Supporting Information

Study on drug-mediated protein-protein interaction in single living cells by fluorescence cross-correlation spectroscopy

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Materials and reagents

All plasmids were constructed by Beijing Hitrobio Biotechnology Co., Ltd (China). Rhodamine green and rhodamineB were from Sigma-Aldrich (USA). Alexa647 was bought from Thermo Fisher (USA).The human embryonic kidney (HEK293T) cell lines. Fetal bovine serum was from Corning (USA). Everolimus, deforolimus, rapamycin and temsirolimus were purchased from Shanghai Titan Scientific Co., Ltd (China). Cell culture and a variety of culture additives, protease inhibitor, lipofectamine3000 and trypsin were acquired from Thermo Fisher Scientific (USA). Glolysis buffer was applied by Promega Biotech Co., Ltd (China). EGFP antibody, mCherry antibody, FRB antibody, FKBP12 antibody and goat anti-rabbit IgG H&L (HRP) was bought from Abcam (UK). The β-actin antibody was gained from Santa Cruz Biotechnology (USA). Ultrapure water was from Millipore Simplicity Systems (USA). All other chemicals were from Sigma-Aldrich (UAS). Before using without further purification. Cell culture dish wish cover glass bottom was bought from MatTek (USA).

Cell culture

Human embryonic kidney cells (HEK293T cells) were from our laboratory in this study. Cells were cultured in growth media which was made of Dulbecco's modification of Eagle's medium (DMEM). The medium was mainly composed of 1% penicillin/streptomycin and 10% fetal bovine serum. During the cell culture period, the cells were cultured in a constant temperature incubator at 37 ℃ and 5% CO_2.

When the cells were in a good state of growth, passage was carried out or subsequent experiments needed.

Plasmid stable transfection

In this study, HEK293T cells were used as host cells, and cell lines stably expressed FRB-EGFP, EGFP, FKBP12-mCherry and mCherry were constructed. The above stably transfected cell lines were constructed in a similar way. And the general process included lentivirus packaging and collection, target cell infection, and screening of stably expressed cell lines. First, added with 1 μg lentiviral plasmid vector containing target gene and 3 μg lentiviral packaging plasmid (psPAX2, 2.25 μg; pMD2.G, 0.75μg) and added with 10 μL transfection reagent lipofectamine3000, incubate for 6 h, then polybrene (final concentration was 8 μg /mL) solution was added. Next, prepared using a complete medium containing screening resistance (1 μg/mL puromycin), and cultured in a cell incubator at 37 ℃ and 5% CO_2.

Western blotting

Living HEK293T cells were lysed by using Glo Lysis Buffer with phosphatase inhibitors. And then cell lysates were separated by sodium dodecyl sulfate-polyacrla-mide gel electrophoresis (SDS-PAGE). Next, transferred to polyvinylidene difluoride membranes (PVDF) and blocking buffer for 1 h, primary and secondary antibodies for 12 h and 1h, respectively. The PVDF membrane was soaked with ECL luminescent solution and imitated using an ultra-sensitive multi-function Imager (Amersham

Imager 600). Proteins were visualized using the Odyssey infrared imaging system.

Cell imaging

In this study, cell imaging was carried out by different microscopes in the laboratory. The inverted microscope (model number IX71) was used to perform fluorescence microscopy imaging on HEK293T cells with stable expression of fluorescent protein EGFP, target protein FRB-EGFP, fluorescent protein mCherry, and target protein FKBP12-mCherry, respectively. Confocal cell imaging of HEK293T cells with stable expression of the target proteins FRB-EGFP and FKBP12-mCherry was performed by laser scanning confocal fluorescence microscopy using a commercially available confocal laser microscope.

Experimental method of FCCS in living cells

The cells used in this study, were HEK293T cells and HEK293T cells containing stable transfected target proteins. Before each FCCS experiment, the detection volume of the 488 nm and 561 nm should be calibrated using the standard fluorescent dyes rhodamineB and rhodamine green commonly used in our laboratory, respectively. We often adjusted the hole to make the instrument laser reach the laser intensity required by the experiment. For each live cell FCCS study, we will examine at least two or three scan sites at the appropriate location for each cell, where each scan site will be repeated four times, and each cell detection time will be maintained for 10 s.

FCCS system

The FCCS system used in this study was successfully built by our laboratory. This FCCS setup is shown in Fig. S1 [1-4]. The 488 nm and the 561 nm lasers were used as the laser sources. The two coupled lasers were focused on the sample, thereby stimulating the sample to produce fluorescence. The fluorescence of the sample was then collected again by the objective lens immersed in ultra-pure water, and continues through the above dichroic mirror, and then through a dichroic mirror (540DRLP) into two fluorescence channels. The emitted light of the two fluorescent channels was passed through two different emission filters (530DF30 and 590DF35) to eliminate the interference of stray light and excitation light. Next, two fluorescence pass through the 60 μm pinhole and were collected through two avalanche photo diodes (APD, model SPCM-AQR-14). Finally, the two collected fluorescence intensity, fluorescence trajectory and other information were recorded by the digital correlation card (model Flex03lq12). Finally, two fluorescence autocorrelation functions and the cross-correlation function of the two fluorescence channels are obtained. Each scanning point was coordinated by a three-dimensional piezoelectric ceramic nano-positioning system (model P-733.2CL/P-721.CLQ), a controller (model E-710.3CD). At the same time, the position of each scan spot detected was combined with the fluorescence intensity of each scan spot, and the scanning laser confocal fluorescence microscopy image of two living cells could be obtained through mat-lab software. Prior to each FACS study, the FCCS system was calibrated with a 10 nm standard fluorescent dye rhodamineB (with a diffusion coefficient of 3.6 x 10^{-6} cm^2/s in water)

and rhodamine green (with a diffusion coefficient of 2.8 x 10^{-6} cm^2/s in water).

Study of brightness per particle (BPP)

The BPP (brightness per particle) value represents the average brightness of a single particle, which is calculated by the following formula [1-4].

$$BPP = \frac{< I >}{< N >} = < I > (G(0) - 1) \quad (7)$$

In equation (7), the <I> represents the average fluorescence signal intensity of moving particles in the detection volume of the confocal microscope, and the <N> represents the average fluorescence particle number of moving particles in the detection volume of the confocal microscope. (G (0)-1) is the intercept of the ordinate, indicating the degree of self-relevance.

Here, the drug mediated single particle brightness of two proteins would be studied from two aspects of drugs with different concentrations at the same time and drugs with the same concentration at different times. And the BPP values will be used to quantitatively evaluate whether drug quenching effect on the two proteins. Incubation time of the four drugs was 2 h, and the concentration gradient of everolimus was 0, 0.16 nM, 1.6 nM, 16 nM, 160 nM, 1600 nM. The concentration gradient of temsirolimus was 0, 0.22 nM, 2.2 nM, 22 nM, 220 nM and 2200 nM. The concentration gradient of deforolimus was 0, 0.25 nM, 2.5 nM, 25 nM, 250 nM, 2500 nM. The concentration gradient of rapamycin was 0, 0.26 nM, 2.6 nM, 26 nM, 260 nM, 2600 nM, respectively. Incubation time gradients of the four drugs were 0, 0.5 h, 1 h, 2 h, 4 h, 6 h, 8 h, 10 h. The incubation concentration was 16 nM, 22 nM, 25 nM,

26 nM in order. The G(0) values and fluorescence intensity (I**)** values were obtained by FCS. And the BPP values of single particle brightness could be obtained by equation (7), so as to evaluate whether the drug had fluorescence quenching effect on the two proteins.

Primer sequence

Primer sequence of FRB-EGFP

CMV-F CGCAAATGGGCGGTAGGTG

PCDH-R CTCTAGGCACCCGTTCAATT

Primer sequence of EGFP

CMV-FCGCAAATGGGCGGTAGGCGTG

PCDH-R CTCTAGGCACCCGTTCAATT

Primer sequence of FKBP12-mCherry

CMV-F CGCAAATGGGCGGTAGGCGTG

PGK-R GGAGGAGTAGAAGGTGGCG

Primer sequence of mCherry

CMV-F CGCAAATGGGCGGTAGGCGTG

PGK-R GGAGGAGTAGAAGGTGGCG

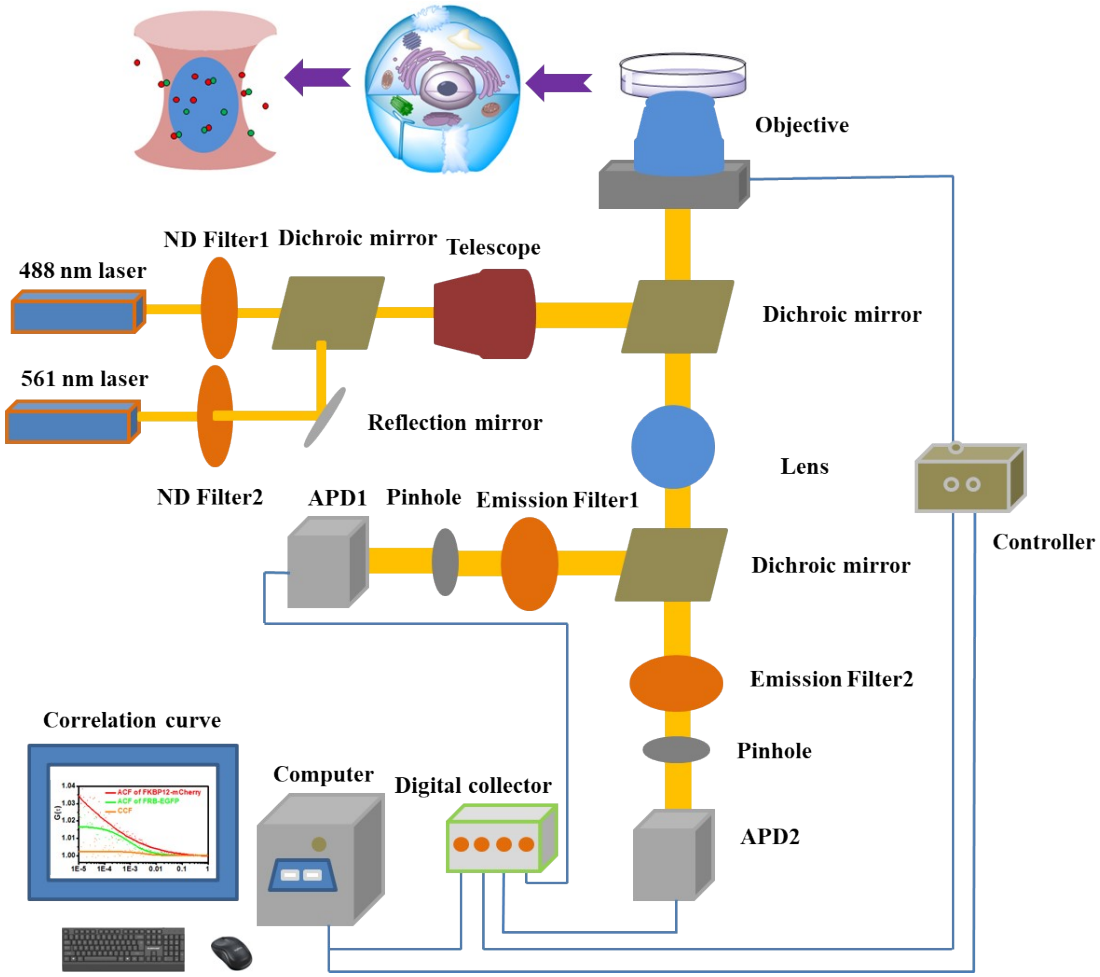

Fig. S1. Schematics diagram of FCCS system.

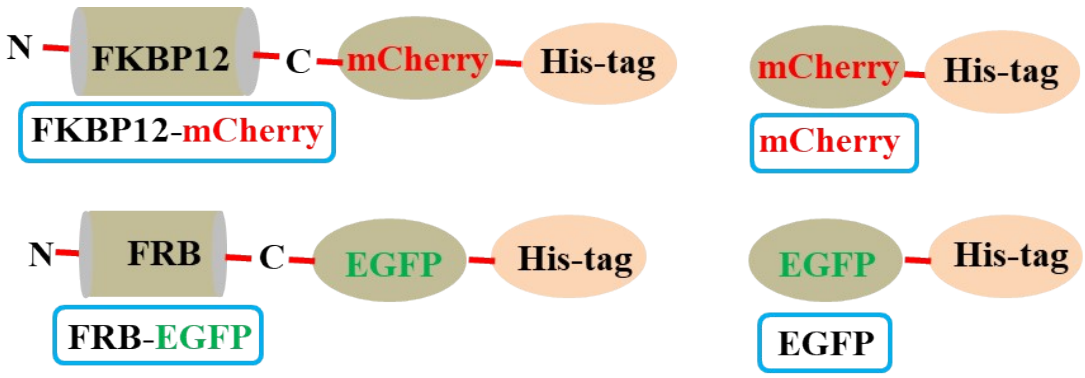

Fig. S2. The structure domains of FKBP12-mCherry and FRB-EGFP [5].

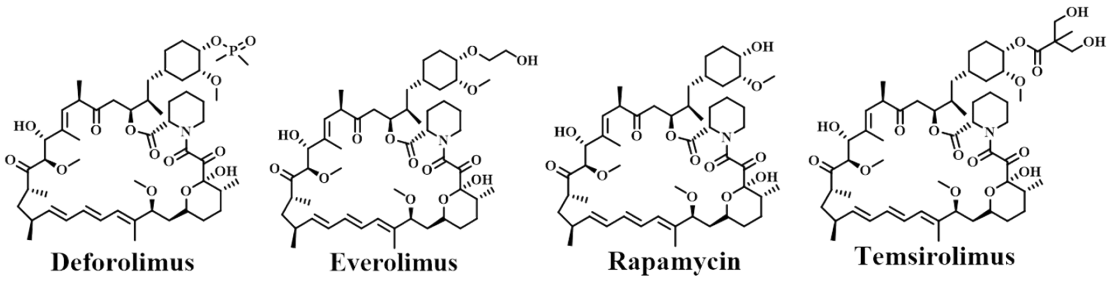

Fig. S3. The structural formulas of four drugs.

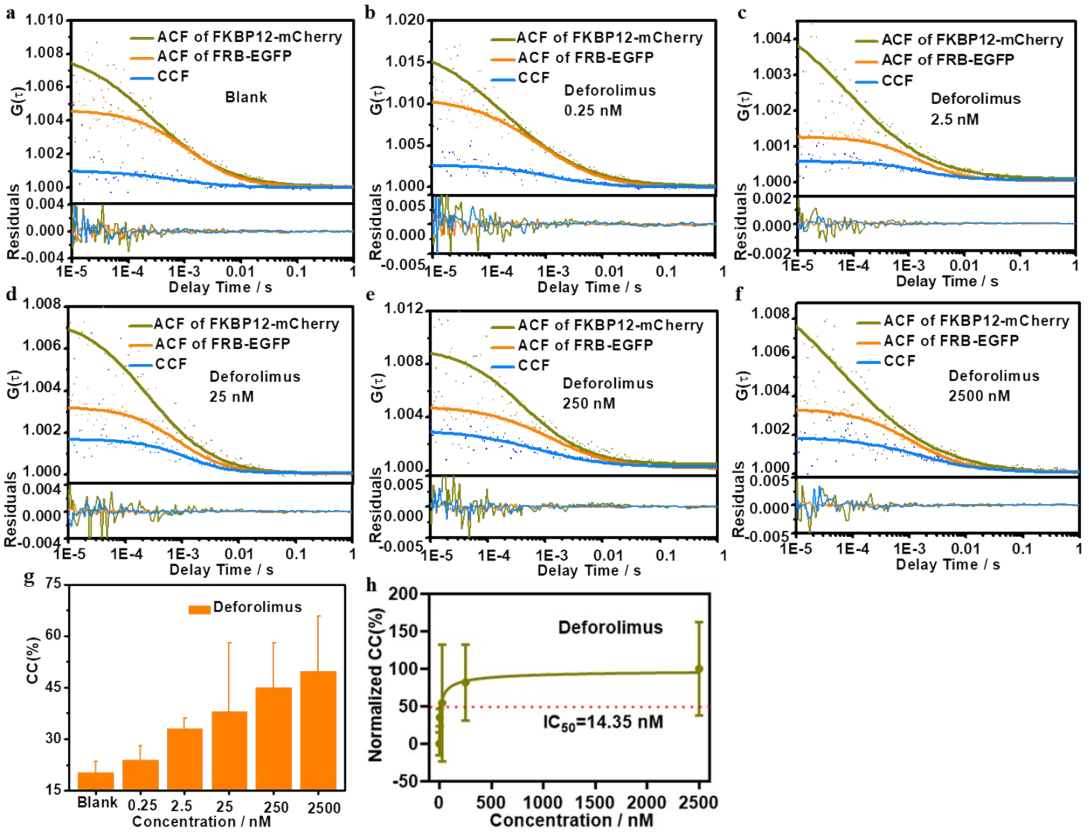

Fig. S4. Effects of deforolimus concentrations on the interaction of target proteins in HEK293T cells. The concentration of deforolimus is (a) 0 nM, (b) 0.25 nM, (c) 2.5 nM, (d) 25 nM, (e) 250 nM, (f) 2500 nM, respectively. The autocorrelation curves and fitting residuals are dark yellow lines (show 561 channel) and orange lines (show 488 channel). The cross-correlation curves and fitting residuals are blue lines. (g) CC% values in the presence of different deforolimus concentration in cells. The 488 channel laser intensity is 17 μw and the 561 channel laser intensity is 25 μw. The error bars represent the standard deviation of four times repeated measurements. (h) Determination of IC_{50} of the semi-inhibitory concentration of deforolimus.

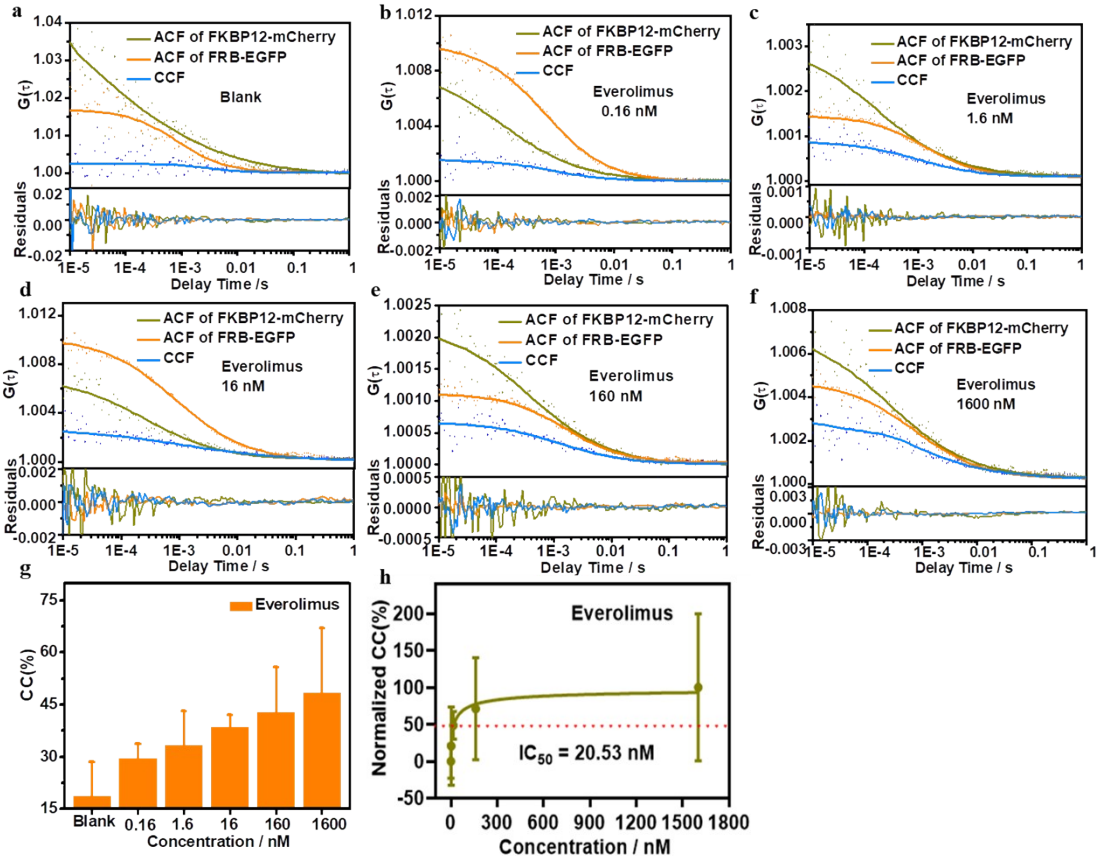

Fig. S5. Effects of everolimus concentrations on the interaction of target proteins in HEK293T cells. The concentration of everolimus is (a) 0 nM, (b) 0.16 nM, (c) 1.6 nM, (d) 16 nM, (e) 160 nM, (f) 1600 nM, respectively. The autocorrelation curves and fitting residuals are dark yellow lines (show 561 channel) and orange lines (show 488 channel). The cross-correlation curves and fitting residuals are blue lines. (g) CC% values in the presence of different everolimus concentration in cells. The 488 channel laser intensity is 23 μw and the 561 channel laser intensity is 35 μw. The error bars represent the standard deviation of four times repeated measurements. (h) Determination of IC_{50} of the semi-inhibitory concentration of everolimus.

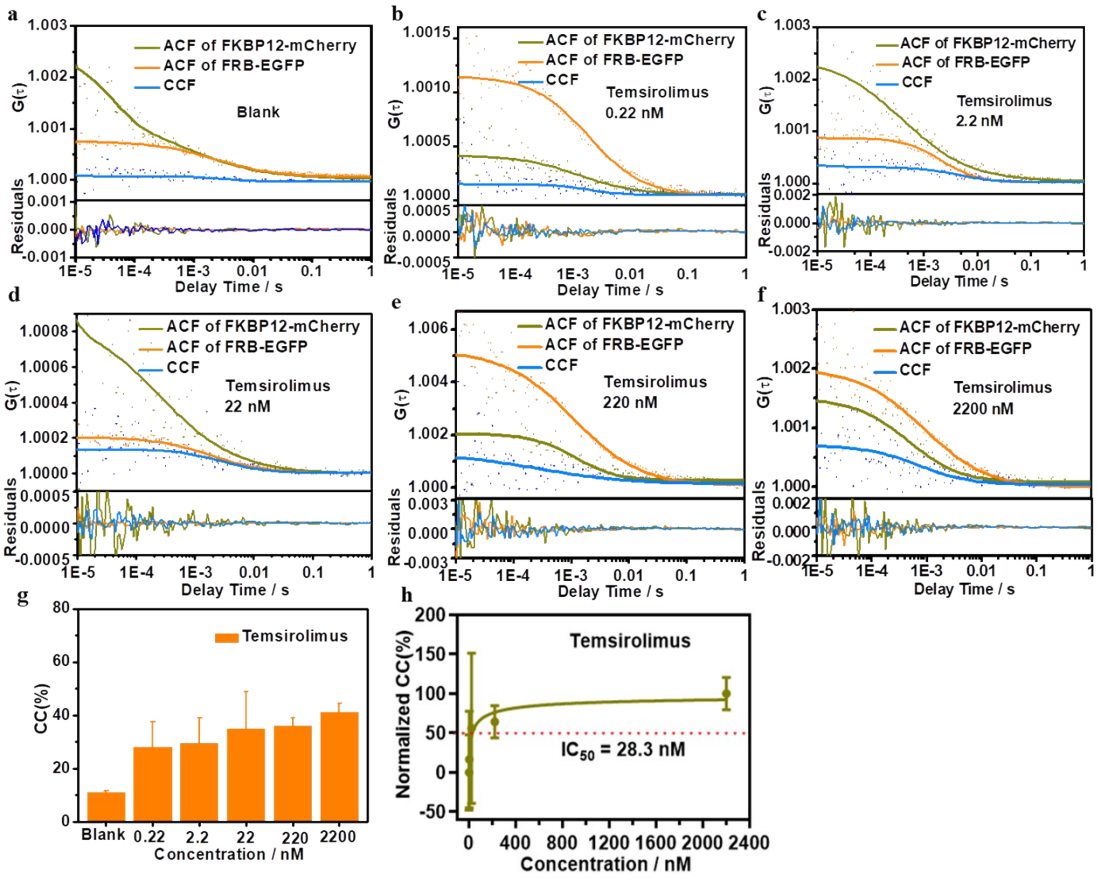

Fig. S6. Effects of temsirolimus concentrations on the interaction of target proteins in HEK293T cells. The concentration of temsirolimus is (a) 0 nM, (b) 0.22 nM, (c) 2.2 nM, (d) 22 nM, (e) 220 nM, (f) 2200 nM, respectively. The autocorrelation curves and fitting residuals are dark yellow lines (show 561 channel) and orange lines (show 488 channel). The cross-correlation curves and fitting residuals are blue lines. (g) CC% values in the presence of different temsirolimus concentration in cells. The 488 channel laser intensity is 8 μw and the 561 channel laser intensity is 23 μw. The error bars represent the standard deviation of four times repeated measurements. (h) Determination of IC_{50} of the semi-inhibitory concentration of temsirolimus.

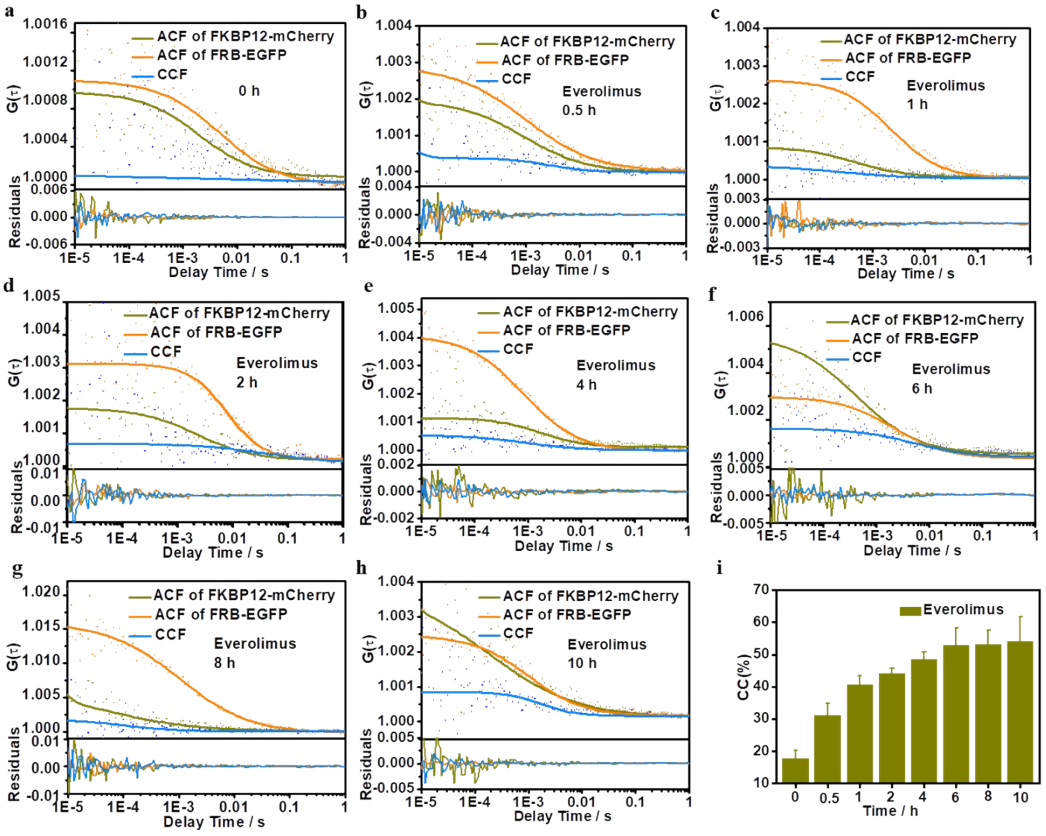

Fig. S7. The effects of incubation time of everolimus on interaction of target proteins in HEK293T cells. The incubation time of everolimus is (a) 0 h, (b) 0.5 h, (c) 1 h, (d) 2 h, (e) 4 h, (f) 6 h, (g) 8 h, (h) 10 h, respectively. The autocorrelation curves and fitting residuals are dark yellow lines (show 561 channel) and orange lines (show 488 channel). The cross-correlation curves and fitting residuals are blue lines. (i) CC% values in different incubation time of everolimus in cells. The error bars represent the standard deviation of four times repeated measurements.

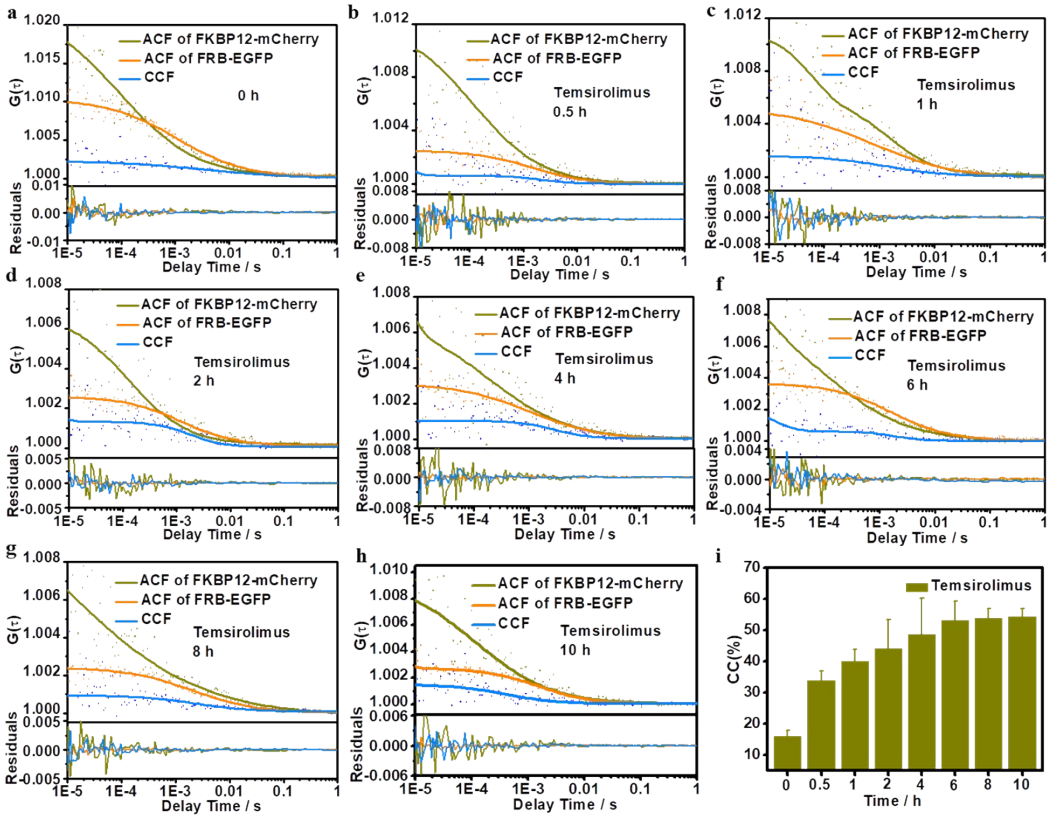

Fig. S8. The effects of incubation time of temsirolimus on interaction of target proteins in HEK293T cells. The interaction of different incubation times of temsirolimus with target proteins in HEK293T cells by FCCS measurement. The incubation time of temsirolimus is (a) 0 h, (b) 0.5 h, (c) 1 h, (d) 2 h, (e) 4 h, (f) 6 h, (g) 8 h, (h) 10 h, respectively. The autocorrelation curves and fitting residuals are dark yellow lines (show 561 channel) and orange lines (show 488 channel). The cross-correlation curves and fitting residuals are blue lines. (i) CC% values in different incubation time of temsirolimus in cells. The error bars represent the standard deviation of four times repeated measurements.

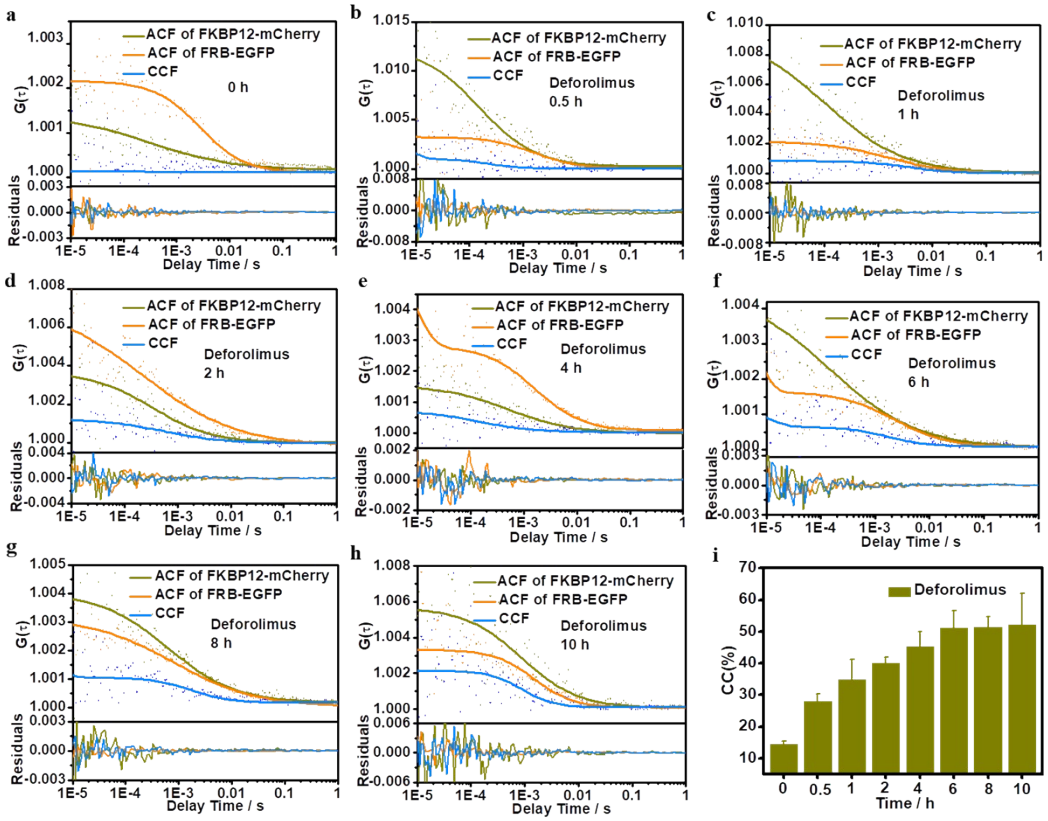

Fig. S9. The effects of incubation time of deforolimus on interaction of target proteins in HEK293T cells. The interaction of different incubation times of deforolimus with target proteins in HEK293T cells by FCCS measurement. The incubation time of deforolimus is (a) 0 h, (b) 0.5 h, (c) 1 h, (d) 2 h, (e) 4 h, (f) 6 h, (g) 8 h, (h) 10 h, respectively. The autocorrelation curves and fitting residuals are dark yellow lines (show 561 channel) and orange lines (show 488 channel). The cross-correlation curves and fitting residuals are blue lines. (i) CC% values in different incubation time of deforolimus in cells. The error bars represent the standard deviation of four times repeated measurements.

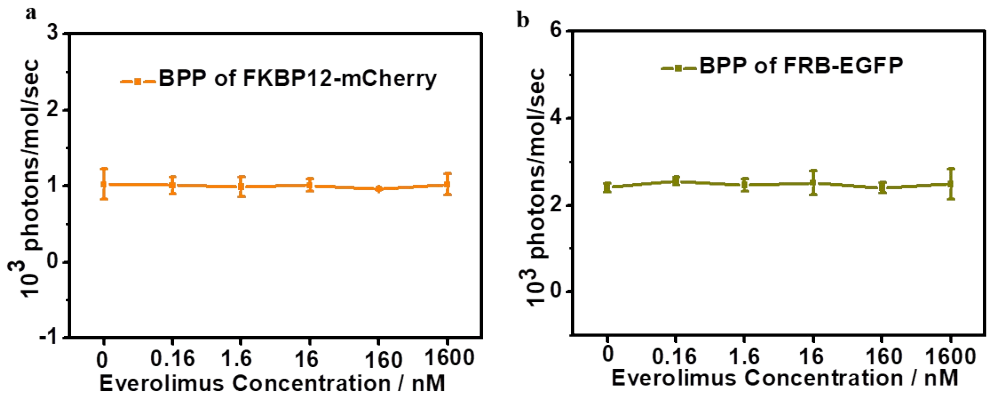

Fig. S10. (a-b) The brightness per particle (BPP) value in different incubation concentration of everolimus in cells. The right side 488 channel laser intensity is 23 μw. The left side 561 channel laser intensity is 35 μw. The error bars represent the standard deviation of four times repeated measurements.

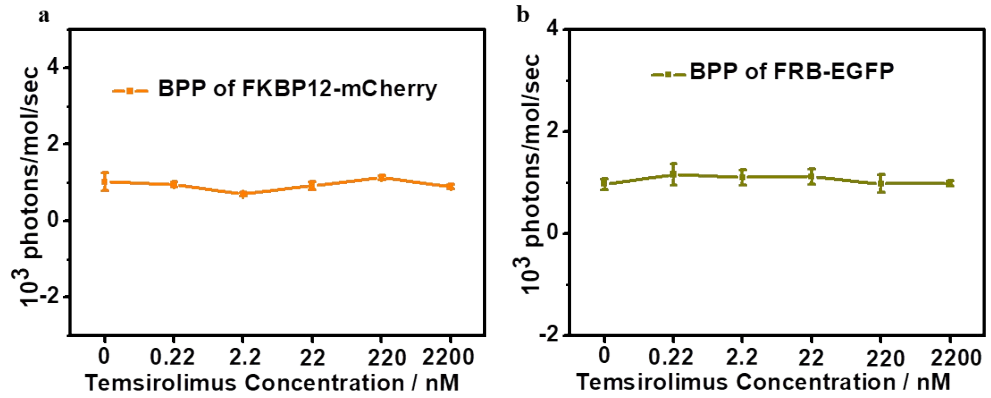

Fig. S11. (a-b) The brightness per particle (BPP) value in different incubation concentration of temsirolimus in cells. The right side 488 channel laser intensity is 8 μw. The left side 561 channel laser intensity is 23 μw. The error bars represent the standard deviation of four times repeated measurements.

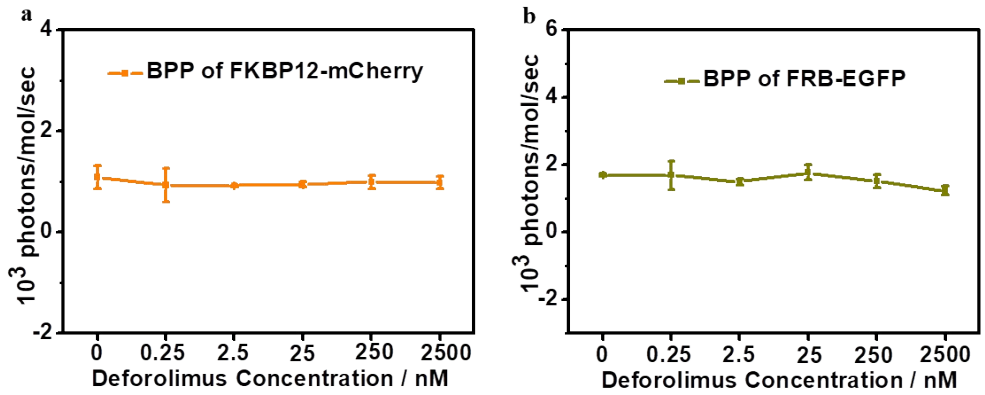

Fig. S12. (a-b) The brightness per particle (BPP) value in different incubation concentration of deforolimus in cells. The right side 488 channel laser intensity is 17 μw. The left side 561 channel laser intensity is 25 μw. The error bars represent the standard deviation of four times repeated measurements.

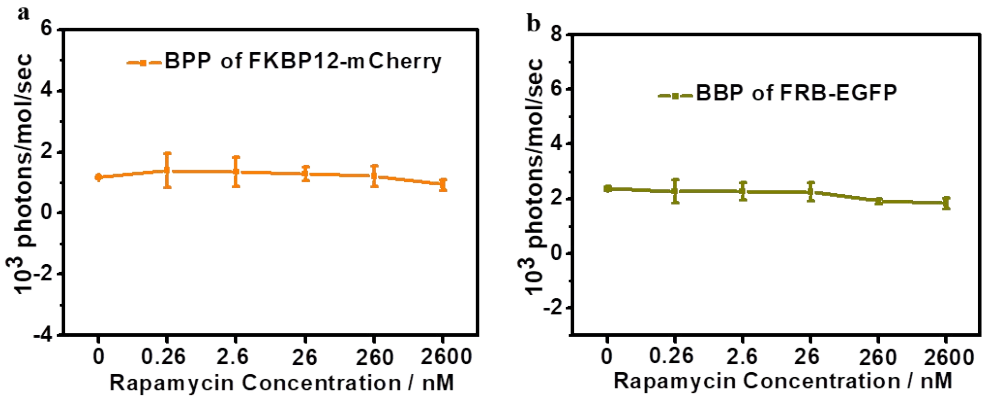

Fig. S13. (a-b)The brightness per particle (BPP) value of incubation concentration with rapamycin was obtained by FCS in cells. The right side 488 channel laser intensity is 23 μw. The left side 561 channel laser intensity is 40 μw. The error bars represent the standard deviation of four times repeated measurements.

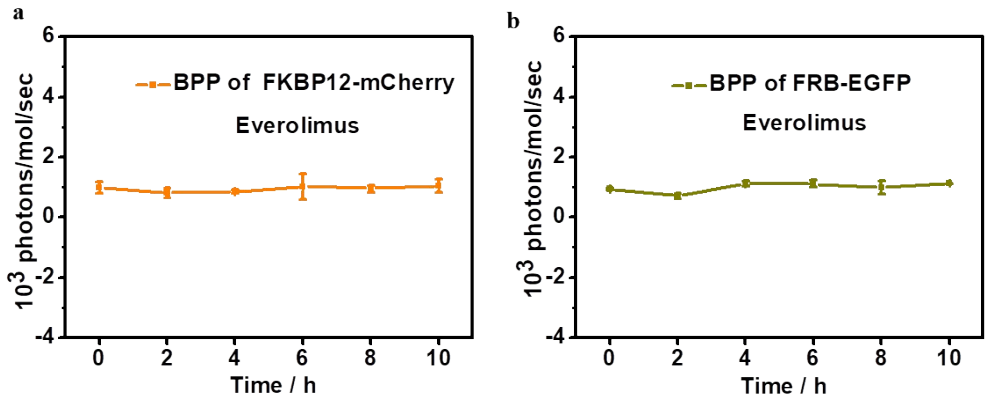

Fig. S14. (a-b) The brightness per particle (BPP) value during different incubation time of everolimus in cells. The 488 channel laser intensity is 33 μw and the left side 561 channel laser intensity is 48 μw. The error bars represent the standard deviation of four times repeated measurements.

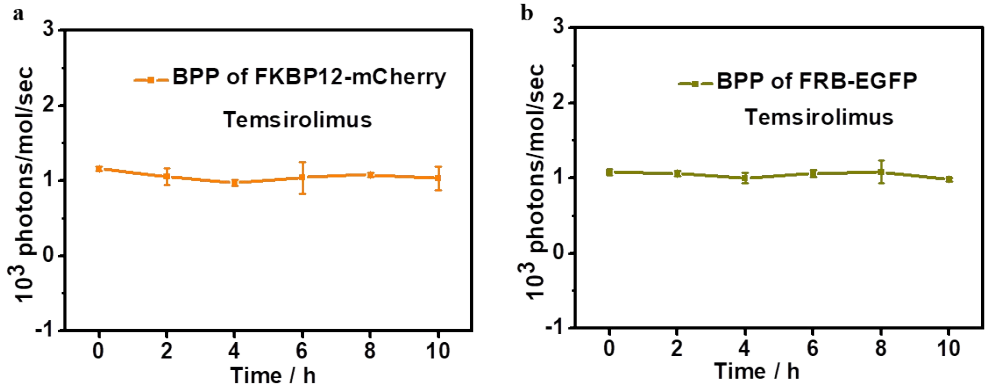

Fig. S15. (a-b) The brightness per particle (BPP) value during different incubation time of temsirolimus in cells. The right side 488 channel laser intensity is 49 μw. The left side 561 channel laser intensity is 36 μw. The error bars represent the standard deviation of four times repeated measurements.

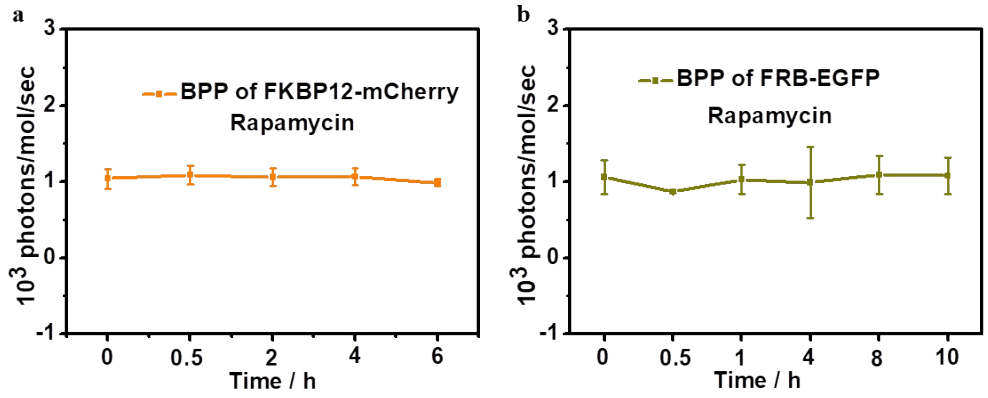

Fig. S16. (a-b) The brightness per particle (BPP) value during different incubation time of rapamycin in cells. The right side 488 channel laser intensity was 46 μw. The left side 561 channel laser intensity is 66 μw. The error bars represent the standard deviation of four times repeated measurements.

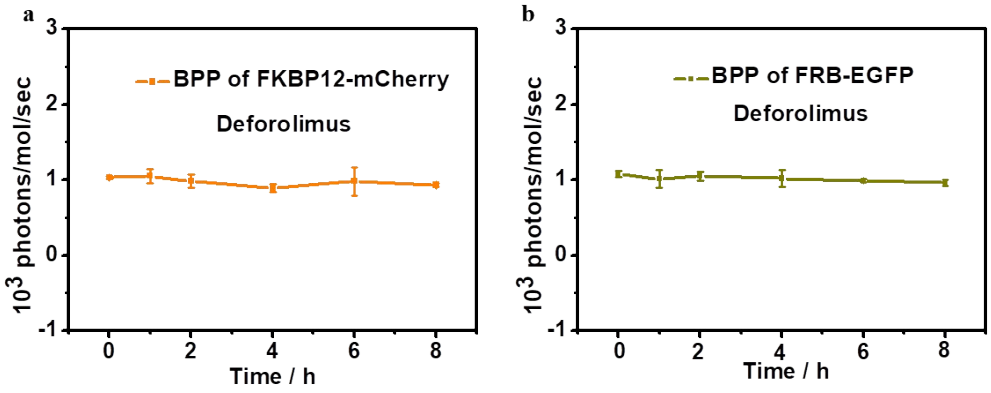

Fig. S17. (a-b) The brightness per particle (BPP) value during different incubation time of deforolimus in cells. The right side 488 channel laser intensity is 46 μw. The left side 561 channel laser intensity is 56 μw. The error bars represent the standard deviation of four times repeated measurements.

References

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