A two-photon lysosome-targeted probe for endogenous formaldehyde in living cells

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1. General information and methods

Measurements of photophysical properties. UV-vis absorption spectra were obtained on a Varian UV-Cary5000 spectrophotometer. In order to get the corrected steady-state excitation and emission spectra, a Hitachi F-7000 spectrofluorimeter was used. Fluorescence emission spectra were collected using Hitachi F-7000 spectrofluorimeter with 1 cm quartz cells. Fluorescein (in 0.1 N NaOH pH = 13, Φ_f = 0.89) were used as fluorescence standard for quantum yield test.[1]

Time-resolved fluorescence spectroscopy. Fluorescence lifetimes were detected by a FLS920. The monitored wavelength was 540 nm. Fluorescence decay histograms

were recorded using the time-correlated single photon counting technique in 4096 channels through a FLS920 spectrometer equipped with a supercontinue white laser (400-700 nm). Histograms of the instrument response functions (using LUDOX scatterer) and sample decays were obtained until it typically reached 1.0×10^4 counts. The fitting parameters (decay times and pre-exponential factors) were decided by minimizing the reduced chi-square χ^2.

Cell culture, fluorescence imaging. To obtain the cell permeability of ***AMNT***, HeLa cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) subjoined with 10% FBS (fetal bovine serum). The cell lines were kept in a moist atmosphere containing 5% CO_2 at 37 °C. Cells were incubated with ***AMNT*** (10 μM) in 1.0 mL of fresh culture medium for 0.5 h after removal of the culture medium. Cells were incubated and rinsed with phosphate-buffered saline (PBS) three times to remove free compound before imaging.

Imaging method	C_{AMNT}	C_{FA}	Cell type	Imaging instrument	Excitation wavelength	Emission wavelength
Single-photon	10 μM	300/500 μM	HeLa	Olympus FV1000-IX81	458 nm	510-580 nm
Two-photon	10 μM	300/500 μM	HeLa	Olympus FV1000 MPE	810 nm	510-550 nm

Cytotoxicity tests. The cytotoxic activity experiment of complex against HeLa cells was tested according to standard 3-(4,5-diethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-etrazolium, inner salt (MTS) assay procedures. HeLa cells were seeded in 96-well assay plates at a density of 10^4 cells per well (100 μL total volume/well) for 24 h. The as-prepared ***AMNT*** (10 μM,) were added in the serum-free medium and incubated with the cells for 24 h after that. The control experiment was finished by detecting the growth culture medium without ***AMNT***. The optical absorbance of the cells was detected at 528 nm through a microplate reader (German Berthold Mithras2LB943).

The limit of detection calculation. The detection limit (DL) for FA was calculated

by the linear function in Figure S2 and the following equation:

$$DL = 3.3\sigma / k$$

Where σ is the standard derivation of fluorescence intensity of ($I_{528\,nm}$) *AMNT* blank solutions; k is the slope of the linear calibration curve in Figure S2; the concentration of *AMNT* is 10 μM.

Table S1. Spectroscopic data of *AMNT* (10 μM) in the absence and addition of FA.
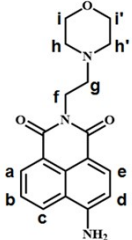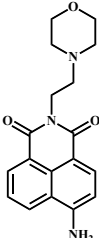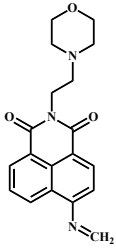
Compounds	Reaction time	λ_{abs} / nm	λ_{ems} / nm	Φ_f
AMNT	-	435	540	0.115
AMNT+ 100 equiv. FA	30 min	435	528	0.217
AMNT				

<table>
<tr><td>
AMNT</td><td>^{1}H NMR (CDCl$_3$) Spectrum of **AMNT** : δ 8.46 (d, 1H, J = 8.0 Hz, H-a), 8.25 (d, 1H, J = 8.0 Hz, H-c), 8.00 (d, 1H, J = 8.0 Hz, H-e), 7.53 (t, 1H, J = 8.0 Hz, H-b), 7.73 (d, 1H, J = 8.0 Hz, H-d), 4.28 (t, 2H, J = 8.0 Hz, H-f), 3.67 (t, 4H, J = 4.0 Hz, H-i+i'), 2.74 (t, 2H, J = 8.0 Hz, H-g), 2.67 (m, 4H, H-h+h').</td></tr>
<tr><td>
AMNT</td><td>MS Spectrum of **AMNT**((C$_{18}$H$_{19}$N$_3$O$_3$): the exact molecular weight calcd for **AMNT** is 325.14, found is 326.19 (M+1).</td></tr>
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AMNT+FA</td><td>MS Spectrum of **AMNT+FA**((C$_{19}$H$_{19}$N$_3$O$_3$): the exact molecular weight calcd for **AMNT+FA** is 337.14, found is 338.36 (M+1).</td></tr>
</table>

Table S2. Photophysical Properties of *AMNT* (10 µM) in the absence and in addition of FA at the same excitation wavelength (435 nm), but at different emission wavelength. Global analyses of decay times τ_i (ns), and the relative amplitude α_i (%), each datum was recorded 0.5 h after FA addition.

Addition of HCHO / µM	Monitored Wavelength / nm	τ_1 (ns)	τ_2 (ns)	α_1 (%)	α_2 (%)	χ^2
0	530	4.13±0.0004	10.33±0.0025	95.5	4.5	1.04
	540			95.4	4.6	1.06
	550			95.7	4.3	1.12
100	520	4.63±0.0010	7.70±0.0005	58.4	41.6	1.07
	530			54.2	45.8	1.02
	540			56.6	43.4	1.00
500	520	4.65±0.0018	7.54±0.0007	37.1	62.9	1.03
	530			40.2	59.8	1.04
	540			43.1	56.9	1.04
1000	520	4.73±0.0026	7.48±0.0007	29.7	70.3	1.05
	530			32.7	67.3	1.04
	540			34.2	65.8	1.02

Table S3. Comparison of the proposed method with other methods for FA detection.

Linear range	Organelle-targeted ability	Detection Limit	Excitation and Emission	React time	Reference
0-100 µM	no	5 µM	λ_{ex} = 645 nm λ_{em} = 662 nm	120 min	J. Am. Chem. Soc. 2015, 137, 10886-10889
0-5000µM	no	10 µM	λ_{ex} = 633 nm λ_{em} = 649 nm	180 min	J. Am. Chem. Soc. 2015, 137,10890-10893
0-200 µM	no	0.71 µM	λ_{ex} = 440 nm λ_{em} = 543 nm	30 min	Angew. Chem. Int. Ed. 2016, 55, 3356-3359
0-3000µM	no	59.6 µM	λ_{ex}= 318 nm λ_{em}= 451/359 nm	240 min	Chem. Commun., 2016, 52, 4029-4032
0 -150 µM	no	0.57 µM	λ_{ex} = 365 nm λ_{em} = 513 nm	180 min	Talanta, 2016, 160, 645-652
0–200 µM	no	0.5 µM	λ_{ex} =405 nm λ_{em} =570/495 nm	120 min	Chem. Sci., 2017, 8, 5616-5621

|					
0–500 µM	no	5 µM	λ_{ex} = 440 λ_{em} = 510 nm	240 min	Sens. Actuators, B, 2017, 241, 1050-1056
2.0-10.0 µM	no	0.77µM	λ_{ex} =530 nm λ_{em} =560 nm	2 min	Chem. Commun., 2016, 52, 9582-9585
0-1000 µM	no	8.3µM	λ_{ex} = 520 nm λ_{em} =620 nm	6 min	Dyes Pigm., 2017, 138, 23-29
0-1000 µM	no	0.104 µM	λ_{ex} = 482 nm λ_{em} = 548 nm	90 min	Talanta 2018, 189, 274-280
0–200 µM	lysosome-targeted	5.02 µM	λ_{ex} = 440 nm λ_{em} = 543 nm	30 min	Anal. Chem. 2016, 88, 9359-9363
10-250 µM	lysosome-targeted	3 µM	λ_{ex} =405 nm λ_{em} =506 nm	40 min	Chem. Commun., 2017, 53, 6520-6523
0-200 µM	lysosome-targeted	0.27 µM	λ_{ex} = 482 nm λ_{em} = 548 nm	60 min	Spectrochim. Acta A Mol. Biomol. Spectrosc., 2021, 245,118949-118955.
0-500 µM	**lysosome-targeted**	**1.77 µM**	**λ_{ex} = 435 nm λ_{em} = 528 nm**	**30 min**	**This work**
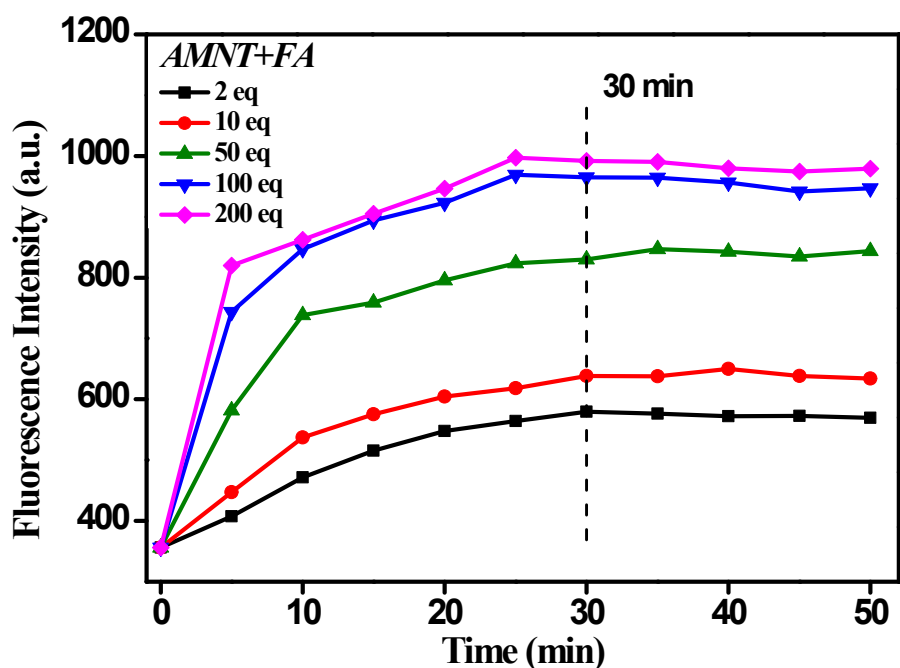
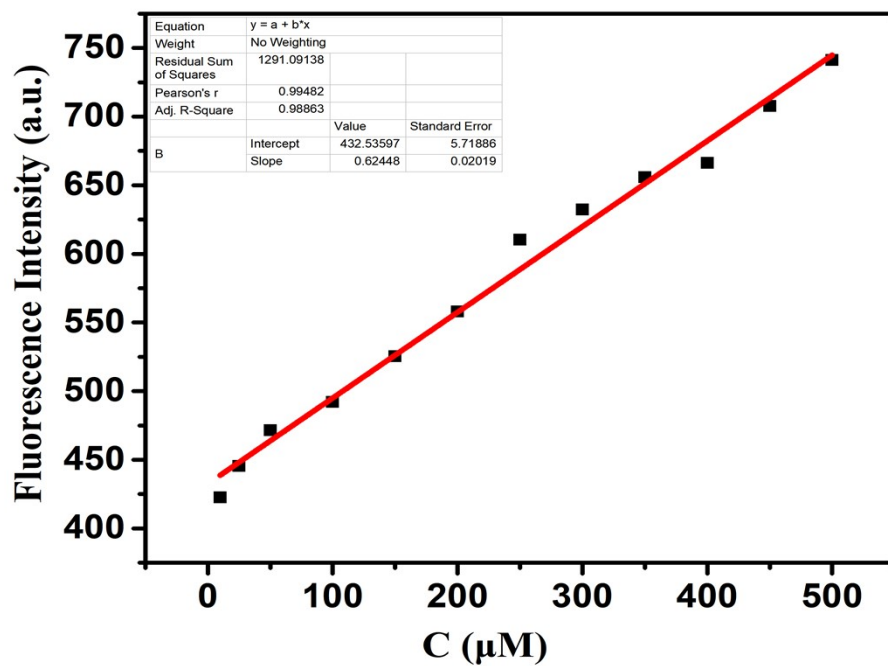**Figure S1.** Time course fluorescence spectra of *AMNT* (10 µM) with different equiv of FA, λ_{ex} = 435 nm.

Figure S2. Linear calibration curve of fluorescence intensity of *AMNT* with FA (0-500 μM), λ_{ex} = 435 nm.
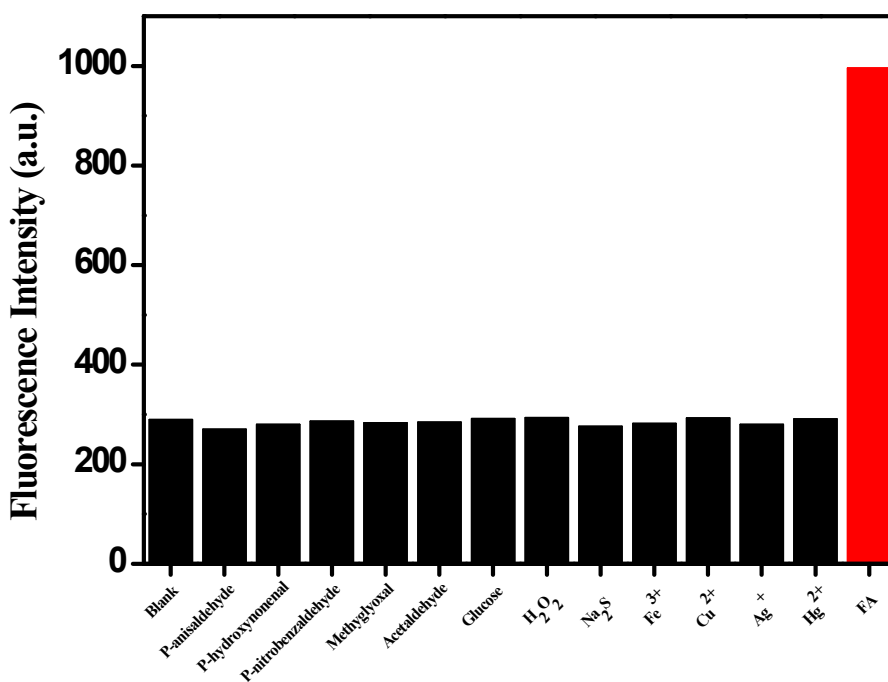

Figure S3. Specific selectivity of *AMNT* (10 μM) reacted with 1 mM different kinds of species. All spectra were obtained after 0.5 h of incubation with different analytes,

λ_{ex} = 435 nm. Each bar represent as: 0) Probe **AMNT**, 1) P-anisaldehyde, 2) P-hydroxynonenal, 3) P-nitrobenzaldehyde, 4) Methyglyoxal, 5) Acetaldehyde, 6) Glucose, 7) H_2O_2, 8) Na_2S, 9) Fe^{3+}, 10) Cu^{2+}, 11) Ag^+, 12) Hg^{2+}, 13) FA.
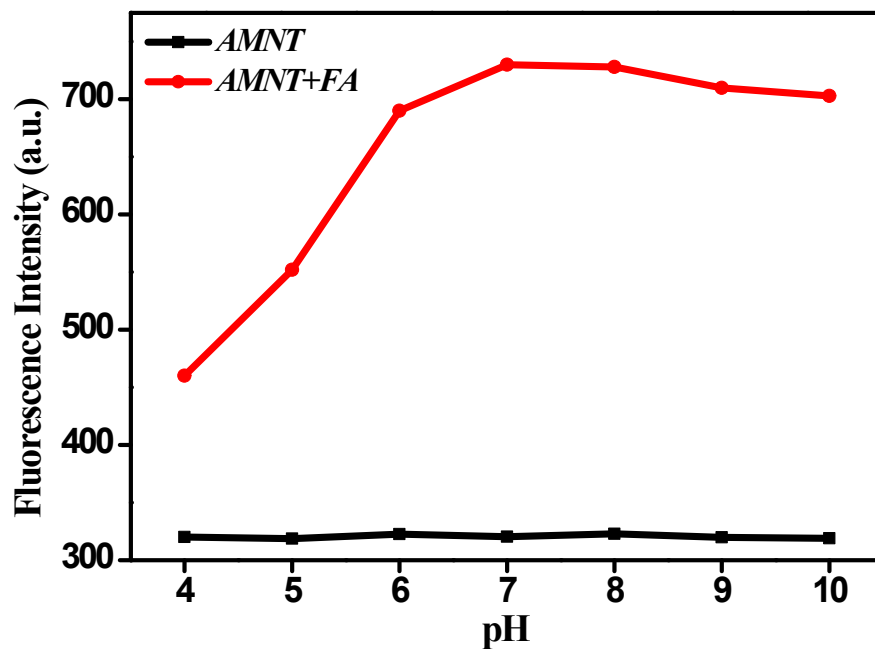

Figure S4. Fluorescence intensity of **AMNT** in the absence and presence of 500 µM FA as a function of pH, Each data point was acquired 0.5 h after addition of FA in PBS buffer at 25 °C, **AMNT** = 10 µM, λ_{ex} = 435 nm.
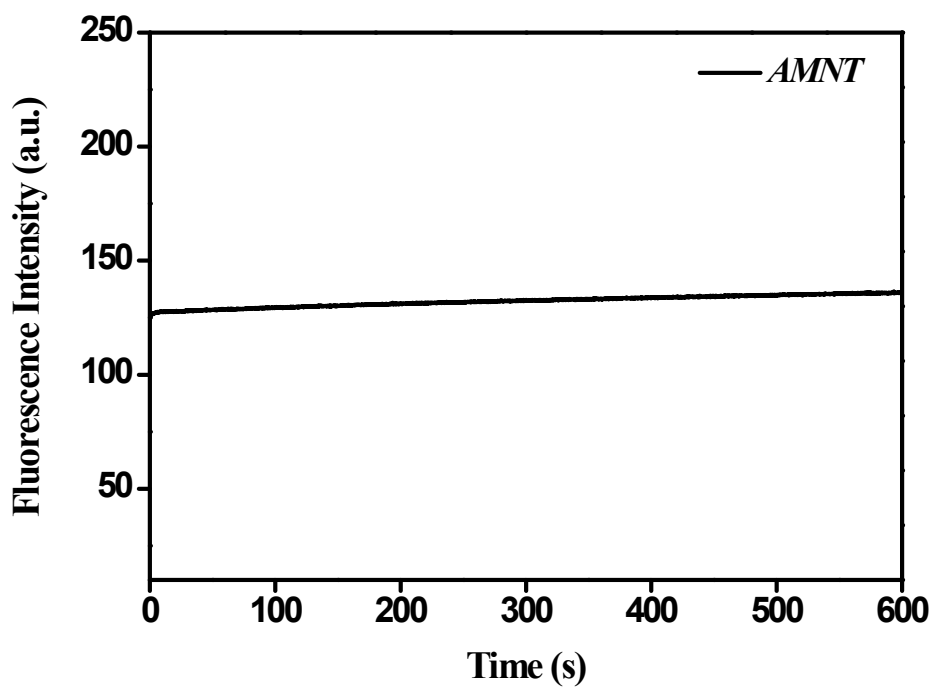
Figure S5. Hitachi F-7000 spectrofluorimeter was used to detection the light stable of *AMNT*.
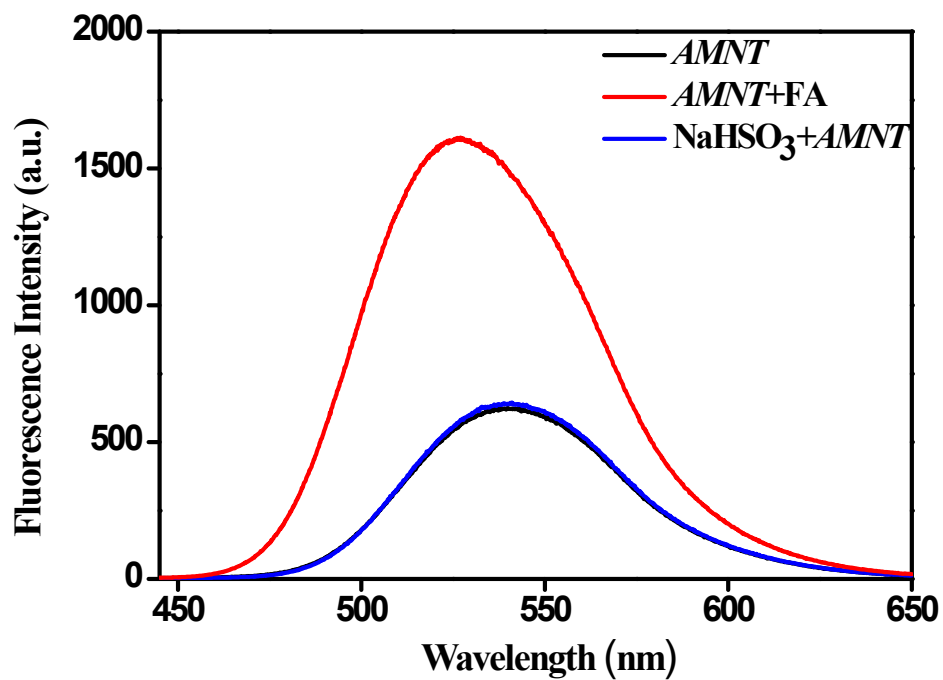
Figure S6. Fluorescence spectra of the free probe *AMNT* (10 μM), *AMNT* (10 μM) treated with FA (1 mM), and *AMNT* (10 μM) treated with NaHSO$_3$ (200 μM), Incubation time: 30 min, λ_{ex} = 435 nm.
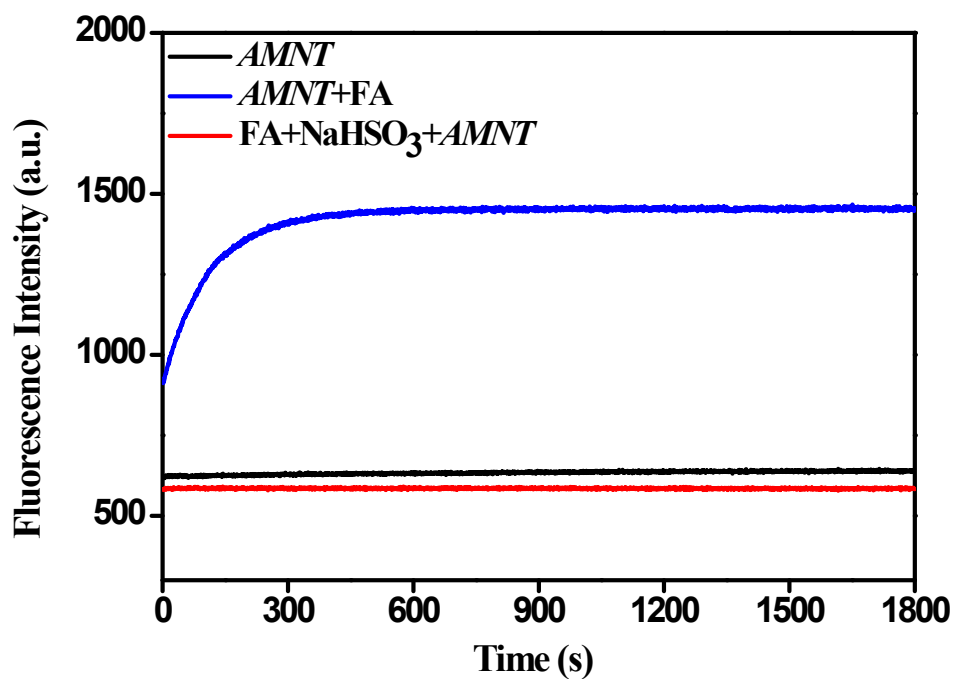
Figure S7. Reaction-time profiles, free probe, 1 mM FA pre-incubated with 10 μM *AMNT*, and FA pre-incubated with 200 μM NaHSO$_3$ then treated with the probe, respectively.
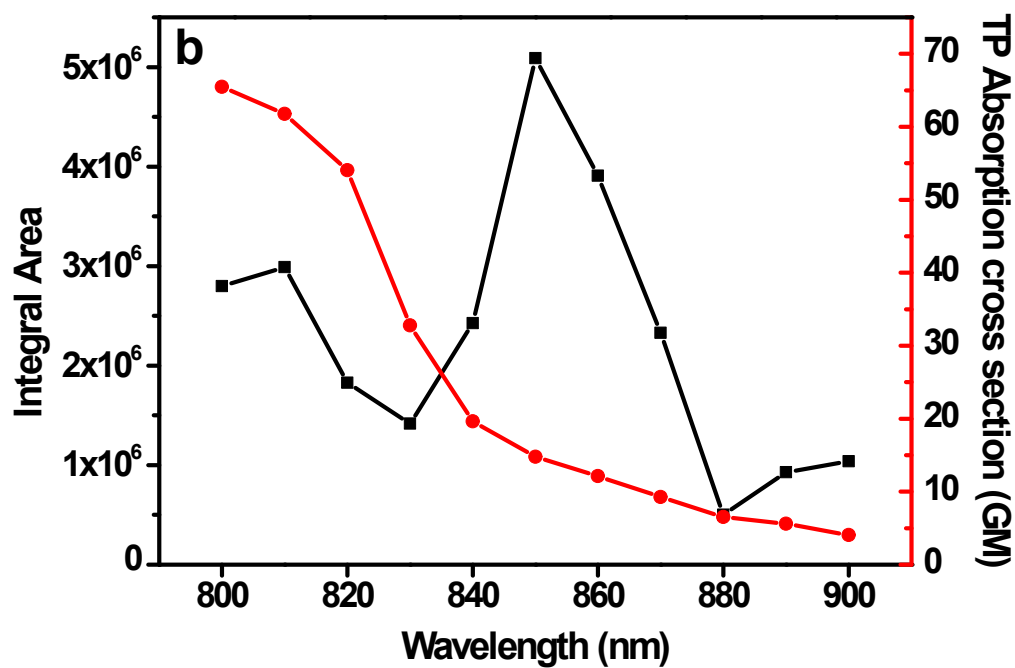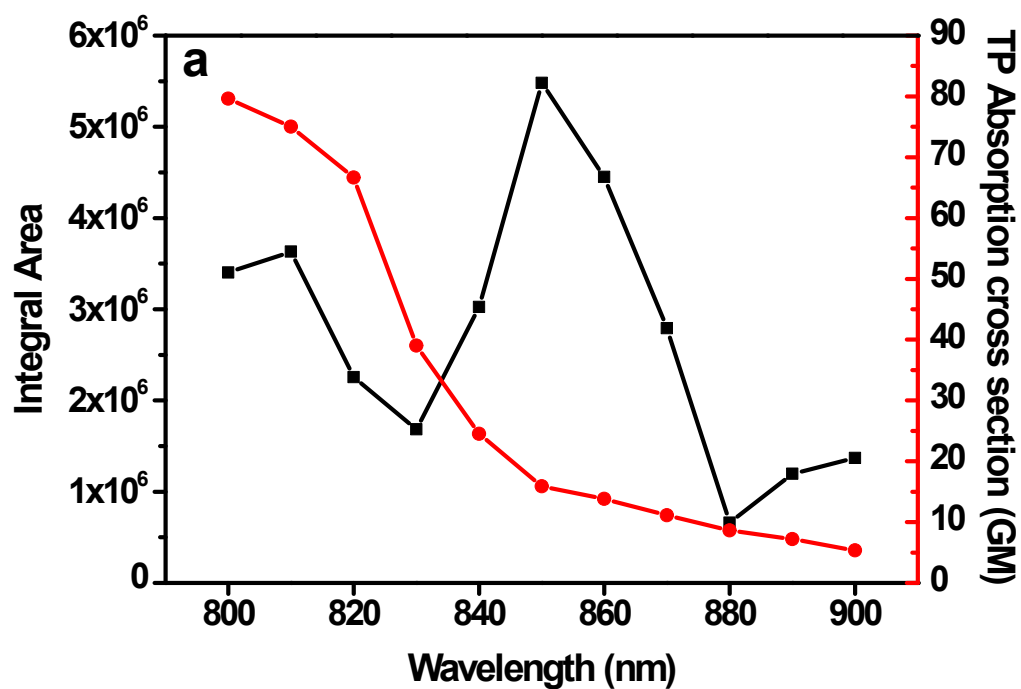
Figure S8. The fluorescence integral area (black line) and two-photon absorption cross-section (red line) spectrum of *AMNT* (a)and *AMNT-FA* (b).

Figure S9. a) Effects of FA and b) Effects of *AMNT* at varied concentrations on the viability of HeLa cells. The cell viability data were checked three times.

0-435-540-ADD.FL

❖ Global Analysis (Reconvolution)

Fitting range   : [249; 4096] channels
Global χ^2 : 1.073
χ^2 : 1.058

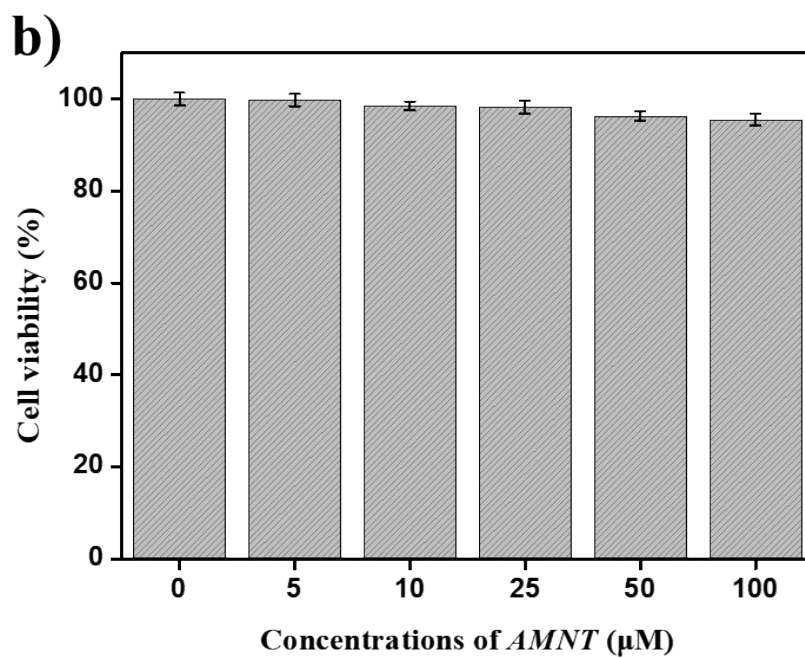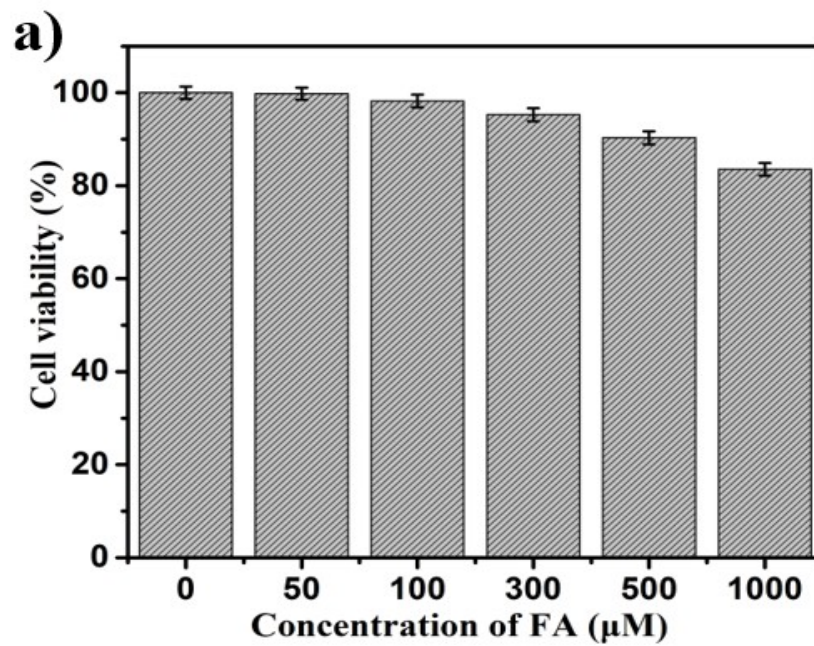|	B_i	ΔB_i	f_i (%)	Δf_i (%)	τ_i (ns)	$\Delta \tau_i$ (ns)
1	0.0340	5.4e-5	95.44	0.1670	4.133 linked	0.0004
2	0.0006	5.3e-5	4.5591	0.3819	10.333 linked	0.0025

Shift   : -0.1714 ns (± 0 ns)
Decay Background : 26.02   (± 0)
IRF Background   : 5.9000

0-435-550-ADD.FL

❖ Global Analysis (Reconvolution)

Fitting range   : [249; 4096] channels
Global χ^2 : 1.073
χ^2 : 1.120

	B_i	ΔB_i	f_i (%)	Δf_i (%)	τ_i (ns)	$\Delta \tau_i$ (ns)
1	0.0336	5.3e-5	95.67	0.1708	4.133 linked	0.0004
2	0.0005	5.2e-5	4.3251	0.3894	10.333 linked	0.0026

Shift   : -0.1464 ns (± 0 ns)
Decay Background : 25.74   (± 0)
IRF Background   : 5.9000

0-435-530-ADD.FL

❖ Global Analysis (Reconvolution)

Fitting range   : [249; 4096] channels
Global χ^2 : 1.073
χ^2 : 1.041

	B_i	ΔB_i	f_i (%)	Δf_i (%)	τ_i (ns)	$\Delta \tau_i$ (ns)
1	0.0338	5.3e-5	95.48	0.1622	4.133 linked	0.0004
2	0.0006	5.2e-5	4.5165	0.3723	10.333 linked	0.0025

Shift   : -0.1772 ns (± 0 ns)
Decay Background : 26.36   (± 0)
IRF Background   : 5.9000

10-435-530-ADD.FL

❖ Global Analysis (Reconvolution)

Fitting range : [250; 4096] channels
Global χ^2 : 1.030
χ^2 : 1.016

	B_i	ΔB_i	f_i(%)	Δf_i(%)	τ_i(ns)	$\Delta \tau_i$(ns)
1	0.0221	0.0002	54.18	0.5665	4.631 linked	0.0010
2	0.0112	0.0002	45.82	0.9601	7.704 linked	0.0005

Shift : -0.1505 ns (± 0 ns)
Decay Background : 33.53 (± 0)
IRF Background : 5.9000

10-435-540-ADD.FL

❖ Global Analysis (Reconvolution)

Fitting range : [250; 4096] channels
Global χ^2 : 1.030
χ^2 : 1.003

	B_i	ΔB_i	f_i(%)	Δf_i(%)	τ_i(ns)	$\Delta \tau_i$(ns)
1	0.0229	0.0002	56.61	0.5555	4.631 linked	0.0010
2	0.0105	0.0002	43.39	0.9480	7.704 linked	0.0005

Shift : -0.1453 ns (± 0 ns)
Decay Background : 33.21 (± 0)
IRF Background : 5.9000

10-435-520-ADD.FL

❖ Global Analysis (Reconvolution)

Fitting range : [250; 4096] channels
Global χ^2 : 1.030
χ^2 : 1.071

	B_i	ΔB_i	f_i(%)	Δf_i(%)	τ_i(ns)	$\Delta \tau_i$(ns)
1	0.0495	0.0004	58.35	0.5302	4.631 linked	0.0010
2	0.0212	0.0005	41.65	0.9453	7.704 linked	0.0005

Shift : -4.3200 ns (± 0 ns)
Decay Background : 32.40 (± 0)
IRF Background : 5.9000

50-435-530-ADD.FL

❖ Global Analysis (Reconvolution)

Fitting range : [240; 4096] channels
Global χ^2 : 1.037
χ^2 : 1.040

	B_i	ΔB_i	f_i(%)	Δf_i(%)	τ_i(ns)	$\Delta\tau_i$(ns)
1	0.0172	0.0004	40.18	0.9663	4.656 linked	0.0018
2	0.0158	0.0004	59.82	1.5803	7.542 linked	0.0007

Shift : -0.0916 ns (± 0 ns)
Decay Background : 35.71 (± 0)
IRF Background : 5.9000

50-435-540-ADD.FL

❖ Global Analysis (Reconvolution)

Fitting range : [240; 4096] channels
Global χ^2 : 1.037
χ^2 : 1.036

	B_i	ΔB_i	f_i(%)	Δf_i(%)	τ_i(ns)	$\Delta\tau_i$(ns)
1	0.0182	0.0004	43.11	0.9422	4.656 linked	0.0018
2	0.0148	0.0004	56.89	1.5628	7.542 linked	0.0007

Shift : -0.1293 ns (± 0 ns)
Decay Background : 34.76 (± 0)
IRF Background : 5.9000

50-435-520-ADD.FL

❖ Global Analysis (Reconvolution)

Fitting range : [240; 4096] channels
Global χ^2 : 1.037
χ^2 : 1.033

	B_i	ΔB_i	f_i(%)	Δf_i(%)	τ_i(ns)	$\Delta\tau_i$(ns)
1	0.0162	0.0004	37.11	0.9765	4.656 linked	0.0018
2	0.0169	0.0004	62.89	1.5768	7.542 linked	0.0007

Shift : -0.1026 ns (± 0 ns)
Decay Background : 36.34 (± 0)
IRF Background : 5.9000

100-435-530-ADD.FL

❖ Global Analysis (Reconvolution)

Fitting range   : [225; 4096] channels
Global χ^2 : 1.038
χ^2 : 1.042

	B_i	ΔB_i	f_i (%)	Δf_i (%)	τ_i (ns)	$\Delta \tau_i$ (ns)
1	0.0141	0.0005	32.70	1.2718	4.734 linked	0.0026
2	0.0184	0.0005	67.30	2.0285	7.477 linked	0.0007

Shift   : -0.0661 ns (± 0 ns)
Decay Background : 36.68   (± 0)
IRF Background   : 5.9000

100-435-540-ADD.FL

❖ Global Analysis (Reconvolution)

Fitting range   : [225; 4096] channels
Global χ^2 : 1.038
χ^2 : 1.018

	B_i	ΔB_i	f_i (%)	Δf_i (%)	τ_i (ns)	$\Delta \tau_i$ (ns)
1	0.0146	0.0005	34.20	1.2454	4.734 linked	0.0026
2	0.0178	0.0005	65.80	1.9954	7.477 linked	0.0007

Shift   : -0.0671 ns (± 0 ns)
Decay Background : 35.81   (± 0)
IRF Background   : 5.9000

100-435-520-ADD.FL

❖ Global Analysis (Reconvolution)

Fitting range   : [225; 4096] channels
Global χ^2 : 1.038
χ^2 : 1.054

	B_i	ΔB_i	f_i (%)	Δf_i (%)	τ_i (ns)	$\Delta \tau_i$ (ns)
1	0.0130	0.0005	29.65	1.2893	4.734 linked	0.0026
2	0.0196	0.0005	70.35	2.0343	7.477 linked	0.0007

Shift   : -0.0547 ns (± 0 ns)
Decay Background : 36.92   (± 0)
IRF Background   : 5.9000

Figure S10. Global analyses of decay times τ_1, τ_2, and the relative amplitude (%).
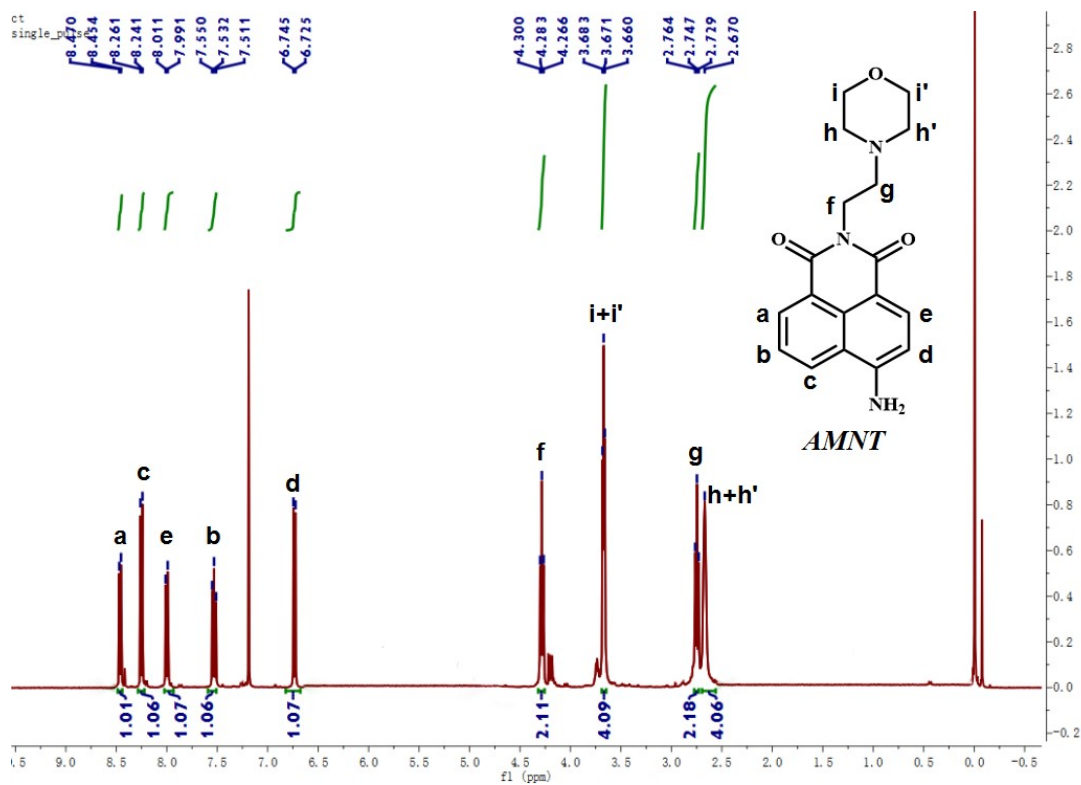
Figure S11. ^{1}H NMR (CDCl$_3$, 400 MHz) spectrum of *AMNT*.

AMNT: δ 8.46 (d, 1H, *J* = 8.0 Hz, H-a), 8.25 (d, 1H, *J* = 8.0 Hz, H-c), 8.00 (d, 1H, *J* = 8.0 Hz, H-e), 7.53 (t, 1H, *J* = 8.0 Hz, H-b), 7.73 (d, 1H, *J* = 8.0 Hz, H-d), 4.28 (t, 2H, *J* = 8.0 Hz, H-f), 3.67 (t, 4H, *J* = 4.0 Hz, H-i+i'), 2.74 (t, 2H, *J* = 8.0 Hz, H-g), 2.67 (m, 4H, H-h+h').
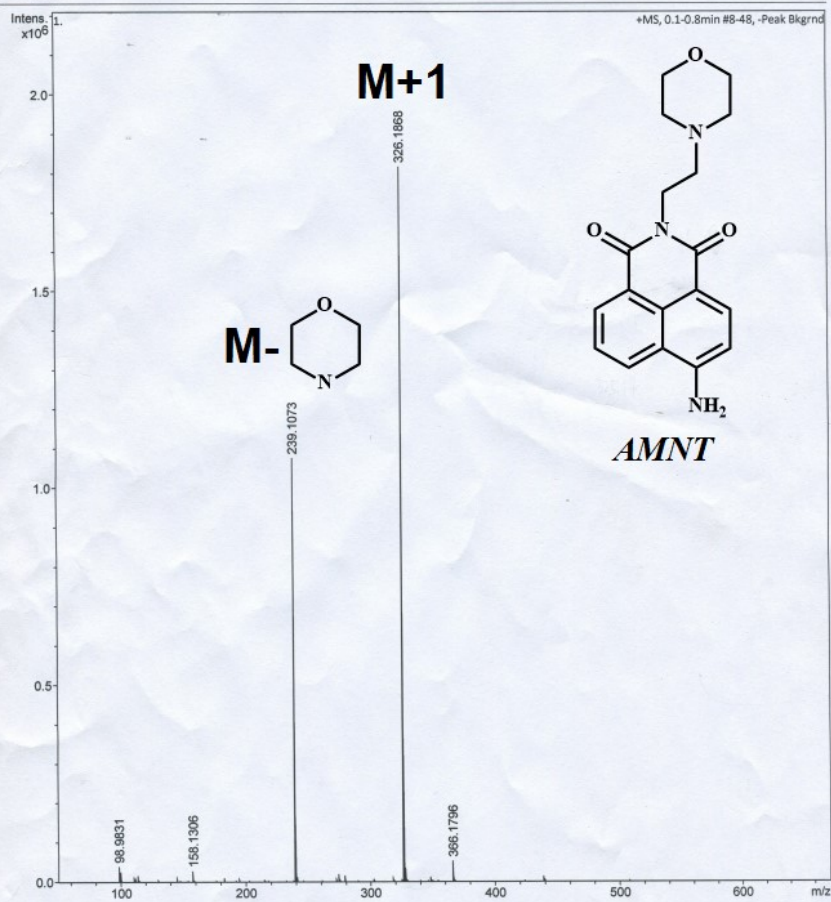
Figure S12. MS Spectrum of **AMNT**((C$_{18}$H$_{19}$N$_3$O$_3$): calcd for **AMNT** (C$_{18}$H$_{19}$N$_3$O$_3$) 325.14, found 326.19 (M+1).
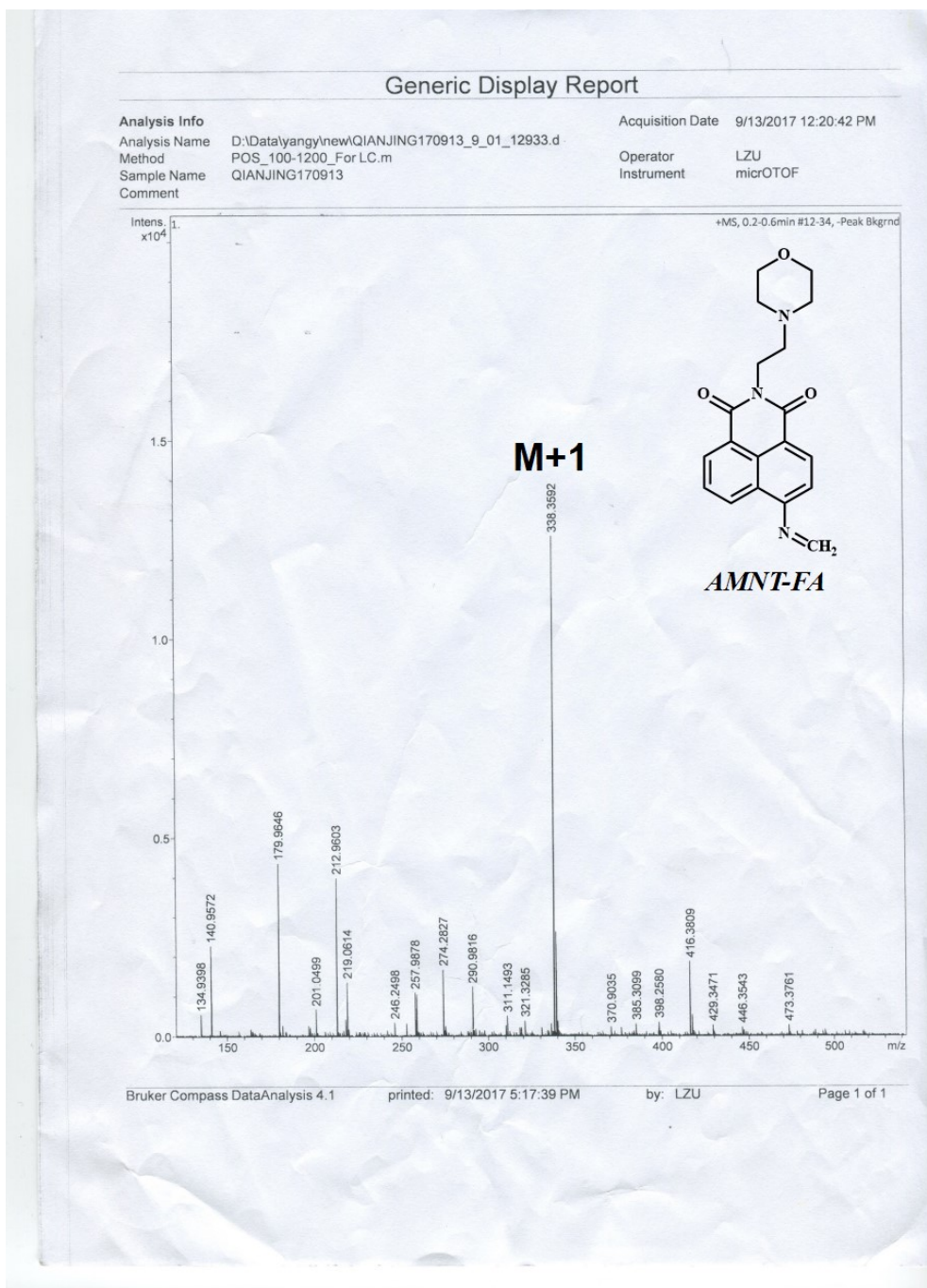
Figure S13. MS Spectrum of *AMNT+FA* ($C_{19}H_{19}N_3O_3$), the exact molecular weight of *AMNT+FA* is 337.14, found is 338.36 (M+1).

References

[1] Lynn W¨urth, Inga J. Wolf, Wolfgang Marquardt, On the Numerical Solution of Discounted Economic NMPC on Infinite Horizons, The International Federation of Automatic Control, **2013,** 18-20.