Ultrafast fluorescence spectroscopy of biologically relevant chromophores using type II difference frequency generation

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Abstract. A novel femtosecond fluorescence experiment based on type II difference frequency mixing is demonstrated. This approach is particularly interesting for near-UV emitting biological chromophores like amino acids and nucleotides, as the fluorescence is converted into the spectral range where CCD have their highest quantum efficiencies. The method is implemented with a 5-kHz amplified Ti:Sapphire laser system and first results obtained with 2,5-diphenyloxazole (PPO) in ethanol are reported.

1. Introduction
In the past years, much research has been conducted on the ultrafast fluorescence dynamics of proteins and oligonucleotides or complexes thereof with the aim of elucidating their structural dynamics. Examples range from the investigation of spatially restricted solvation dynamics [1] to intra-protein energy and charge transfer [2, 3], leading to static and/or collisional quenching. In particular, in the case of static fluorescence quenching the distance-dependence of photo-oxydation or non-radiative resonance energy transfer bears interesting information e.g. about stacking interactions in DNA or in protein/DNA complexes [4, 5], and allows the distinction of different binding motifs [6].

Femtosecond fluorescence up-conversion due to sum frequency generation [7-10] or Kerr gating [11-13] have been the methods of choice for most of these studies. In the present contribution, we explore the use of type II difference frequency mixing (DFG) in a fluorescence down-conversion scheme. DFG is advantageous for the biologically relevant near-UV emitting fluorophores, like tryptophan, tyrosine or 2-aminopurine, in particular when broadband recording of time-resolved spectra with a CCD is aimed for [9, 14, 15]. When using an intense 800 nm gate pulse, most of the DFG signal is in the visible, i.e. in the wavelength range of highest CCD sensitivity.

2. Experiment and methods
Type II DFG is characterized by the following energy conservation and phase-matching conditions

\[ \omega_D = \omega_{fl} - \omega_G \]
\[ \overrightarrow{k}_D^\alpha = \overrightarrow{k}_{fl}^e - \overrightarrow{k}_G^e \]  

UV fluorescence (fl) and gate pulse (G) are both linearly polarized along the extraordinary (e) axis of the non-linear medium, BBO in our case, and the difference frequency mixing yields a VIS signal polarized along the ordinary (o) axis. It is spectrally resolved with a 25-cm focal length spectrometer and detected with a Peltier-cooled low-noise CCD camera (Spec-10, Princeton Instruments). The latter

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operates in 10-pixel horizontal binning, such that one channel integrates ≈3.5 nm of the DFG signal. As background rejection is a major concern in these experiments, it is worth mentioning that the e-polarized gate beam does not fulfill phase-matching conditions for frequency doubling, and that the fundamental close to 800 nm, the gate beam, can be attenuated over several orders of magnitude by a combination of band pass filters and a linear polarizer.

The home-made set-up is sketched in figure 1. A solution of molecules is circulated through a quartz capillary (S) of section 0.5x0.5 mm². The UV fluorescence is excited at 5 kHz, by sub-100 fs, 266-nm pump pulses generated by Third Harmonic Generation (THG) from the fundamental 800 nm beam of a regenerative Ti:Sapphire amplifier. P1 and P2 are two parabolic mirrors of 3-inch diameter, and focal lengths of 5 cm and 30 cm, respectively. The downconversion process occurs in a BBO crystal mounted on a motorized rotary stage by spatially overlapping the UV fluorescence with a ≤35 μJ gate pulse at 800 nm. Both fluorescence and gate beam spots are matched in size and have 0.5-0.6 mm diameter. Rotating the BBO crystal around the vertical axis selects the spectral detection window by optimizing the phase matching condition (1). The gate beam is horizontally polarized, i.e. along the crystal axis, and the type II condition down-converts fluorescence with the same horizontal linear polarization. The 266-nm excitation beam is likewise horizontally polarized. The gate beam can be delayed by up to 2 ns.

With a 0.20 mm-thick BBO crystal, we achieve a 200-fs time resolution similar to what was observed in up-conversion experiments with near-UV emitting chromophores [16, 17]. The time resolution is determined as the FWHM of the time-resolved signal obtained by frequency mixing of the 266 nm pump light scattered from a diluted milk solution. While in the 0.2-mm crystal group velocity mismatch affects the time resolution [16], recent experiments with thinner crystals indicate that it is presently limited by wave front misalignment of fluorescence and gate in our set-up. Data displayed below have been measured with a 0.4-mm BBO crystal.

![Figure 1 A. Layout of the down-conversion experiment. S = sample, THG = third harmonic generation, P1/P2 = parabolic mirrors, SPF: short-pass filter. The gate pulse (red line) and the center of the fluorescence cone (purple) form a ≈7°-angle. Arrows indicate linear polarizations of fluorescence, gate and DFG signal beams. B. Steady-state absorption and emission spectra of 2,5-diphenyloxazole (PPO) in ethanol used for preliminary tests of the experimental set-up. The red curve is the DFG spectrum (transposed into the UV spectral region) at 10-ps time delay.](image)
intensities are kept low enough to operate in a regime of DFG signal linearity. This prevents the BBO crystal to be damaged in a long-term run. In the present contribution we report results for DFG wavelengths shorter than 730 nm only. As a matter of fact, residual (depolarized) scattered light from the intense gate pulse is eliminated by a shortpass filter (Thorlabs FES0750). Hence, any fluorescence emitted around 400 nm (and downconverted around 800 nm) remains undetectable.

3. Results and discussion

Fig. 1.2 displays an overview of the results of the experiment performed with PPO at a fixed BBO angular position, adjusted here for maximum DFG signal at 665 nm. This selects the near-UV fluorescence wavelength range around 363 nm, i.e. close to the maximum of the steady-state spectrum. Note that the spectral width of the DFG signal is 75 nm, translating into a near-UV spectral bandwidth of 20-22 nm. As the steady-state spectrum is almost constant around 363 nm, this value can be taken as the crystal's acceptance bandwidth in the present conditions.

![Figure 2.](image)

**Figure 2.** A. Time-resolved DFG spectra obtained at the maximum of PPO fluorescence. Three panels illustrate different ranges of delay times, as indicated in the legend. B. Experimental kinetic traces at 665 nm (squares) fitted with a sum of 3 (left) or 4 exponentials (right), convoluted with a 200-fs Gaussian representing the instrument response function (red line). Fit residuals (bottom line). Note the break at 10 ps on the time axis.

The first 300 fs display a dramatic spectral blue-shift due to group velocity differences (chirp) of the fluorescence. The only dispersive elements in the set-up are the 0.5 mm ethanol solution and the 0.25-mm fused silica capillary output window. The signal rise and slight red-shift observed over the first 5 picoseconds (cf. fig. 2A center) are attributed to intramolecular or solvent relaxation. Kinetic traces recorded in the range of 580 to 720 nm (fig. 3) underscore this gradual red-shift, as the rise times observed in the long-wavelength traces appear as decay components in the short-wavelength...
counterparts. This initial ≈10-ps phase is followed by a monotonous decay without any significant shift. We expect both anisotropy decay and the excited state lifetime to be responsible for the signal decay.

The signal-to-noise ratio is as good as 2000:1, as can be seen from the kinetic traces in fig. 2B. These have been obtained after ≈30 min data collection and averaging (0.4-mm BBO). The background noise level is defined by the rms of the spurious signal detected at negative times. Note that these traces are produced from a single CCD channel. Further binning and averaging would lead to an even higher dynamic range. The present data quality thus allows for a very accurate determination of the lifetimes in a multi-exponential fitting procedure. Fig. 2B-left shows a tri-exponential fit with two decay times and a single 2-ps rise time. But it is obvious that the recorded short delay time data deviate from this single exponential rise. The fidelity is significantly improved ($\chi^2$ reduced by 40 %, and better residuals) with a fit including a second rise time. Whether the signal rise really is biexponential, possibly indicating two different relaxation mechanisms, or is simply non-exponential (as may be the case for relaxation processes) is beyond the focus here. The point is rather to illustrate the very good signal-to-noise ratio of the time-resolved fluorescence data, and our ability to refine the quantitative analysis by including up to four time constants. For the signal decay we find a 60-ps time constant attributed to rotational diffusion of the PPO molecules in ethanol, as excitation and detection are linearly co-polarized, followed by a time constant of 1.32±0.1 ns. Even though a precise determination of the latter would ideally require a larger time span to be covered, it is in very good agreement with the well-characterized fluorescence lifetime of PPO in ethanol (1.4 ± 0.2 ns, [4]).

Figure 3: Selection of kinetic traces for PPO. DFG signal from 580 nm (red) to 720 m (cyan). The wavelength dependence of the initial sub-5 ps phase supports the interpretation of vibrational and or dielectric relaxation inducing a progressive red-shift of the fluorescence.

4. Conclusions
In summary, we have shown that type II difference frequency mixing is a new way of time-resolving near-UV fluorescence at wavelength < 400 nm with 200-fs resolution and an excellent signal-to-noise ratio. It is an interesting alternative to the wide-spread type I "up-conversion" technique, in particular in view of investigating local structural dynamics of proteins or oligonucleotides. Indeed, when multi-
exponential decay components need to be resolved, the present approach optimized for signal detection using a CCD affords excellent signal-to-background ratio. Further developments of the experiment involve selective near-UV excitation for Tryptophan or Aminopurine and the use of a near-infrared OPA as gate pulses, in view of recording complete time-resolved DFG spectra in the 500-900 nm range.

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