Supporting Information

Discovery of highly selective and ultra-sensitive colorimetric fluorescent probe for malononitrile and its applications in living cells and zebrafish

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1. Materials and instruments

Except for special labels, chemical reagents were obtained from commercial vendor and employed without further purification. High resolution mass spectra (HRMS) were obtained by LC-MS2010A instrument. Absorption spectra were obtained by UV-3101PC spectrophotometer. Fluorescence spectra were obtained by Horiba FluoroMax-4 spectrophotometer (λ_{ex}: 480 nm, Slit widths: $W_{ex} = W_{em} = 5$ nm). Fluorescence imaging of malononitrile in live cells and zebrafish were carried out on an Olympus FV1000-IX81 confocal fluorescence microscope. Except for special instructions, all optical measurements in this work were carried out 60 minutes after the substance was added in the EtOH/PBS aqueous solution (50% ethanol, 10 mM PBS, pH = 8.0).

2. Characterization data of probe DC-Mal

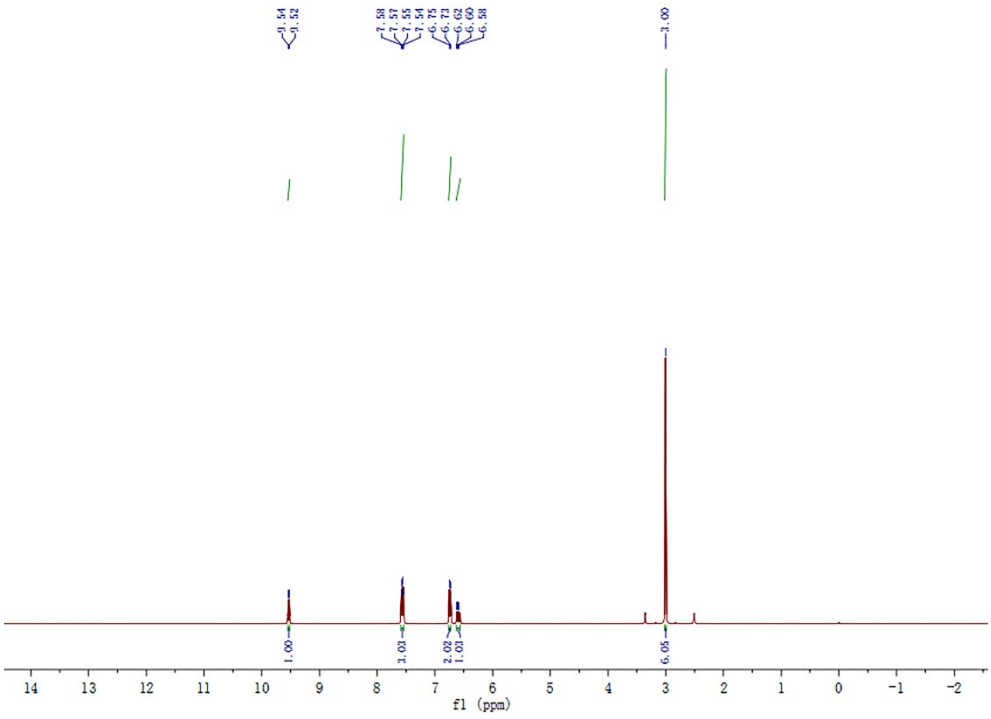

Fig. S1. ^{1}H-NMR data of probe **DC-Mal**.

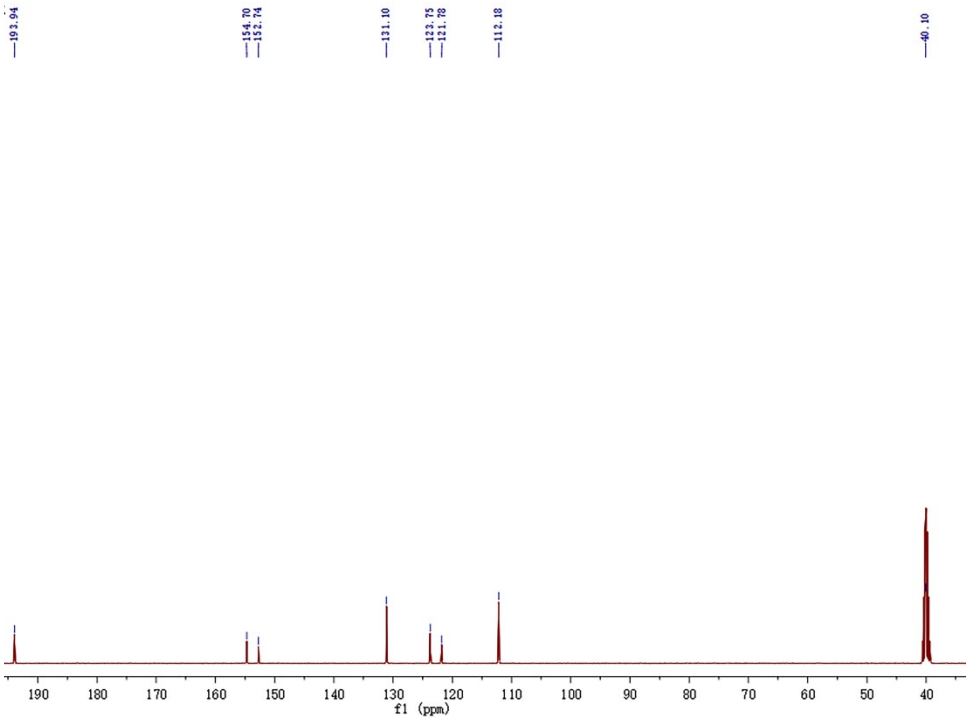

Figure. S2. ^{13}C-NMR data of probe **DC-Mal**.

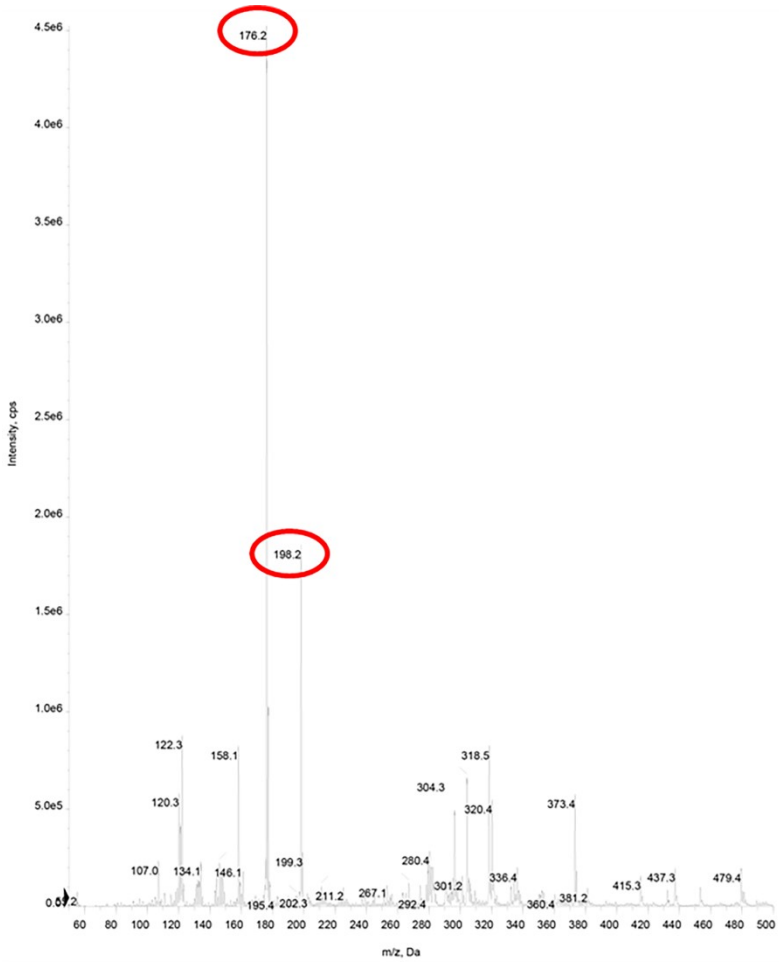

Figure. S3. MS data of probe **DC-Mal**.

3. Recognition mechanism of probe DC-Mal for malononitrile

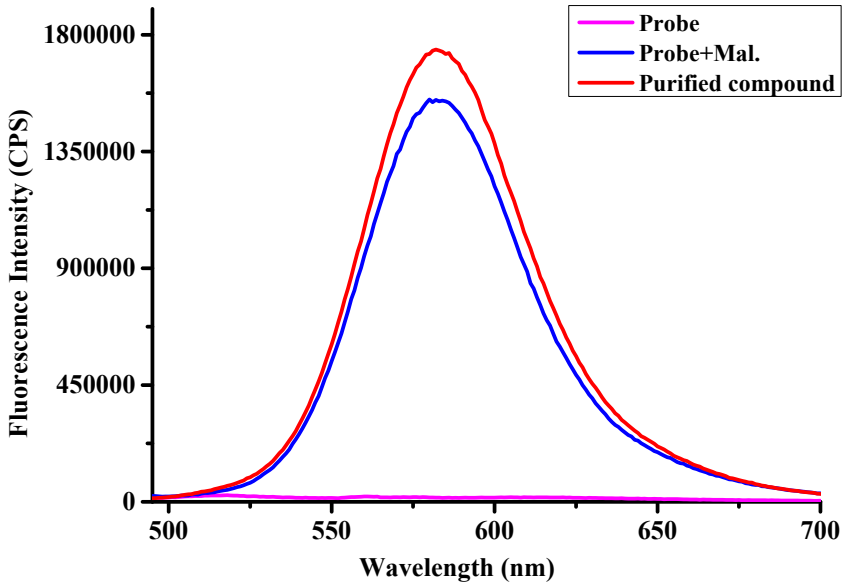

Fig. S4. Fluorescence spectra of the probe **DC-Mal** (purple line), the reaction solution of probe **DC-Mal** with malonitrile (blue line) and the purified product of probe with malonitrile (red line). λ_{ex} = 480 nm, λ_{em} = 580 nm. Slit widths: W_{ex} = 5 nm, W_{em} = 5 nm.

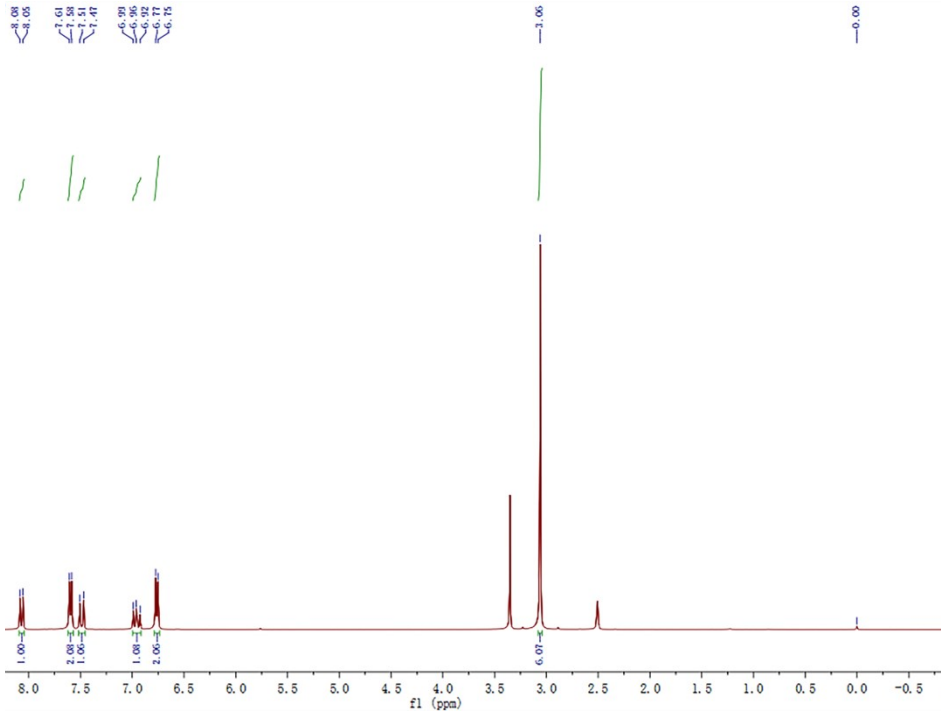

Fig. S5. ^{1}H-NMR data of reaction product of probe **DC-Mal** and malononitrile.

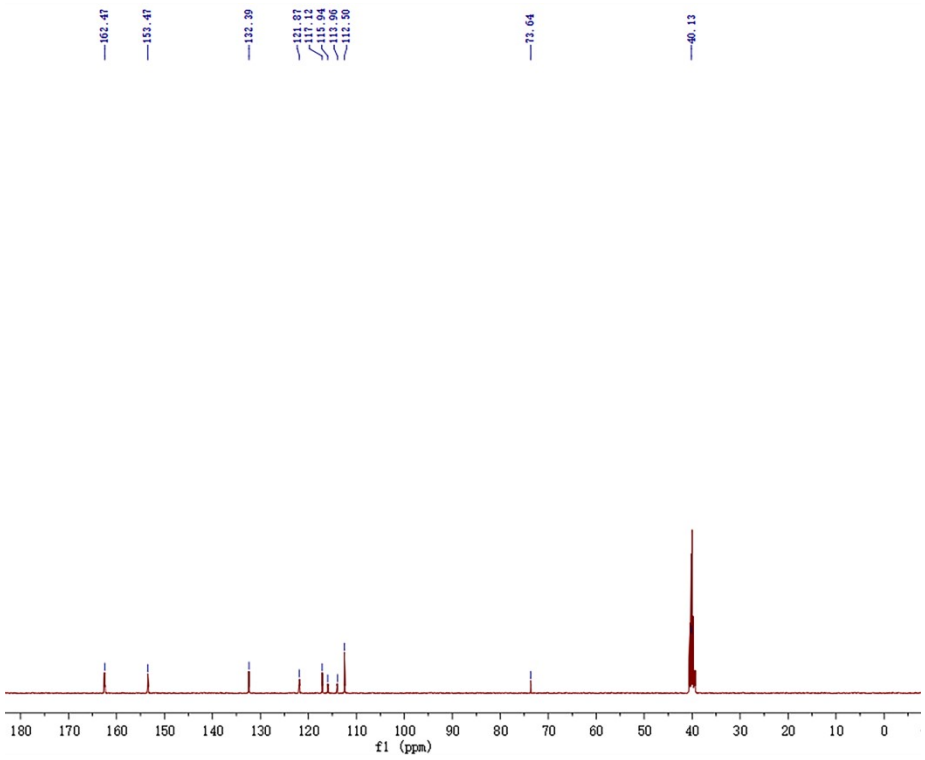

Figure. S6. ^{13}C-NMR data of reaction product of probe **DC-Mal** and malononitrile.

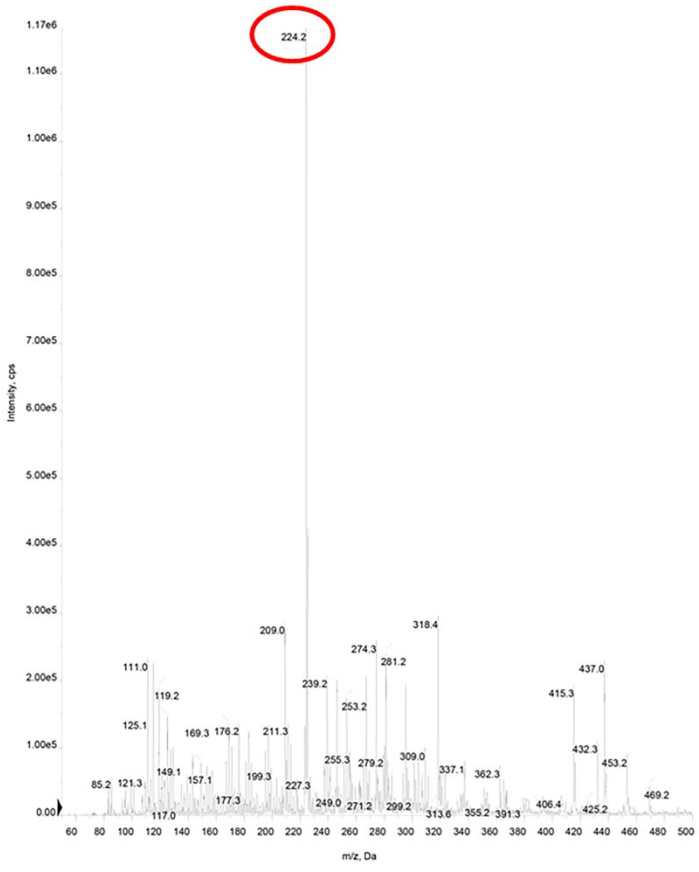

Figure. S7. MS data of reaction product of probe **DC-Mal** and malononitrile.

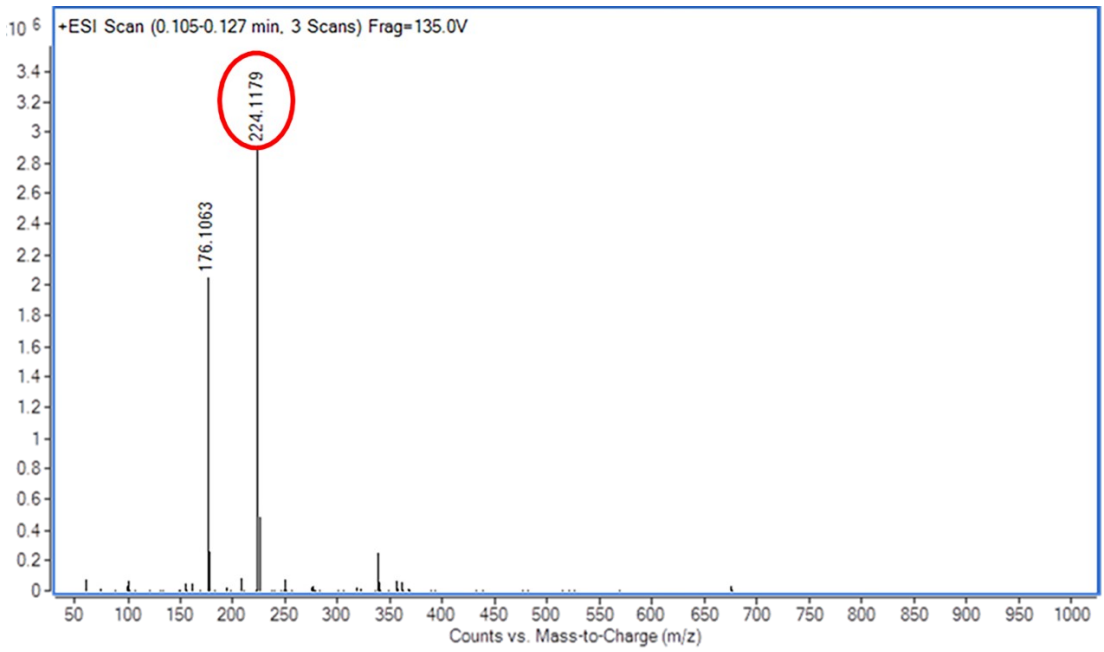

Figure. S8. HRMS data of reaction solution of probe **DC-Mal** and malononitrile.

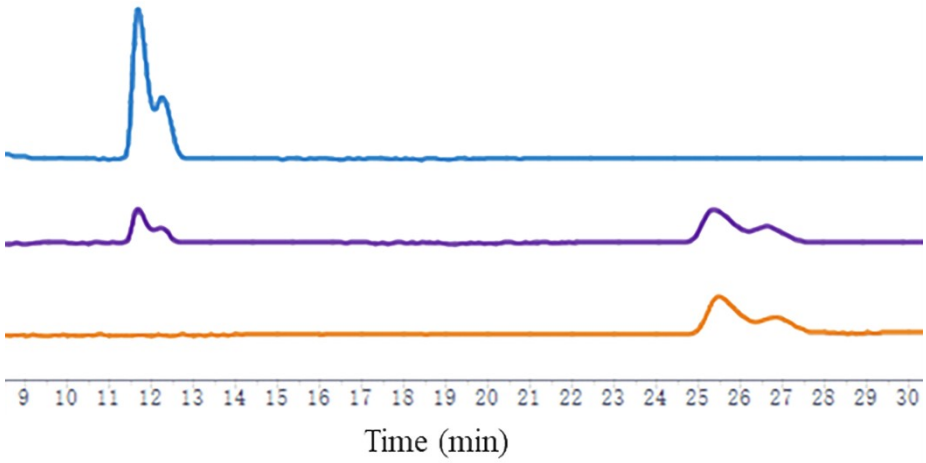

Figure. S9. HPLC results (detection band at 450 nm) for probe **DC-Mal** (blue line), the reaction solution of probe and malonitrile (purple line), the product of probe and malonitrile (orange line) ; Mobile phase: Methanol/ ultrapure water (45/55, v/v).

4. Determination of molar absorption coeffcients and quantum yields

Molar absorption coeffcient was determined in 10 mM PBS buffer-EtOH solution (5:5, v/v, PBS 8.0) and the molar absorption coeffcient was calculated using the following equation:

$$\varepsilon = A/bc$$

Where, A is the absorbance, b is thickness of liquid layer in absorption tank, and c is molarity of a solution.

Molar absorption coeffcient of probe **DC-Mal**: ε = 32500 L/mol • cm

Molar absorption coeffcient of probe **DC-Mal** with malononitrile: ε = 69000 L/mol • cm

Fluorescence quantum yield of probe **DC-Mal** was determined in 10 mM PBS buffer-EtOH solution (5:5, v/v, pH 8.0) with optically matching solutions of fluorescein (Φ = 0.95 in 0.1 M NaOH solution) as the standard and the quantum yield was calculated using the following equation:

$$\Phi_s = \Phi_r (A_r F_s / A_s F_r) (n_s^2 / n_r^2)^2$$

Where, s and r denote sample and reference, respectively. A is the absorbance. F is the relative integrated fluorescence intensity and n is the refractive index of the solvent.

Quantum yield of probe DC-Mal: Φ = 0.015

Quantum yield of reaction solution: Φ = 0.18

5. Kinetic studies of probe DC-Mal

In the kinetic experiment, we first prepared a probe solution with probe **DC-Mal** concentration of 10 μM, and then the spectral data of the solution were collected at 580 nm. Then we added malononitrile (500 μM) and measured the change of fluorescence intensity at certain intervals. It can be found that at about 60 min, the fluorescence intensity reached the platform with almost no significant change.

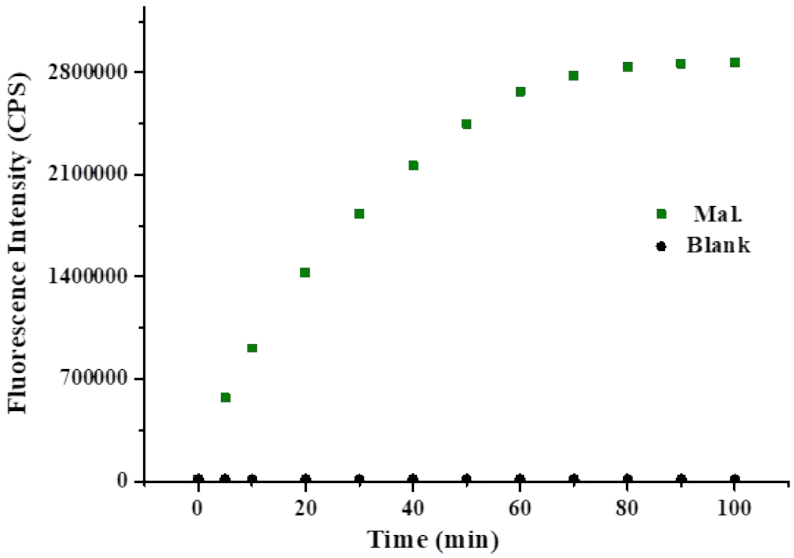

Fig. S10. Fluorescence intensity changes with time at 580 nm after adding malononitrile (0 μM / 500 μM) into the probe **DC-Mal** solution (10 μM).

6. Spectral experiments of probe DC-Mal

The fluorescence data were recorded in the EtOH/PBS aqueous solution (50% ethanol, 10 mM PBS, pH = 8.0). Unless otherwise stated, the emission of probe **DC-Mal** at 580 nm was measured after adding malononitrile in solution for 60 min at room temperature, and λ_{ex} = 480 nm, slit widths: $d_{ex} = d_{em}$ = 5 nm.

In the UV/vis absorption spectra, we prepared a probe solution with probe **DC-Mal** concentration of 10 μM, then we added different concentrations of malononitrile (0-8 μM). After 60 min, we collected the absorption spectra of different groups. In the fluorescence titration experiment, the probe solution with **DC-Mal** concentration of 10 μM was prepared and divided into several groups. According to a certain concentration gradient, malononitrile (0-1 mM) was added to the solution. When the reaction between malononitrile and probe **DC-Mal** is complete, we recorded and saved all spectral data. At the same time, we set a low concentration group and found

a linear relationship between malononitrile concentration and fluorescence intensity (0-8 μM).

7. Determination of the detection limit

The detection limit was calculated based on the fluorescence titration.The fluorescence spectra of free probe **DC-Mal** were measured by five times and its standard deviation was obtained. To gain the slope, the fluorescence intensities at 580 nm were plotted as the increasing concentrations of the corresponding malononitrile. So, the detection limit was calculated with the following equation:

$$LOD = 3\sigma/k,$$

where σ is the standard deviation of the blank solution, and k is the slope between the fluorescence intensities versus the concentrations of malononitrile.

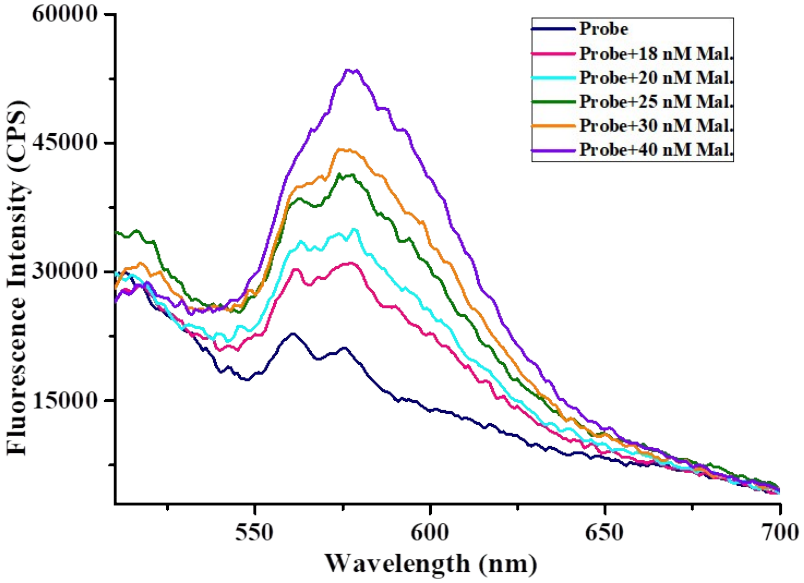

Figure. S11. Fluorescence spectra of probe **DC-Mal** (10 μM) with the continuous addition of malononitrile in the aqueous solution (50% ethanol, 10 mM PBS, pH = 8.0) for 60 min at room temperature. λ_{ex} = 480 nm, λ_{em} = 580 nm. Slit widths: W_{ex} = 5 nm, W_{em} = 5 nm.

8. Selectivity study of DC-Mal

In the selective experiment, we first prepared a series of analyte solutions, such as Fe^{3+}, Cu^{2+}, I^-, F^-, HSO_3^-, Ca^{2+}, Na^+, K^+, Lys, Asp, Gln, GSH, Hcy, Cys (Fig. 3). At the same time, the response of the probe to the compounds with similar structure to malonitrile was also studied (Fig. S12). We prepared a probe solution with probe **DC-Mal** concentration of 10 μM, and the solution was divided into several groups. The above analytes were added to the probe solution.

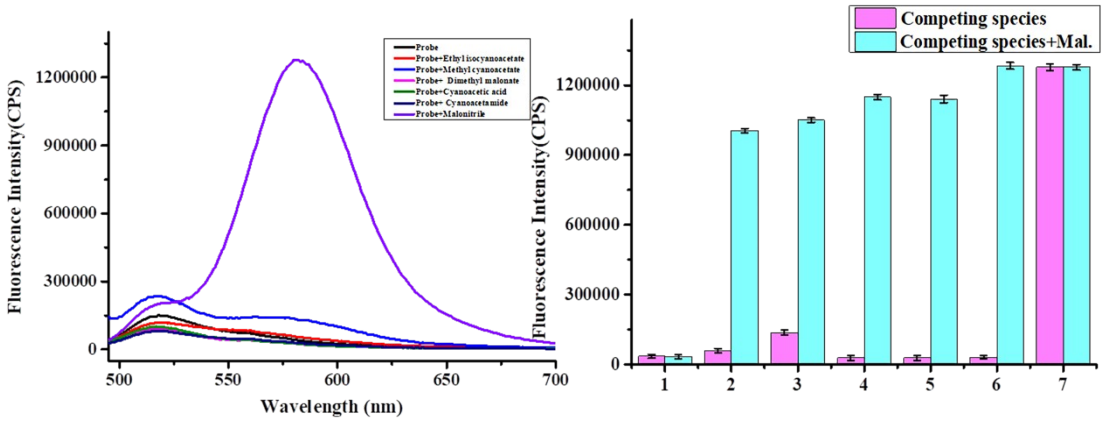

Fig. S12. Fluorescence response of probe **DC-Mal** (10 μM) to different analytes (100 μM) in the aqueous solution (50% ethanol, 10 mM PBS, pH = 8.0). 1. probe, 2. ethyl isocyanoacetate, 3. methyl cyanoacetate, 4. dimethyl malonate, 5. cyanoacetic acid, 6. cyanoacetamide, 7. malonitrile.

Then we added malononitrile to the probe solution, and then added different kinds of ions or amino acids. After that we collected the spectral data at 580 nm.

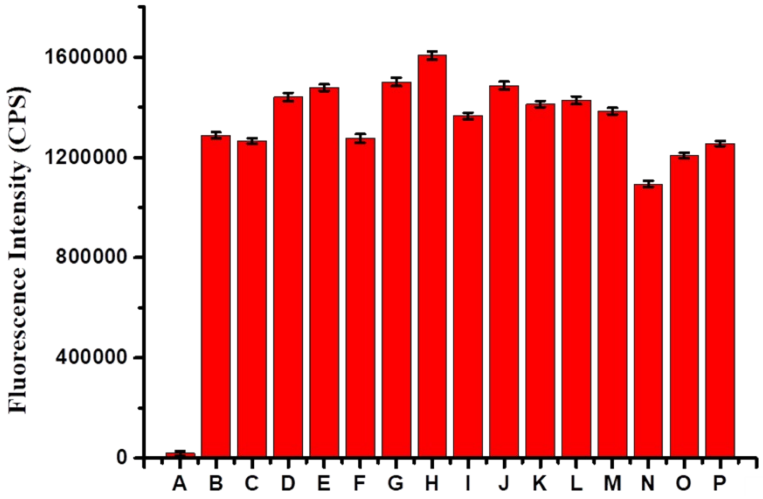

Fig. S13. Fluorescence response of probe **DC-Mal** (10 μM) and malononitrile to different analytes (100 μM). A. Black, B. Fe^{3+}, C. Cu^{2+}, D. I^-, E. F^-, F. HSO_3^-, G. Ca^{2+}, H. Na^+, I. K^+, J. Lys, K. Asp, L. Gln, M. GSH, N. Hcy, O. Cys, P. Malononitrile .

9. Live subject statement

All experimental procedures were carried out in strict accordance with the National Institute of Health (NIH) guidelines for the Care and Use of Laboratory Animals and the regulations of Qilu University of Technology on the ethical use of animals. All experimental procedures were approved by the faculty Ethical Committee of the Biology Institute of the Shandong Academy of Sciences. All efforts were made to minimize the number of animals used and their suffering. The sources of biological samples in our experiments were all from Shanghai Institute of Biochemistry and Cell Biology, China Academy of Sciences (Shanghai, China).

10. Cytotoxicity assays of DC-Mal and malononitrile

The cell viability of HeLa cells, treated with probe **DC-Mal**, was assessed by a cell counting kit-8 (CCK-8; Dojindo Molecular Technologies, Tokyo, Japan). Briefly, HeLa cells, seeded at a density of 1×10^6 cells·mL^{-1} on a 96-well plate, were

maintained at 37 °C in a 5% CO_2 / 95% air incubator for 12 h. Then the live HeLa cells were incubated with various concentrations (0, 5, 10, 20 and 30 μM) of probe **DC-Mal** suspended in culture medium for 24 h. The other group of cells were incubated with different concentrations of malononitrile (10, 20, 50, 100, 200 μM) suspended in culture medium for 16 h. Subsequently, CCK-8 solution was added into each well for 2 h, and absorbance at 480 nm was measured.

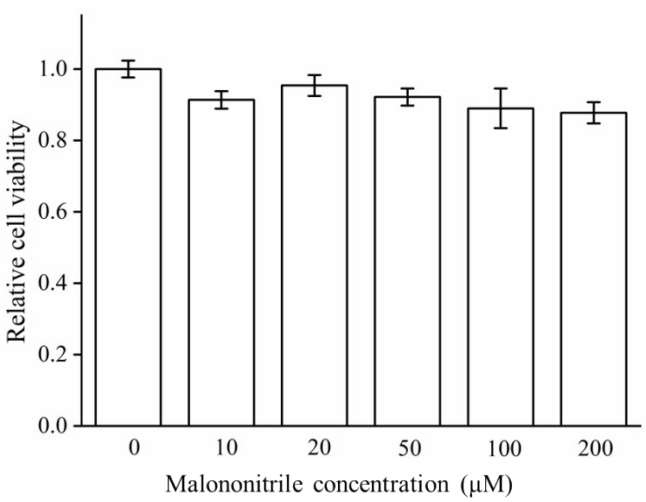

Fig. S14. Toxicity analysis of different concentrations of malononitrile to HeLa cells.

11. pH effects on the detection of probe to malononitrile

The fluorescence intensity of the probe and malononitrile reaction system under different PH conditions was shown in Fig. S13. The change of PH had little effect on the probe itself, but the fluorescence was obviously enhanced with the increasing alkalinity of the reaction system.

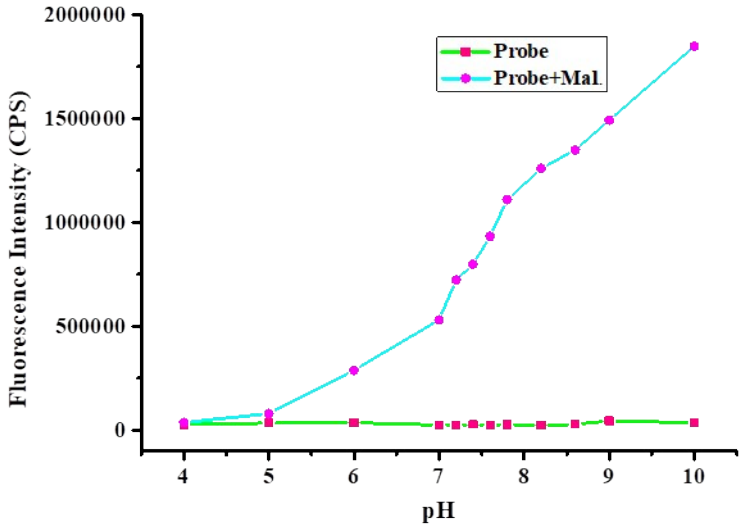

Fig. S15. Recognition of malononitrile (100 μM) by **DC-Mal** (10 μM) at different pH conditions in the EtOH/PBS aqueous solution (50% ethanol, 10 mM PBS) at 25 °C. λ_{ex} = 480 nm, λ_{em} = 580 nm.

12. Imaging studies of live cells

The HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and incubated under an atmosphere containing 5% CO_2 at 37 °C humidified air for 24 h. DMEM contains 10% fetal bovine serum and 1% penicillin-streptomycin.

Control cells were imaged by confocal fluorescence microscope. Then, probe **DC-Mal** (10 μM) was used to incubate HeLa cells for 20 min, the culture medium was then removed and rinsed with phosphate buffer saline for three times before fluorescence imaging was performed. The other groups of cells were incubated with probe **DC-Mal** (10 μM) for 20 min, washed with culture water, and then treated with different concentrations of malononitrile (5, 20, 50 μM) for 60 min for imaging.

13. Imaging studies of zebrafish

Behavioral test was conducted to evaluate the safety of probe **DC-Mal**. The

normal zebrafish larvae at 72 hpf were randomly divided into 2 well plates and exposed to various concentrations of probe **DC-Mal** (0, 10 μM) dissolved in the bathing medium. The larvae were transferred and maintained in a 14 h light (~1000 lux): 10 h dark (LD) cycle at 28 °C. Then the zebrafish larvae were cleaned in bathing medium and placed in 48-well plates (one larva per well) at 96 hpf. After a 10 min acclimation period, the locomotor activity of each larva was monitored for 20 min in a silent room using an automated computerized video-tracking system (Viewpoint, Lyon, France), and the detailed track was recorded with Zebralab software (Viewpoint). The swimming duration, movement distance and speed were analyzed. Additionally, to reduce possible diurnal factors on level of locomotor activity, all behavioral tests were performed at zeitgeber time 8-12 (ZT8-12).

Healthy male and female zebrafish (AB stain) were maintained in different tanks with a 14 h light / 10 h dark cycle at 28 °C. Then, sexually mature zebrafish were selected to induce spawning in tanks and the zebrafish eggs were obtained by giving light stimulation in the morning. After sterilizing and cleaning, the fertilized eggs were added to zebrafish embryo culture water (5 mM NaCl, 0.17 mM KCl, 0.4 mM $CaCl_2$, 0.16 mM $MgSO_4$) and cultured in illumination incubator at 28 °C.

The 5-day-old zebrafish were incubated with 10 μM probe **DC-Mal** for 20 min, and then washed with culture water to remove the remaining probe and imaged by confocal fluorescence microscope. After that, the zebrafish w were first incubated with the probe (10 μM) for 20 minutes and then with different concentrations of malononitrile (5, 20, 50 μM) for 60 minutes and washed with culture water. Then the

fluorescence imaging of zebrafish was carried out. λ_{ex} = 480 nm, and red channel at 500-600 nm.