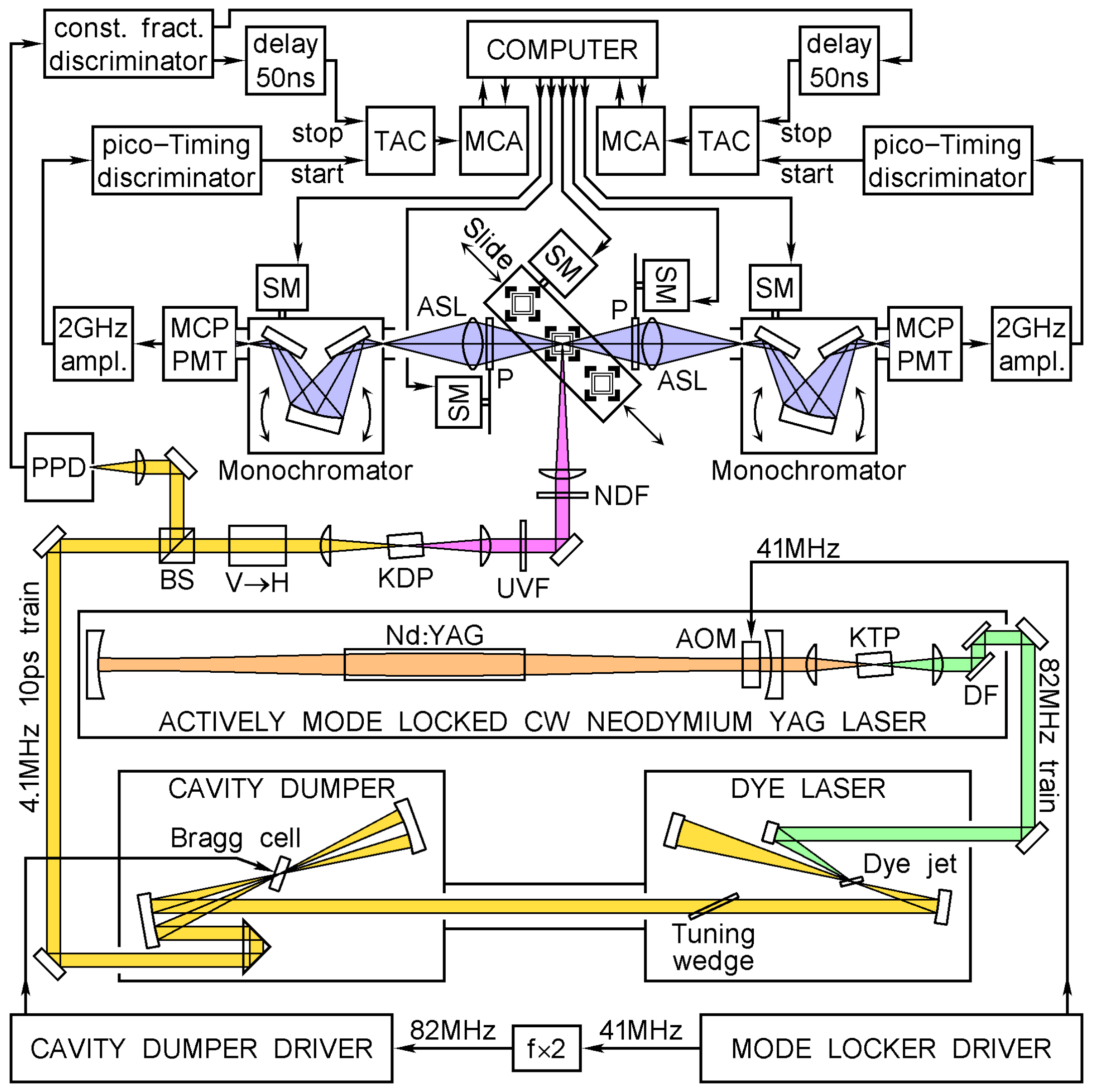
EXPERIMENTAL RECORDING OF TIME-RESOLVED TRYPTOPHAN FLUORESCENCE SPECTRA

Dmitri Toptygin and Ludwig Brand

Experimental Setup

The experimental setup is based on the Time-Correlated Single-Photon Counting (TCSPC) technique. Fluorescence is excited by a train of picosecond pulses from a laser system shown in the schematic diagram below. The inclusion of the computer-controlled polarizers and monochromators in the emission paths makes it possible to measure fluorescence intensity as a function of the emission wavelength, polarization, and the time after excitation. Time resolution ≈50 picoseconds FWHM.



2GHz ampl.	Low-noise amplifier with 2GHz bandwidth.
AOM	Acousto-Optical Modulator.
ASL	ASpheric condenser Lens.
BS	Beam Splitter; diverts 1% to PPD, transmits 99% straight through.
DF	Dielectric Filter, consists of two dielectric mirrors with high reflectivity at 532nm and low reflectivity at 1064nm (at 45° angle of incidence).
KDP	Potassium dihydrophosphate nonlinear optical crystal. Frequency-doubles the dye laser output (572 ... 638nm) to UV radiation (286 ... 319nm).
KTP	Potassium thallium phosphate nonlinear optical crystal. Frequency-doubles the infrared laser radiation (1064nm) to visible light (532nm).
MCA	MultiChannel Analyzer.
MCP-PMT	MicroChannel Plate PhotoMulTiplier.
NDF	Neutral Density Filter, helps to avoid protein photobleaching.
Nd:YAG	Neodymium-doped Yttrium-Aluminum Garnet crystal.
P	Polarizers in the fluorescence emission paths.
PPD	Picosecond PhotoDetector, a passivated non-avalanche photodiode.
Slide	A linear translational stage with three thermostatted cuvette holders.
SM	Synchronous Motors, computer-controlled via the computer serial port.
TAC	Time-to-Amplitude Converter.
V→H	A polarization rotator, converting Vertical polarization to Horizontal.
UVF	UltraViolet Filter. Blocks visible light, transmits UV radiation.

Mathematical Model

The Time-Correlated Single-Photon-Counting (TCSPC) data are fitted by the numerical convolution of the quasi-simultaneously measured Impulse Response Function (IRF) with the following model function:

$$F_m(t) = b_m + s_m\delta(t) + \theta(t)\sum_{n=1}^{N_E}\frac{f_{mn}}{\tau_n}\exp(-t/\tau_n) \tag{1}$$

t	time, measured from δ-excitation
m	the serial number of the wavelength at which the TCSPC data were collected
$\delta(t)$	Dirac delta function
$\theta(t)$	Heaviside step function
N_E	the number of exponential terms
b_m	photomultiplier background (dark counts)
s_m	the intensity of scattered exciting radiation (including Raman)
f_{mn}	the intensities associated with the exponential terms
τ_n	the time constants (the same set of time constants τ_n applies to all wavelengths)

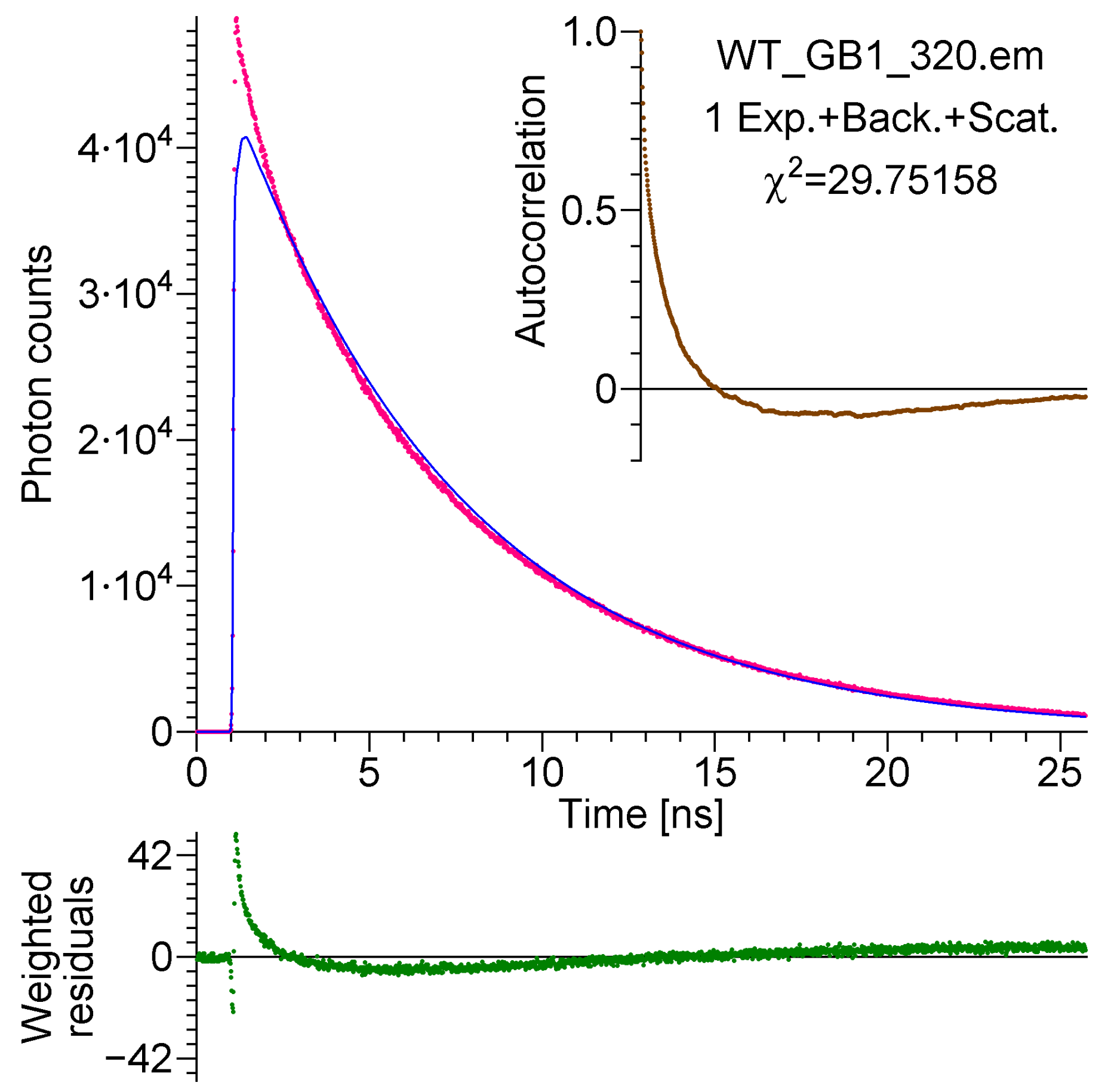
Experimental TCSPC data are shown by the dots of magenta color

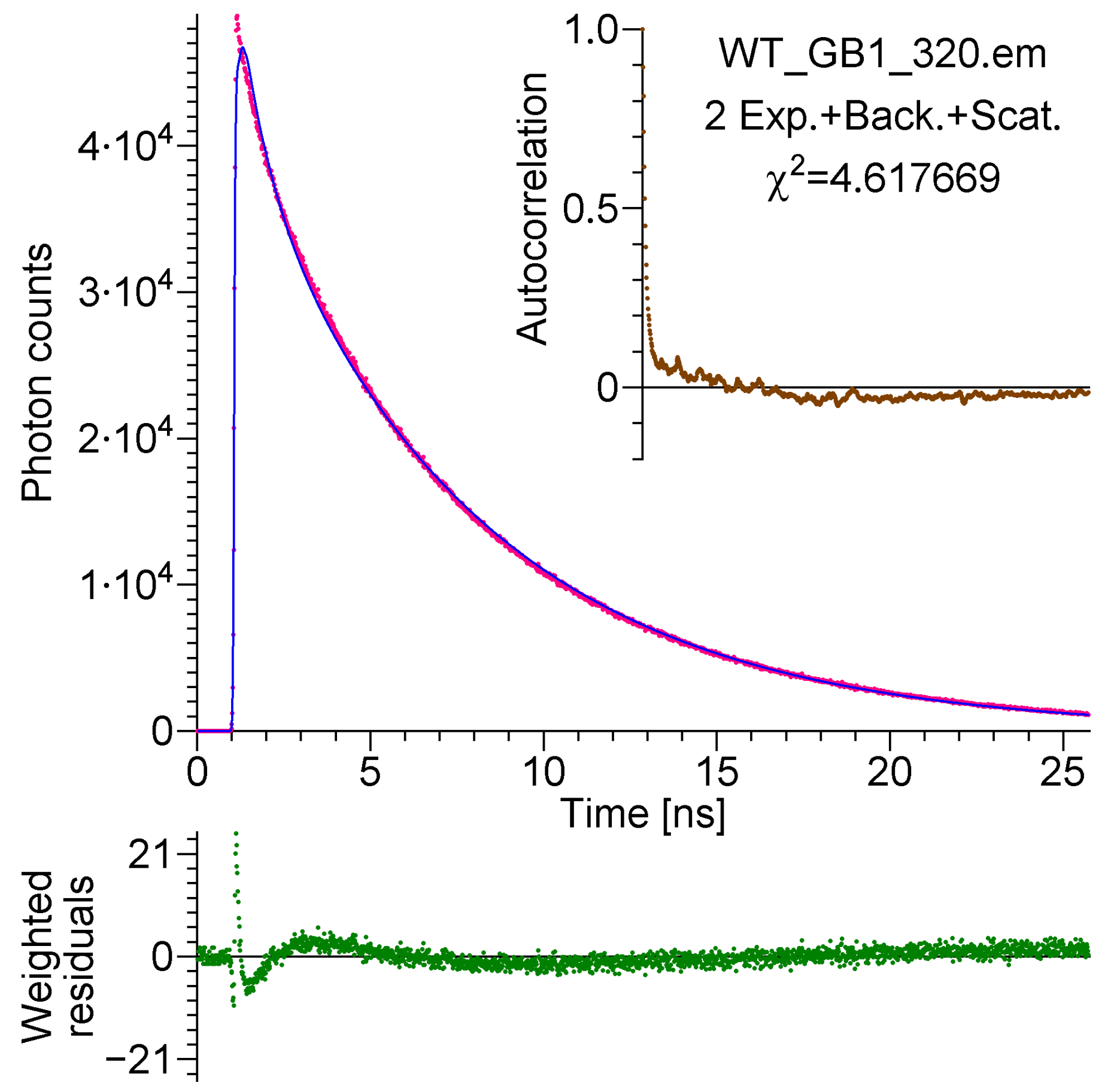
The fit by the numerical convolution of the quasi-simultaneously measured IRF with the model function (1) is shown by the blue line

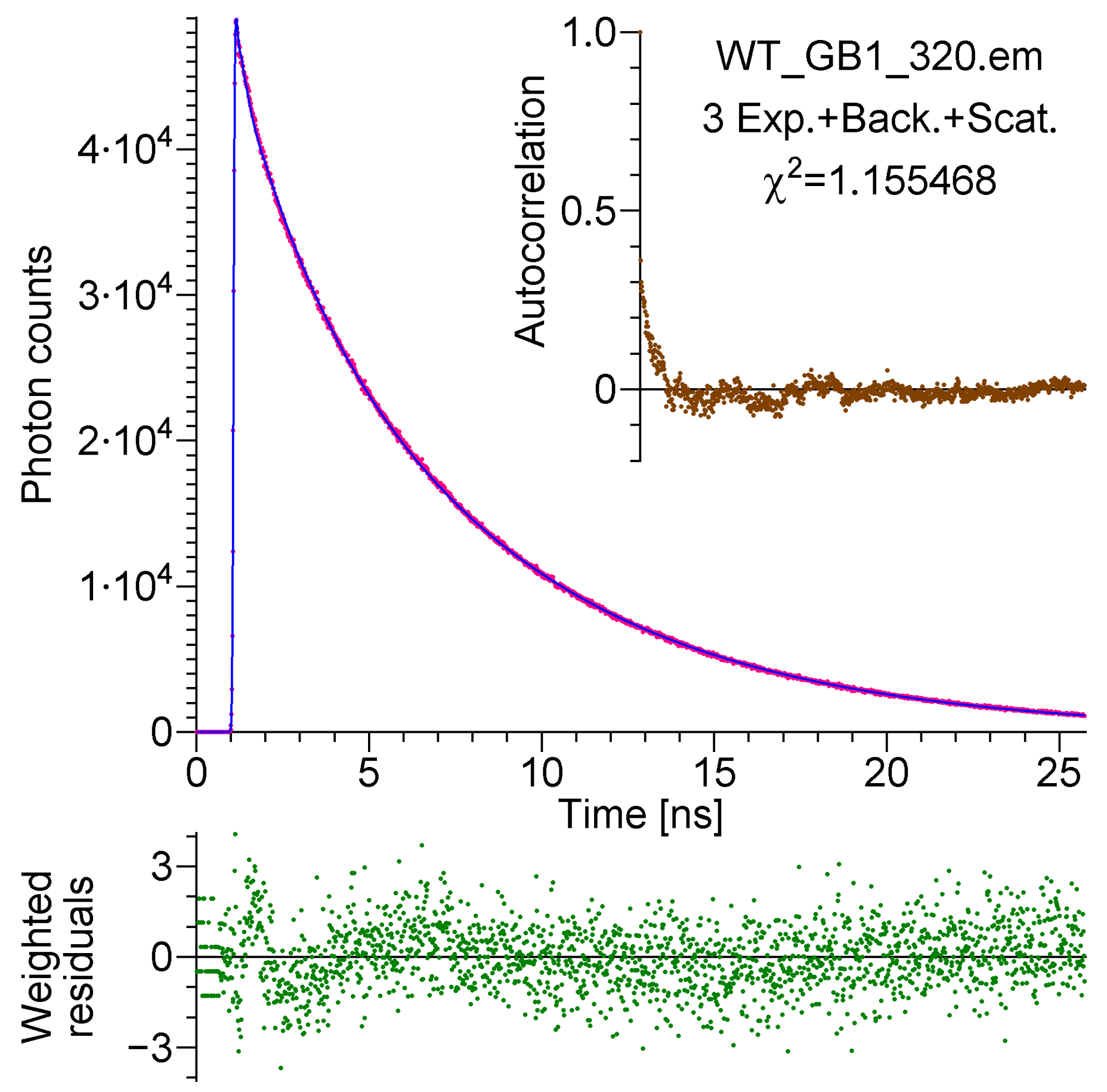
The differences between the data and the fit, divided by the corresponding standard deviations, called the Weighted Residuals, are depicted by the dots of green color

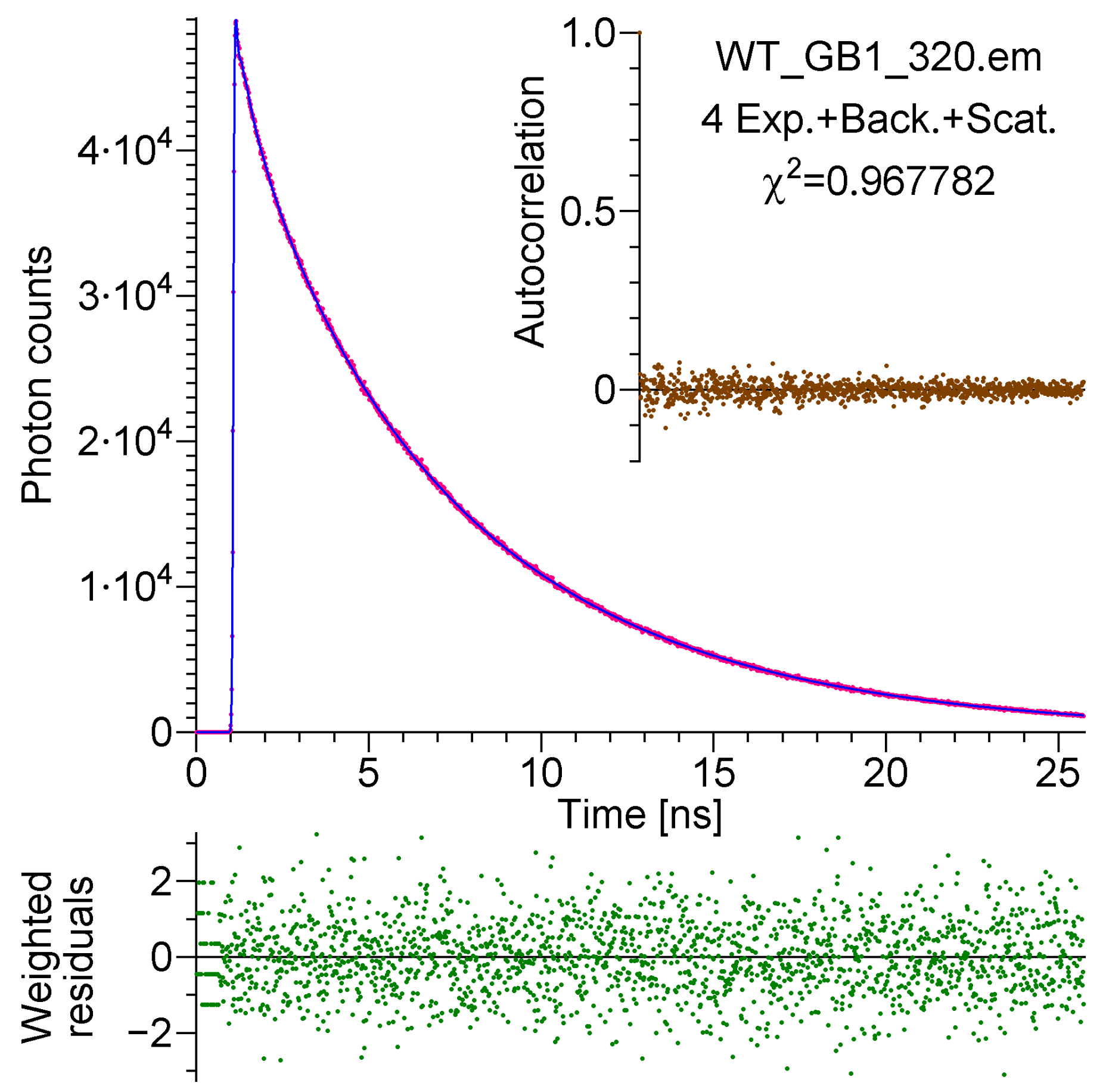
Peak-normalized autocorrelation of unweighted residuals (plain differences between the data and the fit) is depicted by the dots of brown color

Single-Curve Fitting



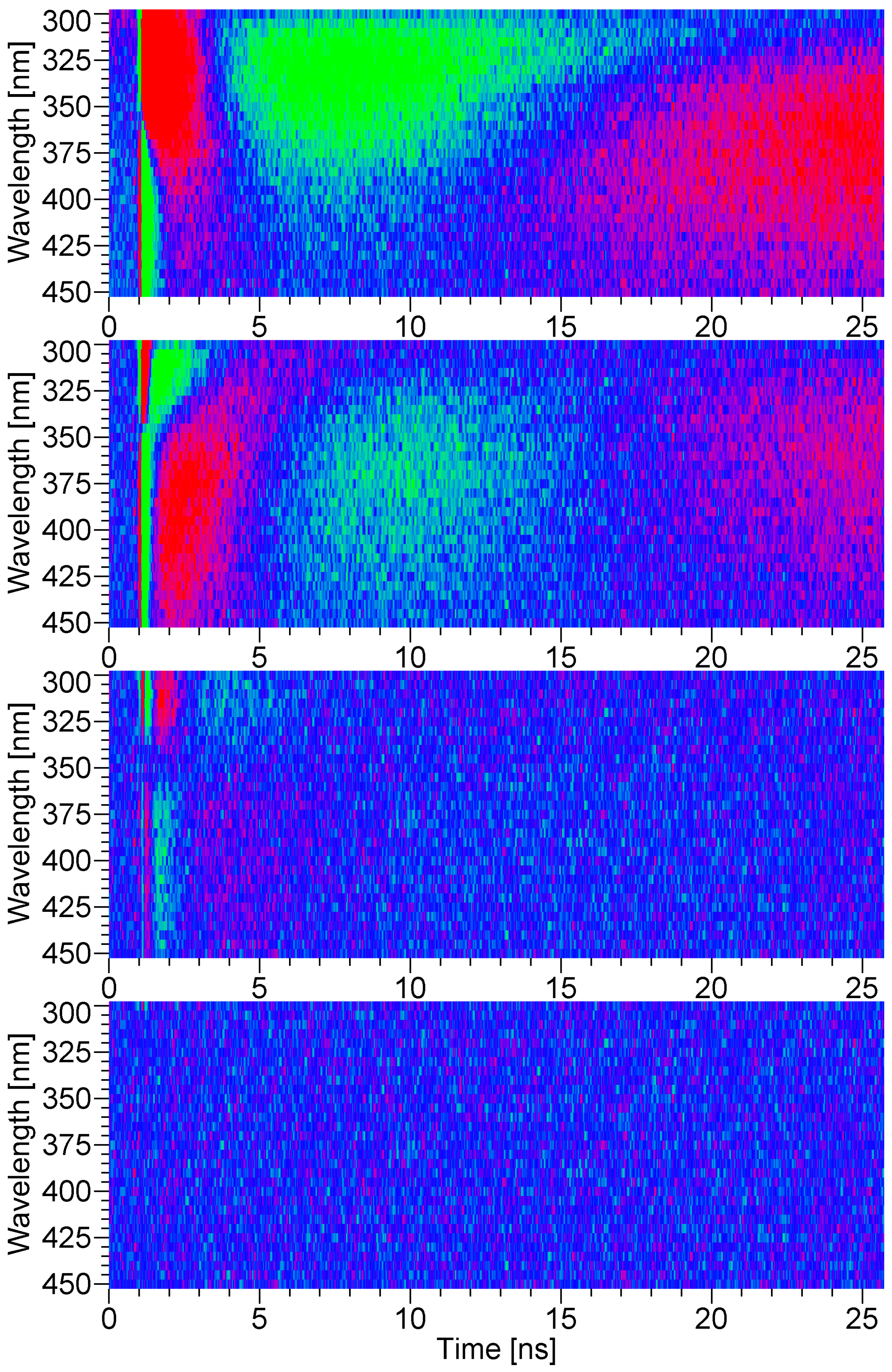






Global Fitting

Single-curve fitting is used mainly to generate the plots of the data, fit, weighted residuals, and autocorrelation, like the ones shown to the right. To obtain time-resolved emission spectra, we collect TCSPC data sets at multiple emission wavelengths and analyze all the data sets simultaneously (this is necessary because the global fitting parameters τ_n apply to all data sets). The quality of the global fit is judged by the reduced global χ^2 and also by the color map of weighted residuals, four examples of which are shown below. The 31 TCSPC data sets were obtained at emission wavelengths from 300nm to 450nm at 5nm wavelength intervals; fluorescence emission was from the single Trp residue of the B1 domain of Streptococcal protein G (GB1) at +5°C and 296nm excitation, data from *J. Phys. Chem. B* **2006,** *110,* 26292. The four color maps (top to bottom) represent the fits to the same data with the models of one, two, three, and four exponentials, respectively. Corresponding reduced χ^2 values are: 4.956, 2.134, 1.075, and 1.004.



The weighted residuals (WR) are calculated as the differences between the data and the fit, divided by the corresponding standard deviations, and depicted using the following color scheme: blue for near-zero WR, red for positive WR, green for negative WR. Brighter green and red colors correspond to greater magnitudes of the WR.

The most important results of the global fitting are the values of the fitting parameters f_{mn} and τ_n that are then used to reconstruct the amplitude spectra and the time-resolved emission spectra shown to the right. Minute mathematical details of this procedure will be soon published in the volume chapter entitled *Analysis of Time-Dependent Red Shifts in Fluorescence Emission from Tryptophan Residues in Proteins,* in the series *Methods in Molecular Biology*, volume title: *Fluorescence Spectroscopy and Microscopy: Methods and Protocols*. The amplitude spectra (the first and the third figure from the top in the right column) represent the spectral variation of the amplitudes corresponding to different exponential terms as a function of the emission wavenumber. The choice of the wavenumber rather than the wavelength for the X axis is dictated by the fact that energy is linearly related to the wavenumber (or frequency), but not to the wavelength. The presence of negative amplitudes in some spectral regions is a direct evidence of relaxation (heterogeneity alone cannot result in negative amplitudes). The time-resolved emission spectra (the second and the fourth figure from the top in the right column) represent instantaneous emission spectra at different times after the excitation by a short pulse (δ-excitation). The top two figures in the right column represent the single Trp residue of the B1 domain of Streptococcal protein G (GB1) at +5°C and 296nm excitation, data from *J. Phys. Chem. B* **2006,** *110,* 26292. The bottom two figures in the right column represent the single Trp residue of the E21W variant of $\mathrm{IIA}^{\mathrm{Glc}}$ protein from *Escherichia coli* at +5°C and 289nm excitation, data from *J. Phys. Chem. B* **2001,** *105,* 2043.

Amplitude Spectra and Time-Resolved Spectra