SUPPORTING INFORMATION

A fluorene-thiophene oligomer turn-on fluorescence probe with high fold fluorescent enhancement for acetaldehyde dehydrogenase detection in cells

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Experimental section

Materials and Reagents

Potassium acetate, Bis(pinacolato)diboron, Fluorescein sodium salt (CAS: 518-47-8), 4-Diethylaminobenzaldehyde, *N*,*N*-Dimethylformamide, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT), 1,4-Dioxane, HEPES, 2,7-Dibromofluorene, p-Toluenesulfonyl chloride (PTSC), and Tetraethylene glycol monomethyl ether were purchased from Aladdin Industrial Inc (China). Sodium hydride was obtained from Zhengzhou Alpha Chemical Co. LTD (China). Dimethyl sulfoxide (DMSO), potassium carbonate, anhydrous ethanol, Silver nitrate, tetrahydrofuran, magnesium sulfate, and sodium hydroxide were purchased from Sinopharm Chemical Reagent Co., Ltd (China). Tetrakis (triphenylphosphine) palladium (0) was supplied by J&K (Beijing) Scientific Co., Ltd (China). 5-Bromo-2,2'-bithiophene-5'-carboxaldehyde was purchased from TCI Shanghai (China). Acetaldehyde dehydrogenase (A9770-25UN), Penicillin-streptomycin solution, 0.25% trypsin, fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), and Kanamycin were provided by Sigma-Aldrich Co., Ltd (USA). Plasmid pEGFP-N1-ALDH 1A1, 4S GelRed 10, 000 X in water, dithiothreitol (DTT), nicotinamide adenine dinucleotide (NAD), and SanPrep Column PCR Product Purification Kit were obtained from Sangong Bioengineering (Shanghai) Co., LTD. G418 was purchased from Procell Life Science&Technology Co., Ltd (China). ApaL I was provided by NEB (Beijing) Co., LTD. EndoFree Mini Plasmid Kit II was obtained from TIANGEN BIOTECH (Beijing) CO., LTD. Human colon carcinoma cell lines (MCF-7 and SK-BR-3), Human colon cancer cell lines (HCT-116) were purchased from the Type Culture Collection of the Chinese Academy of Sciences (China). All solutions were treated with purified water (18.2 MΩ cm, Millipore Milli-QA-10 water purification system, Merck KGaA, Darmstadt, Germany). Chemicals and solvents during the experiment were of analytical grade and used without purification.

Instrumentations

DNA concentration was measured on a NanoDrop One spectrophotometer (Thermo Fisher Scientific Co., Ltd.). UV-Vis absorption spectra were obtained on a TU-1901 Dual-beam UV-visible spectrophotometer (Beijing Purkinje General Instrument Company, Ltd., China). The fluorescence spectra were recorded on an F-7000 fluorescence spectrophotometer (Hitachi High-Tech Corporation, Japan). Cell viability was obtained by a Bio Tek Epoch microplate reader (Bio Tek Instruments, Inc., USA). Plasmid linearization results are recorded in a Gel Doc ZE gel imaging system (Bio-Rad Laboratories Co., Ltd, USA). The plasmid transfection validation experiment was measured on a BD FACSCalibur Flow Cytometer (USA). Fluorescence images were recorded on an Olympus FV-1200 confocal laser scanning microscope (Olympus Corporation, Japan). Microscopic pictures were recorded on an Electronic eyepiece microscope camera (5 million pixels) (Beijing Century Science Instrument Co., LTD). Electrospray ionization high-resolution mass spectra (ESI-HRMS) and ^{1}H NMR spectra were operated on Bruker Maxis ESI-Q-TOF instrument and Bruker Ascend 600 NMR spectrometer (Bruker Corporation, Germany).

Synthesis of precursor compounds and F-TCO

Synthesis of Compound 1: 1-(p-Tolylsulfonyl)-3,6,9,12-tetraoxotridecane (Compound 1) was synthesized following the literature.[S1]. The synthesis route was showed in Figure S1. A dropping funnel and a nitrogen inlet were fitted to a 100 mL three-necked reaction flask and purged with nitrogen. NaOH (36.6 mmol) was dissolved in distilled H_2O (5 mL), while tetraethylene glycol monomethyl ether (20.0 mmol) was dissolved in THF (5 mL). These two solutions were introduced into the reaction flask and cooled to 3°C with rapid stirring. A THF (6 mL) solution containing p-toluenesulfonyl chloride (20.1 mmol) was then transferred to the dropping funnel for 15 min with maintaining the temperature between 3-10°C. The mixture was slowly heated to room temperature with stirring for 12 h. Then the mixture was extracted with Et_2O (20 mL), and the aqueous phase was extracted with Et_2O (20 mL) as well. The extract was back-extracted with H_2O (100 mL) until the pH of the extract was neutral. Finally, the extract was dried with $MgSO_4$, and rotary evaporated (without heating) to obtain Compound 1 (5.9003 g, 81%) as a pale-yellow oil.

Synthesis of Compound 2: 2,7-Dibromo-9,9-bis-(1-{2-[2-(2-methoxy-ethoxy)-ethoxy]-ethoxy}-ethyl)-fluorene (Compound 2) was synthesized following the literature procedure.[S2] The synthesis route was showed in Figure S1. The anhydrous DMF (12.5 mL) solution containing 2,7-dibromofluorene (2.3 mmol) was added to NaH (6.3 mmol, 60% w/w dispersion in mineral oil) under a nitrogen atmosphere with stirring for 1 h at room temperature. Subsequently, Compound 1 (5.75 mmol) was added to the mixture and stirred for 24 h. After quenching the reaction with water, the mixture was extracted with CH_2Cl_2. The organic portion was dried with $MgSO_4$, and the solvent was removed under reduced pressure. The crude product was purified by column chromatography (CH_2Cl_2: CH_3OH = 9:1) to give Compound 2 (1.5 g, 91%) as a colorless oil. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.59 (d, *J* = 1.5, 2H), 7.56 (s, 2H), 7.49 (dd, *J* = 1.5, 6.9, 2H), 3.60 - 3.47 (m, 16H), 3.39 - 3.36 (m, 10H), 3.19 (dd, *J* = 3.4, 4.1, 4H), 2.79 - 2.78 (m, 4H), 2.32 (t, *J* = 6.3, 4H).

Synthesis of Compound 3: 2,7-Bis-(4,4,5,5-tetramethyl-[1,3,2]-dioxaborolan-2-yl)-9,9-bis-(1-{2-[2-(2-methoxy-ethoxy)-ethoxy]-ethoxy}-ethyl)-fluorene (Compound 3) were synthesized following the literature procedure.[S2] The synthesis route was shown in Figure S1. The anhydrous 1,4-dioxane (4 mL) solution containing Compound 2 (0.43 mmol) was added by Bis(pinacolato)diboron (1.06 mmol), KOAc (1.06 mmol), and tetrakis(triphenylphosphine)palladium (0) (0.009 mmol) under argon atmosphere. The mixture was stirred at 80°C for 24 h. After allowing the mixture to cool to room temperature, the crude product was purified by column chromatography (CH_2Cl_2: MeOH = 99/1) to give a colorless solid Compound 3 (132 mg, 44%).^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.83 (s, 2H), 7.80 - 7.78 (m, 2H), 7.69 - 7.68 (m, 2H), 3.58 - 3.49 (m, 16H), 3.38 - 3.34 (m, 10H), 3.17 (dd, *J* = 4.1, 4.8, 4H), 2.68 - 2.65 (m, 4H), 2.43 - 2.41 (m, 4H), 1.38 (s, 24H).

Synthesis of F-TCO: The silver nitrate (3.186 mmol) was slowly added to a quick-stirring sodium hydroxide solution (10%, 3 mL). F-TAO (0.054 mmol) was added to the mixture solution with stirred vigorously for 3 h. Then the solution was poured into 60 mL of water and the pH of the

solution was adjusted to 2.0. This acidic aqueous phase was extracted by ethyl acetate (60 mL) and evaporated to dryness. The solid crude product was suspended in dichloromethane (30 mL) and filtered through a bush funnel to obtain a bright yellow solid F-TCO. ^{1}H NMR (600 MHz, $(CH_3)_2CO$): δ = 8.01 (s, 2H), 7.91 (d, *J* = 7.8, 2H), 7.77-7.76 (m, 4H), 7.65 (s, 2H), 7.51 (s, 2H), 7.41 (s, 2H), 3.48-3.42 (m, 16H), 3.33 (s, 4H), 3.25-3.13 (m, 10H), 2.93-2.85 (m, 4H), 2.56-2.54 (m, 4H). HRMS-ESI (m/z): $(M + Na)^+$ Calculated mass for $C_{49}H_{54}O_{12}S_4$ = 985.2390, found mass = 985.2396.

Synthesis of F-TAO

The synthesis procedures of precursor compounds (Compound 1, 2, 3) and F-TCO were depicted in supporting information. For synthesis of F-TAO, firstly, the mixture of 2,7-Bis-(4,4,5,5-tetramethyl-[1,3,2]-dioxaborolan-2-yl)-9,9-bis-(1-{2-[2-(2-methoxy-ethoxy)-ethoxy]-ethoxy}-ethyl)-fluorene (Compound 3) (0.125 mmol) and 5-bromo-2,2'-bithiophen-5'-formaldehyde (0.25 mmol) in 6 mL THF and 2 mL potassium carbonate solution (2 M) was degasified in nitrogen atmosphere. Then, tetra(triphenylphosphine) palladium (0) (0.0052 mmol) was added to the mixture with stirring at 80°C for 48 h. After cooling to room temperature, the organic phase was extracted with 20 mL saturated sodium chloride solution, then the water phase was extracted with 60 mL dichloromethane three times. Then the crude product was dried with anhydrous magnesium sulfate, and purified by column chromatography (eluent: CH_2Cl_2: CH_3OH = 99: 1, v/v). The obtained orange solid product was F-TAO. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 9.98 (s, 2H), 7.71 (dd, *J* = 1.8, 6.0, 4H), 7.64 (t, *J* = 7.8, 4H), 7.38 (dd, *J* = 3.6, 8.4, 4H), 7.31 (d, *J* = 3.6, 2H), 3.56-3.47 (m, 16H), 3.38 (t, *J* = 4.2, 4H), 3.34 (d, *J* = 9.6, 6H), 3.22 (t, *J* = 4.8, 4H), 2.85 (t, *J* = 7.2, 4H), 2.47 (t, *J* = 2.4, 4H). HRMS-ESI (m/z): $(M + Na)^+$ Calculated mass for $C_{49}H_{54}O_{10}S_4$ = 953.2492, found mass = 953.2483.

UV-Visible absorption spectra and Fluorescence spectra measurement experiments

The F-TAO and F-TCO were dissolved in DMSO to obtain a 10 mM stock solution. The working solution (10 μM) was prepared by diluting the stock solution with HEPES (200 mM, pH = 7.5, KCl 100 mM). The ALDHs was diluted in Tris-HCl buffer (0.02% BSA, pH = 7.4) at different concentrations.

For absorption and fluorescence spectra detection, 50 μL 200 μM F-TAO and F-TCO were diluted in 950 μL PBS (10 mM).

For assessment of F-TAO, 10 μL F-TAO (200 μM), 2.5 μL DTT (160 mM), and 2.5 μL NAD^+ (80 mM) were mixed in 165 μL HEPES buffer. Then 20 μL 10 U/mL ALDHs was added to the mixture. UV-Visible absorption and fluorescence spectra were recorded after the reaction of ALDHs and F-TAO at 37℃ for 2 hours.

For response time study, 10 μL F-TAO (200 μM), 2.5 μL DTT (160 mM) and 2.5 μL NAD^+ (80 mM) were mixed in 165 μL HEPES buffer. Then 20 μL 10 U/mL ALDHs was added to the mixture solution. The fluorescence spectra were recorded at the time points 0, 5, 10, 15, 30, 60, and 120 min after the reaction of F-TAO and ALDHs at 37°C.

For sensitivity determination, ALDHs was diluted by Tris-HCl buffer (0.02% BSA, pH = 7.4) to various concentrations (0.001, 0.002, 0.005 U, 0.01 U/mL). The fluorescence intensity was recorded after 2 h post reaction of F-TAO and ALDHs by three times, and the standard deviation of blank measurement was achieved. The calculation formula for the detection limit was LOD = 3σ/s (σ represents the standard deviation of Blank, and s represents the slope of the linear regression equation).

For inhibition assay, DEAB was dissolved in anhydrous ethanol at different concentrations (0.625, 1,25, 2.5, 5, 10, 25, 50, 100 mM). 20 μL ALDHs (10 U/mL), 2.5 μL DTT (160 mM) and 2.5 μL NAD^+ (80 mM) were mixed in 163 μL HEPES buffer. After that, 10 μL F-TAO and 2μL DEAB were added to the mixture solution (pH 7.4). The fluorescence intensity was measured after 2 h post-reaction at 37°C.

For selectivity experiments, 10 μL F-TAO (200 μM), 2.5 μL DTT (160 mM) and 2.5 μL NAD^+ (80 mM) were mixed in 165 μL HEPES buffer. Then, ALDHs, oxidants (H_2O_2, HClO), a reductant (GSH), metal salts ($CaCl_2$, $MgCl_2$, $CuSO_4$, $ZnCl_2$), and amino acids (Cysteine, Glucose, Serine) was added to the mixture solution respectively. The concentration we used were as follow: ALDHs (1.0 U/mL), $CaCl_2$ (2 mM), $MgCl_2$ (2 mM), $CuSO_4$ (50 μM), $ZnCl_2$ (100 μM), glucose (10 mM), cysteine (1 mM), serine (1 mM), H_2O_2 (100 μM), HClO (100 μM) and glutathione (100 μM). The fluorescence intensity was measured after 2 h post-reaction at 37°C.

The UV-Visible absorption spectra were recorded by a TU-1901 Dual-beam UV-visible spectrophotometer. The fluorescence spectra were measured by an F-7000 fluorescence spectrophotometer with excitation wavelengths of 397 nm. The slit width was 10 nm for excitation and emission.

Study on the relative fluorescence quantum yields of F-TAO and F-TCO

The relative fluorescence quantum yield was determined by a reference method. The fluorescein sodium salt (φ = 0.92, 0.1 M NaOH) was selected as the reference standard material.[24] The fluorescein sodium, F-TAO, and F-TCO were diluted by 0.1M NaOH respectively to make sure the absorbance was < 0.05. The fluorescence spectra were measured and the fluorescence integral intensity was obtained. For the fluorescein sodium salt sample, the excitation wavelength was 489 nm, and for the samples, the excitation wavelength was 397 nm. The relative quantum yield was calculated by the following equation:

$$\varphi_{sample} = \varphi_{standard} \times \frac{F_{sample}}{F_{standard}} \times \frac{A_{standard}}{A_{sample}}$$

In this equation, φ represents the quantum yield; A represents the absorbance, and F represents the fluorescence integral intensity.

Computational studies of F-TAO and F-TCO

The ground state geometry of all molecules was optimized at B3LYP/def2-SVP[S3] (def2/J[S4]) level, and single point energy was calculated at B3LYP/def2-TZVP[S3] (def2/J[S4]) level with tight convergence criteria and atom-pairwise dispersion correction (Becke-Johnson damping scheme,

D3BJ[S5,S6]) in a solvent of water (SMD solvation model[S7]). ORCA 4.1.1 program package was used for all calculations[S8]. Frontier orbitals were visualized with Multiwfn 3.8[S9] and VMD 1.9.3 program package[10]. Since methoxytetrakis (ethylene glycol) side chains in the fluorene did not contribute to the conjugation structure, thus only two methyl groups were reserved at the 9 positions of fluorene in all calculations to avoid unnecessary consumption of computational resources.

Cell culture and cytotoxicity assay

Human breast cancer MCF-7 and SK-BR-3, and human colon cancer HCT-116 cells were cultured in DMEM with 1% penicillin/streptomycin and 10% FBS in an atmosphere of 5% CO_2 at 37°C.

The cytotoxicity of F-TAO was verified by an MTT assay. HCT-116 cells were seeded at a density of 5, 000 cells per well in a 96-well plate, and incubated for 24 h in DMEM complete medium. Post 24 h incubation, F-TAO was added to a culture medium with different concentrations (0, 2.5, 5, 10, 20, 50 μM), and incubated for 4 h at 37°C. The cells were then incubated with 20 μL MTT solution (5 mg/mL in PBS) for 4 h in darkness at 37°C. Subsequently, 150 μL of DMSO was added to each well. After vibrating for 10 min, the absorbance at 490 nm was measured by Bio Tek Epoch Plate Readers.

Construction of ALDH1A1-MCF-7 cells through electroporation

According to the cDNA fragment (NM_000689.3) of the ALDH 1A1 gene, the gene sequence of ALDH 1A1 was integrated into a eukaryotic cell expression vector pEGFP-N1 to construct a pEGFP-N1-ALDH1A1 expression plasmid (Figure S2). To increase the transfection efficiency of the pEGFP-N1-ALDH1A1 into MCF-7 cells, the pEGFP-N1-ALDH1A1 plasmid was linearized. Then the pEGFP-N1-ALDH1A1 plasmid was transfected into MCF-7 cells through electroporation to generate ALDH1A1-MCF-7 cells.

Firstly, the cells were digested with 0.25% trypsin and subcultured to obtain 50%-70% fusion. Nest, the cells were digested and resuspended in precooled PBS in 1×10^6 cells/ml. To increase the transfection efficiency of the pEGFP-N1-ALDH1A1 into MCF-7 cells, the pEGFP-N1-ALDH1A1 plasmid was linearized. ApaLI restriction endonuclease was used to digest the plasmid with an enzyme digestion system at 37°C for 4 h to generate a linear plasmid. The linear plasmid was purified and recovered by a PCR product purification kit, and subsequently electrophoresed in 0.7% agarose gel (Gel-Red). The image of the results was taken in a gel imaging system. The linearized plasmids (10 μg) were fully mixed with cell suspension in a pre-cooled electrode cup. The blank control group was added to 10 μL sterile water instead plasmid. Then, the electroporation cup was quickly inserted into the gene introducer for electroporation transfection. After the electric shock, the electroporation cup was placed on ice for 2 minutes and subsequently at 37°C for 8 minutes. After that, the cells were resuspended in DMEM complete medium (10% FBS) and cultured at 37°C. After 24 h, the DMEM complete medium was changed to the selective medium with 400 μg/ml G418 and cultured at 37°C continually. The screening time was expected to be 2-3 weeks to select ALDH1A1-MCF-7 cells. The flow cytometry was applied to verify the efficiency of transfection. The ALDH1A1-MCF-7 cells after G418 selection were trypsinized and resuspended in HEPES

buffer (10 mM, 0.5% FBS) with a concentration of 1×10^6 cells/mL and studied on a BD FACSCalibur Flow Cytometer based on co-expression of EGFP. The untransfected MCF-7 cells were used as a control.

Cell imaging

For the co-location of EGFP and F-TAO experiment, ALDH1A1-MCF-7 and MCF-7 cells were stained with F-TAO (10 μM) for 30 min at 37°C. The cells were observed and imaged under a blue channel (for F-TAO, E_x = 405 nm, E_m = 425-475 nm) and a green channel (for EGFP, E_x = 488 nm, E_m = 500-600 nm) using a confocal laser scanning microscopy.

For imaging of ALDHs in cells, SK-BR-3 and HCT-116 cells were incubated with F-TAO (10 μM) for 30 min at 37°C. The cells were then imaged under a green channel (E_x = 405 nm, E_m = 425-475 nm), and images were obtained using an Olympus FV-1200 confocal laser scanning microscopy. As the control group, DEAB (1 mM) was added to the culture medium in advance for 30 min. The main text of the article should appear here with headings as appropriate.

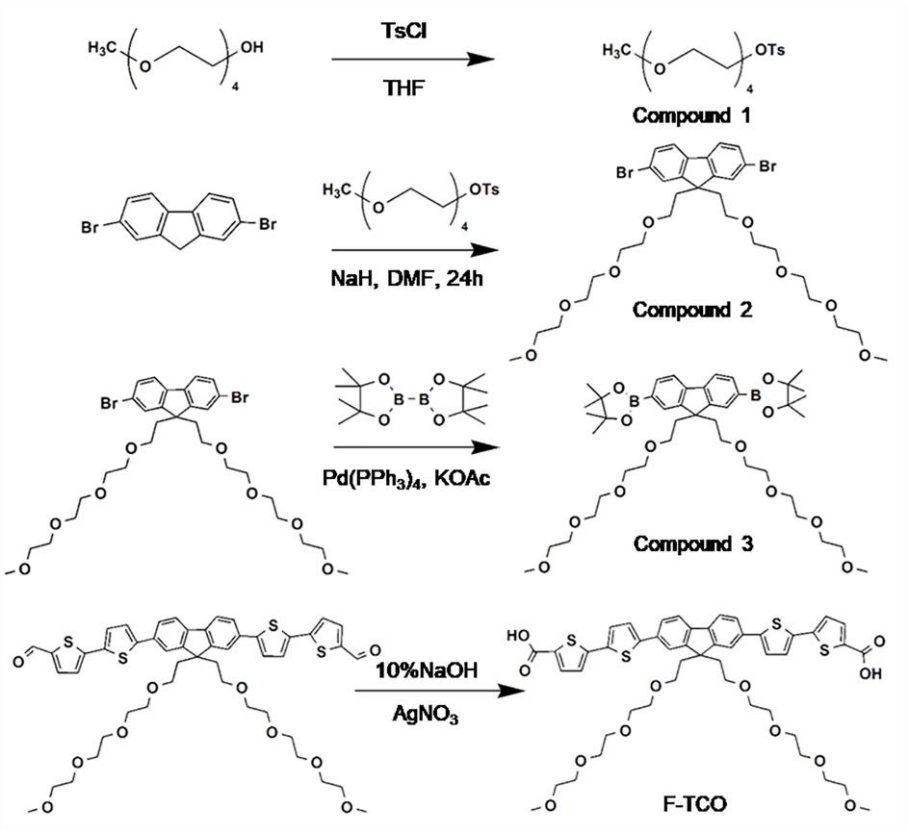

Figure S1. Synthetic routes of Compound 1-3 and F-TCO.

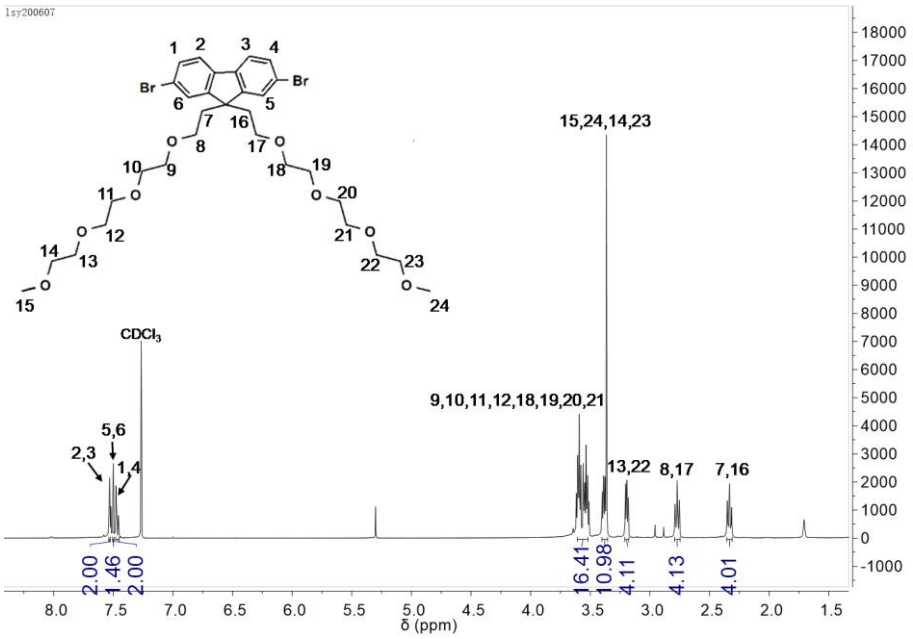

Figure S2. The ^{1}H NMR spectrum of Compound 2.

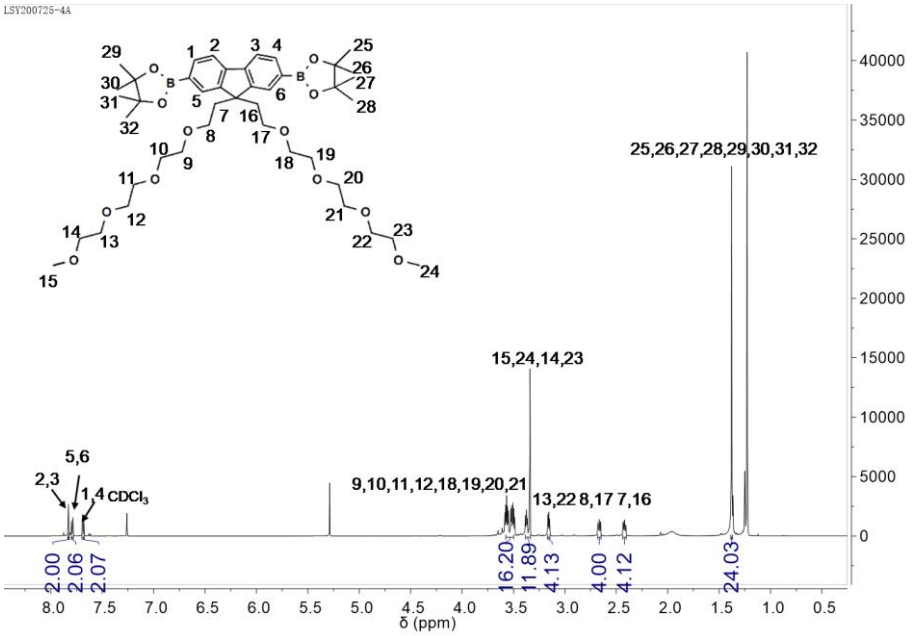

Figure S3. The ^{1}H NMR spectrum of Compound 3.

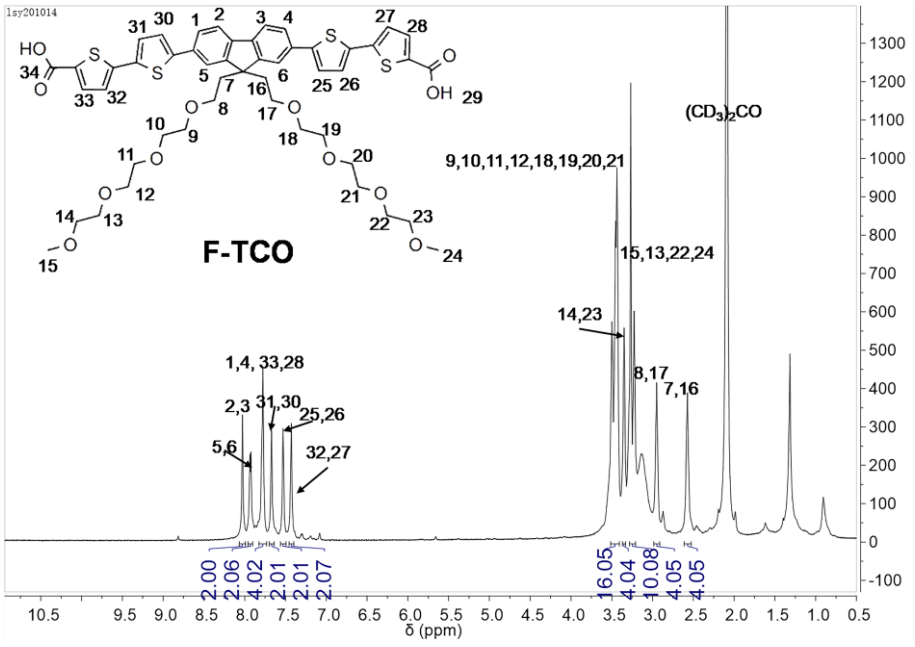

Figure S4. The ^{1}H NMR spectrum of F-TCO.

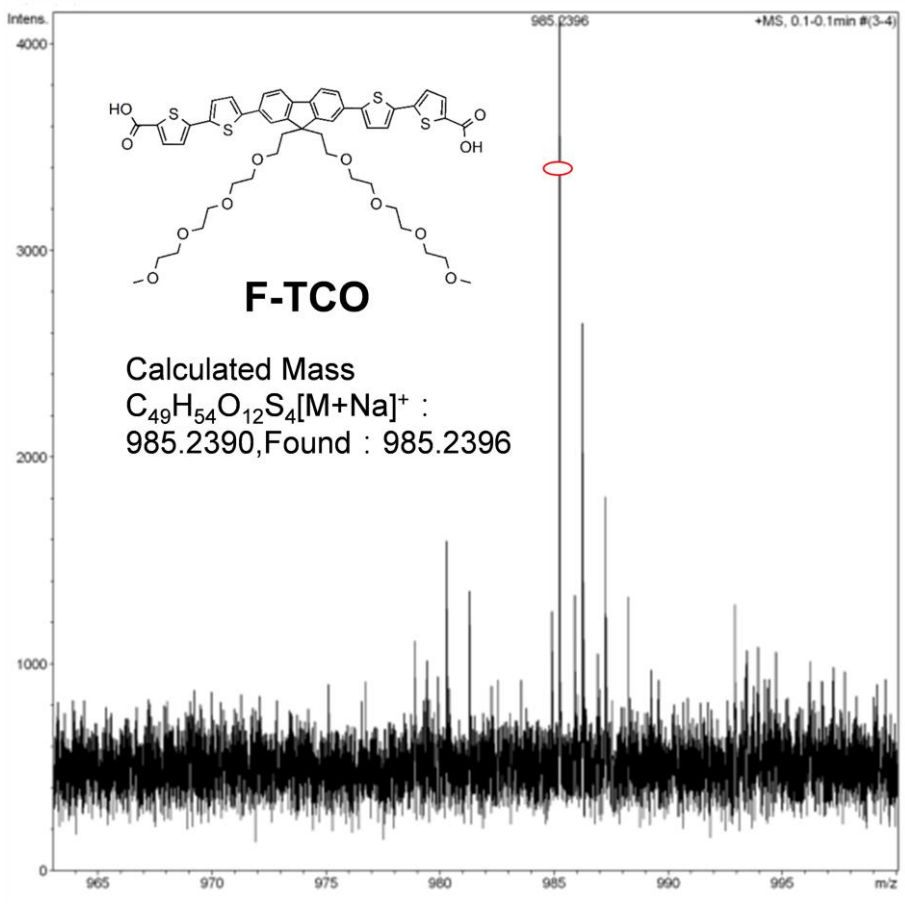

Figure S5. The HR MS spectrum of F-TCO.

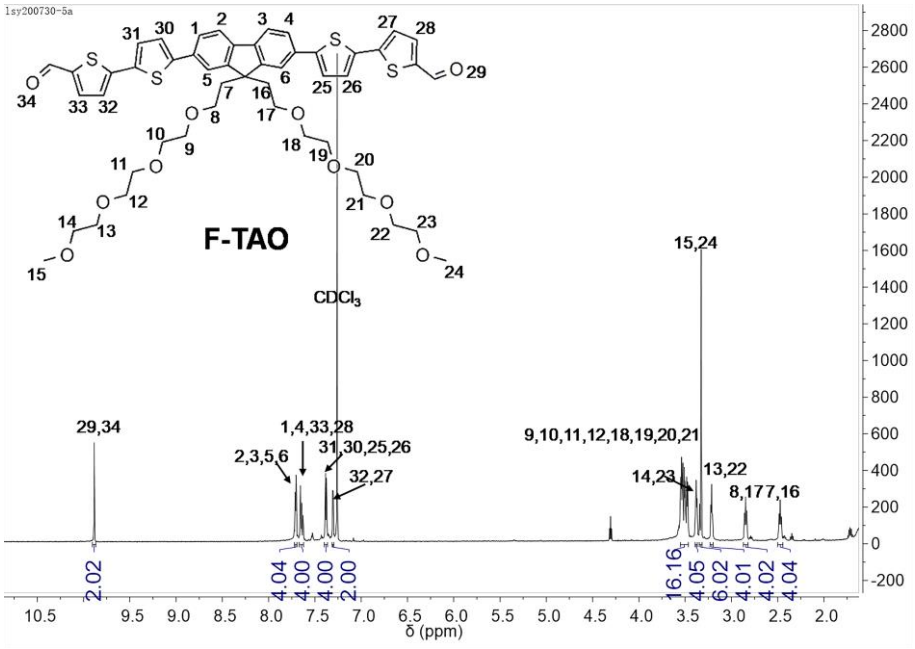

Figure S6. The ^{1}H NMR spectrum of F-TAO.

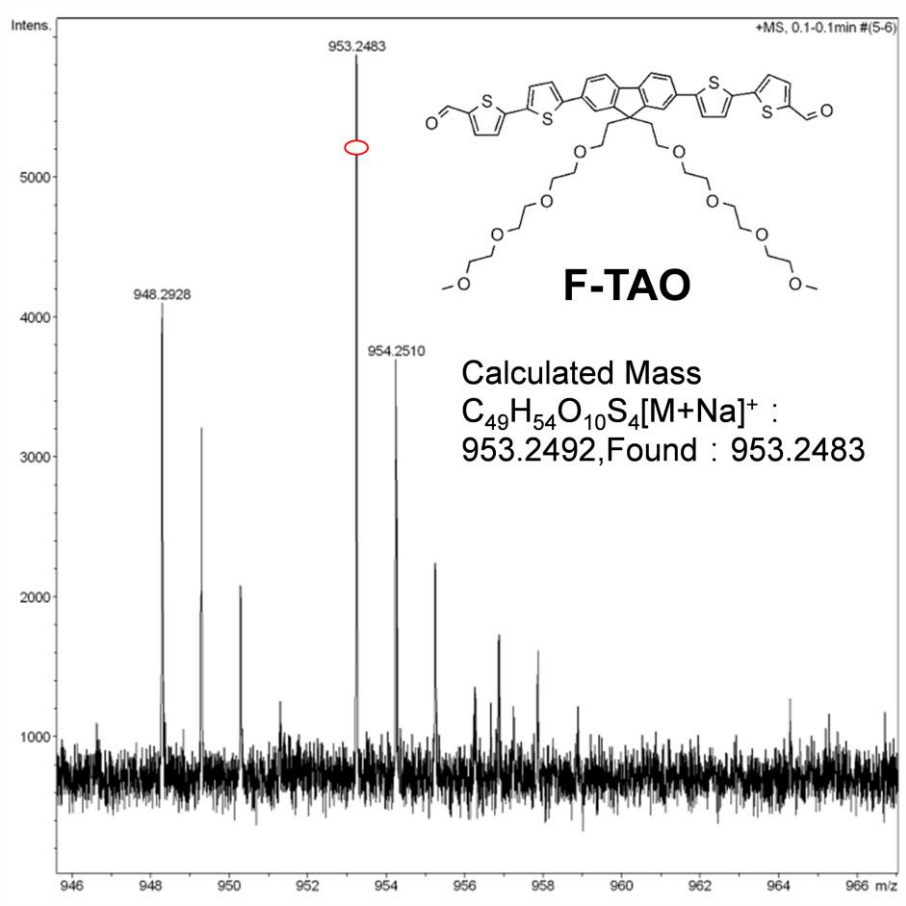

Figure S7. The HR-MS spectrum of F-TAO.

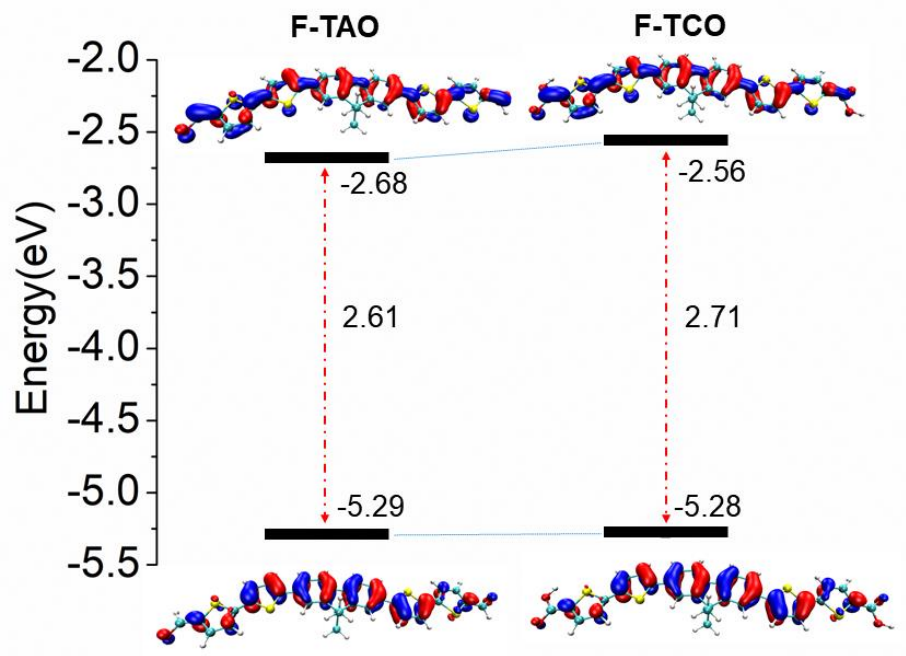

Figure S8. Frontier orbital pictures. (Top two): F-TAO (LUMO and HOMO). (Bottom two): F-TCO (LUMO and HOMO) calculated at B3LYP/def2-TZVP level.

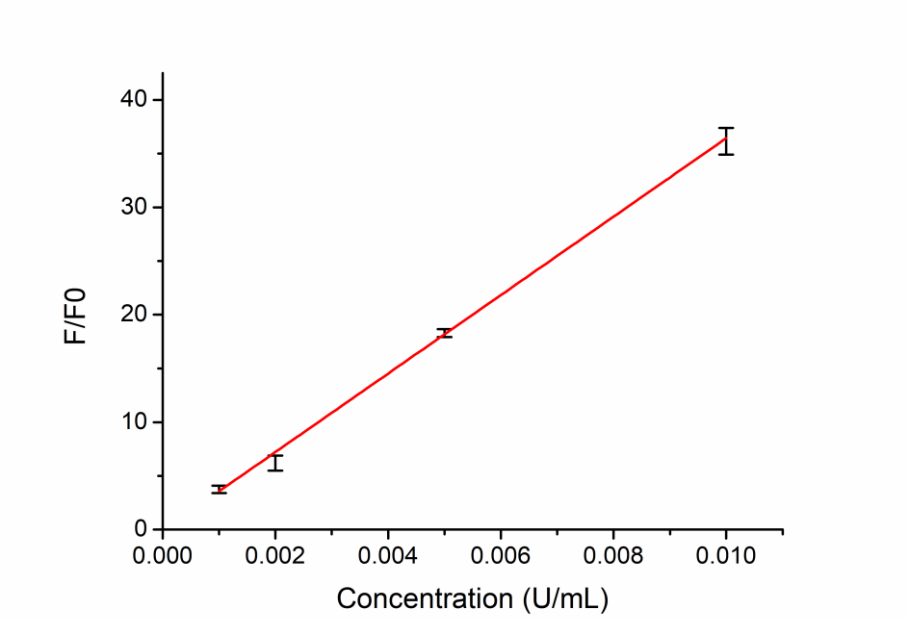

Figure S9. Linear regression fitting diagram of the relationship between F/F0 and concentrations of ALDH. y = 3651x – 0.08437, R^2 = 0.9969.

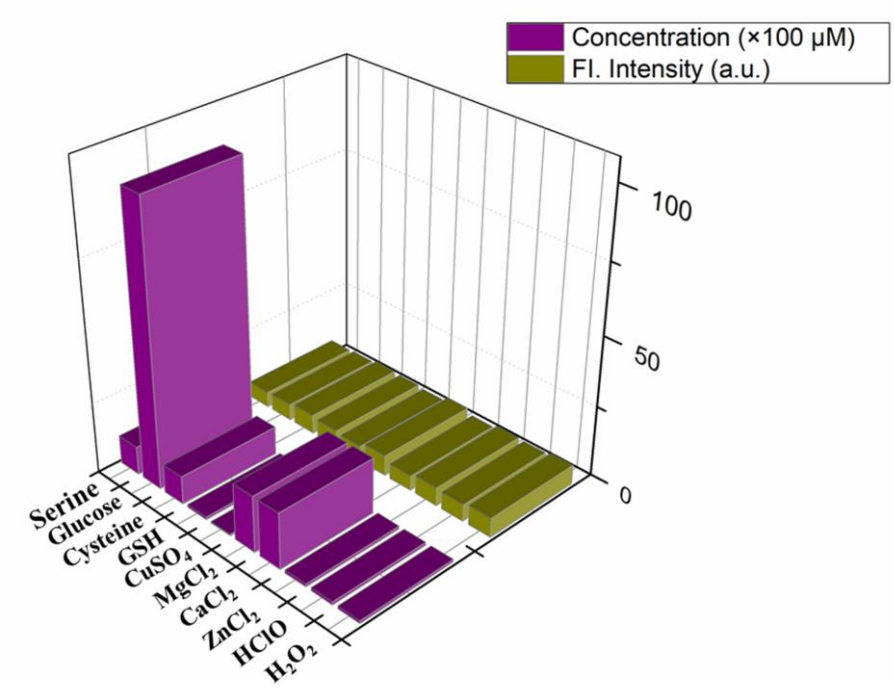

Figure S10. 3D-graph of fluorescence intensity of F-TAO and concentration of interfering substances ($CaCl_2$ 2 mM, $MgCl_2$ 2 mM, $CuSO_4$ 50 μM, $ZnCl_2$ 100 μM, glucose 10 mM, cysteine 1 mM, serine 1 mM, H_2O_2 100 μM, HClO 100 μM, and glutathione 100 μM) in HEPES buffer (200 mM, pH = 7.5, KCl 100 mM) under the excitation wavelength of 397 nm.

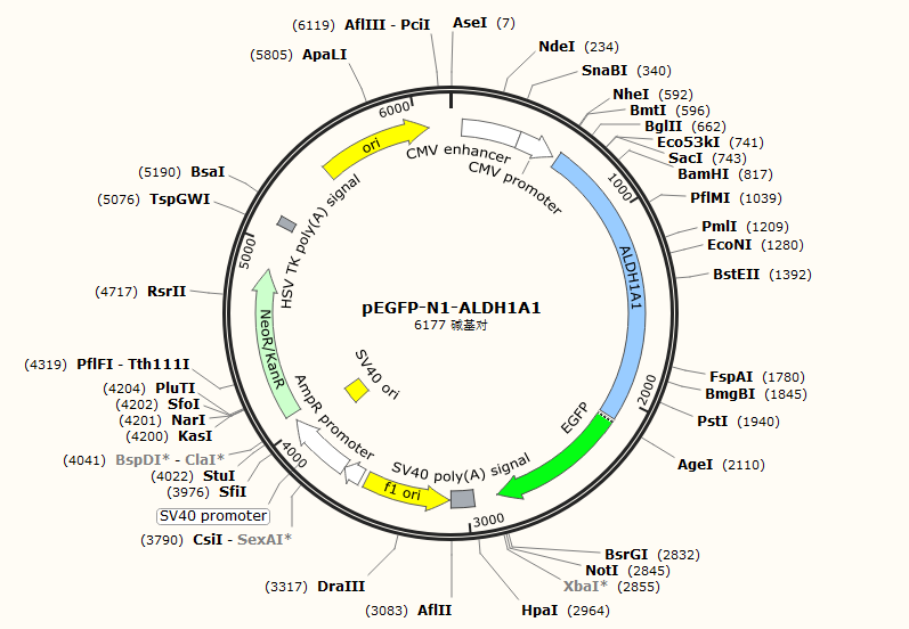

Figure S11. Schematic diagram of pEGFP-N1-ALDH1A1 expression vector.

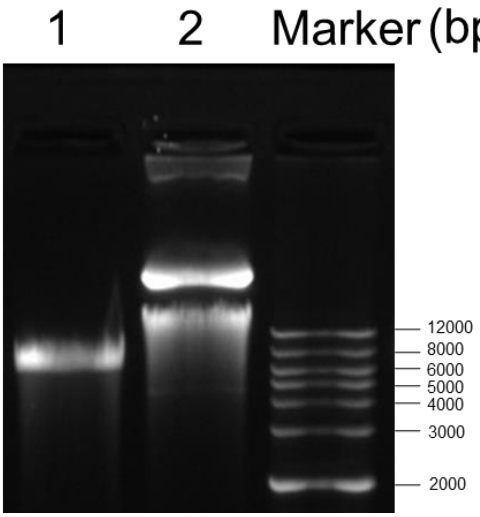

Figure S12. Electrophoretic results of pEGFP-N1-ALDH1A1 plasmids and linearized plasmids. Electrophoresis was performed at 100 V for 40 min in 0.7% agarose Gel (including Gel-Red). Line 1 showed the linearized plasmids and line 2 was the unlinearized plasmids. The marker was a DNA ladder from 2000 to 12000 bp.

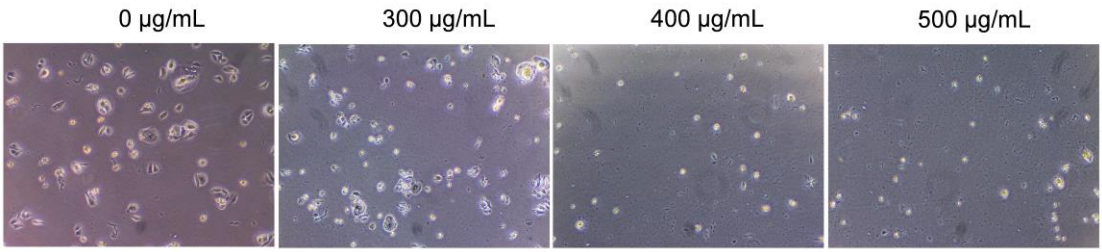

Figure S13. G418 concentration selection.

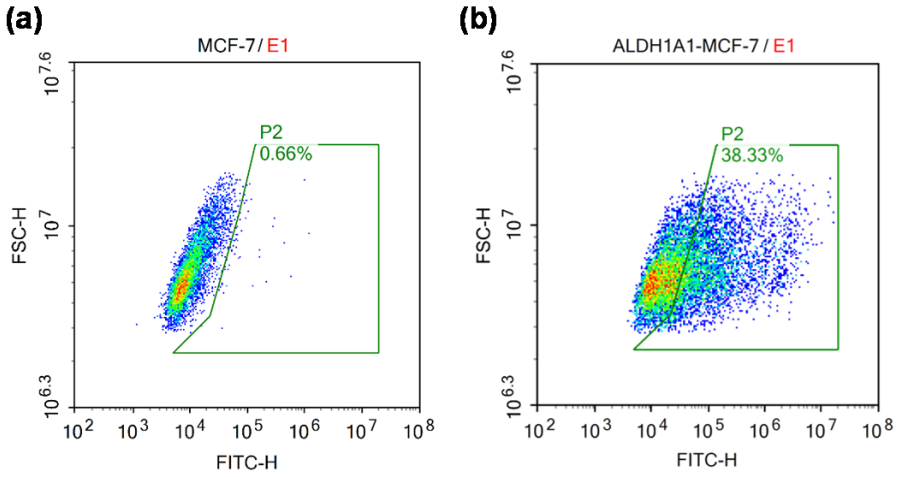

Figure S14. Transfection of MCF-7 cells with pEGFP-N1-ALDH1A1 plasmids was verified by flow cytometry. MCF-7 cells (a) and ALDH1A1-MCF-7 cells (b) were treated with PBS (10 mM, 0.1%FBS) to adjust the cell density to 10^6 cells/mL, and flow cytometry was performed at an excitation wavelength of 488 nm based on EGFP expression.

References

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