

Supporting Information

Engineering Quantum Dots with Different Emission Wavelengths and Specific Fluorescence Lifetimes for Spectrally and Temporally Multiplexed Imaging of Cells

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Analysis of Fluorescence Decays

The fluorescence decay curves of DAPI, CdSe and ZAIS QDs were fitted using an incomplete decay model with the correction method proposed in a previous report.[1] In this model, the measured incomplete fluorescence decay is a summation of the fluorescence decays under excitation of current and previous pulses:

$$I(t) = \lim_{n \rightarrow \infty} [I_0(t) + I_0(t + T) + \dots + I_0(t + nT)] \quad (s1)$$

where $I(t)$ is the measured fluorescence decay, $I_0(t)$ is original fluorescence decay, T (50 ns in this study) is the repetition period of the laser pulses.

Given the original fluorescence decay described as,

$$I_0(t) = \sum_{i=1}^N A_{0i} e^{-t/\tau_{0i}} \quad (s2)$$

and the measured fluorescence decay fitted to,

$$I(t) = \sum_{i=1}^N A_i e^{-t/\tau_i} \quad (s3)$$

where τ_{0i} , τ_i is the i th lifetime components of the original and measured decay curves, and A_{0i} and A_i is the amplitude of the corresponding lifetime components. By substituting Equation s2 and s3 into Equation s1 and considering $n \rightarrow \infty$, the parameters of original decays can be obtained by correction of the measured parameters,

$$A_{0i} = (1 - e^{-T/\tau_i})A_i \quad (s4)$$

$$\tau_{0i} = \tau_i \quad (s5)$$

For mono-exponential fitting with only one lifetime component, the original fluorescence lifetime is theoretically equal to the that calculated from the measured lifetime. For bi-exponential fitting, the values of lifetime components are unchanged according to Equation s5. However, the amplitudes can be significantly different before and after corrections, especially when the corresponding lifetime component is comparable or longer than the repetition period of laser. Table S1 gives the measured and corrected lifetime components and amplitudes of DAPI, CdSe and ZAIS QDs by bi-exponential fitting.

Table S1. Lifetime components and amplitudes before and after corrections by bi-exponential fitting.

	DAPI	Green CdSe	Red CdSe	Green ZAIS	Red ZAIS
A_1 / A_{01}	0.32 / 0.32	0.24 / 0.24	0.27 / 0.27	0.11 / 0.11	0.08 / 0.08
τ_1 / τ_{01} [ns]	0.75	3.30	5.03	0.50	0.98
A_2 / A_{02}	0.68 / 0.68	0.76 / 0.58	0.73 / 0.44	0.89 / 0.15	0.92 / 0.10
τ_2 / τ_{02} [ns]	2.61	34.1	55.2	275	425

The corrected lifetime components and amplitudes were used for calculating the fluorescence lifetimes. The average lifetime of the bi-exponential function can be computed as,[2, 3]

$$\tau_{avg} = (A_{01}\tau_{01}^2 + A_{02}\tau_{02}^2)/(A_{01}\tau_{01} + A_{02}\tau_{02}) \quad (s6)$$

In mono-exponential fitting, the calculated lifetime is equal to the time that the fluorescence intensity drops to 1/e of its peak intensity, $\tau_{1/e}$. For decay curves fitted by bi-exponential functions, we also calculated this $\tau_{1/e}$ by solving t from the formula,

$$A_{01}e^{-t/\tau_{01}} + A_{02}e^{-t/\tau_{02}} = 1/e (A_{01} + A_{02}) \quad (s7)$$

From Table S2, the lifetime τ (*i.e.*, $\tau_{1/e}$) obtained from mono-exponential fitting has no significant difference compared to the $\tau_{1/e}$ calculated from the corrected bi-exponential models, which proves that the fluorescence lifetimes calculated by using mono-exponential model are reasonable. The average lifetimes τ_{avg} obtained from bi-exponential models are close to the long fluorescence component when there is a large difference between two lifetime components.

Table S2. Fluorescence lifetimes calculated using the mono-exponential and bi-exponential fitting models using corrected parameters.

		DAPI	Green CdSe	Red CdSe	Green ZAIS	Red ZAIS
Mono-exponential	τ [ns]	1.93	23.5	32.4	127	188
Bi-exponential	$\tau_{1/e}$ [ns]	1.82	22.2	28.8	122	179
	τ_{avg} [ns]	2.39	32.9	52.5	275	424

Simulation of the Fluorescence Lifetime Distributions

To simulate the process of resolving fluorescence lifetime decays with different lifetimes, two mono-exponential lifetime decays with an order of magnitude difference in their lifetimes (1 and 10 ns, 2 and 20 ns, ... 64 and 640 ns) were generated. The amplitudes of the complete lifetime decay curves were obtained by normalizing their accumulated intensity over time (*i.e.*, area under the lifetime curves). The obtained values were substituted to Equation s4 as A_{0i} for calculating the amplitudes of the incomplete decay curves. For N photons recorded by the time-correlated single-photon counting (TCSPC) technique, the data with shot noise obey Poisson statistics with a standard deviation \sqrt{N} and signal-to-noise (SNR) ratio $N/\sqrt{N} = \sqrt{N}$. [4] Herein, Gaussian noise (a close approximation to Poisson noise for large photon numbers)

was added to the incomplete decay curves at an SNR ratio of \sqrt{N} . The simulated lifetime decays were fitted by mono-exponential functions using a trust-region algorithm. The resolvability of the two lifetime distributions was calculated as the distance between the right tail of the short lifetime distribution and the left tail of the long lifetime distribution relative to that between the mean of two lifetimes.

Processing of Fluorescence Lifetime Images

The fluorescence lifetime images were analyzed using custom Matlab scripts. First, raw data was imported as a matrix A with 256 rows (time channels) and 256 x 256 columns (pixels). The integrated intensity of each pixel was calculated by summation of the photon counts in the 256 time channels and normalized as a matrix B. After subtraction of the background signal (estimated from pixels with low intensity in the same image), the fluorescence decay in each pixel was fitted by standard mono-exponential ('exp1') or bi-exponential ('exp2') functions using a trust-region algorithm. For mono-exponential fitting, the calculated lifetimes were normalized and capped to [0,1], linearly projected to the hue space ([0-1] to [240°-0°]) and recorded in a matrix C. The values in matrices C and B were taken as the hue and value components in an a hue-saturation-value (HSV) image and the saturation component was set as 1. Then the HSV images were converted to RGB images and plotted as a 256 x 256 pixel image. By using a similar procedure, the average fluorescence lifetimes calculated from the corrected bi-exponential models were correlated with the hue component of the HSV image.

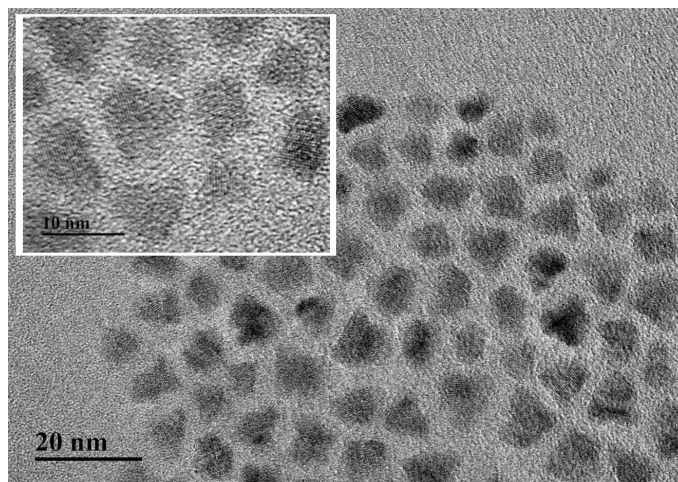


Figure S1. TEM image of organically-capped red CdSe QDs.

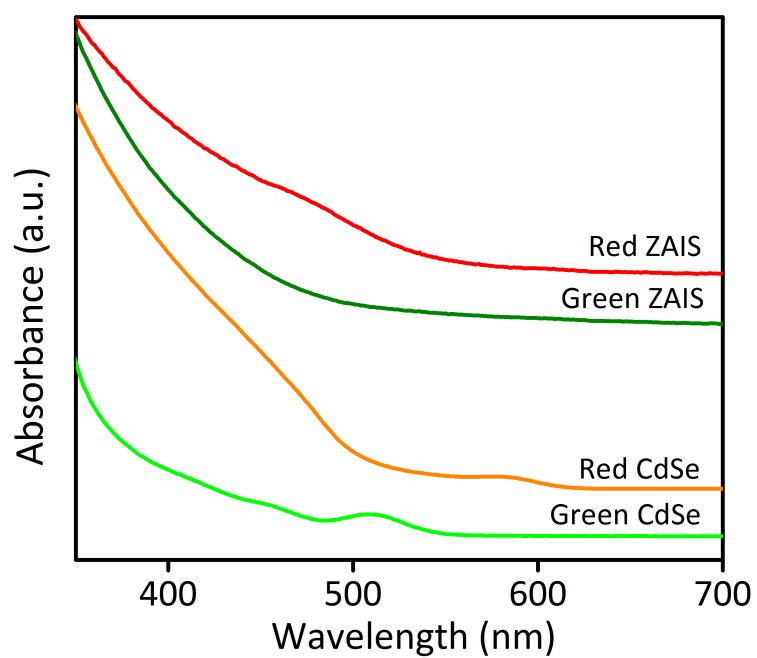


Figure S2. Absorption spectra of green CdSe, red CdSe, green ZAIS and red ZAIS QDs.

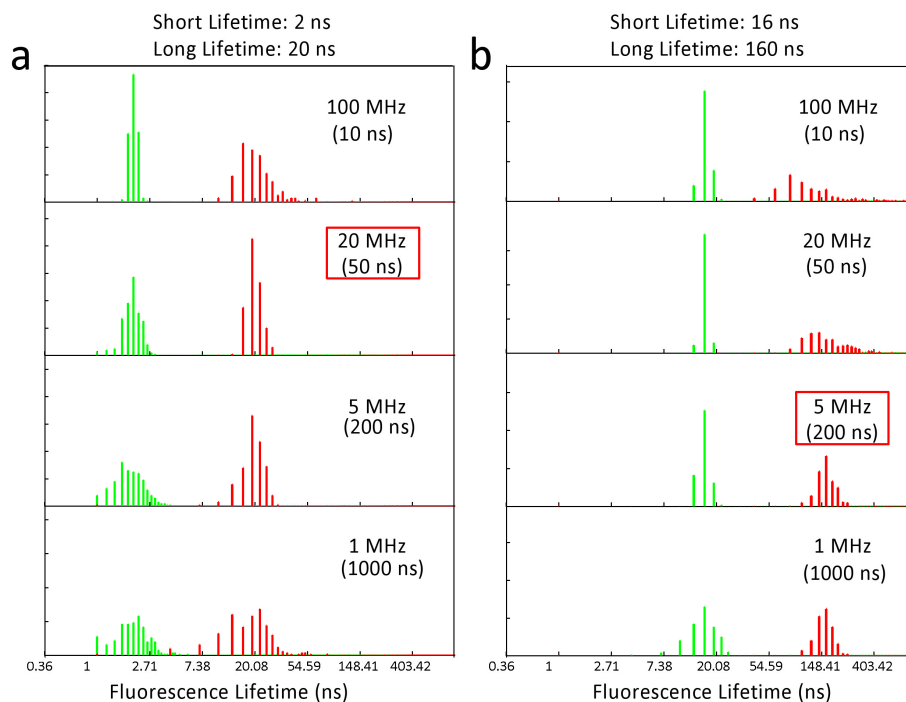


Figure S3. Simulated fluorescence lifetime distributions at laser frequency of 1, 5, 20 and 100 MHz. 20 MHz and 5 MHz (red outline) are calculated to be the optimal frequencies for resolving the fluorescence lifetime of (a) 2 and 20 ns and (b) 16 and 160 ns, respectively.

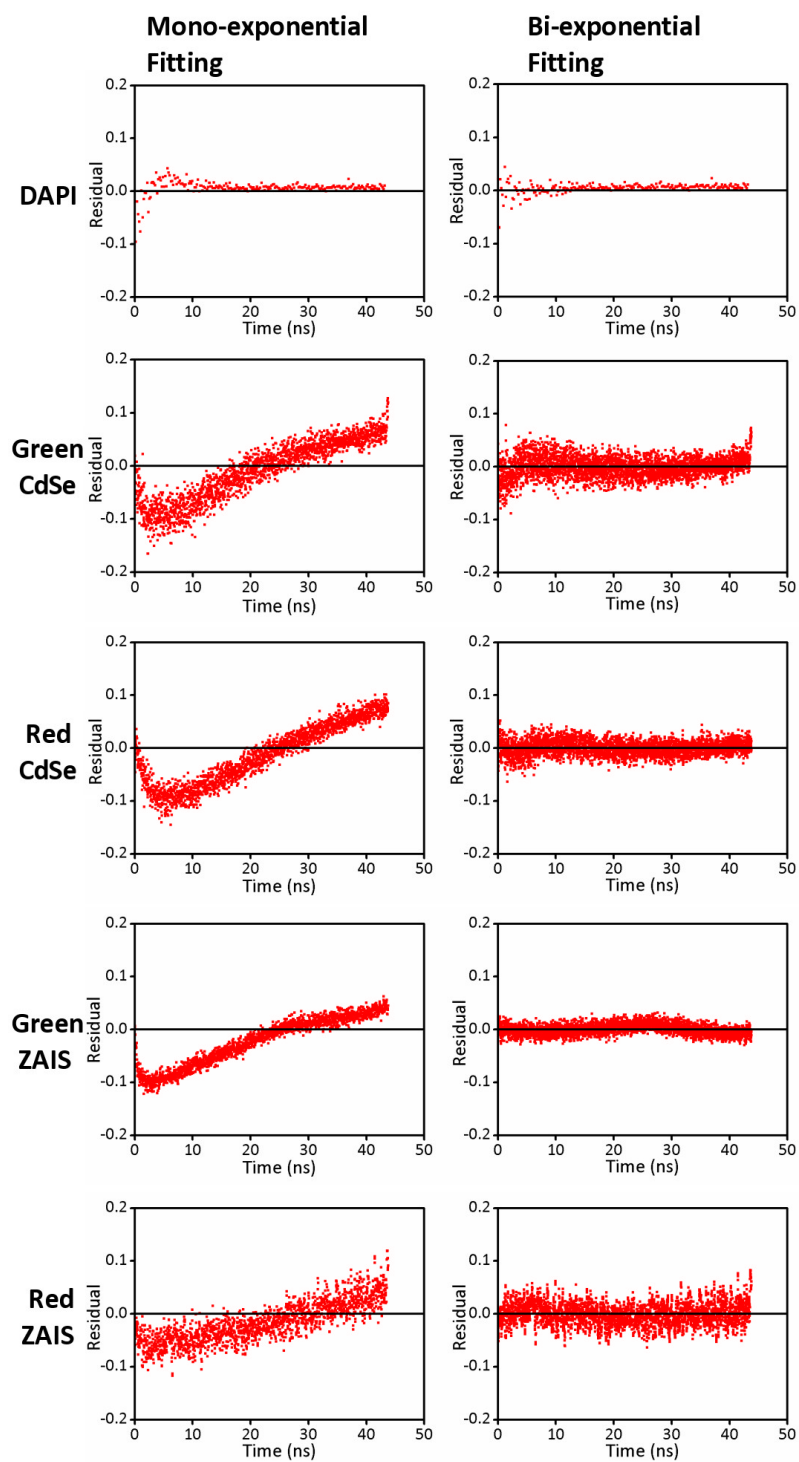


Figure S4. Residuals for mono-exponential (left column) and bi-exponential (right column) fitting of time-resolved PL decays shown in Figure 2f.

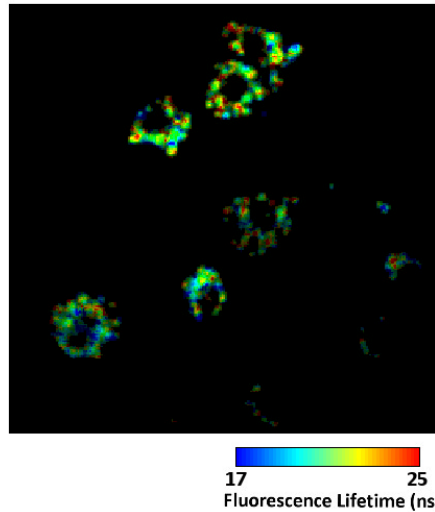


Figure S5. Fluorescence lifetime imaging of RAW264.7 cells treated with red CdSe QDs (same raw data with that of Figure 3). The fluorescence lifetimes were obtained by mono-exponential fitting and mapped to a false-color scale from 17 ns (blue) to 25 ns (red).

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- [4] J. Philip and K. Carlsson, "Theoretical investigation of the signal-to-noise ratio in fluorescence lifetime imaging," *JOSA A*, vol. 20, pp. 368-379, 2003.