Supplementary Information (SI) for Nanoscale.

Supporting Information

Assessing the Overflowing Pile-up Effect in the Photoluminescence Lifetime of Nanomaterials

Shagun Sharma[a], Aditya Yadav[a], Kush Kaushik[a], Abdul Salam[a], and Chayan Kanti Nandi[a]*
[a]School of Chemical Sciences, Indian Institute of Technology (IIT) Mandi, H.P.- 175075, India

(I) Materials

All glassware were washed with aqua regia, followed by rinsing several times with double distilled water. Bovine serum albumin (BSA) was purchased from Sigma Aldrich (Cat no. A2153-10G), having purification ≥ 96 %. Sodium borohydride ($NaBH_4$), Gold (III) chloride trihydrate ($HAuCl_4.3H_2O$), Cadmium chloride hemipentahydrate ($CdCl_2.2.5H_2O$), and Mercaptosuccinic acid (MSA) were purchased from Sigma Aldrich. Zinc acetate dihydrate ($Zn(OAc)_2.H_2O$), 4-(diethylamino)-2-hydroxybenzaldehyde and diaminomaleonitrile were also purchased from Sigma Aldrich. Sodium tellurite was purchased from Alfa-Aesar. Sodium Hydroxide (NaOH) and Silver Nitrate ($AgNO_3$) were purchased from Merck Chemicals. Copper sulphate pentahydrate was purchased from SD Fine Chemicals whereas hydrazine hydrate (85%) and L-Arginine were purchased from Loba Chemie. 6-Aza-2-thiothymin (ATT) was purchased from Thermo Scientific Chemicals. Cyanine 5 (Cy5) dye was purchased from Lumiprobe Life Science Solution. All chemicals were used without further purification. Double-distilled (18.3 mΩ) deionized water (ELGA PURELAB Ultra) was used throughout the entire process.

(II) Instrumentation

UV-Visible absorption spectra were measured with Shimadzu U-2450 UV-Visible spectrophotometer from wavelength range of 200-800 nm. The spectra were collected using a quartz cuvette having a 10 mm path length and 1 mL volume. **Steady-state fluorescence spectra** were recorded using an Agilent Technologies Cary Eclipse fluorescence spectrophotometer. Spectra were collected using a quartz cuvette of path length 10 mm and 1 mL volume. **Fluorescence lifetime** was performed using a time-correlated single photon counting (TCSPC) system PicoHarp 300, PDL-800D and two PMA Hybrid detectors (from PicoQuant Germany) integrated with Nikon Confocal Microscope. PDL-800D can tune laser frequency from 80 MHz to 31.25 kHz. A pulsed diode laser, LDH-P-C-405B, PicoQuant, 405 nm (Pulse FWHM <50 ps and Max Repetition rate 40 MHz) was used for sample excitation, and fluorescence signals were collected by choosing a proper dichroic-filter set. In addition, the fluorescence lifetime was also measured using **Horiba Scientific Delta Flex TCSPC** system with Pulsed LED Sources.

(III) Experimental

Preparation of Zn Salen complex (ZnL_1)

ZnL_1 was synthesized by a modified method[1] in which a mixture of 98 mg (0.51 mmol) of 4-(diethylamino)-2-hydroxybenzaldehyde, 27.5 mg (0.25 mmol) of diaminomaleonitrile, and 55.5 mg (0.25 mmol) of $Zn(OAc)_2.H_2O$ were dissolved in 50 ml of ethanol and the solution was kept for reflux overnight. The solvent was evaporated through rotary evaporators, washing of crude sample was done by iced ethanol to give 65 mg (50% yield) of ZnL_1.

Preparation of BSA protected Silver Nanoclusters (BSA-Ag NCs).

The BSA-Ag NCs were prepared by a previously reported study.[2] In this protocol, 4 ml of 10 mM $AgNO_3$ solution diluted with 15.5 ml of ultrapure water was added to 2 ml of BSA (50 mg/ml) solution under vigorous stirring. After 2 min, 0.2 ml aqueous solution of 1M NaOH was added to the mixture, which was followed by dropwise addition of freshly prepared NaBH4 solution (0.3 ml, 10 mM) under continuous stirring. The formation of Ag NCs was confirmed by the colour change from colourless to brown. For further characterisation, it was kept at 4 ˚C.

Preparation of BSA protected Gold Nanoclusters (BSA-Au NCs).

The BSA-Au NCs utilized in this study were synthesized by a previously reported protocol given by Xie et. al.[3] For BSA-Au NCs, 5 ml of 10 mM $HAuCl_4$ was added to 5 ml of 50 mg/ml BSA. After NaOH addition (0.5 ml, 1M), the mixture was kept at 37 ˚C overnight in the incubator. The clusters formed were stabilized by the thiol group of cysteine in BSA and composed of Au_{25} atoms.

Preparation of ATT-protected Gold Nanoclusters (ATT-Au NCs).

ATT-Au NCs was prepared by the facile one pot approach given by Hao-Hua et al.[4] Here, ATT is 6 aza 2 thiothymine, which is a thiolate ligand. In a typical synthesis, ATT (80 mM, 15 ml) in 0.2 M NaOH solution was added to $HAuCl_4$ (10 mg/ml, 15 ml) with continuous stirring at room temperature for 1 hour in dark condition. The clusters formed were kept at 4 ˚C for further studies.

Preparation of BSA protected Copper Nanoclusters (BSA-Cu NCs).

BSA-Cu NCs were synthesized following the protocol described by Wang et al.[5] Briefly, 100 mg of BSA was added to $CuSO_4$ solution (5 ml, 5 mM) which was stirred for 10 min. at room temperature. Using 1M NaOH The pH was adjusted to 12 M NaOH, forming a transparent purple solution. After the slow addition of hydrazine (1 ml) and stirring at room temperature for 4 hrs, the solution turned to light yellow, indicating the formation of Cu NCs.

Preparation of CdTe Quantum Dots (QDs).

CdTe Quantum Dots (QDs) were synthesized using the reported protocol given by Jiawei Tan et al.[6] with slight modification. For our study, we used CdTe QD, which had an emission maxima of 580 nm. In a typical synthesis, 0.3425 g (1.5 mmol) $CdCl_2.2.5H_2O$ and 0.2477 g (1.65 mmol) methane sulphonic acid (MSA) were dissolved in deionized water. This solution was deaerated for 25-30 minutes. Further, the solution pH was set to 9-10 using 5 M NaOH. The resulting solution was called as solution I. Additionally, 0.0554 g (0.25 mmol) of sodium tellurite was dissolved in 10 ml deionised water and named as solution II. Then, 0.0662 g (1.75 mmol) of sodium borohydride was added to the solution I and II mixture. The obtained solution was stirred and a yellow-colored solution was formed after 3-4 minutes, which changes to deep orange after 15 minutes. For getting different coloured QDs, the final solution is divided into five fractions of 10 ml each and varied amount of Hydrazine hydrate was added to these fractions. 450 mM hydrazine hydrate (0.225 g or 218.9 μl) is required for 580 nm emitting QDs which are of our interest. This fraction (in test tube) was kept on water bath at 90-95° Celsius for 30 minutes which then allowed to cool at room temperature.

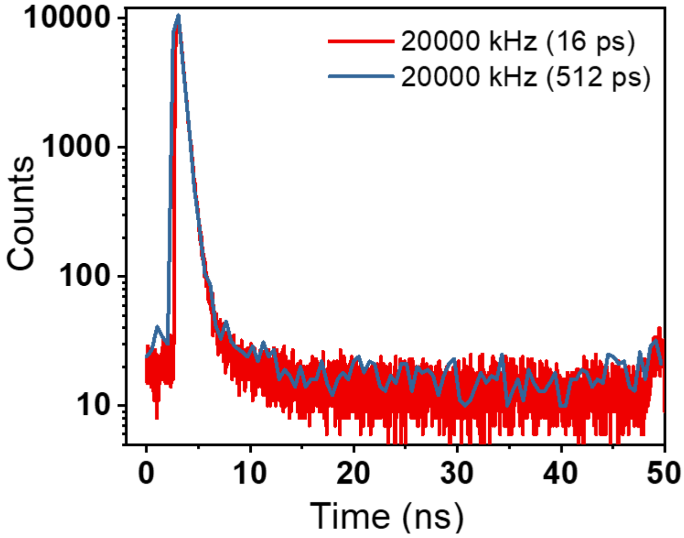

Figure S1. Represents the lifetime decay of Cyanine 5 with same repetition rate and different time resolution.

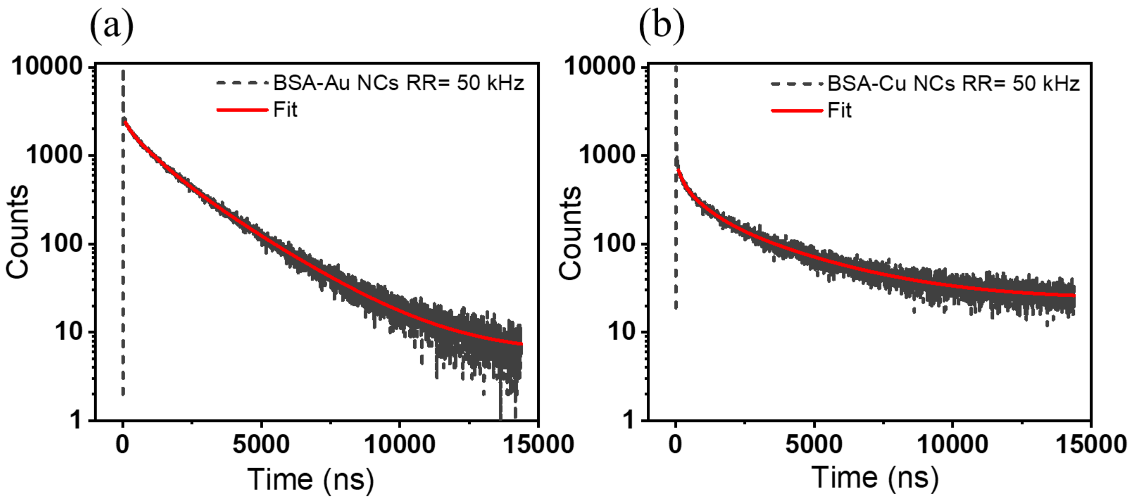

Figure S2. Represents the lifetime decay (black) and the tail fit result (red) of (a) BSA-Au NCs and (b) BSA-Cu NCs at same repetition rate of 50 kHz. The excitation wavelength (λ_{exc}) is same for both the measurements i.e., 454 nm.

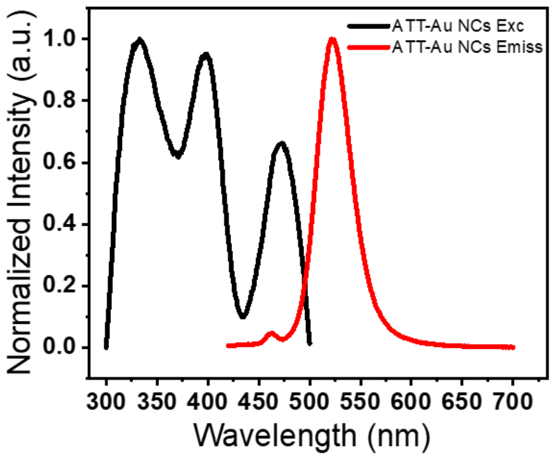

Figure S3. Represents the fluorescence excitation and emission spectra of ATT-Au NCs. The excitation spectra showing three peaks centred at 335 nm, 400 nm, and 470 nm. The emission spectra is centred at a wavelength of 530 nm.

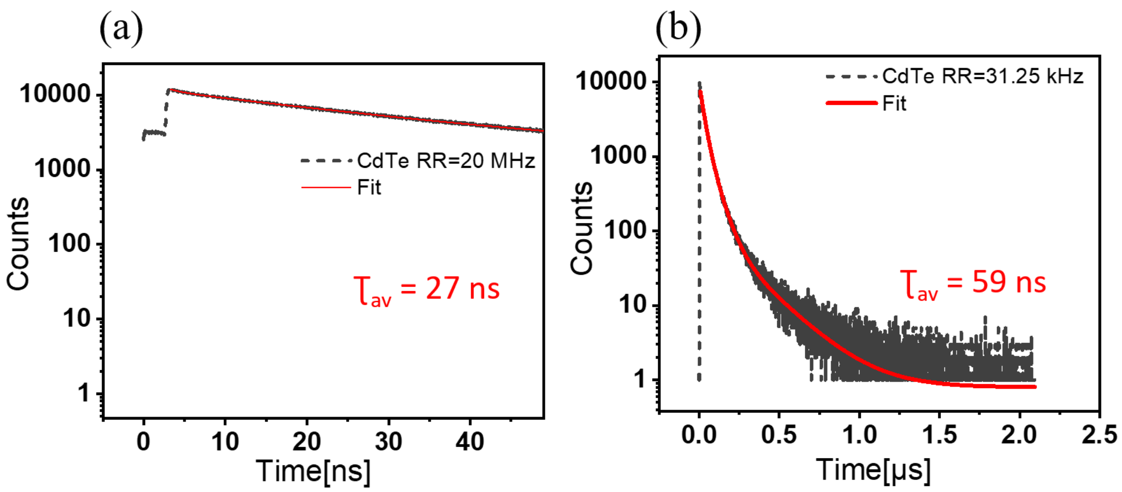

Figure S4. Represents the lifetime decay (black) and the tail fit result (red) of CdTe at different repetition rate. The measurement is done at the repetition rate (RR) of (a) 20 MHz and (b) 31.25 kHz. The excitation wavelength (λ_{exc}) is same for both the measurements i.e., 400 nm.

Table S1. Represents the fitting parameters of Zn-Salen complex including individual lifetime component with their amplitudes specifying time resolution, repetition rate, pulse separation and photon count rate. The parameters are listed by varying the time resolution and keeping the repetition constant.

Zn-Salen Complex

S. No.	τ_i (ns)			A_i (%)			τ_{av}(ns)	Time Resolution	Repetition Rate (kHz)	Pulse Separation	Photon Count Rate
	τ_1	τ_2	τ_3	A_1	A_2	A_3					
1.	3.4	0.7	5.1	83	6	10	**3.4**	16 ps	10000	100 ns	30 Hz
2.	4.4	0.7	3.3	27	5	67.6	**3.4**	32 ps	10000	100 ns	50 Hz
3.	3.4	0.7	5.7	88	6.4	5	**3.4**	64 ps	10000	100 ns	30 Hz
4.	3.3	0.7	4.6	75	6	18	**3.4**	128 ps	10000	100 ns	40 Hz
5.	4.3	0.7	3.2	32	5.2	62.3	**3.4**	256 ps	10000	100 ns	80 Hz

Table S2. Represents the fitting parameters of BSA-Ag NCs including individual lifetime component with their amplitudes specifying time resolution, repetition rate, pulse separation, and photon count rate. The parameters are listed by varying the repetition rate and keeping the time resolution constant.

BSA-Ag NCs

S. No.	τ_i (ns)			A_i (%)			τ_{av}(ns)	Time Resolution	Repetition Rate (kHz)	Pulse Separation	Photon Count Rate
	τ_1	τ_2	τ_3	A_1	A_2	A_3					
1.	5.80	1.90	0.43	22.0	47.1	30.8	**2.31**	512 ps	20000	50 ns	5130 Hz
2.	5	1.30	0.32	31.2	44.6	24.2	**2.20**	512 ps	2500	400 ns	2290 Hz
3.	6.3	1.90	0.44	20.1	50.4	29.4	**2.40**	512 ps	125	8 µs	1390 Hz

Table S3. Represents the fitting parameters of BSA-Ag NCs including individual lifetime component with their amplitudes specifying time resolution, repetition rate, pulse separation and photon count rate. The parameters are listed by varying the time resolution and keeping the repetition rate constant.

BSA-Ag NCs

S. No.	τ_i (ns)			A_i (%)			τ_{av}(ns)	Time Resolution	Repetition Rate (kHz)	Pulse Separation	Photon Count Rate
	τ_1	τ_2	τ_3	A_1	A_2	A_3					
1.	5	1.30	0.32	31.2	44.6	24.2	**2.20**	512 ps	2500	400 ns	2290 Hz
2.	5.50	1.60	0.37	30.2	46.5	23.2	**2.46**	256 ps	2500	400 ns	2240 Hz
3.	6	1.70	0.39	21.4	46.4	32.1	**2.25**	128 ps	2500	400 ns	2370 Hz
4.	6	1.82	0.41	21.3	46.5	32	**2.30**	64 ps	2500	400 ns	2170 Hz
5.	6.20	1.89	0.42	19.3	46.9	33.6	**2.24**	32 ps	2500	400 ns	2200 Hz

Table S4. Represents the fitting parameters of standard dye Cyanine 5 including individual lifetime component with their amplitudes specifying time resolution, repetition rate, pulse separation and photon count rate. The measurement is performed by varying the time resolution and keeping the repetition rate constant.

Cyanine 5

S. No.	τ_i (ns)		A_i (%)		τ_{av}(ns)	Time Resolution	Repetition Rate (kHz)	Pulse Separation	Photon Count Rate
	τ_1	τ_2	A_1	A_2					
1.	-	-	-	-	**NA**	512 ps	2500	400 ns	1240 Hz
2.	-	-	-	-	**NA**	256 ps	2500	400 ns	1220 Hz
3.	0.49	4	96	3.9	**0.63**	64 ps	2500	400 ns	1150 Hz
4.	0.47	3.90	96.4	3.5	**0.59**	32 ps	2500	400 ns	1250 Hz

Table S5. Represents the fitting parameters of BSA-Au NCs including individual lifetime component with their amplitudes specifying time resolution, repetition rate, pulse separation and photon count rate. The measurement is performed by varying the time resolution and keeping the repetition rate constant.

BSA-Au NCs

S. No.	τ_i (ns)			A_i (%)			τ_{av}(ns)	Time Resolution	Repetition Rate (kHz)	Pulse Separation	Photon Count Rate
	τ_1	τ_2	τ_3	A_1	A_2	A_3					
1.	6.90	253	1.60	24.2	62.8	12.8	**162**	512 ps	2500	400 ns	13 kHz
2.	6.30	226	1.30	28.	58.8	13	**135**	256 ps	2500	400 ns	13 kHz
3.	5.90	208	1.20	30.4	56.6	12.9	**120**	128 ps	2500	400 ns	12 kHz
4.	5.50	86	1.00	47.6	34	18.3	**32**	64 ps	2500	400 ns	13 kHz
5.	4.09	20	0.83	55.9	27.2	16.8	**8**	32 ps	2500	400 ns	14 kHz

Table S6. Represents the fitting parameters of ATT-Au NCs including individual lifetime component with their amplitudes specifying time resolution, repetition rate, pulse separation and photon count rate. The measurement is performed by varying the repetition rate and keeping the time resolution constant.

ATT-Au NCs

S. No.	τ_i (ns)			A_i (%)			τ_{av}(ns)	Time Resolution	Repetition Rate (kHz)	Pulse Separation	Photon Count Rate
	τ_1	τ_2	τ_3	A_1	A_2	A_3					
1.	11	3.80	1.12	51	40	9.6	**7.2**	16 ps	20000	50 ns	1509 kHz
2.	12	4.3	1.2	44	46.8	8.6	**7.6**	16 ps	10000	100 ns	773 kHz
3.	12	4.4	1.3	45.5	46.5	8	**7.7**	16 ps	5000	200 ns	383 kHz
4.	13	5	1.5	42	49	9	**7.9**	16 ps	2500	400 ns	191 kHz

Table S7. Represents the fitting parameters of ATT-Au NCs including individual lifetime component with their amplitudes specifying time resolution, repetition rate, pulse separation and photon count rate. The measurement is performed by varying the time resolution and keeping the repetition rate constant.

ATT-Au NCs

S. No.	τ_i (ns)			A_i (%)			τ_{av}(ns)	Time Resolution	Repetition Rate (kHz)	Pulse Separation	Photon Count Rate
	τ_1	τ_2	τ_3	A_1	A_2	A_3					
1.	12	4.3	1.2	44	46.8	8.6	**7.6**	16 ps	10000	100 ns	773 kHz
2.	16	5.6	1.6	29	55	15	**8.0**	32 ps	10000	100 ns	780 kHz
3.	16	5.6	1.6	30.1	55	14.5	**8.0**	64 ps	10000	100 ns	780 kHz
4.	16	5.50	1.5	31.2	55	13.5	**8.0**	128 ps	10000	100 ns	777 kHz
5.	15	5.3	1.5	33	54	13	**8.0**	256 ps	10000	100 ns	777 kHz
6.	16	5.5	1.50	32	55	12.3	**8.2**	512 ps	10000	100 ns	773 kHz

Table S8. Represents the fitting parameters of BSA-Cu NCs including individual lifetime component with their amplitudes specifying time resolution, repetition rate, pulse separation and photon count rate. The parameters are listed by varying the repetition rate and keeping the time resolution constant.

BSA-Cu NCs

S. No.	τ_i (ns)			A_i (%)			τ_{av}(ns)	Time Resolution	Repetition Rate (kHz)	Pulse Separation	Photon Count Rate
	τ_1	τ_2	τ_3	A_1	A_2	A_3					
1.	15	3.30	0.70	23.2	56.3	20.4	**5**	512 ps	20000	50 ns	7250 Hz
2.	12	3.20	0.86	30.3	50.6	18.9	**5.30**	512 ps	2500	400 ns	2800 Hz
3.	15	1569	163	13.3	77.3	9.3	**1234**	512 ps	125	8 µs	2260 Hz

Table S9. Represents the fitting parameters of CdTe including individual lifetime component with their amplitudes specifying time resolution, repetition rate, pulse separation and photon count rate. The measurement is performed by varying the time resolution and keeping the repetition rate constant.

CdTe

S. No.	τ_i (ns)			A_i (%)			τ_{av}(ns)	Time Resolution	Repetition Rate (kHz)	Pulse Separation	Photon Count Rate
	τ_1	τ_2	τ_3	A_1	A_2	A_3					
1.	79	30	5.4	33.9	64.1	1.9	**46**	512 ps	2500	400 ns	109 kHz
2.	76	29	4.3	36.3	62	1.5	**46**	256 ps	2500	400 ns	111 kHz
3.	81	30	6.70	33.5	63.9	2.4	**47**	128 ps	2500	400 ns	113 kHz
4.	61	26	3	49.4	49.6	0.9	**43**	64 ps	2500	400 ns	112 kHz
5.	37	12	1.30	91.4	8.1	0.3	**35**	32 ps	2500	400 ns	110 kHz

Table S10. Represents the fitting parameters of CdTe including individual lifetime component with their amplitudes specifying time resolution, repetition rate, pulse separation and photon count rate. The measurement is performed by varying the repetition rate and keeping the time resolution constant.

CdTe

S. No.	τ_i (ns)			A_i (%)			τ_{av}(ns)	Time Resolution	Repetition Rate (kHz)	Pulse Separation	Photon Count Rate
	τ_1	τ_2	τ_3	A_1	A_2	A_3					
1.	30.7	2.7	4.6	98.6	0.6	0.7	**30**	512 ps	20000	50 ns	787 kHz
2.	37	12	1.3	91.4	8.1	0.3	**35**	512 ps	2500	400 ns	109 kHz
3.	25	235	57	43.7	8.5	47.7	**58**	512 ps	125	8 µs	7560 Hz
4.	25	238	56	42.9	8.2	48.8	**58**	512 ps	62.5	16 µs	4830 Hz
5.	26	256	60	46.5	7.6	45.8	**59**	512 ps	31.25	32 µs	3700 Hz

Table S11. Represents the measurement of the average lifetime (τ_{av}) with fitting error ($\Delta\tau$) of CdTe at the same repetition rate of 2.5 MHz and different time resolution.

S.No.	Time resolution	$\tau_{av} \pm \Delta\tau$
1.	512 ps	46±0.49
2.	256 ps	46±0.37
3.	128 ps	47±0.17
4.	64 ps	43±0.15

Table S12. Summarizes the optimized acquisition parameters (repetition rate, time resolution and photon count rate) for the lifetimes ranging from nanoseconds to microseconds.

Lifetime Range	**Repetition Rate**	**Time Resolution (ps)**	**Photon Count Rate**
2-10 ns	20 MHz - 5 MHz	< 20	< 10%
10-30 ns	5 MHz - 2.5 MHz	64-512	< 10%
30-100 ns	2.5 MHz - 125 kHz	128-512	< 10%
> 100 ns	125 kHz - 50 kHz	128-512	< 10%
1-10 μs	50 kHz - 10 kHz	≥ 512	< 10%

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