Supporting Information

for

Solid-Phase Single Molecule Biosensing Using Dualcolor Colocalization of Fluorescent Quantum Dot Nanoprobe

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Experimental Section

1. Preparation of QDs Nanoprobe

Two different fluorescent color hydrophobic QDs emission at 560 nm and 650 nm were fabricated via a hot-injection organic phase synthetic procedures. Their encapsulation with Biotin-PEG2000-DSPE and Met-PEG2000-DSPE were performed as following. 1.0 mg Biotin-PEG2000-DSPE and 9.0 mg Met-PEG2000-DSPE were dispersed in 2.0 mL of chloroform in a 10 mL round-bottom flask. Then, a 1.0 mL portion of QDs dispersion (1.0 μM) was added. QDs micelles were prepared by evaporating the organic solvent in a rotary evaporator under vacuum for 30 min and then flushing with a N₂ stream to remove any residual traces of organic solvent. The lipid film, deposited on the reaction vial, was hydrated with 1.0 mL of water at 80 °C. The resulting dispersion was filtered through a 0.2 μm
membrane filter and subjected to ultrasonication for 10 min. Bioin functionalized phospholipid encapsulated QDs micelles (biotin-QDs) can be obtained and kept at 4 °C for further use.

2. Calculation of the Förster radius

The Förster radius $R_0$, defined as the distance between the donor and acceptor that yields 50% energy-transfer efficiency, can be calculated as

$$R_0^e = 8.8 \times 10^{22} \kappa^2 n^{-3} Q_D J$$  \hspace{1cm} (1)

Where $Q_D$ is the quantum yield of the donor in the absence of an acceptor, $n$ is the refractive index of the medium, and $\kappa^2$ is an orientation factor, depending on the relative orientation of the donor and acceptor dipoles. $\kappa^2 = 2/3$ is the value for randomly oriented dipoles, which is suited for the systems studied here. The overlap integral $J$ is a quantitative measure of donor-acceptor spectral overlap integrated over all wavelengths $\lambda$, and is defined as

$$J = \int f_D(\lambda) \varepsilon_A(\lambda) \lambda^4 d\lambda$$  \hspace{1cm} (2)

Where $f_D$ is the normalized fluorescent emission spectrum of donor and $\varepsilon_A(\lambda)$ is the molar extinction coefficient of acceptor. The overlap of the absorption spectra of QD650 with the fluorescence emission spectra of QD560 is shown in Figure S2. $J$ could therefore be calculated by integrating the overlap spectra and Förster radius $R_0$ for the QDs donor-acceptor pair was calculated as 9.7 nm with the help of the software Matlab.

3. Threshold imaging processing

The algorithm for identifying spatially correlated bright spot requires a threshold for detecting significant signal intensity above background. This intensity threshold, $I_{th}$, is
defined as some multiple, $n_{th}$, of the standard deviation of the background, $\sigma_B$, above the mean background, $\mu_B$:

$$I_{th} = \mu_B + n_{th} \sigma_B \quad (3)$$

For each set of images, $\mu_B$ and $\sigma_B$ were determined by fitting a Gaussian distribution to a histogram of photoelectron counts. The threshold for counting spots was usually set $n_{th} \geq 3.0$. This leads to a small probability ($< 0.3\%$) of any pixel being above the threshold. Generally, for images with low SNR, adaptive or localized image segmentation technique should be usually used. For images with high SNR, the threshold for counting spots was usually set relative large $n_{th}$. This leads to a small probability of misclassification.

**Legends**

Figure S1. Experimental setup for fluorescence imaging with dual channels. An inverted microscope (TE2000U, Nikon) was used with a solid state laser excitation source operating at 532 nm (25 mW). The fluorescence emission from the specimen was collected with a high refractive index oil-immersion objective lens (Apo TIRF 60×, NA 1.49, Nikon). The green color was collected through Cy3 filter (Emission filter FF01-593/40-25, Dichroic mirrors, FF562-Di02-25×36, Semrock), and red color was collected through Txred filter (Emission filter FF01-624/40-25, Dichroic mirrors, FF593-Di02-25×36, Semrock). The green and red images were alternately collected side by side on a back-thinned EM-CCD camera (C9100913, Hamamatsu).
Figure S2. Absorbance and fluorescence spectra of both QD560 and QD650 nanoprobes. The spectra illustrated their good spectral resolution and no obvious spectral overlap. The arrow (Excitation) indicated the sample was excited with a solid state laser operating at 532 nm.

Figure S3. Transmission spectra of Cy3 and Txred filters. This displayed that the fluorescence of both QDs nanoprobes could be separately collected through the two filter channels, respectively.
Figure S4. Electrophoresis analysis of the conjugation of QDs with aptamer stained by Sybr@Gold. Under the electric field at 100 V constant voltages for 1.0 h, the QDs in the lane (a) were retained in the sample wells; but aptamer-QDs complex in the lane (b) moved to the positive electrode. The faster mobility of aptamer-QDs complex than QDs was ascribed to a higher negative potential after aptamers grafting on their surface.

Figure S5. Fluorescent imaging of glass slides immobilized different concentration of QD560-TBA I nanoprobe (From A-E: 10 nM, 1 nM, 0.1 nM, 0.01 nM, 0.001 nM); F: The linear dependence between the amounts of fluorescent spots and the QDs molar concentration range from 0.001 nM to 0.1 nM; G: representative time trajectories of single fluorescent spot, and the single fluorescent spot was marked in red arrow in Fig. 5C.
Figure S6. Fluorescent imaging of PLL assembled glass slides, following by BSA blocking (A) and FITC-BSA blocking (B). This supported that BSA protein successfully adsorbed on the sticky PLL layer.

Figure S7. Signal noise ratio of QD560 (A) and QD650 (B) nanoprobes.

Figure S8. Fluorescence images from different channels. (A) Fluorescence image from Cy3 channel; (B) Fluorescence image from Txred channel; (C) merge of images from Cy3 channel and Txred channel; (D) Colocalization of images from Cy3 channel and Txred channel. Bar: 1.0 µm.
Figure S9. (A) Single molecular detection of thrombin at different target concentrations. (B) Selectivity of thrombin detection, protein concentration at 12.5 nM.