Electronic Supplementary Material (ESI) for Analyst.

Competitive Aptasensor for Ultrasensitive Multiplexed Detection of Cancer Biomarkers by Fluorescent Nanoparticle Counting

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Contents

1. Experimental section

1.1. Conjugation of amino-DNA on FNPs

This procedure has been published in *Anal. Chem.*, 2018, **90**, 1376-1383.[1] In this work, the exact procedure was followed. Herein, we provide again for the convenience of readers.

The preparation of the capture DNA conjugated fluorescent nanoparticle (DNA-FNP) was carried out by 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide hydrochloride (EDC) coupling chemistry. Specifically, 10 μL of 10 mg/mL FNP suspension was washed three times with 100 μL of 50 mM MES buffer (pH 5.0). Then, 10 μL of 50 mg/mL freshly prepared EDC solution was added and mixed, an aliquot (5 μL) of 100 μM amino-DNA was added and mixed by vortexing. The solution with total volume of 100 μL was incubated for 30 min at room temperature with slow agitation, and 10 μL of 50 mg/mL freshly prepared EDC in ice-cold MES buffer was added and mixed well by vortexing. The mixture was stirred overnight and followed centrifugation. Afterward, FNPs were washed three times with 100 μL of 10 mM PBS to remove excess amino-DNA, re-suspended in 1 mL of PBS, and stored at 4 °C for further use.

1.2. Modification of MBs with biotinylated DNA

This procedure has been published in *Anal. Chem.*, 2018, **90**, 1376-1383.[1] In this work, the exact procedure was followed. Herein, we provide again for the convenience of readers.

The capture DNA functionalized magnetic beads (DNA-MBs) were prepared via streptavidin-biotin conjugation. Specifically, 200 μL of Dynabeads™ magnetic beads were transferred to1 mL of PBS and washed three times with PBS buffer. Then, 25 μL of 100 μM biotinylated DNA (biotin-DNA) was added at room temperature by gentle rotation. After 15 min, the resulting DNA-MBs were washed five times with PBS containing 0.1% Triton X100 to remove the excess biotin-DNA, and re-suspended in 1 mL PBS containing 0.1% Triton X100 and stored at 4 °C for further use.

1.3. Image processing and particle counting

This procedure has been published in *Anal. Chem.*, 2018, **90**, 1376-1383.[1] In this work, the exact

procedure was followed. Herein, we provide again for the convenience of readers.

The 3 colors of FNPs were recognized and enumerated automatically using the software developed in C# programming language based on our previous work.[2] The general idea of the automatic counting was to recognize the FNPs referred sequentially by shape and color characteristics. Specifically, at first, Gaussian blur was applied to smooth the image, and, sharpen edges was used to enhance the edge of the FNPs. The high-pass filtering step described in our previous works was then applied to eliminate off-focus FNPs signals interfering with the recognition. The shape-based segmentation was then used to divide the image into subimages with each containing a single object to be identified. The shape (area and axial ratio) and color judgments were sequentially applied to each subimage to identify FNPs, and last the number of FNPs was counted. The three colors of FNPs were found to be best separated in the CIELCh color space, with C^* (chroma) and $h°$ (hue angle) components. To simplify the identification, an average color was generated for each subimage. The average color had color differences (calculated with CIEDE2000 algorithm) less than a specified threshold with more than a half of the pixels of the object in a subimage. Average colors obtained from the images of each single type of FNPs were considered as reference colors. Linear boundaries of the reference color in $C^*– h°$ chart were then calculated and used as the criteria for color judgments.

Table S1. The sequences used in this study.

Name	DNA sequences
MB-Universal	TC TGC TAC CCA CAG CCG GTT AAAAAAAAAA
FNP-CEA	AAAAAAAAAAAAAAAAAAAACCAGCTTATTCA
aptamer-CEA	ATACCAGCTTATTCAATT
Linker-CEA	AAC CGG CTG TGG GTA GCA GA TGA ATA AGC TGG TTT
FNP-PSA	AAAAAAAAAAAAAAAAAAAA GCT CGC CAT CAA
aptamer-PSA	ATT AAA GCT CGC CAT CAA ATA GC
Linker-PSA	AAC CGG CTG TGG GTA GCA GA TTG ATG GCG AGC TT
FNP-Thr-2	AAAAAAAAAAAAAAAAAAAAGTAGGGCAGGTTGG
aptamer-Thr	AGTCCGTGGTAGGGCAGGTTGGGGTGACT
linker-Thr	AAC CGG CTG TGG GTA GCA GA CCA ACC TGC CCT AC CA

2 Supplemental results

Table S2. Spike Recoveries (%) from fetal bovine serum (n = 3).

Target	Singleplex	Multiplex（10% diluted）		
Protein	CEA	PSA	CEA	thrombin
Fetal bovine serum	101.1±7.7	107.4±6.9	92.2±16.9	101.4±9.0

2.1. Fluorescence microscopic images for biomarker detection

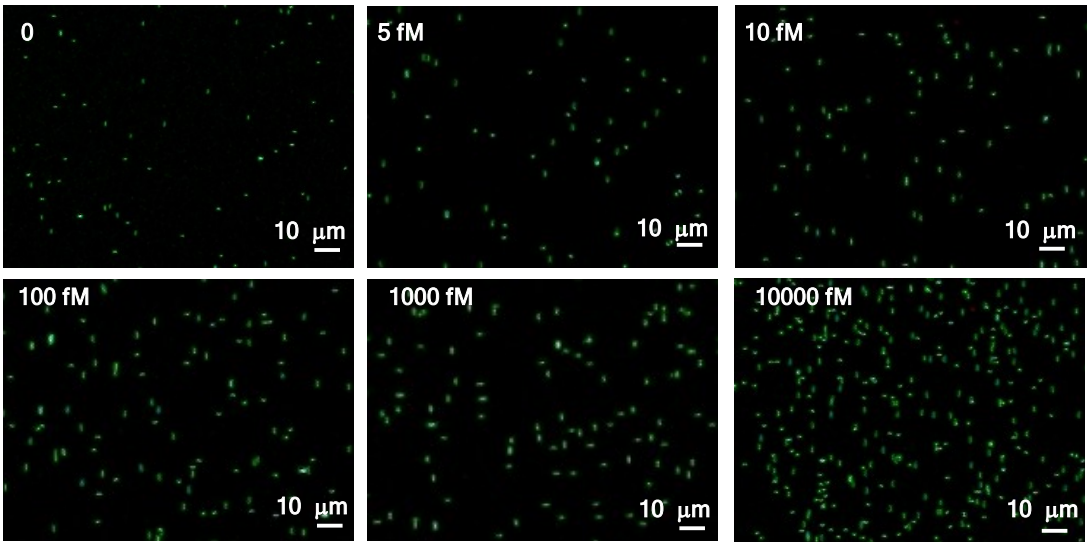

Fig. S1 The fluorescence microscopic images of FNPs for thrombin detection.

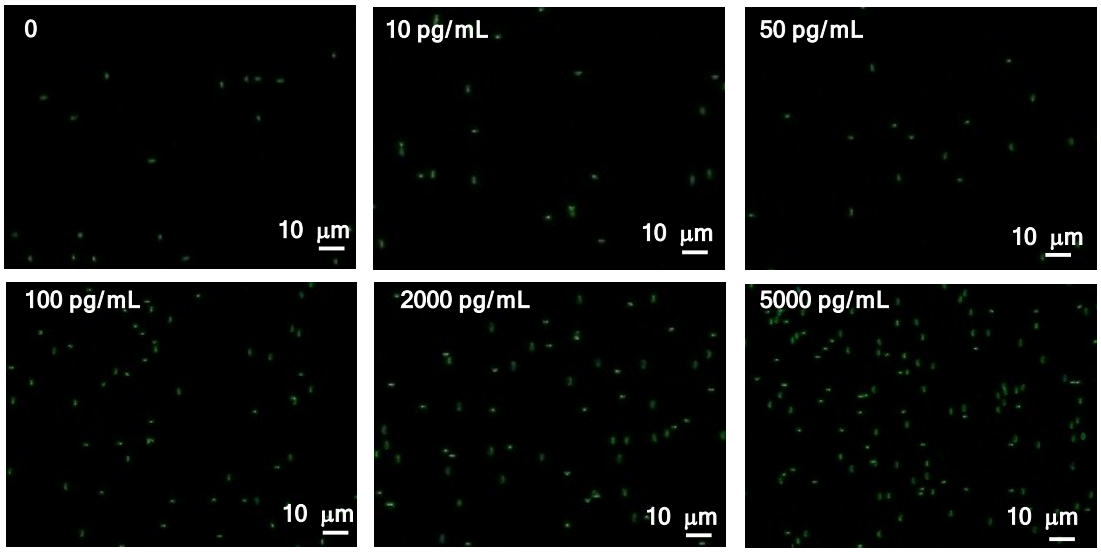

Fig. S2 The fluorescence microscopic images of FNPs for PSA detection.

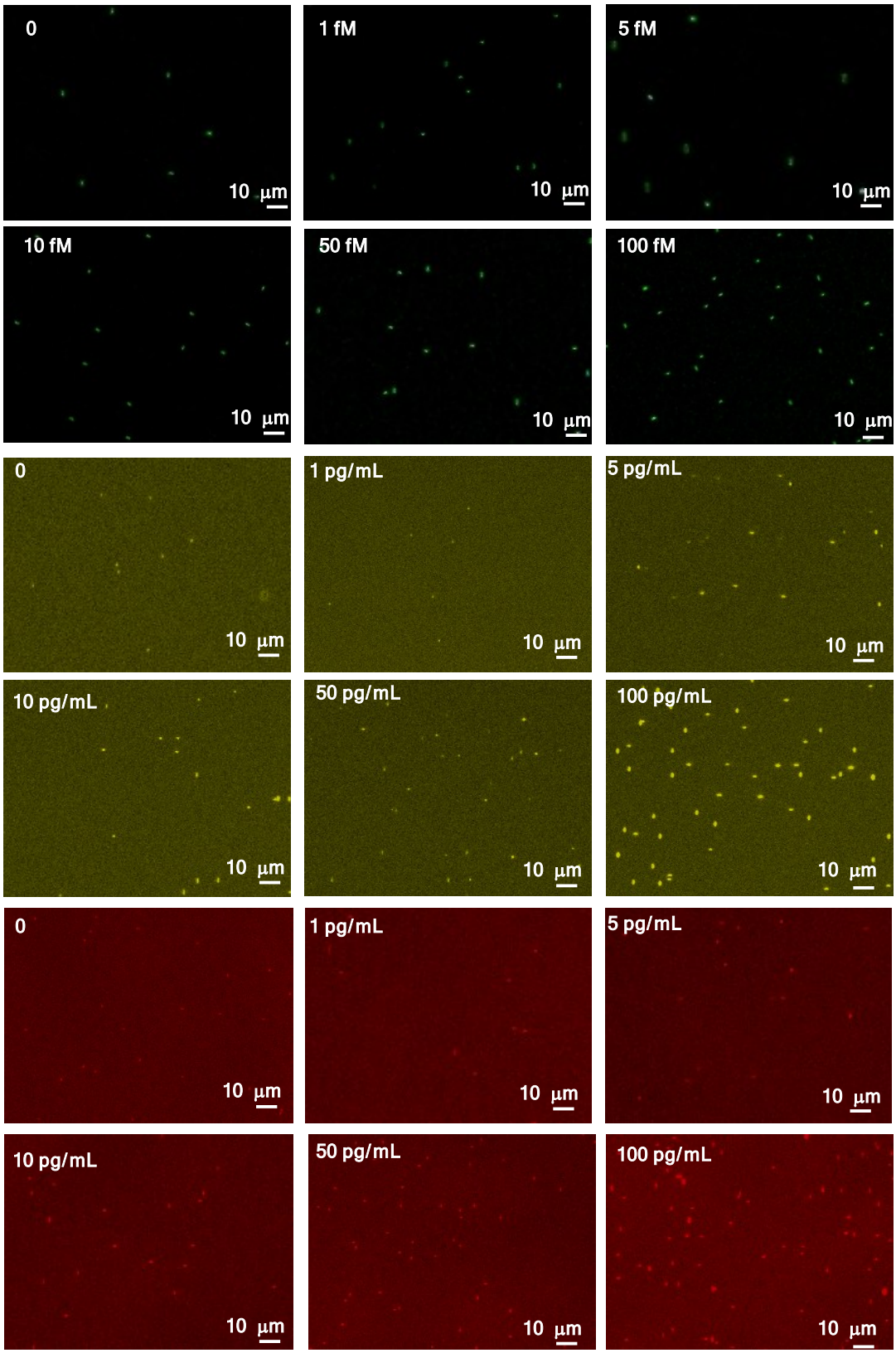

Fig. S3 The fluorescence microscopic images of FNPs for multiplexed detection of thrombin (green), CEA (yellow) and PSA (red) at different concentrations. The fluorescence microscopic images of CEA and PSA are Pseudo-color images.

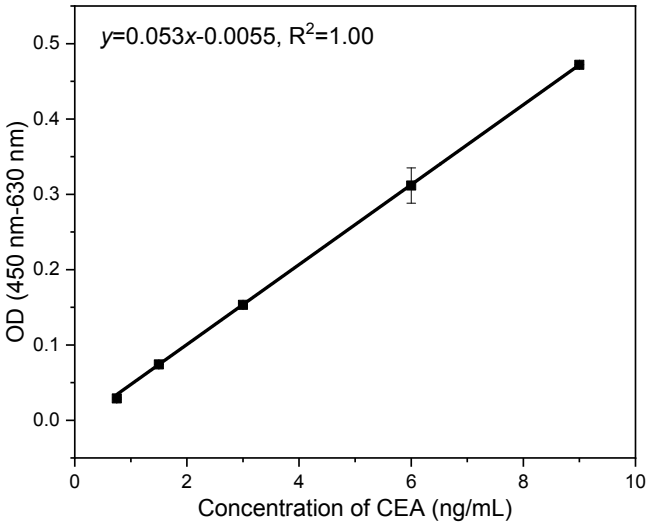

Fig. S4 Calibration curve for CEA by ELISA.

3. References

1. Pei, X., Yin, H., Lai, T., Zhang, J., Liu, F., Xu, X., Li, N., *Anal. Chem.* 2018, **90**, 1376-1383.

2. Xu, X., Li, T., Xu, Z., Wei, H., Lin, R., Xia, B., Liu, F., Li, N., *Anal. Chem.* 2015, **87**, 2576-2581.