Electronic Supplementary Material (ESI) for Analyst.

In situ study of RSK2 kinase activity in a single living cell by combining single molecule spectroscopy with activity-based probes

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Experimental Section

Materials and Reagents. FMK were purchased from Shanghai Taoqin Chemical Co., Ltd. (Shanghai, China). BODIPY^{TM} FL C3 NHS Ester and Cy3 NHS ester were products of Life Technologies Inc. and Lumiprobe (USA), respectively. Recombinant RSK2 was obtained from BioVisionInc (USA). Lipofectamine2000 transfection reagents were provided by Life Technologies Inc. Glo lysis buffer were purchased from Promega Corporation (USA). EGF was purchased from Thermo Fisher Scientific (USA). Anti-RSK2 antibody was purchased from Abcam (no. ab32133), anti-β-actin antibody was purchased from Santa Cruz Biotechnology (no. SC-813). Alexa Fluor 680-conjugated goat anti-rabbit antibody was purchased from Thermo Fisher Scientific (no. A21076), Alexa Fluor 680-conjugated goat anti-mouse antibody was purchased from Thermo Fisher Scientific (no. A21058). All other chemicals were products of 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). UVA-365 nm lamp was purchased from Uvata Precision Optoelectronics Co., Ltd (Shanghai, China).

Labeling of RSK2 with Probes in Solution and in Cell Lysate. The recombinant RSK2 were incubated with the Probe1 or Probe2 for 1.5 h at 37 °C in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). The final concentration of recombinant RSK2 was 200 nM, and the final concentration of Probe1 or Probe2 were 2 nM. The labeling product is used for FCS measurements, and the free Probe1 (without RSK2) and BODIPY or free Probe2 (without RSK2) and

Cy3 were used as the negative control experiments.

The MCF-7 cells were lysed using Glo Lysis Buffer, the lysate was purified with 30 kDa ultrafiltration tube (Millipore, U.S.A) by centrifugation at 14,000 g for 20 min at 4 °C according to the instructions of the manufacture, and then the 50 μL samples were incubated with the Probe1 or Probe2 (final concentration 6 nM) for 1.5 h at 37 °C. The BODIPY or Cy3 added into MCF-7 cells lysate were used as the negative control. After the incubation, samples were measured at 37 °C by FCS setup.

Cell Culture and Cell Labeling with Probes. MCF-7 cells, HEK293T cells and Tu686 cells were cultured in DMEM (Corning) in a 37 °C humidified incubator with 5% CO_2. All media were supplemented with 10% fetal bovine serum (FBS, Corning) and 1% Penicillin/Streptomyc (Life Technologies). Prior to FCS measurements, coverglass-bottom imaging dish (MatTek, USA) was treated with 1 mL culture medium for 15 min at 37 °C humidified incubator with 5% CO_2. The dish was washed with PBS, approximate 10^5 cells were seeded into the dish in medium and then were growth in a 37 °C humidified incubator with 5% CO_2 overnight. Thereafter, the cells were incubated with the probes. The medium was discarded, then the cells were rinsed with PBS twice, followed by culturing the cells in a medium without phenol red, and finally, the cells were analyzed by FCS and FCCS methods.

Cellular imaging. MCF-7 cells were seeded in glass-bottom dishes (Mattek) and cultured for 24 h at 37 °C humidified incubator with 5% CO_2. For imaging experiments with the Probe1, cells were incubated with the Probe1 (final concentration 1 μM) in DMEM growth medium for 2 h. The cells were then washed

with PBS twice and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature, washed with PBS three times (5 min each wash), and then permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature. Cells were then washed four times with PBS. Images were obtained using our home-built FCCS setup.

Stimulation Experiments of MCF-7 Cells. MCF-7 cells were seeded into coverglass-bottom imaging dish and cultured for 24 h at 37 °C humidified incubator with 5% CO_2. Thereafter, the cells were deprived of serum for 24 h and then stimulated with EGF, U0126 inhibitor and UV for different time. After the stimulation, the cells were incubated with the indicated concentrations of Probe1. The cells were analyzed by FCS and FCCS methods.

Construction of Stable Cell Lines. The plasmids of wild-type RSK2 (pLVX-EGFP-RPS6KA3-PGK-Puro) were obtained from Fit Gene BioTechnology Inc. (China). Two RSK2 CTD mutants (C436V and T493M) were constructed. In C436V, the Cys436 was replaced with Val, and in T493M, the Thr493 was replaced with Met. C436V and T493M were created by site directed mutagenesis. The plasmids of shRNA (pLVX-ShRNA-Puro-hRSK2) were obtained from Biowit Technologies Ltd. (China), and the target sequences were as follows: 5'-GGGAGGAGAUUUGUUUACACGCUUA-3' (RSK2) and 5'-GCUCGCCUGUCUACUAACUAA-3' (scramble control). To generate recombinant lentiviral particles, the plasmids were transfected into HEK293T cells with the corresponding auxiliary plasmids via Lipofectamine 2000 reagent. HEK293T cells were treated with lentiviral particles, produced and collected in the cell culture

medium. Then, lentiviruses with polybrene were used to infect host cells, thereby a stable cell line was obtained.

Western Blot. The monoclonal stable cells were lysed using Glo Lysis Buffer with protease inhibitors. Then the total protein levels were quantified by using bicinchoninic acid assay (BCA). Equal amounts of proteins were separated by 10% SDS-PAGE, and then transferred to PVDF membrane. The membrane was blocked with blocking buffer for 1 h, and incubated overnight at 4 °C with RSK2 antibodies or β-actin antibodies, then washed, and incubated with secondary antibodies labeled with Alexa Fluor 680 at room temperature for 1.5 h. Protein levels were visualized using the Odyssey infrared imaging system (LI-COR, USA).

Theory Section

In this study, FCS and FCCS combine with ABPP are applied to real-time monitor protein kinase activity in living cells. FCS and FCCS are based on measuring the fluorescence fluctuations in a small detection volume (<1fL) due to Brownian motion of single fluorescent molecules. In FCS, the fluorescence fluctuations of $\delta F(t)$ around the average fluorescence $\langle F \rangle$ are recorded in real time, and the normalized auto-correlation $G(\tau)$ is calculated as follow Equation (S1).

$$G(\tau) = \frac{< \delta F(t) \delta F(t+\tau) >}{< F(t) >^2} \quad \text{(S1)}$$

Here, $\delta F(t) = F(t) - < F(t) >$, and τ is the delay time.

In this study, the auto-correlation curve of fluorescent molecules (probes) was fitted to a three-dimensional Gaussian diffusion model using single-components model. The raw FCS data were analyzed with the standard Equation (S2) and nonlinearly fitted with the Origin Lab8.0.

$$G(\tau) = \frac{1}{N} \frac{1}{\left(1 + \frac{\tau}{\tau_D}\right)} \frac{1}{\sqrt{1 + \left(\frac{w_0}{z_0}\right)^2 \frac{\tau}{\tau_D}}} \quad \text{(S2)}$$

Here, N and τ_D are the average number of fluorescent molecules in the focal detection volume and characteristic diffusion time, respectively. The ω_0 and z_0 are the lateral and axial radii of the focal detection volume, respectively. If the three-dimensional Gaussian profile in the focal detection volume is assumed and the triplet state of a fluorescence molecule is considered, the auto-correlation curve of fluorescent molecule diffusing in solution was fitted using the Equation (S3)

$$G(\tau)=\frac{1}{N}\left(1+\frac{Te^{-\tau/\tau_{tr}}}{1-T}\right)\frac{1}{\left(1+\frac{\tau}{\tau_D}\right)}\frac{1}{\sqrt{1+\left(\frac{\omega_0}{z_0}\right)^2\frac{\tau}{\tau_D}}} \quad \text{(S3)}$$

T and τ_{tr} are the fraction of the fluorescent molecules in triplet state and its delay time.

The diffusion coefficient D of the species can be obtained from Equation (S4).

$$D=\frac{{w_0}^2}{4\tau_D} \quad \text{(S4)}$$

In this study, the components of Probe1 and Probe1-RSK2 complexes can be represented by the two-component diffusion model, given by Equation (S5) as

$$G(\tau)=\frac{1}{N}\left(1+\frac{Te^{-\tau/\tau_{tr}}}{1-T}\right)\left[\frac{1-Y}{\left(1+\frac{\tau}{\tau_{free}}\right)\sqrt{1+\left(\frac{\omega_0}{z_0}\right)^2\frac{\tau}{\tau_{free}}}}+\frac{Y}{\left(1+\frac{\tau}{\tau_{bound}}\right)\sqrt{1+\left(\frac{\omega_0}{z_0}\right)^2\frac{\tau}{\tau_{bound}}}}\right] \quad \text{(S5)}$$

The τ_{free} and τ_{bound} are the characteristic diffusion times of the free probe and the bound probe, and Y is the fraction of the bound probe. To obtain τ_{free} and τ_{bound} in living cells, a multicomponent model called MEMFCS[1] is used to study the probes distribution of different diffusion times.

MEMFCS is a useful approach for studying multicomponent diffusers in a heterogeneous system.[2-4] In MEMFCS, the data analysis is based on minimizing quantitative parameter χ^2 as well as maximizing entropy S to obtain an optimal fit when the characteristic diffusion times of the different components are involved in the detection volume. $G(\tau)$ in Equation (S2) can be rewritten to obtain a continuous distribution of characteristic diffusion times as follow Equation (S6).

$$G(\tau)=\int \alpha(\tau_D)\frac{1}{\left(1+\frac{\tau}{\tau_D}\right)}\frac{1}{\sqrt{1+\left(\frac{w_0}{z_0}\right)^2\frac{\tau}{\tau_D}}}d\tau_D \quad \text{(S6)}$$

Here, the diffusion time τ_D is considered to be a variable and $\alpha(\tau_D)$ is the amplitude associated with τ_D.

The χ^2 is defined as follow Equation (S7).

$$\chi^2=\frac{1}{M}\sum_{i=1}^{M}r_i^2$$
$$r_i=\frac{G^c(\tau_i)-G^e(\tau_i)}{\sigma_i} \quad \text{(S7)}$$

Where, M is the number of FCS data points. σ_i is the inverse of weight for the i^{th} data. The value of $G^c(\tau_i)$ is calculated using Equation (S6) and the $G^e(\tau_i)$ is the experimental value.

The S is defined as follow Equation (S8).

$$S=-\sum_i p_i \ln p_i \quad \text{(S8)}$$

Where $p_i=\alpha_i/\sum\alpha_i$. The definitions of χ^2 and S in MEMFCS were in details described by Maiti et al[1]. Herein, the distribution of the characteristic diffusion times is used to distinguish the different components of Probe1 in the living cells.

In FCCS, the 488 nm and 561 nm laser beams were used to respectively excite the green and red fluorophores, cross-correlation function from the recorded fluorescence intensity traces detected in the green and red channels were calculated as

$$G_{gr}(\tau)=\frac{\langle \delta F_g(t)\delta F_r(t+\tau)\rangle}{\langle F_g(t)\rangle\langle F_r(t)\rangle} \quad \text{(S9)}$$

Where $\delta F(t)=F(t)-\langle F(t)\rangle$, the < > denotes time average, F_g and F_r are the fluorescence intensities in the EGFP (green) and the Cy3 (red) detection channels,

respectively.

The cross-correlation curves were fitted using Equation (S10) according to the reference.[5]

$$G_{gr}(\tau)=\frac{N_{gr}}{N_g N_r}\frac{1}{\left(1+\frac{\tau}{\tau_D}\right)}\frac{1}{\sqrt{1+\left(\frac{\omega_0}{z_0}\right)^2\frac{\tau}{\tau_D}}} \quad \text{(S10)}$$

Here, N_g, N_r and N_{gr} are the average numbers of green fluorescent molecules, red fluorescent molecules and bound complexes (both green and red fluorescence) in the detection volume, respectively. They are defined as Equations (S11), (S12) and (S13).

$$N_g=\frac{1}{G_g(0)} \quad \text{(S11)}$$

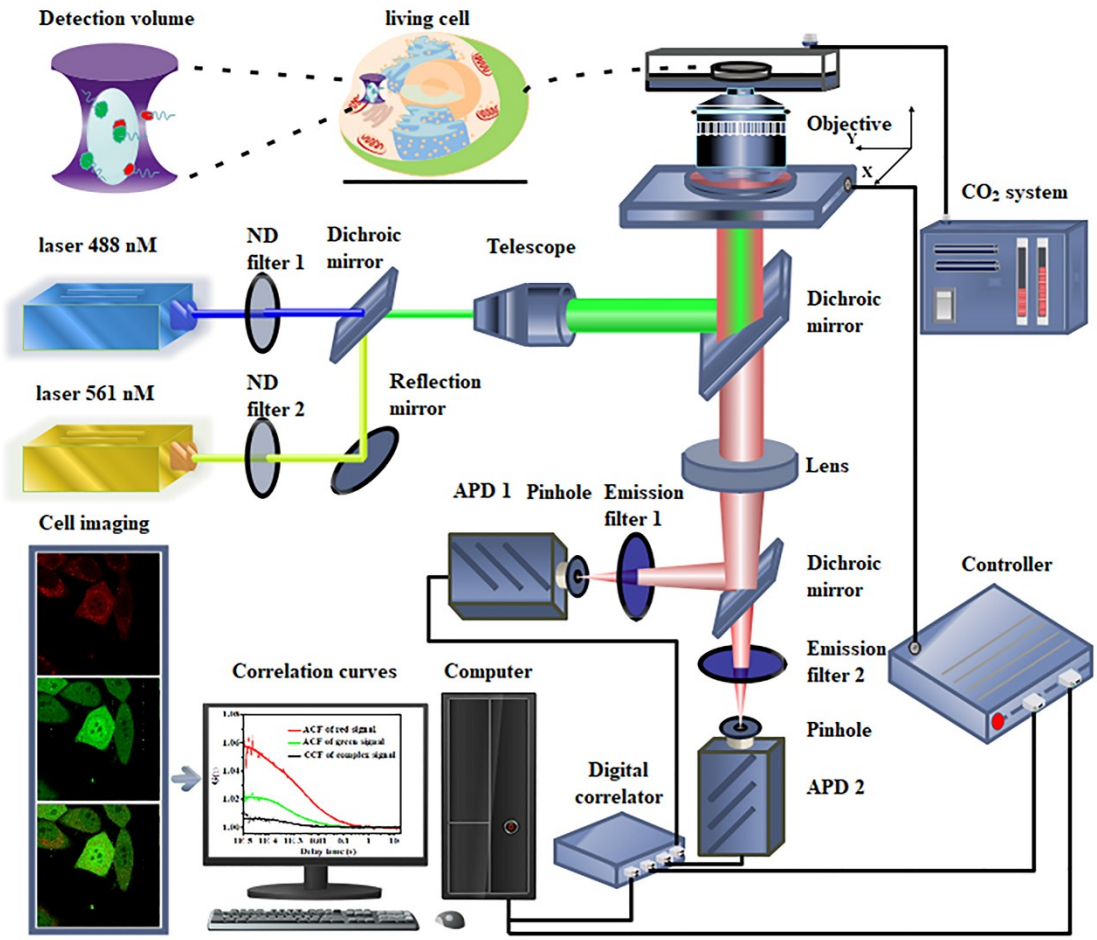
$$N_r=\frac{1}{G_r(0)} \quad \text{(S12)}$$

$$N_{gr}=N_g N_r G_{gr}(0) \quad \text{(S13)}$$

In this study, "relative cross-correlation amplitude" (CC value) was used to quantify the RSK2 activity, and CC value is defined in Equation S14.

$$\mathrm{CC}=\frac{N_{gr}}{N_r}=\frac{G_{gr}(0)}{G_g(0)}, or=\frac{N_{gr}}{N_g}=\frac{G_{gr}(0)}{G_r(0)} \quad \text{(S14)}$$

The CC value is calculated by choosing the lower one from G_g (0) and G_r (0). As shown in equation S14, the CC value is positively correlated to N_{gr} (number of bound complexes) and the increased CC value thus indicates an increased RSK2 activity.



FigureS1. Schematic diagram of FCS and FCCS setup.

Synthesis of Compounds

Compound 1. Imidazole (9.94 mg，0.146 nmol) was added to a CH_2Cl_2 (500 μL) solution containing1-[4-amino-7-(3-hydroxypropyl)-5-(4-methylphenyl)- 7H-pyrrolo[2,3-d] pyrimidin-6-yl]-2-fluoro-Ethanone (FMK, 50.0 mg, 0.146 nmol) at 0 ^oC. TBSCl (44.02 mg, 0.292 nmol) was quickly added, and then the mixture was stirred for 1 h at 0 ^oC. The reaction mixture was diluted with CH_2Cl_2 (3 mL) and washed with saturated NaCl (1 mL×3). The organic fraction was dried over Na_2SO_4, filtered, and concentrated *in vacuo,* the crude product was purified by preparative flash chromatography (1:1 ethyl acetate/petroleum ether) to afford **compound 1** (56.7 mg, yield: 85%) as a yellow solid: R_f 0.70 (1:1 ethyl acetate/petroleum ether); ^1H NMR (400 MHz, $CDCl_3$) δ 8.34 (s, 1H), 7.33 (m, 4H), 5.31 (s, 2H), 4.67 (t, 2H), 3.72 (t, 2H), 2.47 (s, 3H), 2.04 (s, 2H), 0.91 (s, 9H), 0.05 (s, 6H).

Compound 2. To a solution of **compound 1** (56.2 mg, 0.123 nmol) in THF (500 μL) was slowly added DMAP (4.49 mg, 0.037 nmol) followed by $(Boc)_2O$ (107.06 mg, 0.490 nmol) at 0 ^oC. After stirring for 1 h at 0 ^oC, the reaction mixture was diluted with ethyl acetate (3 mL×5) and washed with saturated $NaHCO_3$ (1.5 mL). The organic fraction was dried over Na_2SO_4, filtered, and concentrated *in vacuo,* the crude product was purified by preparative flash chromatography (3:1 ethyl acetate/petroleum ether) to afford compound 2 (72.5 mg, yield: 90%) as a yellow solid: R_f 0.75 (4:1 ethyl acetate/petroleum ether); ^1H NMR (400 MHz, $CDCl_3$) δ 8.94 (s, 1H), 7.28 (m, 4H), 4.76 (t, 2H), 4.52 (t, 2H), 3.73 (t, 2H), 2.46 (s, 3H), 2.07 (m, 2H), 1.35 (s, 18H), 0.94 (s, 9H), 0.09 (s, 6H).

Compound 3. The **compound 2** was dissolved in THF (500μL) and TBAF (57 mL, 0.21 nmol) was added. After reaction for 3 h at room temperature, the reaction mixture was diluted with water (2 mL) and extracted with ethyl acetate (3 mL×5). The organic fraction was dried over Na_2SO_4, filtered, and concentrated *in vacuo*, the crude product was purified by flash chromatography (1:1 ethyl acetate/petroleum ether) to afford **compound 3** (50.2 mg, yield: 88%) as a yellow solid: R_f 0.72 (1:1 ethyl acetate/petroleum ether); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.92 (s, 1H), 7.23 (m, 4H), 5.29 (s, 2H), 4.66 (t, 2H), 3.45 (t, 2H), 2.43 (s, 3H), 2.03 (m, 2H), 1.32 (s, 18H).

Compound 4. To a solution of **compound 3** (50.0 mg, 0.092 nmol) in CH_2Cl_2 (600 μL) was added DIPEA (0.020 Ml, 0.11 nmol), and followed by CDI (15.58 mg, 0.11 nmol) at room temperature. After stirring for 2 h at room temperature, N-Boc-1,4-butanediamine (34.7 mg, 0.184 nmol) was added and the reaction was stirred for 3 h at room temperature. The reaction mixture was diluted with water (2 mL) and extracted with ethyl acetate (3 mL×5). The organic fraction was dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The crude product was purified by preparative flash chromatography (1:1 ethyl acetate/petroleum ether) to afford **compound 3** (50.2 mg, yield: 72%) as a yellow solid: R_f 0.60 (1:1 ethyl acetate/petroleum ether); ^{1}H NMR (400 MHz, $CDCl_3$) □ 8.94 (s, 1H), 7.27 (d, 2H), 7.23 (d, 2H), 4.79 (t, 2H),4.69 (t, 2H), 4.10 (t, 2H), 3.07 (m, 4H), 2.46 (s, 3H), 1.82 (m, 2H), 1.43 (s, 9H), 1.34 (s, 18H), 1.26 (m, 4H).

Compound 5. Compound 4 (10.0 mg, 0.0132 nmol) was dissolved in CH_2Cl_2 (200 μL) and transferred to 1 L brown reaction bottle, TFA (200 μL) was added at 0 ^{o}C.

After slowly warming the reaction mixture to room temperature over a period of 2 h, the solvent was removed *in vacuo* and the crude bocdeprotected product was afforded.

Compound 6 (Probe1 or Probe2). The crude bocdeprotected product (1.00 mg, 2.19 μmol) was dissolved in DMF (200 μL) at 1 L brown reaction bottle. BODIPY^{TM} FL C3 NHS Ester (0.85 mg, 2.63 μmol) or Cy3 NHS ester (1.29 mg, 2.19 μmol) was added, followed by DIPEA (1.2 μL, 6.57 μmol). The mixture was stirred for 2 h at room temperature and protected from light. The reaction mixture was purified by preparative HPLC (gradient 40-60% CH_3OH, 0.1 % TFA, over 15 min, 120 mL/min flow rate, C18 column (10×250 mm) Agilent) to afford **Probe1** (1.12mg, yield: 70%) or **Probe2** (1.19mg, yield: 62%). **Probe1** was assigned the molecular formula ($C_{37}H_{42}BF_3N_8O_4$) on the basis of HR-ESI-MS (*m/z* 731.3472 $[M+H]^+$). **Probe2** was assigned the molecular formula ($C_{53}H_{64}FN_8O_4^+$) on the basis of HR-ESI-MS (*m/z* 896.5095 $[M+H]^+$).

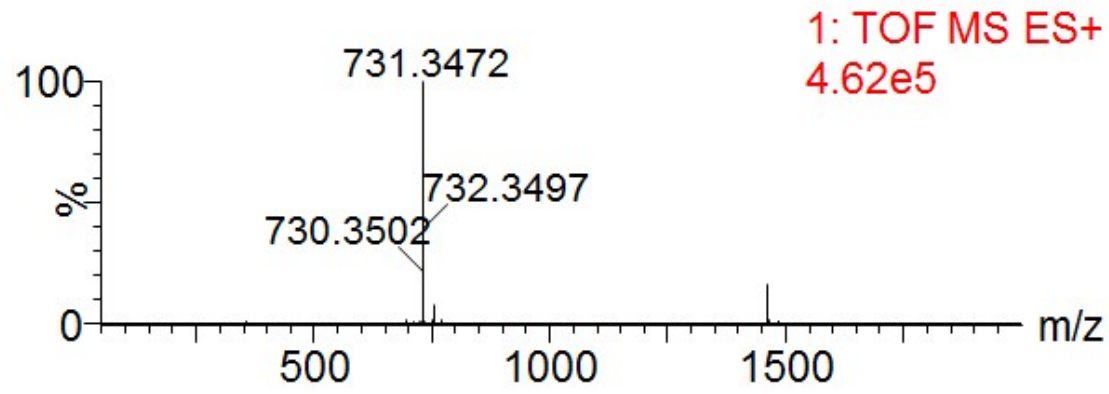


Figure S2. MS analysis results of Probe1.

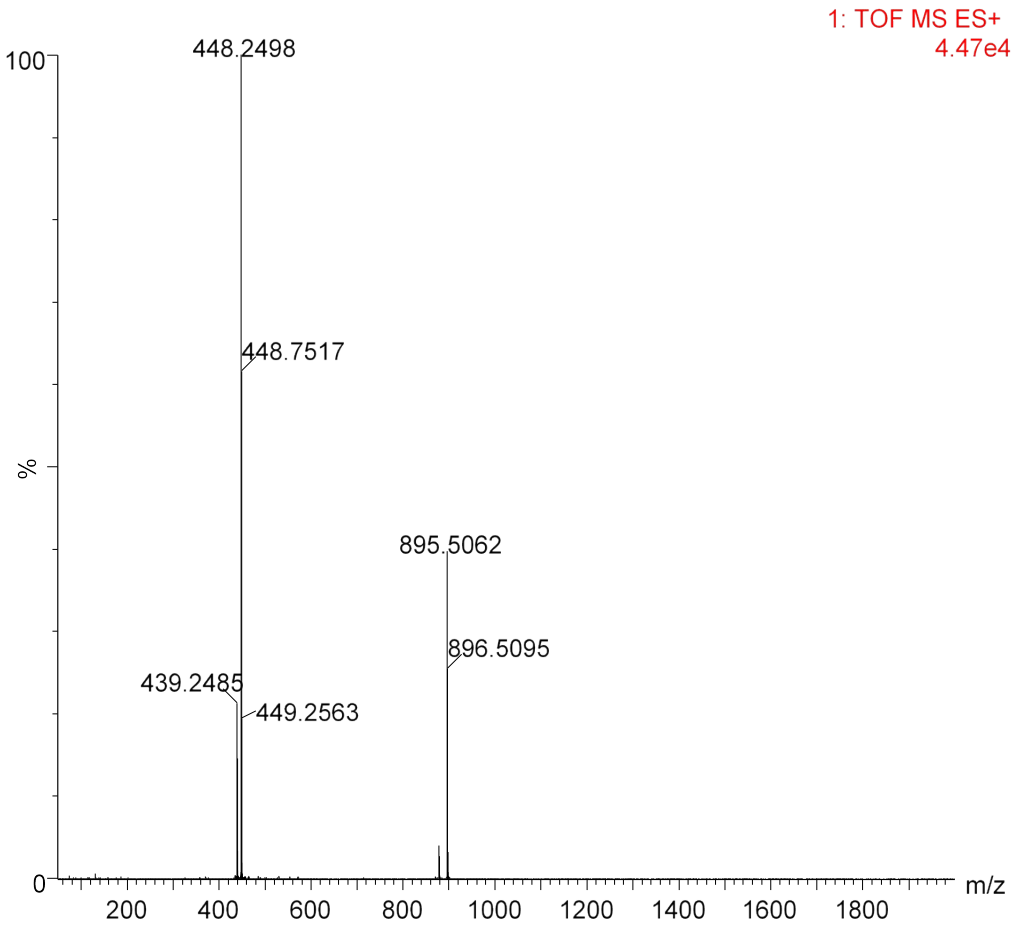


Figure S3. MS analysis results of Probe2.

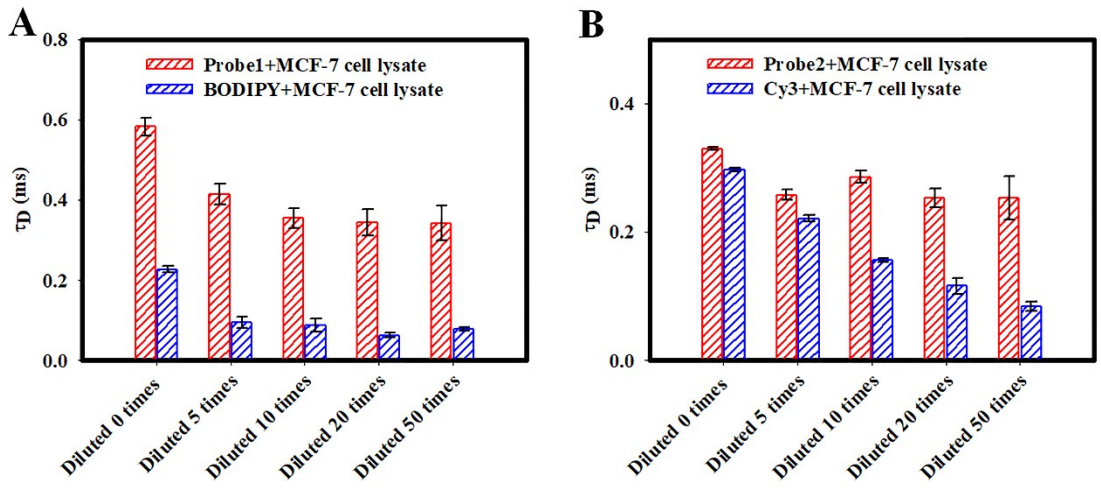


Figure S4. The results of Probe1 (A) and Probe2 (B) covalent modified RSK2 in MCF-7 cell lysate by FCS measurements.

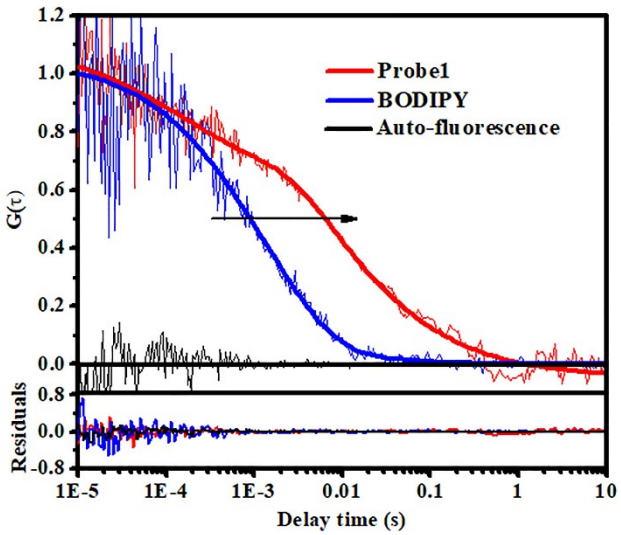


Figure S5. Typical normalized auto-correlation curves of Probe1, BODIPY and auto-fluorescence in MCF-7 cell, their fitting curves and corresponding fit residuals using single-component model.

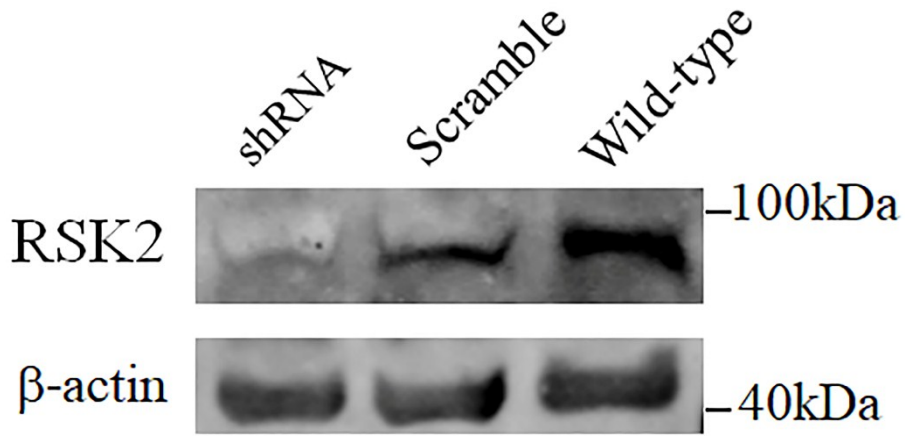


Figure S6. Western blotting analysis for shRNA interference in MCF-7 cell. Expression of endogenous RSK2 in MCF-7 cells were knocked down using RNA interference. The wild-type cells and scramble control cells were used as the control experiments. The western blot confirmed that the expression of endogenous RSK2 was knocked down using shRNA interference.

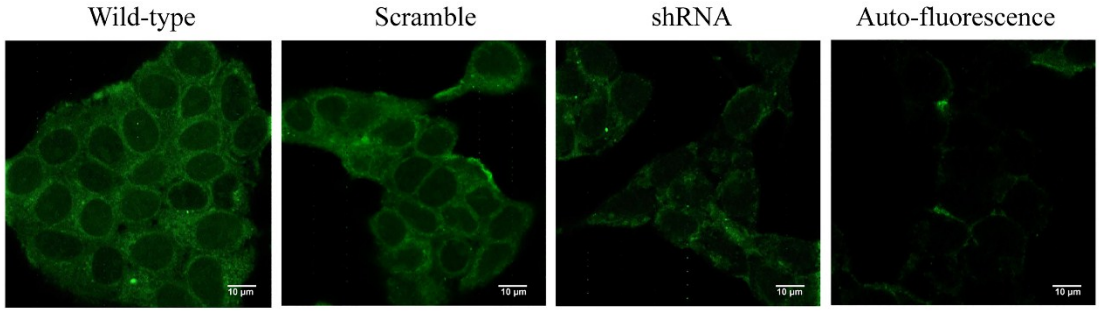


Figure S7. The specificity of the Probe1 to RSK2 was characterized by cell fluorescence images in MCF-7 cells. The green shows the fluorescence signal of Probe1, excited at 488 nm. The RSK2 containing cells (Wild-type), shRNA knockdown cells and scramble control cells were treated with 1 μM Probe1 for 2 h at 37 ^{o}C, followed by washing away the unbound probes. The confocal imaging results show that the significant decrease in fluorescence intensity of shRNA knockdown cells compared to the wild-type and scramble control cells, and cell auto-fluorescence is very low and should be ignored in this case. The results confirmed that the Probe1 specifically bound to RSK2 in the cytoplasm and nucleus. The scale bars are 10 μm.

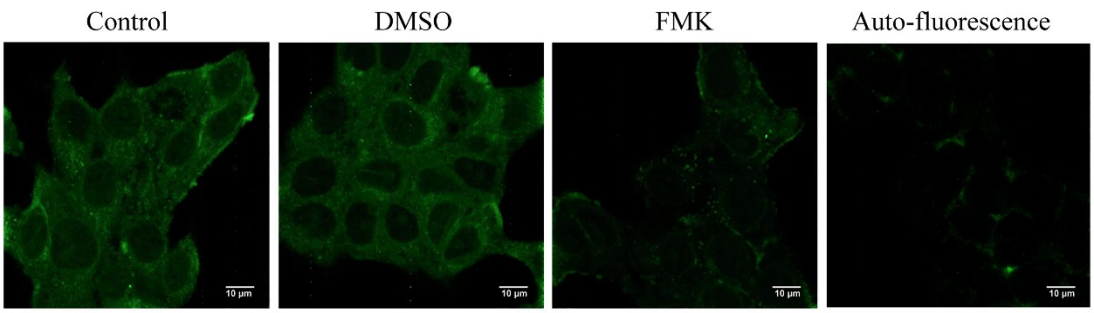


Figure S8. The specificity of the Probe1 to active RSK2 was characterized by cell fluorescence confocal images in MCF-7 cells. The green shows the fluorescence signal of Probe1, excited at 488 nm. MCF-7 cells were treated with the 100 μM FMK for 2 h followed by 1 μM Probe1 for 2 h at 37 °C, and then followed by washing away the unbound probes. The untreated cells and the DMSO treated cells were used as the control experiments. The confocal imaging results show that the significant decrease in fluorescence intensity of FMK treated cells compared to the untreated cells and the DMSO treated cells, and the cell auto-fluorescence is very low and should be ignored in this case. The results showed that the Probe1 specifically bound to active RSK2 in the cytoplasm and nucleus but not to the inactive RSK2. The scale bars are 10 μm.

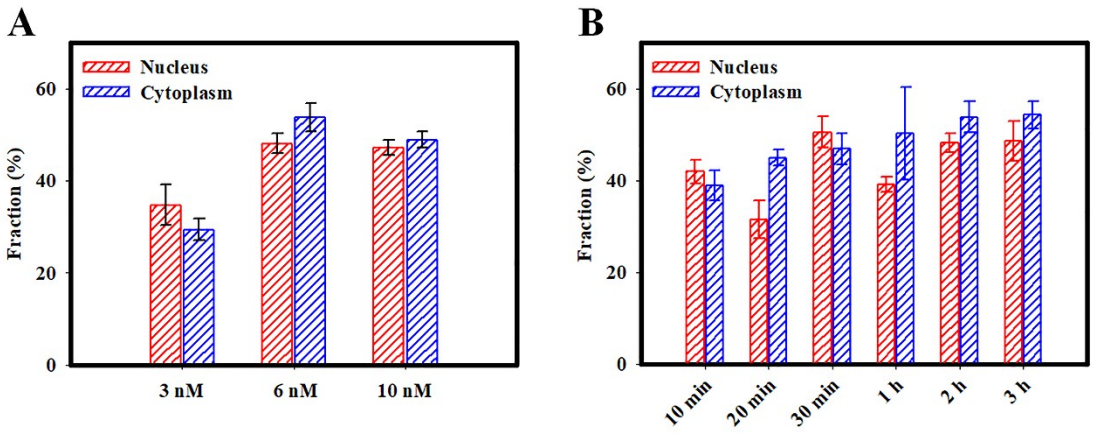


Figure S9. The optimization of living cell assay experiments. MCF-7 cells were treated with the different concentration (A) and different incubation time (B) of Probe1, Numerical values in the graph indicate the mean value ± SEM (n=20 cells).

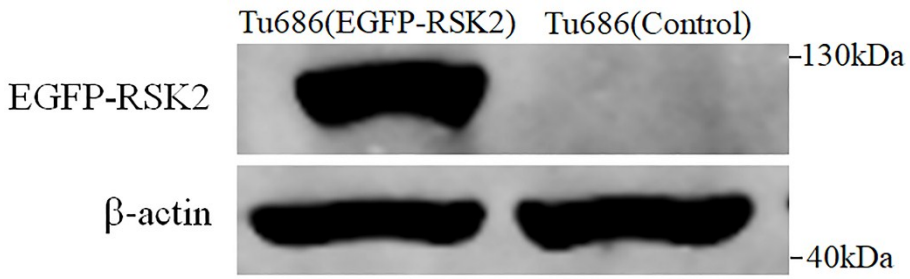


Figure S10. Western blotting analysis. Tu686 clone stably expressing full-length EGFP-RSK2 fusion protein is detected by anti-RSK2 and anti β-actin antibodies.

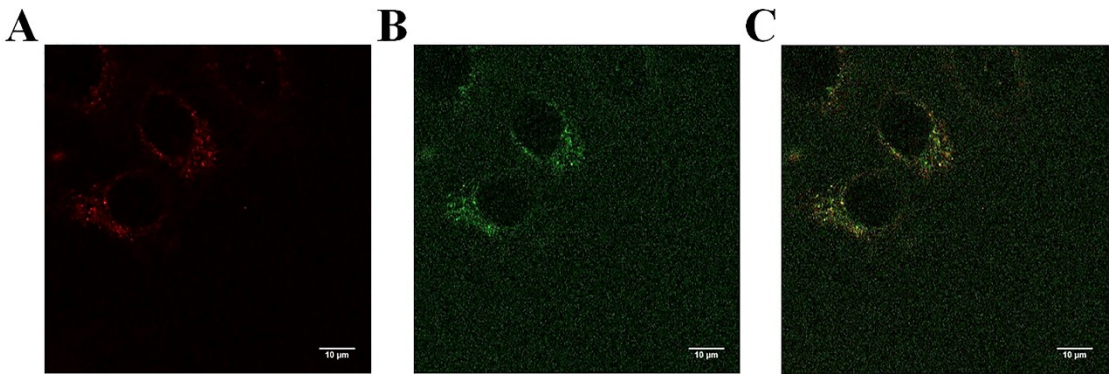


Figure S11. The typical auto-fluorescence imaging of Tu686 cells at 488 nm (A) and at 561 nm (B) excitation, and their merge (C).

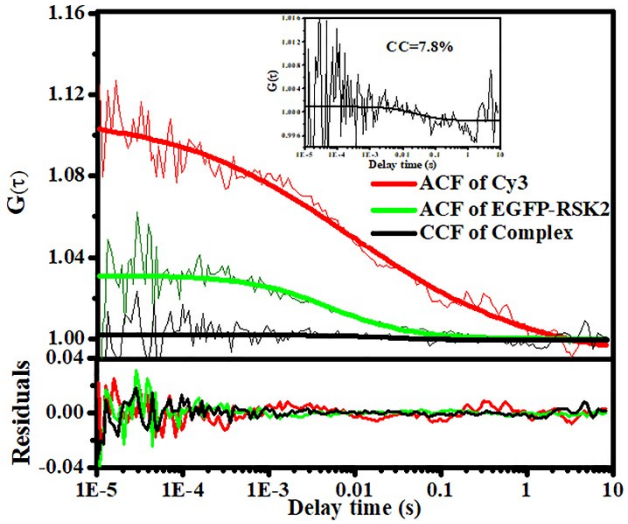


Figure S12. Representative FCCS curve of the negative control experiment.

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