest gate time of 200 ps full width at half-maximum (FWHM). The gate can be shifted in time in steps of 25 ps or longer. An intensified CCD camera consists of a CCD detector coupled with a phosphor plate as an image intensifier. For each electron that hits the phosphor plate, 200 green photons are emitted, which are focused on the CCD detector. The use of an image intensifier has several advantages compared to using only the CCD: (1) ultimate sensitivity: it is possible to measure single photons, (2) UV extended spectral sensitivity down to 200 nm (with quartz entrance window), and (3) most importantly: an extremely fast shutter. Detection is triggered by a reflection of the laser beam on a photodiode. The intensity decay was recorded in 25 ps steps over a range of 12 ns, resulting in 480 data points per decay curve. Image collection and image processing was performed under control of DaVis software v6.2 (LaVision, Göttingen, Germany).

**Time-Correlated Single Photon Counting.** Time-correlated single photon counting was used to record fluorescence decay curves with high time-resolution. For this, a TCSPC-630 system (Becker & Hickl GmbH, Berlin, Germany) was used with a time resolution of about 15 ps. A reflection of the laser pulse focused on a photodiode provides the synchronization signal for the TCSPC. The fluorescence emission from the sample, after being dispersed by the monochromator, is collected on a photomultiplier tube and analyzed. Decays were recorded at wavelengths between 330 and 440 nm in 10 nm steps.

**Chemicals.** L-Tryptophan (98%) and N-acetyl-tryptophan amide were both purchased from Sigma and used without further purification. Solutions of both compounds were prepared in Milli-Q water. In the TCSPC experiments a concentration of $1 \times 10^{-5}$ M of tryptophan and NATA was used. Concentrations down to $1 \times 10^{-8}$ M could be measured with good intensity, but at the cost of long measurement times. For the ICCD camera experiments, concentrations of $1 \times 10^{-5}$ M of tryptophan and NATA were also used. Concentrations down to $1 \times 10^{-7}$ M (gate 300 ps) gave sufficient intensity, but like TCSPC, the measurement times became very long.

**Data Analysis.** Fluorescence decay curves of both TCSPC and ICCD camera data were analyzed using a global fit procedure based on the Levenberg–Marquardt algorithm. This method uses the instrument response signal for deconvolution of the recorded decays and determination of lifetimes and wavelength dependent amplitudes. The instrument response (TCSPC) and gate function (ICCD) were obtained by measuring the signal from scattered laser light. The basis of the global fit procedure is the assumption of constant lifetimes for the decays included, but with variable amplitudes. This assumption is correct in many cases: for tryptophan and NATA it can be confirmed by individual fits of the decays with the curve-fitting program FluoFit v3.1 (PicoQuant GmbH, Berlin, Germany). It is generally accepted that a multi-exponential fit of a decay curve only gives reliable results if the decay times involved differ by a factor of three. With a global fit of ten decays or so, this requirement can probably be relaxed. With our fitting procedure, i.e., convolution of a multi-exponential decay with either the instrument response function (TCSPC) or the appropriate gate function (ICCD), and fitting the result to the experimental decay, reproducible results are obtained. We try to keep the set of time constants at a minimum and normally do not include more than three. The quality of the fit ("goodness of fit") was assessed on the basis of $\chi^2$, the covariance matrix, and distribution of residuals. Fluorescence emission spectra were fitted to Gaussian functions using Origin 7.0 (OriginLab, Northampton, MA) to determine the emission maxima.

**RESULTS AND DISCUSSION**

With the intensified CCD camera used in our experiments, both time and spectral data of tryptophan and NATA could be obtained in a single experiment with a 200 ps gating time. However, since intensity was quite low, a gate width of 300 ps was used for the experiments discussed in this paper. The results obtained with both gate widths are comparable. The three-dimensional plot in Fig. 2, recorded in 10 minutes, shows the experimental data of $10^{-3}$ M tryptophan in water.

The relation between time and spectral data is illustrated in this graph (Fig. 2): fluorescence emission spectra along the x-axis, decay curves along the y-axis. Changes

![Fig. 1. Structural formulas of tryptophan (left) and N-acetyl-tryptophan amide (right).](image-url)
in the emission spectra of tryptophan and NATA were monitored during decay of the fluorescence and lifetimes could be monitored on a short time-scale.

**Time-Resolved Emission Spectra.** In Fig. 2, time-resolved emission spectra (TRES) of tryptophan are shown along the x-axis. To illustrate the changes in the spectra more clearly, a selection of TRES are depicted in Fig. 3. Interestingly, the largest shift is observed within the first nanosecond of decay.

Whereas using the fast-gated ICCD camera TRES are measured directly, this is not possible for the TCSPC method. In the latter case TRES had to be constructed from intensity decays by plotting the intensity recorded at twelve different wavelengths (330–440 nm, 10 nm steps) at different times.

Time-resolved emission spectra of both the ICCD camera and TCSPC data were fitted to a double Gaussian curve to determine the emission maximum. The changes of the emission maxima in time of tryptophan and NATA of data recorded with both detection methods are shown in Fig. 4 on a short (1 ns) and a longer (8 ns) timescale. Figures 4a and 4b were obtained with ICCD camera detection, and Figs. 4c and 4d with the TCSPC technique.

The results obtained for both methods are quite similar: the emission spectrum of tryptophan shifts several nanometers, whereas the emission maximum of NATA is constant. For tryptophan, the total shift towards longer wavelength is about 4 nm, of which about 2 nm takes place within the first nanosecond.

**Fluorescence Lifetimes.** From the ICCD camera results shown in Fig. 2, fluorescence decay curves of tryptophan and NATA can also be derived (seen along the y-axis). To determine the lifetimes, twelve decay curves (each corresponding to 50 pixels, i.e., to a spectral band-
width of 7 nm) over the range from 330 to 440 nm were considered. They were fitted to mono- or multi-exponential curves (Eq. 1) using the procedures for global fitting described in the Experimental section:

\[ I(t) = \sum_i A_i e^{-t/\tau_i} \]  

For NATA the expected single lifetime of 3.1 ns (TCSPC: 3.0 ns) was found, but for a good fit of the tryptophan decays three exponents were needed, revealing lifetimes of 0.5 ns, 3.2 ns, and 7.0 ns. Surprisingly in view of the available literature, the TCSPC data of tryptophan also had to be fitted with a third lifetime, the lifetimes found being 0.5 ns, 3.1 ns, and 7.5 ns. Thus, the sets of lifetimes provided by ICCD and TCSPC compare quite well.

As noted above, the longest lifetime (7 ns) of tryptophan was unexpected: it has been reported before,\textsuperscript{10} but only for pH > 8. To our knowledge this is the first time this 7 ns lifetime has been reported for tryptophan in water, although Fleming et al.\textsuperscript{15} did observe a 5.4 ns lifetime with detection >370 nm at pH 7, together with a 2.1 ns lifetime. These results were not mentioned in later papers, but may be a combination of the three lifetimes found in our experiments.

The small difference observed for the longest lifetime using ICCD and TCSPC detection is probably due to the fact that with the camera, only decays of up to 12 ns could be recorded, not long enough for the intensity to completely return to zero. For the 3 ns component this effect will already be close to negligible. In the TCSPC experiments, 40 ns decays were recorded, which makes determination of the longer lifetimes more accurate. It is possible to record longer decays with the ICCD camera when the time step is increased, but at the cost of accuracy in the lifetimes: the total number of points per decay cannot exceed 480.

Recently, a paper was published in which lifetimes of tryptophan were recorded using a third technique, a streak camera.\textsuperscript{9} This instrument was able to record 2.2 ns of fluorescence decay with a time response of 4 ps. Decay times above 2.2 ns could only be estimated. For tryptophan, lifetimes of 13 ps, 500 ps, and 3.8 ns were found. This agrees reasonably well with our results: the 0.5 and 3.8 ns lifetimes are comparable (the second lifetime being somewhat too long, probably because of the limited recording time), and the 13 ps lifetime is too short to be observable with our ICCD camera, while the 7 ns lifetime observed with ICCD and TCSPC is beyond the scope of the streak-camera.

**Decay-Associated Spectra.** Decay-associated spectra (DAS) provide information on the fluorescence emission per lifetime component. This is of particular interest for tryptophan, where DAS can be calculated for each of the three lifetimes, whereas for NATA, the single-lifetime DAS is of course identical to the total emission spectrum. DAS are constructed by dividing the total intensity per decay curve among the lifetimes that make up the total intensity according to their ratio (amplitudes are obtained by fit). The relative fluorescence intensity of lifetime component \( \tau_j \) at wavelength \( \lambda \) can be expressed by the following equation:

\[ I_j(\lambda) = \frac{A_j \tau_j}{\sum_i A_i \tau_i} \]
Obviously, the three lifetimes are associated with clearly distinct emission spectra, as shown in Fig. 5.

The emission maximum of the total fluorescence spectrum of tryptophan is seen at about 360 nm. The 0.5 ns lifetime has an emission spectrum with a maximum around 330 nm, blue-shifted by about 30 nm compared to the total spectrum. The 3.2 ns lifetime has the largest contribution to the total emission spectrum, so the highest overall intensity, and has its maximum at about 360 nm, the same as the total spectrum. The decay-associated spectra of these two lifetime components were already presented by Szabo and Rayner. The maximum of the DAS of the 7.0 ns lifetime is red-shifted to about 400 nm, so this lifetime has its main contribution to the total spectrum at long wavelengths.

From the decay-associated spectra the observed red-shift of the emission spectrum of tryptophan as a function of time can be explained: the longer lifetimes have an emission maximum at longer wavelength, so the total spectrum of all lifetime components together will also shift with time. This shift is the largest on the short timescale, since the 0.5 ns lifetime has the most blue spectrum, and its main contribution will be in the first 0.5 ns of decay. The contribution of the 7.0 ns lifetime is on the red side of the spectrum and is relatively small. Therefore, it does not cause a shift in the emission maximum of the total spectrum, but it broadens the red side of the spectrum. The shift in the time-resolved emission spectra of tryptophan can be seen both from spectra recorded with the ICCD camera and when reconstructed from TCSPC data. In the same way, the absence of a shift in NATA can be rationalized: there is only a single DAS.

**Evaluation of the Camera Performance.** The possibilities of the fast-gated intensified CCD camera for time-resolved fluorescence experiments are mainly found in recording time-resolved emission spectra. For exact determination of fluorescence lifetimes it is less suitable; the resolution provided in the time-domain by TCSPC is much better: 4096 data points per decay curve are obtained. The ICCD camera typically only acquires 480 points per curve due to the minimum step size of 25 ps in the gate shift. The single exponential decay of NATA could be fitted well, but determination of the multiple fluorescence lifetimes of tryptophan is less accurate than with TCSPC. So, for accurate determination of fluorescence lifetimes TCSPC is the preferred method.

However, for recording time-resolved fluorescence emission spectra (TRES), the fast-gated ICCD camera is extremely useful because the spectra are recorded directly and not constructed from intensity decay measurements. Determination of the emission maximum is more accurate since there are about 840 data points per spectrum (120 nm, so 7 pixels per nm), compared to 12 wavelengths in our TCSPC experiments (1 per 10 nm): experiments with the same spectral resolution would take about 70 times longer with TCSPC than with the fast-gated ICCD camera. Recording a decay curve with TCSPC requires at least one minute, so recording a plot such as the one in Fig. 2 would take about 840 minutes. The long duration of such an experiment makes the TCSPC method much more sensitive to changes in intensity due to photodegradation of the sample or due to the instability of the laser system. On the contrary, using the ICCD camera, a three-dimensional plot, as shown in Fig. 2, containing both the intensity decays and the spectral information, may be obtained within 10 minutes. Shifts of the TRES on very short timescales can be seen with the ICCD camera. Such effects can also be observed in TRES constructed from TCSPC decays, but, as stated above, this method is much more laborious.

Finally, it should be noted that for the fast-gated ICCD camera two measurement corrections were needed: one to correct for the spatial sensitivity, which is not constant over the whole chip, the other for the fact that the gate width was up to 100 ps longer in the long wavelength region of the chip. Some technical improvements of the CCD chip are therefore desirable.

**CONCLUSION**

The results discussed above unambiguously demonstrate the appropriateness of the novel fast-gated ICCD camera for time-resolved emission spectroscopy dealing with short fluorescence lifetimes (down to 0.5 ns) and small wavelength shifts. Lifetimes longer than typically 5 ns are somewhat less precise, since the recording time is limited to 12 ns when using the shortest time step of 25 ps. Comparison of ICCD measurements on tryptophan...
and NATA with other techniques, i.e., the TCSPC method and the streak camera,underline the accuracy of the ICCD technique. It is especially useful for recording time-resolved emission spectra that need a resolution of up to 1 nm. The required data can be obtained in a short time (typically 10 minutes), so that long-term sample and instrumental instabilities are less significant.