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A novel hydrosoluble near-infrared fluorescence probe for specifically monitoring of tyrosinase and application in living systems

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1. Apparatus and reagents

Ultraviolet-visible (UV-vis) absorption spectra in this work was recorded on Hitachi U-3010 spectrophotometer (Kyoto, Japan). Fluorescence intensity was collected on a Hitachi F-7000 with excitation and emission slit widths of 10 nm. Electrospray ionization mass spectrometry (ESI-MS) was performed in a Shimadzu LC-MS 2010A instrument (Kyoto, Japan). ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded with Bruker DMX-600 spectrometer (Dissolved in CD_3OD). Fluorescence quantum yield was measured with Hamamatsu C9920-02G Quantum Efficiency Measurement. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) analysis was recorded on a microplate reader (BioTek Synergy HT, Winooski, VT, U.S.A.). Fluorescence imaging of cells was conducted on a confocal laser scanning microscope (Leica, Germany) with 635 nm excitation. In vivo imaging was performed on IVIS Lumina LT Series III.

IR-783 iodide was purchased from Sigma-Aldrich. Phosphorus tribromide and 3-Hydroxybenzyl alcohol were purchased from J&K. The MTT were obtained from Invitrogen. RPMI 1640, fetal bovine serum (FBS), phosphate buffered saline (PBS) solution, penicillin (100 μg/mL), and streptomycin (100 μg/mL) were purchased from HyClone (South Logan, UT, U.S.A.). Flash column chromatography and thin layer chromatography (TLC) analysis were performed with silica gel (200-300 mesh). The water used in the experiment is Milli-Q ultra-pure water, and all other reagents used are analytical pure. B16 cells, HepG2 cells and BALB/c mice were obtained from the Experimental Animal Center of the Fourth Military Medical University (Xi'an, China). All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Shaanxi Normal University and approved by the Animal Ethics Committee of Shaanxi Normal University.

2. Synthesis of probe 1

2.1 Synthesis of fluorophore 2. To a stirred solution of resorcinol (110 mg, 1.0 mmol) in N,N-Dimethylformamide (DMF, 5 mL) was added potassium carbonate (K_2CO_3 , 138 mg, 1.0 mmol)

at 40 °C under nitrogen atmosphere, and the resulting mixture was stirred for 20 min. Then, a solution of IR783 (374.5 mg, 0.5 mmol) in DMF (5 mL) was added to the above mixture via a syringe, and the reaction mixture was heated at 50 °C for 5 h. Eventually the solvent was evaporated under reduced pressure, and the crude product was purified by flash column chromatography (dichloromethane/MeOH = 20:1) on silica gel, affording the desired compound **2** as a violet solid. The fluorophore was characterized by ESI-MS m/z: calculated for fluorophore ($C_{29}H_{32}NO_5S^+$, [M]+), 506.19; found, 506.20 (Fig. S1).

2.2 Synthesis of compound 3. First of all, the 248 mg (2.0 mmol) 3-Hydroxybenzyl alcohol were added to the 5 mL trichloromethane, then dropped a solution of 270 mg (1.0 mmol) phosphorus tribromide in reaction mixture via a syringe over a period of 30 min at 0 °C. After complete addition, the reaction mixture was warmed up to ambient temperature and stirred for 2 h. Then the reaction mixture was diluted with trichloromethane (20 mL). The organic layer was separated, washed with water and brine three times, and then dried over dry Na_2SO_4. The solution was dried under reduced pressure to yield yellow oil that was used without further purification.

2.3 Synthesis of probe 1. First of all, dissolve the fluorophore **2** (253 mg, 0.5 mmol) and K_2CO_3 (103.5 mg, 0.75 mmol) in DMF and the above mixture was stirred at 40°C for 20 min under the nitrogen atmosphere. After that, the solution of compound **3** (92.5 mg, 0.5 mmol) in DMF was added to the mixture dropwise via a syringe. The reaction mixture was allowed to stir at 50 °C for 5 h. Following, the crude product was then purified using flash chromatography (dichloromethane/MeOH = 20:1) on silica gel to yield (*E*)-2-(2-(6-((3-hydroxybenzyl) oxy)-2,3-dihydro-1*H*-xanthen-4-yl) vinyl)-3,3-dimethyl-1-(4-sulfobutyl)-3*H*-indol-1-ium (**1**) as a violet solid. The probe **1** was characterized by NMR (Fig. S2 and S3). ESI-MS m/z: calculated for probe **1** ($C_{36}H_{38}NO_6S^+$, [M]+), 612.24; found, 612.25 (Fig. S4).

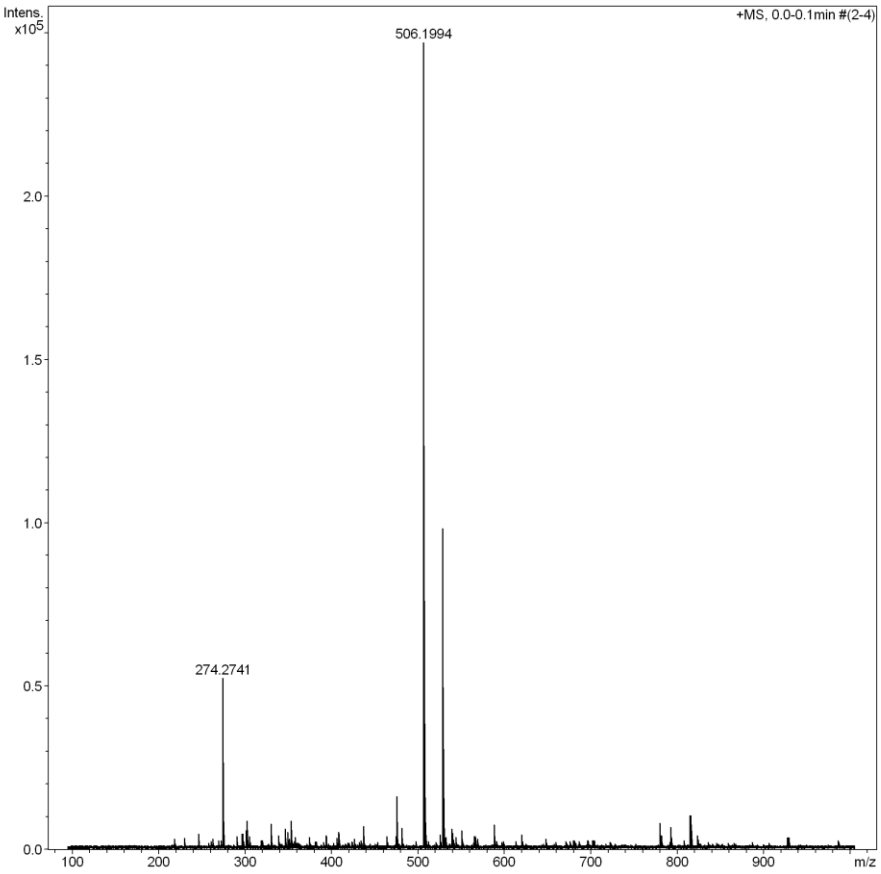

Fig. S1 ESI-MS of the fluorophore **2**

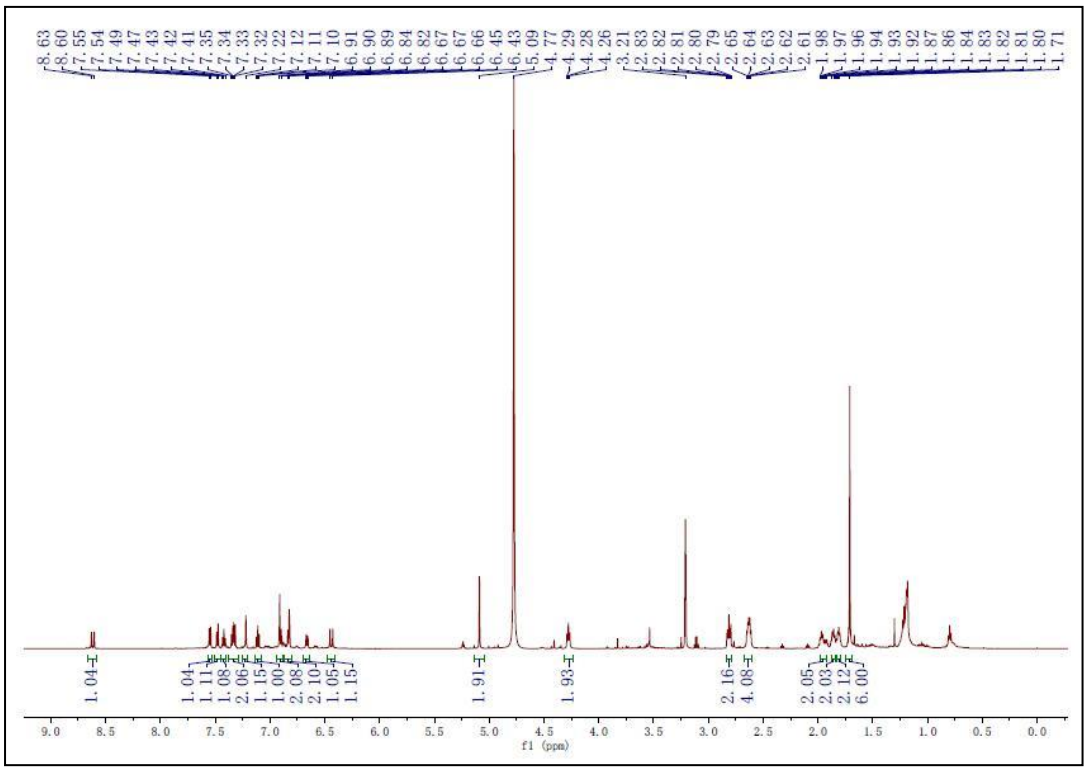

Fig. S2 ^{1}H NMR spectrum of probe **1** in CD_3OD.

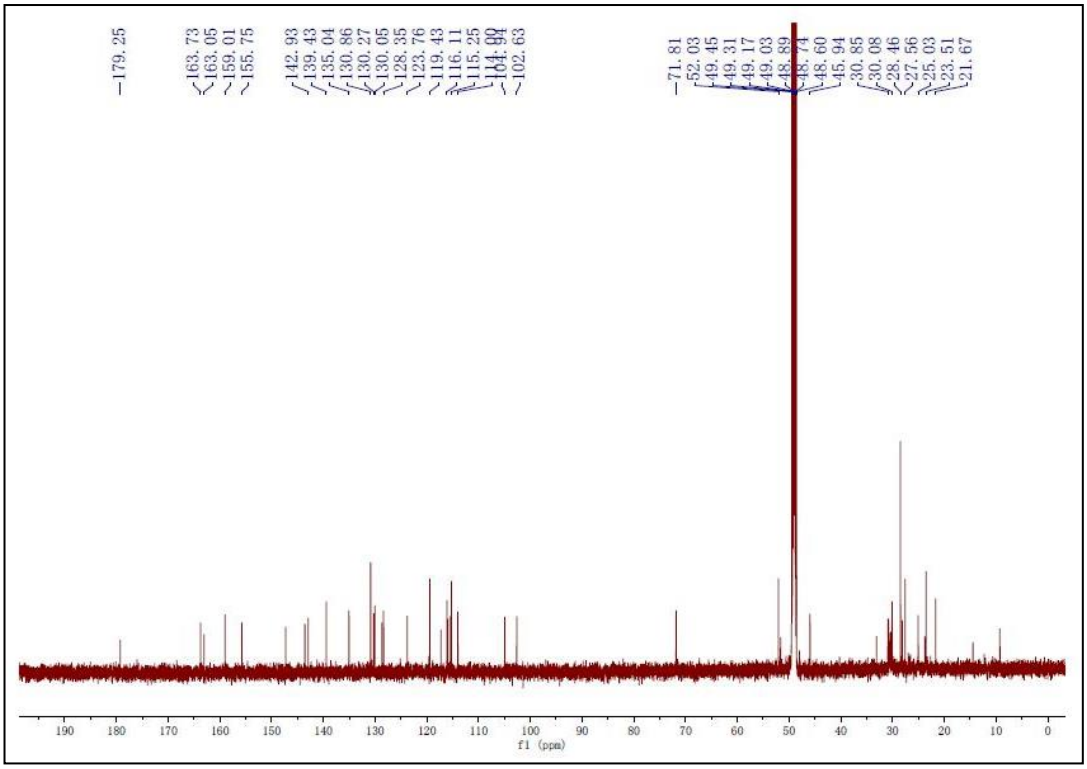

Fig. S3 ^{13}C NMR spectrum of probe **1** in CD_3OD.

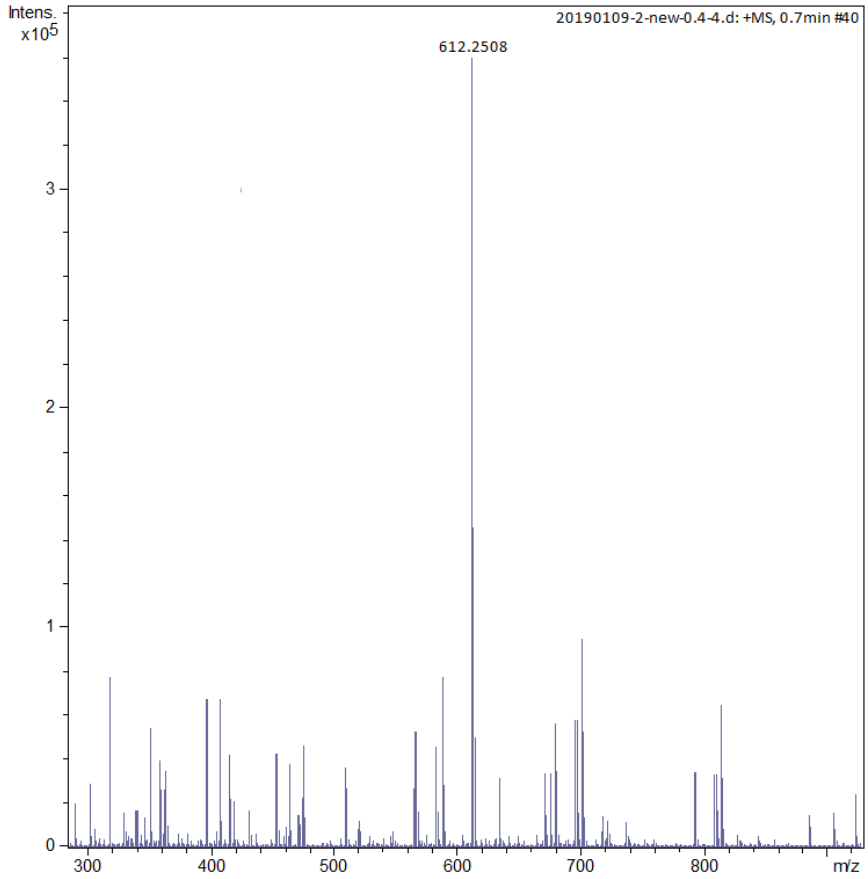

Fig. S4 ESI-MS of the probe **1**

3. General Procedure for TYR Detection

Fluorescent property of probe **1** toward TYR was investigated in 10 mM PBS buffer (pH 7.4). The stock solutions of probe **1** were dissolved in 4 mL of PBS, followed the tested sample was added. Add appropriate PBS up to 5 mL, and fluorescence spectra were recorded after incubating at 37 °C for 3 h. Excitations was at 670 nm and emission was detected at 708 nm.

4. Electrospray ionization mass spectrum of the reaction solution of probe 1

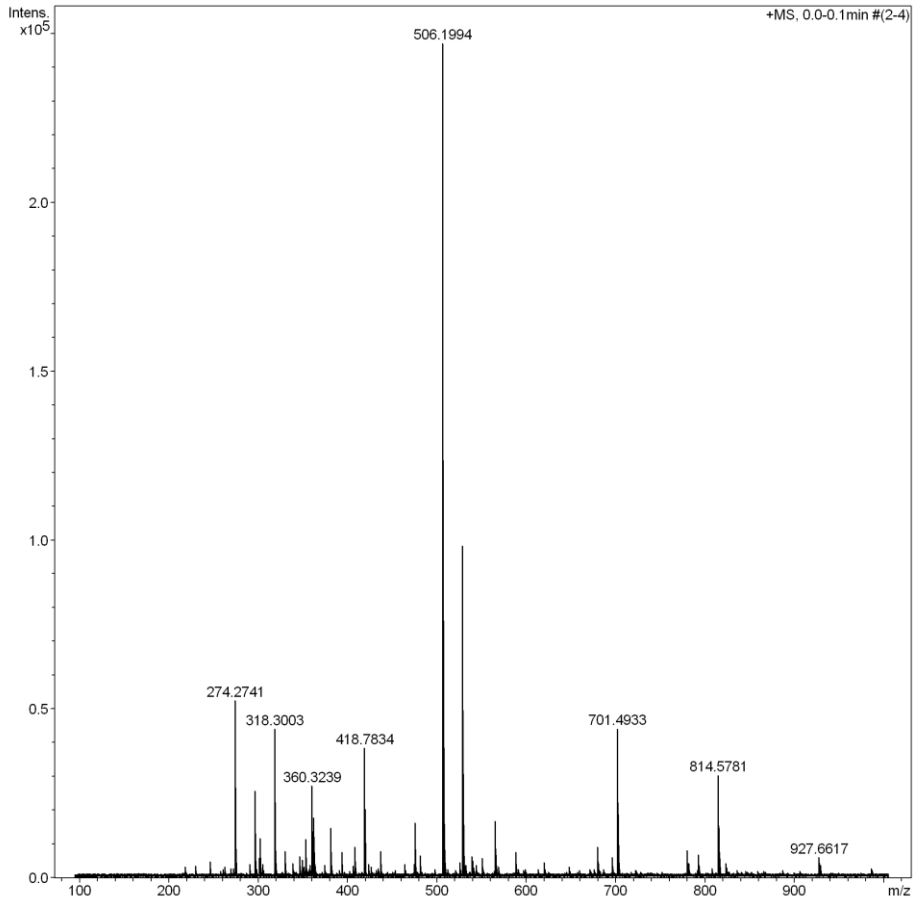

Fig. S5 ESI-MS of the reaction solution of probe **1** (10 μM) with TYR (80 μg/mL).

5. Fluorescence kinetic curves of probe 1 reacting with TYR

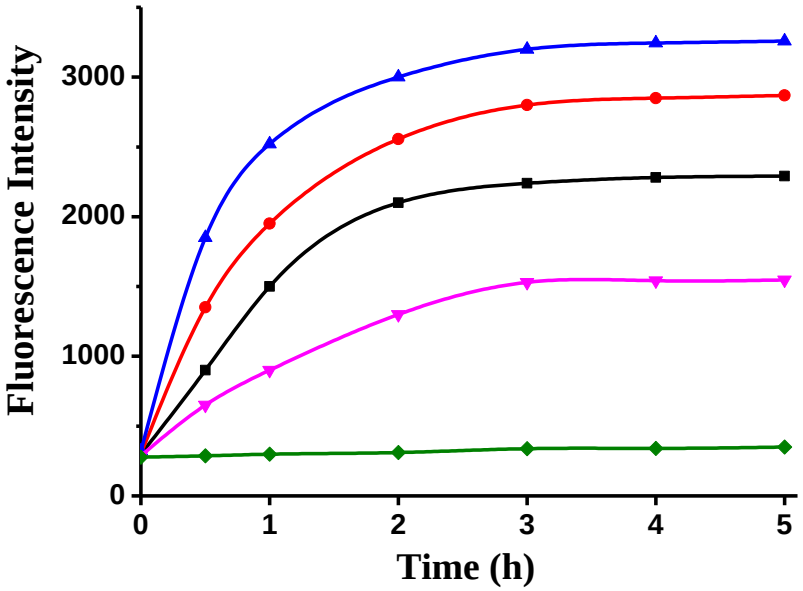

Fig. S6 Plots of fluorescence intensity of probe **1** (10 μM) vs.the reaction time in the presence of varied concentrations of TYR (from bottom to top): 0 (control), 20, 40, 60 and 80 μg/mL. The measurements were performed at 37 °C in 10 mM PBS (pH=7.4) with λex/em= 670/708 nm.

6. Effects of pH and temperature

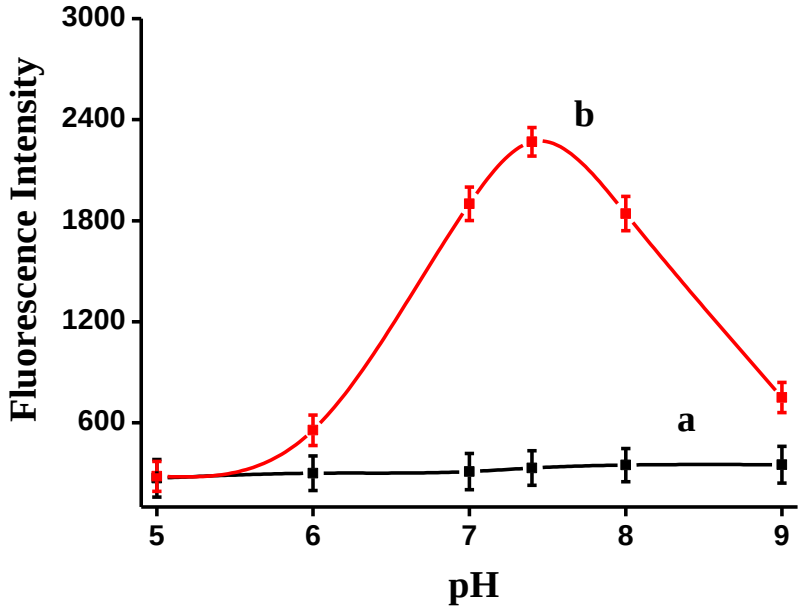

Fig. S7 Effects of pH on the fluorescence of 10 μM probe **1** (a) before and (b) after reaction with TYR (40 μg/mL). The results are the mean ± standard deviation of three separate measurements; λex/em= 670/708 nm.

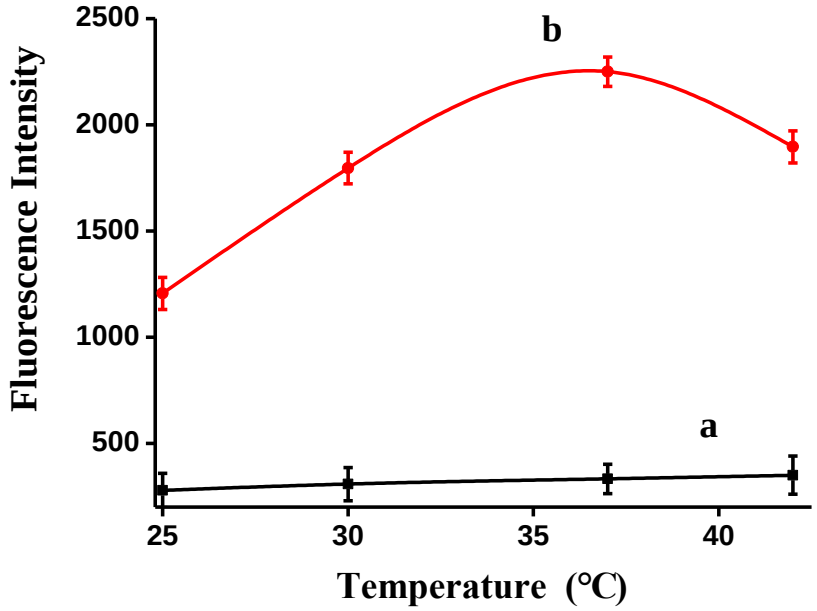

Fig. S8 Effects of temperature on the fluorescence of 10 μM probe **1** (a) before and (b) after reaction with TYR (40 μg/mL). The results are the mean ± standard deviation of three separate measurements; λex/em= 670/708 nm.

7. Selectivity of the probe 1

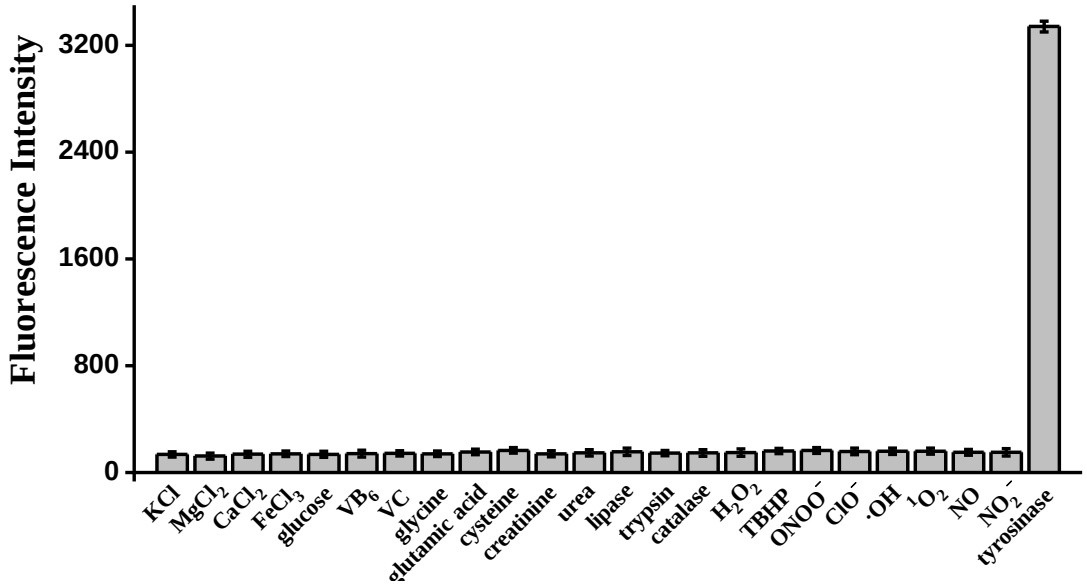

Fig. S9 Fluorescence responses of probe **1** (10 μM) in the presence of various species: 30 μM of KCl, $MgCl_2$, $CaCl_2$, $FeCl_3$, 5 mM of glucose, VB_6, VC, glycine, glutamic acid, cysteine, creatinine, urea, lipase, trypsin, catalase, 30 μM of H_2O_2, TBHP, $ONOO^-$, ClO^-, ·OH, $^1O_2$, NO, NO_2^-. 80 μg/mL of TYR. The results are the mean ± standard deviation of three separate measurements. λex/em= 670/708 nm.

8. Compared with some known TYR fluorescent probes

Authors	Excitation/ Emission	Detection limit	Biological application	Title
Zhou et al.	428/547 nm	7 U L^{-1}	B16 cells	Detection of misdistribution of tyrosinase from melanosomes to lysosomes and its upregulation under psoralen/ultraviolet A with a melanosome-targeting tyrosinase fluorescent probe.
Li et al.	550/583 nm	0.5 U mL^{-1}	B16, HepG2 cells	Highly selective fluorescent probe based on hydroxylation of phenylboronic acid pinacol ester for detection of tyrosinase in cells.
Li et al.	650/720 nm	0.01 U mL^{-1}	/	A near-infrared fluorescent probe for monitoring tyrosinase activity.
Teng et al.	350/610 nm	0.006 U mL^{-1}	/	Ratiometric fluorescence detection of tyrosinase activity and dopamine using thiolate-protected gold nanoclusters.
Wu et al.	550/586 nm	0.04 U mL^{-1}	B16, HepG2, MCF-7 cells	A highly sensitive and selective fluorescence off-on probe for the detection of intracellular endogenous tyrosinase activity.
Li et al. (Present Research)	670/708 nm	0.11 μg mL^{-1}	B16, HepG2 cells, Zebrafish, Mouse	A novel hydrosoluble near-infrared fluorescence probe for specifically monitoring of tyrosinase and application in living systems

Table 1 Compared with some known TYR fluorescent probes.

9. Effects of inhibitors on enzymes and probe 1

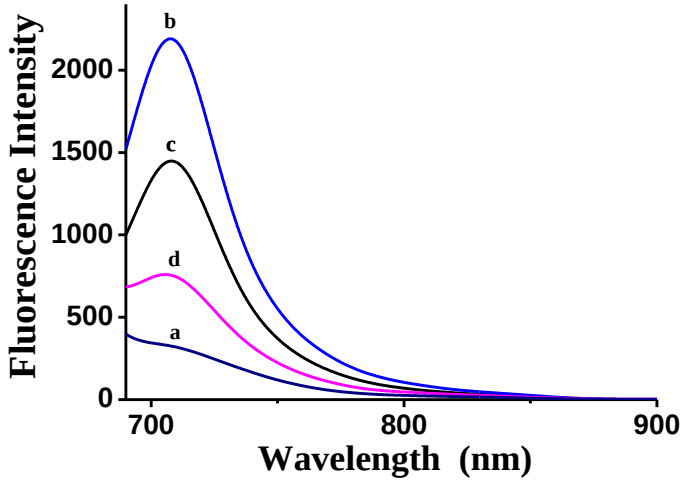

Fig. S10 Fluorescence intensity of different reaction systems. (a): Control (10 μM probe **1** in PBS of pH 7.4); (b): system (A) +TYR (40 μg/mL); (c): system (b)+ kojic acid (100 μM); (d): system (b) + kojic acid (200 μM) The results are the mean ± standard deviation of three separate measurements. λex/em= 670/708 nm.

10. Cytotoxicity assay

Cytotoxicity of probe **1** or fluorophore **2** for B16 cells were evaluated at different concentrations via MTT assay according to the previous report.

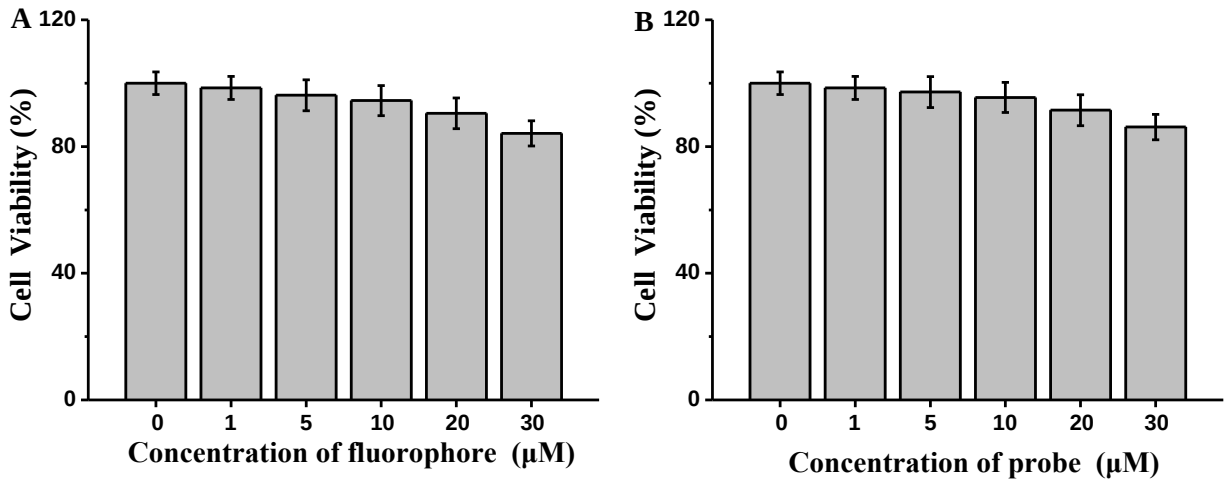

Fig. S11 Effects of (A) fluorophore **2** and (B) probe **1** with varied concentrations (10 μM) on the viability of B16 cells. The viability of the cells without probe **1** or fluorophore **2** is defined as 100%. The results are the mean ± standard deviation of six separate measurements.

11. Fluorescence Imaging of TYR in Cells

The cells (B16, HepG2) were cultured on the glass-bottom culture dishes in RPMI 1640 medium supplemented with heat-inactivated fetal bovine serum (10%), penicillin (100 μg/mL) and streptomycin (100 μg/mL) in a humidified environment containing 5% CO_2 and 95% air at 37 °C, respectively. Prior to use, the adherent cells were washed with fetal bovine serum (FBS)-free RPMI 1640. For imaging of TYR in living cells, we took the fluorescent images of the cells that incubated with probe **1** (10 μM) at different time (0, 0.5, 1, 2, 3 and 4 h) individually.

In order to verify whether the fluorescence came from the reaction of probe with TYR, we conducted the following kojic-acid-involved experiments. In brief, the cells were incubated with 200 μM of kojic acid for 1 h at 37 °C before fluorescence imaging, then washed with PBS three times,

and further incubated with 10 μM probe **1** for 3 h. The pixel intensity collected from fluorescence image at least 10 cells were measured using Image J software.

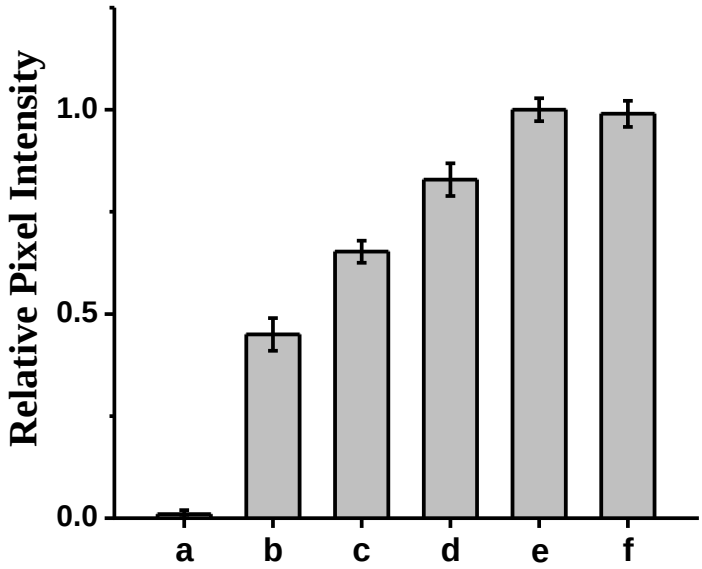

Fig. S12 Relative pixel intensity measurements obtained from the images of B16 cells: the B16 cells were incubated with the 10 μM probe **1** for (a) 0 h. (control). (b) 0.5 h. (c) 1 h. (d) 2 h. (e) 3 h. (f) 4 h. The strongest fluorescence intensity from the image (e) is defined as 1.0. The results are the mean ± standard deviation of three separate measurements.

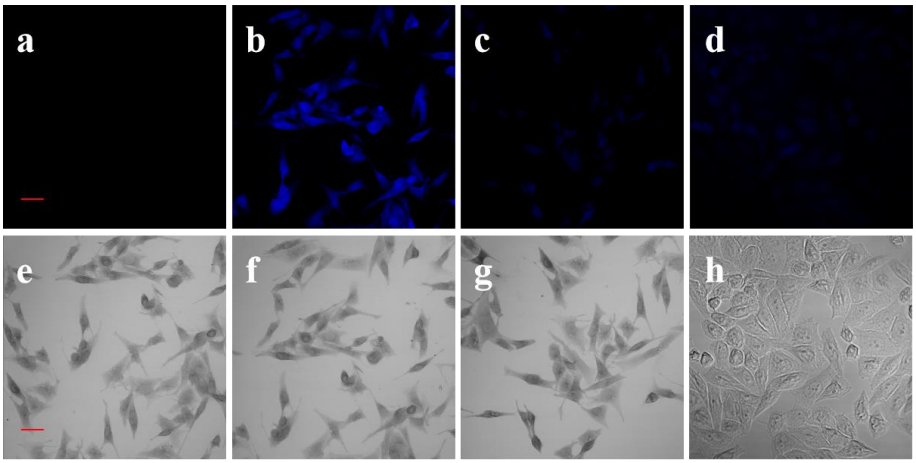

Fig. S13 Confocal fluorescence images of endogenously stimulated TYR in B16 cells. (a) B16 cells only (control). (b) B16 cells were incubated with only 10 μM probe **1** for 3 h. (c) B16 cells were pretreated with 200 μM kojic acid for 1 h, then incubated with 10 μM probe **1** for 3 h. (d) HepG2 cells treated with 10 μM probe **1** for 3 h. The differential interference contrast (DIC) images of the corresponding samples are shown below them (panels e-h). Scale bar: 20 μm.

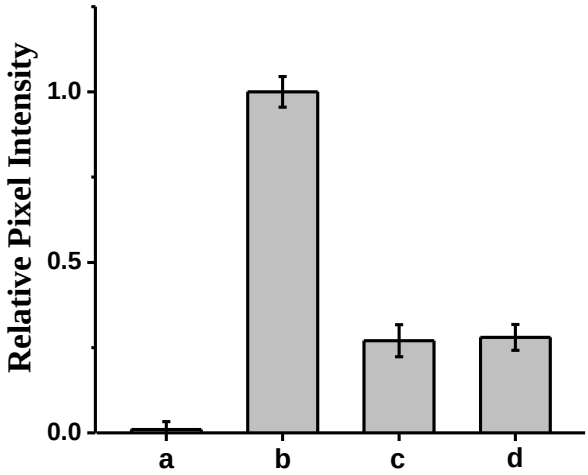

Fig. S14 Relative pixel intensity measurements obtained from the images of cells: (a) B16 cells only (control); (b) B16 cells incubated with probe **1** (10 μM) for 3 h; (c) B16 cells were pretreated with kojic acid (200 μM) for 1 h, and then incubated with probe **1** (10 μM) for 3 h. (d) HepG2 cells treated with 10 μM probe **1** for 3 h. The strongest fluorescence intensity from the image (b) is defined as 1.0. The results are the mean ± standard deviation of three separate measurements.

12. Fluorescence Imaging of TYR in living zebrafish

All zebrafish experiments were in full compliance with international ethics guidelines. In fluorescence imaging experiments. Then the zebrafish were divided into 3 groups. The first group of zebrafish treated with probe **1** (10 μM) for 3 h. Then the second group zebrafish were preincubated with 100 μM kojic acid for 1 h and treated with probe **1** (10 μM) for 3 h. The last group of zebrafish were preincubated with 200 μM kojic acid for 1 h and treated with probe **1** (10 μM) for 3 h. The pixel intensity collected from fluorescence image were measured using Image J software.

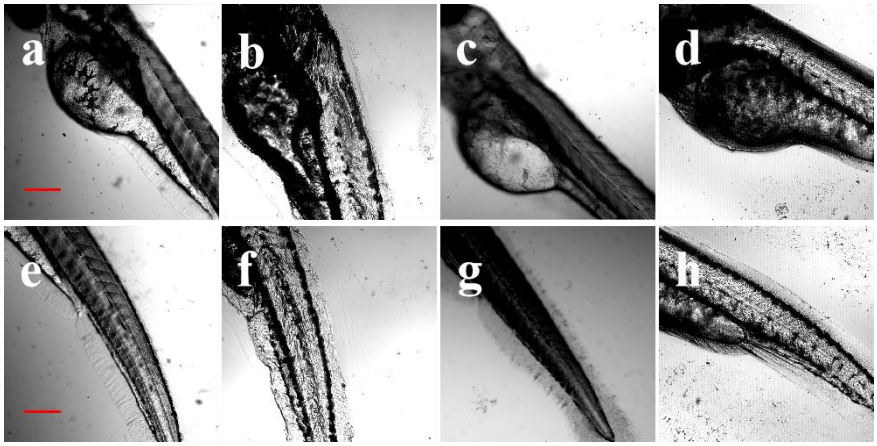

Fig. S15 The DIC images of the corresponding zebrafish are shown in panels a-h. Scale bar=200 μm.

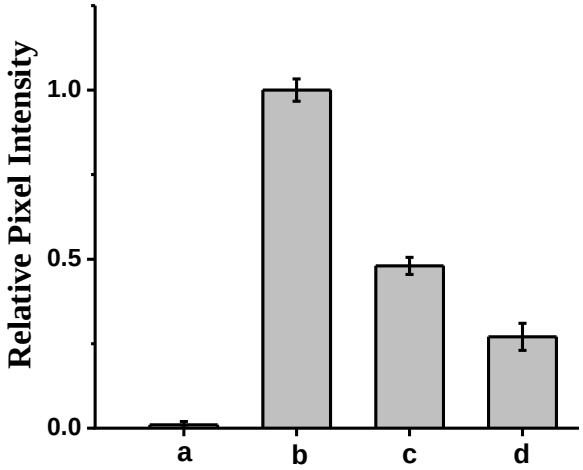

Fig. S16 Relative pixel intensity measurements obtained from the images of zebrafish: (a) Zebrafish only (control); (b) The zebrafish were incubated with probe **1** (10 μM); (c) The zebrafish were pretreated with kojic acid (100 μM) for 1 h and followed incubated with probe **1** (10 μM) for 3 h; (d) The zebrafish were pretreated with kojic acid (200 μM) for 1 h and followed incubated with probe **1** (10 μM) for 3 h. The strongest fluorescence intensity from the image of the zebrafish were incubated with probe **1** (10 μM) is defined as 1.0. The results are the mean ± standard deviation of three separate measurements.

13. Fluorescence imaging of TYR in mouse models

To generate tumor xenografts in mouse, B16 cells were subcutaneously implanted under the right rear thigh of each five-week old BALB/c nude mouse. Then the mouse was divided into 2 groups. The first group of mouse received an intravenous injection of probe **1** (1 mM, 100 μL) at different times (0, 60, 90, 120, 180 min). Mouse in group 2 was pretreated with kojic acid (1 mM, 100 μL) for 60 min, and followed by intravenous injection of probe **1** (1 mM, 100 μL) at different times (0, 60, 90, 120, 180 min). All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Shaanxi Normal University and approved by the Animal Ethics Committee of Shaanxi Normal University.

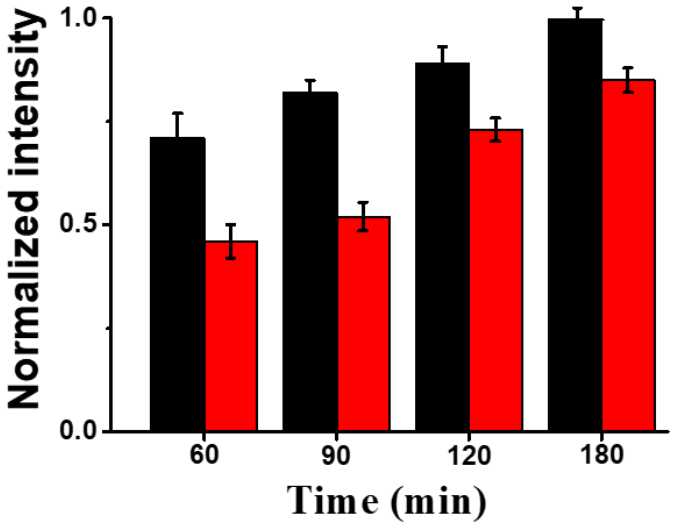

Fig. S17 The quantified fluorescence intensity measurements obtained from the images of mouse: The first group of mouse (black bar) received an injection of probe **1** (1 mM, 100 μL) by tail vein at different times (0, 60, 90, 120, 180 min). The second group of mouse (red bar) was pretreated with kojic acid (1 mM, 100 μL) for 60 min, and followed by injection of probe **1** (1 mM, 100 μL) into the same region at different times (0, 60, 90, 120, 180 min). The results are the mean ± standard deviation of three separate measurements.