Electronic Supplementary Material (ESI) for Analyst.

Supporting Information

Probing DNA-Stabilized Fluorescent Silver Nanocluster Spectral Heterogeneity by Time-Correlated Single Photon Counting

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Synthesis procedure and description of the samples

The synthesis protocol for making samples with buffer and using a heat treatment step is as follows:

1. The buffer was added to the new DNA vial
2. The vial was heated for 2 min at 80-85 °C and stirred for 2 min at 500 rpm
3. The volume of DNA needed was transferred to a different container and cooled down for 1 min at room temperature
4. The $AgNO_3$ solution was added and the mixture stirred for 2 min at 1000 rpm
5. 10-15 min after addition of $AgNO_3$, the $NaBH_4$ solution was prepared and immediately added. The mixture was stirred for 2 min at 1000 rpm. The DNA concentration at this stage is [DNA] = 80 μM.
6. After 10 min the sample was diluted to [DNA] = 10 μM for spectroscopic measurements

If the DNA vial was used several times, the procedure was started from step 2.

Adding the buffer to the DNA vial ensures a control of the pH environment from the first step of the synthesis. The reason of following step 2 is to melt the DNA in order to "reset" its configuration and to make sure that it is homogeneously diluted in the media. This turned out to be especially important when using buffers. In fact, when we used buffer and did not follow the heat treatment step (step 2), the amount of DNA taken from the vial showed large fluctuations (see for example S6 in Figure SI3 where too much was taken). When too little was taken, very little fluorescence and a very broad plasmon band of silver nanoparticles was observed in the absorbance spectra. The latter results of too low DNA amount were discarded. For the samples with Milli-Q water, Milli-Q water was used in step 1 instead of buffer.

SAMPLE	BATCH	NEW	MEDIA	HEATING
S1	1	Yes	Milli-Q	No
S2	1	No	Milli-Q	No
S3	2	Yes	Milli-Q	No
S4	3	Yes	Buffer	Yes
S5	3	No	Buffer	No
S6	4	No	Buffer	Yes*
S7	5	Yes	Buffer	Yes
S8	5	No	Buffer	Yes
S9	5	No	Buffer	Yes
S10	6	No	Buffer	Yes
S11	6	No	Buffer	Yes

Table SI1: Description of the samples. Different DNA vials received from Eurogentec are identified with the batch number, the field "Sample" names the samples in chronological order. "New" indicates if the DNA was extracted from the vial the first time, "Media" indicates the media where the DNA was diluted before first usage of the batch, and "Heating" indicates whether the heating procedure was used. The lines shaded in blue (green) indicate that the samples are classified into group 1 (group 2). *The heating procedure was followed; however the amount of DNA used was too high, since the vial was used before without the heating procedure to make S5.

Decay curve fitting with multiexponential model

The measured decay curves were fitted to a four exponential decay model with free background convoluted with the instrument response function (IRF), which showed in the majority of the cases a χ^2 parameter well below 1.1 and residuals randomly distributed around zero. An integration time of 80 s per curve ensured that the counts were at least 40000 at the peak emission wavelength. In order to ensure the quality of the data, decay curves with less than 10000 counts in the maxima, corresponding to the red tail of the spectra are not included in the calculation of the average decay time spectra. A given dataset is labeled by the excitation wavelength, time after preparation and sample number. Each dataset consists of 40-70 decay curves (5 nm steps of the emission monochromator) plus an IRF. We fitted the dataset globally, linking the decay times and allowing the amplitude to change freely. An example of a decay time fit is shown in Figure SI1.

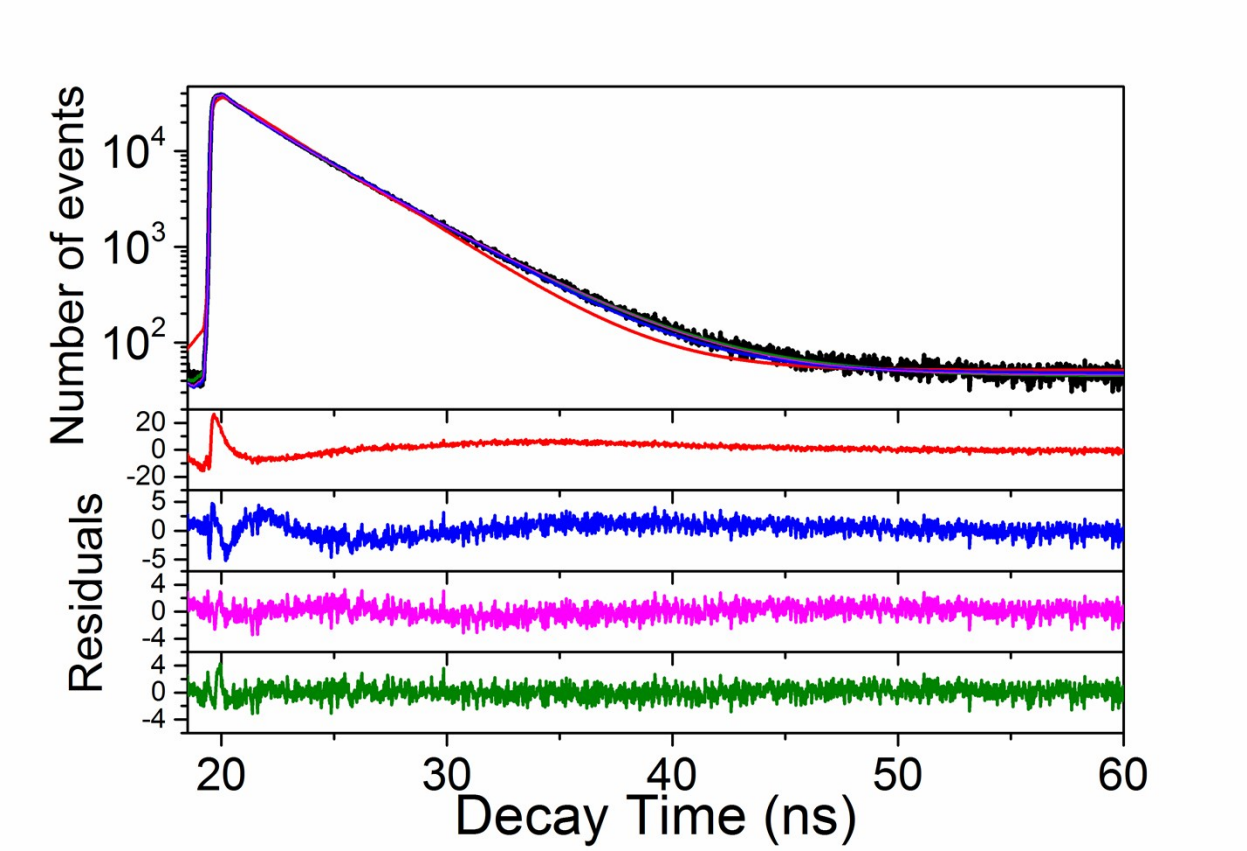

Figure SI1: 1 (red), 2 (blue), 3 (magenta) and 4 (green) exponential decay curve fit of sample S8, excited at 560 nm and detected at 660 nm. The χ^2 values of the fit shown were 15.23, 1.82, 1.09 and 1.03 for the 1, 2, 3 and 4 exponential decay curve fit. This fits yielded an average lifetime of 2.87 ns, 3.03 ns, 3.05 ns and 3.04 ns respectively. The χ^2 values of the global fits to which the shown fits belong were 17.33, 2.49, 1.22 and 1.08 for the 1, 2, 3 and 4 exponential decay curve fit.

Alternative decay curve fitting with Gaussian model

In our study, a multiexponential model was used to calculate the average decay time spectra, although the different amplitudes and decay times do not necessarily represent the physical model. The reason is that the average decay time is virtually independent of the model used to fit

the decay curves, as long as the fit is good enough. Table SI2 shows a comparative of different models used to fit three different decay curves.

λ_{exc} (λ_{em})	510 nm (612 nm)			560 nm (667 nm)			635 nm (704 nm)		
Model	G1	G2	E4	G1	G2	E4	G1	G2	E4
Av. decay time τ_{av} (ns)	4.27	4.23	4.23	2.99	2.91	2.91	3.37	3.36	3.38
χ^2	3.87	1.16	1.09	2.52	1.13	1.13	1.31	1.11	0.997

Table SI2: Values of the average decay time of three different decay curves from sample S7 and for different models, along with the goodness of the fit (χ^2). The models are noted as G (Gaussian) or E (Exponential) followed by the number of normal distributions or exponents in the fit. The average decay time values from the Gaussian model are calculated numerically by the FluoFit software (there referred to as intensity weighted average lifetime).

A particularly good candidate of a physical model is a Gaussian distribution of decay times, based on the single molecule studies of C24-AgNCs by Hooley et al.[1] In that article, upon 635 nm excitation, the distribution of decay times for different single molecules is in excellent agreement with a Gaussian distribution centered around 2.7 ns and with a full width half maximum (FWHM) of 1.4 ns. Figure SI2C shows the distribution of decay times after a fit with two Gaussian distributions for sample S7 at 635 nm excitation and 704 nm emission wavelength (around the peak emission). A sharp distribution centered at 0.22 ns (which could be interpreted as a single exponential component) and a broad distribution centered at 3.2 ns with FWHM of 1.8 ns are observed. The broad distribution gives values comparable with the single molecule studies, and the differences in the values could be due to the immobilization of the molecules in PVA for single molecule studies versus the solution case. Similar fits with 2 Gaussian functions

were also performed for the data at 510 and 560 nm and the results are shown in Figure SI2A and SI2B. The fit with 2 Gaussian functions gives similar good χ^2 values to the 4-exponential model (see Table SI2).

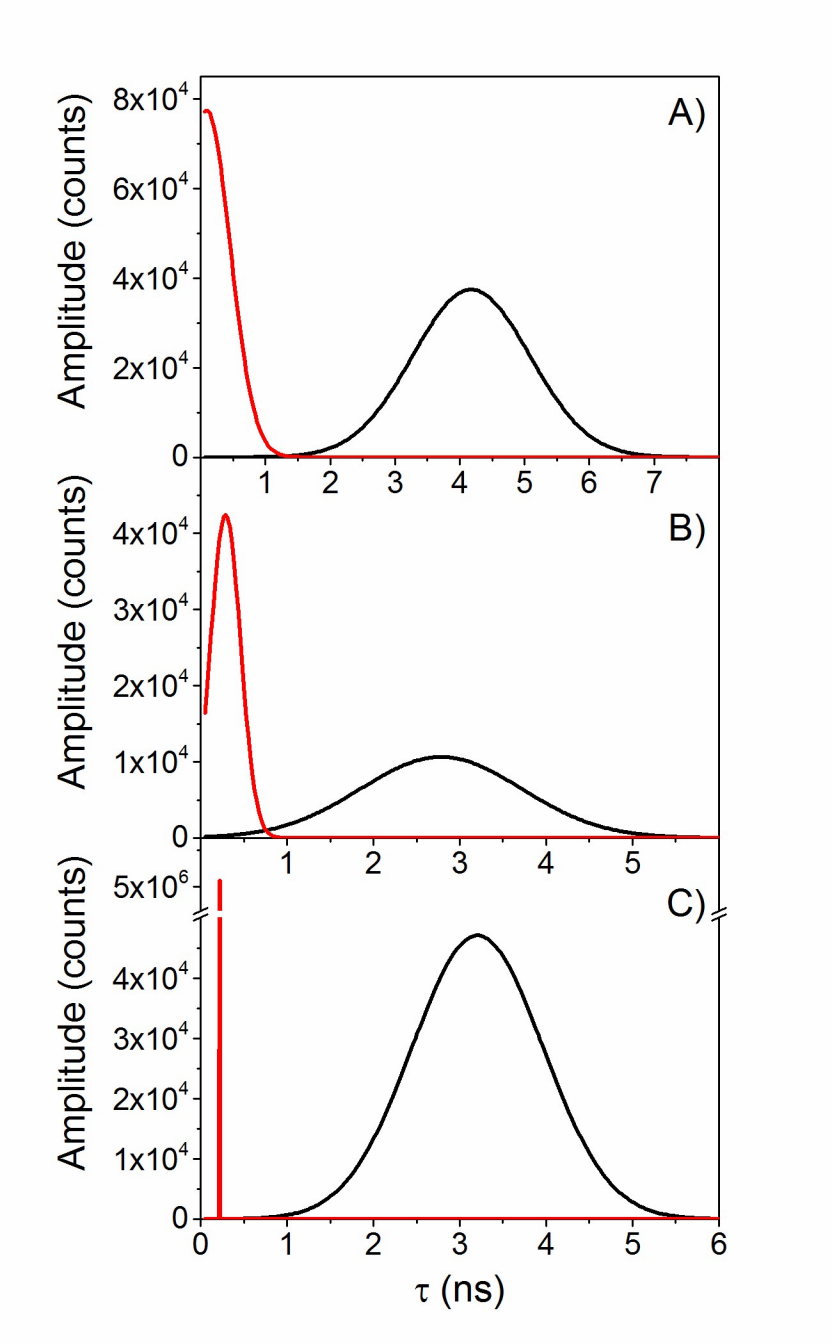

Figure SI2: Distribution of decay times after fits with 2 Gaussians for A) 510 nm, B) 560 nm and C) 635 nm excitation wavelengths, around the peak emission wavelength (612 nm, 667 nm and 704 nm respectively). The values of χ^2 and average decay times are shown in Table SI2.

Absorbance spectra of all the samples

Figure SI3 shows the absorbance of all the samples. The main purpose of this data is to compare the actual amount of DNA that was used in each of the sample preparations. A deviation of the DNA concentration can be observed for S1, S4 and S6 compared to the other samples, which most likely occurred during preparation (taking out the DNA from the vial). Despite this, the average decay time spectra of S6 still look similar to these of S5 (see Figure 3).

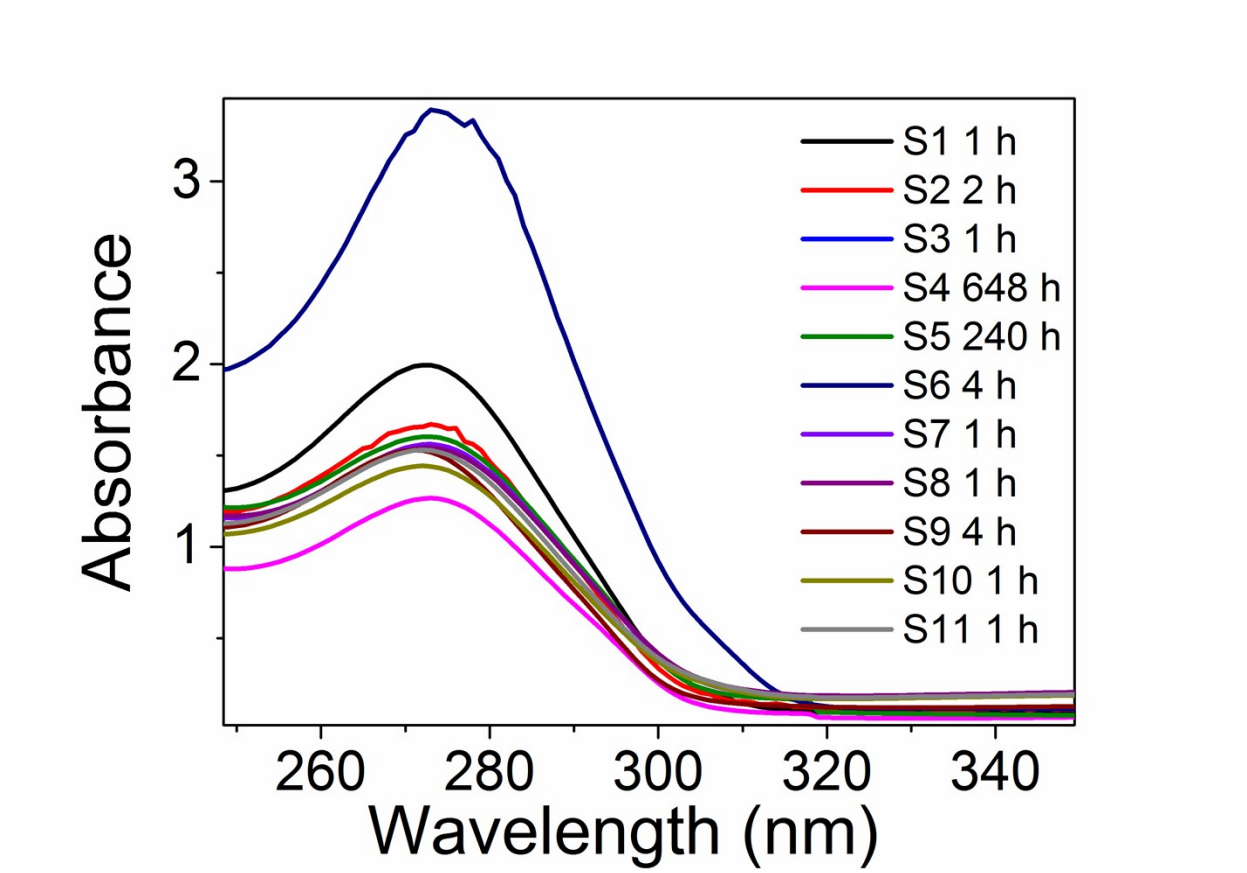

Figure SI3: Absorbance of the DNA for all the samples used in the experiments, together with the approximate times of acquisition after preparation.

Average decay time spectra of a mixture of two Rhodamines.

In Figure SI4, a control experiment of our experimental system and the analysis method is presented. We measured the average decay time spectra of two Rhodamine fluorophores with known decay times (Rhodamine B and Rhodamine 6G in water[2]) and of a mixture of both. The Rhodamine 6G has an average decay time of 4 ns over its emission spectra (Figure SI4A). Rhodamine B has an average decay time of 1.6 ns over its emission spectra (Figure SI4B). Figure SI4C shows the average decay time spectra of the mixture of the two dyes. The average

decay time of the mixture is nearly flat in the region below 550 nm where the Rhodamine B practically does not contribute to the emission while it features a clear slope in the region where the spectra of the two fluorophores overlap and where their relative contributions change drastically. Above 575 nm, the average decay time becomes fairly flat since the two dyes have similar contribution to the total emission. The average decay time in this red-edge region is in between the average decay times of the two Rhodamines. The reason is that the average decay time of the mixture is the (intensity) weighted average value of the average decay times of the individual dyes.

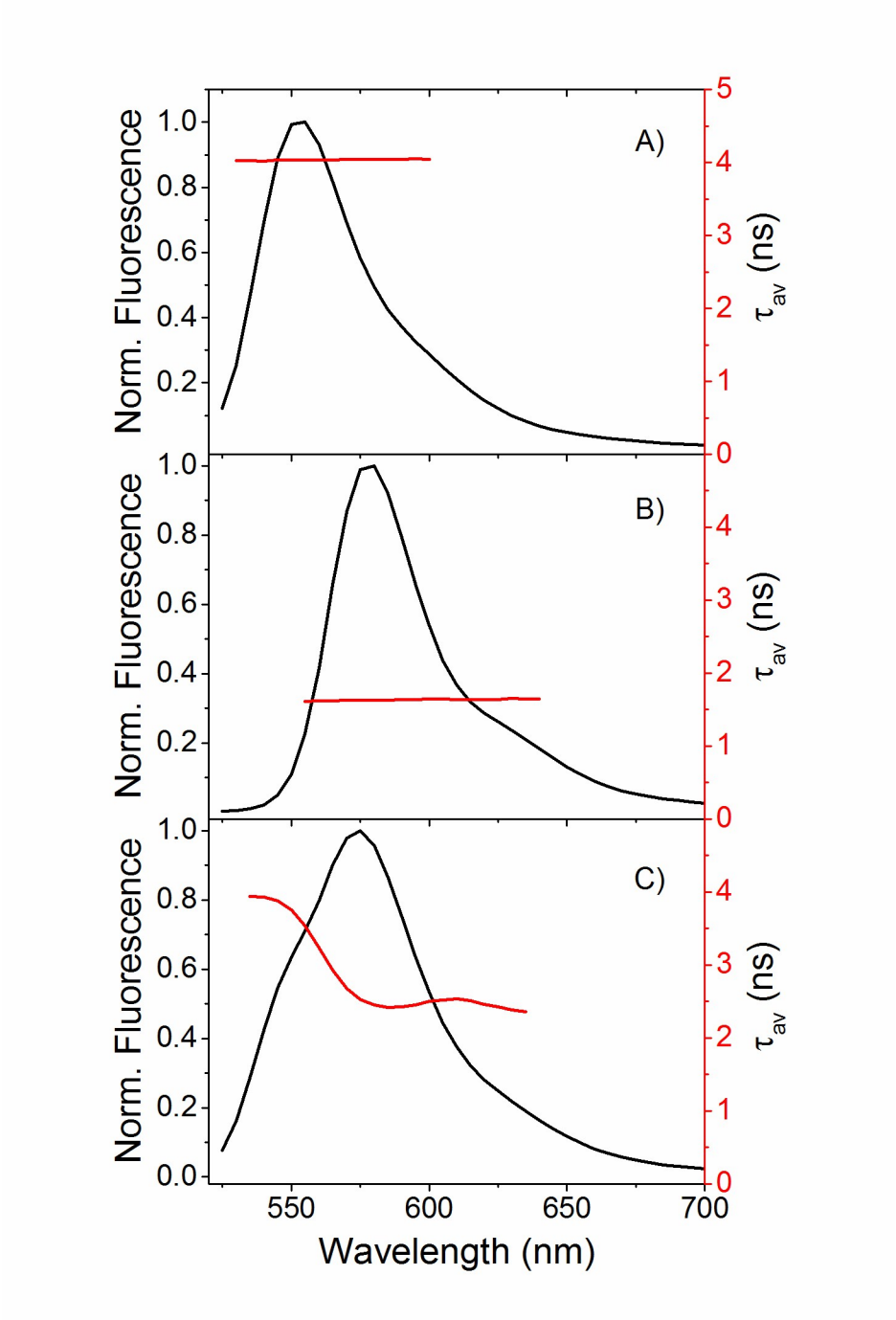

Figure SI4: Fluorescence spectra and average decay time spectra of Rhodamine 6G (A), Rhodamine B (B) and a mixture of both (C). The left axis shows the fluorescence spectra (black) and the right axis shows the average decay time spectra (red).

Average decay time spectra (large figures).

In this section we show the subfigures from Figure 3 individually. In Figure 3, the figures showing data from the same excitation wavelength have the same scale, in order to compare easily the average decay time spectra of all the experiments. In the figures shown in this section, the scale has been adapted in order to distinguish the details of the different curves within one plot. Similarly as in Figure 3, the data acquired on the day of preparation is shown as solid lines, the day after as dashed lines and subsequent days are shown as dotted lines. The starting time of the acquisition since the preparation is shown in the legend.

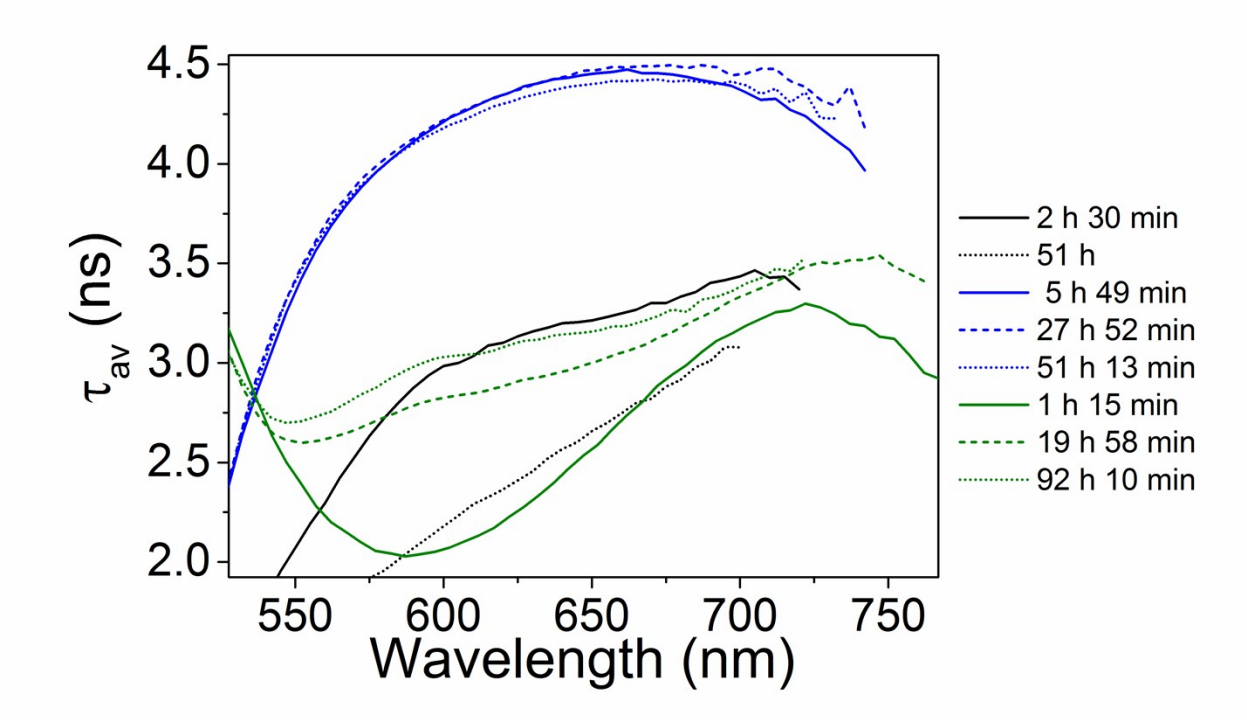

Figure SI5: Average decay time spectra of samples S1 (black), S2 (blue), and S3 (green) for 510 nm excitation. Large version of Figure 3A.

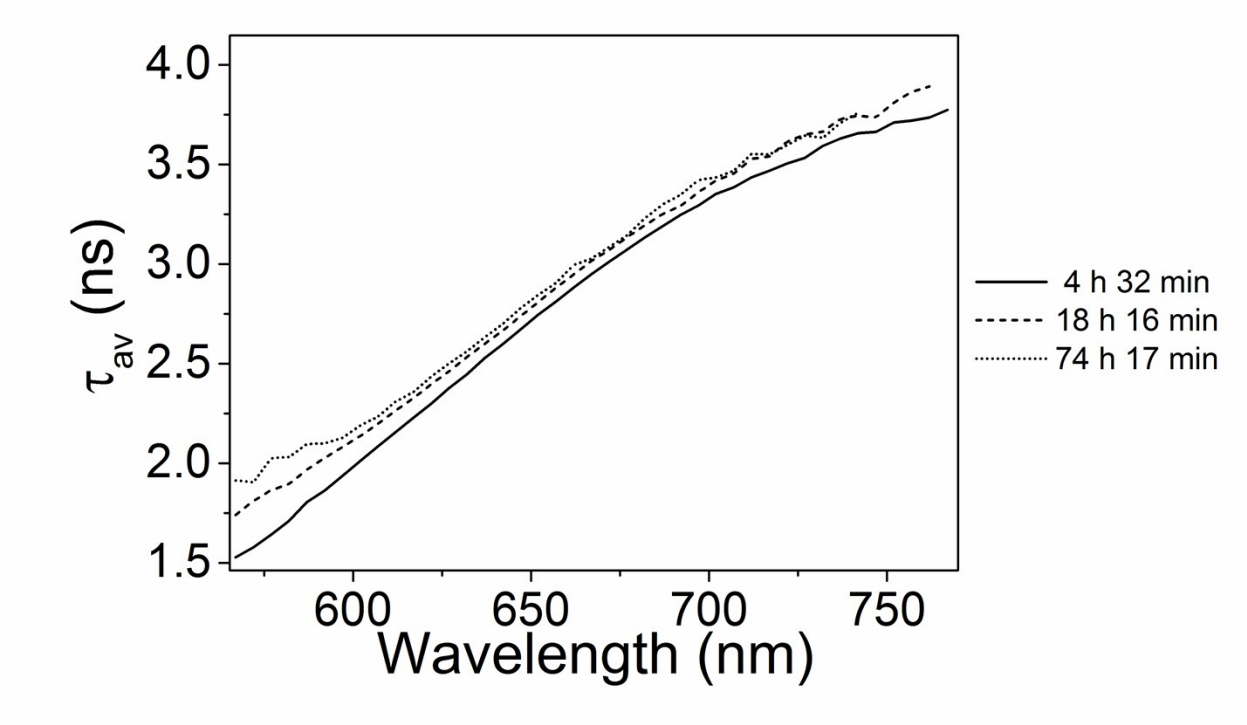

Figure SI6: Average decay time spectra of sample S3 for 560 nm excitation. Large version of Figure 3B.

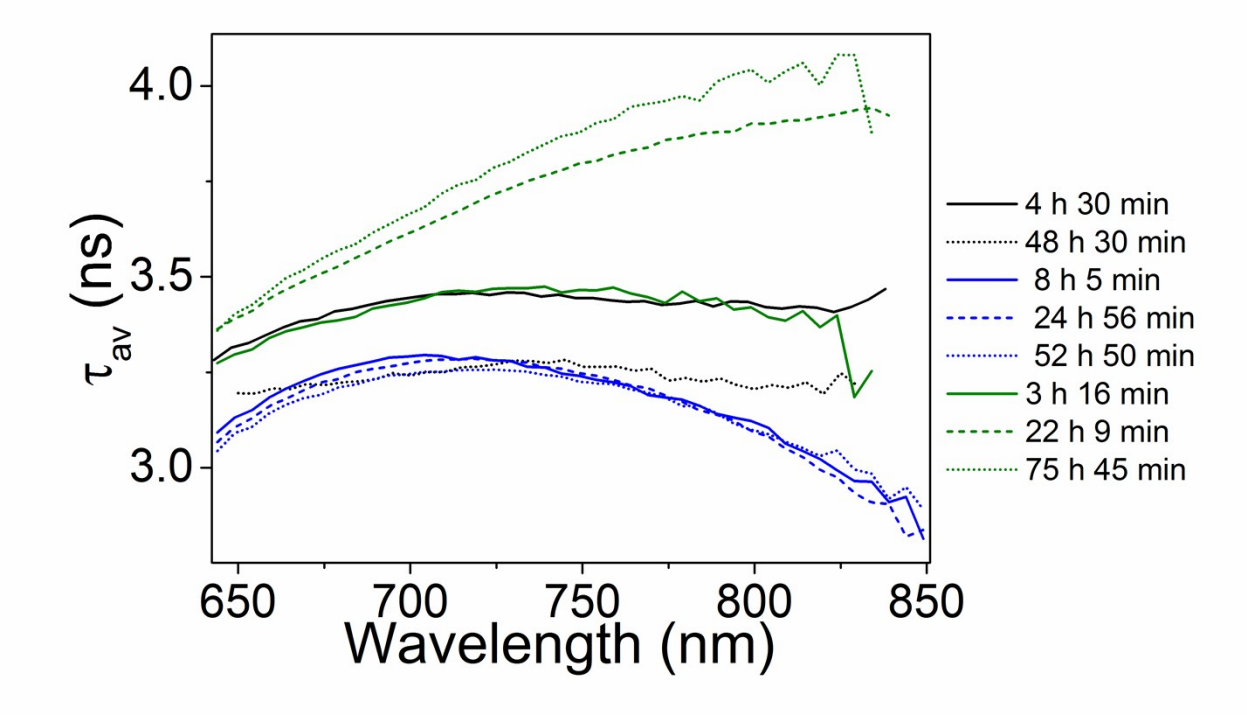

Figure SI7: Average decay time spectra of samples S1 (black), S2 (blue), and S3 (green) for 635 nm excitation. Large version of Figure 3C.

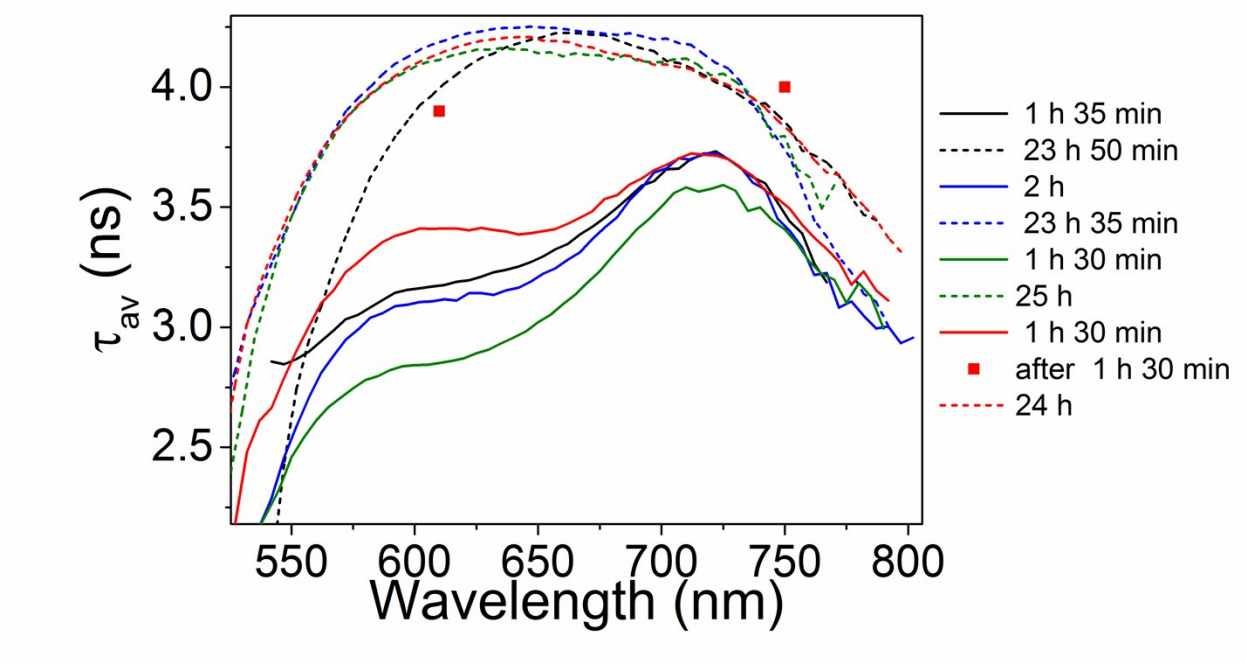

Figure SI8: Average decay time spectra of samples S4 (black), S7 (blue) S8 (green) and S9 (red) for 510 nm excitation. Large version of Figure 3D.

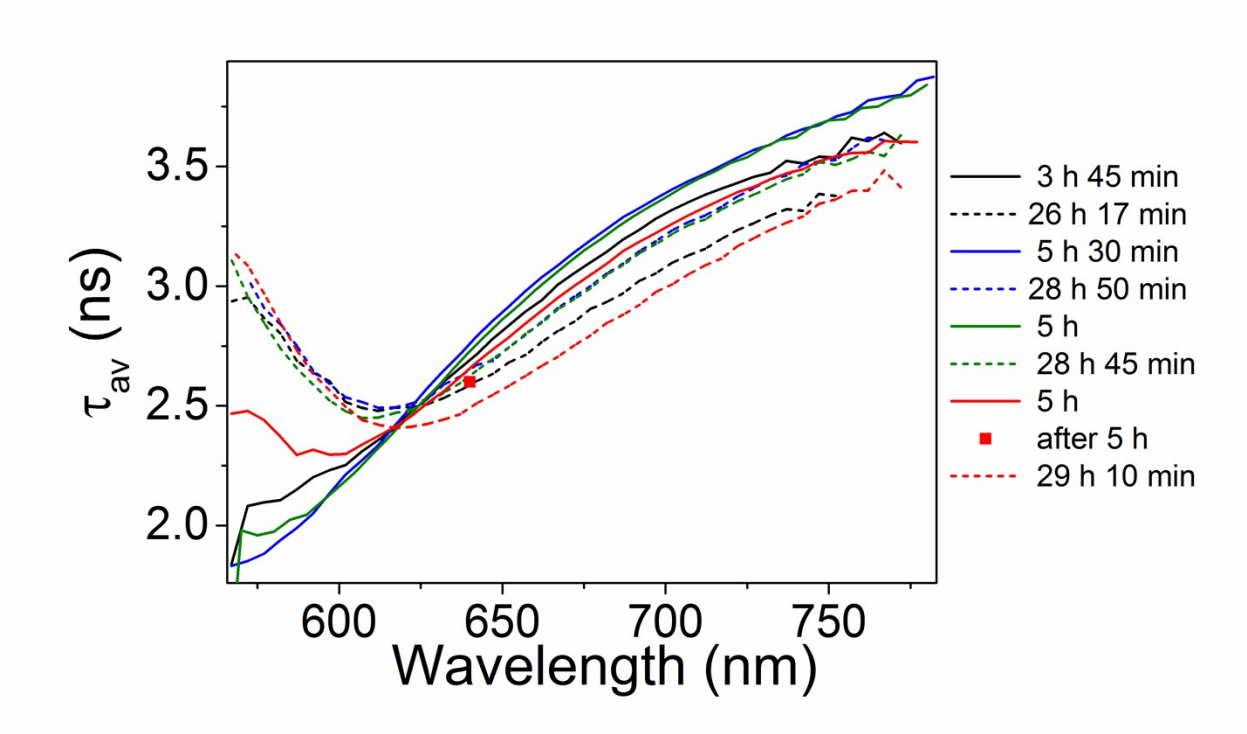

Figure SI9: Average decay time spectra of samples S4 (black), S7 (blue) S8 (green) and S9 (red) for 560 nm excitation. Large version of Figure 3E.

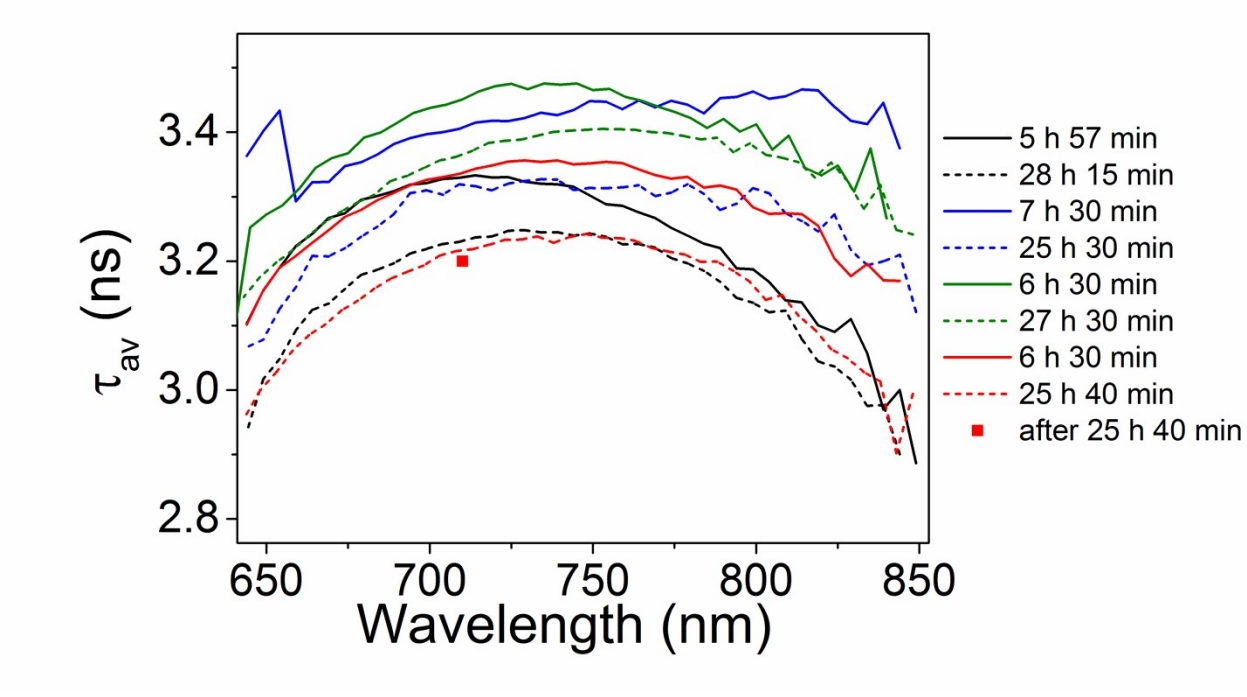

Figure SI10: Average decay time spectra of samples S4 (black), S7 (blue) S8 (green) and S9 (red) for 635 nm excitation. Large version of Figure 3F.

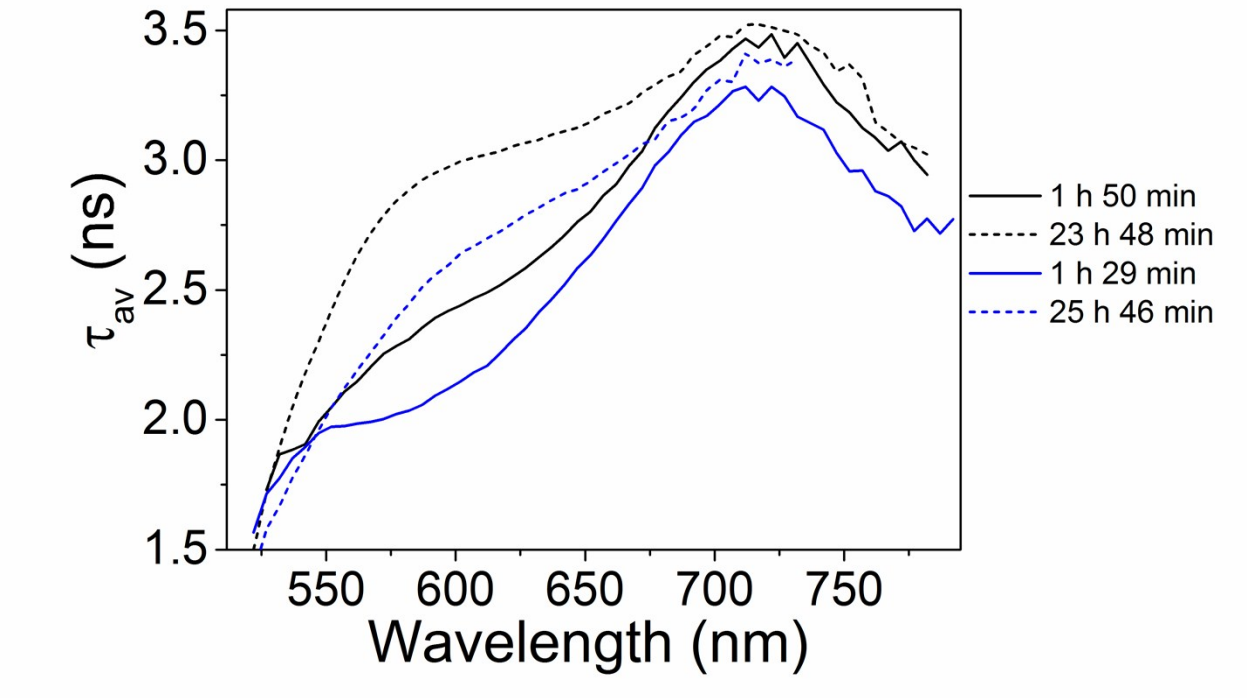

Figure SI11: Average decay time spectra of samples S5 (black) and S6 (blue) for 510 nm excitation. Large version of Figure 3G.

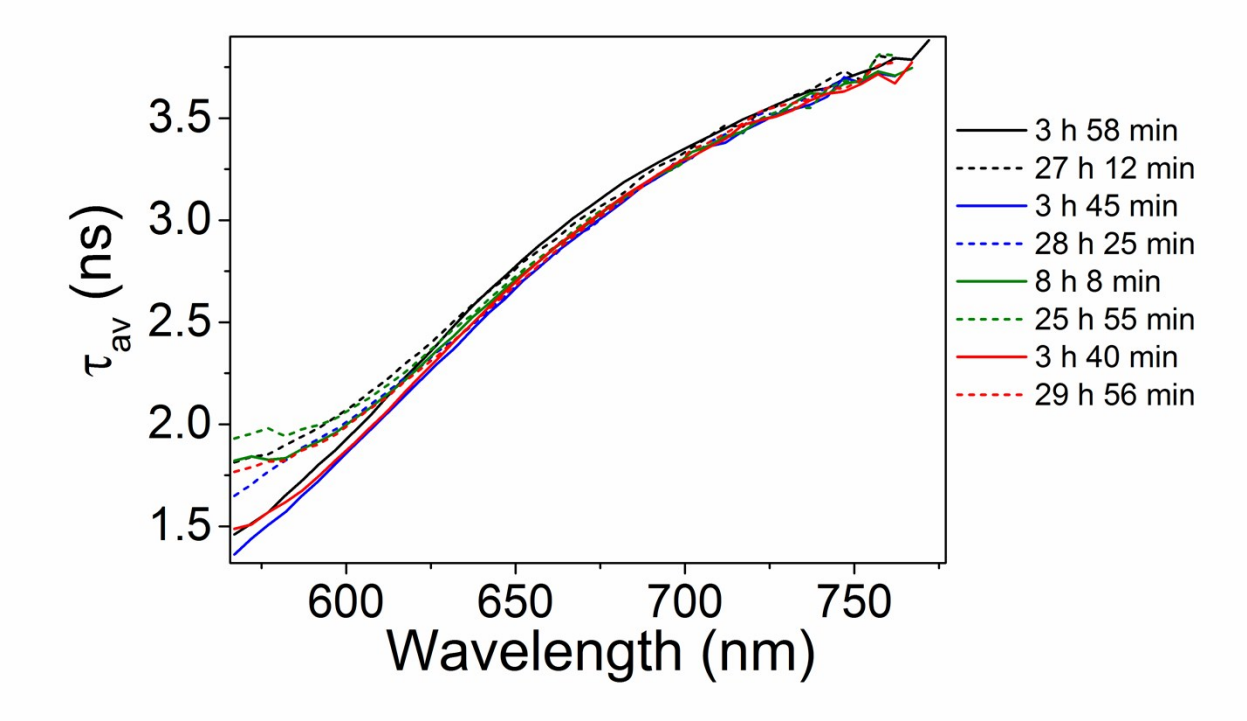

Figure SI12: Average decay time spectra of samples S5 (black), S6 (blue) S10 (green) and S11 (red) for 560 nm excitation. Large version of Figure 3H.

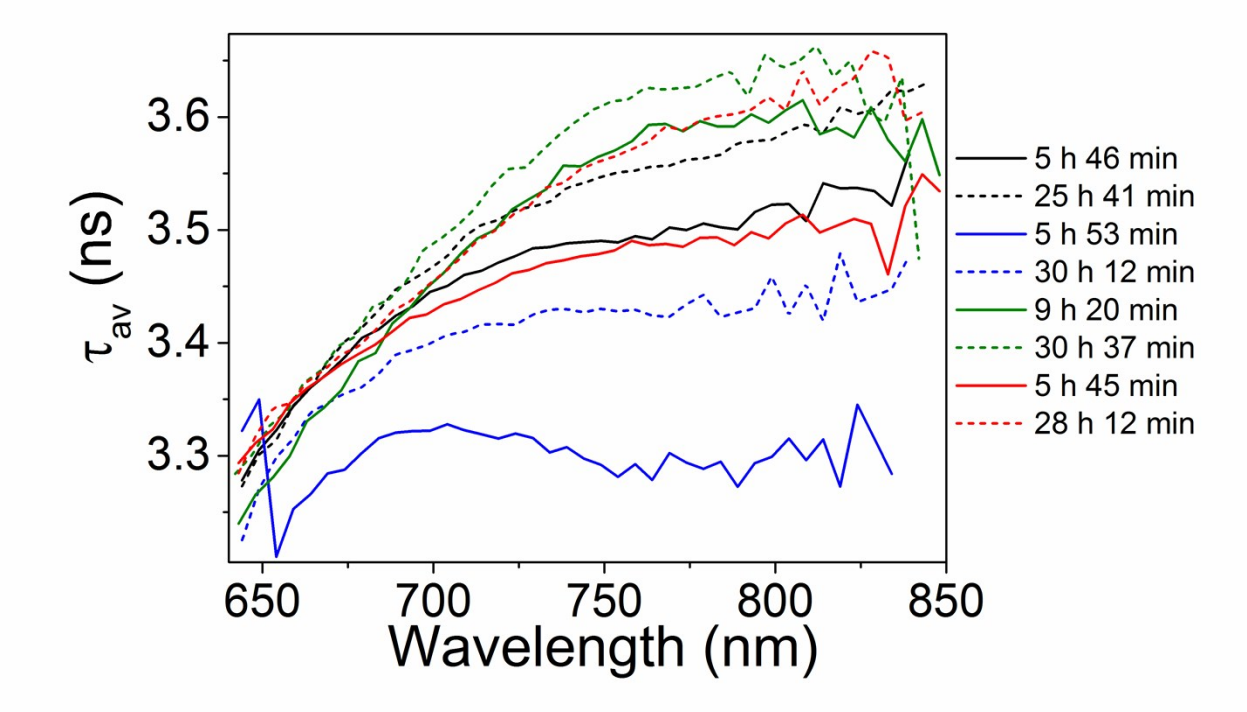

Figure SI13: Average decay time spectra of samples S5 (black), S6 (blue) S10 (green) and S11 (red) for 635 nm excitation. Large version of Figure 3I.

REFERENCES

1. Hooley, E. N.; Paolucci, V.; Liao, Z.; Carro-Temboury, M. R.; Vosch, T., Single Molecule Characterization of near-Infrared Emitting Silver Nanoclusters. *Adv. Opt. Mater.* **2015**, *3*, 1109-1115.

2. Magde, D.; Rojas, G. E.; Seybold, P. G., Solvent Dependence of the Fluorescence Lifetimes of Xanthene Dyes. *Photochem. Photobiol.* **1999**, *70*, 737-744.