Electronic Supporting Information (ESI)

Fast Tyrosinase Detection in Early Stage Melanoma with Nanomolar Sensitivity using Naphthalimide-based Fluorescent Read-out Probe

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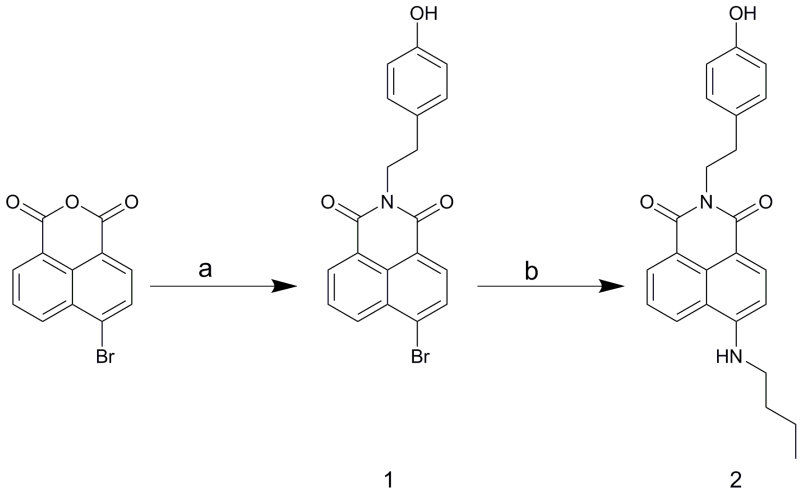
Experimental Methods:



1 2

(a) 4-(2-aminoethyl)phenol, EtOH, Reflux, 6 h, 78 %; (b) butan-1-amine, dry DMSO, 110^{o}C, overnight, 56 %

Synthesis:

100 mg (0.36 mmol) of 4-Bromo-1,8-naphthalic anhydride (**1**) and 52 mg (0.37 mmol) of 4-(2-aminoethylphenol) were added in a round bottom flask and then to it 10 ml of dry ethanol was added. After the addition of dry ethanol, the reaction was allowed to reflux for 6 h. The progress of the reaction was monitored by TLC. After completion of the reaction the ethanol was evaporated under reduced pressure and the pure compound was isolated by column chromatography with 20 % ethyl acetate-hexane mixture. Finally, 105 mg of white solid

compound (**2**) was isolated with 78% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.65 (dd, J = 7.3, 0.8 Hz, 1H), 8.58 (dd, J = 8.5, 0.9 Hz, 1H), 8.41 (d, J = 7.9 Hz, 1H), 8.05 (d, J = 7.9 Hz, 1H), 7.85 (dd, J = 8.4, 7.4 Hz, 1H), 7.21 (d, J = 8.4 Hz, 2H), 6.76 (d, J = 8.4 Hz, 2H), 4.60 (s, 1H), 4.39 – 4.30 (m, 2H), 2.99 – 2.90 (m, 2H).HRMS (APCI) m/z [M + H^+] calculate for $C_{20}H_{14}BrNO_3$ is 396.0157 Da and found 396.0230 Da.

To a biotage microwave vial 35 mg (0.088 mmol) of compound **2** was taken and the vial was sealed with aluminium cap fitted with teflon by maintaining proper nitrogen atmosphere. Then to the vial 2 ml of dry DMSO was added, to that solution 32 mg (0.45 mmol) of the butan-1-amine was added by using a Hamilton microsyringe. After that the reaction was heated up to 283 K and allowed to react for overnight. The reaction was monitored by TLC and after completion of the reaction the whole reaction mixture was poured in ice-cold water and the crude product was extracted with $CHCl_3$. Finally, 19.2 mg of yellow solid pure compound (**3**, **Tyro1**) was isolated by column chromatography with 20% ethylacetate- hexane mixture (isolated % yield 56).^{1}H NMR (400 MHz, $CDCl_3$) δ 8.56 (d, J = 7.3 Hz, 1H), 8.45 (d, J = 8.4 Hz, 1H), 8.05 (d, J = 8.3 Hz, 1H), 7.60 (t, J = 7.9 Hz, 1H), 6.73 (dd, J = 12.4, 8.4 Hz, 3H), 5.18 (s, 1H), 4.63 (s, 1H), 4.34 – 4.25 (m, 2H), 3.39 (dd, J = 12.3, 6.8 Hz, 2H), 2.96 – 2.86 (m, 2H), 1.83 – 1.72 (m, 2H), 1.00 (t, J = 7.3 Hz, 3H). HRMS (APCI) m/z [M + H^+] calculate for $C_{24}H_{24}N_2O_3$ is 389.1787 Da and found 389.1860 Da.

Compound **5** (**Tyro2**) was synthesized and purified according to the reported procedure.[1]

Enzyme assay:

Tyrosinase from mushroom (Sigma Life Sciences, T3824-25KU, Lot-SLBM7158V), Lyophilized powder, 2687 units/mg of solid. A stock of 500 U/ml was prepared in Potassium Phosphate buffer, pH 6.5. All the measurements have been carried out in Potassium Phosphate buffer, pH 6.5.

For the enzyme inhibition assay with kojic acid, the kojic acid stock was prepared in PBS (50 mM, pH 6.3, 0.2% DMSO). For the inhibition of tyrosinase, we incubated the enzyme with the kojic acid for 20 minutes at room temperature.[2] Followed by the enzyme inhibition, we checked the fluorescence quenching of the probes.

Fluorescence kinetics measurements:

Horiba jobin yvon fluorolog used for steady-state and fluorescence kinetics measurements the integration time is 0.5 seconds. For kinetics measurements the integration time is 0.2 seconds. The fluorescence spectra measurements were started with the addition of dyes. After few seconds of scanning, the programme was paused followed by the addition of mentioned concentration of enzyme. The kinetics spectra were measured thereafter with the least possible time duration to avoid the time lag between the enzyme addition and the spectra measurement.

UV-Vis. measurements:

Shimadzu UV-1800 dual-beam spectrophotometer was used for UV-Vis. measurements. The readings were captured at the interval of 0.2 nm.

Cell culture:

The B16F10 and HeLa cells were grown with regular supplementation of DMEM+10% FBS medium at 310 K and 5% CO_2. For microscopy, the cells were seeded on glass bottom dishes. The **Tyro1** to a final concentration of 1 μM was added to cells and incubated for 15 minutes. Thereafter, the cells were washed with PBS followed by imaging. The LASER of 488 nm was used for the probe excitation and images were captured using Olympus Fluoview FV 3000 confocal microscope.

For the tyrosinase inhibition assay, the cells were incubated 24 hours with kojic acid. Afterward, the cells were washed and treated with **Tyro1** for fluorescence imaging.

Cytotoxicity assay (MTT):

Around 10,000 of B16F10 cells per well were seeded in a 96 well-plate and grown for 24 h in DMEM medium with 10% serum condition. After that compound **Tyro1** and **Tyro2** were added accordingly to get the desired concentration. After 24 h of incubation 20 μL (5 mg/ mL in PBS buffer) of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye solution was added to each well and incubated for 4 h. The media was gently removed from the well and 200 μL of dimethyl sulfoxide (DMSO) was added to each well and allowed to dissolve the purple color crystal. The absorption value at 570 nm was measured in microplate reader. All the measurements were performed in triplicate.

Table S1: Comparative table for tyrosinase detection

S. No.	Reference	Amount of enzyme used / U mL^{-1}	Time required by the probe/ min
1	L. J. Chai, J. Zhou, H. Feng, C. Tang, Y. Y. Huang and Z. S. Qian, *ACS Appl. Mater. Interfac.*, 2015, **7**, 23564-23574	14	20
2	J. Zhou, W. Shi, L. H. Li, Q. Y. Gong, X. F. Wu, X. H. Li and H. M. Ma, *Anal. Chem.*, 2016, **88**, 4557-4564	100	200
3	K. N. Bobba, M. Won, I. Shim, N. Velusamy, Z. G. Yang, J. L. Qu, J. S. Kim and S. Bhuniya, *Chem. Commun.*, 2017, **53**, 11213-11216	100	120
4	X. F. Wu, X. H. Li, H. Y. Li, W. Shi and H. M. Ma, *Chem. Commun.*, 2017 **53**, 2443-2446	100	180
5	C. Y. Zhan, J. T. Cheng, B. W. Li, S. L. Huang, F. Zeng and S. Z. Wu, *Anal Chem.*, 2018, **90**, 8807-8815	100	60
6	F. M. Zhang, L. B. Xu, Q. N. Zhao, Y. Sun, X. H. Wang, P. Y. Ma and D. Q. Song, *Sens. Actuator B Chem.*, 2018, **256**, 1069-1077	~0.4	35
7	**This work**	**5**	**4**

NMR characterization of Tyro1

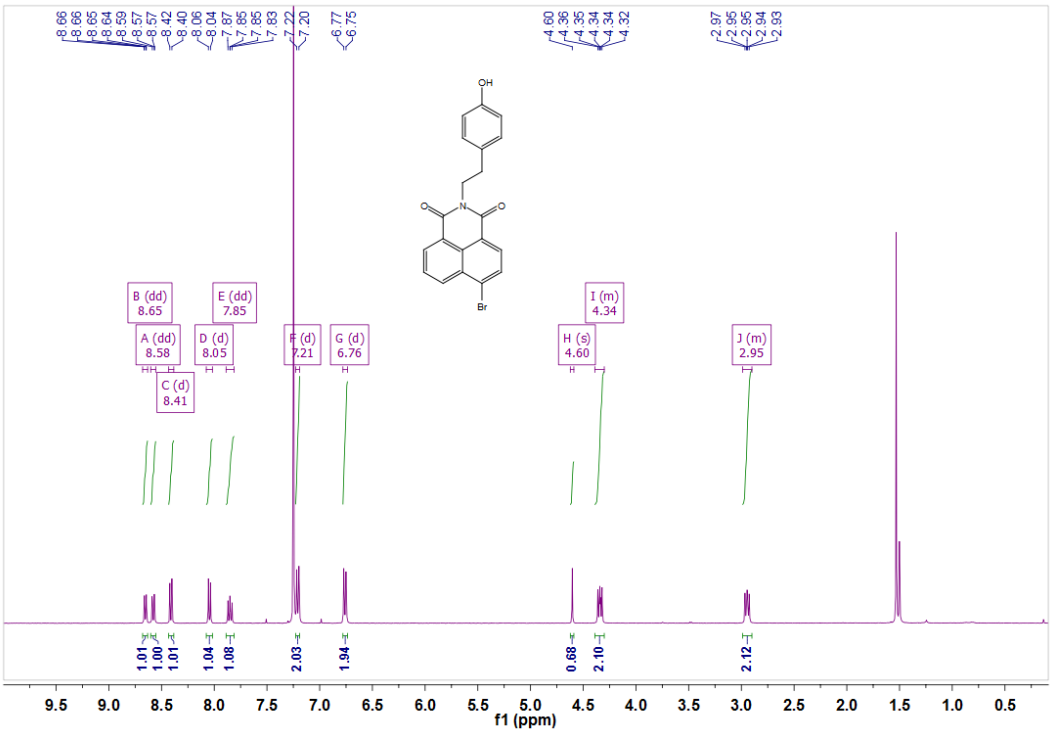


Fig. S1 ^{1}H NMR spectrum of compound **2**

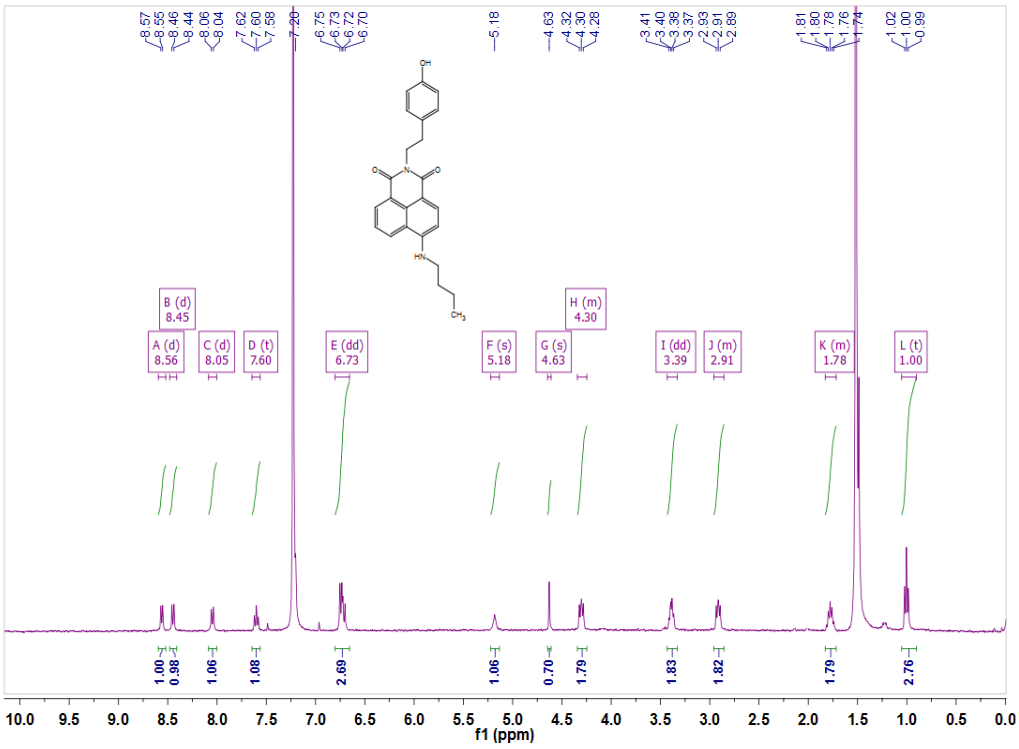


Fig. S2 ^{1}H NMR spectrum of compound **3 (Tyro1)**

Characterization of Tyro1 by mass spectrometry:

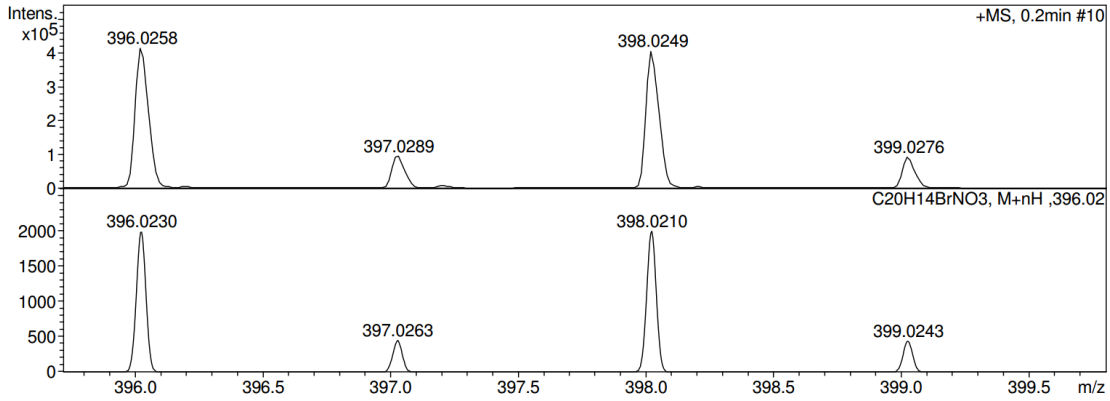


Fig. 3 APCI- HRMS mass spectrum of compound **2**

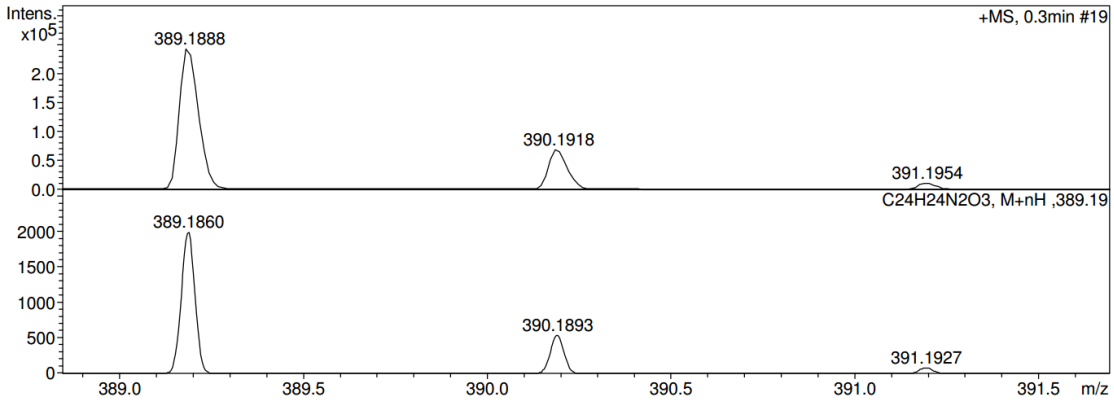


Fig. 4 APCI- HRMS mass spectrum of compound **3 (Tryo1)**

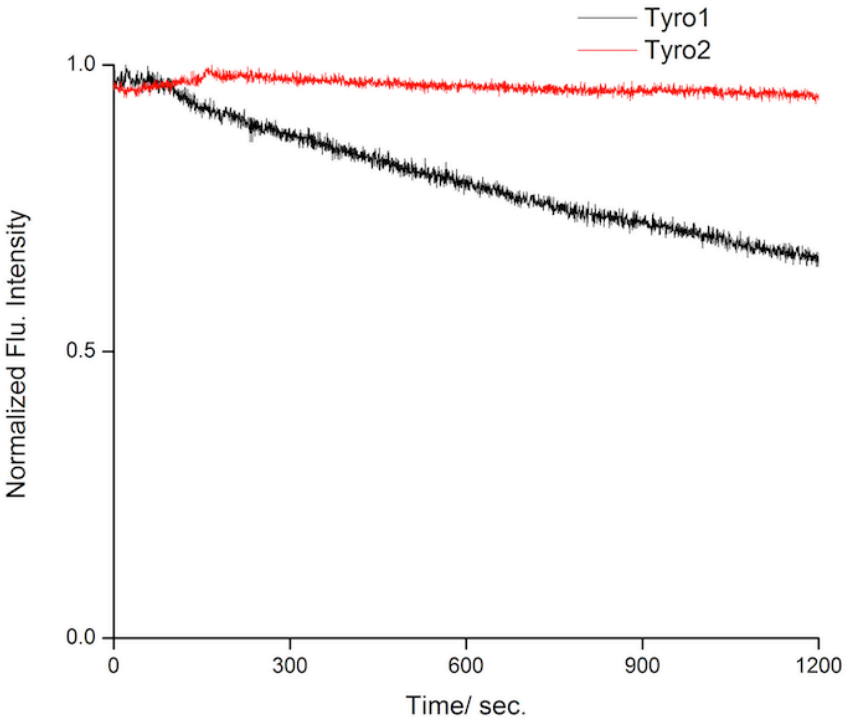


Fig. S5 Photostability of both the dyes upon continuous illumination of Xenon-arc lamp. The concentration of the dyes is 10 μM at temperature 298 K.

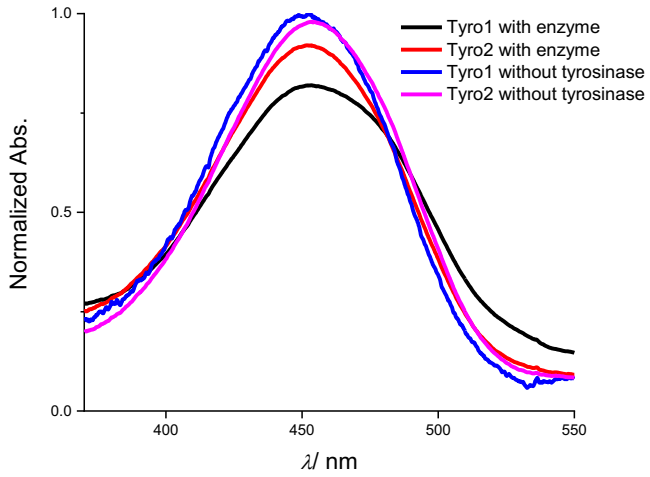


Fig. S6 UV-vis. spectra of with and without probes. The UV-vis. spectra of the dyes, before and after the addition of enzyme. For the dyes only, the PPS buffer is the baseline and for the dyes, the enzyme in the potasium phosphate buffer is the baseline.

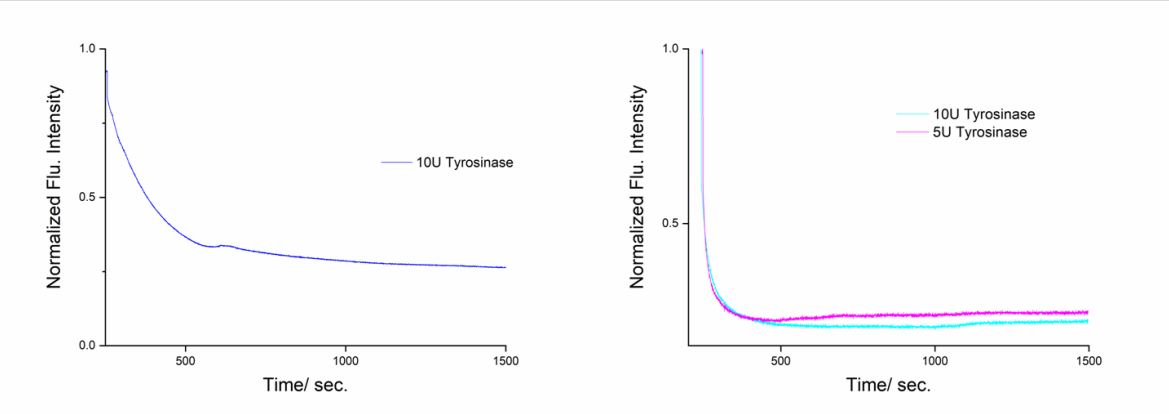


Fig. S7 Fluorescence kinetics for higher units of enzyme at 298 K. The concentration of the probe is kept at 10 μM. The probes are **Tyro1** (left) and **Tyro2** (right).

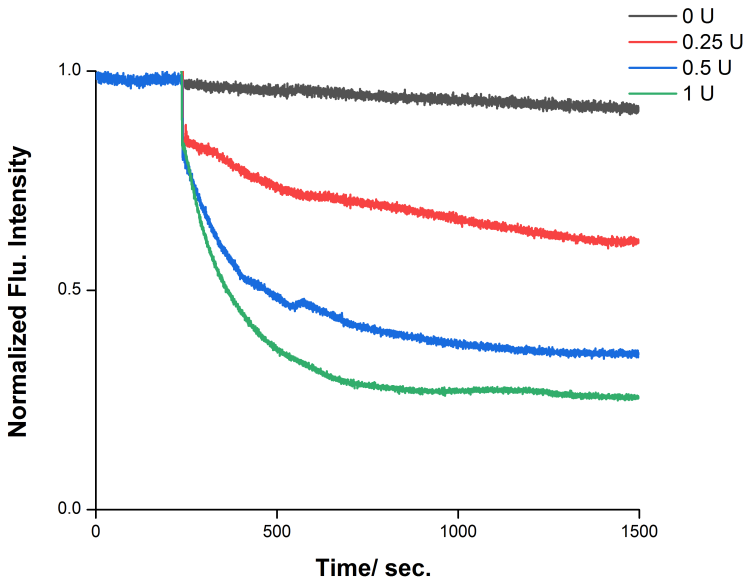


Fig. S8 Fluorescence kinetics for **Tyro2** in absence and presence of different concentration of tyrosinase at 298 K. The concentration of the probe is kept at 10 μM.

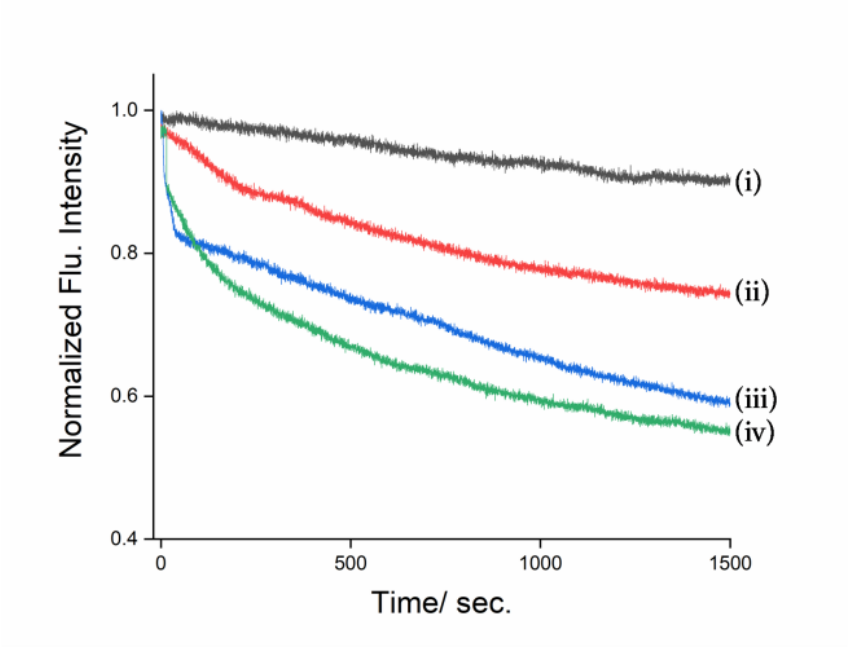


Fig. S9 Tyrosinase inhibition assay using **Tyro2** upon treatment with different concentration of kojic acid. The temperature of the measurement is 298 K. The concentrations of the kojic acid used for the enzyme inhibition are 7.00 mM (i), 3.50 mM (ii), 1.75 mM and (iii) 0.87 mM followed by fluorescence kinetics study.

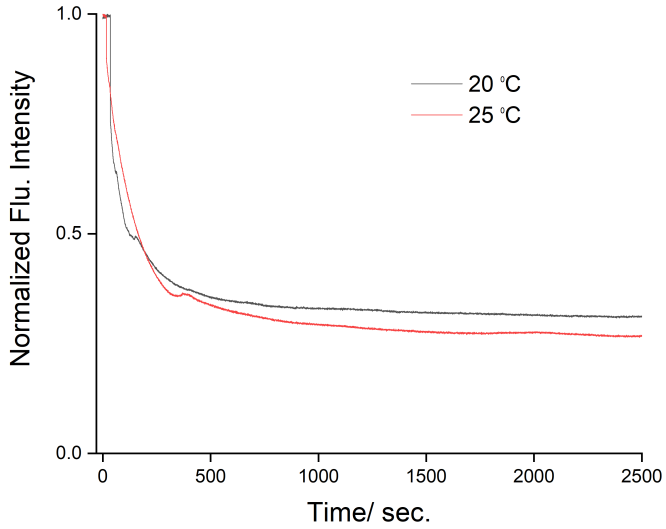


Fig. S10 Temperature-dependent reaction kinetics (10 U tyrosinase) for **Tyro1**

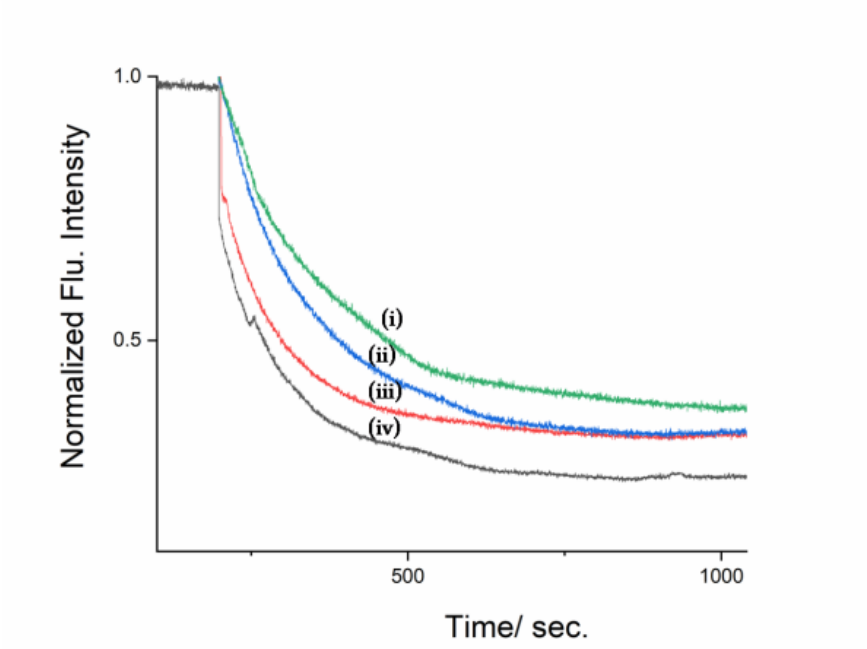


Fig. S11 Temperature dependent activity of 1 U tyrosinase towards fluorescence quenching of 10 μM **Tyro2**. Different temperatures for the fluorescence kinetics are (i) 293 K, (ii) 298 K, (iii) 303 K and (iv) 308 K.

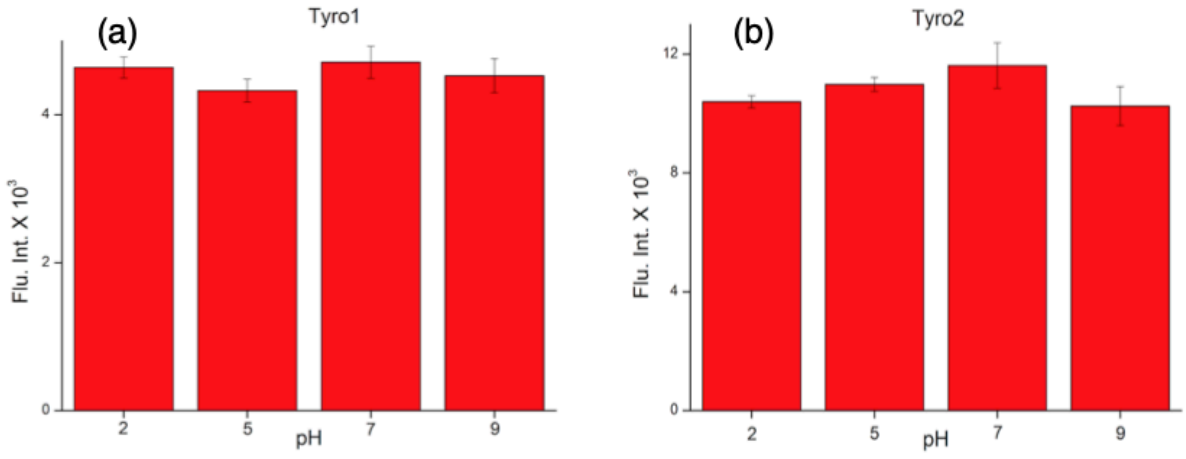


Fig. S12 Effect of pH on the fluorescence intensity of (a) **Tyro1** and (b) **Tyro2**

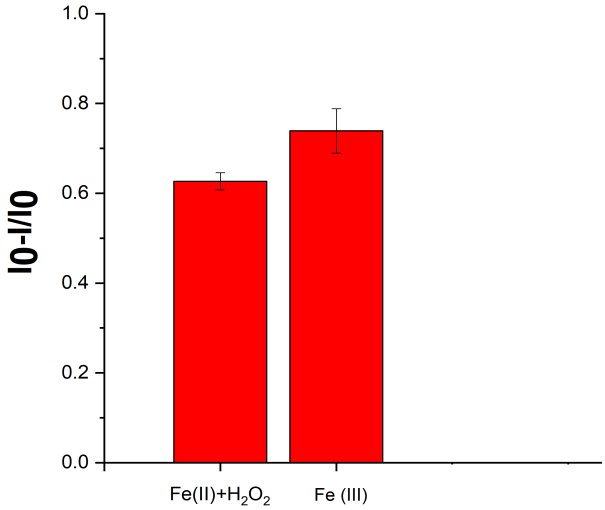


Fig. S13 The fluorescence quenching of **Tyro2** in the presence of Fe^{3+}.

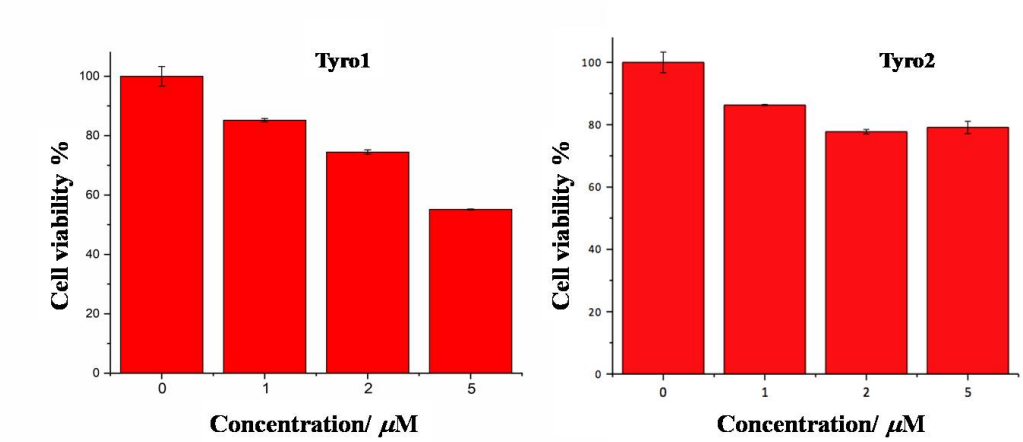


Fig. S14 Cytotoxicity assay of **Tyro1** and **Tyro2.** The plot of cell viability against the concentration of the probe using MMT assay suggest the IC_{50} value is greater than 5 μM for both **Tyro1** and **Tyro2**.

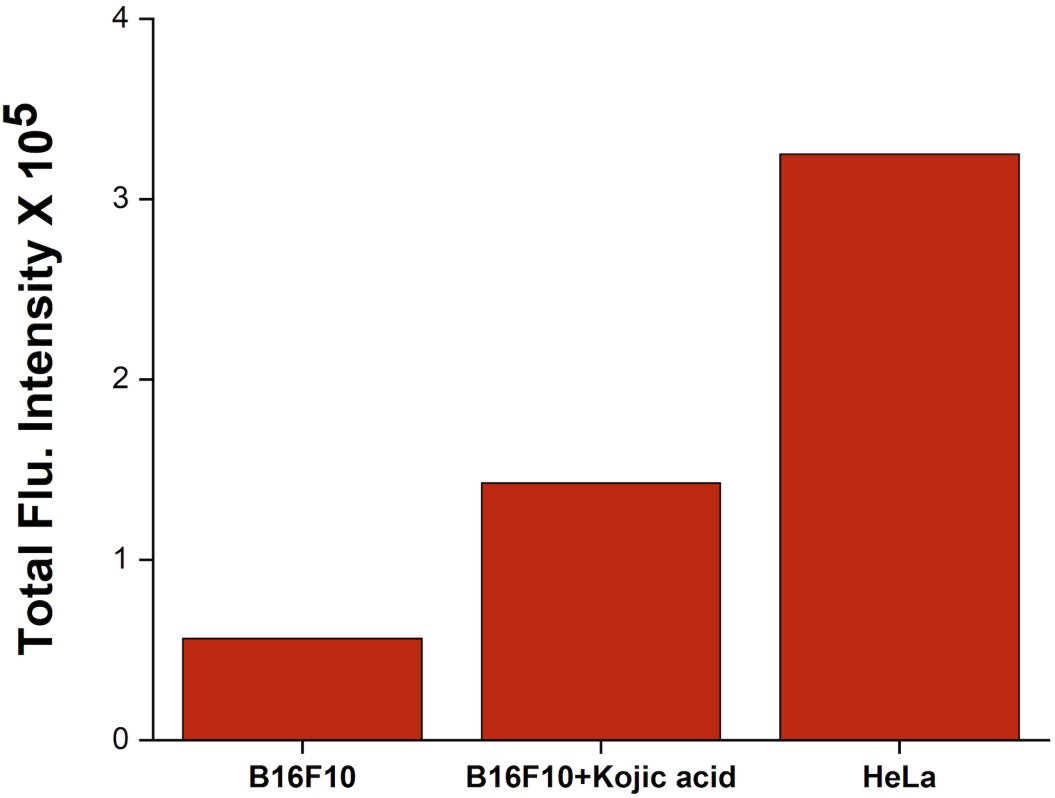


Fig. S15 Figure shows the total fluorescence intensity plot from the cells after incubating with **Tyro1.** There is very less fluorescence intensity observed in the B16F10 due to the tyrosinase activity on the probe. However, in the B16F10 cell treated with kojic acid, the tyrosinase is inhibited significantly (*ca.* 2.5 times) which results in the high fluorescence intensity. Moreover, in the negative control, HeLa, which has very minimal tyrosinase expression shows the highest fluorescence intensity.

References:

1. S. Biswas, V. Sharma, P. Kumar and A. L. Koner, *Sens. Actuator B Chem.*, 2018, **260**, 460-464.
2. T. I. Kim, J. Park, S. Park, Y. Choi and Y. Kim, *Chem. Commun.*, 2011, **47**, 12640-12642.