Supporting Information

Fluorescence and scattering light cross correlation spectroscopy and its applications in homogeneous immunoassay

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The characterization of SNPs

The characterization of SNPs was shown in Figure S1, and the average diameter of SNPs was 17.4 nm ± 2.3 nm. Transmission electron micrograph (TEM) images were taken with a JEM-2100 transmission electron microscope (JEOL, Japan). UV-vis absorption spectra of nanoparticles were obtained by using a UV-3501 spectrophotometer (Tianjin Gangdong Sci. & Tech. Development Co. Ltd., China).

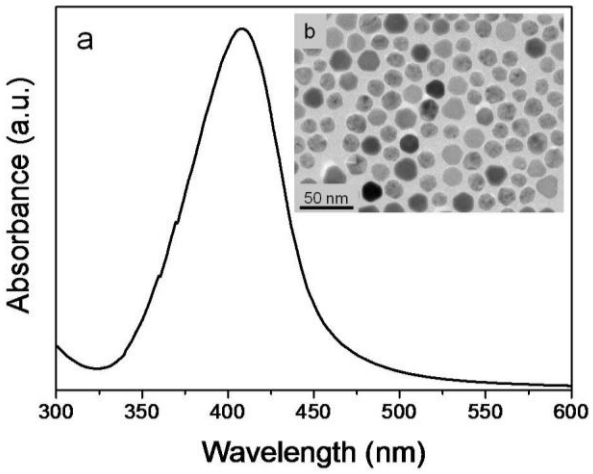

Figure S1. (a) UV-vis absorption spectra of silver nanoparticles solution. (b) TEM image of silver nanoparticles. Scale bar: 50 nm.

Under the identical condition, a series of SNPs solutions with different concentrations were measured by the resonance light scattering correlation spectroscopy (RLSCS) system. The fitted value of *G(0)* was proportional to the dilution times of SNPs with good linearity, which was shown in Figure S2, and the coefficient of determination (R^2) is 0.999, and the standard error of slope is 0.023 while the standard error of intercept is 0.026. This result further manifested that the RLSCS method was very sensitive to the concentration of particles, which was similar to FCS.

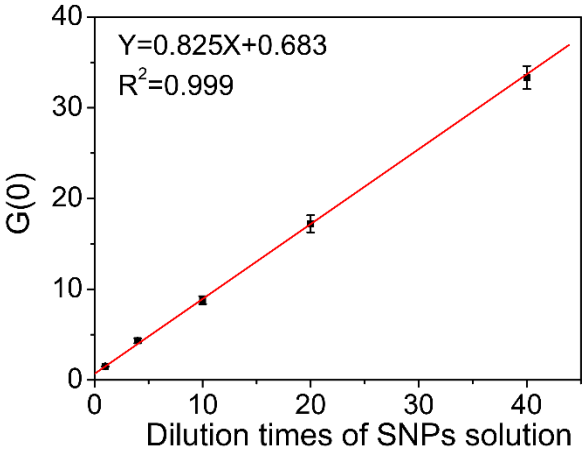

Figure S2. A linear relationship between the fitted *G(0)* value and dilution times of silver nanoparticles solution. The error bars represent the standard deviation of 3 time measurements. The original concentration of SNPs solution was about 1.6 $\times 10^{-9}$M.

Preparation and purification of fluorescent probes

Approximately 1.0 mg/mL of protein was dissolved in a solution containing 0.1 M Na_2CO_3 (pH 8.3). An appropriate amount of Alexa was added. Reaction ratio of Alexa to AFP was 100:1. The mixture was incubated in a separate tube for 1 h at room temperature under gentle stirring, protected from light.

To remove the free dye molecules from the solution, a size exclusion column (Sephadex G50, GE Healthcare, Sweden) was used. After filtering, the Alexa-protein was concentrated to a final concentration of 0.5 mg/mL using 30 kDa molecular weight cutoff centrifuge concentrators (Microcon YM-30), and then stored at 4 ^{o}C.

Characterization of AFP antibodies conjugation to SNPs

The conjugation ratio of SNP to antibody was approximately calculated by the following formula:

$$SNP: Ab = [SNPs]: ([Ab]_{before\ reaction} - [Ab]_{after\ reaction}) \quad (S1)$$

Where $[Ab]_{before\ reaction}$ and $[Ab]_{after\ reaction}$ were the concentrations of antibodies before and after reaction respectively, and [SNPs] was the concentration of SNPs.

Table S1. The ratio of SNP to antibody (Ab) calculated by FCS.

	C_{SNPs}	Before Reaction C_{Ab}	After Reaction C_{Ab}	The ratio of SNP to Ab
Alexa-Ab1	0.5 nM	10.2 nM	7.3 nM	1:5.9
Alexa-Ab2	0.5 nM	10.6 nM	7.5 nM	1:6.2

The stability of SNPs-Ab1/Ab2 bioconjugates was examined by FCS. The desorption percentage of antibodies from SNP (free antibodies) in 0.1% BSA-PBS and 10 times diluted serum sample was calculated by the following formula.

$$Free\ Ab\% = \frac{I_{after\ reaction} - I_{background}}{I_{beforereaction} - I_{background}} \times 100\% \quad (S2)$$

The low and stable concentrations of free antibodies confirm that the desorption was very low. This data illustrated that SNPs bioconjugates were stable at least 5 days.

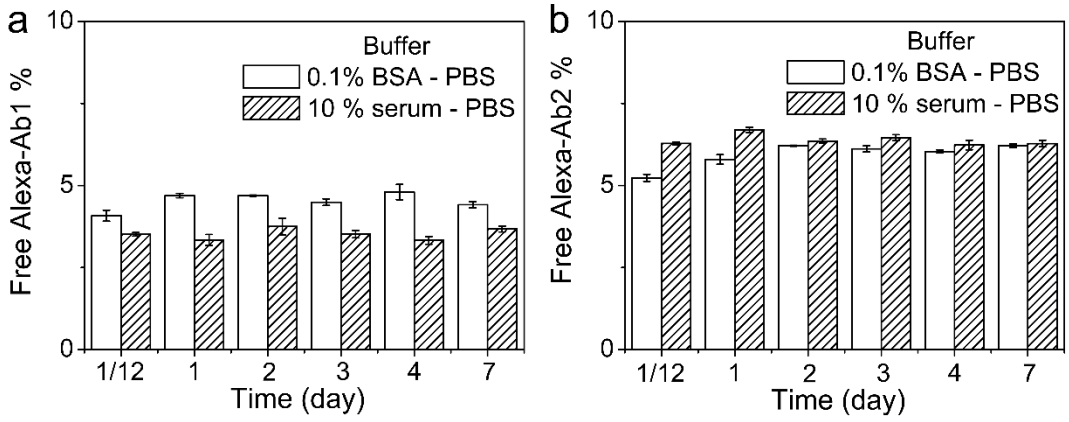

Figure S3. Stability of SNPs-(Alexa-Ab1) conjugates (a) and SNPs-(Alexa-Ab2) conjugates (b) in 0.1% (w/w) BSA-PBS solution or in 10% (v/v) serum sample (diluted from serum with PBS buffer). The percentages of free Alexa-Ab1 or Alexa-Ab2 in the SNPs-(Alexa-Ab1) or SNPs-(Alexa-Ab2) conjugates were measured at different store time at 4 ^{o}C after SNPs-(Alexa-Ab) conjugates were prepared with centrifugal purification.

Preparation of different SNPs-Alexa models

The procedures for preparation of different SNPs-Alexa models were shown in Figure 1.

Model A was formed by mixing 1 nM SNPs with 1 nM Alexa-Ab2.

Model B was formed by mixing 250 pM SNPs-Ab1 and 250 pM Alexa-AFP.

Model C was formed by mixing 250 pM SNPs-Ab1, 250 pM Alexa-AFP and 250 pM SNPs-Ab2.

Model D was formed by mixing 250 pM SNPs-AFP1, 400 pM AFP and 250 pM Alexa-AFP2.

All samples mentioned above were incubated at 25 ^{o}C for 2 h. The effects of SNPs on the intensity of Alexa were studied by four SNPs-Alexa models using FCS. Relative intensity was compared to the free Alexa-labeled protein with same concentration.

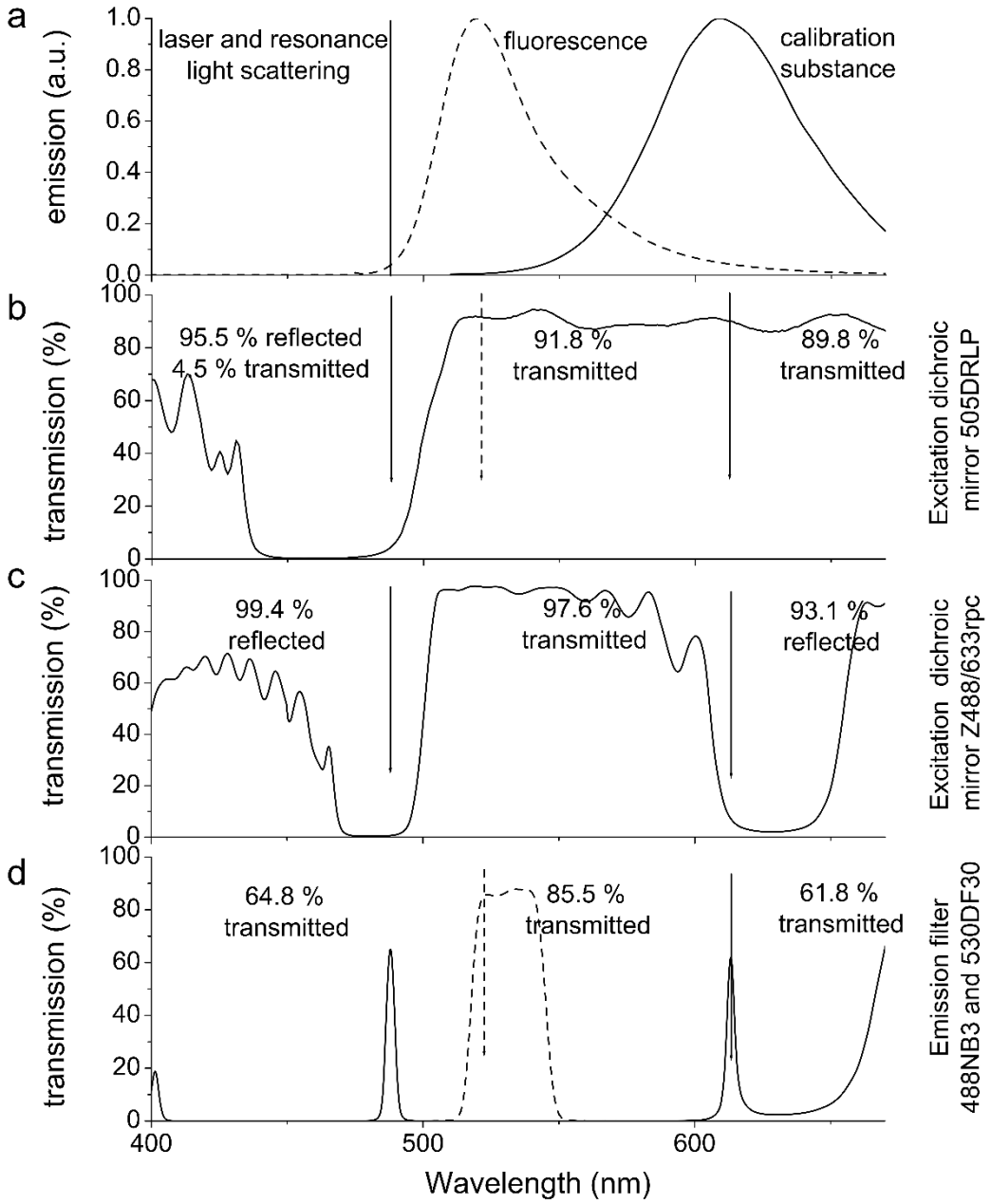

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Figure S4. Spectral separation of excitation and emission wavelengths. (a) Emission, resonance light scattering spectra of the 488nm laser line and Alexa (dash line) and the calibration substance QD610(solid line). Calibration substance was used to calibrate the resonance light scattering channel. (b) Wavelength selection by the excitation dichroic mirror 505DRLP (DM1 in figure 2). The laser line was reflected by the mirror from the laser sources to the objective while 4.5% scattering light and 97.6% fluorescence emission from the sample traverses the mirror. (c) Wavelength selection by the dichroic mirror Z488/633rpc (DM2). While the emission of the resonance light scattering was reflected by the emission dichroic mirror, fluorescence passes the mirror. (d) Absorption filters for residual scattered laser emission, 488NB3 (Fliter2) and 530DF30 (Fliter1).

V_S calculation method

The average number of molecules in the detection volume can be described as $N = V \bullet C$. When the same SNPs sample was used, the detection volume in scattering signal channel (V_S) was approximately calculated by the following formula:

$V_S = N_S \bullet V / N$

The mean numbers of SNPs (N) was measured in the detection volume (V) calculated by Rhodamine Green using RLSCS system. N_S was measured in V_S using FSCCS system.

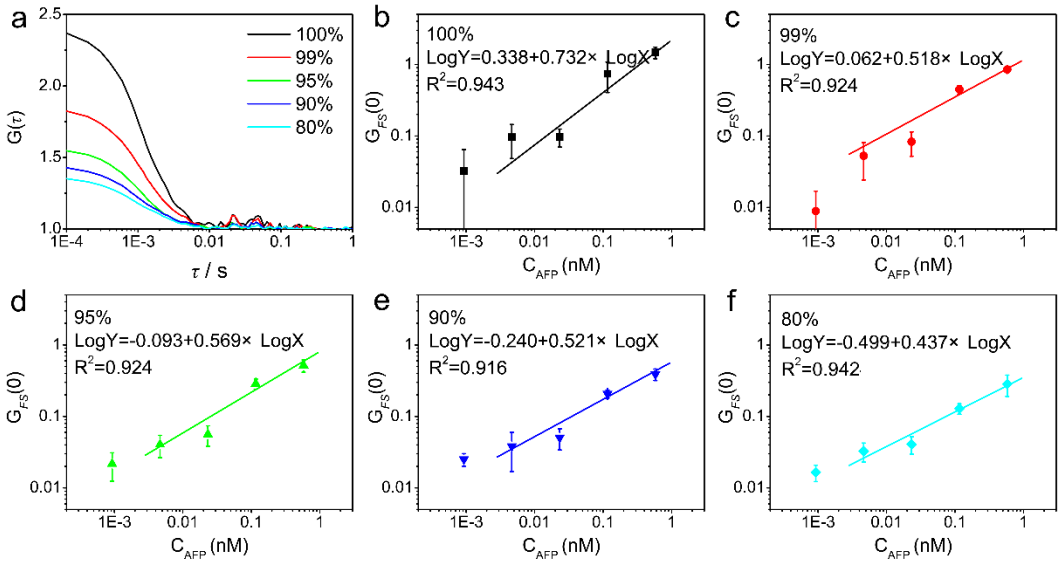

Figure S5. Analysis of cross-correlations using aggregate exclusion method. (a) When excluding the slices with the top 1.0% (red line) and 5.0% (green line) of signal intensities, the correlation drops dramatically compared to 0% excluded. And the cross-correlation drops slightly as more time slices are excluded. (b-f) The linear relations between the cross-correlation and logarithm AFP concentration when different percentage of time slices are excluded. The detection method was homogeneous sandwich immunoassay. The concentrations of SNPs-Ab1 and Alexa-Ab2 were 25 pM and 250 pM. The incubation temperature was 25 ^{o}C, and the immune reaction time was 2 h. The reaction and detection buffers were 0.1% BSA-PBS. The measurement time was 300 s. The error bars represent the standard deviation of 3 time measurements.

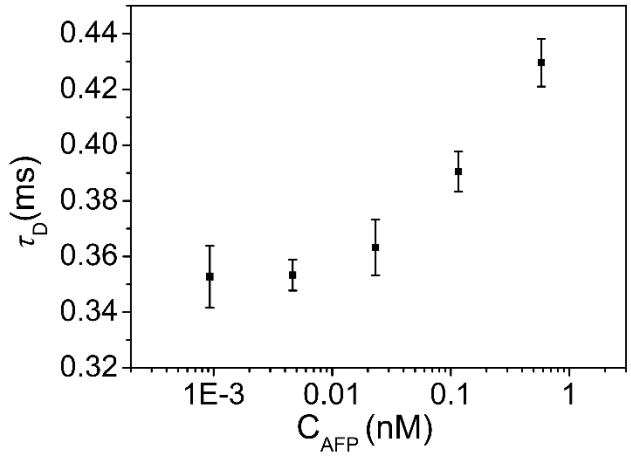

Figure S6 Relationship between the characteristic diffusion time (τ_D) of Alexa-Ab2 and Log AFP concentration in homogeneous sandwich immunoassay corresponding to Figure 6a. The error bars represent the standard deviation of 3 time measurements. The excluded fraction of the aggregate exclusion method is 0%.

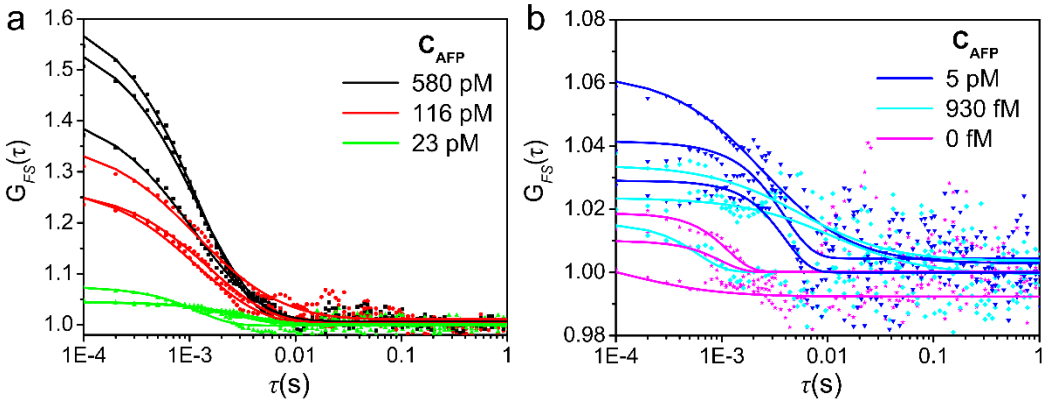

Figure S7 Cross-correlation curves, $G_{FS}(\tau)$, calculated from AFP of various concentrations in homogeneous sandwich immunoassay. We measured three times for each sample. Amplitudes of the cross-correlation curves increase with increasing concentrations of AFP. The cross-correlation curves were shown at two $G_{FS}(0)$ scale (a, 0.98-1.6. b, 0.98-1.08.). The concentrations of SNPs-Ab1 and Alexa-Ab2 were 25 pM and 250 pM. The immune reaction temperature and time were 25 ^{o}C and 2 h, respectively. The reaction and detection buffer were 0.1% BSA-PBS. The measurement time was 300 s. The excluded fraction of the aggregate exclusion method is 5%.

Table S2. Recovery results of AFP homogeneous sandwich immunoassay by FSCCS.

Sample	Original founded amount (pM) [a]	Added amount (pM)	Founded amount (pM)	Recovery (%)	RSD (%)[b]
4	29	116	147	101.4	25.8
5	1	116	109	93.2	16.3

[a] Original founded amount is calculated by dividing the sample concentration obtained by ELISA by the dilution factor.
[b] RSD represents the ratio of the standard deviation to the mean of 3 time measurements.