Supplementary Information

FRET Based Dual Emission Nanoprobe (FREDEN) with Improved Blinking Behavior for Single Molecule Localization Imaging

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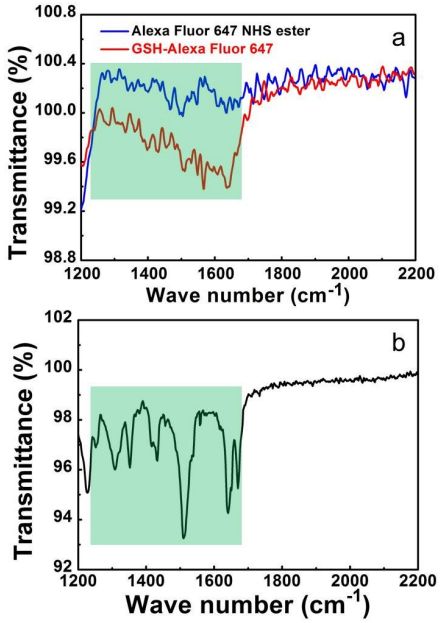

Figure S1. (a) FTIR spectra of Alexa Fluor 647 NHS ester and GSH-A647. (b) FTIR spectrum of pure GSH.

Determine the number of A647 molecules on each FREDEN particle

The ratio of radiant power transmitted (I) by a sample to the radiant power incident (I_0) on the sample is called the transmittance T:

$T = I/I_0$

Absorbance (A) is defined as the logarithm of the reciprocal of the transmittance:

$A = -\ln T = \ln (1/T)$

In a spectrophotometer, monochromatic plane parallel light enters a sample at right angles to the plane

surface of the sample. In these conditions, the transmittance and absorbance of a sample depends on the molar concentration (C), light path length in centimeters (L), and molar absorptivity (ε) for the dissolved substance.

$T = e^{-\varepsilon CL}$ or $A=\varepsilon CL$

Beer's Law states that molar absorptivity is constant and the absorbance is proportional to concentration for a given substance dissolved in a given solute and measured at a given wavelength. Which means the absorbance (A) is proportional to molar concentration (C), and we can use the absorbance/extinction spectra to determine the concentration of A647 in the FREDEN by comparing the absorbance value with A647 standard solutions.

The concentration of the original oil phase QDs is 8 μM. In the phase transfer procedures, 100 μL of oil phase QDs was precipitated and suspended in 200 μL of chloroform. Then 50 μL of QDs in chloroform was taken out and used for phase transfer, the final product was suspended in 1 mL of water. Before extinction spectra measurements, the water phase QDs was diluted for 4 times. Hence, the concentration of QDs in the final water phase is determined as follows:

$$C_{QDs} = \frac{8\mu M \times 100\mu L}{200\mu L} \times \frac{50\mu L}{1mL} \times \frac{1}{4} = 0.05\mu M$$

The concentration of A647 in FREDEN is determined from the extinction spectra in Figure S2 by the following equation:

A_1=0.1853 A_2=0.025 A_3=0.0006

$$C_{A647-FREDEN} = \frac{A_2 - A_3}{A_1} \times 1\mu M = 0.1317\mu M$$

$$N_{A647-FREDEN} = \frac{0.1317}{0.05} = 2.634$$

Where A_1 is the absorbance of 1 μM A647 standard solution at 650 nm, A_2 is the absorbance of 0.05 μM FREDEN at 650 nm, A_3 is the absorbance of pure GSH QDs at 650 nm, $C_{A647\text{-}FREDEN}$ is the molar concentration of A647 in FREDEN, $N_{647\text{-}FREDEN}$ is the number of A647 molecules on each FREDEN. As a result, on average there are 2-3 A647 molecules on a single quantum dot.

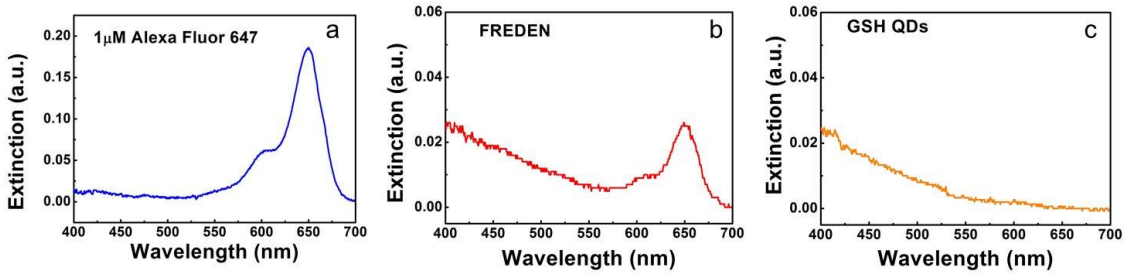

Figure S2. Extinction spectra of (a) 1μM Alexa Fluor 647, (b) 0.4 μM FREDEN, (c) 0.4 μM GSH QDs.

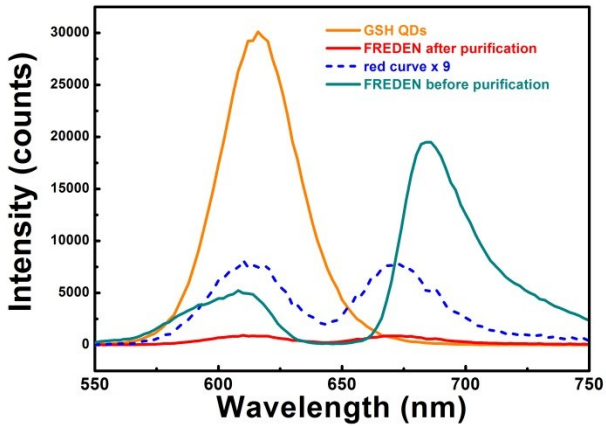

Figure S3. PL spectra of FREDEN and GSH QDs before normalization and smoothing. The concentrations of FREDEN and GSH QDs were the same.

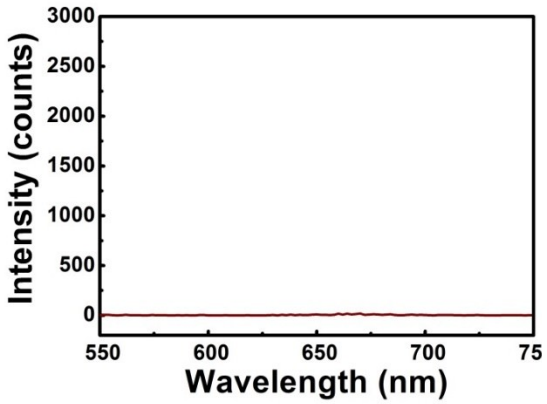

Figure S4. PL spectrum of Alexa Fluor 647 dye solution using 405 nm excitation.

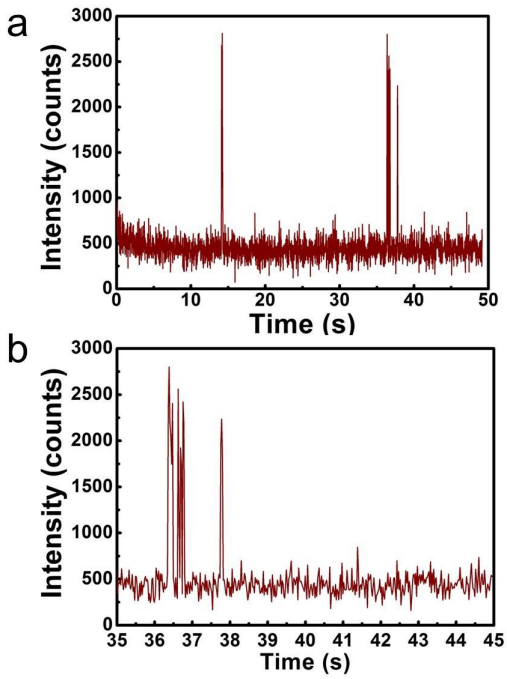

Figure S5. (a) Time dependent fluorescence intensity profile of Alexa Fluor 647 under 405 nm and 640 nm excitation. Laser power: 640 nm 17 W/cm^2, 405 nm 0.69 mW/cm^2. Alexa Fluor 647 was immersed to imaging buffer containing mercaptoethanol [1]. (b) The enlarged region from 35 to 45 s.

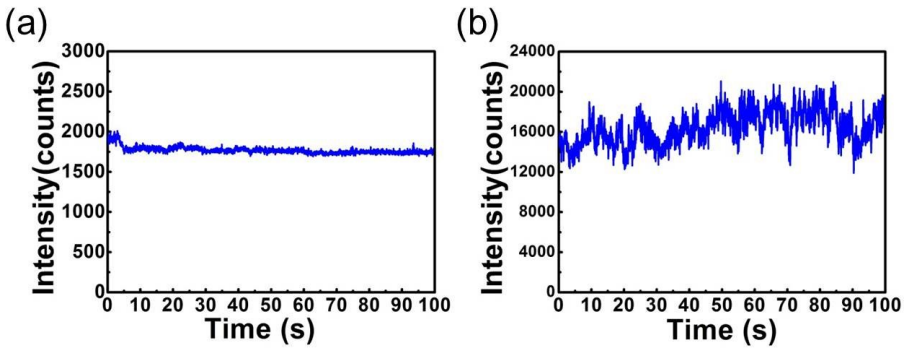

Figure S6. Time dependent fluorescence intensity profiles of GSH QDs under 405 nm excitation with a laser power of 54 mW/cm^2 (a) and 108 mW/cm^2 (b).

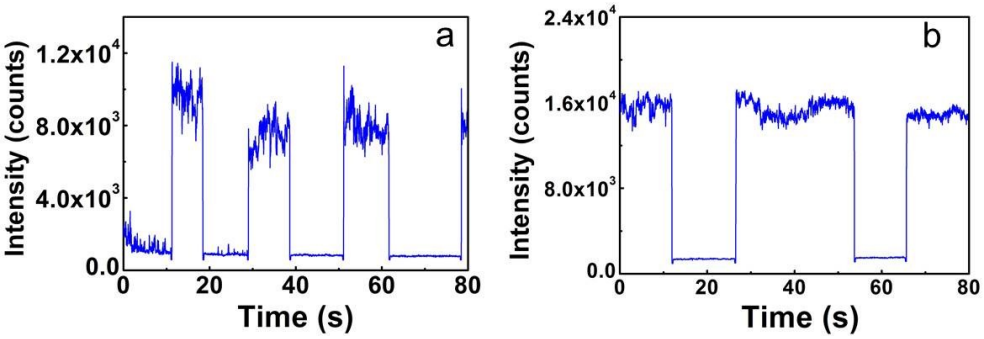

Figure S7. Time dependent fluorescence intensity profiles of individual FREDEN (a) and GSH QDs (b) obtained while artificially switching the filters.

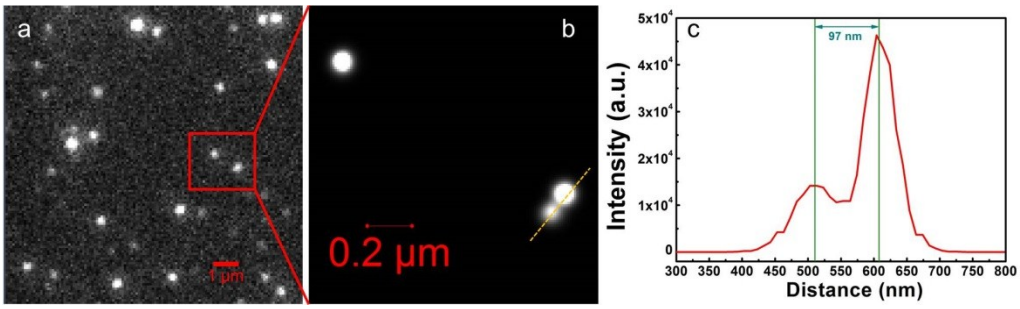

Figure S8. (a) WF image of FREDEN nanoprobes immobilized on chamber slide. (b) PALM/STORM image of FREDEN nanoprobes located in the red box region in (a). (c) Intensity profile along the yellow dashed line in (b). The experimental parameters in PALM/STORM imaging were the same to those used in Figure 4f.

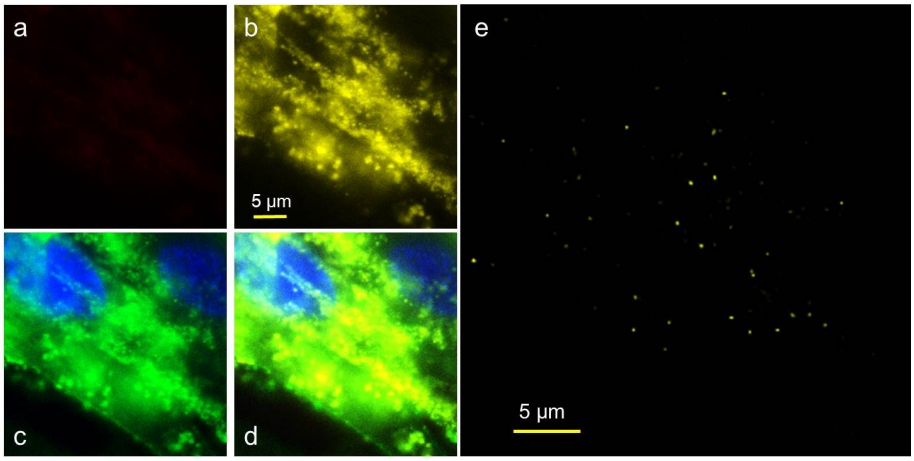

Figure S9. WF TIRF images of MRC-5 cells incorporated with GSH QDs, (a) A647 channel, (b) GSH QDs channel, (c) cell membrane and cell nucleus channels, (d) merged image of (a-c). (e) PALM/STORM image of the GSH QDs channel.

PALM/STORM imaging of exosomes using FREDEN

To better show the availability of the FREDEN, we imaged tumor derived exosomes using PALM/STORM. Exosomes are small vesicles secreted by cells and play a vital role in intercellular communications. The diameter of exosomes is around 30-100 nm, which is much smaller than the diffraction limit. Consequently, imaging of exosomes using conventional optical microscopes is quite problematic. Recently, we have reported imaging and intracellular tracking of tumor derived exosomes using PALM/STORM technique, where Alexa Fluor 647 is used as the fluorophore [2]. Here, to better show the applicability of FREDEN, we also imaged tumor derived exosomes. In the experiment, exosomes of human cervical cancer cells (HeLa cells) were first extracted from conditioned cell culture media and the membrane of the collected exosomes were stained with CM-Dil according to our previously published work [2]. Then 50 μL of BSA coated FREDEN, 50 μL of exosome extractions and 0.5 μL of 50% glutaraldehyde were mixed together and shaken under room temperature for 3 h. Glutaraldehyde was used as the linker to allow covalent binding of BSA coated FREDEN to exosomes, because BSA on FREDEN and proteins on exosome membranes contain abundant amino groups which can be cross-linked with glutaraldehyde. After that, exosomes attached with FREDEN were purified by ultrafiltration to removed unbound FREDEN. Finally, these FREDEN attached exosomes were immobilized onto Nunc™ Lab-Tek™ II Chamber Slide™ via

electrostatic interactions and imaged using our Elyra P.1 system. The results are presented in the following Figure S10.

Figure S10 shows a typical imaging result, where three individual exosomes were visualized. As can be seen in the merged WF image (Figure S10d1), the well colocalization of Alexa Fluor 647, QDs and CM-Dil channels confirmed that FREDEN has been successfully attached to exosomes. Moreover, as shown in Figure S10(a2-d2), dual color PALM/STORM imaging of exosomes has also been accomplished with FREDEN using a single 405 nm excitation laser. Intensity profile measurements indicated that the obtained FWHM of individual exosomes is around 50-70 nm, which is way beyond the diffraction limit and more precisely reports the actual size of exosomes. The above experimental results prove that the FREDEN shows a fine applicability in imaging of small biological targets.

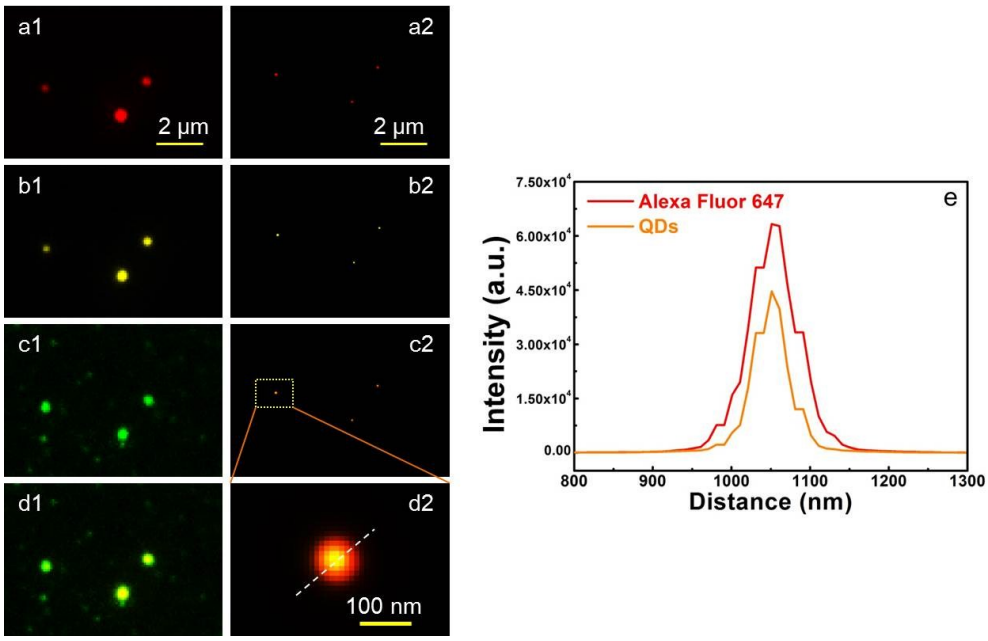

Figure S10. Imaging of FREDEN decorated tumor derived exosomes. (a1) WF image: Alexa Flour 647 channel, (b1) WF image: QDs channel, (c1) WF image: CM-Dil channel, (d1) merged image of a1-c1. (a2) PALM/STORM image: Alexa Fluor 647 channel, (b2) PALM/STORM image: QDs channel, (c2) merged image of a2 and b2, (d2) enlarge image of an individual exosome as indicated by the dashed box in c2. (e) Intensity profiles along the dashed white line in d2, red curve: Alexa Fluor 647 channel, yellow curve: QDs channel. PALM/STORM images were reconstructed from 5000 frames, the exposure time for each frame is 20 ms. Excitation laser for FREDEN is 405 nm and that for CM-Dil is 561 nm.

References

1. G. T. Dempsey, J. C. Vaughan, K. H. Chen, M. Bates and X. Zhuang, *Nature Methods*, 2011, **8**, 1027-1036.
2. C. Chen, S. Zong, Z. Wang, J. Lu, D. Zhu, Y. Zhang and Y. Cui, *ACS Applied Materials & Interfaces*, 2016, DOI: 10.1021/acsami.6b09442.