Supporting Information

Objective Scanning-based Fluorescence Cross-Correlation Spectroscopy (Scan-FCCS) for Studying Fusion Dynamics of Protein Phase Separation

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Materials and Reagents.

The construct encoding mCherry, EGFP, mCherry-CRDBP, EGFP-CRDBP or mCherry-β-catenin cloned into pet28a vector with the C-terminal of His6 tag were from HITRO Bio Tech (Beijing, China). The Qdot™ 605 ITK™ Carboxyl Quantum Dots were purchased from Thermo Fisher Scientific (USA). The coverslips (24×40, 0.17 mm thickness) were provided by Thermo Fisher Scientific. All other chemicals were from Sigma-Aldrich (USA) at the highest available purity. All materials were used without further purification. All solutions were prepared with ultrapure water (18.2 MΩ/cm) purified by Millipore simplicity (Millipore, USA).

Purification of protein in solution.

The plasmid was transformed into *E. coli* by heat transformation. The strains successfully transformed were selected and inoculated into 2 mL liquid LB medium containing 50 mg/mL kanamycin, and cultured overnight at 37 °C. The overnight culture was inoculated into the liquid LB medium containing 50 mg/mL kanamycin at a ratio of 1:100 to expand the culture to OD600=0.6. IPTG with 0.5 nM final concentration was added and cultured at 16 °C for 12 h to induce protein expression. Centrifuged (12,000 rpm, 10 min) to collect the bacteria, and used 40 mL of ice-cold lysis buffer (20 mM Tris-HCl, 300 mM NaCl, 5 mM imidazole, 5 mM β-ME, 1 mM PMSF, 10% glycerol, pH 7.9) were resuspended. The suspension was ultrasonically treated on ice and centrifuged at 4 °C (12000 rpm, 30 min) to collect the supernatant. The supernatant was loaded into a Ni NTA resin column at 4 °C. And used 150 mL of washing buffer (300 mM NaCl, 20 mM Tris-HCl, 5 mM β- ME, 10% glycerin, 1 mM PMSF and 50 mM imidazole, pH 7.9) to wash Ni NTA resin column. Use 5 mL elution buffer (20 mM Tris-HCl, 300 mM NaCl, 5 mM β-ME, 10% glycerol, 1 mM PMSF, 200 mM imidazole, pH 7.9) to elute the protein from the chromatographic column. The eluted protein was analyzed by 15% SDS-PAGE and Coomassie brilliant blue staining. The BSA solution was used as the standard solution to draw the standard curve and determine the concentration of the purified protein. The protein buffer was changed into storage buffer (50 mM Tris-HCl, 500 mM KCl, 1 mM TCEP, pH 7.4) by ultrafiltration at 4 °C, and then sub-packed, frozen with liquid nitrogen, and stored at - 80 °C.

Setup of the Scan-FCS/Scan-FCCS system.

Scan-FCS/Scan-FCCS measurements are performed on a home-made FCCS system (shown as in Fig. S9). FCCS system is built on the basis of the inverted microscope (IX71, Olympus, Japan). The 488 nm continuous solid-state laser (Sapphire LP USB, Coherent, Germany) and 561 nm continuous solid-state laser (Cobolt Jive, 04-01, Sweden) are selected as the excitation light sources. After the laser beam is adjusted by the reflector, the spatial photobeam is conducted through the dichroic mirror. The beam is expanded through the beam expander. Then the beam is reflected by the dichroic mirror (485-565DBDR, OmegaOptical, USA) and enters the water immersion objective lens (UplanApo, 60 × NA 1.2, Olympus, Japan), the laser focused by the objective lens shines on the sample on the cover glass, and the focused detection volume is less than 1 fL. The fluorescence signals generated by laser excited samples are collected by the same objective lens, and then passed through the same 485-560DBDR dichroic mirror. The fluorescence signals generated by two laser excited fluorescence molecules are divided into two paths by dichroic mirror (560DCLP, OmegaOptical, USA), and the stray light and excitation light are removed by 530DF30 (Omega Optical, USA) and 625QM50 (Omega Optical, USA) band-pass filters respectively. Finally, the fluorescence generated by the sample is collected by the single photon counter (SPCM-AQR16, Perkin Elmer EG&G, Canada) of the respective channel after passing through a 50 μm diameter pinhole respectively. The collected signals are recorded by the digital correlation card (Flex02-12D/C, Correlator. com, USA), and the respective fluorescence fluctuation correlation digital signals of the two fluorescent molecules are obtained, and the respective autocorrelation curves of the fluorescent molecules and the cross-correlation curves between them are obtained through correlation calculation. In addition, the objective lens was mounted on a nanopositioner (moving distance: 100 μm (X)x100 μm (Y), P-733.2CD, PI, Germany). The reciprocating scanning and scanning rate in X direction was controlled by the digital piezoelectric controller (E-712, PI, Germany). During the scanning of objective, the sample solution was immobile by fixing the sample container to the sample stage of microscope.

Table S1 The R-Square of mCherry-CRDBP at different concentrations, using conventional FCS and Scan-FCS.

The mode of FCS	R-Square of mCherry-CRDBP at different concentrations							
	5 nM	10 nM	25 nM	50 nM	75 nM	100 nM	250 nM	500 nM
Conventional FCS	0.915	0.951	0.960	0.963	0.969	0.974	0.978	0.978
Scan-FCS	0.916	0.957	0.995	0.996	0.998	0.999	0.999	0.999

Table S2 The photon burst number of mCherry-CRDBP at different concentrations, using conventional FCS and Scan-FCS.

The mode of FCS	The photon burst number of mCherry-CRDBP at different concentrations							
	5 nM	10 nM	25 nM	50 nM	75 nM	100 nM	250 nM	500 nM
Conventional FCS	—	—	1.8 ± 0.4	3.0 ± 1.1	4.3 ± 0.9	4.5 ± 1.3	5.5 ± 0.5	8.5 ± 2.2
Scan-FCS	—	—	9.3 ± 0.9	18.9 ± 1.6	23.9 ± 1.4	32.3 ± 3.2	52.0 ± 2.2	86.9 ± 4.1

Table S3 The radii of mCherry-CRDBP at different concentrations, using negative-stain transmission electron microscopy, conventional FCS and Scan-FCS. (Unit: nm)

The mode of FCS	The radii (nm) of mCherry-CRDBP at different concentrations				
	25 nM	50 nM	100 nM	250 nM	500 nM
Conventional FCS	25.9 ± 8.9	61.8 ± 21.0	184.9 ± 76.8	262.8 ± 105.5	390.0 ± 82.9
Scan-FCS	21.6 ± 1.7	52.9 ± 7.1	139.5 ± 41.8	210.5 ± 27.4	301.1 ± 24.2
TEM	20.0 ± 0.2	48.6 ± 1.0	134.6 ± 2.2	197.6 ± 2.0	293.1 ± 4.6

Table S4 The characteristic diffusion time (τ_D) of the interaction between mCherry and EGFP or the interaction between mCherry-CRDBP fused with EGFP-CRDBP measured by Scan-FCCS at different concentrations of proteins in the buffer. (Unit: ms)

The interaction of the proteins	τ_D (ms) of the interaction at different protein concentrations							
	5 nM	10 nM	25 nM	50 nM	75 nM	100 nM	250 nM	500 nM
mCherry and EGFP	0.08	0.11	0.12	0.12	0.13	0.13	0.15	0.17
mCherry-CRDBP and EGFP-CRDBP	0.09	0.13	1.09	2.67	3.94	7.05	10.03	15.31

Table S5 The characteristic diffusion time (τ_D) of mCherry-CRDBP fused with EGFP-CRDBP measured by Scan-FCCS over time in different buffer conditions. (Unit: ms)

The Conditions	τ_D (ms) of mCherry-CRDBP fused with EGFP-CRDBP over time					
	10 min	20 min	30 min	40 min	50 min	60 min
25 nM protein, 100 mM NaCl	1.01	0.80	0.67	0.43	0.08	0.07
10 nM protein, 30% PEG	0.08	0.07	0.08	0.07	0.54	2.08

Table S6 The characteristic diffusion time (τ_D) of 500 nM fused proteins measured by Scan-FCCS over time in the buffer. (Unit: ms)

The Conditions	τ_D (ms) of fused proteins over time									
	1 min	10 min	20 min	30 min	40 min	50 min	60 min	70 min	80 min	90 min
EGFP-CRDBP fused with mCherry	0.45	0.48	0.48	0.47	0.48	0.48	0.49	0.48	0.49	0.48
EGFP-CRDBP fused with mCherry-CRDBP	0.72	2.22	3.22	4.79	7.17	9.81	12.95	15.13	15.71	15.67
EGFP-CRDBP fused with mCherry-β-catenin	0.27	0.87	1.52	2.56	3.49	6.68	10.41	15.91	15.86	15.99

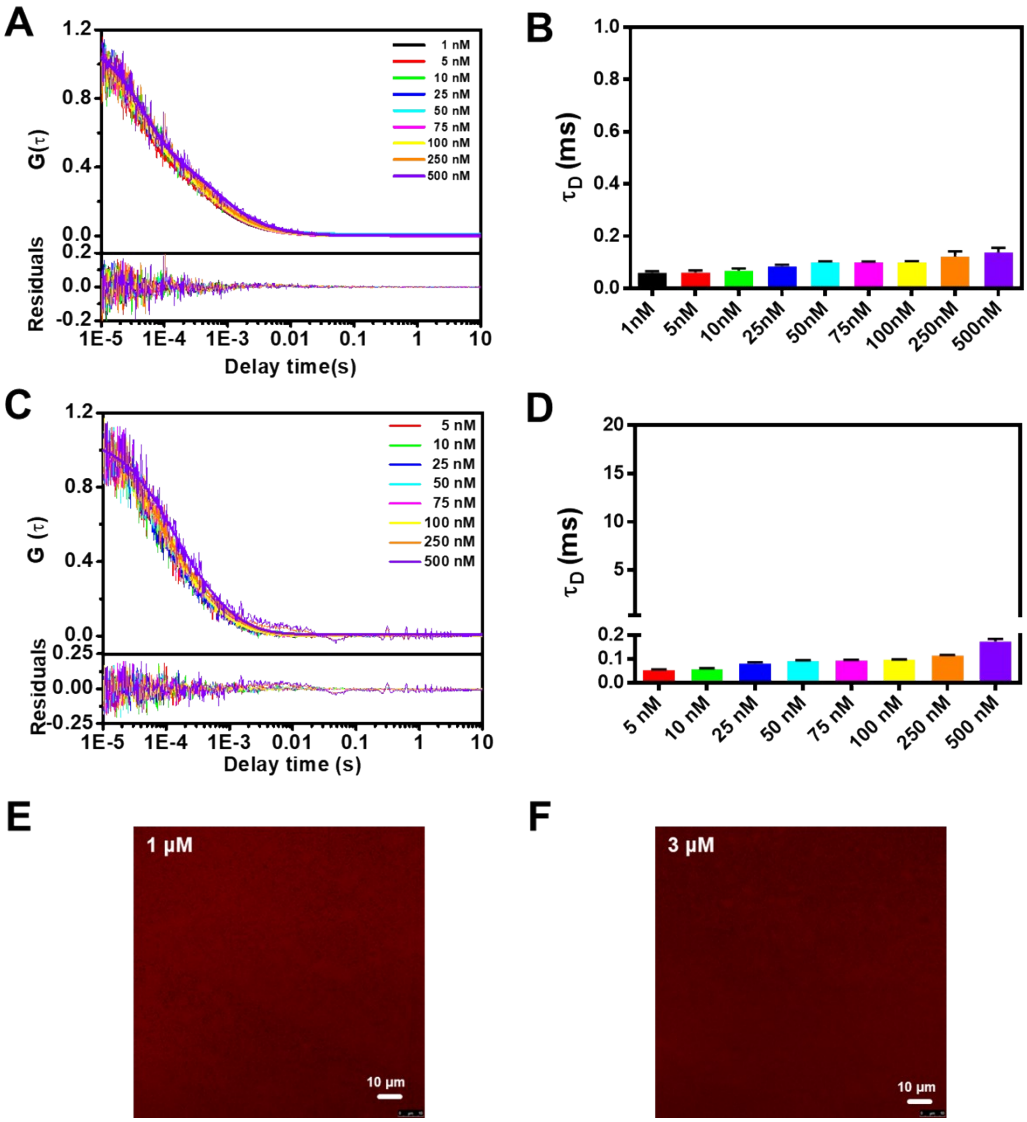

Fig. S1 Typical conventional FCS, Scan-FCS results and confocal fluorescence images of mCherry in solution. (A) (C) Typical normalized autocorrelation curves, fitting curves, and corresponding fitting residuals of mCherry in solution by using conventional FCS and Scan-FCS, respectively. (B)(D) Diffusion times of mCherry in solution by using conventional FCS and Scan-FCS, respectively. (E) (F) Confocal fluorescence images of mCherry at 1 μM and 3 μM concentrations, respectively. Scale bar, 10 μm. Numerical values in the graph indicate the mean value ± SEM. n ⩾ 5. The measurement time was 120 s.

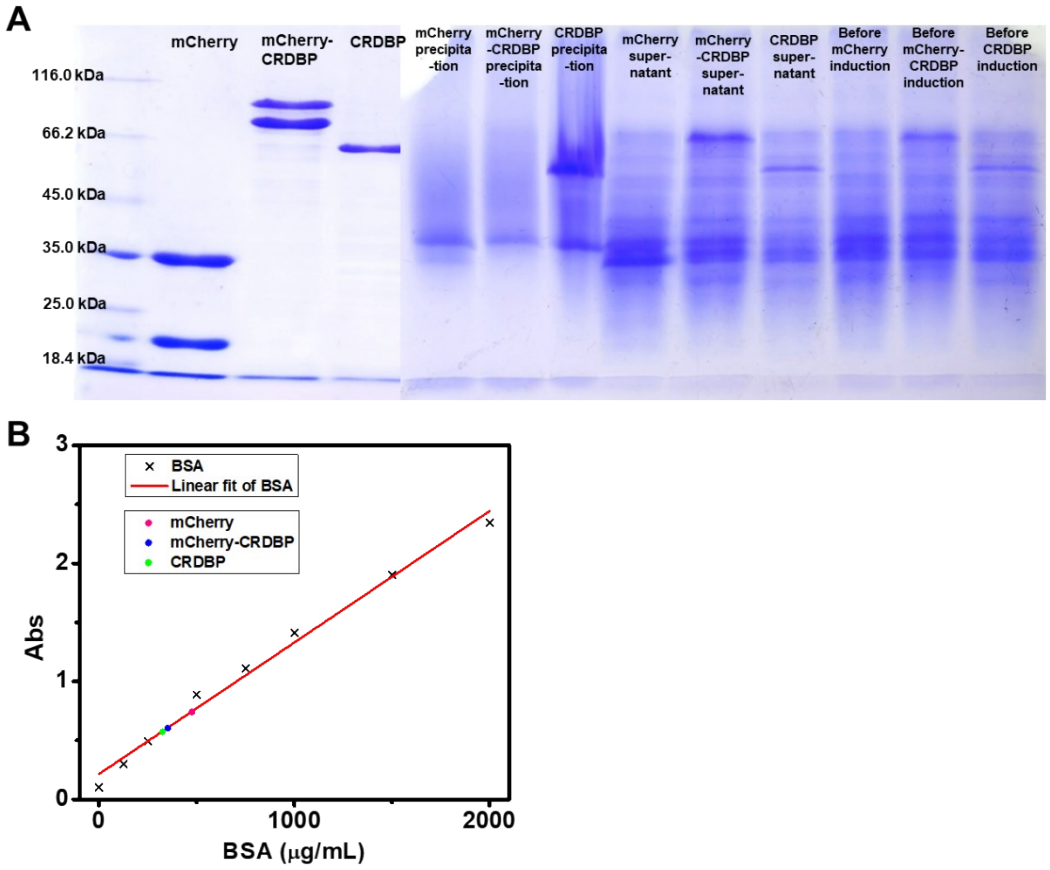

Fig. S2 Purification and concentration determination of mCherry, mCherry-CRDBP and CRDBP. (A) After mCherry, mCherry-CRDBP and CRDBP were purified in Escherichia coli system, the results were stained with Coomassie brilliant blue. (B) BCA method was used to determine the concentration of mCherry, mCherry-CRDBP and CRDBP.

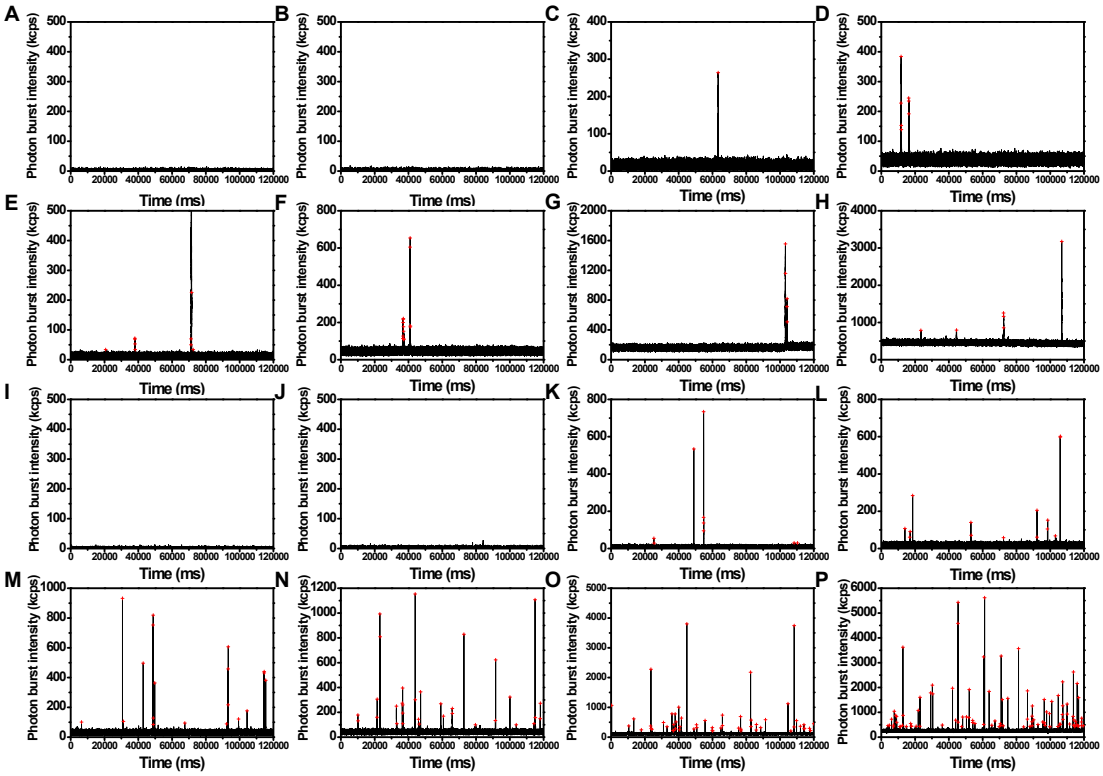

Fig. S3 The typical photon burst trace of mCherry-CRDBP in solution. (A-H) are typical photon burst trace of mCherry-CRDBP at 5 nM, 10 nM, 25 nM, 50 nM, 75 nM, 100 nM, 250 nM and 500 nM by conventional FCS, respectively. (I-P) are typical photon burst trace of mCherry-CRDBP at 5 nM, 10 nM, 25 nM, 50 nM, 75 nM, 100 nM, 250 nM and 500 nM by Scan-FCS, respectively.

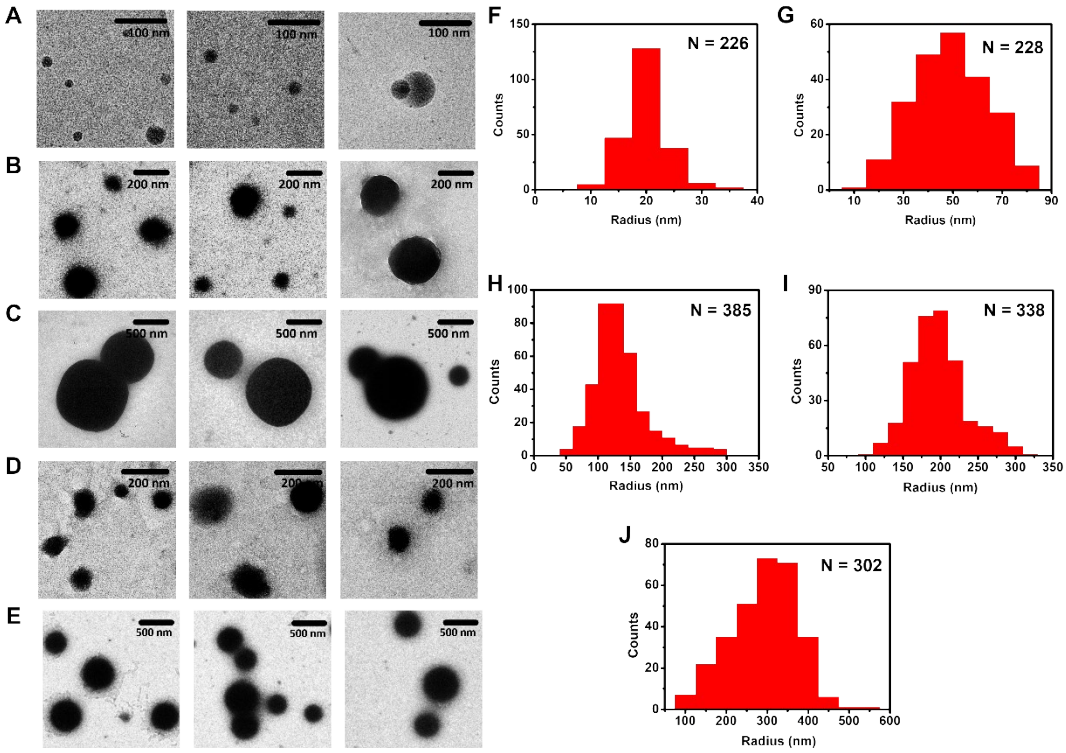

Fig. S4 mCherry-CRDBP condensates examined by negative-stain transmission electron microscopy. (A) (B) (C) (D) (E) are the TEM image of mCherry-CRDBP at 25 nM, 50 nM, 100 nM, 250 nM, 500 nM, respectively. Scale bars are listed. (F-J) are the size distributions of mCherry-CRDBP at 25 nM, 50 nM, 100 nM, 250 nM, 500 nM, respectively.

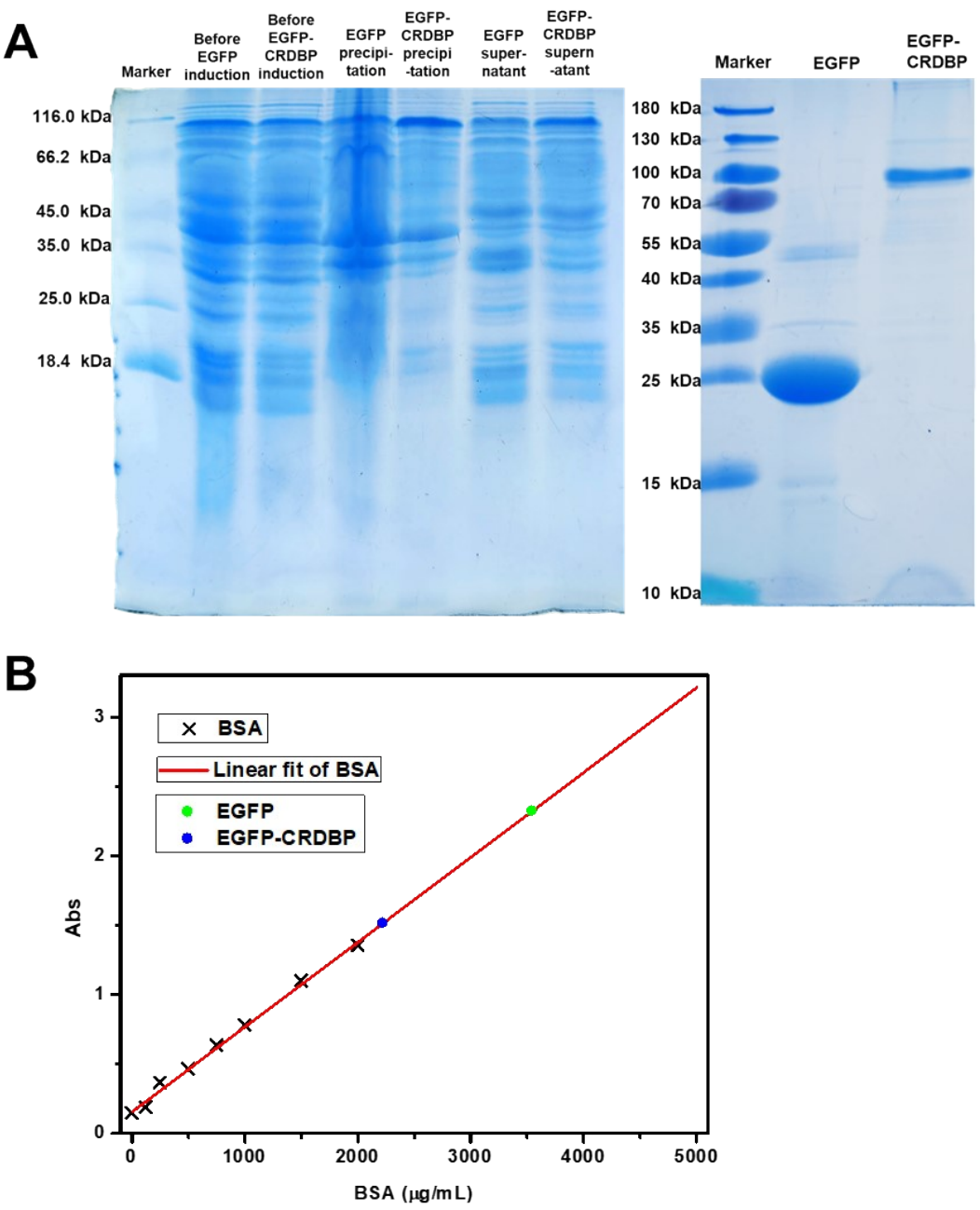

Fig. S5 Purification and concentration determination of EGFP and EGFP-CRDBP. (A) After EGFP and EGFP-CRDBP were purified in Escherichia coli system, the results were stained with Coomassie brilliant blue. (B) BCA method was used to determine the concentration of EGFP and EGFP-CRDBP.

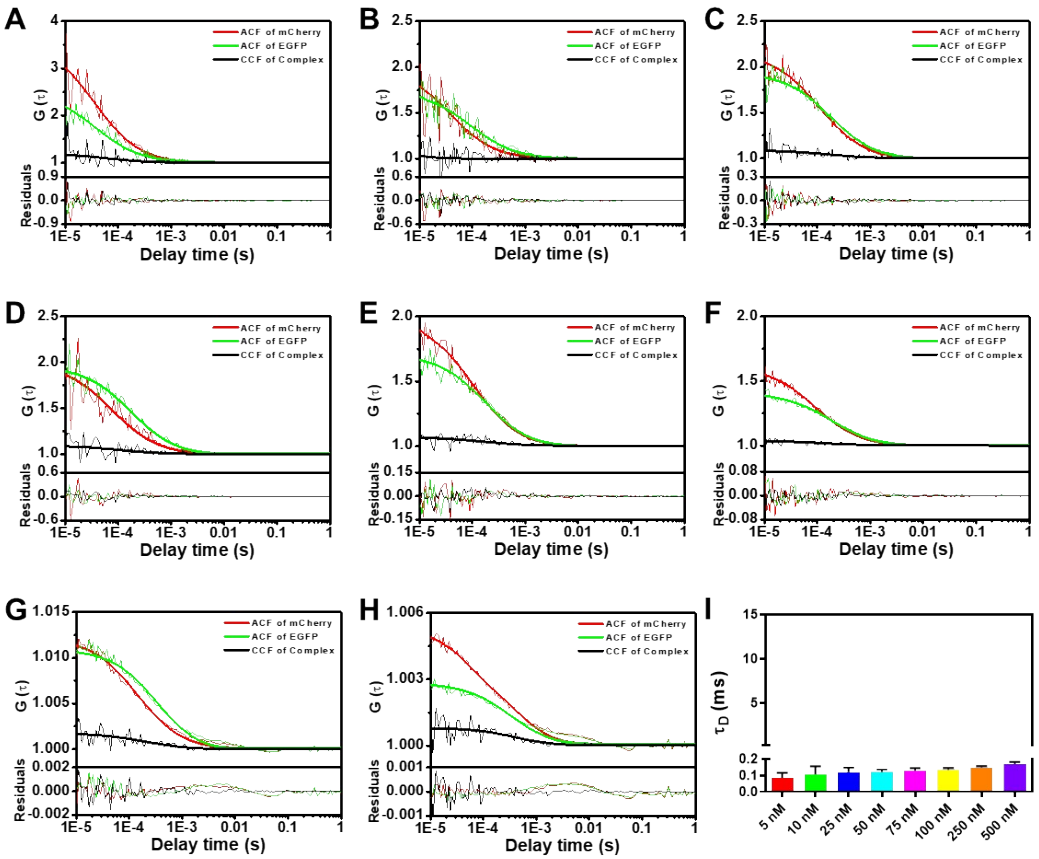

Fig. S6 The FCCS results of the interaction between mCherry and EGFP at various concentrations in solution by Scan-FCCS. (A-H) are typical cross-correlation curves, their fitting curves, and corresponding fitting residuals of the interaction between mCherry and EGFP at 5 nM, 10 nM, 25 nM, 50 nM, 75 nM, 100 nM, 250 nM and 500 nM, respectively. (I) The diffusion time of the interaction between mCherry and EGFP at different concentrations. Numerical values in the graph indicate the mean value ± SEM. n ⩾ 5. The measurement time was 120 s.

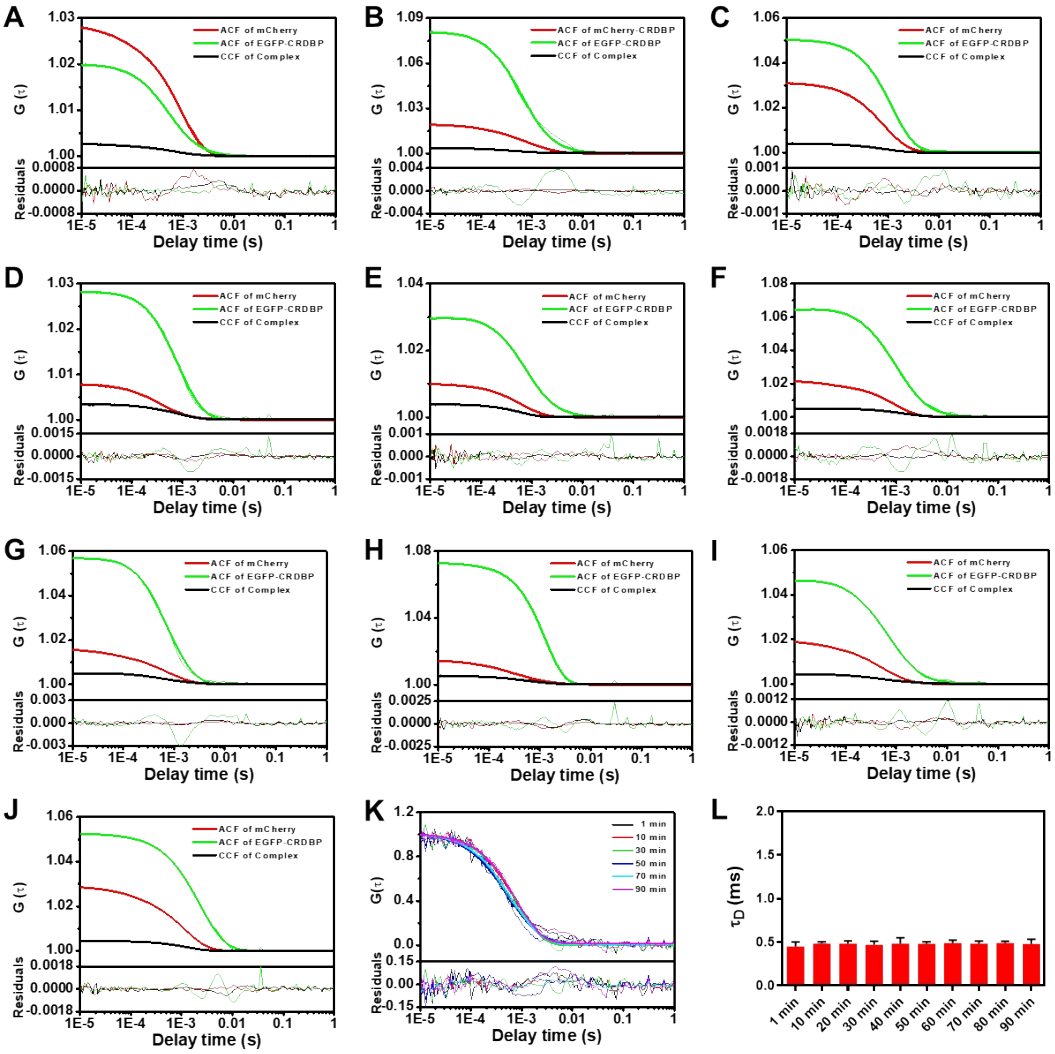

Fig. S7 The FCCS results of the recruitment behaviors of EGFP-CRDBBP to mCherry over time in solution by Scan-FCCS. (A-J) are the typical cross-correlation curves, their fitting curves, and corresponding fitting residuals of EGFP-CRDBP recruiting mCherryt in 500 nM at 1 min, 10 min, 20 min, 30 min, 40 min, 50 min, 60 min, 70 min, 80 min, and 90 min, respectively. (K) The typical normalized cross-correlation curve, their fitting curves, and corresponding fitting residuals of EGFP-CRDBP recruiting mCherry in 500 nM over time. (L) The diffusion time of EGFP-CRDBP recruiting mCherry in 500 nM over time. Numerical values in the graph indicate the mean value ± SEM. n ⩾ 5. The measurement time was 120 s.

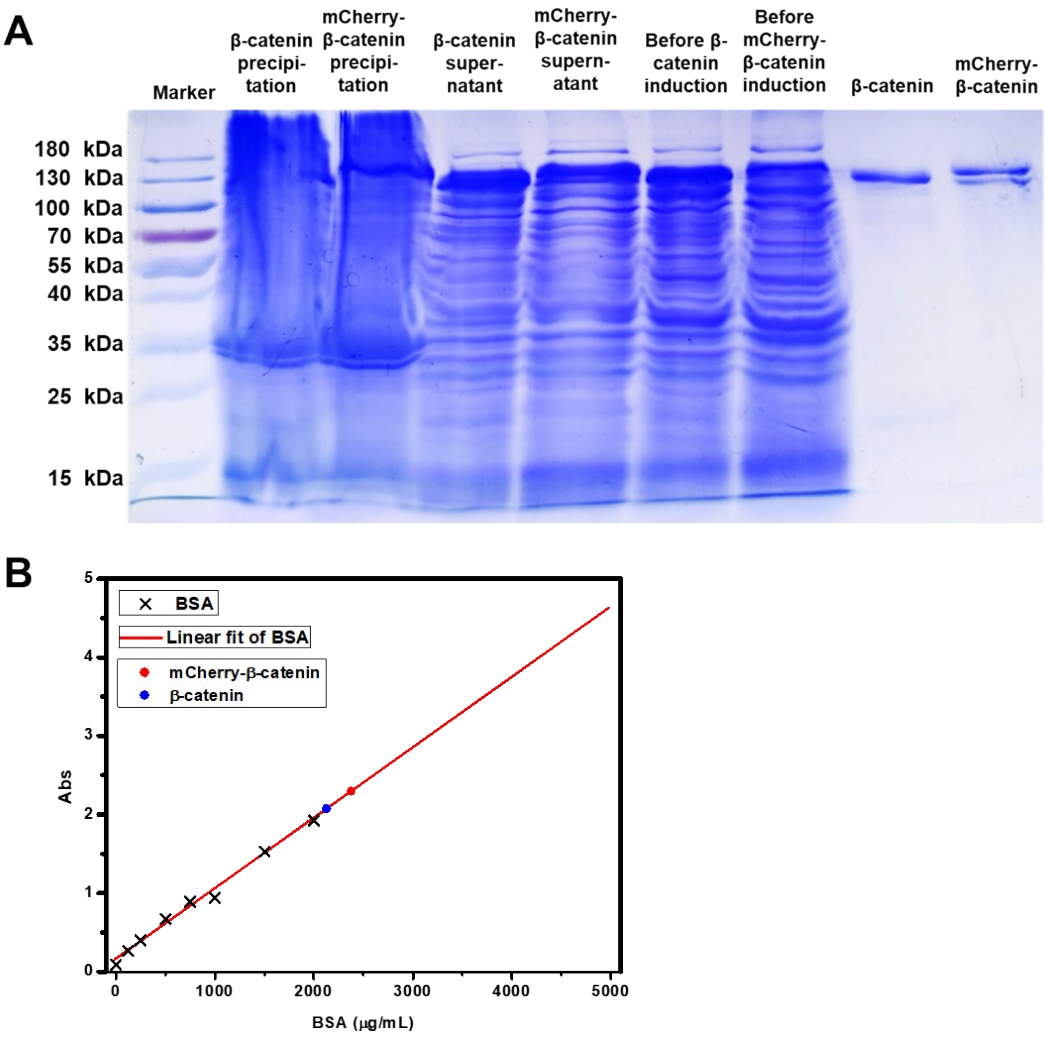

Fig. S8 Purification and concentration determination of β-catenin and mCherry-β-catenin. (A) After β-catenin and mCherry-β-catenin were purified in Escherichia coli system, the results were stained with Coomassie brilliant blue. (B) BCA method was used to determine the concentration of β-catenin and mCherry-β-catenin.

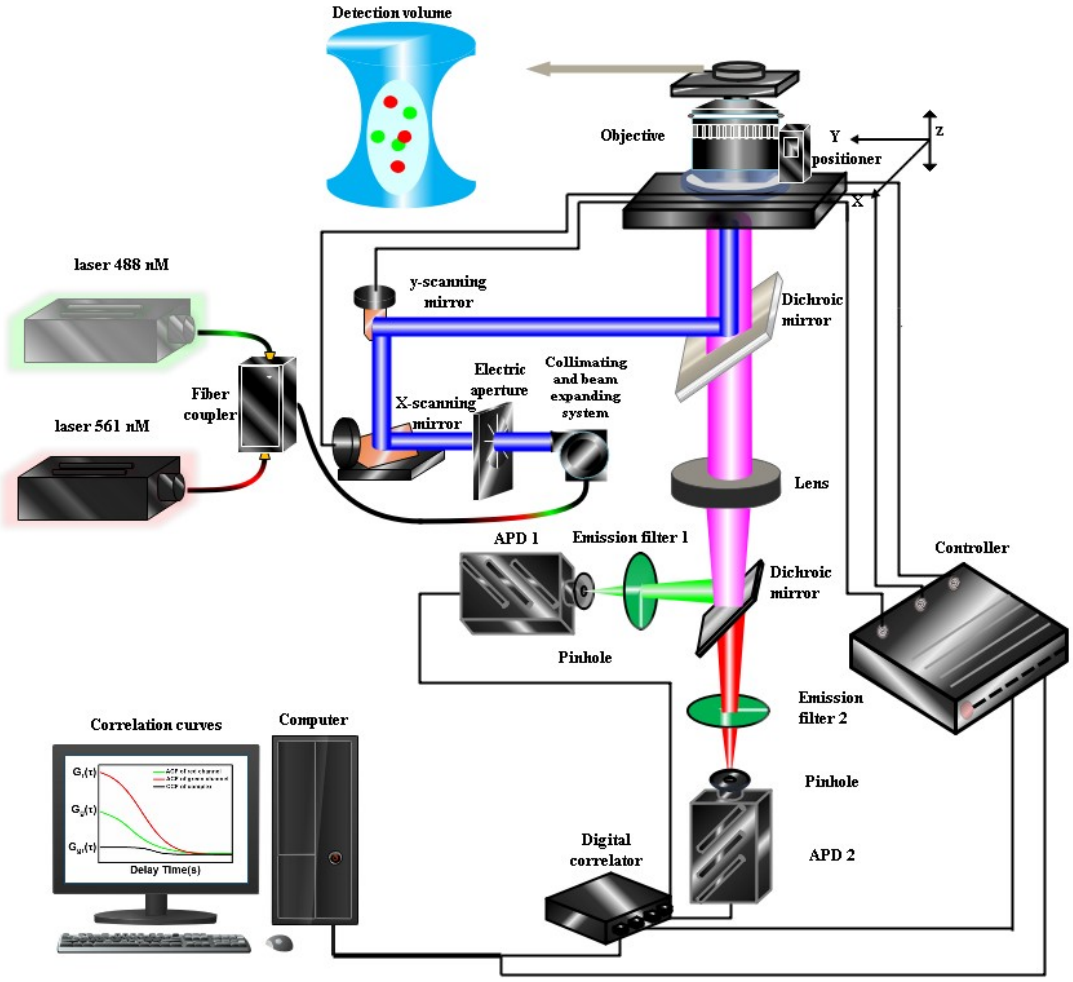

Fig. S9 The setup of the San-FCS/San-FCCS system.

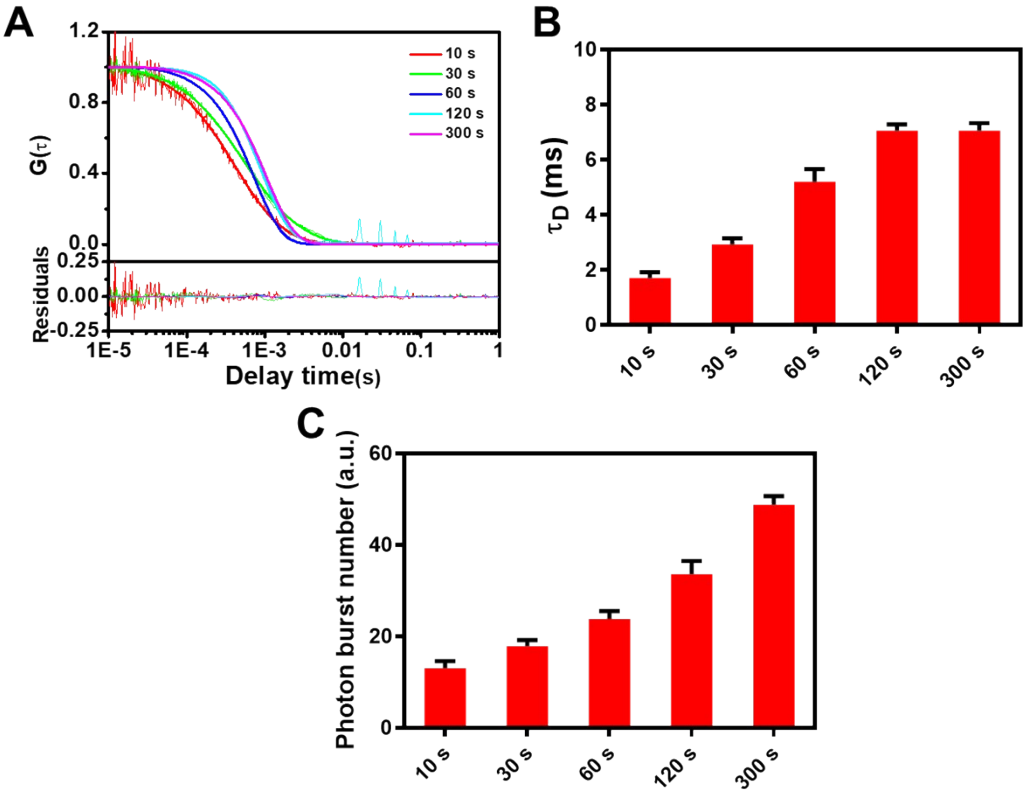

Fig. S10 Optimization of detection time. (A) Typical normalized autocorrelation curves, fitting curves, and corresponding fitting residuals using by Scan-FCS for mCherry-CRDBP at detection times of 10 s, 30 s, 60 s, 120 s, and 300 s, respectively. (B) The diffusion time of mCherry-CRDBP at detection times of 10 s, 30 s, 60 s, 120 s, and 300 s, respectively. (C) The photon burst number of mCherry-CRDBP at detection times of 10 s, 30 s, 60 s, 120 s, and 300 s, respectively. As the detection time prolonged, the diffusion time of mCherry CRDBP gradually increased and stabilized at 120 s.

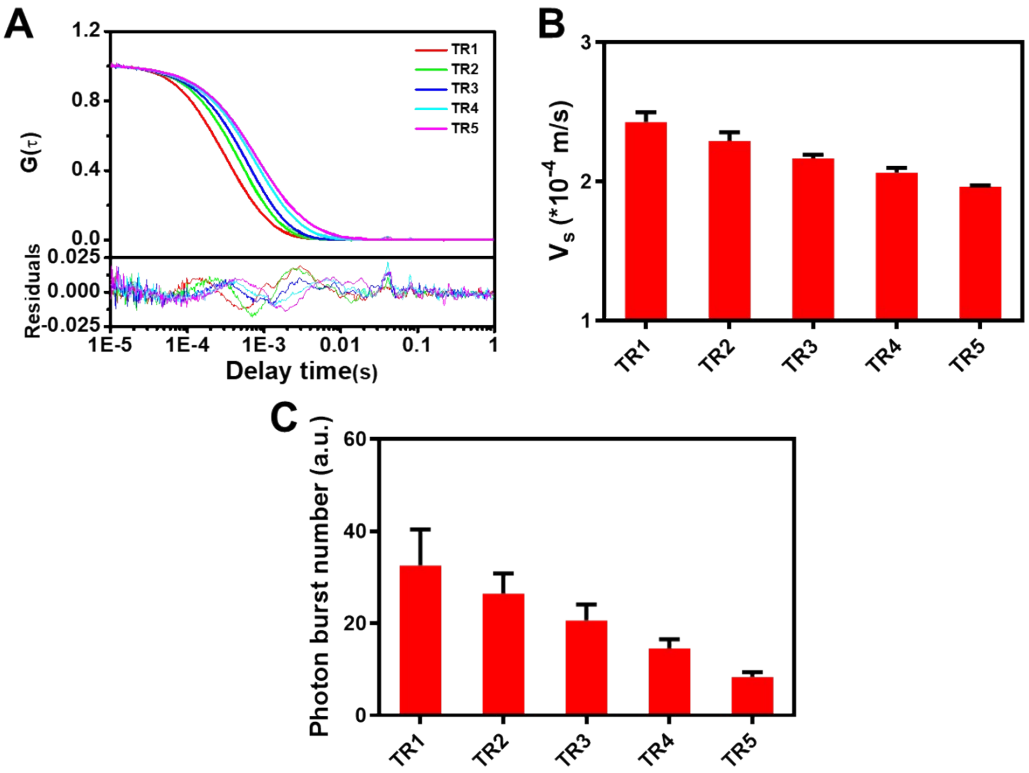

Fig. S11 Optimization of different scanning speeds. (A) Typical normalized autocorrelation curves, fitting curves, and corresponding fitting residuals using by Scan-FCS for Qdot™ 605 ITK™ Carboxyl Quantum Dots at scanning speeds of TR1, TR2, TR3, TR4, and TR5, respectively. (B) The v_s of objective lens at scanning speeds of TR1, TR2, TR3, TR4, and TR5, respectively. (C) The photon burst number of mCherry-CRDBP at scanning speeds of TR1, TR2, TR3, TR4, and TR5, respectively. The detection time is 120 s. TR1 is the maximum scanning speed of the instrument, and we use TR1 as the fixed scanning speed for the experiment.

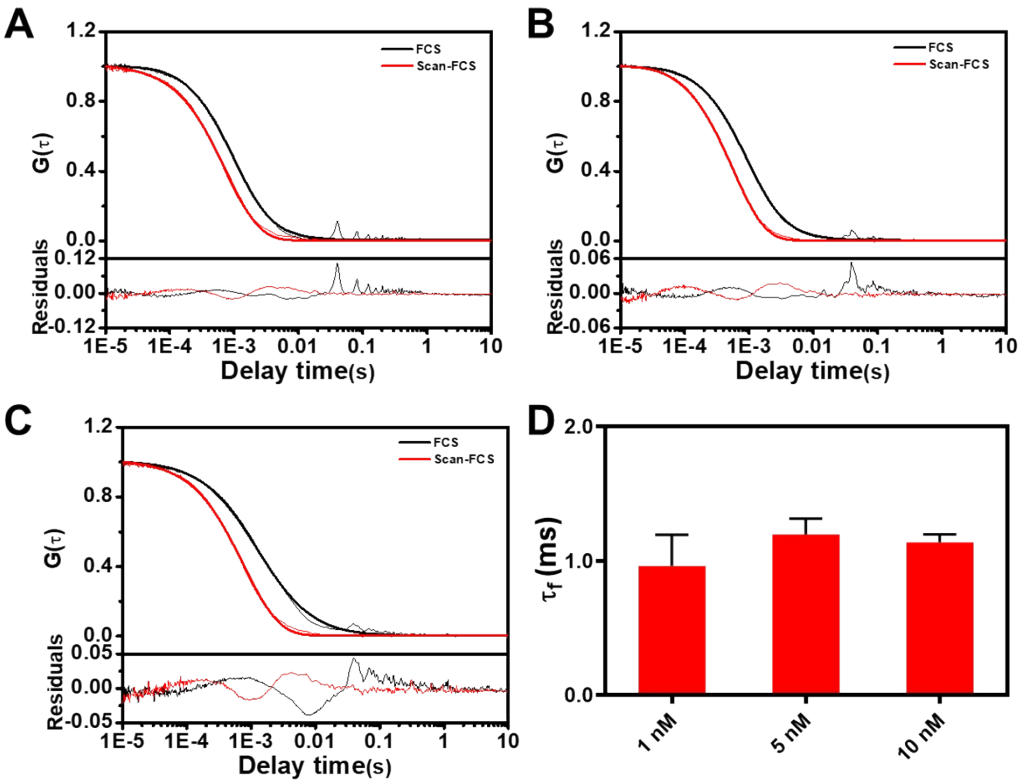

Fig. S12 The FCS results of Qdot™ 605 ITK™ Carboxyl Quantum Dots in solution by conventional FCS and Scan-FCS. (A-C) are the typical normalized auto-correlation curves, their fitting curves and corresponding fitting residuals of Qdot™ 605 ITK™ Carboxyl Quantum Dots in solution by conventional FCS and Scan-FCS at 1 nM, 5 nM and 10 nM, respectively. (D) the time of Qdot™ 605 ITK™ Carboxyl Quantum Dots passing through the radial radii of the detection micro area relative to the action of objective lens at 1 nM, 5 nM and 10 nM. Total average τ_f is 1.16 ms. Numerical values in the graph indicate the mean value ± SEM. $n \geq 5$. The measurement time was 120 s.