# Supporting Information

# Asynchrony of spectral blue-shift of quantum dots based digital homogeneous immunoassay

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## Experimental Section

**Chemical and material.** Transmission grating with 70 lines per mm was bought from Edmund Scientific (Barrington, NJ, USA). Capture antibody to carcino-embryonic antigen (CEA, L1C00202), detection antibody to CEA (L1C00205), capture antibody to alpha-fetoprotein (AFP, L1C00301), detection antibody to AFP (L1C00302), CEA (L2C01001), and AFP (L2C00202) were ordered from Shanghai Linc-Bio Science Co., LTD (Shanghai, China). Carboxylic QD 655 (Q21321MP), positively charged slides (4951PLUS4), phosphate buffer saline (PBS, 10010-023), and cover slips (12-541A) were obtained from Thermo Scientific (Waltham, MA, USA). The radius of the QD 655 is in the range of 7.5-10.0 nm according to the manufacturer. Chromatography resin of Superose 6 pre grade and Superose 12 prep grade was bought from GE Healthcare Bio-Sciences (PA, USA). N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC， E6383) were from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals were obtained from local vendors.

**Single-particle fluorescence spectral imaging.** Single-particle spectral imaging was conducted on an Olympus IX71 microscope as described in our previous study [16]. A transmission grating is fixed between a filter and a CMOS scientific camera as shown in Scheme 1B. The grating splits a fluorescence beam from a single emitter into two beams. The dot-shaped beam in direct transmission is termed as the zeroth-order dot, and the streak-shaped beam deviating from direct transmission is termed as the first-order diffraction streak. The wavelength of the emitter (λ) follows the equation, $\lambda = L \times d/S$ (S, the distance between the grating and the camera chip; d, the grating constant; L, the distance between the zeroth-order dot and the corresponding first-order streak). In our optical setup, the value of d/S was measured to be 2.80 nm/pixel using QD 655 as standard. Multi-image mode (video) was used to acquire images. Both exposure time and interval time were set as 100 ms. Imaging acquisition was stopped until all QDs were bleached. The time required for the complete QD bleaching is dependent on the power of light source. The shorter the lamp is used, the more energy the lamp has and hence, the less time is needed for bleaching. For example, it would need approximately 5 min and 17 min to completely bleach QDs with a lamp of 1 hr usage and 200 hr usage, respectively. Therefore, the optimal time points for a lamp with 1 hr and 200 hr usages were 5 min and 17 min, respectively. All the obtained videos were processed by the software of Image J. The counting of the number of the split first-order streak was sped up through fast forwarding video.

**Antibody-modified QD (A-QD) preparation.** Capture antibodies (CAs) were conjugated to carboxylic QD 655 following the manufacture's instruction. A 10.0 μL aliquot of freshly prepared 1.0 mg/mL EDC was mixed with a 5.0 μL aliquot of QD 655 and a 35.0 μL aliquot of 50.0 mM $NaHCO_3$. After 30.0 min incubation, CAs were added into the above solution at a CA-to-QD ratio of 40:1. The reaction was allowed for 2 h at room temperature. Detection antibodies (DAs) were conjugated to carboxylic QD 655 following the same procedure, except for the DA-to-QD ratio of 20:1. In order to obtain pure A-QDs, the conjugation solutions were sequentially passed through a 250.0 mm×4.6 mm size exclusion column packed with chromatography resin from GE Healthcare Bio-Sciences. The detection wavelength and injection volume were set as 224.00 nm and 7.00 μL, respectively. The peak summit of A-QD was collected and used as stock solution.

A-QD in the stock solution was quantified according to the fluorescence intensity of QD. The fluorescence intensity was measured on Hitachi F-4600 spectrophotometer (Hitachi, Japan). The excitation and emission wavelength were set as 400 and 655 nm, respectively. The working curve is measured to be y=1331.1x-545.25 with a regression coefficient of 0.998.

**Homogeneous immunoassay.** All experiments were done in triplicate and the error bars referred to the standard error of the mean of the triplicated experiments unless specified. The stock solution of the protein was prepared by dissolving calculated amount of pure protein in 500.00μL aliquot of PBS buffer. A series of protein standard solutions were diluted from this stock solution. For TEM imaging, the test solution was prepared by mixing a 5.26 μL aliquot of QD-CA stock solution, a 14.30 μL aliquot of DA-QD stock solution, a 95.27 μL aliquot of PBS buffer, and a 26.70 μL aliquot of protein standard

solution for 30 min. The final concentrations of both QD-CA and DA-QD in the test solution were 0.20 nM. A 4.00 μL aliquot of the test solution was dropped on a TEM grid and dried for TEM imaging. The TEM imaging was conducted on a Tecnai G2 F20 (FEI, USA). Note: prior to use, the grid was immersed in 0.5% poly(diallyldimethylammonium chloride) solution (v/v) for several tens of seconds and dried at room temperature.

For the single particle spectral imaging, the test solution was prepared by mixing a 1.30 μL aliquot of QD-CA stock solution, a 3.53 μL aliquot of DA-QD stock solution, a 110.00 μL aliquot of PBS buffer, and a 26.70 μL aliquot of protein standard solution for 30 min. The final concentrations of QD-CA and DA-QD in the test solution were each 0.05 nM. A 1.80 μL aliquot of the test solution was dropped onto a positively charged slide and covered with a cover slip. Immediately, the glass slide and the cover slip were glued with nail polishing oil for microscope observation. The sealed device can be stored for months without evaporation of the trapped solution according to our experience..

**Clinical sample test.** The plasma from healthy donor was chosen as model clinical sample. Plasma samples were handled according to the approved protocols (IRB#M0423) by Institutional Review Board (IRB) of Jiangsu Normal University. The plasma samples were provided by Xuzhou Center Hospital. All subjects offered written informed consents. A series of calculated amount of standard protein was added into a series of aliquots of plasma, respectively. Those aliquots of plasma were then mixed with PBS buffer, QD-CA stock solution, and DA-QD stock solution to make an optimized test solution. The final concentrations of the added protein in the test solutions ranged from 0.20 pM to 1.50 pM. After 30 min incubation, the test solutions were analyzed using single particle spectral imaging.

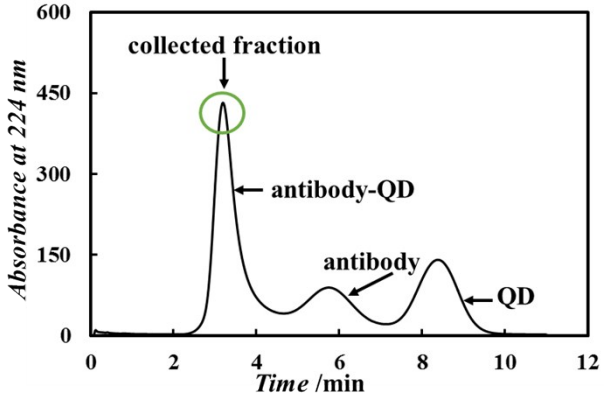


**Figure S1.** Typical size exclusion chromatogram of the conjugation solution of antigen (CEA) CA and QD. The flow rate, detection wavelength, and injection volume were set as 1.00 mL/min, 224.00 nm, and 7.00 μL, respectively.

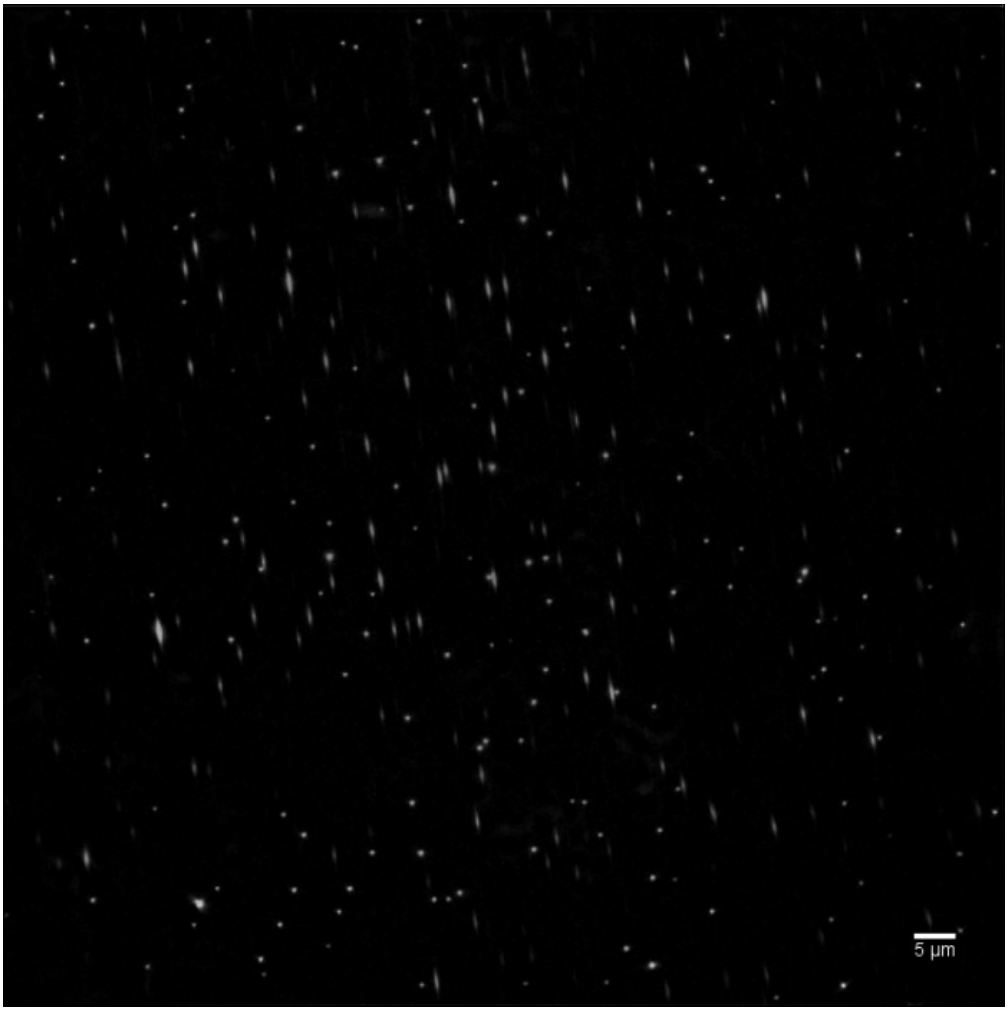


Figure S2. Typical transmission grating-based single particle spectral image.

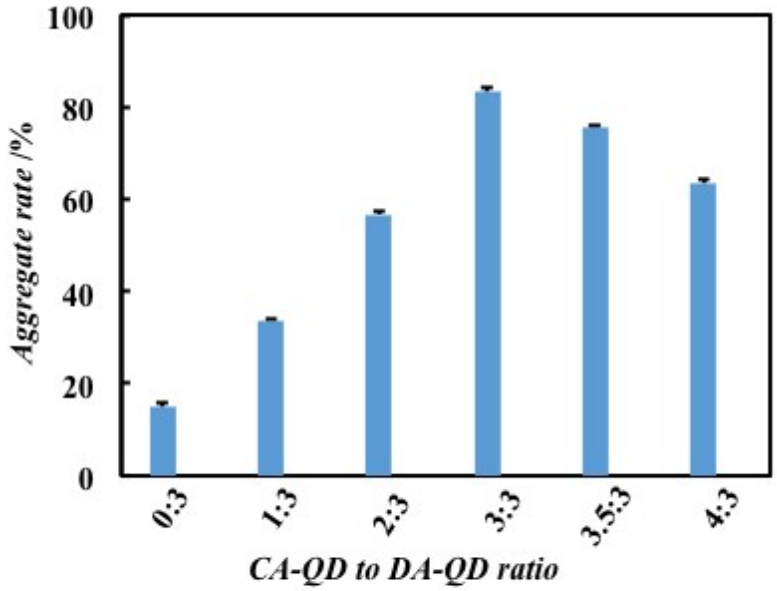


**Figure S3. Optimization of QD-CA to DA-QD ratio.**

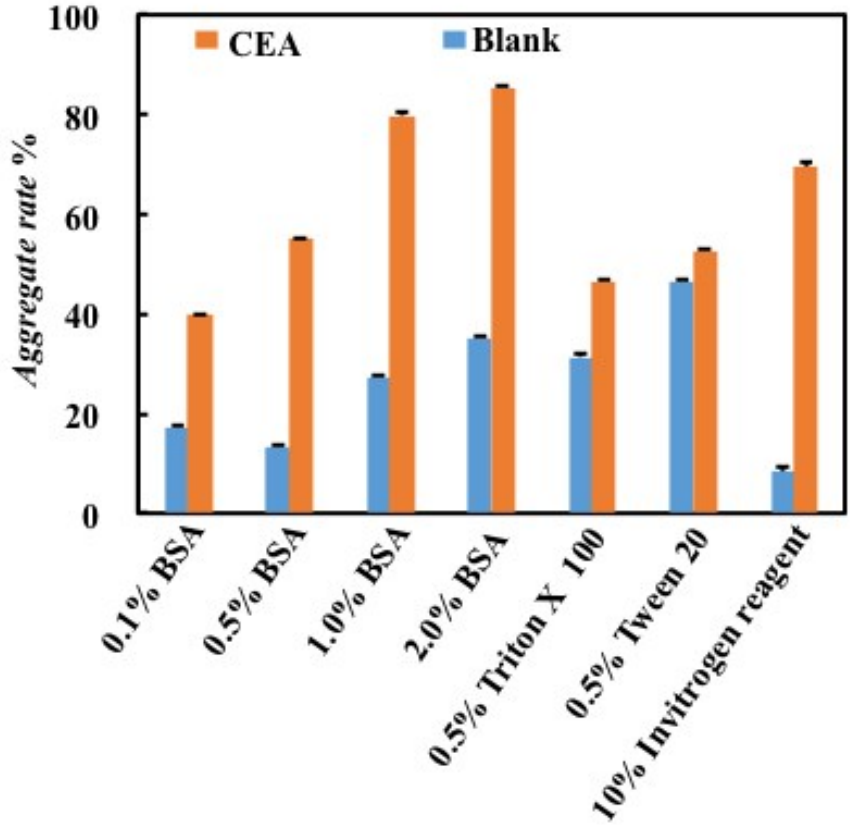


**Figure S4. Optimization of blocking efficiency.**