Electronic Supporting Information

Simultaneously monitoring endogenous MAPK members in single living cell by multi-channel fluorescence correlation spectroscopy

Table of Contents

Preparation of Compounds. ····S3

Binding Assay of Three Probes in Kinases Solution by FCS. ····S4

Heterogeneous Expression and Affinity Purification of the BRAF Protein. ····S4

Cell Lysis and Binding Assay of Probes in Cell Lysate by FCS. ····S5

Data Analysis for Cross Correlation Curves (CCFs) and Brightness Per Particle (BPP). ····S5

Cell Imaging. ····S6

Figure S1. Schematic diagram of the multi-channel FCS system. ····S7

Figure S2. Characterization of **Compound 1** by mass spectrometry. ····S8

Figure S3. Characterization of **Probe 1** by mass spectrometry. ····S9

Figure S4. Characterization of **Compound 2** by mass spectrometry. ····S10

Figure S5. Characterization of **Probe 2** by mass spectrometry. ····S11

Figure S6. Characterization of **Compound 3** by mass spectrometry. ····S12

Figure S7. Characterization of **Probe 3** by mass spectrometry. ····S13

Figure S8. The autocorrelation curves, fitting residuals and brightness per molecule (BPP) values of probes and free dyes obtained by FCS. ····S14

Figure S9. Normalized FCS curves and fit residuals of probes at different concentrations of corresponding kinases. ····S15

Figure S10. Normalized FCS curves and fit residuals of probes and dyes in cells lysate. ····S16

Figure S11. CLSM images of HeLa cells for FCCS measurements. ·····S17

Figure S12. CLSM images and the fluorescence intensity of cells under different incubation conditions. ·····S18

Figure S13. CLSM images and the fluorescence intensity of cells cultured with medium containing different volume fraction of serum. ·····S19

Figure S14. Transformation of recombinant plasmid in yeast colony judged by PCR and the protein BRAF-HIS idendified by SDS-PAGE and stained with Coomassie Brilliant Blue. ·····S20

Notes and references. ·····S20

Preparation of Compounds.

Compound 1. 4-Boc-chloroglycine (11.0 mg, 0.05 mmol) and KOH (5.0 mg, 0.08 mmol) were added to a solution of vemurafenib (20.0 mg, 0.04 mmol) dissolved in DMF (0.5 mL). The mixture was stirred at 10 ºC for 3 h. Then the reaction mixture was washed three times with saturated NaCl solution (2 mL) and dried with Na_2SO_4. The crude mixture was purified by silica flash chromatography (1:3 ethyl acetate/petroleum ether) to afford **compound 1** (19.5 mg, yield: 72.3%) as a light yellow solid. **Compound 1** was assigned the molecular formula ($C_{31}H_{31}ClF_2N_4O_7S$) on the basis of HR-MS (ESI): m/z = 677.1633 $[M+H]^+$. MS analysis result is shown in Fig. S2.

Probe 1. Compound 1 (15.0 mg, 0.03 mmol) was dissolved in dichloromethane (0.8 mL) and then TFA (0.2 mL) was added slowly in ice water bath. After stirring for 2 h, the solvent was removed under vacuum and deprotected product was obtained. The product (1.0 mg, 0.002 mmol), BODIPY TR NHS Ester (0.2 mg, 0.0005 mmol) and TEA (0.2 μL) were dissolved and mixed in DMF (0.3 mL). The mixture is protected from light and stirred at room temperature for 5 h. The crude product was purified by semi-prepared reversed-phase HPLC (C18 column, Agilent). **Probe 1** was assigned the molecular formula ($C_{48}H_{37}BClF_4N_5O_7S_2$) on the basis of HR-MS (ESI): m/z = 983.1982 $[M+H]^+$. MS analysis result is shown in Fig. S3.

Compound 2. To a solution of selumetinib (25.0 mg, 0.05 mmol) dissolved in DMAC (0.5 mL), Boc-glycine (10.0 mg, 0.06 mmol), HOBT (7.5 mg, 0.05 mmol) and TEA (10 μL) were added and stirred with nitrogen protection. After 5 minutes, EDCl (15.0 mg, 0.08 mmol) was added to the mixture. The mixture was stirred overnight at room temperature and then was poured slowly to 3 mL deionozied water with stirring for 1 h. After filtering through a funnel, the cake was washed with water and tert-butyl methyl ether successively. The tert-butyl methyl ether layer was dried with Na_2SO_4 and was purified by silica flash chromatography (2:5 ethyl acetate/petroleum ether) to afford **compound 2** (19.2 mg, yield: 62.6%) as yellow solid. **Compound 2** was assigned the molecular formula ($C_{24}H_{26}BrClFN_5O_6$) on the basis of HR-MS (ESI): m/z = 614.0920 $[M+H]^+$. MS analysis result is shown in Fig. S4.

Probe 2. Compound 2 (12.0 mg, 0.02 mM) was added to dichloromethane (1 mL) and TFA (0.2 mL) was added slowly in ice water bath. The mixture was stirred for 3 h and crude product was dried under vacuum. The product (1.0 mg, 0.002 mmol) was added to a solution of BODIPY 493 NHS Ester (0.4 mg, 0.001 mmol), TEA (0.3 μL) and DMF (0.2 mL). The reaction and purification processes were similar to that of **Probe 1**. **Probe 2** was assigned the molecular formula

($C_{35}H_{35}BBrClF_3N_7O_5$) on the basis of HR-MS (ESI): m/z = 816.03930 [*M*+H]$^+$. MS analysis result is shown in Fig. S5.

Compound 3. Ulixertinib (18.0 mg, 0.04 mmol), 6-(Boc-amino) hexanoic acid (20.0 mg, 0.08 mmol) and PPh3 (26.0 mg, 0.1 mmol) were dissolved in DMSO (0.5 mL). DEAD (0.05 mL) was added slowly to the above solution in dry nitrogen atmosphere. The mixture was stirred for 5 h at room temperature, washed with saturated NaCl solution (2 mL) and dried with Na_2SO_4. The crude product was purified by silica flash chromatography (2:3 ethyl acetate/petroleum ether) to afford compound 1 (15.6 mg, yield: 60.5%) as a yellow solid. **Compound 3** was assigned the molecular formula ($C_{32}H_{41}Cl_2N_5O_5$) on the basis of HR-MS (ESI): m/z = 646.1496 [*M*+H]$^+$. MS analysis result is shown in Fig. S6.

Probe 3. TFA (0.2 mL) was added slowly to a solution of **Compound 3** (12.0 mg, 0.02 mmol) dissolved in dichloromethane (1 mL) in ice water bath. The crude product was dried under vacuum after stirring for 2 h. The product (1.0 mg, 0.002 mmol), ATTO 647N NHS Ester (0.4 mg, 0.0005 mmol) and TEA (0.2 μL) were dissolved and mixed in DMF (0.4 mL). The reaction and purification process was similar to that of **Probe 1** and **Probe 2**. **Probe 3** was assigned the molecular formula ($C_{69}H_{83}Cl_2N_8O_5^+$) on the basis of HR-MS (ESI): m/z = 1190.5940 [*M*+H]$^+$. MS analysis result is shown in Fig. S7.

Binding Assay of Three Probes in Kinases Solution by FCS. Three probes (final concentration 1 nM) were added to the corresponding kinases solution of different concentrations (1 nM, 10 nM, 50 nM, 100 nM, 150 nM, 300 nM and 500 nM,), respectively. The mixtures were incubated at 37 ºC for 1 h and then were detected by FCS. The single measurement time was 30 s and the measurement repeated five times. FCS curves and BPP values are shown in Fig. S9.

Heterogeneous Expression and Affinity Purification of the BRAF Protein. Plasmid pESC-HIS-BRAF was transformed into the BY4741 yeast cells using electroporation protocol[1]. PCR-analysis of BY4741 recombinant transformants were conducted using the primers as follows: 5'-GGGGGTCGACGGATGGCGGCGCTGAGCGGT-3', 5'-GGGAAGCTTTCAGTGGACAGGAAA CGCACCATATCCCCC-3' and confirmed the existence of recombinant plasmid in yeast cells (Fig. S14*A*). The BY4741 strains were inoculated in 15 mL YPD medium and cultured at 30 ºC until

OD600 = 2. Then the cultures were transferred into 200 mL YPD medium containing 2% galactose instead of 2% glucose and cultured overnight. Cells were collected and resuspended with 30 mL ice-cold lysis buffer (20 mM Tris-HCl, 300 mM NaCl, 5 mM imidazole, 5 mM β-mercaptoethanol (β-ME), 1 mM phenylmethanesulfonyl fluoride (PMSF), 10% glycerol, pH 7.9). The cell solution was sonicated on ice, and then the supernatant was collected by centrifugation at 4 °C (12,000 rpm, 30 min). The cell supernatants were loaded in affinity chromatography of Ni-NTA resin at 4 °C and washed with 150 mL washing buffer (300 mM NaCl, 20 mM Tris-HCl, 5 mM β-ME, 10% glycerol, 1 mM PMSF, and 50 mM imidazole, pH 7.9). The recombinant protein was eluted from the column using 5 mL of elution buffer (20 mM Tris-HCl, 300 mM NaCl, 5 mM β- ME, 10% glycerol, 1 mM PMSF, 200 mM imidazole, pH 7.9) and analyzed by 15% SDS-PAGE followed by staining with Coomassie Brilliant Blue (Fig. S14*B*).

Cell Lysis and Binding Assay of Probes in Cell Lysate by FCS. Cells (approximate 1×10^6) were harvested by centrifugation at 1000 rpm for 4 min and suspended with 500 μL ice-cold PBS. The cell suspension was frozen at -80 ºC for 10 min and thawed at 37 ºC. After freezing and thawing for three times, the supernatant was transferred into a tube after centrifugation (12000 rpm, 10 min, 4 ºC) and was diluted ten times with PBS for FCS measurements.

Probes and corresponding dyes (final concentration 1.0 nM) added to equal volume of cell lysate, respectively. The mixtures were incubated at 37 ºC for 1 h and then were measured by FCS. The single measurement was 30 s and repeated five times. FCS curves are shown in Fig. S10.

Data Analysis for Cross Correlation Curves (CCFs) and Brightness Per Particle (BPP). Cross correlation data obtained from two channels (channel 1 and channel 2) simultaneously were fitted with the model described as eqn (S1)[2]:

$$G_{1,2}(\tau) = \frac{N_{1,2}}{N_1 N_2} \frac{1}{\left(1+\frac{\tau}{\tau_D}\right)} \frac{1}{\sqrt{1+\left(\frac{\omega_0}{z_0}\right)^2 \frac{\tau}{\tau_D}}} \qquad \text{(S1)}$$

Here, N_1 and N_2 are the average numbers of fluorescent molecules corresponding to channel 1 and channel 2 in the detection volume, respectively. They are defined as eqn (S2) and (S3):

$$N_1 = \frac{1}{G_1(0)} \tag{S2}$$

$$N_2 = \frac{1}{G_2(0)} \tag{S3}$$

$N_{1,2}$ is the average numbers of molecules with two fluorescent dyes in the detection volume, which is defined as eqn (S4):

$$N_{1,2} = N_1 N_2 G_{1,2}(0) \tag{S4}$$

In this study, cross-correlation amplitude (CC value) is defined as eqn (S5):

$$CC = \frac{N_{1,2}}{N_1} = \frac{G_{1,2}(0)}{G_2(0)}, \text{or} = \frac{N_{1,2}}{N_2} = \frac{G_{1,2}(0)}{G_1(0)} \tag{S5}$$

where $G_1(0)$ and $G_2(0)$ represent the auto-correlation amplitudes of fluorescent molecules in channel 1 and fluorescent molecules in channel 2, and $G_{1,2}(0)$ represents the cross-correlation amplitude in channel 1 and channel 2. The CC value is calculated by taking the lower value of $G_1(0)$ and $G_2(0)$. The CC value is in positive correlation with $N_{1,2}$ (the number of kinase-probe complexes).Thus, the increased CC value illustrates an increase in kinases activity.

The BPP represents the average particle brightness, which is defined as eqn (S6):

$$BPP = \frac{\langle I \rangle}{\langle N \rangle} = \langle I \rangle G(0) \tag{S6}$$

Here, $\langle I \rangle$ is average detected fluorescent intensity and $\langle N \rangle$ is the average number of molecules in the detection volume.

Cell Imaging. HeLa cells were seeded on cover slides in 6-well plates at a density of 1×10^5 cells per well and cultured for 24 h. Cells were incubated with three probes (both final concentrations were 8.0 nM) for 2 h, respectively, rinsed with PBS twice and fixed with 4% paraformaldehyde for 20 min at room temperature. Then the slides were washed with cold PBS three times, treated with 0.1% Triton X-100 in PBS for 10 min and rinsed three times with PBS. The treated cells were observed under fluorescence microscope (LEICA TCS SP8, Germany).

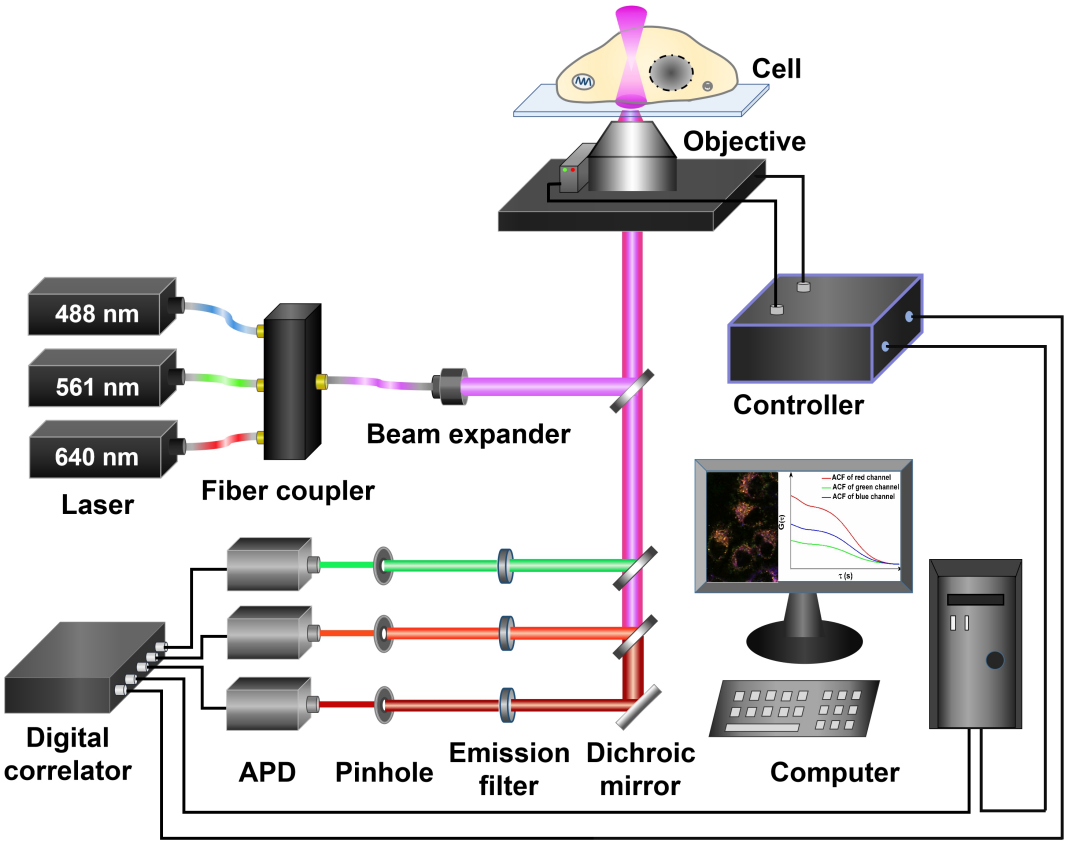

Fig. S1 Schematic diagram of the multi-channel FCS system.

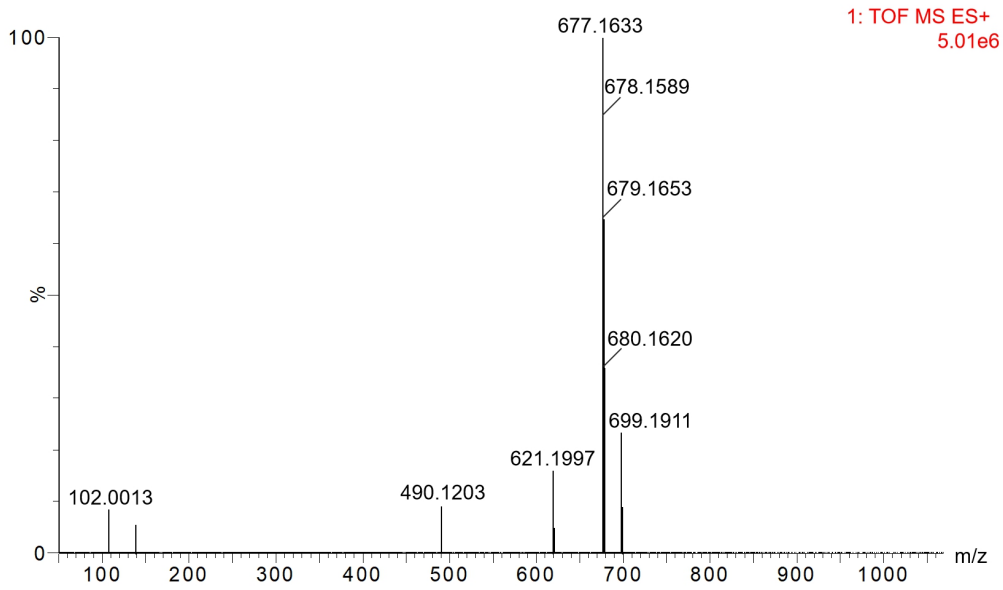

Fig. S2 Characterization of **Compound 1** by mass spectrometry.

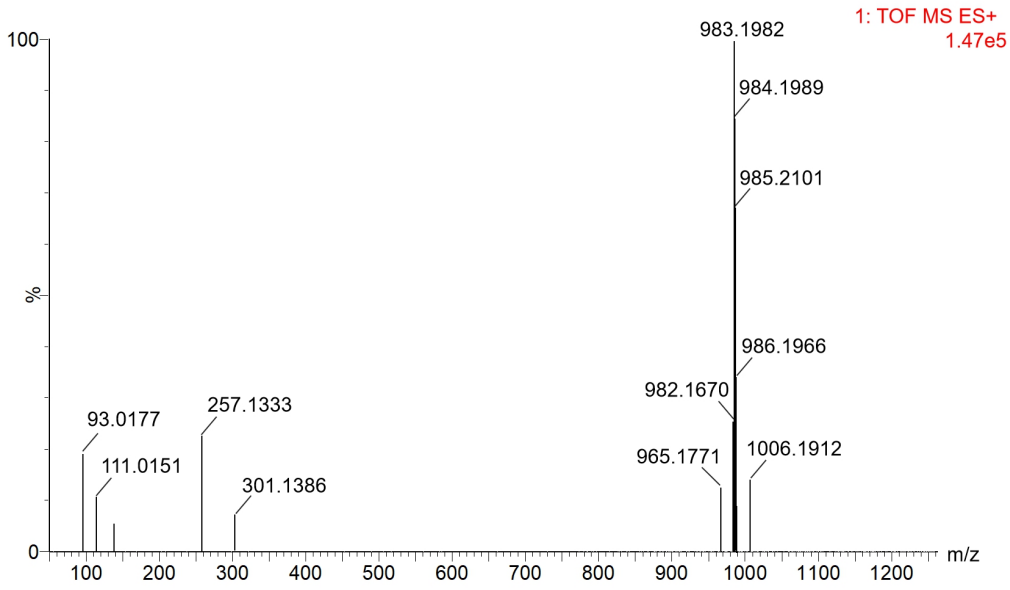

Fig. S3 Characterization of **Probe 1** by mass spectrometry.

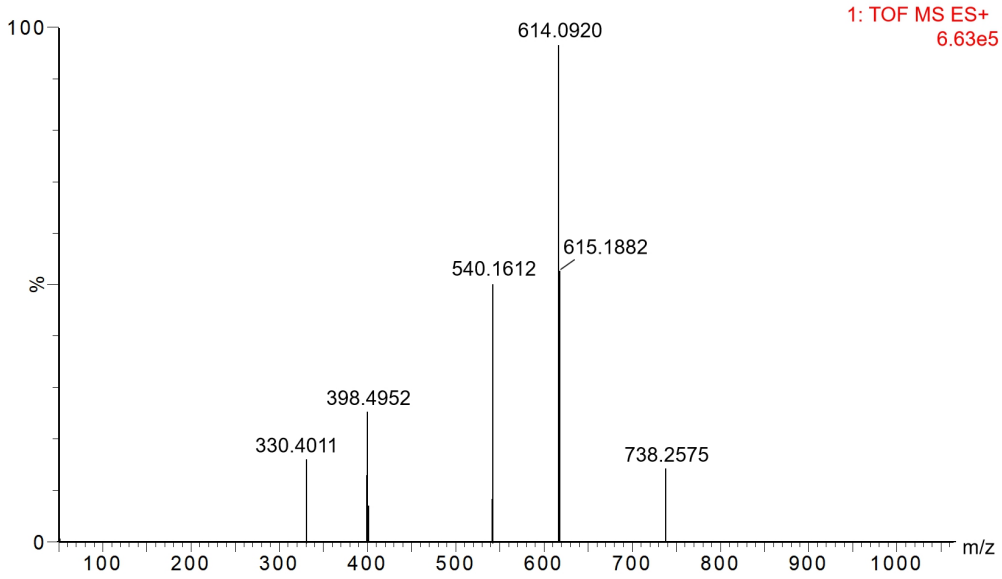

Fig. S4 Characterization of **Compound 2** by mass spectrometry.

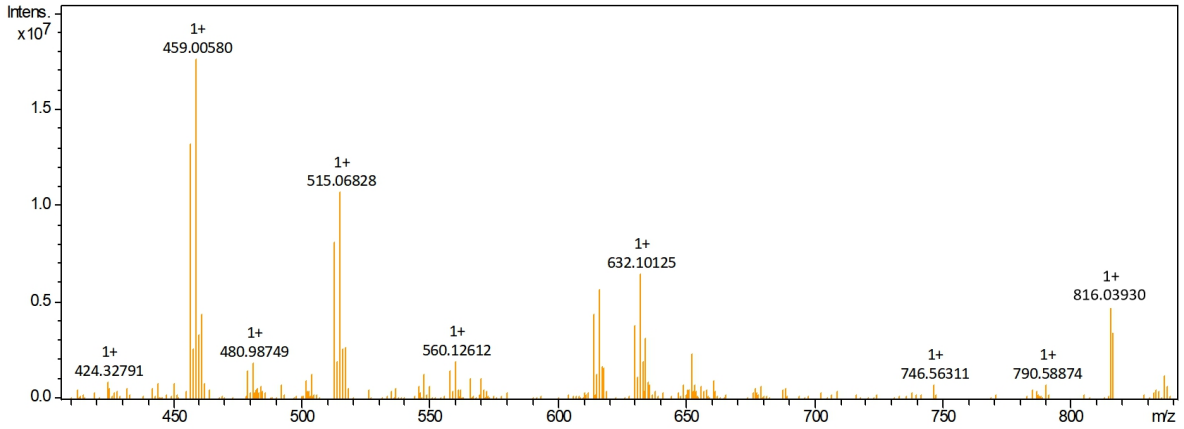

Fig. S5 Characterization of **Probe 2** by mass spectrometry.

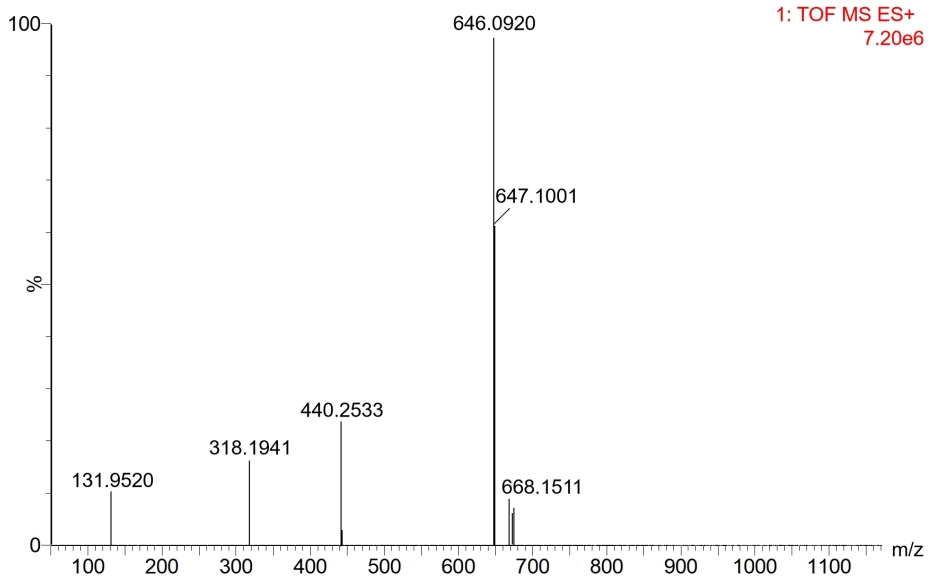

Fig. S6 Characterization of **Compound 3** by mass spectrometry.

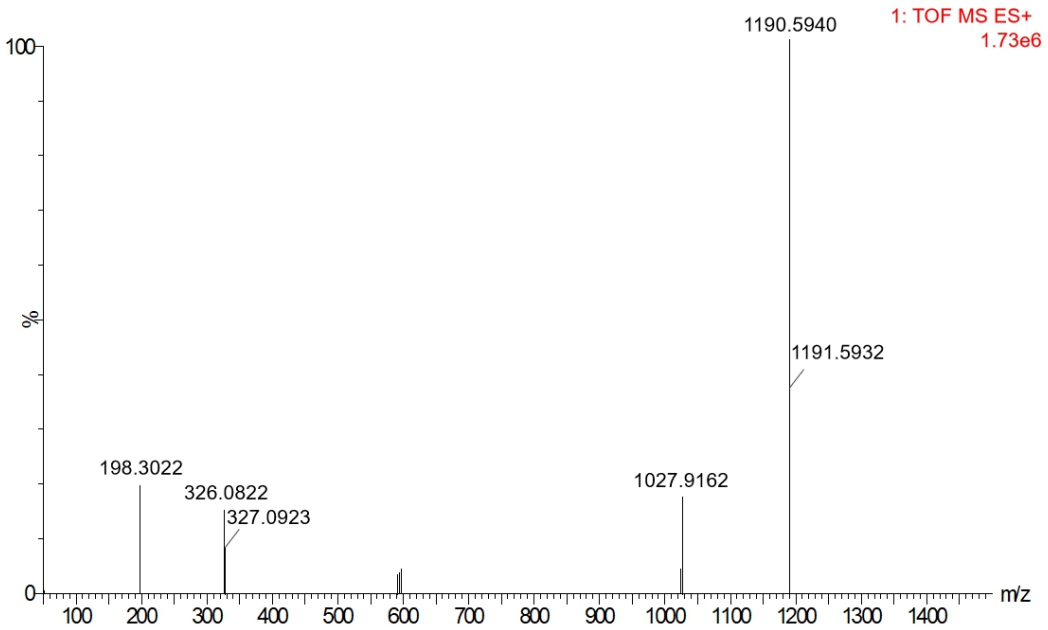

Fig. S7 Characterization of **Probe 3** by mass spectrometry.

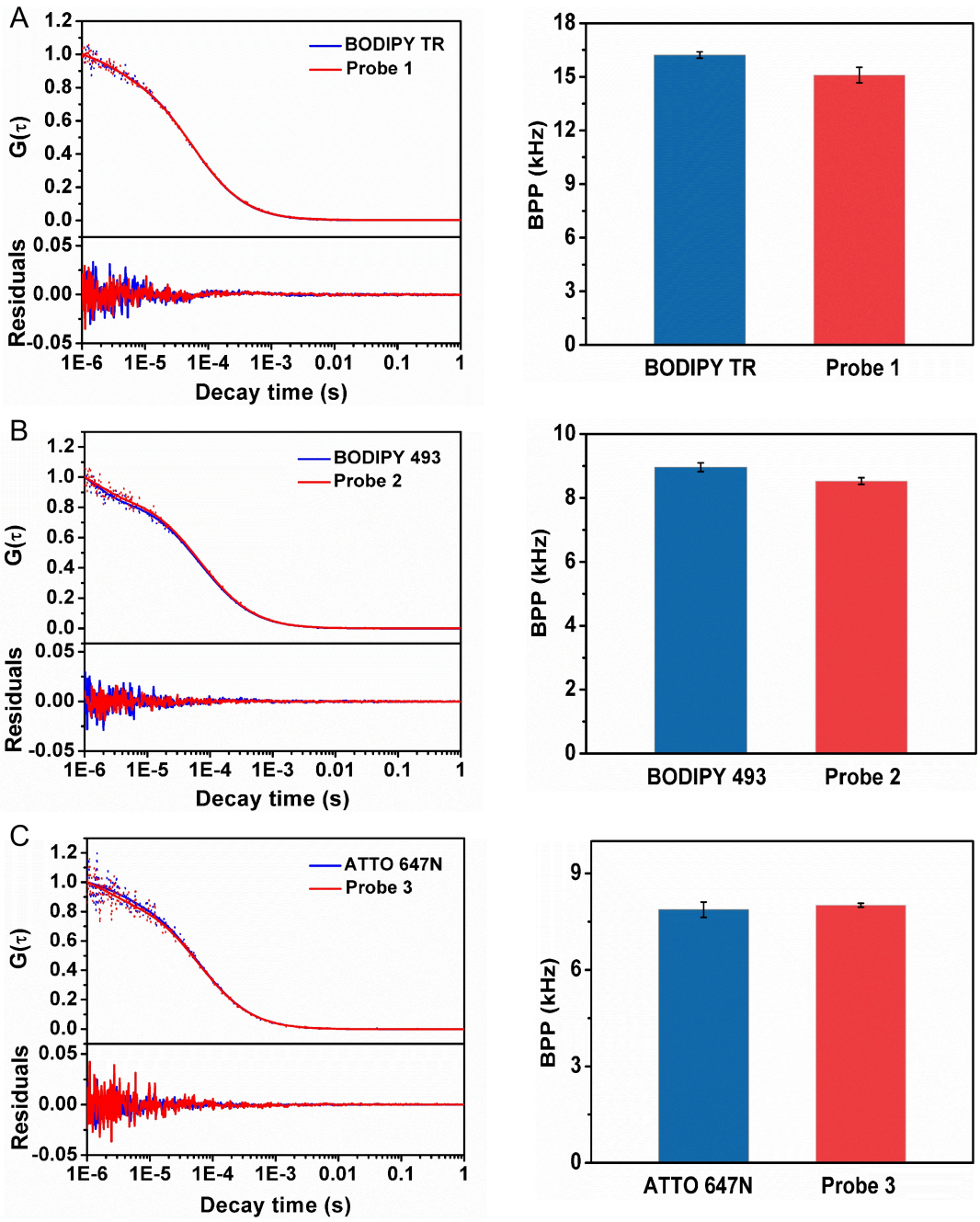

Fig. S8 The autocorrelation curves, fitting residuals and brightness per molecule (BPP) values of probes and free dyes obtained by FCS. (A) Probe 1 and BODIPY TR; (B) Probe 2 and BODIPY 493; (C) Probe 3 and ATTO 647N. The error bars represent the standard deviation of 5 repeated measurements.

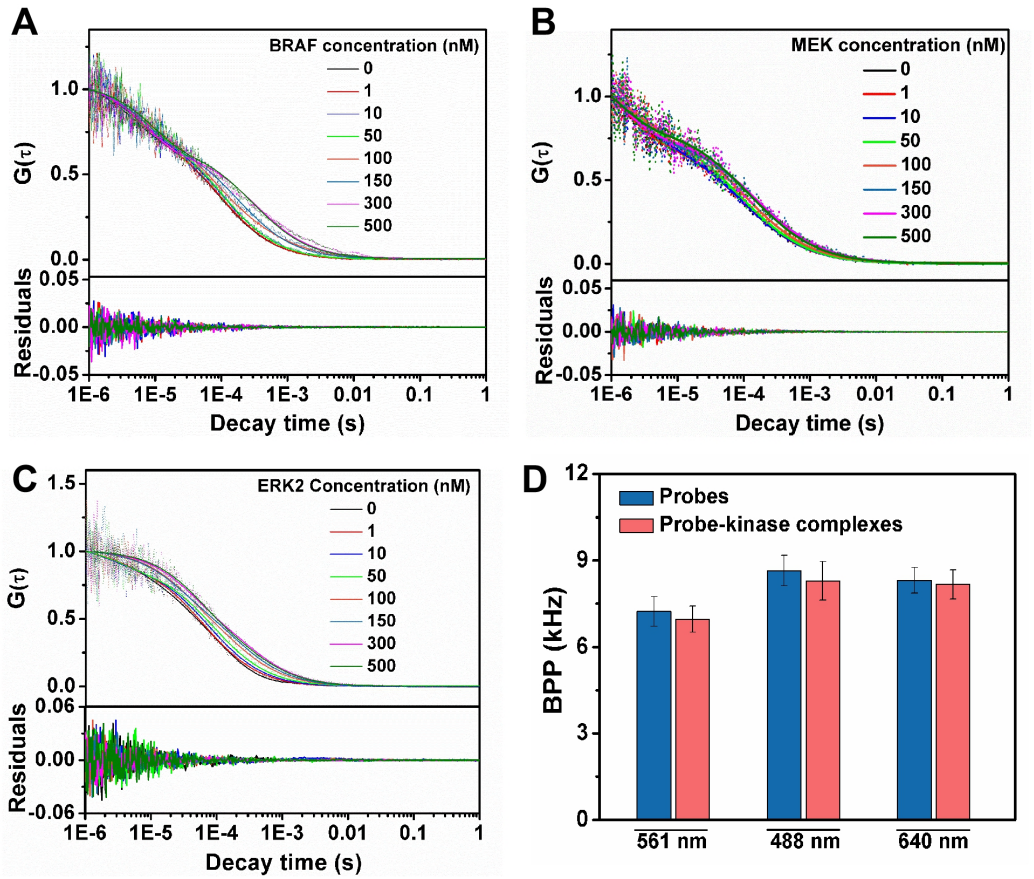

Fig. S9 Normalized FCS curves and fit residuals of probes at different concentrations of corresponding kinases. (A) Probe 1 and BRAF; (B) Probe 2 and MEK1; (C) Probe 3 and ERK2. (D) Comparison of the BPP values between probes and probe-kinase complexes, the error bars represent the standard deviation of 5 repeated measurements.

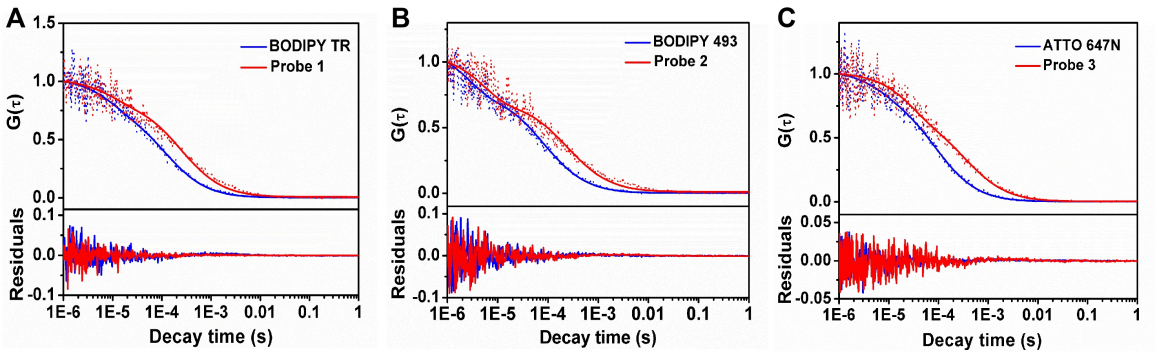

Fig. S10 Normalized FCS curves and fit residuals of probes and free dyes in HeLa cells lysate. (A) Probe 1 and BODIPY TR; (B) Probe 2 and BODIPY 493; (C) Probe 3 and ATTO 647N.

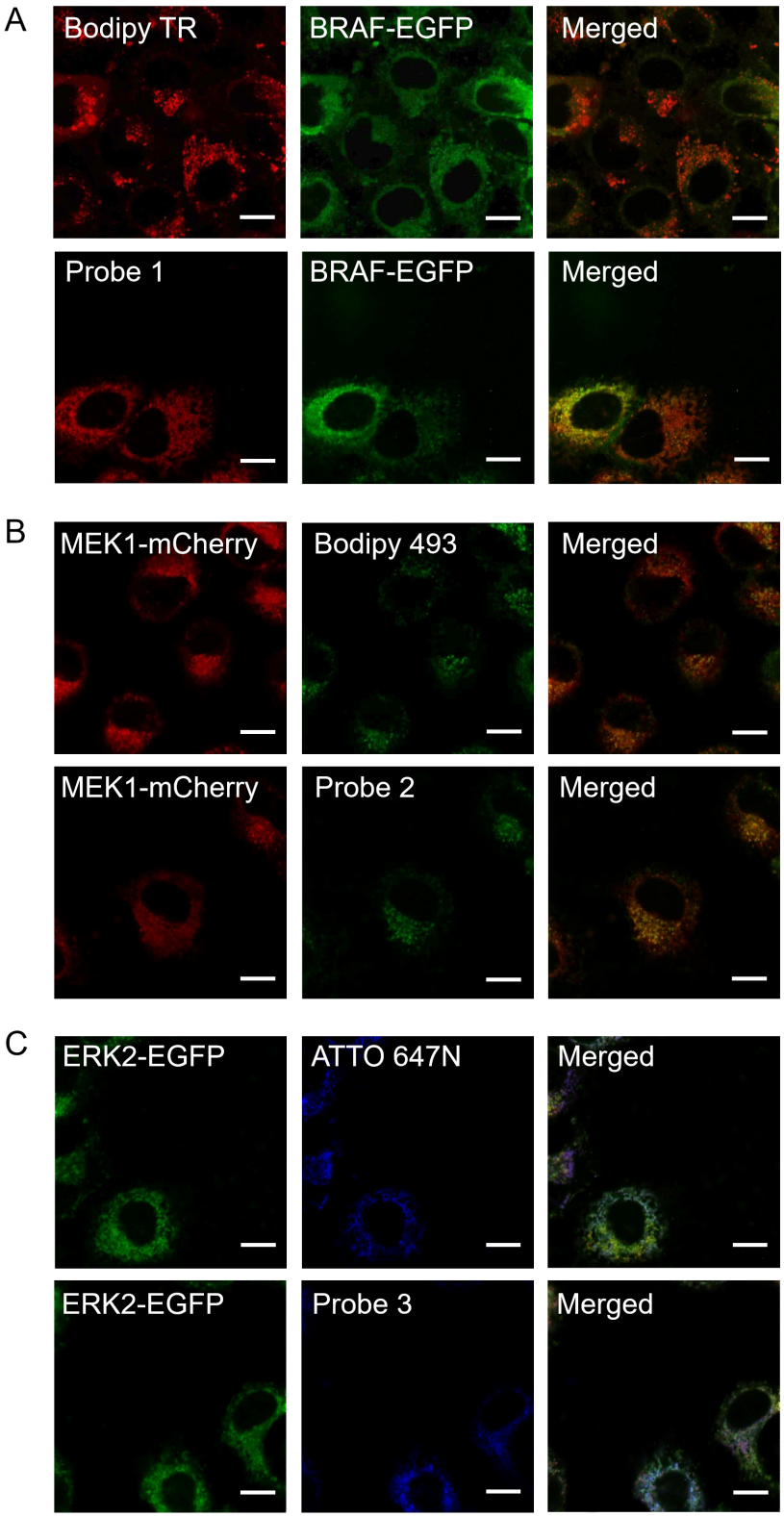

Fig. S11 CLSM images of HeLa cells for FCCS measurements. Scale bar = 10 μm.

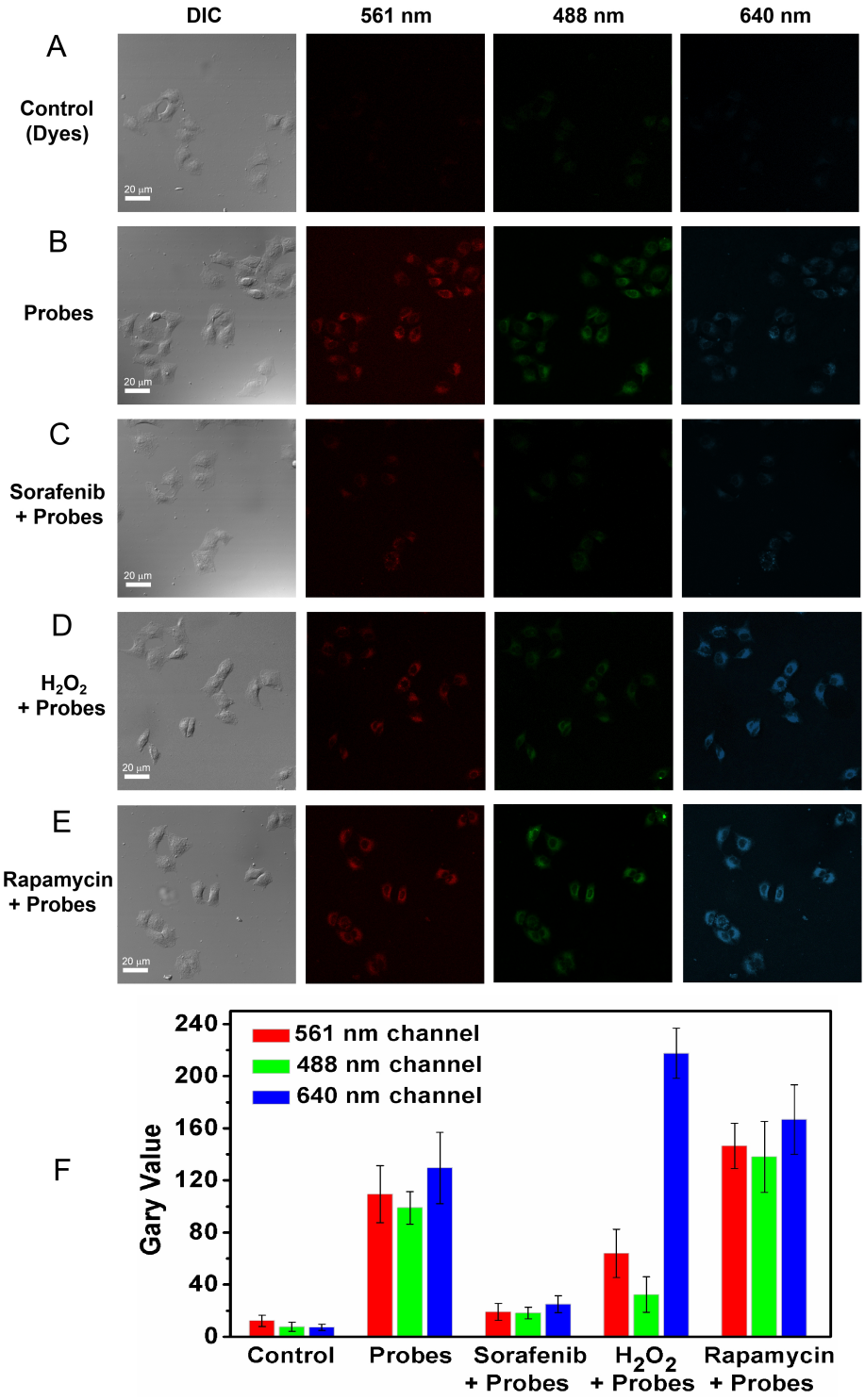

Fig. S12 CLSM images of HeLa cells incubated with (A) BODIPY TR, BODIPY 493 and ATTO 647N; (B) Probe 1, 2 and 3; (C) sorafenib and three probes; (D) H_2O_2 and three probes; (E) rapamycin and three probes; (F) the fluorescence intensity of cells under different incubation conditions.

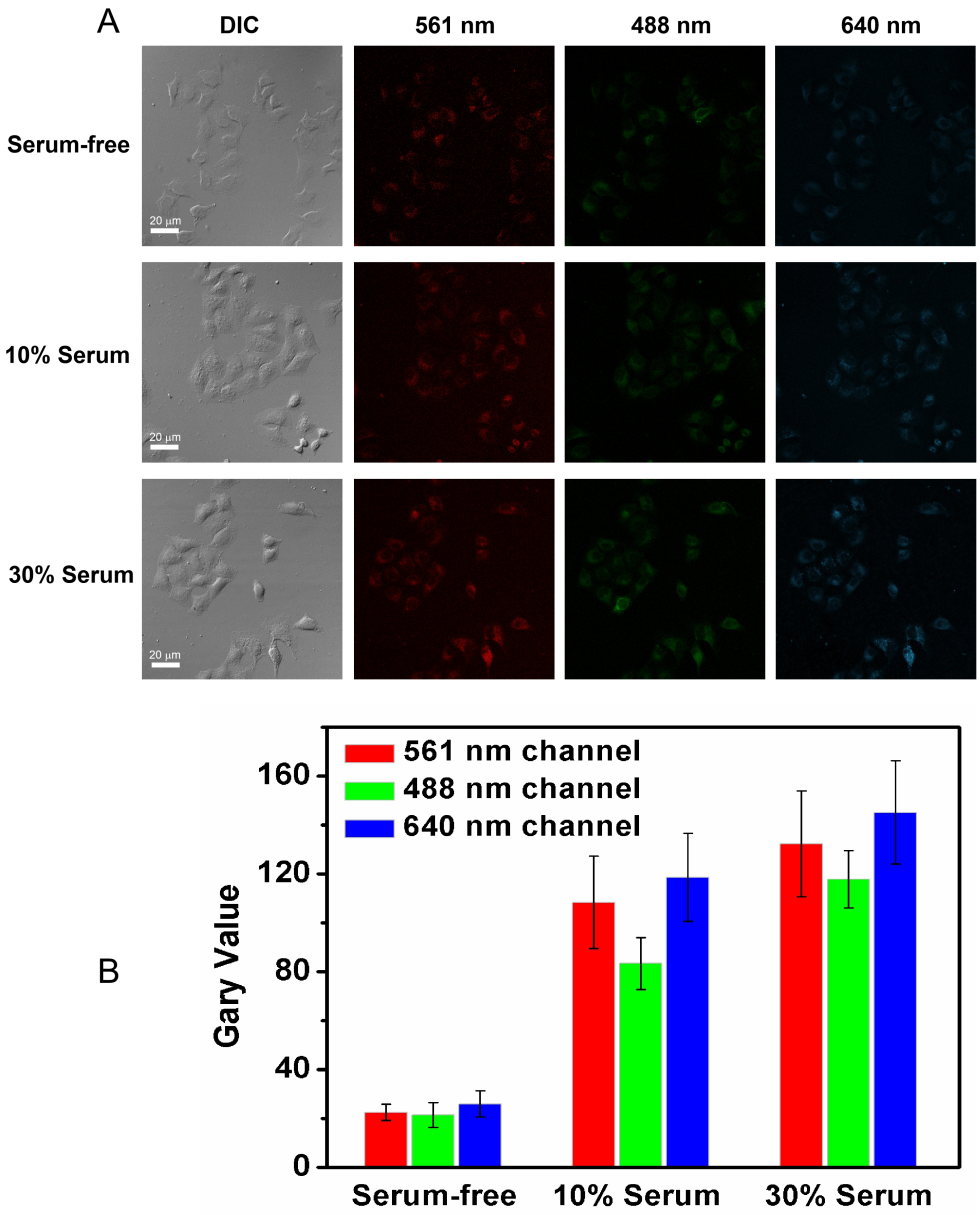

Fig. S13 (A) CLSM images of HeLa cells cultured with medium containing different volume fraction of serum; (B) the fluorescence intensity of cells incubated with medium containing different volume fraction of serum.

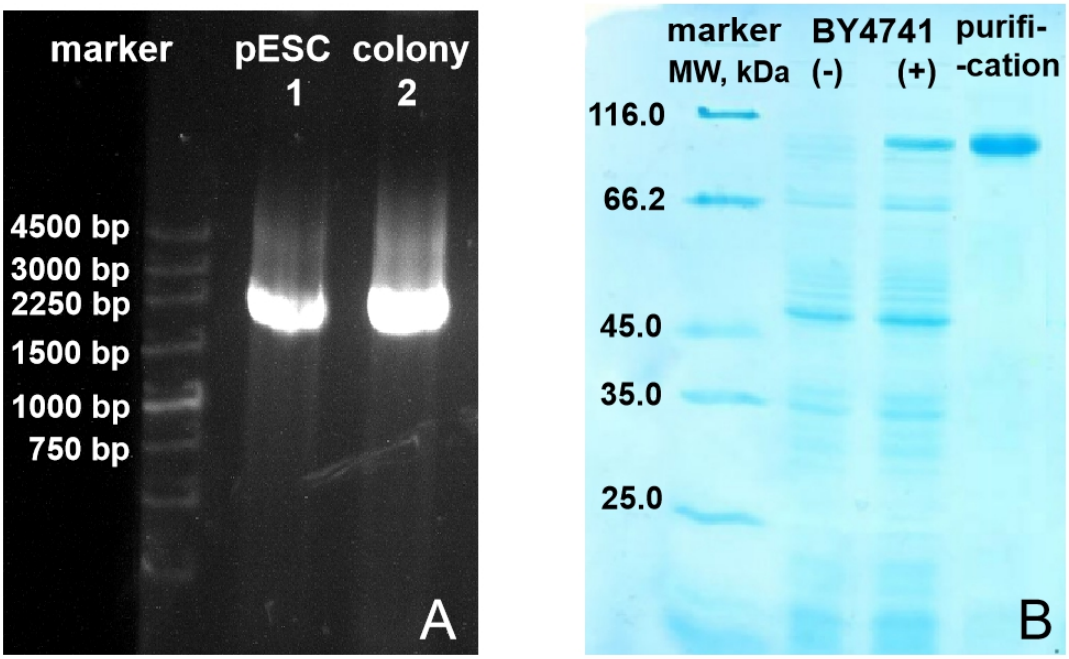

Fig. S14 (A) Transformation of recombinant plasmid pESC-HIS-BRAF in BY4741 yeast colony judged by PCR. (B) The recombinant protein BRAF-HIS idendified by SDS-PAGE and stained with Coomassie Brilliant Blue (+: cultued with 2% galactose to induce protein expression, -: cultured without galactose).

Notes and References

1 M. Helmuth, W. Altrock, T. M. Böckers, E. D. Gundelfinger and M. R. Kreutz, *Anal. Biochem.,* 2001, **293**, 149-152 .
2 K. Bacia and P. Schwille, *Nat. Protoc*., 2007, **11**, 2842-2856.