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Multiplexed fluorescence lifetime imaging by concentration-dependent quenching

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Supporting Information

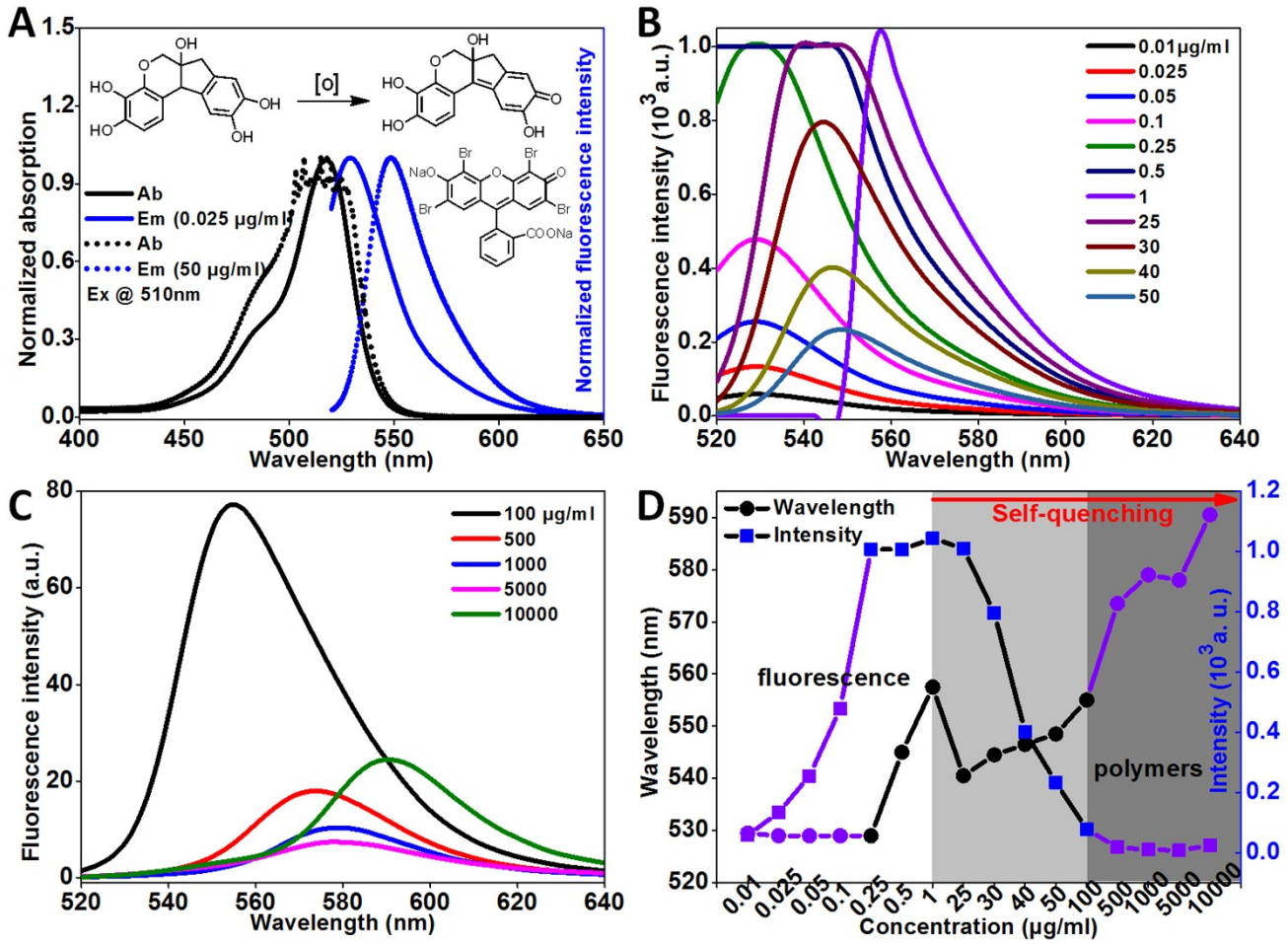

Fig. S1 Characterization of eosin solution (cytoplasm staining, KeyGen H&E staining kit). (A) Normalized absorption (Ab) and emission (Em) spectra of 0.025 and 50 μg/ml eosin solution. (B) Fluorescence emission spectra versus 0.01–50 μg/ml eosin solution. (C) Fluorescence emission spectra versus 0.1–10 mg/ml eosin solution. (D) Fluorescence emission peaks and fluorescence intensity versus concentration. AF: autofluorescence; Ab: absorption; Ex: excitation; Em: emission

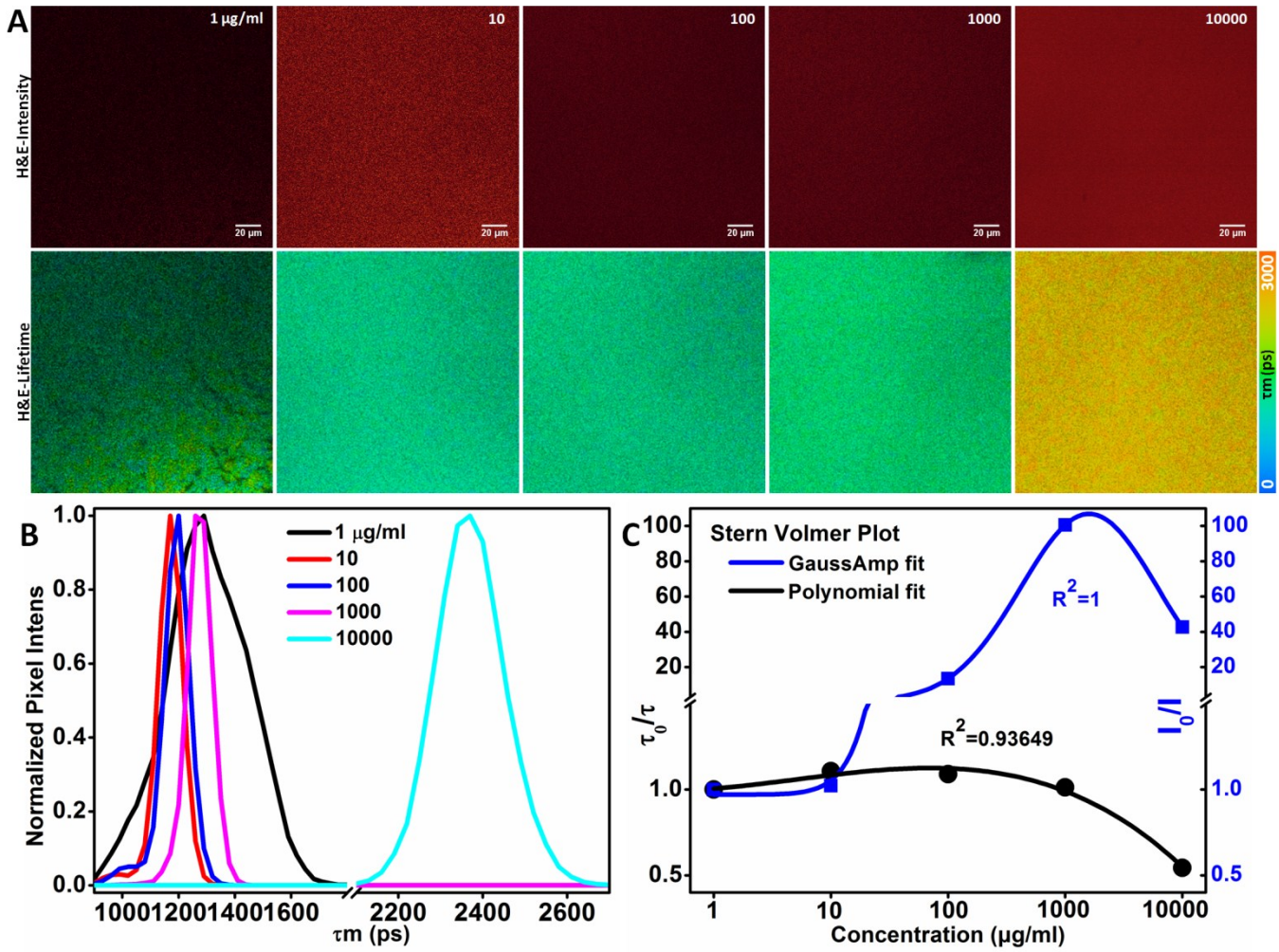

Fig. S2 Concentration-dependent quenching of hematoxylin and eosin (H&E). (A) Images of H&E fluorescence emission (upper row) and pseudocolor average fluorescence lifetimes (τm) (lower row) versus different concentrations of H&E solution (1, 10, 100, 1000, 10000 μg/ml). (B) τm histogram corresponds to A. (C) Stern-Volmer plot of self-quenching efficiency versus concentration of H&E solution, the threshold concentrations of H&E were calculated and the threshold I and τm were determined, where I_0 and τ_0 are the gray intensity in A and the the mean values (μ) of τm histogram (τm_μ) in B, respectively. Correlation coefficients, R^2, were calculated using least squares regression analysis.

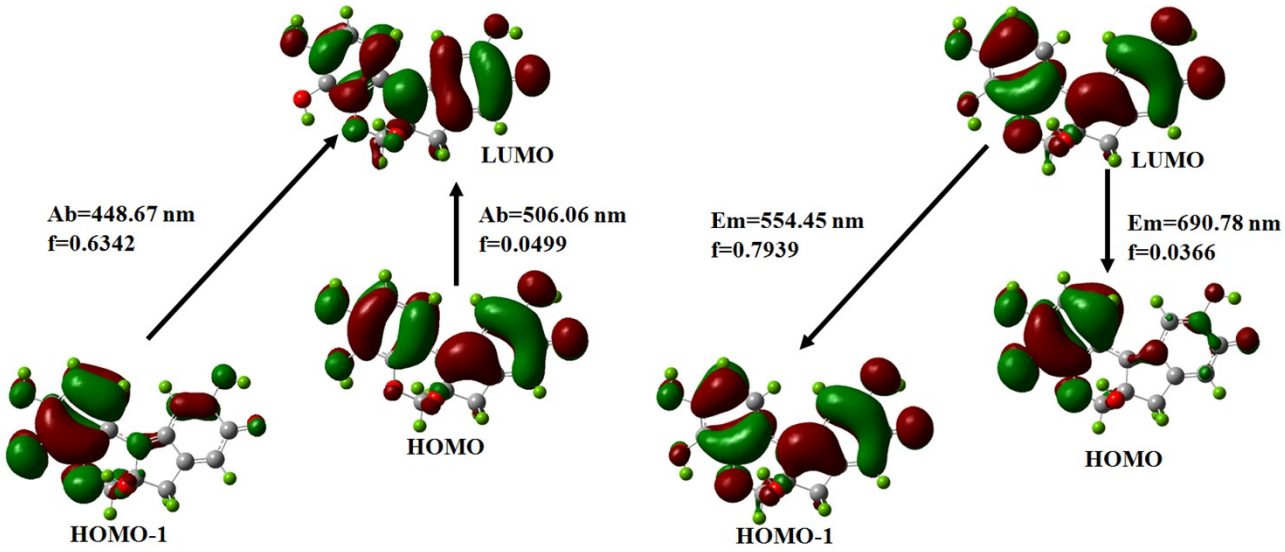

Fig. S3 Density functional theory (B3LYP/6-31G*) study of haematein in its excited state and the emission pathway. HOMO and LUMO for haematein (Ab=448 nm) and haematein (Ab=506 nm).

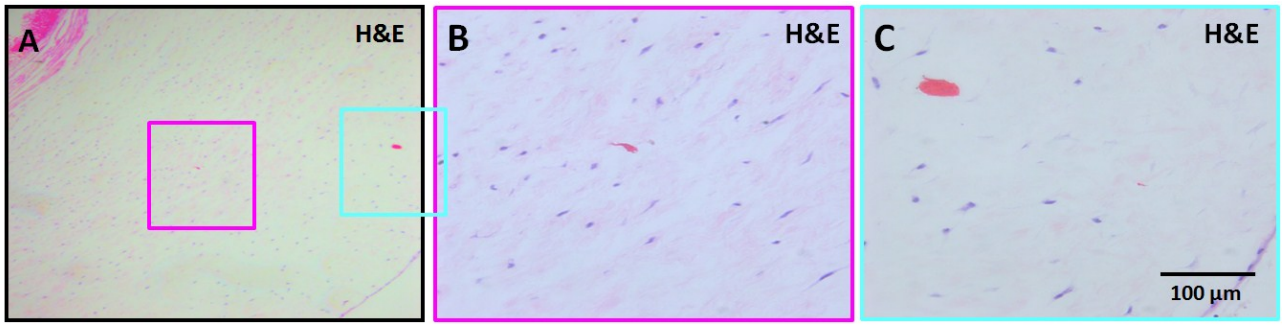

Figure S4.Certain artificial structures in a microscopic section. H&E stained umbilical artery (A). The pink and cyan rectangle in A corresponds to B and C, respectively.

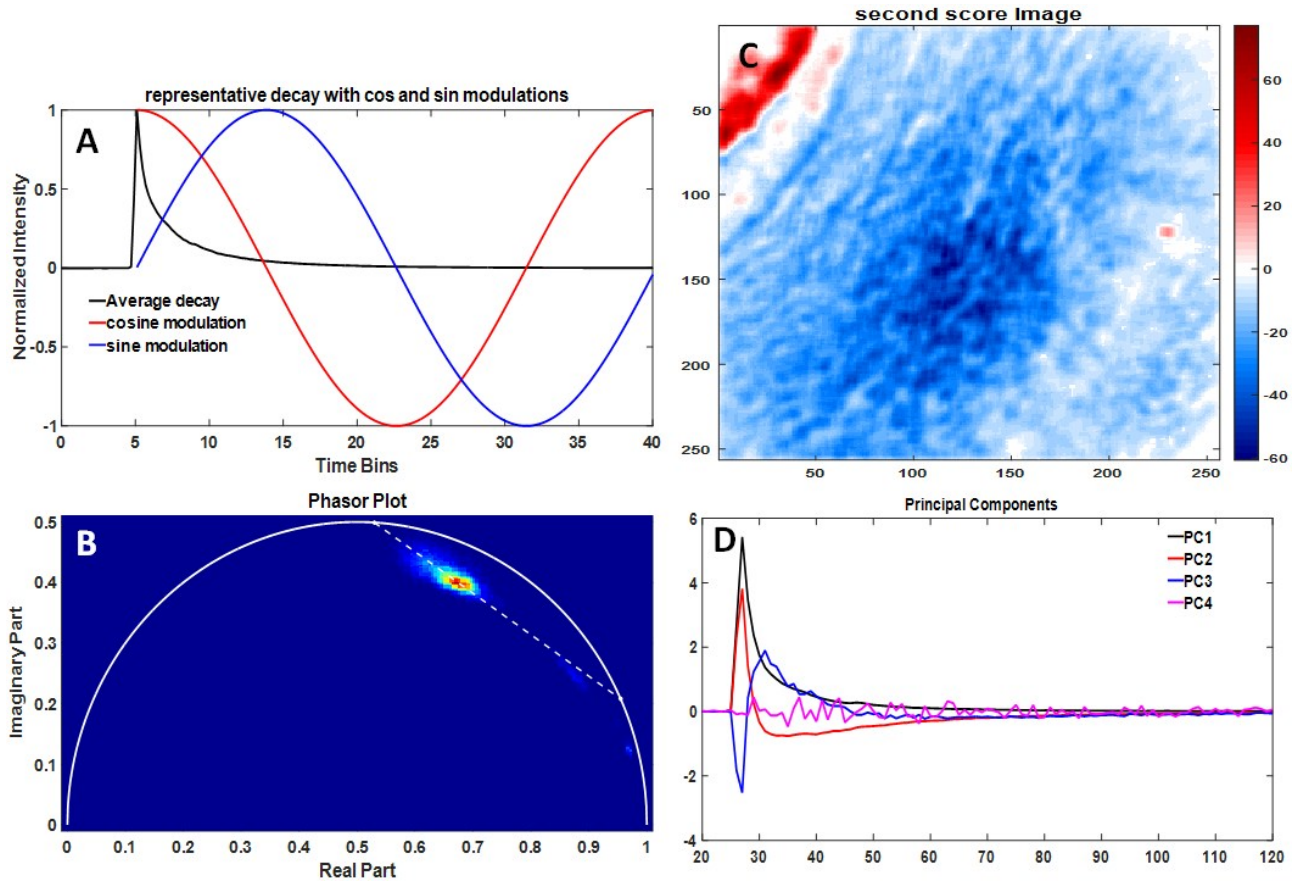

Fig. S5 Noise-corrected principal component analysis (NC-PCA) of fluorescence lifetime imaging data (Figure 3B2) verifies that simultaneous multicolor fluorescence lifetime imaging of erythrocytes, smooth muscles and collagen in H&E stained umbilical artery can be realized based on their different affinities to eosin. (A) Representative decay with cos and sin modulations. (B) Phasor plot of the data; under a two-component assumption the cloud was fitted to a straight line and intersection points with the universal circle found. (C) Represents the phasor second score image from NC-PCA. (D) Four first eigenvectors obtained with NC-PCA.

S4 Experimental section

The phasor approach to FLIM data analysis

The measurement of the fluorescence decay time in FLIM can be performed by several methods. Usually, the analysis of FLIM data collected in the time domain is performed

by fitting the decay at each pixel using multi-exponentials[8]. The phasor method transforms the histogram of the time delays at each pixel into a pair of sine cosine polar coordinates (phasor). Each pixel is then plotted in two-dimensional phasor space (phasor plot). The principal advantages of the phasor analysis, as compared to multi-exponential decay fitting, are its requirements of less initial fitting assumptions, iterative calculations, and its provision of a graphical overview of fluorescence decay at each pixel. Moreover, the phasor approach has been well established for the separation of clusters of pixels with distinctly different lifetimes. Thus, populations having similar lifetimes can be selected in the phasor plot and the fluorescence image can be obtained accordingly.

The fluorescence collected from each pixel of the image was transformed to the Fourier space. The phasor plot, a graphical representation of intensity decays for a FLIM image was constructed. Points in the two-dimensional phasor plot are defined by the values of sine (S) and cosine (G) transforms derived by the following equations:

$$s_{i,j}(\omega) = \frac{\int_0^\infty I(t)\sin(n\omega t)dt}{\int_0^\infty I(t)dt} \qquad (1)$$

$$g_{i,j}(\omega) = \frac{\int_0^\infty I(t)\cos(n\omega t)dt}{\int_0^\infty I(t)dt} \qquad (2)$$

where the indices i and j identify a pixel of the image and $s_{i,j}(\omega)$ and $g_{i,j}(\omega)$ are the y and x coordinates of the phasor plot, respectively; $\omega = 2\pi f$ where f is the laser

repetition frequency (i.e.,76 MHz in our experiment); and n is the harmonic frequency. The analysis of the phasor distribution is performed by cluster identification. There is a direct relationship between a phasor location and lifetime. Every possible lifetime can be mapped into this universal representation of the decay (phasor plot). All possible single exponential lifetimes lie on the "universal circle," defined as the semicircle going from point (0, 0) to point (1, 0), with radius 1/2. Point (1, 0) corresponds to $\tau=0$, and point (0, 0) to $\tau=\infty$. In the phasor coordinates the single lifetime components add directly because the phasor follows the vector algebra. A mixture of two distinct single lifetime components, each of which lie separately on the single lifetime semicircle, does not lie on the semicircle. Clusters of pixel values are detected in specific regions of the phasor plot. The clustering assignment is performed by taking into account not only the similar fluorescence properties in the phasor plot but also exploiting the spatial distribution and localization in cellular substructures or tissues.

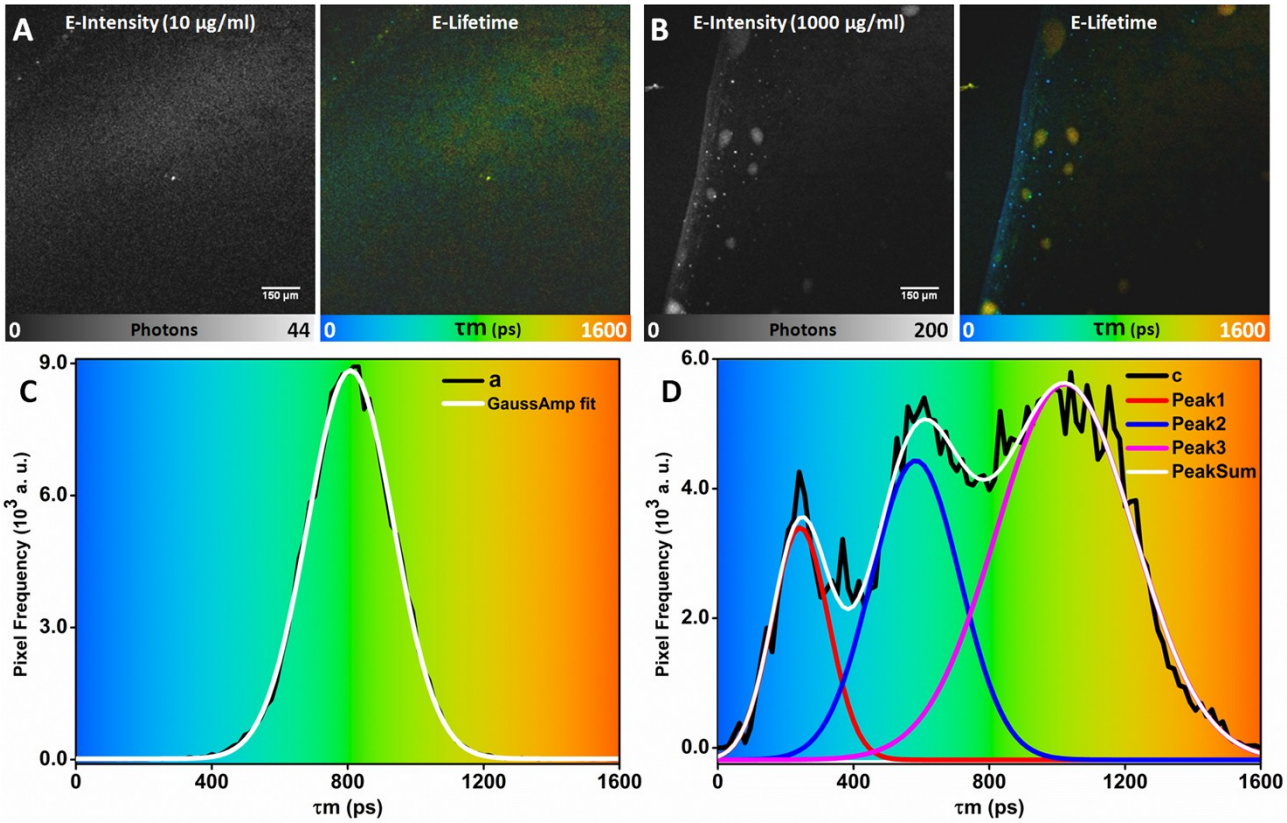

Fig. S6 H&E solution is dropped on glass slide. After the solvent evaporates, fluorescence lifetime imaging is performed. (A) and (B) are the fluorescence lifetime and τm histogram of 10 µg/ml H&E solution, respectively. (C) and (D) are the fluorescence lifetime and τm histogram of 10 mg/ml H&E solution, respectively.