Electronic Supplementary Material (ESI) for RSC Advances

Supporting Information

Two-photon excitation of the fluorescent nucleobase analogues 2-AP and tC

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Supporting Materials and Methods

a) Fluorescence spectroscopy of 2-AP

Time-resolved fluorescence spectroscopy was performed with the technique of time-correlated single photon counting (TCSPC), using an Edinburgh Instruments spectrometer equipped with TCC900 photon counting electronics, as described previously.[1] The repetition rate was 4.75 MHz and the instrument response function (IRF), measured using a dilute Ludox scatterer, was *ca.* 70 ps full width at half maximum. Magic angle detection was employed. The pulsewidth and spectrum were measured with an autocorrelator (Mini, APE) and spectrometer (Wavescan, APE), respectively. The laser spectrum was continuously monitored to ensure pulse stability. The laser power was varied with neutral density filters and measured with a power meter (Fieldmaster with LM10 head, Coherent). The laser was focused onto the sample with a 4×, NA=0.1 objective. Powers were low to avoid saturation effects (*ca.* 1 mW for 3P, *ca.* 1 μW for 1P). Peak counts of 4000 (3P) and 10000 (1P) were recorded. A BG39 filter blocked the excitation for 3P excitation. Emission was detected at 380 nm in a front face configuration to avoid inner filter effects. The 3P excitation spectrum was corrected for the average power cubed.

Decay curves were analyzed using a standard iterative reconvolution method using Edinburgh Instruments software, assuming a multiexponential decay function.

$$I(t) = \sum_{i=1}^{n} A_i \exp\left(\frac{-t}{\tau_i}\right)$$

where A_i is the fractional amplitude (the "A-factor") and τ_i is the fluorescence lifetime of the *i*th decay component.

Steady-state emission spectra following excitation at 300, 584 and 900 nm were recorded with the same setup, with 9 nm emission bandpass. Corrected 1P excitation spectra were recorded using a spectrofluoremeter (Fluoromax, HORIBA Jobin Yvon).

b) Fluorescence spectroscopy of tC

Power dependence and cross-section measurements. A broadband Ti:sapphire laser (Mantis, Coherent) and compressor (CPC, Coherent) provided 2P excitation at 800 nm; this produced output pulses of *ca.* 20 fs at 80 MHz. Reflective neutral density filters attenuated the beam, which passed through a dichroic mirror (650DCSPXR, Chroma) and was focused with a 40× objective (PF, NA = 0.60, Nikon) onto the sample, which was in a 1cm pathlength cuvette. The incident power was monitored throughout (Uno meter and PH100-Si head, Gentech). The sample fluorescence was collected by the same objective and reflected from the dichroic mirror, passed through a shortpass filter (625SP, Chroma) to remove residual excitation light and detected by a fibre-coupled spectrometer (Ocean Optics QE65000).

Lifetime measurements. Time-resolved fluorescence spectroscopy of tC was performed using TCSPC. A Mai Tai Ti:Sapphire laser at 80 MHz was pulse picked (PulseSelect Dual, APE) to 8 MHz. The fundamental at 800 nm was used for 2P excitation or was frequency-doubled (from 810 nm) to give an output at 405 nm (Harmonic Generator 9300, Coherent). Emission at 515 nm was detected through a monochromator (1870C,

Spex) with bandpass of 6 nm using a microchannel plate detector (R3809U-50, Hamamatsu). Powers were low to avoid saturation effects (*ca.* 10 mW for 2P, *ca.* 5 μW for 1P). Peak counts of 4000 (2P) and 10000 (1P) were recorded. A 625SP filter blocked the excitation for 2P measurements, while a 420LP filter was used for 1P excitation. The instrument response function (IRF), measured using a dilute Ludox scatterer, was *ca.* 100 ps full width at half maximum. Magic angle detection was employed. For low-resolution measurement, 256 detection channels were employed (0.195 ns/channel). For high-resolution measurements, 4096 channels were used (0.012 ns/channel). Decay curves were analysed as described above by tail fitting.

Two-photon steady-state spectra. 2P emission and excitation spectra were collected in the same way as for the power dependence measurements above, but using the MaiTai laser at 80 MHz as the source. The excitation spectrum of tC was collected using this setup and was corrected for the average power squared. The pulsewidth was not monitored; however, we do not expect this to change significantly as a function of wavelength for this laser.

One-photon steady-state spectra. Absorption spectra were measured on a Perkin Elmer Lambda 1050 spectrometer. 1P emission spectra were recorded on the same setup as used above for the power dependence measurements, but using a supercontinuum laser source (SC400-PP, Fianium) tuned to 405 nm using an acousto-optic tuneable filter (AOTF VIS 5L, Crystal Technology). Corrected excitation spectra were collected, under magic angle conditions, using a spectrofluorometer (Fluorolog FL3-iHR, HORIBA Jobin Yvon) with

R928P PMT. The absorbance of the sample was low (< 0.05) so that inner-filter effects were negligible.

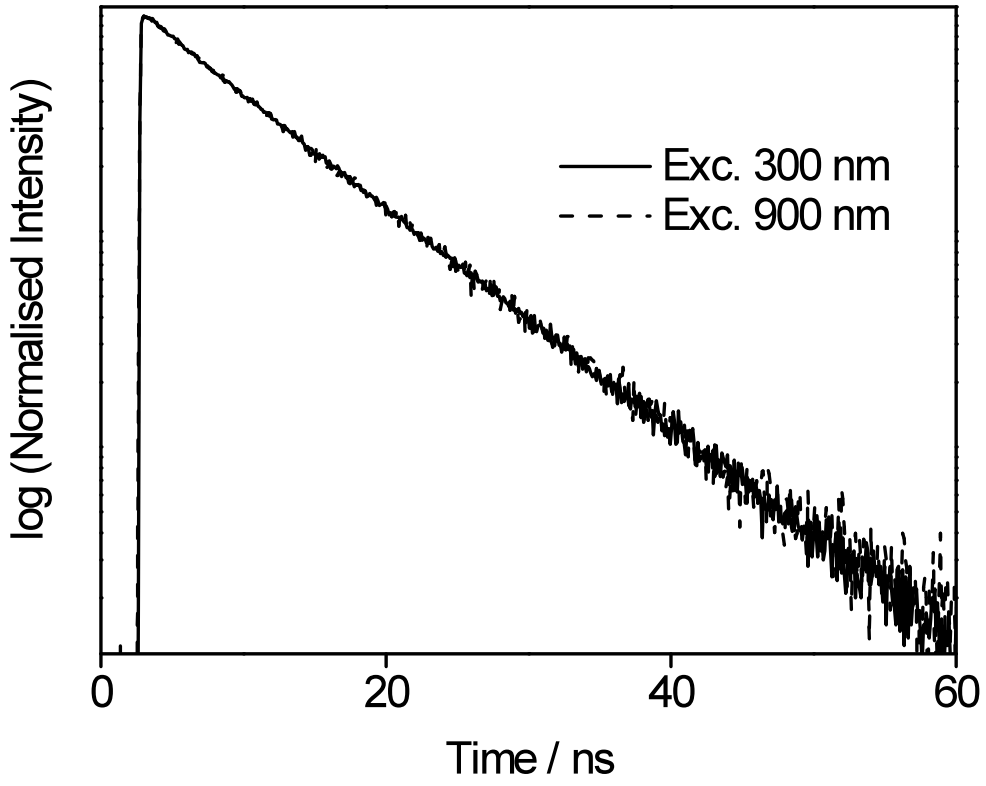

Figure S1. Normalised time-resolved decays of 2-AP following excitation at 300 nm and 900 nm. The overlaid decays are indistinguishable.

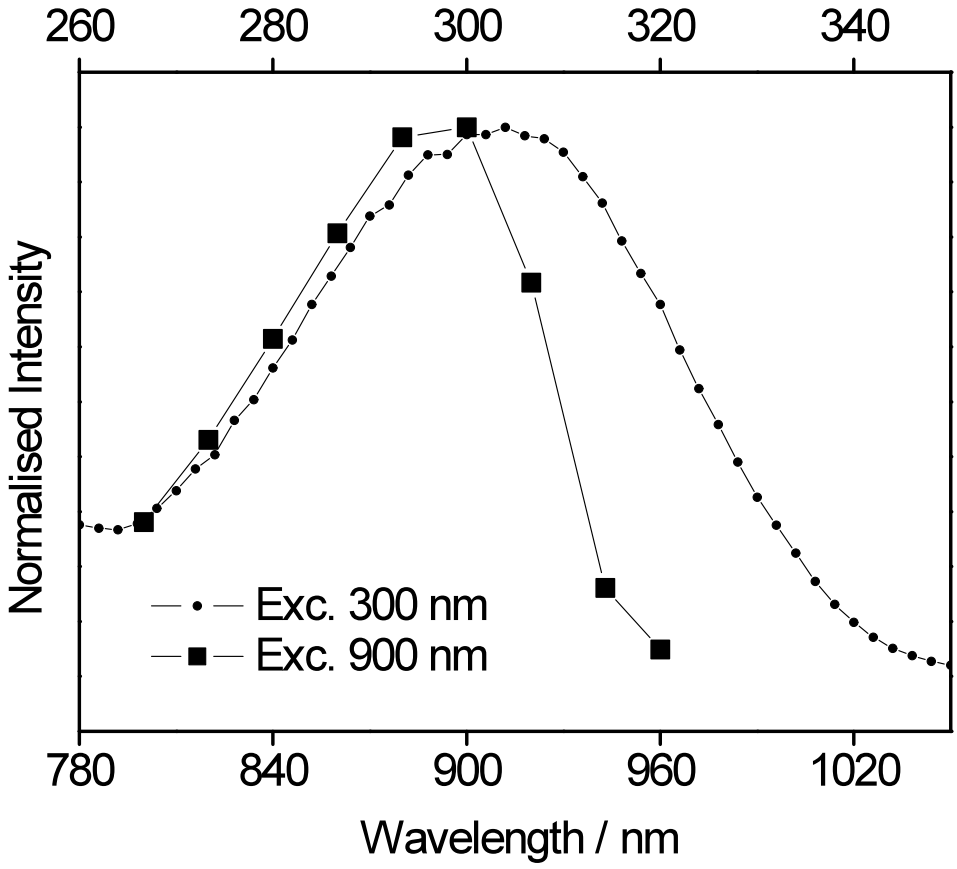

Figure S2. Excitation spectra of 2-AP following one-photon excitation (circles) and three-photon excitation (squares).

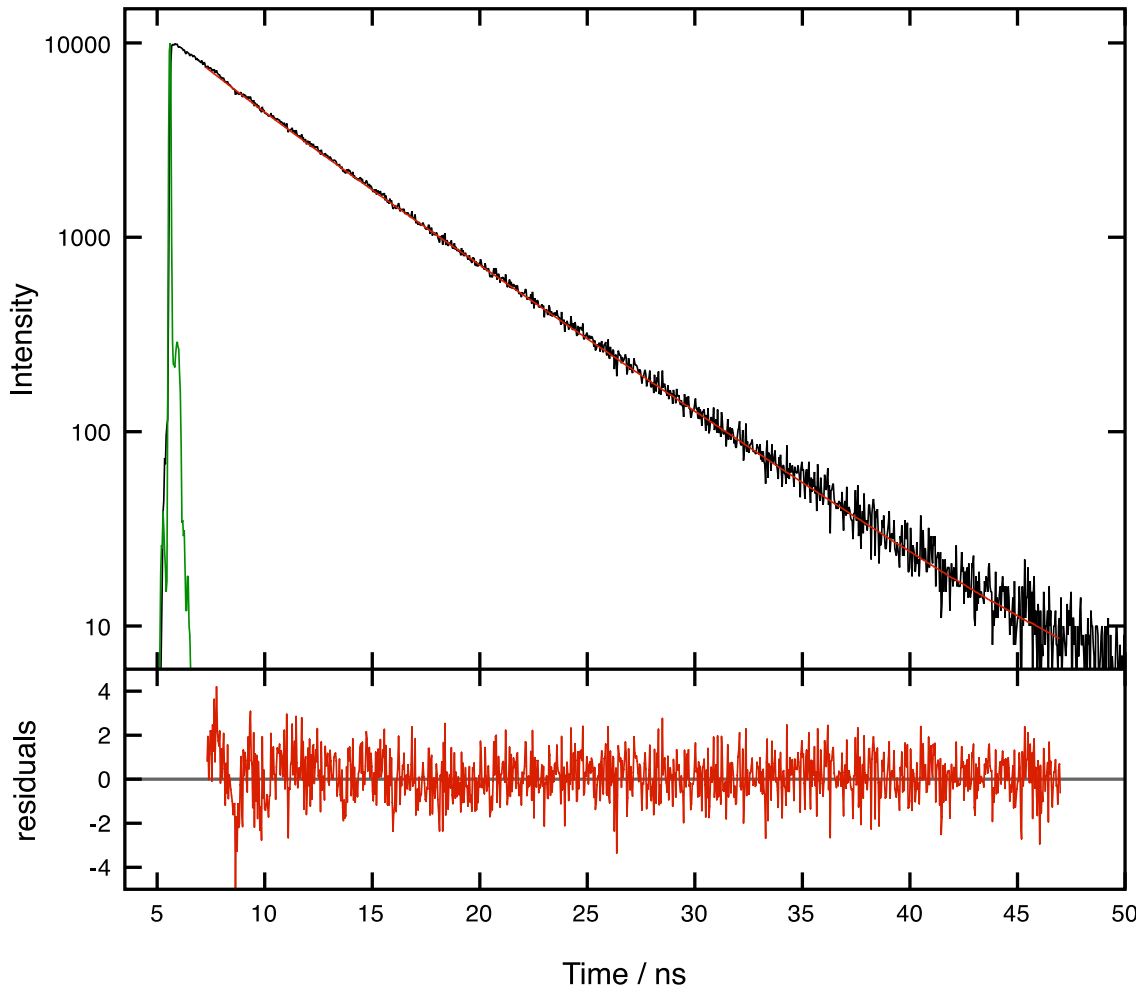

Figure S3. Time-resolved decay of tC (oligo **3**, 100 μM) at high resolution (4096 channels). The red line shows a biexponential fit with lifetimes of 2.86 ns and 5.75 ns, with A-factors of 0.17 and 0.83, respectively; χ^2 = 1.15. A single exponential fit (not shown) gave a lifetime of 5.48 ns; χ^2 = 1.40. The instrument response function is shown in green.

References

1. R. K. Neely, S. W. Magennis, D. T. F. Dryden and A. C. Jones, *J. Phys. Chem. B*, 2004, **108**, 17606-17610.