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SUPPORTING INFORMATION

Toward Live-Cell Imaging of Dopamine Neurotransmission with Fluorescent Neurotransmitter Analogues

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I. MATERIALS AND METHODS

Materials. Dopamine hydrochloride (DA) (Alfa Asesar), L-norepinephrine hydrochloride (L-NE), serotonin hydrochloride (5-HT), ethylamine (EA), 1-[2-(diphenylmethoxy)ethyl]-4-(3-phenylpropyl)piperazine dihydrochloride (GBR 12935), reserpine, (±)-nicotine, fluorescein isothiocyanate (FITC), collagen from rat tail (Type I), potassium chloride (KCl) and sodium bicarbonate ($NaHCO_3$) (Sigma), benzylamine (BA) hydrochloride, cyclohexylamine (CA), cadmium chloride hydrate ($CdCl_2$), *N,N*-dimethylformamide (DMF, anhydrous 99.8%) and triethylamine (Sigma-Aldrich), γ-aminobutyric acid (GABA) (Fluka), mouse nerve growth factor (NGF, 7.0S) and Dulbecco's phosphate-buffered saline (D-PBS) (Invitrogen) were obtained from the indicated sources.

Cell culture. Rat adrenal pheochromocytoma (PC12) cells, human hepatoblastoma (HepG2) cells, human cervical epithelioid carcinoma (HeLa) cells and mouse neuroblastoma (NB41A3) cells were obtained from the Bioresource Collection and Research Center (BCRC, Taiwan). Human neuroblastoma (SK-N-SH) cells and Chinese hamster ovary cells (CHO-K1) were obtained from the American Type Culture Collection (ATCC, U.S.A.). All cells were cultured according to protocols reported in literatures.[1-7] Briefly, PC12 cells were plated in a collagen-coated glass-base dish, and were cultured for another day before use. Differentiation of PC12 cells was induced by loading the medium with NGF (50 ng/mL) for designed durations. HepG2, HeLa, NB41A3, SK-N-SH and CHO-K1 cells were plated onto glass-base dishes, and were cultured for another two days before use.

Uptake of fluorescent analogues by PC12 cells (Figures 1). The solution of varied fluorescent analogues of neurotransmitters or monoamines was prepared with culture medium immediately before experiments. PC12 cells were incubated with the designated solution (final concentration 10 μM) for 10 min at 37 °C in 5 % CO_2 and imaged. The control was PC12 cells loaded with the same amount of culture medium containing no fluorescent analogue.

Competitive and Inhibitory assays for the uptake of FITC-DA by PC12 cells (Figure 2). To examine the competition between natural dopamine and FITC-DA, PC12 cells were incubated with a solution containing FITC-DA (final concentration 100 nM) and dopamine of varied concentrations (0, 100, 500 and 1000 nM) for 10 min at 37 °C in 5 % CO_2 before imaging. To examine the inhibitory effect of GBR 12935 on the uptake of FITC-DA, PC12 cells were pretreated with a GBR 12935 solution of varied concentrations (0, 50 and 100 nM) for 90 min, and then were loaded with a solution of FITC-DA (final concentration 100 nM) for 10 min at 37 °C in 5 % CO_2 before imaging. The data displayed in **Figure 2** have a background subtracted, which was determined from PC12 cells loaded with solvent containing no FITC-DA, dopamine or GBR 12935.

Cytotoxicity of FITC-DA on the differentiation of PC12 cells (Figure S2). To examine the toxicity of FITC-DA, undifferentiated PC12 cells were loaded with FITC-DA (10 μM) for 10 min. The medium was replaced with a new medium containing NGF (50 ng/mL), and then incubated for another 96 h at 37 °C in 5 % CO_2 before imaging. The control is PC12 cells loaded with the same amount of solvent containing no FITC-DA for 10 min, and then incubated a medium containing NGF (50 ng/mL) for another 96 h at 37 °C in 5 % CO_2 before imaging.

Uptake of FITC-DA by cells of varied types (Figure 3). Cells of the designated type were incubated with FITC-DA (10 μM, 10 min) at 37 °C in 5 % CO_2 and imaged. The control comprised cells of the designated type loaded with solvent containing no FITC-DA.

Evoked release of FITC-DA from PC12 cells (Figure 4). To examine the evoked release of FITC-DA by potassium ions, PC12 cells were prepared by incubation with NGF for 72 h, and loaded with FITC-DA (10 μM, 10 min). Before measurements, the cells were washed three times with 1× D-PBS and supplied with a fresh 1× D-PBS buffer. The cell dishes were then injected with a solution of KCl (20 uL, 70 mM). The time-varying intensity of fluorescence was measured concurrently with a setup built on an inverted optical

microscope (X71, Olympus, Japan) equipped with a video system (**Figure 4a**, to be described). The sham experiment was performed on injection of a PBS solution containing no potassium ions. The untreated control corresponds to cells subject to no injection. The result is displayed in **Figure 4b** in which the arrow denotes the time point of the injection (blue: with an injection of KCl; dark grey: sham; light grey: untreated control).

A diode-pumped solid laser (Sapphire-488, Coherent, U.S.A.) served as excitation. Guided with a video system, the Petri dish that contained cells was positioned with a translational stage such that the laser focus was located in the cytoplasm of a cell. The fluorescence signal was collective by an objective lens (UPLSAPO 60XW NA1.20; Olympus, Japan), filtered and then detected with a fiber-coupled photomultiplier (R928, Hamamatsu, Japan). To increase the signal-to-noise ratio, a scheme of phase-sensitive detection was employed. In brief, the intensity of the laser was modulated with an optical chopper at 2.5 kHz. The signal produced from the photomultiplier was demodulated and amplified with a lock-in amplifier (SRS 850, Stanford Research Systems, U.S.A.). The output signal from the lock-in amplifier was digitized with a data acquisition card (PCI6221, National Instruments, U.S.A.) at a sampling rate of 1 kHz, and averaged every 200 data-points that corresponded to a temporal resolution of 0.2 s. With this setup, we were able to keep the laser power below 2 μW throughout the measurements while maintaining a good signal-to-noise ratio.

To examine the effect of an inhibitor of VMAT 2, PC12 cells were pretreated with a solution of reserpine (20 μM) for 90 min before the measurements. The cells were then injected with a solution of KCl in the same way as the preceding experiment (**Figure 4c**; blue: cells pre-treated with reserpine; grey: cells with no pretreatment of reserpine). To examine if the evoked release of FITC-DA depended on calcium ions, we examined cells pretreated with $CdCl_2$ (100 μM) for 20 min before measurements, and then injected with a solution of KCl (**Figure 4d**; blue: cells pre-treated with $CdCl_2$; grey: cells with no pretreatment of $CdCl_2$).

The result displayed in **Figure 4** is representative and has been normalized to the data recorded at the time zero.

Animals and feeding protocols. Male Wistar rats (BioLASCO Experimental Animal Center, Taiwan) aged ~8 weeks, mass ~300 g were used. All animals were allowed free access to standard diet and drinking water, and were maintained under cycles with light for 12 h and darkness for 12 h. The animals were fasted for 24 h and then anesthetized by intraperitoneal injection of pentothal (2.5 %, 70 mg/kg) before experiments. At the end of the experiment, the rat was euthanized by injection of excessive pentothal.

Staining and mapping of acute brain slices of rats (Figure 5). The whole brain was removed from a deeply anesthetized rat, and cut into thin slices (thickness ~ 1 mm). The acute brain slices were transferred immediately to a culture medium containing FITC-DA (20 μM) for 10 min at 37 °C in 5 % CO_2. Before imaging, the slices were washed three times with 1× D-PBS. The whole process from removal of the whole brain to imaging of the brain slice was completed within 15 min. The control was prepared by loading a medium containing no FITC-DA.

Pharmacological activity of FITC-DA on the heart rate of rats (Figure S3). To examine if FITC-DA increased the heart rate in a way similar to dopamine, a dose of FITC-DA (4.5 μmol/kg) was injected intravenously at an injection rate around 20 μg/kg/min after anesthesia, and the heart rate was measured for three min at the end of the injection. The experiment of dopamine was performed in the same way. The two sham experiments were implemented with an injection of normal saline or normal saline containing FITC (4.5 μmol/kg) or FITC-EA(4.5 μmol/kg). The control is rats subject to no injection.

Optical imaging. Before imaging, the cells were washed three times with 1× D-PBS and then supplied with a medium free of serum. All images were obtained with a modified laser-scanning confocal microscope (FV300; Olympus, Japan) equipped with an objective lens (UPLSAPO 60XW NA1.20; Olympus, Japan). A diode-pumped solid laser

(Sapphire-488, Coherent, U.S.A.) served for excitation. The laser power, measured before the back aperture of the objective lens, was kept below 0.3 mW for all measurements, except the cotreatment of natural dopamine and inhibitory assay **(Figure 2)** for which a power of 0.5 mW was employed. The emission between 510 nm and 650 nm was recorded. Bright-field and fluorescent images were obtained simultaneously. All images shown in this work were produced on averaging results of five consecutive scans.

Quantitative analysis of images. Quantitative analysis of the image was performed with commercial software (FluoView; Olympus, Japan) according to a protocol illustrated in **Figure S6** The region of individual cells was first identified as a region of interest (ROI). The integrated intensity and number of pixels of the ROI were obtained from the software.

The intensity of all images, except those in **Figures 3** and **Figure S4**, was represented as average intensity per pixel that was calculated by dividing the integrated intensity of an ROI with the number of pixels for that ROI; the result was then averaged over an indicated number of ROI for each experiment. The intensity of the images in **Figures 3** and **Figure S4** are presented as average intensity per cell.

Unless explicitly specified, all data have the background subtracted, determined on cells treated with solvent of the same amount containing no fluorescent analogue.

Statistical analysis. All data are expressed as mean ± SEM. Comparison between the means of two groups was made using the *two-tailed Student's t* test. The levels of statistical significance were set at $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$, respectively.

II. SYNTHESIS AND CHARACTERTERIZATION OF ALL PRODUCES

Synthesis and characterization of fluorescent analogues. The synthesis of the fluorescent analogue of neurotransmitters (DA, L-NE, 5-HT and GABA) or monoamines (BA, CA and EA) was achieved through conjugation of the isothiocyanate reactive group (-N=C=S) of FITC to the primary amine group of the neurotransmitters or the monoamines **(Scheme 1)**. For instance, to conjugate FITC to dopamine, FITC (2×10^{-5} mol) was mixed with dopamine (2×10^{-5} mol) in an anhydrous DMF solution (5 mL) containing triethylamine (5 μL). After stirring under nitrogen for 24 h at 25 °C, distilled H_2O (1 mL) was added and the resultant precipitate was collected. The solid was then washed three times with distilled H_2O (3 x 3 mL). Proton NMR was used to check the purity of the product. If the purity was not satisfied, flash column chromatograph was used to remove the impurities. FITC-DA was obtained quantitatively as an orange solid. CH_2Cl_2/MeOH (10/1, v/v) was used for gel purification of FITC-DA. The purify of the product was verified with an electrospray-ionization mass spectrometer (ESI-MS), and ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra. The emission spectrum (Excitation = 488 nm) of FITC-DA is nearly identical to that of FITC (**Figure S5**).

Data for novel compounds:

FITC-dopamine (FITC-DA)

^{1}H NMR (400 MHz, DMSO-d_6, ppm): δ = 10.15 (brs, 2H), 9.96 (brs, 1H), 8.80 (s, 1H), 8.69 (s, 1H), 8.23 (s, 1H), 8.00 (brs, 1H), 7.71 (d, J = 7.6 Hz, 1H), 7.16 (d, J = 8.4 Hz, 1H), 6.45-6.75 (m, 9H), 3.66 (brs, 2H), 2.72 (t, J = 7.2 Hz, 2H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm): δ = 180.3, 168.7, 160.0, 152.1, 146.6, 145.2, 143.8, 141.3, 129.9, 129.2, 127.1, 124.3, 119.4, 116.8, 116.1, 115.7, 113.0, 110.0, 102.3, 45.7, 33.8; HRMS (ESI) calculated for $C_{29}H_{22}N_2O_7S$ [M+H$^+$] requires 543.11, found 543.09.

FITC-L-norepinephrine (FITC-L-NE)

^{1}H NMR (400 MHz, DMSO-d_6, ppm): δ = 10.12 (brs, 2H), 8.93 (brs, 2H), 8.36 (s, 1H), 8.01 (s, 1H), 7.72 (d, J = 8.0 Hz, 1H), 7.16 (d, J = 8.4 Hz, 1H), 6.81 (s, 1H), 6.49-6.74 (m, 9H), 5.53 (brs, 1H), 4.68 (brs, 1H), 3.84 (brs, 1H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm): δ = 180.6, 168.9, 159.8, 152.1, 147.1, 145.2, 144.6, 141.6, 134.6, 129.6, 129.3, 126.8, 124.3, 117.0, 116.7, 115.4, 113.6, 113.0, 110.2, 110.0, 102.5, 70.5, 52.0; HRMS (ESI) calculated for $C_{29}H_{22}N_2O_8S$ [M+H$^+$] requires 559.11, found 559.07.

FITC-serotonin (FITC-5-HT)

^{1}H NMR (400 MHz, DMSO-d_6, ppm): δ = 10.53 (s, 1H), 10.07 (brs, 1H), 8.63 (brs, 1H), 8.26 (s, 1H), 8.18 (brs, 1H), 7.73 (d, J = 8.0 Hz, 1H), 7.16 (dd, J = 8.4, 8.4 Hz, 2H), 7.10 (s, 1H), 6.95 (s, 1H), 6.54-6.72 (m, 7H), 3.78 (brs, 2H), 2.95 (t, J = 7.2 Hz, 2H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm): δ = 180.3, 168.6, 160.0, 152.1, 150.2, 146.5, 141.4, 130.9, 129.1, 127.9, 127.0, 124.2, 123.4, 116.7, 112.9, 111.7, 111.4, 110.5, 109.9, 102.5, 102.3, 44.5, 24.6; HRMS (ESI) calculated for $C_{31}H_{23}N_3O_6S$ [M+H$^+$] requires 566.13, found 566.13.

FITC-γ-aminobutyric acid (FITC-GABA)

^{1}H NMR (400 MHz, DMSO-d_6, ppm): δ = 10.10 (brs, 2H), 8.35 (brs, 1H), 8.24 (s, 1H), 7.76 (brs, 1H), 7.16 (d, J = 8.0 Hz, 1H), 6.53-6.69 (m, 6H), 3.51 (brs, 2H), 2.28 (t, J = 7.2 Hz, 2H), 1.80 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm): δ = 180.5, 174.4, 168.6, 159.6, 151.9, 146.9, 141.5, 129.5, 129.0, 126.6, 124.0, 116.5, 112.6, 109.8, 43.2, 31.4, 24.1; HRMS (ESI) calculated for $C_{25}H_{20}N_2O_7S$ [M+H$^+$] requires 493.10, found 493.29.

FITC-benzylamine (FITC-BA)

^{1}H NMR (400 MHz, DMSO-d_6, ppm): δ = 10.26 (brs, 1H), 8.73 (brs, 1H), 8.29 (s, 1H), 7.75 (dd, J = 8.4, 0.8 Hz, 1H), 7.22-7.44 (m, 4H), 7.16 (d, J = 8.4 Hz, 1H), 6.52-6.70 (m, 6H), 4.77 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm): δ = 180.9. 168.6, 160.0, 152.1, 146.5, 141.4, 138.7, 129.5, 129.1, 128.5, 128.3, 127.8, 127.4, 127.0, 124.2, 117.0, 112.9, 109.9, 102.3, 47.1; HRMS (ESI) calculated for $C_{28}H_{20}N_2O_5S$ [M+H$^+$] requires 497.11, found 497.17.

FITC-cyclohexylamine (FITC-CA)

^{1}H NMR (400 MHz, DMSO-d_6, ppm): δ = 10.26 (brs, 2H), 9.76 (s, 1H), 8.30 (s, 1H), 7.99 (d, J = 7.2 Hz, 1H), 7.73 (d, J = 8.0 Hz, 1H), 7.14 (d, J = 8.4 Hz, 1H), 6.53-6.69 (m, 6H), 4.10 (brs, 1H), 1.11-1.99 (m, 10H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm): δ = 179.4, 169.0, 159.5, 152.2, 146.9, 141.8, 129.6, 129.3, 126.8, 124.2, 116.6, 113.0, 110.0, 102.5, 52.5, 32.0, 25.4, 24.7; HRMS (ESI) calculated for $C_{27}H_{24}N_2O_5S$ [M+H$^+$] requires 489.14, found 489.19.

FITC-ethylamine (FITC-EA)

^{1}H NMR (400 MHz, DMSO-d_6, ppm): δ = 10.14 (brs, 1H), 10.01 (s, 1H), 8.21 (s, 2H), 7.73 (d, J = 7.6 Hz, 1H), 7.17 (d, J = 8.4 Hz, 1H), 6.56-6.69 (m, 6H), 3.52 (m, 2H), 1.15 (t, J = 7.2 Hz, 2H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm): δ = 180.2, 168.6, 160.0, 152.0, 146.5, 141.4, 129.1, 127.0, 124.2, 116.7, 112.9, 109.0, 102.3, 38.7, 14.0; HRMS (ESI) calculated for $C_{23}H_{18}N_2O_5S$ [M+H$^+$] requires 435.09, found 435.14.

III. REFERENCES

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IV. SUPPORTING DATA

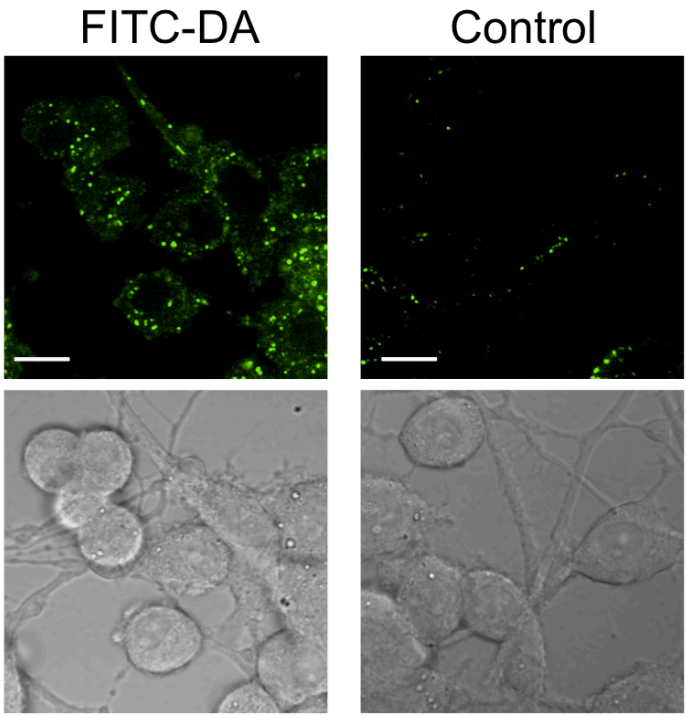


Figure S1. High-resolution images of PC 12 cells showing numerous punctate structures of FITC-DA stored vesicles.

Left: PC12 cells loaded with FITC-DA (5 μM, 5 min).

Right: PC12 cells loaded with medium containing no FITC-DA. Scale bar: 10 μm.

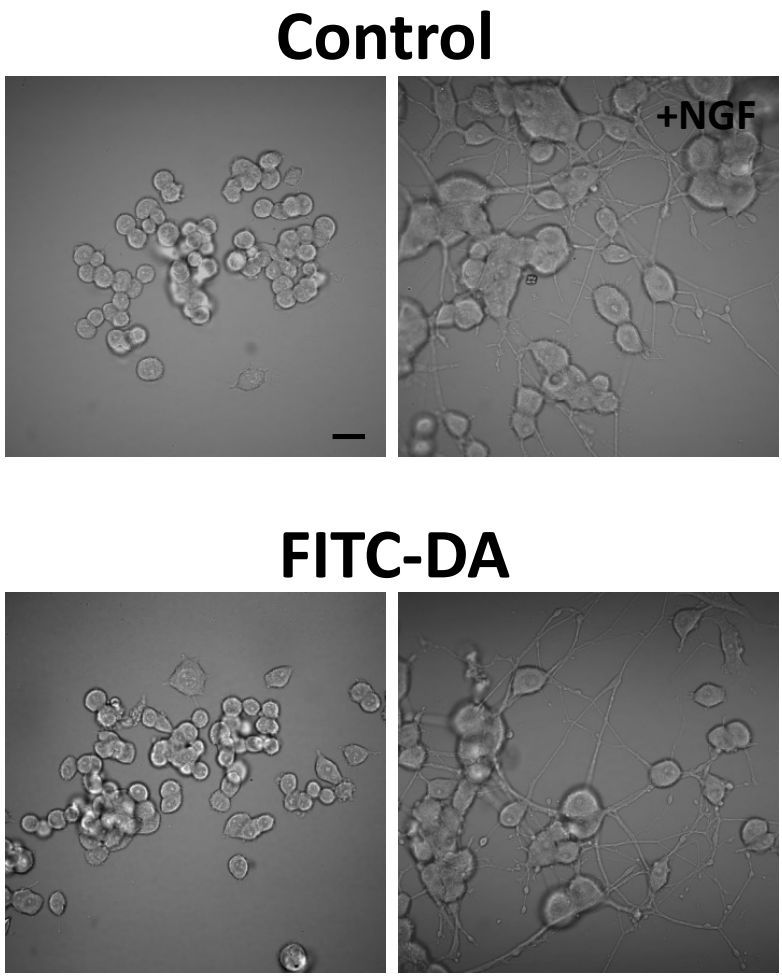


Figure S2. Test of the cytotoxicity of FITC-DA.

Upper panel: bright-field images of PC12 cells obtained before (left) and after (right) adding neural growth factors (NGF) for 96 h.

Lower panel: images obtained from PC12 cells before adding NGF (left), and from cells that were sequentially loaded with FITC-DA and then incubated with NGF for 96 h (right). Scale bar: 20 μm.

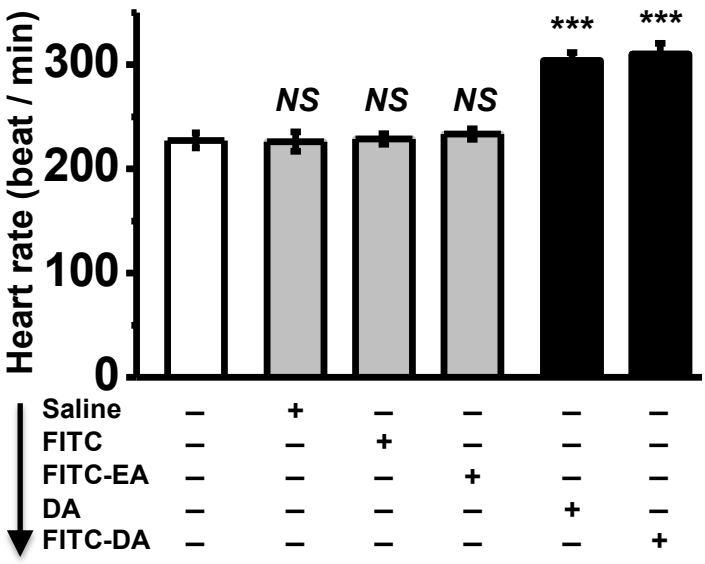


Figure S3. Testing of the pharmacological activity of FITC-DA on the heart rate of rats. ***$P < 0.001$, *NS*: not significant ($n = 5$).

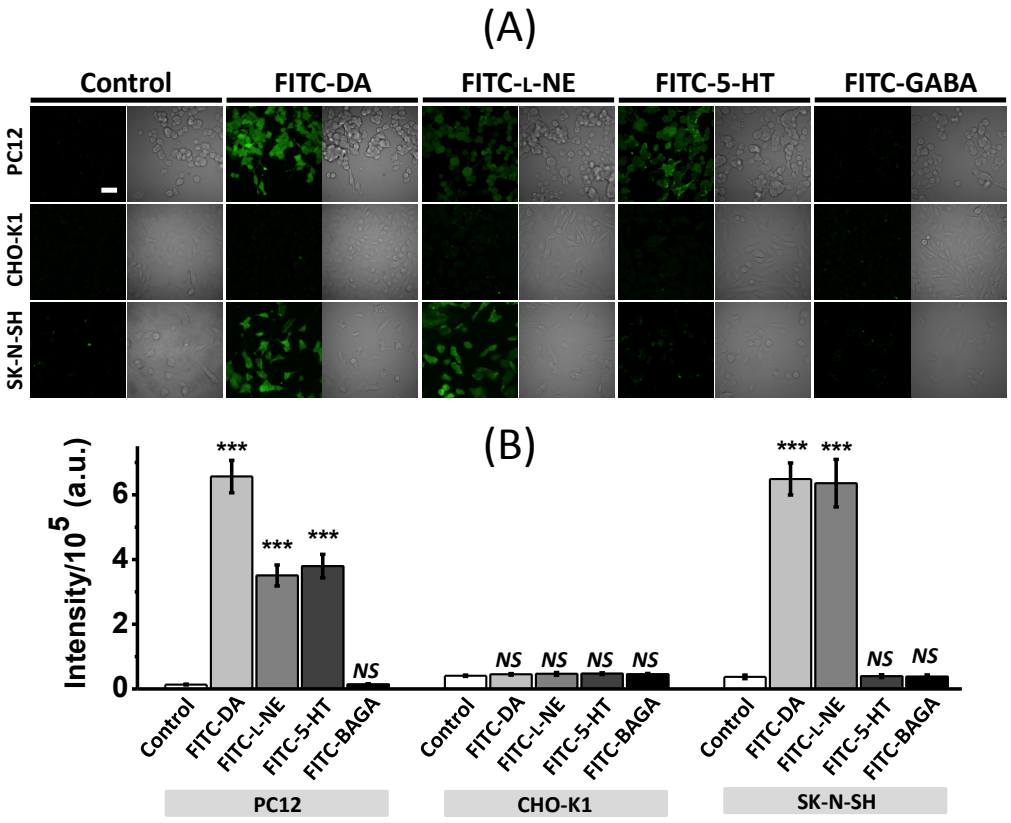


Figure S4. Uptake of fluorescence neurotransmitter analogues by cells of varied types.
(A) Fluorescent (green) and bright-field (grey) images obtained from the untreated control and from the cells loaded with FITC-DA.
(B) Results of quantitative analysis. ***$P < 0.001$, *NS:* not significant ($n = 25$). Scale bar: 40 μm.

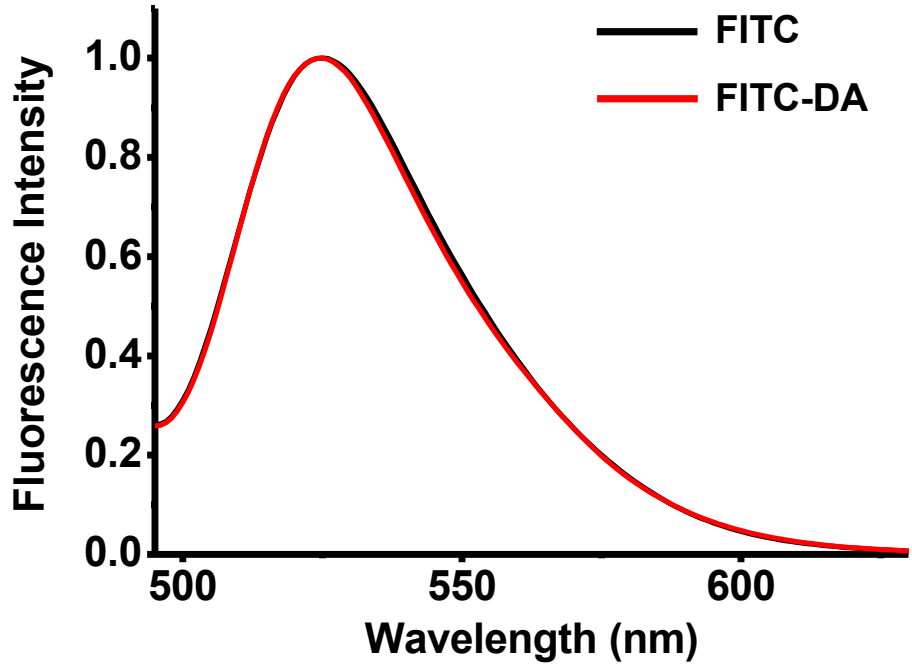
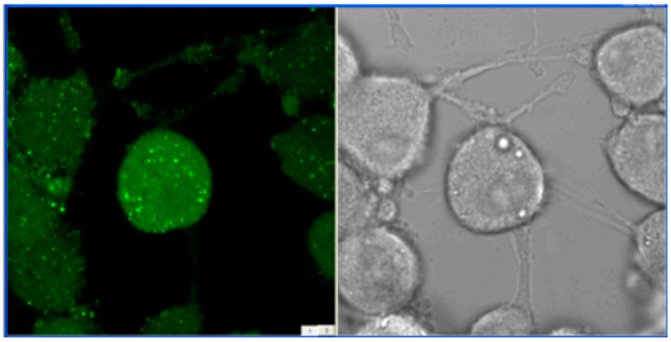
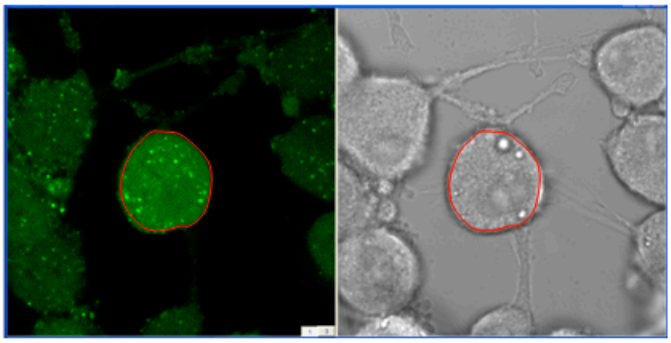


Figure S5. Comparison of the emission spectra of FITC and FITC-DA.
The solution was prepared in DPBS buffer maintained at pH 7.4. The excitation wavelength is 488 nm.

(A)



(B)



(C)

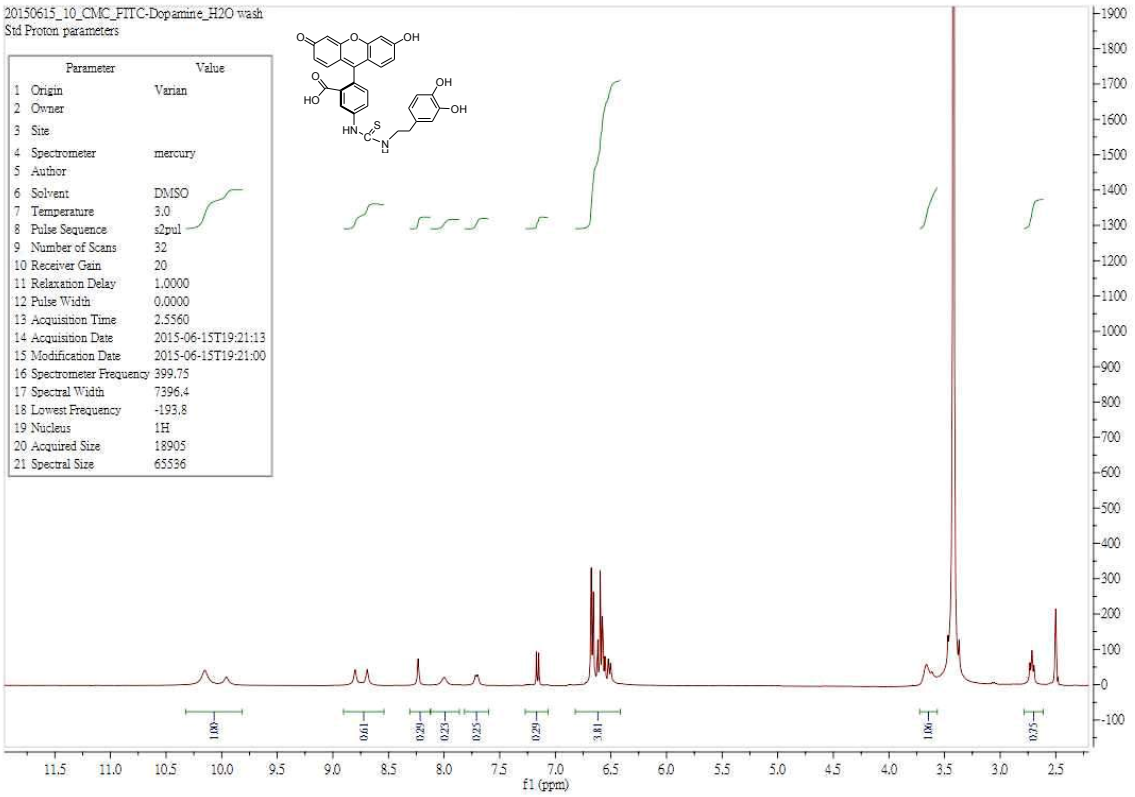
Measurement Results

Perimeter = 64.16 µm
X extent = 15.997 µm
Y extent = 17.954 µm
Area = 217.01 µm^2
Intensity Statistics
Total = 1.8401e+006, 3.9858e+007
Average = 112.31, 2432.8
Std Dev = 28.35, 307.7

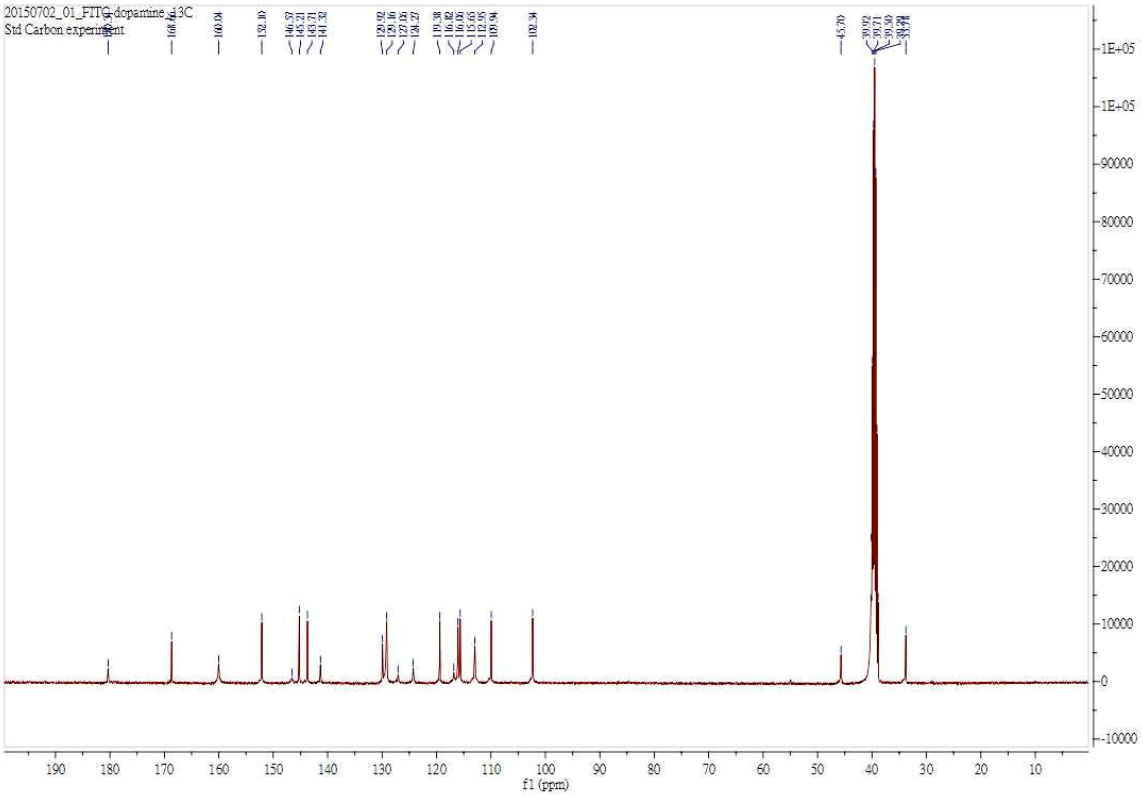
Figure S6. Protocols for quantitative analysis of images.

(A) Representative images obtained at 4X Zoom. (B) Identification of a region of interest (ROI). (C) Parameters calculated from the ROI.

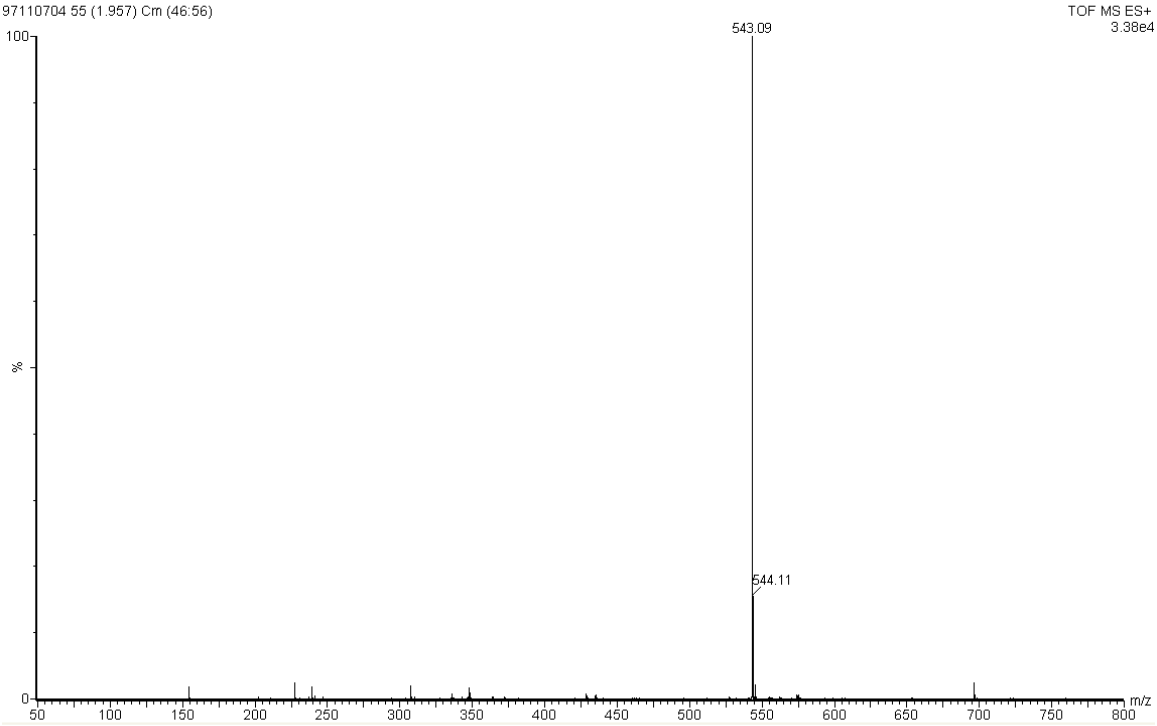
^{1}H NMR spectrum of **FITC-DA** in DMSO-d_6



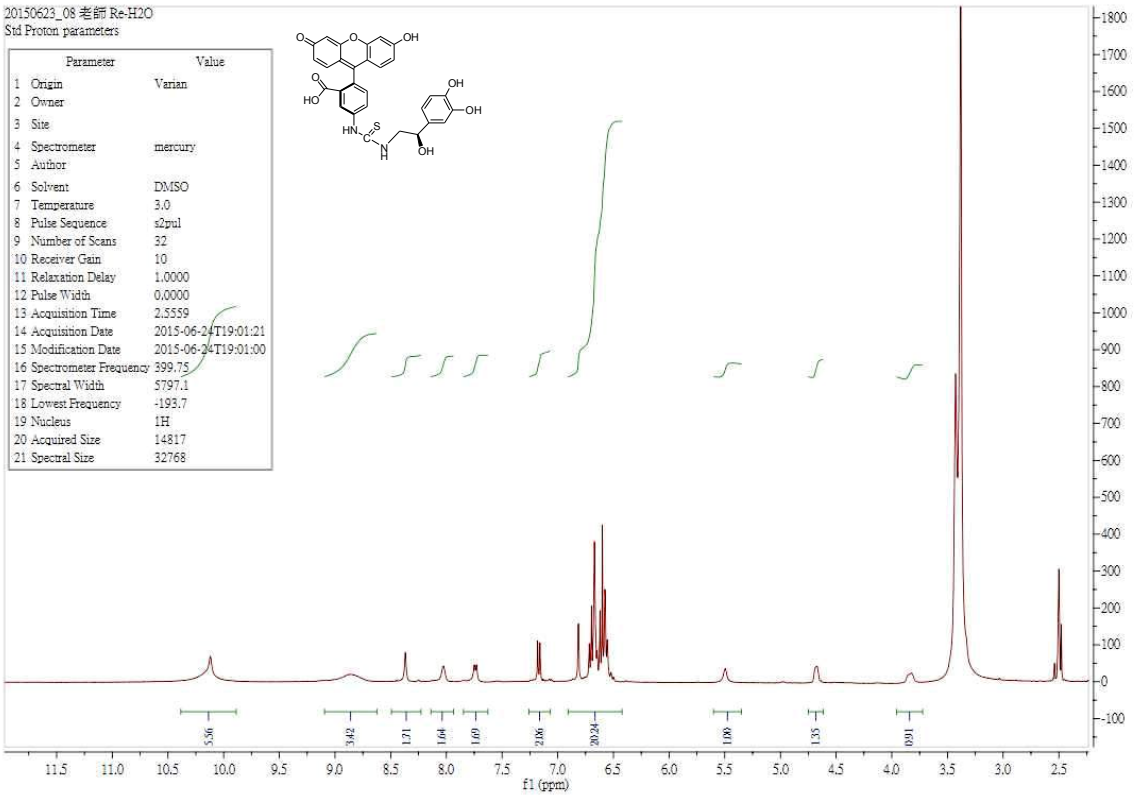
^{13}C NMR spectrum of **FITC-DA** in DMSO-d_6



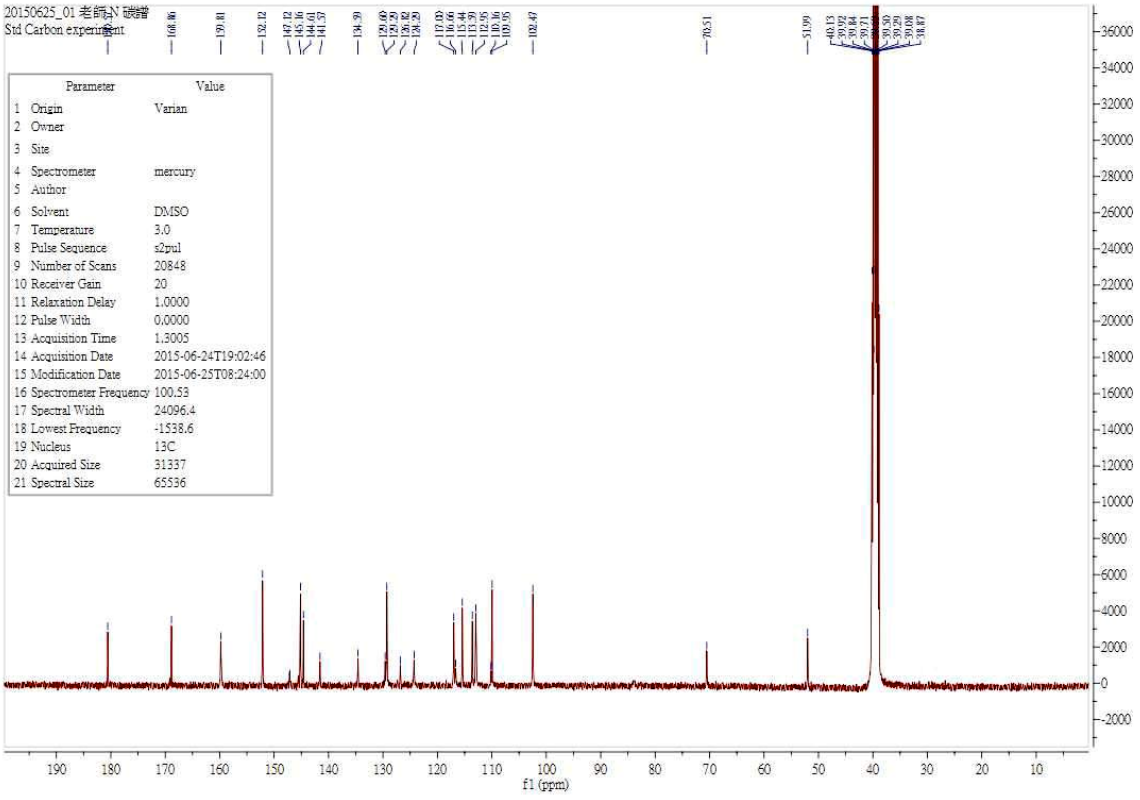
Mass spectrum of **FITC-DA**



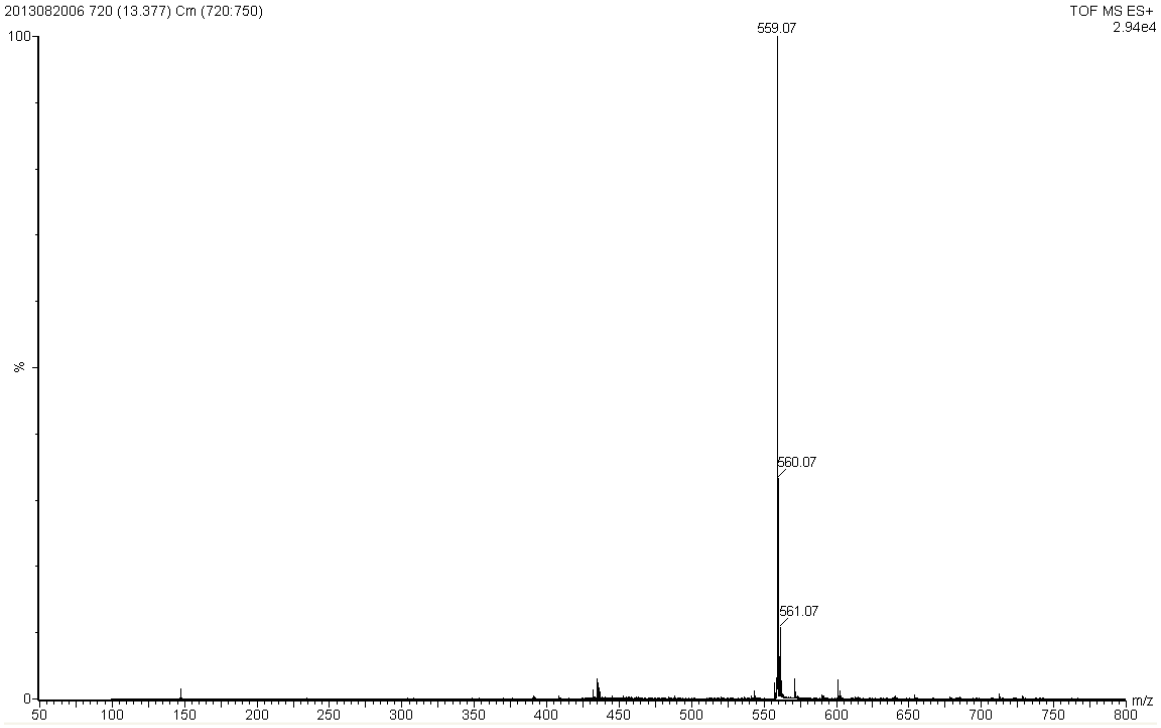
[1]H-NMR spectrum of **FITC-L-NE** in DMSO-d_6



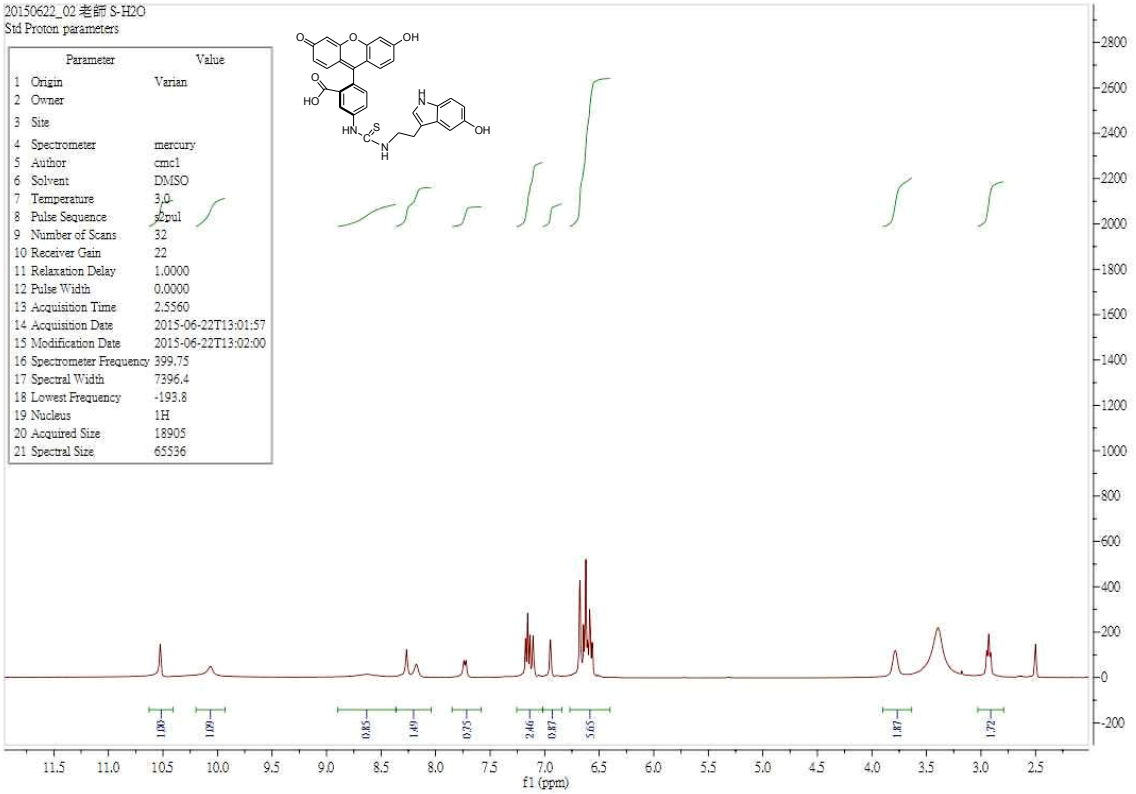
[13]C NMR spectrum of **FITC-L-NE** in DMSO-d_6



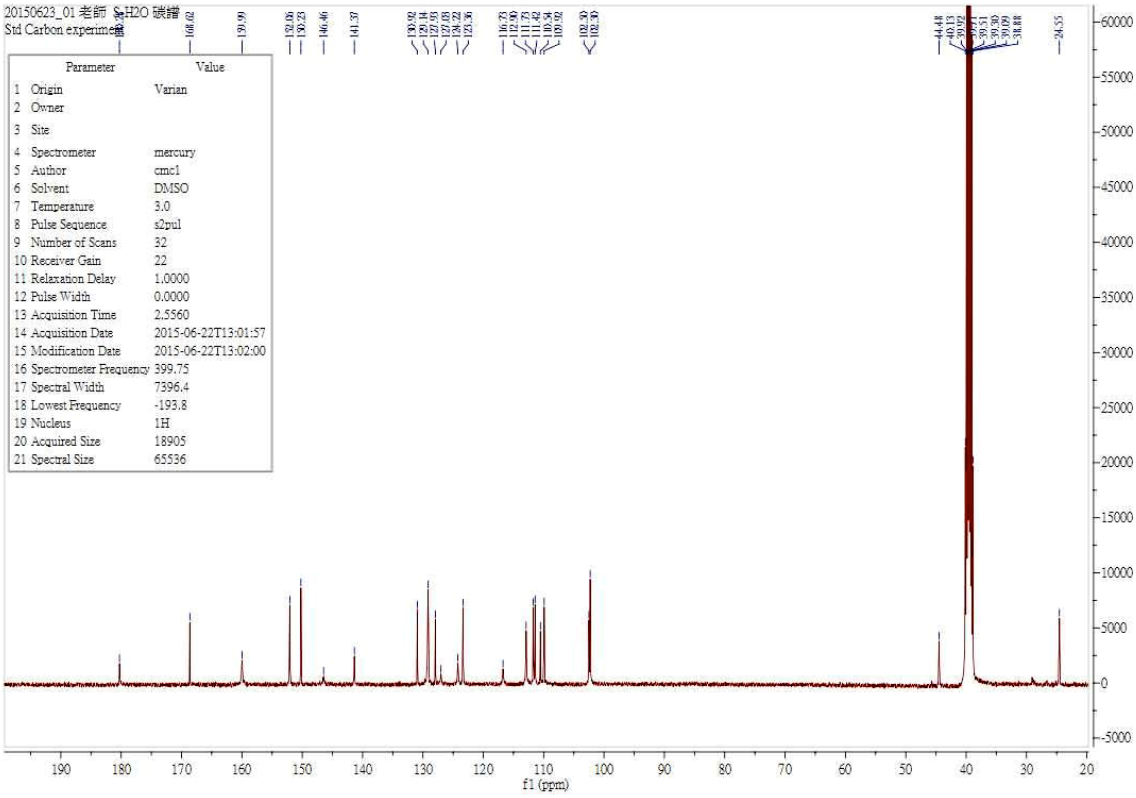
Mass spectrum of **FITC-L-NE**



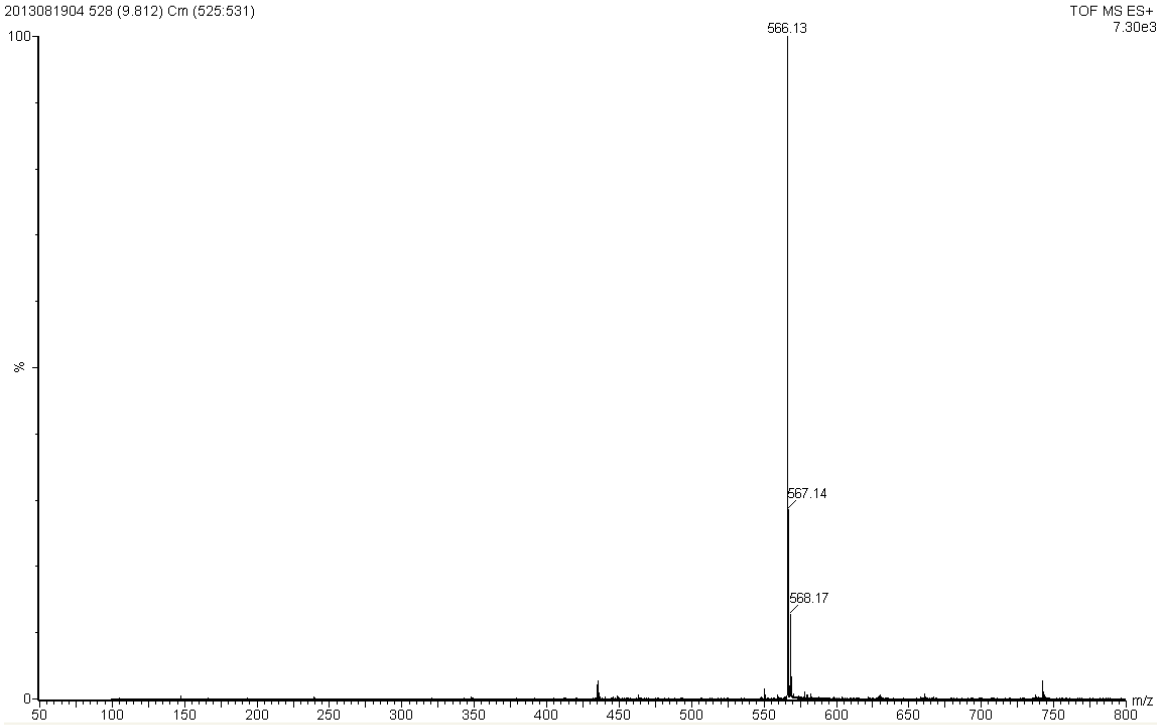
[1]H-NMR spectrum of **FITC-5-HT** in DMSO-d_6



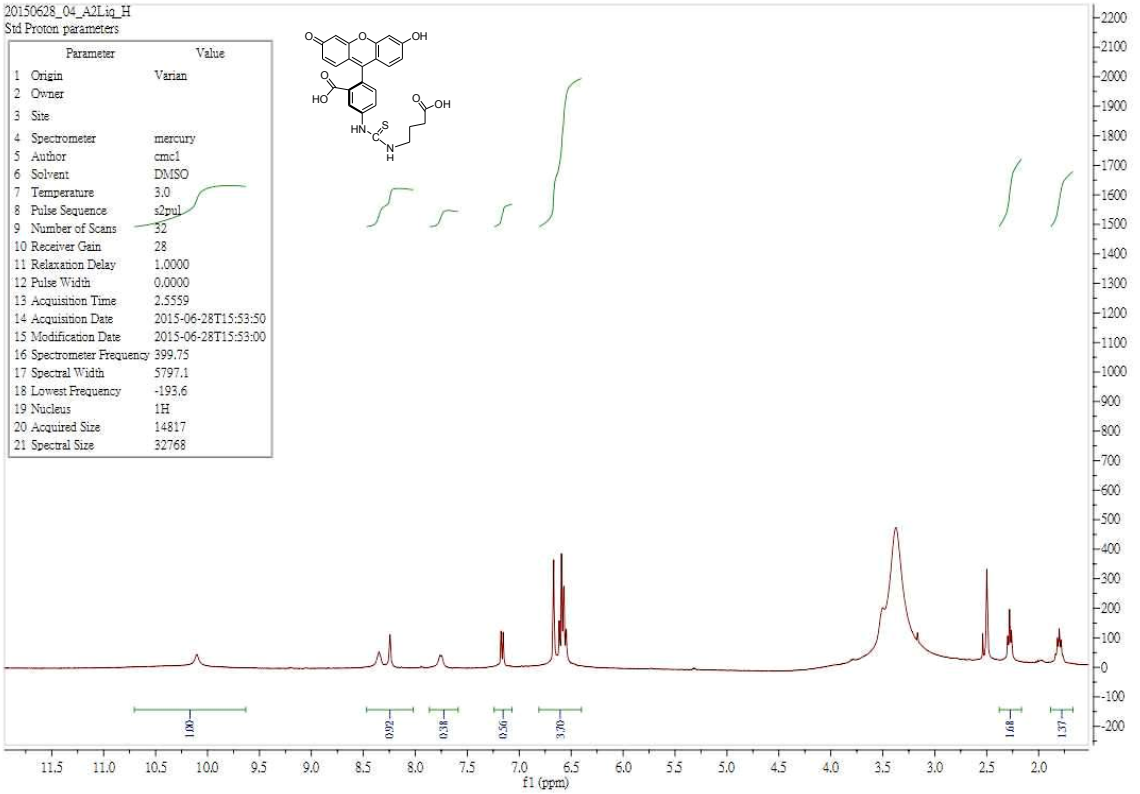
[13]C NMR spectrum of **FITC-5-HT** in DMSO-d_6



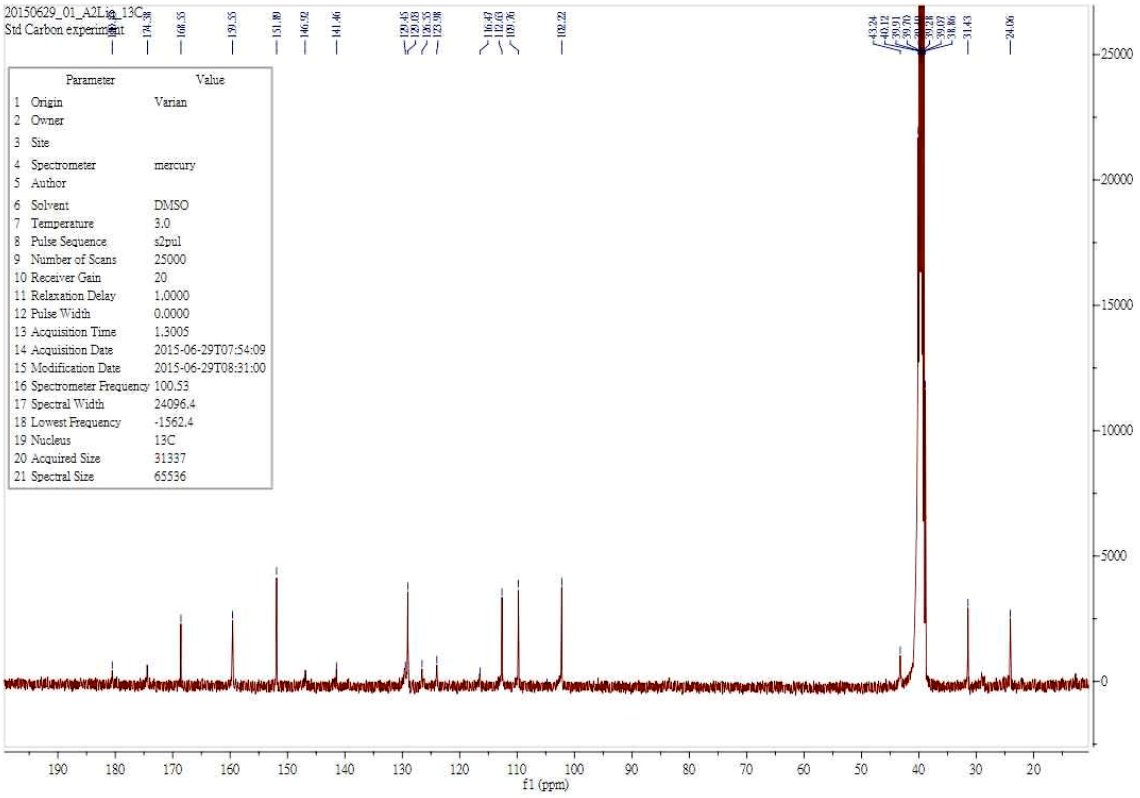
Mass spectrum of **FITC-5-HT**



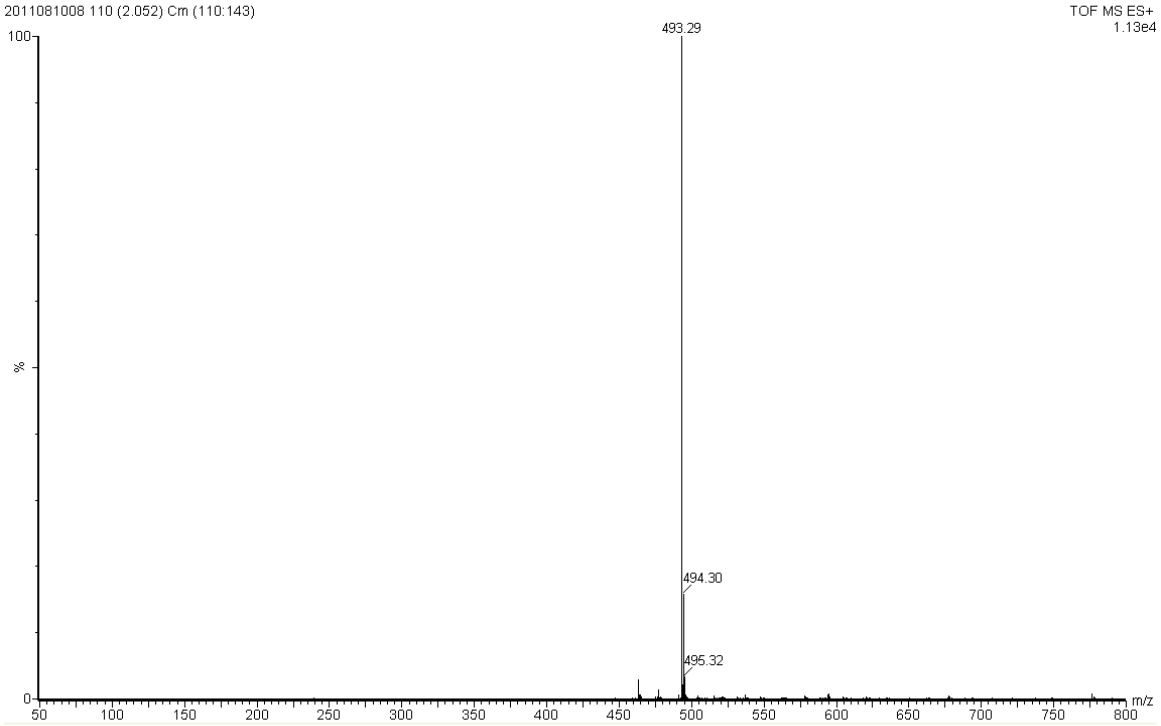
^{1}H-NMR spectrum of **FITC-GABA** in DMSO-d_6



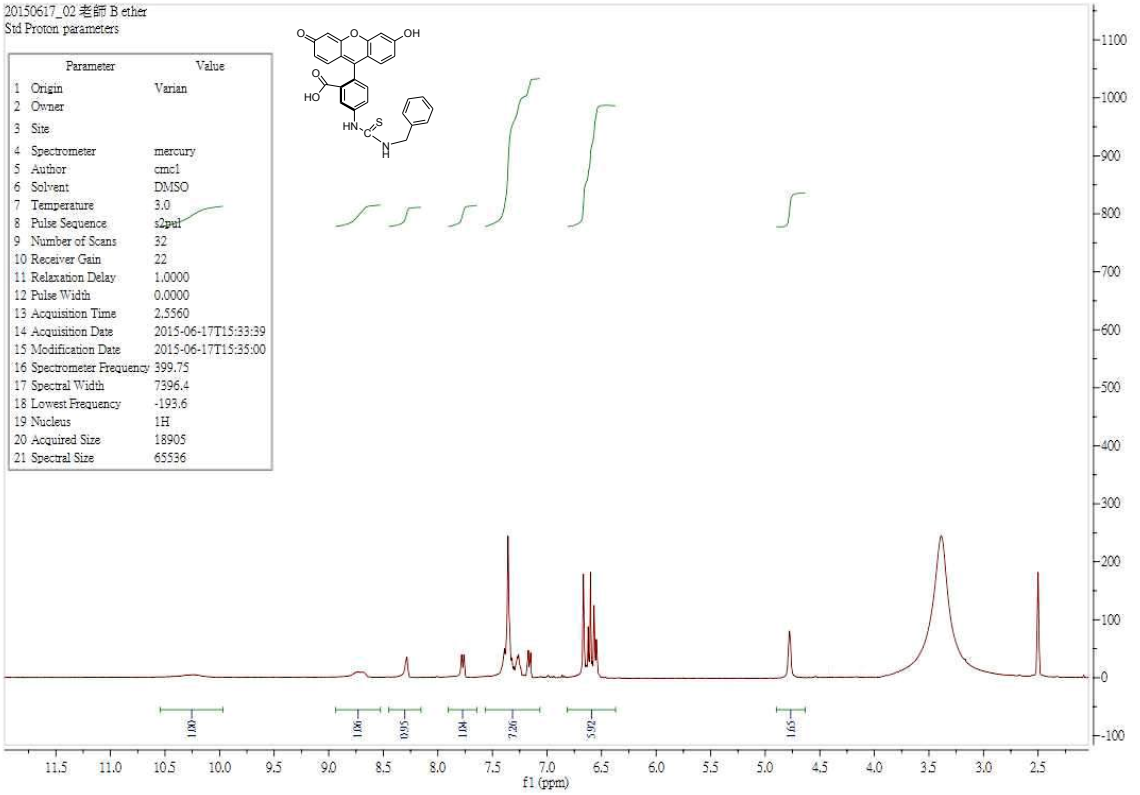
^{13}C NMR spectrum of **FITC-GABA** in DMSO-d_6



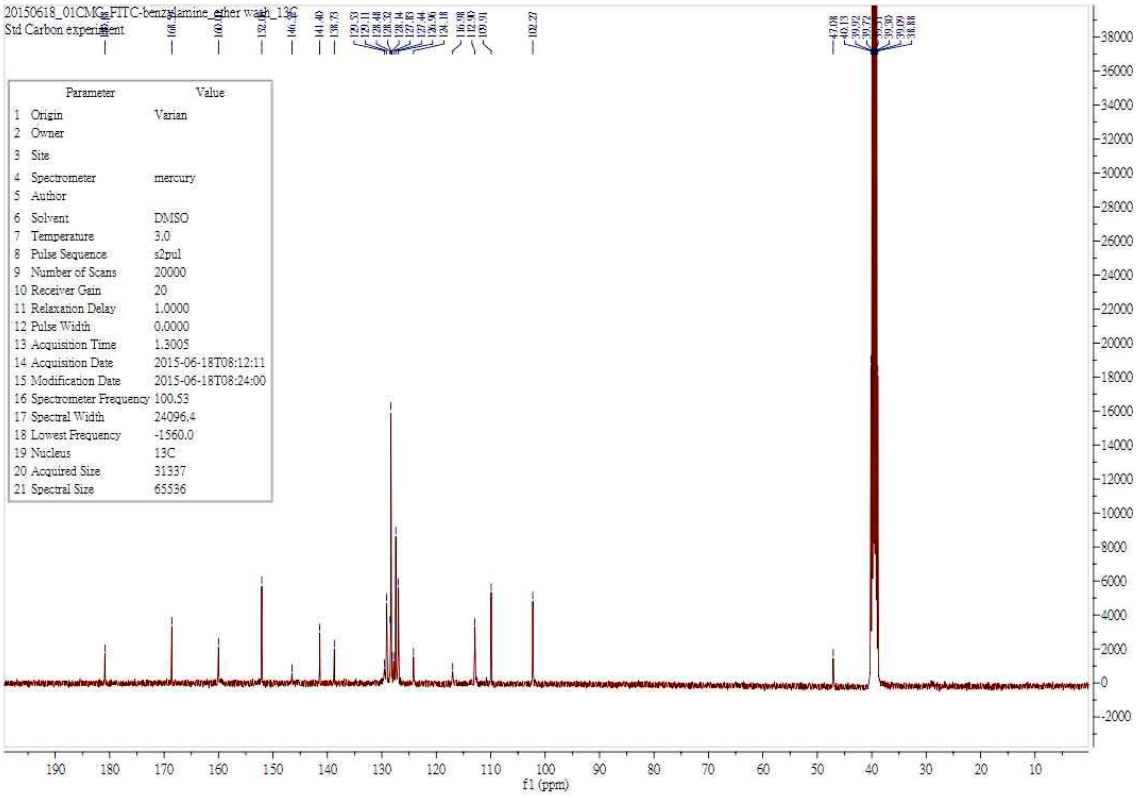
Mass spectrum of **FITC-GABA**



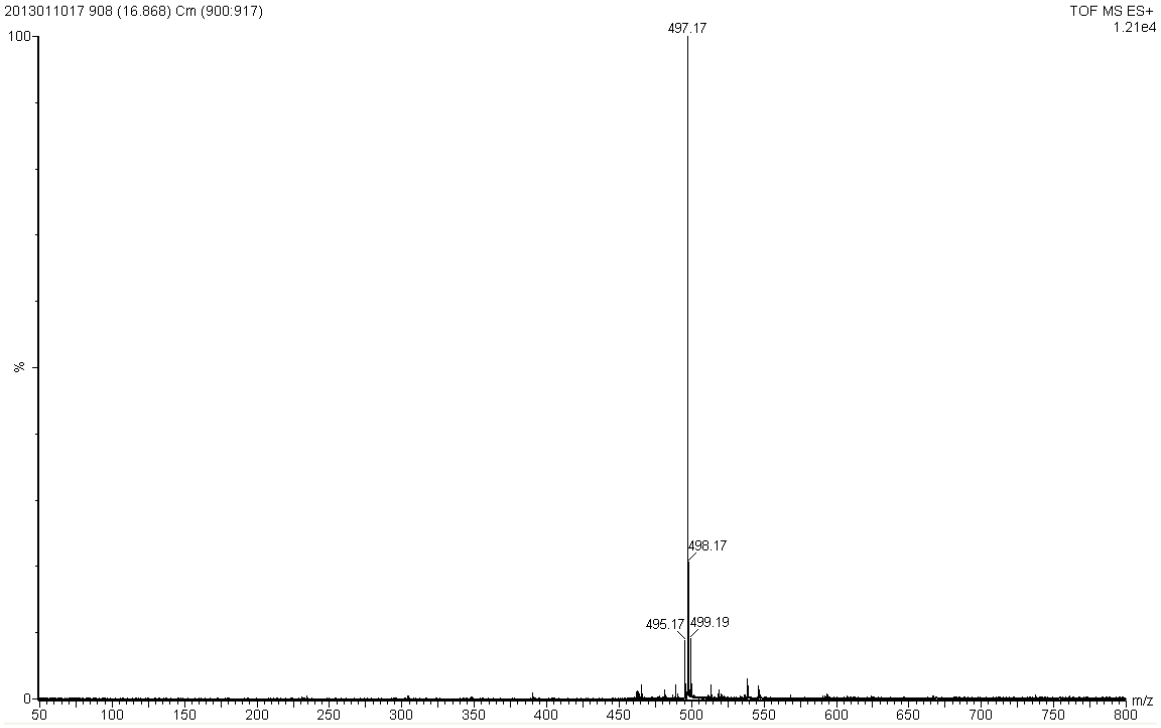
^{1}H-NMR spectrum of **FITC-BA** in DMSO-d_6



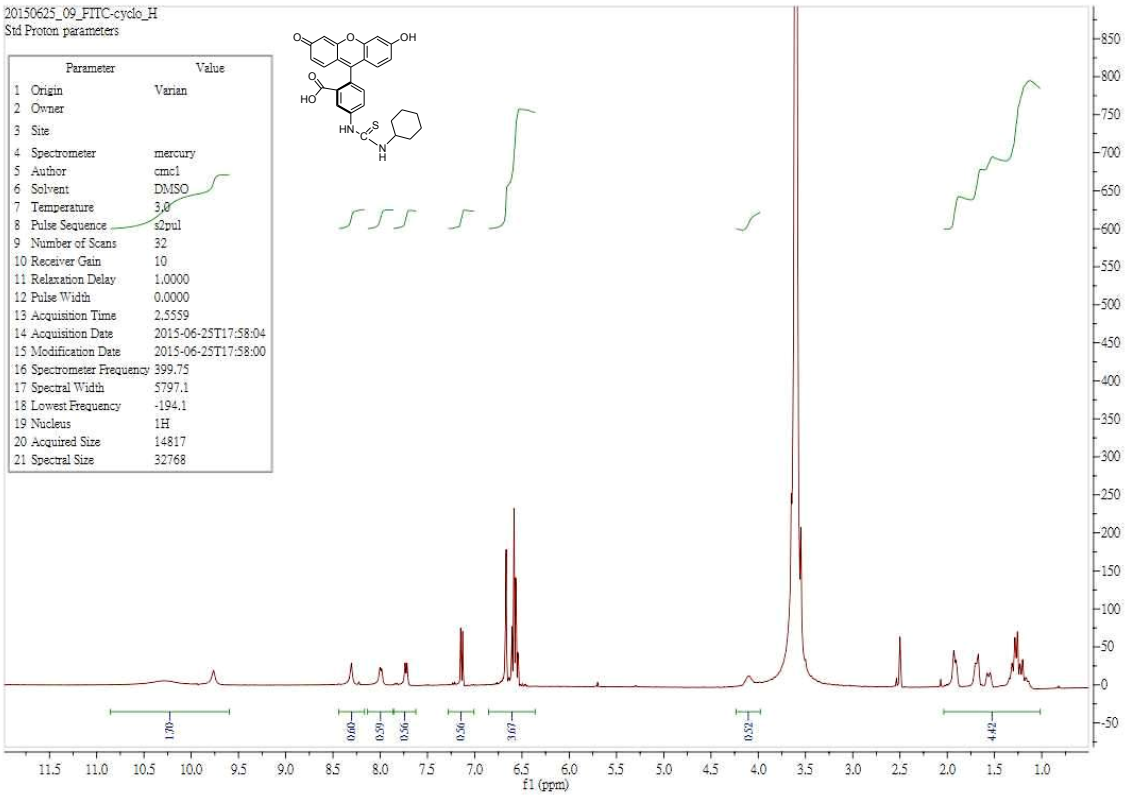
^{13}C NMR spectrum of **FITC-BA** in DMSO-d_6



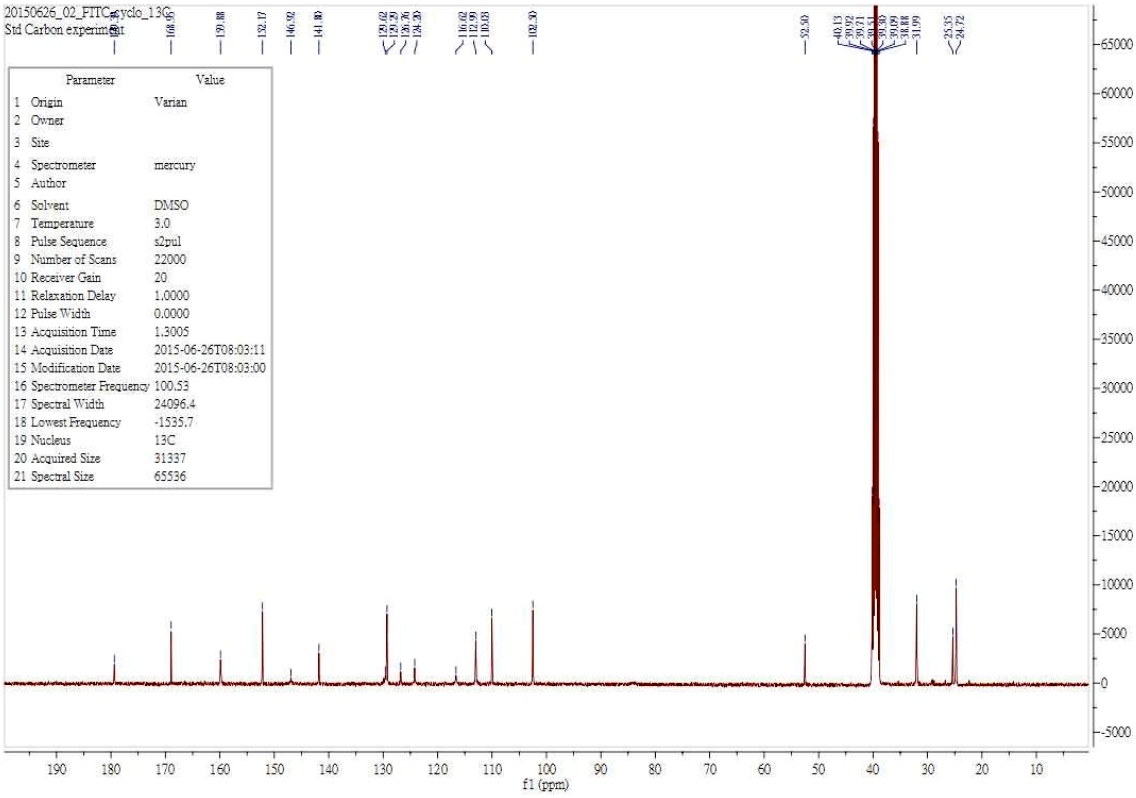
Mass spectrum of **FITC-BA**



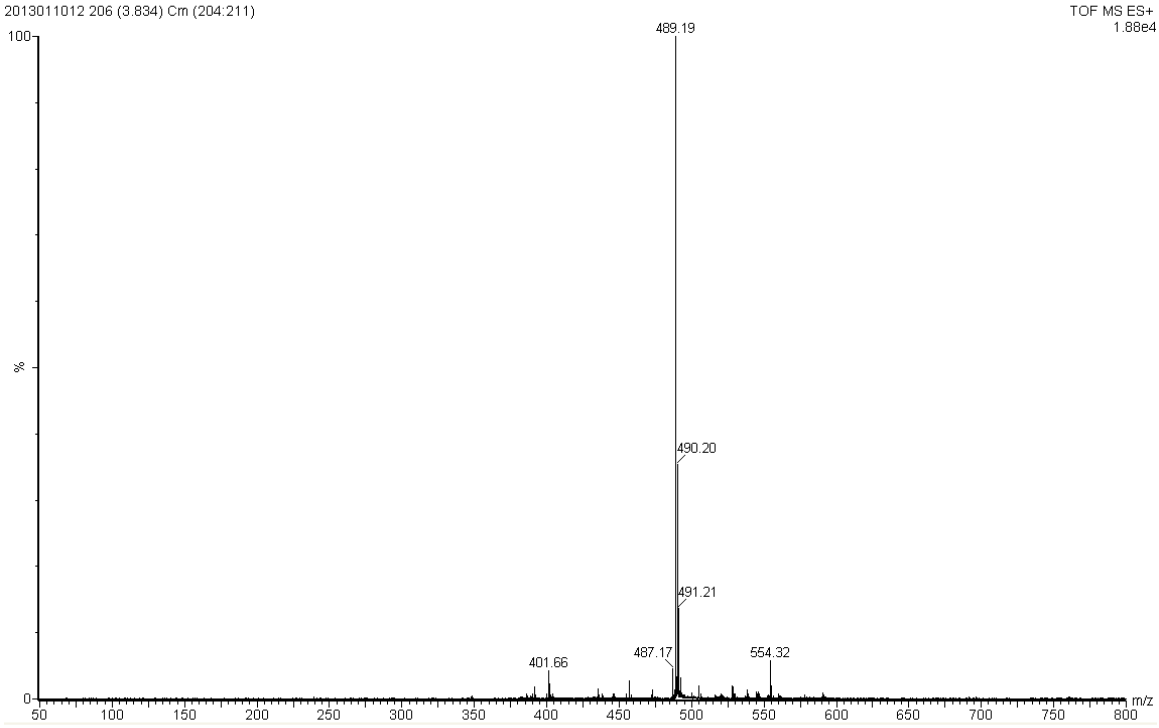
^{1}H-NMR spectrum of **FITC-CA** in DMSO-d_6



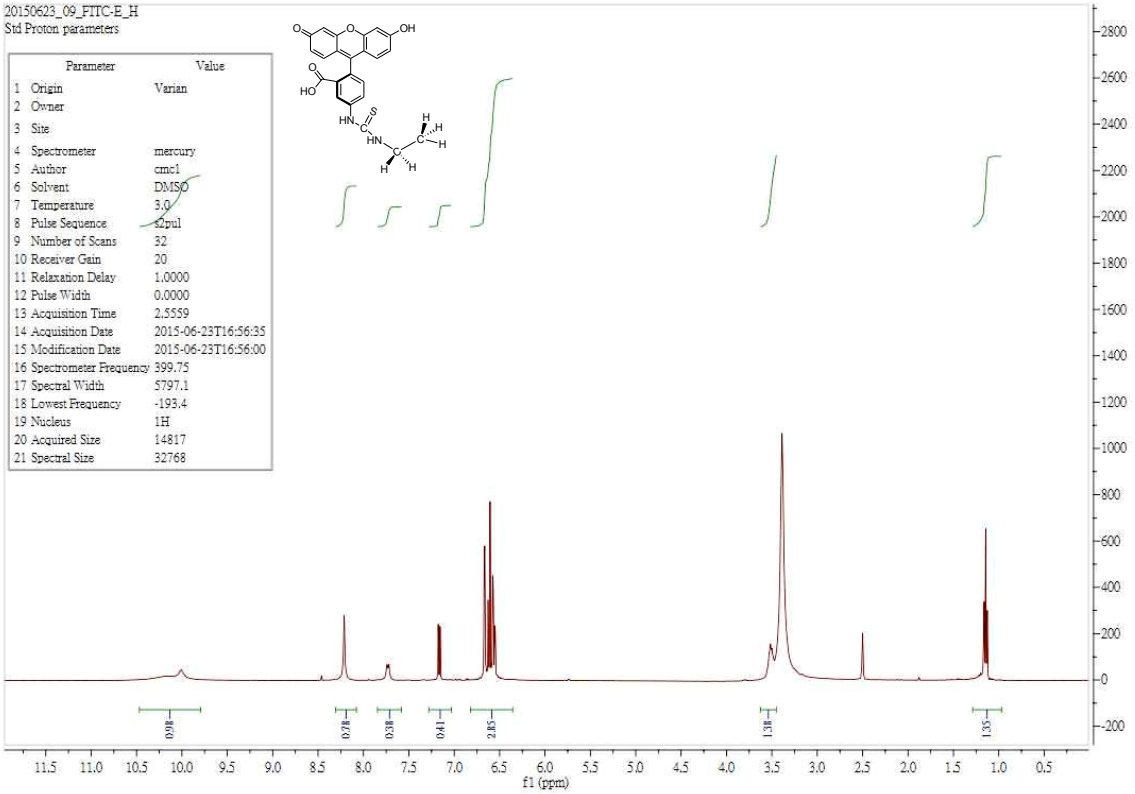
^{13}C NMR spectrum of **FITC-CA** in DMSO-d_6



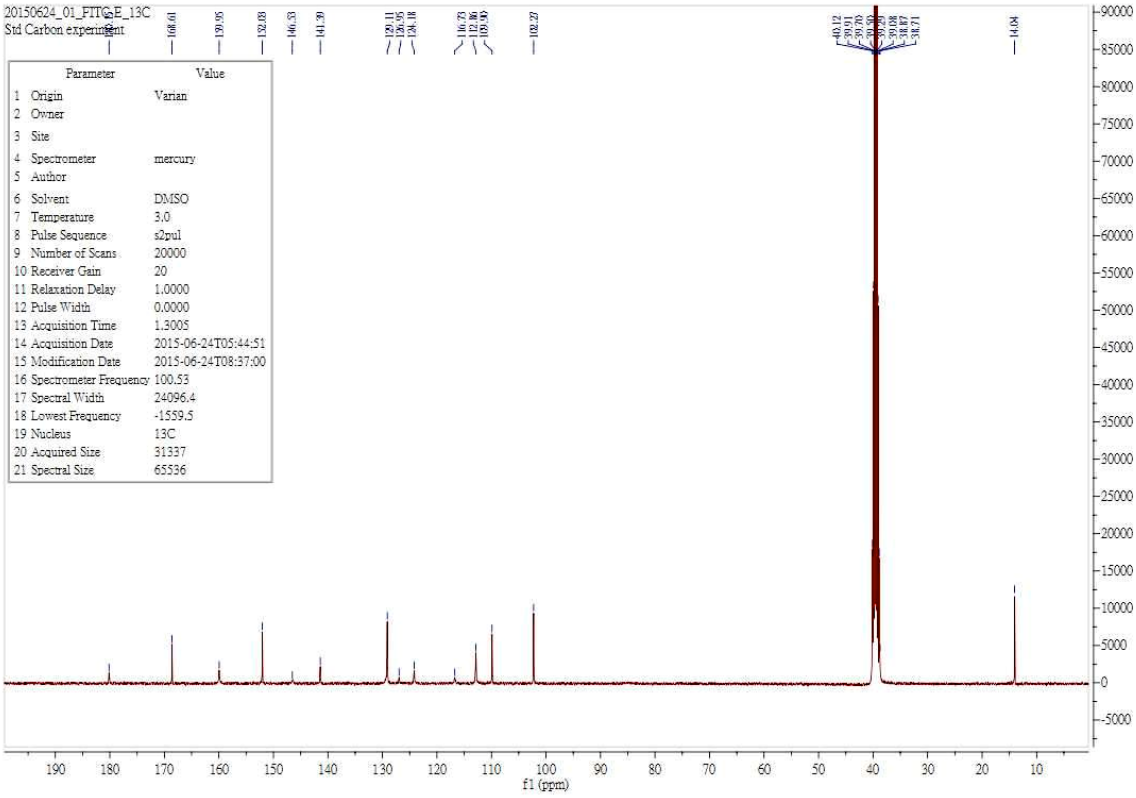
Mass spectrum of **FITC-CA**



^{1}H-NMR spectrum of **FITC-EA** in DMSO-d_6



^{13}C NMR spectrum of **FITC-EA** in DMSO-d_6



Mass spectrum of **FITC-EA**