Electronic Supplementary Information (ESI)

Polarity-sensitive pyrene fluorescent probes for multi-organelles imaging in living cells

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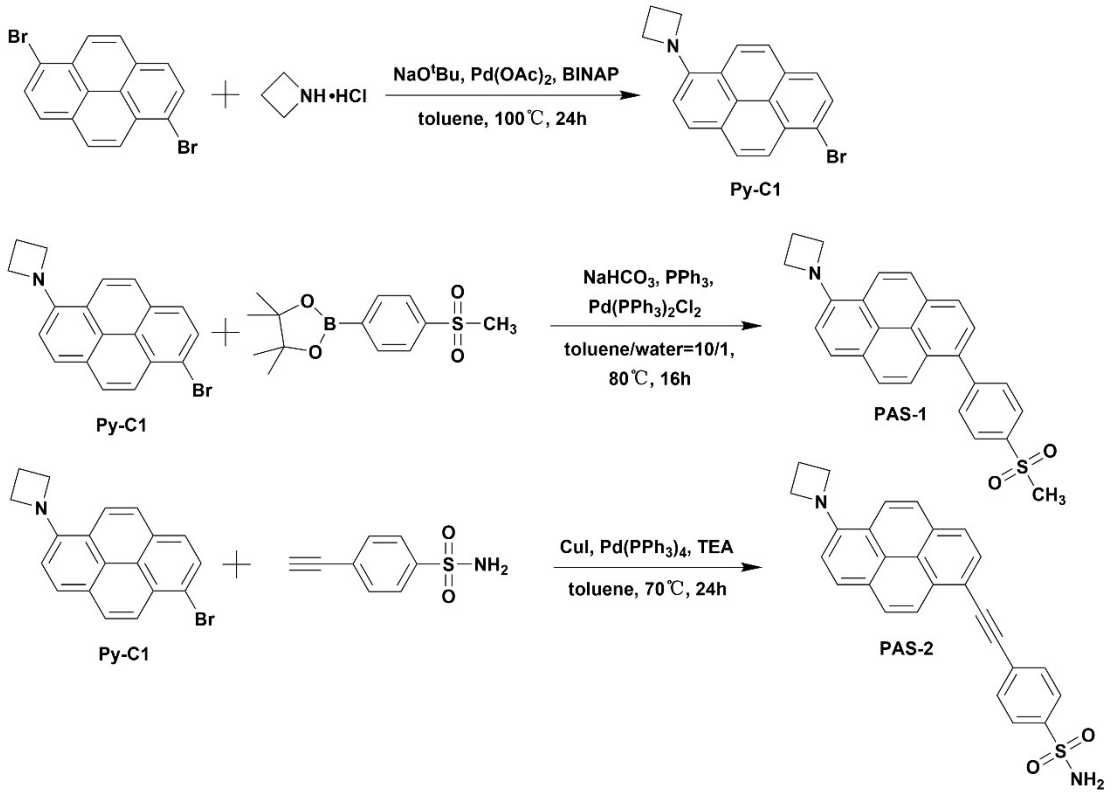
1. General information

All solvents and reagents were purchased from Shanghai BiDe PharmaTech Co. or J&K Scientific Ltd. and used as received unless otherwise mentioned. All synthesis steps were carried out under a nitrogen ambient atmosphere. Silica gel (200-300 mesh) procured from Titan Technology Discovery Platform was used for column chromatography. Liquid nuclear magnetic resonance (NMR) spectra were recorded on a Bruker NMR spectrometer at 400 MHz or 600 MHz. ^{1}H-NMR spectra at 400 MHz or 600 MHz and ^{13}C-NMR spectra at 101 MHz were recorded in deuterated chloroform or deuterated acetone using TMS as an internal standard. High-resolution mass spectra (HR-MS) were obtained using a Thermo Scientific high-resolution mass spectrometer. Melting points were measured on glass slides using SGW X-4 Melting Point Apparatus. UV-Vis absorption spectra were recorded with a Shimadzu WV-2550 spectrophotometer. Fluorescence emission spectra were recorded with a Shimadzu RF-5301 fluorescence spectrophotometer. Fluorescence lifetime measurements in different solvents in vitro were performed using an Edinburgh FLS980 series fluorescence spectrometer. The relative fluorescence quantum yield was determined using quinine sulfate in 0.5 M H_2SO_4 solution (λ_{ex}=350 nm, Φ_f=55%) as a standard. The quantum yield was calculated as $\Phi_s = \Phi_r (A_r F_s / A_s F_r) (n_s^2 / n_r^2)$, where s is the sample, r is the reference, A is the absorbance, F is the relative integrated fluorescence intensity, and n is the refractive index of the solvent. Cell fluorescence imaging was recorded using a spectral confocal laser scanning microscope (Olympus Fluoview FV-1000). Cell super-resolution imaging was collected using a structured light illumination microscope (Airy PolarSIM). Cell fluorescence lifetime imaging was recorded using a super-resolution confocal microscope (TCS SP8 STED 3X).

2. Synthetic scheme



Scheme S1 Synthetic routes of Py-C1, PAS-1 and PAS-2.

3. Synthesis and characterization

Synthesis of Py-C1

A solution of NaO^{t}Bu (0.48 g, 5.0 mmol), Pd(OAc)$_2$ (31 mg, 0.14 mmol), and BINAP (0.26 g, 0.42 mmol) in toluene (10 mL) was stirred for 5 min under nitrogen. Subsequently, 1,6-dibromo pyrene (1.0 g, 2.78 mmol) was added and stirred for another 10 minutes. Then, azetidine hydrochloride (0.39 g, 4.17 mmol) was added and heated to 100 ℃ for 24 hours of reaction. The reaction was quenched by the addition of water, and then, the mixture was extracted with dichloromethane, and the organic layer was dried over Na_2SO_4, filtered, and remove the solvent under vacuum. The residue was purified by silica gel column chromatography (petroleum ether: dichloromethane = 2:1) to give the product Py-C1 (0.30 g) as a yellow solid. Yield: 32 %; ^{1}H NMR (600 MHz, Chloroform-*d*) δ 8.20 – 8.11 (m, 3H), 8.06 (d, *J* = 8.4 Hz,

1H), 8.00 (d, *J* = 9.1 Hz, 1H), 7.87 – 7.80 (m, 2H), 7.16 (d, *J* = 8.4 Hz, 1H), 4.40 (t, *J* = 7.3 Hz, 4H), 2.52 (p, *J* = 7.3 Hz, 2H). ^{13}C NMR (101 MHz, Chloroform-d) δ 147.63, 131.12, 130.70, 130.04, 129.19, 126.92, 126.37, 125.75, 124.37, 123.81, 123.51, 123.21, 122.12, 118.05, 111.69, 55.80, 17.45. HRMS $[M]^+$: Calcd. for $C_{19}H_{14}BrN$: 335.0310, found: 335.0309.

Synthesis of PAS-1

4,4,5,5-tetramethyl-2-(4-(methylsulfonyl)phenyl)-1,3,2-dioxaborolane (0.10 g, 0.36 mmol), Py-C1 (0.10 g, 0.30 mmol), $NaHCO_3$ (0.13 g, 1.49 mmol), $Pd(PPh_3)_2Cl_2$ (6.3 mg, 0.009 mmol) and triphenylphosphine (4.7 mg, 0.018 mmol) were added into the reaction flask with 5 mL of mixed solvent (toluene: water=10:1). Then the mixture was reacted at 80°C for 16 hours under the protection of nitrogen. Before completion, the reaction was monitored by a TLC plate. Add water to quench the reaction, then extract the mixture three times with dichloromethane, combine the organic layer dried over Na_2SO_4, filter, and remove the solvent under vacuum. The crude product was purified by silica gel column chromatography (ethyl acetate: petroleum ether = 1:2) to give the yellow powder product 50 mg. The full name is 1-azetidine-6-(4-(methylsulfonyl)phenyl) pyrene (PAS-1) according to the systematic nomenclature; m.p. 234.4 - 236.2 ℃; Yield: 41 %; ^{1}H NMR (400 MHz, Chloroform-d) δ 8.20 (d, *J* = 9.2 Hz, 1H), 8.10 (d, *J* = 8.4 Hz, 2H), 8.03 (d, *J* = 8.4 Hz, 2H), 7.90 (dd, *J* = 16.0, 9.2 Hz, 2H), 7.81 (d, *J* = 8.4 Hz, 4H), 7.17 (d, *J* = 8.4 Hz, 1H), 4.40 (s, 4H), 3.18 (s, 3H), 2.52 (p, *J* = 7.3 Hz, 2H). ^{13}C NMR (101 MHz, Chloroform-d) δ 147.76, 138.94, 133.65, 131.91, 131.49, 129.58, 128.50, 127.48, 126.35, 126.05, 124.70, 123.69, 122.93, 120.48, 111.67, 55.92, 44.74, 17.55. HRMS $[M]^+$: Calcd. for $C_{26}H_{21}NO_2S$: 411.1293, found: 411.1278.

Synthesis of PAS-2

4-ethynylbenzenesulfonamide (67 mg, 0.37 mmol), Py-C1 (50 mg, 0.15 mmol), copper(I) iodide (10 mg, 0.05 mmol), $Pd(PPh_3)_4$ (43 mg, 0.037 mmol) and triethylamine (1mL) were added into the reaction flask with 5 mL of toluene as the solvent. Then the mixture was reacted at 70°C for 24 hours under the protection of nitrogen. Add water to quench the reaction, then extract three times with saturated

saline and dichloromethane, combine the organic layer dried over Na_2SO_4, filter, and remove the solvent under vacuum. The crude product was purified by silica gel column chromatography (dichloromethane: methanol = 50:1) to give the orange powder product 20 mg. The full name is 1-azetidine-6-(((4-sulfamido)phenyl)ethynyl)pyrene (PAS-2) according to the systematic nomenclature; m.p. 248.6 - 250.2 ℃; Yield: 31 %; ^{1}H NMR (400 MHz, Acetone-d_6) δ 8.37 (d, J = 9.0 Hz, 1H), 8.30 (d, J = 9.3 Hz, 1H), 8.21 – 8.09 (m, 3H), 8.04 (d, J = 8.0 Hz, 1H), 7.99 (d, J = 8.5 Hz, 2H), 7.93 (dd, J = 15.3, 8.9 Hz, 3H), 7.21 (d, J = 8.4 Hz, 1H), 6.74 (s, 2H), 4.46 (t, J = 7.4 Hz, 4H), 2.53 (p, J = 7.3 Hz, 2H). ^{13}C NMR (101 MHz, Acetone-d6) δ 131.78, 129.96, 129.28, 127.33, 126.48, 124.66, 124.21, 123.40, 122.54, 120.85, 114.46, 111.69, 55.64, 17.11. HRMS $[2M]^+$: Calcd. for $C_{27}H_{20}N_2O_2S$: 872.2490, found: 872.2462.

4. Optical experiments

Preparation of Large Unilamellar Vesicles (LUVs) model solution. DOPC, DOPC/Chol, and SM/Chol solutions were prepared via the thin-film hydration method, and the stock solution concentration was 10 mM. Take the preparation of DOPC solution as an example. Initially, an appropriate amount of DOPC was weighed and dissolved in ethanol, after which the solvent was evaporated under reduced pressure and dried under vacuum for 3h to form a lipid film. Subsequently, lipid film was hydrated by adding water, and the obtained solution was sonicated for 30 min to disperse into a homogeneous liposome suspension, which was extruded 10 times through a polycarbonate membrane with a pore size of 200 nm, promptly used for subsequent experiments. The other two vesicle solutions were prepared according to the same method (DOPC/Chol=70/30, SM/Chol=55/45). All vesicle model experiments were performed at a working concentration of 100 μM.

Optical measurement. Stock solutions of PAS-1, and PAS-2 in DMSO were prepared separately, with concentrations of 5 mM. The three probes were diluted with toluene, dioxane, ethyl acetate, chloroform, acetone, acetonitrile, ethanol, methanol,

and water, respectively, making them with a final working concentration of 5 μM, and the absorption spectra and emission spectra under the excitation of 405 nm laser were measured. Subsequently, a mixed solution of the three probes was prepared in the model film solution to achieve a working concentration of 5 μM, and the UV absorption spectra and fluorescence emission spectra under 405 nm laser excitation were recorded.

5. Cell experiments

Cell culture. HeLa cell lines were obtained from the National Infrastructure of the Cell Line Resource. HeLa Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM medium, Invitrogen Corp) supplemented with 10% fetal bovine serum (FBS, Gibco), penicillin (100 U/mL), and streptomycin (100 ug/mL). The cells were incubated in a CO_2 incubator at a temperature of 37 ℃ with a CO_2 concentration of 5%. The cells were seeded in con-focal dishes 24 hours before the experiment.

Cytotoxicity. The cytotoxicity of PAS-1 and PAS-2 was evaluated by CCK8 assay. Regarding PAS-1 as an example, HeLa cells were seeded in 96-well plates at a density of 1×104 cells/well, supplemented with Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL), which was incubated in an incubator with 5% CO_2 at 37 ℃ for 24 h. After the medium was removed, HeLa cells were incubated with medium containing 0, 0.1, 0.2, 0.5, 1, and 2 μM PAS-1 for 12/24 h, then replaced with medium containing CCK8 (10 μL) for 1 h. The absorbance at 450 nm was measured using a Biotek/800TS microplate reader (Berten, USA). Cell survival rate = (OD value of experimental group - OD value of blank group)/(OD value of control group - OD value of blank group)*100%.

6. Additional tables and figures

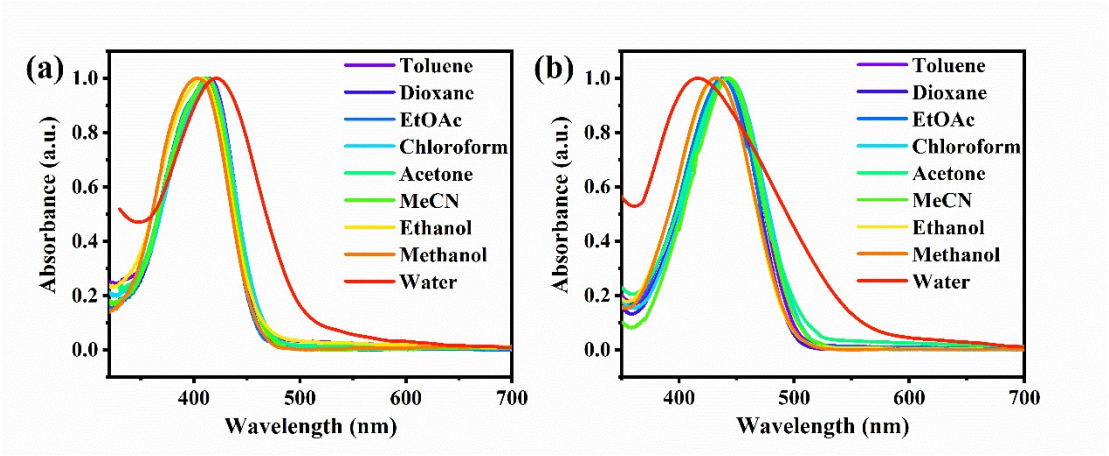


Fig. S1 The absorption spectra of PAS-1 (a) and PAS-2 (b) in different solvents.

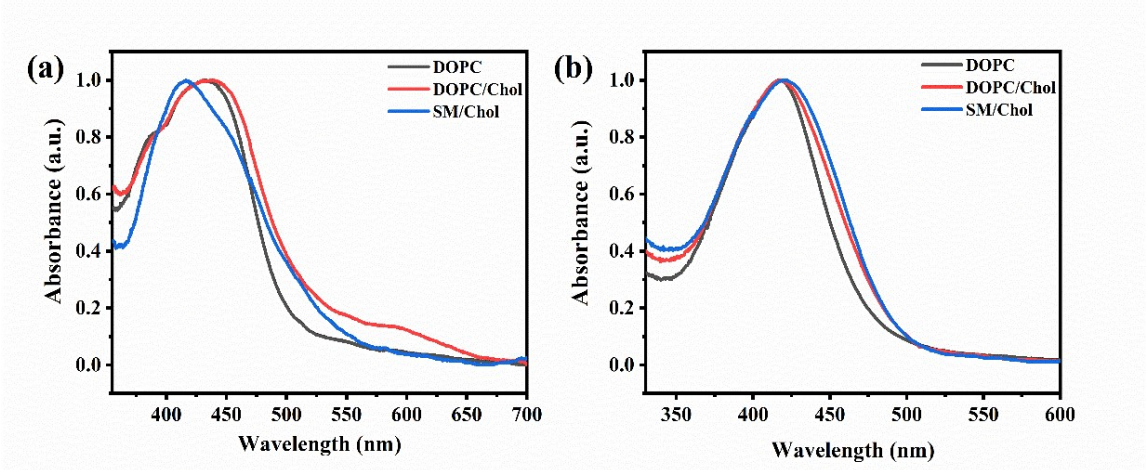


Fig. S2 The absorption spectra of PAS-1 (a) and PAS-2 (b) in liposomes with different lipid compositions.

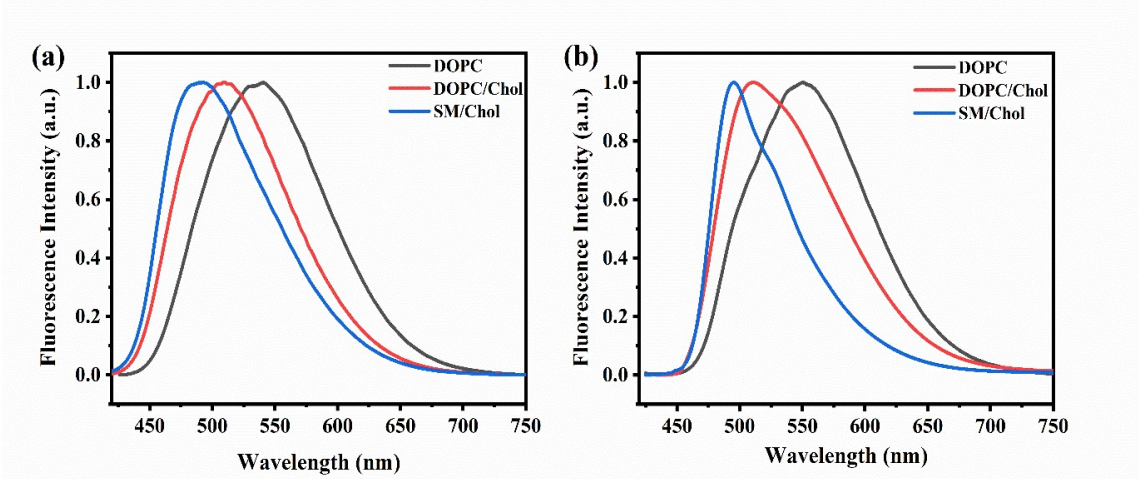


Fig. S3 The emission spectra of PAS-1 (a) and PAS-2 (b) in liposomes with different lipid compositions.

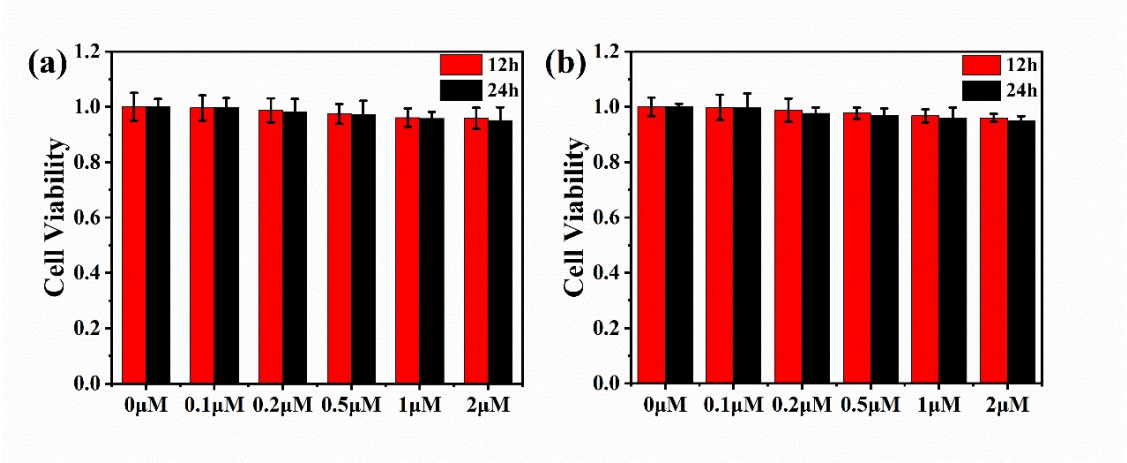


Fig. S4 Cytotoxicity assay of PAS-1 (a) and PAS-2 (b) after 12 h/24 h incubation quantified by the CCK-8 assay.

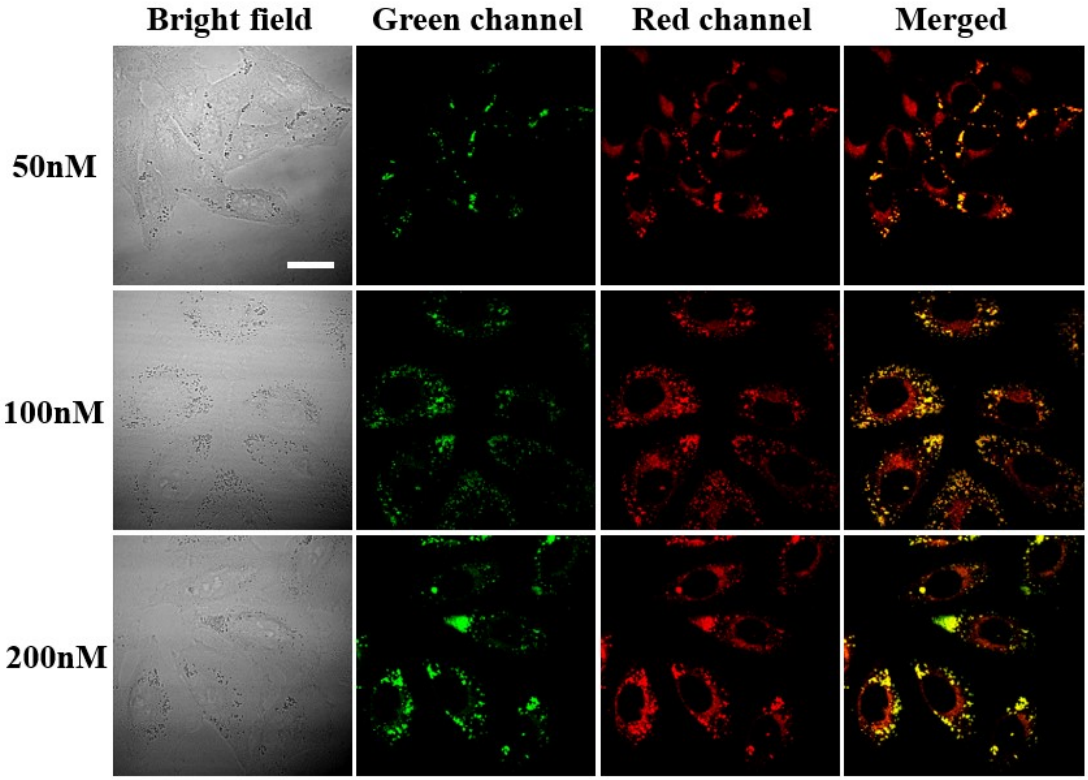


Fig. S5 Dual-channel CLSM images of HeLa cells incubated with different concentrations of PAS-1. λ_{ex} = 405 nm, Green channel: λ_{em} = 430–480 nm. Red channel: λ_{em} = 500–550 nm. Scale bar: 10 μm.

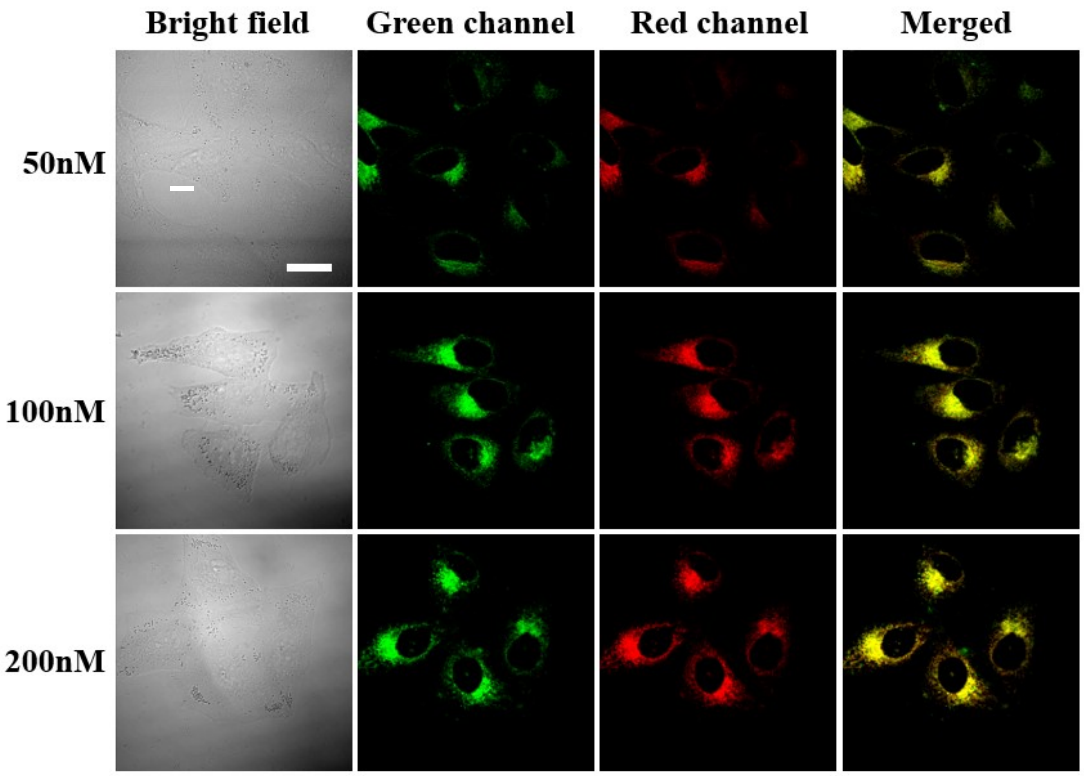


Fig. S6 Dual-channel CLSM images of HeLa cells incubated with different concentrations of PAS-2. λ_{ex} = 405 nm, Green channel: λ_{em} = 450–500 nm. Red channel: λ_{em} = 550–600 nm. Scale bar: 10 μm.

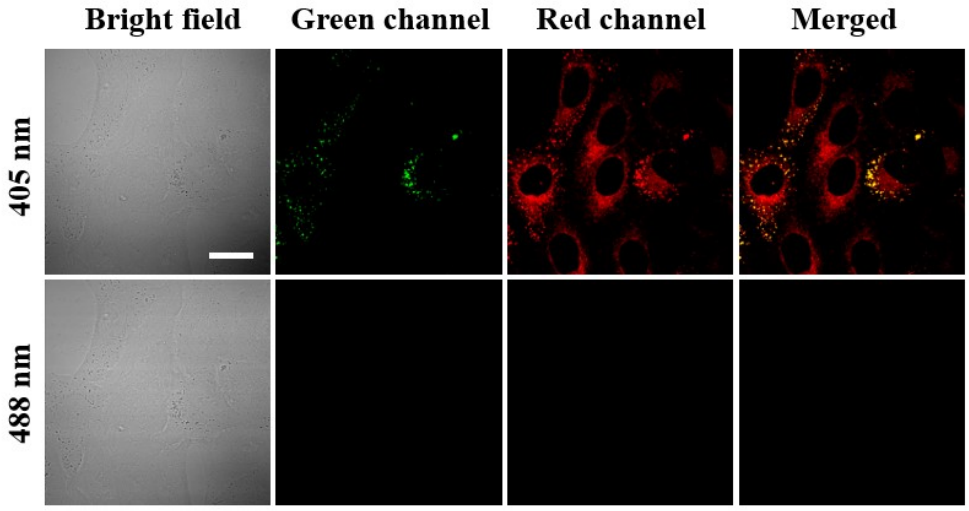


Fig. S7 Dual-channel CLSM images of HeLa cells incubated with PAS-1 (200 nM). Green channel: λ_{em} = 430–480 nm. Red channel: λ_{em} = 500–550 nm. Scale bar: 10 μm.

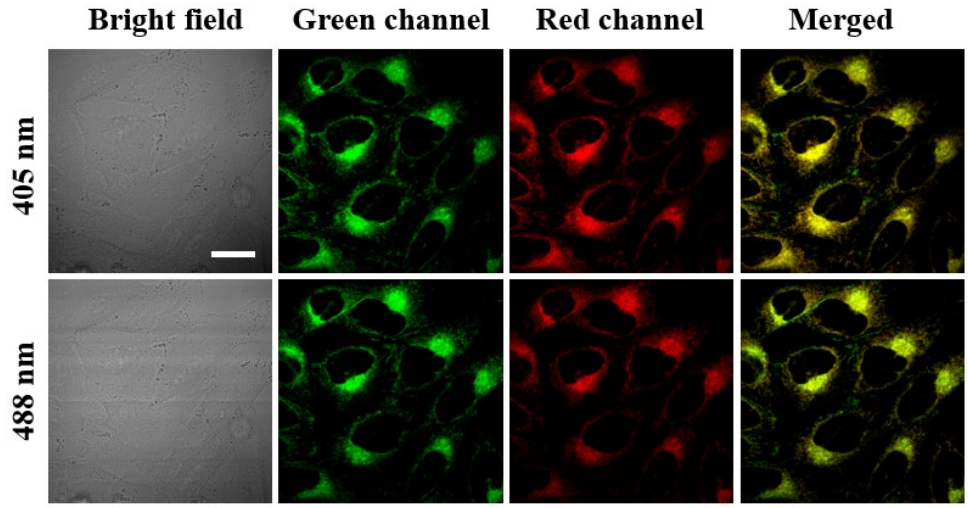


Fig. S8 Dual-channel CLSM images of HeLa cells incubated with PAS-2 (200 nM). Green channel: λ_{em} = 450–500 nm. Red channel: λ_{em} = 550–600 nm. Scale bar: 10 μm.

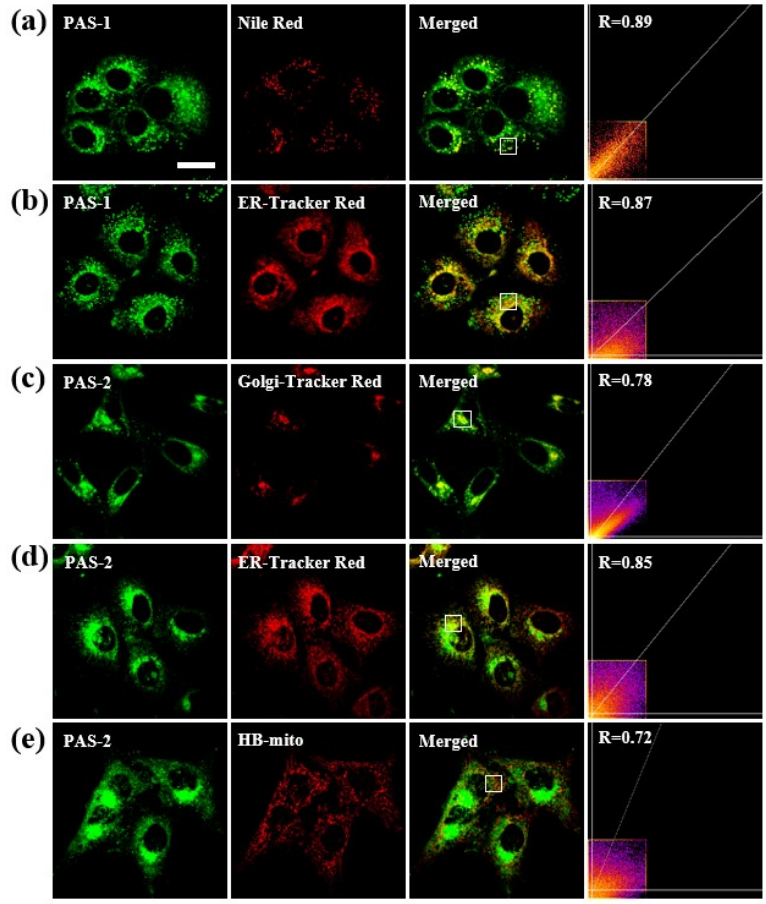


Fig. S9 Colocalization imaging of PAS-1 (200 nM) with Nile Red (a) and ER-Tracker Red (b). Colocalization imaging of PAS-2 (200 nM) with Golgi-Tracker Red (c), ER-Tracker Red (d), and HBmito Crimson (e). Calculated the co-localization coefficient of the white rectangular area. Scale bar: 10 μm.

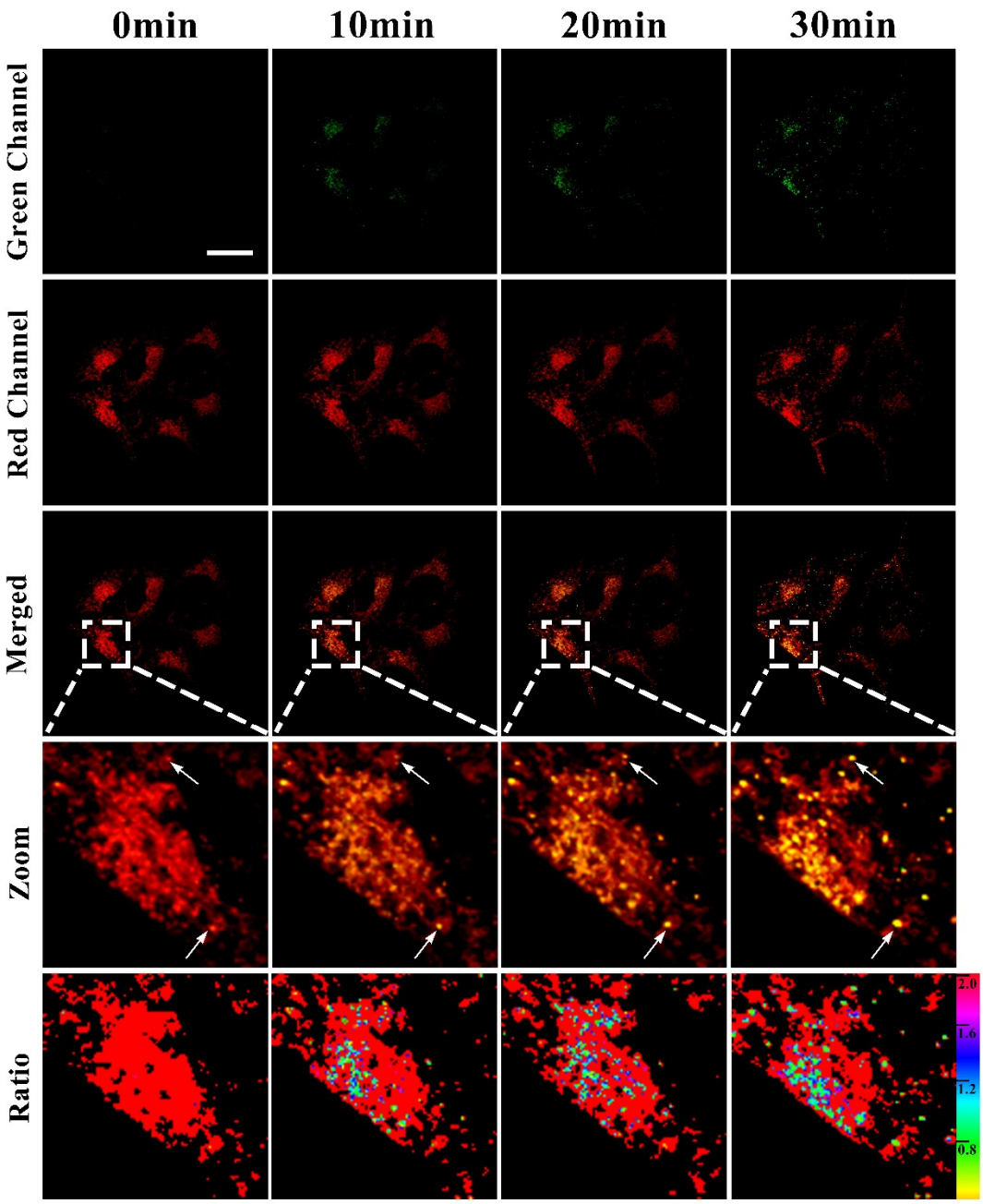


Fig. S10 Real-time tracking of Lipid droplets and endoplasmic reticulum dynamics in oleic acid pretreated HeLa cells by PAS-1 (200 nM). λ_{ex} = 405 nm. Green channel: λ_{em} = 430–480 nm. Red channel: λ_{em} = 500–550 nm. The arrow displays the dynamics of a single lipid droplet. Ratio: Red/Green ratio images of white rectangular area. Scale bar: 10 μm.

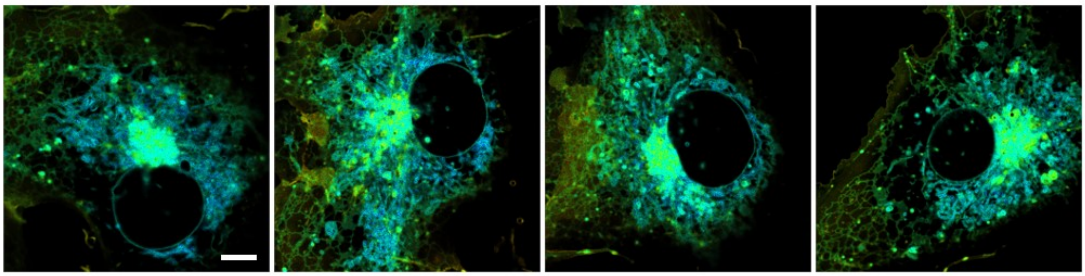


Fig. S11 Fluorescence lifetime images of COS-7 cells stained with PAS-2 (200 nM). λ_{ex} = 470 nm, Scale bar: 5 μm.

Table S1 The pyrene fluorescent probes summary.

Name	Maximum absorption (nm)	Applicable lasers (nm)	Localization	SIM imaging	FLIM imaging	Applications	References
PAS-1	415	405	LDs and ER	N.A.	N.A.	Cell lines (HeLa)	This work
PAS-2	442	405/488	Golgi, ER and Mito	Yes	Yes	Cell lines (HeLa, COS-7)	This work
PA	422	405	LDs, ER and cell membrane	N.A.	Yes	Cell lines (HeLa)	Y. Niko et al., 2016, Sci Rep.[1]
PK	402	405	LDs, ER and Mito	N.A.	N.A.	Cell lines (HeLa)	J. Valanciunaite et al., 2020, Anal. Chem. [2]
LW-1	409	405	LDs	N.A.	N.A.	Cell lines (3T3, 4T1, HL-7702, HepG2)	C. Liu et al., 2021, SNB Journal. [3]
1P2N	450	405	LDs, ER, Mito and lysosome	N.A.	N.A.	Cell lines (HeLa)	Y. Liu et al., 2023, Chem. Eng. J. [4]

Table S2 Photophysical properties of PAS-1 in different solvents.

Solvent	$E_T(30)$	λ_{ex} (nm)	λ_{em} (nm)	Stokes Shift (nm)	τ (ns)	Φ_f (%)	k_r (10^9 s^{-1})	k_{nr} (10^9 s^{-1})
Toluene	33.9	415	499	84	3.06	96	0.315	0.012
Dioxane	36.0	413	504	91	3.54	89	0.250	0.032
EtOAc	38.1	411	521	110	3.63	83	0.228	0.048
Chloroform	39.1	414	521	107	3.75	81	0.216	0.051
Acetone	42.2	413	541	128	3.78	50	0.132	0.132
MeCN	45.6	412	555	143	4.17	43	0.102	0.138
Ethanol	51.9	408	551	143	4.18	30	0.071	0.169
Methanol	55.4	403	559	156	4.27	28	0.065	0.170
Water	63.1	420	560	140	6.15	3	0.004	0.158

Table S3 Photophysical properties of PAS-2 in different solvents.

Solvent	$E_T(30)$	λ_{ex} (nm)	λ_{em} (nm)	Stokes Shift (nm)	τ (ns)	Φ_f (%)	k_r (10^9 s^{-1})	k_{nr} (10^9 s^{-1})
Toluene	33.9	442	516	74	2.72	76	0.280	0.087
Dioxane	36.0	440	518	78	3.10	66	0.212	0.111
EtOAc	38.1	438	533	95	3.11	46	0.149	0.172
Chloroform	39.1	444	542	98	3.16	45	0.143	0.174
Acetone	42.2	441	556	115	3.21	33	0.104	0.207
MeCN	45.6	444	577	133	3.24	29	0.089	0.220
Ethanol	51.9	433	553	120	3.52	23	0.064	0.220
Methanol	55.4	432	562	130	3.54	19	0.053	0.230
Water	63.1	417	609	192	5.18	1	0.002	0.191

Table S4 Photophysical properties of PAS probes in different Lipid compositions.

Lipid Composition	PAS-1			PAS-2		
	λ_{ex} (nm)	λ_{em} (nm)	Stokes Shift (nm)	λ_{ex} (nm)	λ_{em} (nm)	Stokes Shift (nm)
DOPC	417	541	124	432	551	119
DOPC/Chol	418	510	92	437	510	73
SM/Chol	420	493	73	419	492	73

Table S5 Fluorescence lifetime statistics for multiple organelles.

	Mito	Golgi	ER
1	3.268	3.429	3.879
2	3.312	3.569	3.934
3	3.257	3.406	3.937
4	3.349	3.518	3.846
5	3.308	3.486	3.947
6	3.353	3.445	3.853
7	3.334	3.585	3.973
8	3.372	3.597	3.965
Average	3.319	3.504	3.917

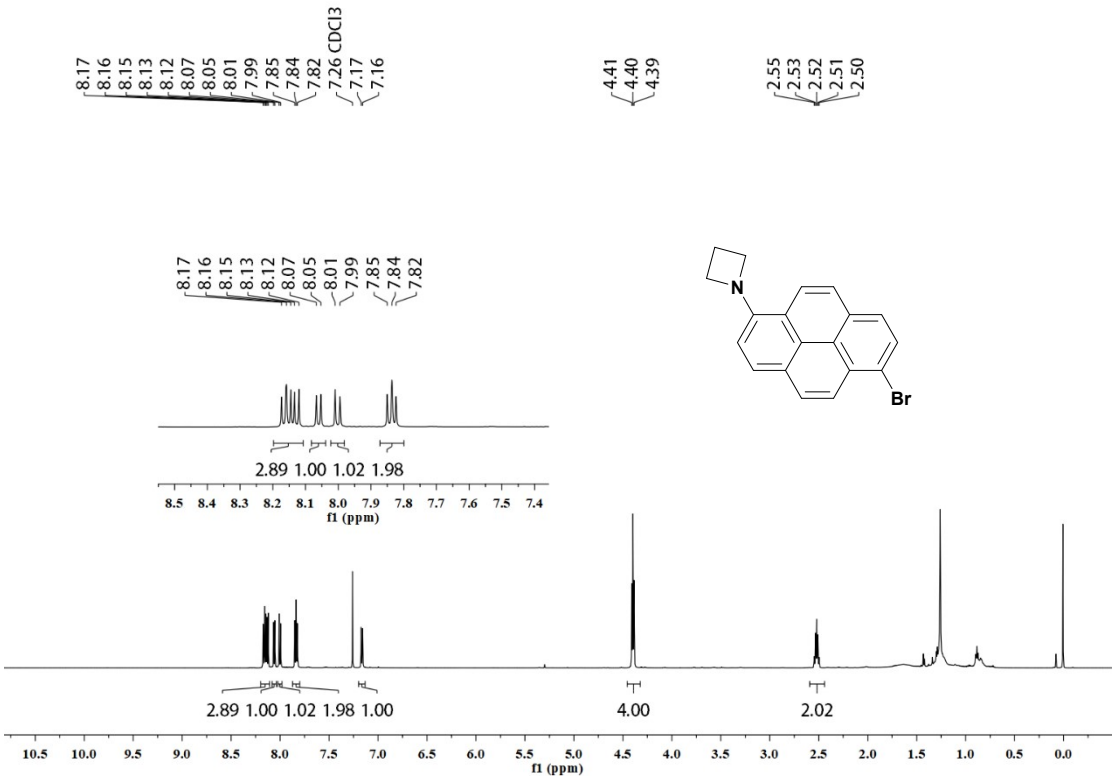


Fig. S11 ^{1}H-NMR spectra of compound Py-C1 ($CDCl_3$).

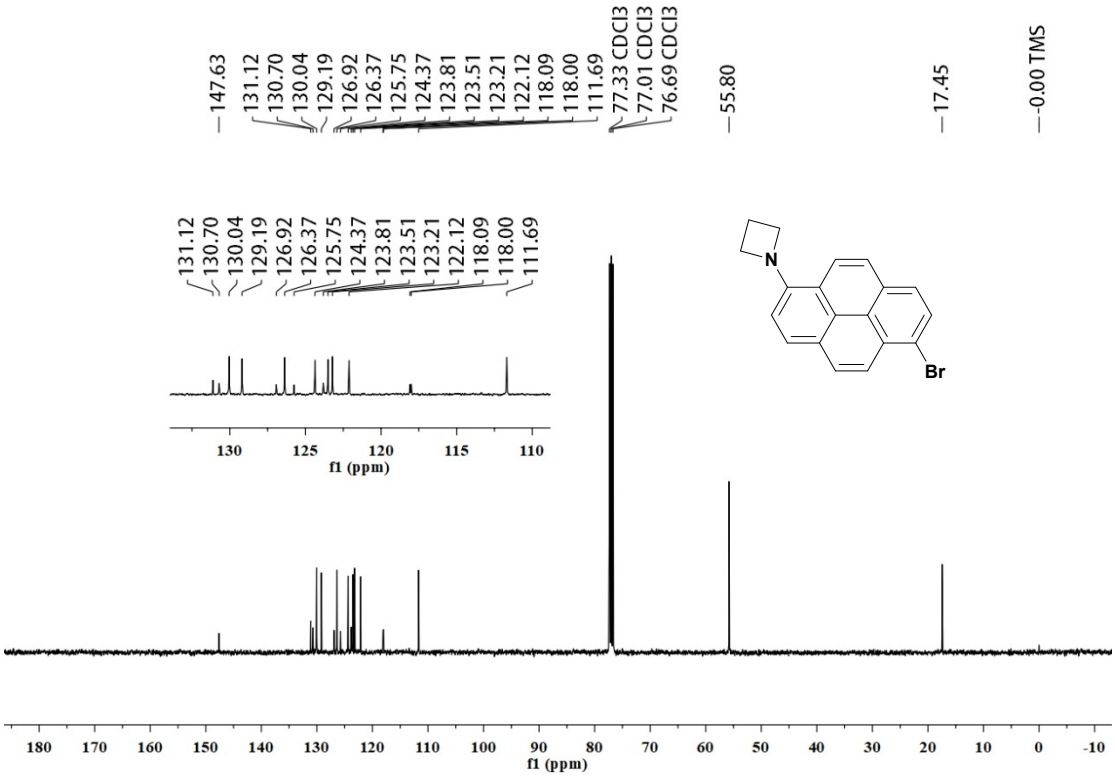


Fig. S12 ^{13}C-NMR spectra of compound Py-C1 ($CDCl_3$).

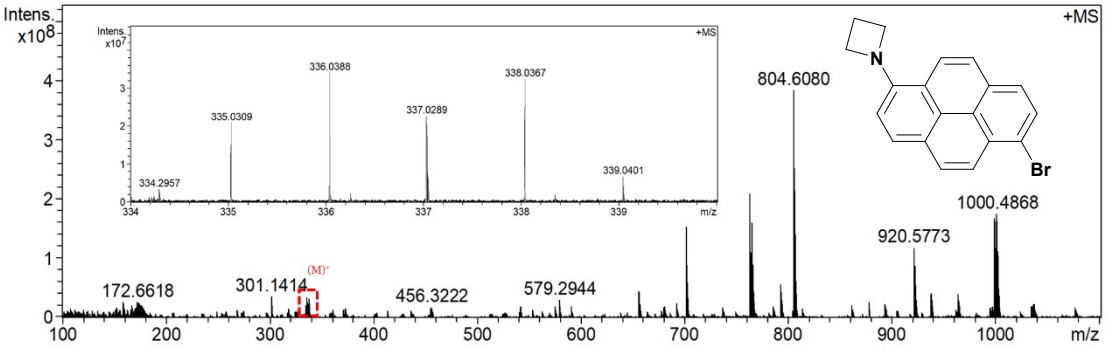


Fig. S13 HRMS spectra of compound Py-C1.

([M]$^+$: Calcd. for $C_{19}H_{14}BrN$: 335.0310 found: 335.0309)

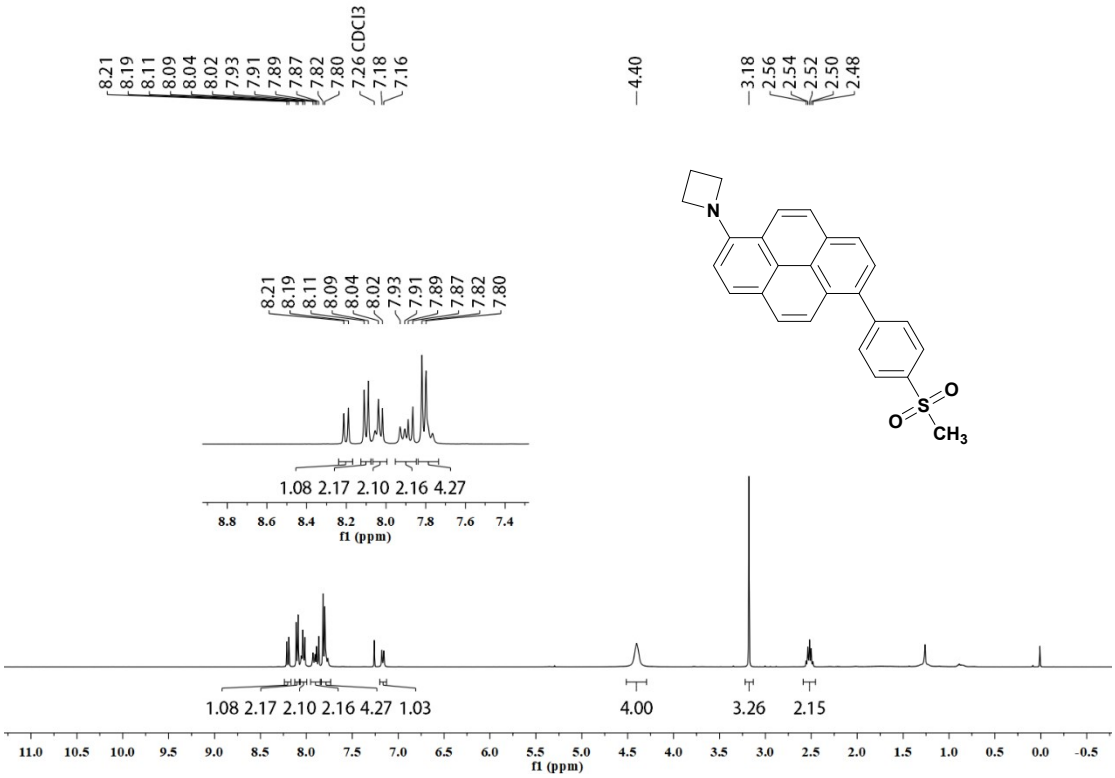


Fig. S14 ^{1}H-NMR spectra of compound PAS-1 ($CDCl_3$).

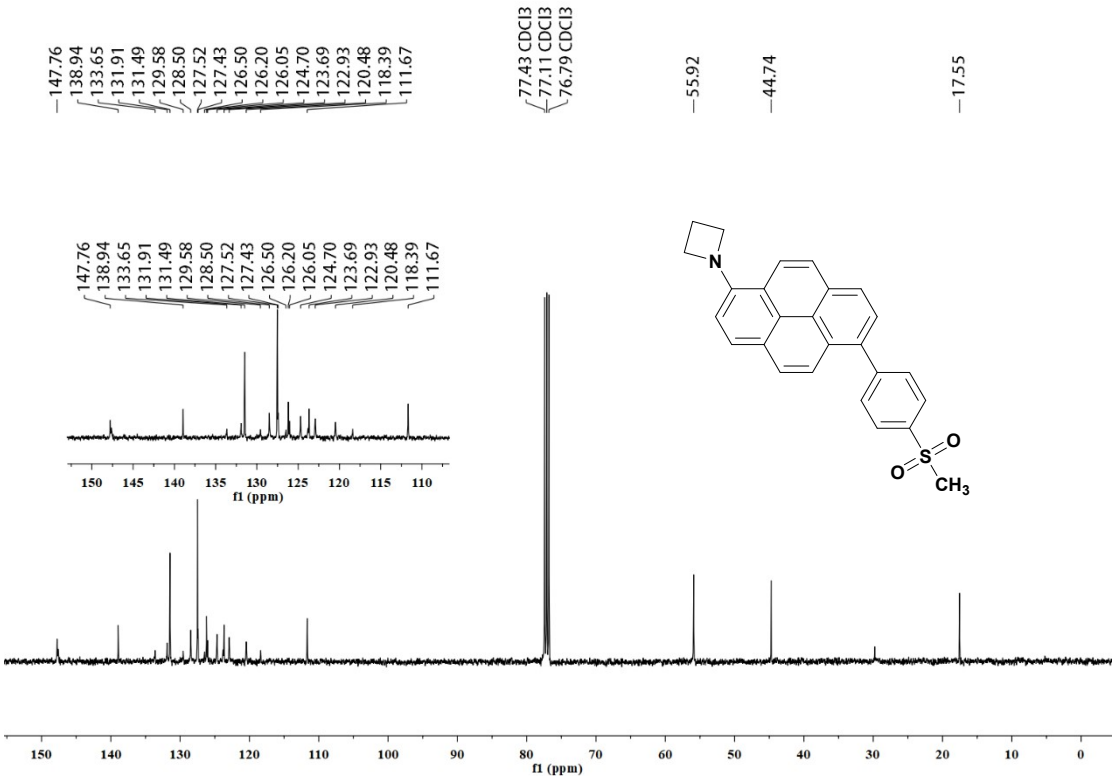


Fig. S15 ^{13}C-NMR spectra of compound PAS-1 ($CDCl_3$).

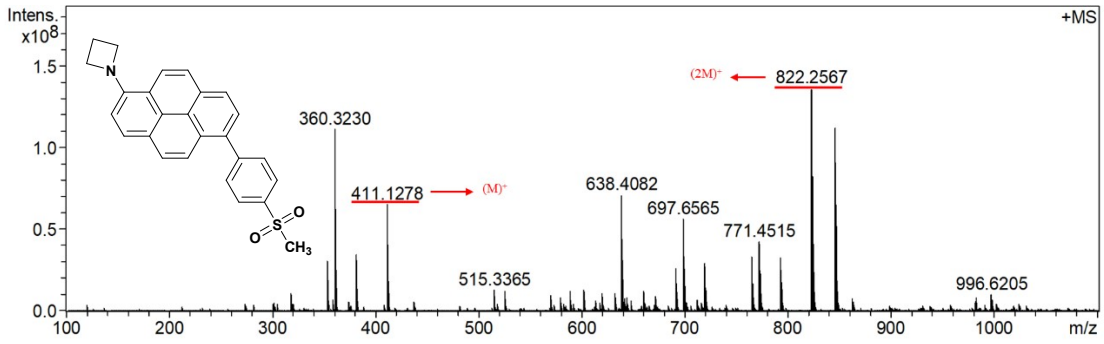


Fig. S16 HRMS spectra of compound PAS-1.

([M]$^+$: Calcd. for $C_{26}H_{21}NO_2S$: 411.1293 found: 411.1278)

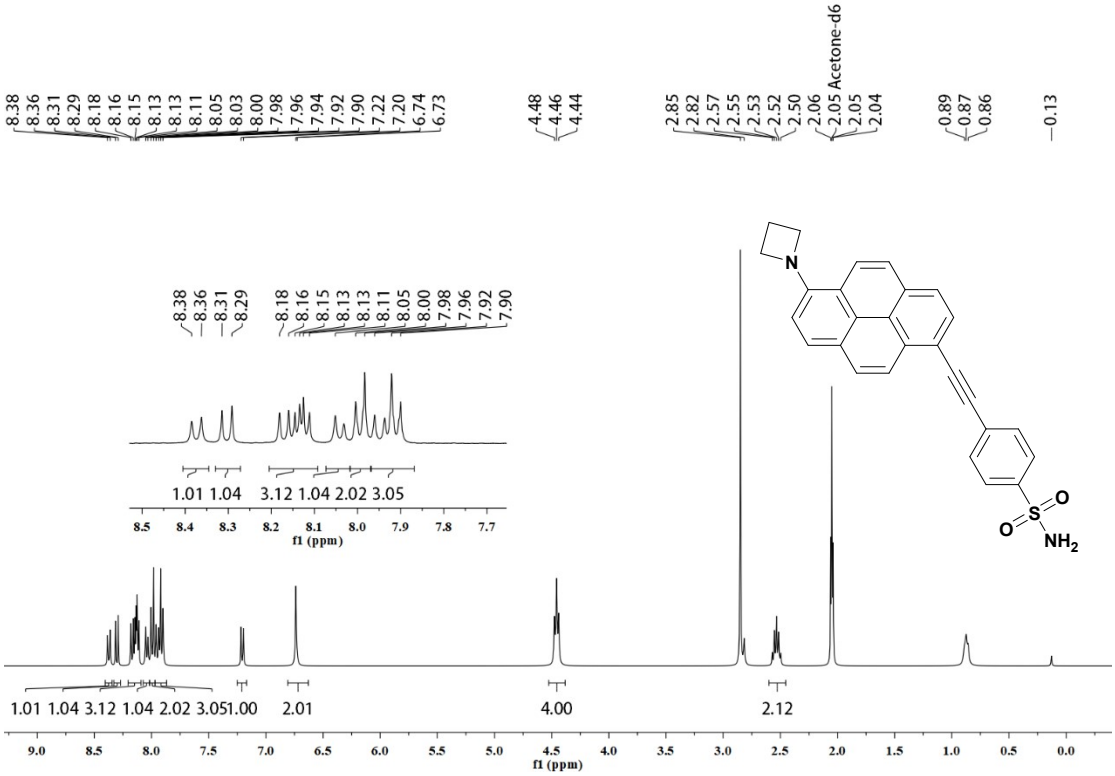


Fig. S17 [1]H-NMR spectra of compound PAS-2 (Acetone-d6).

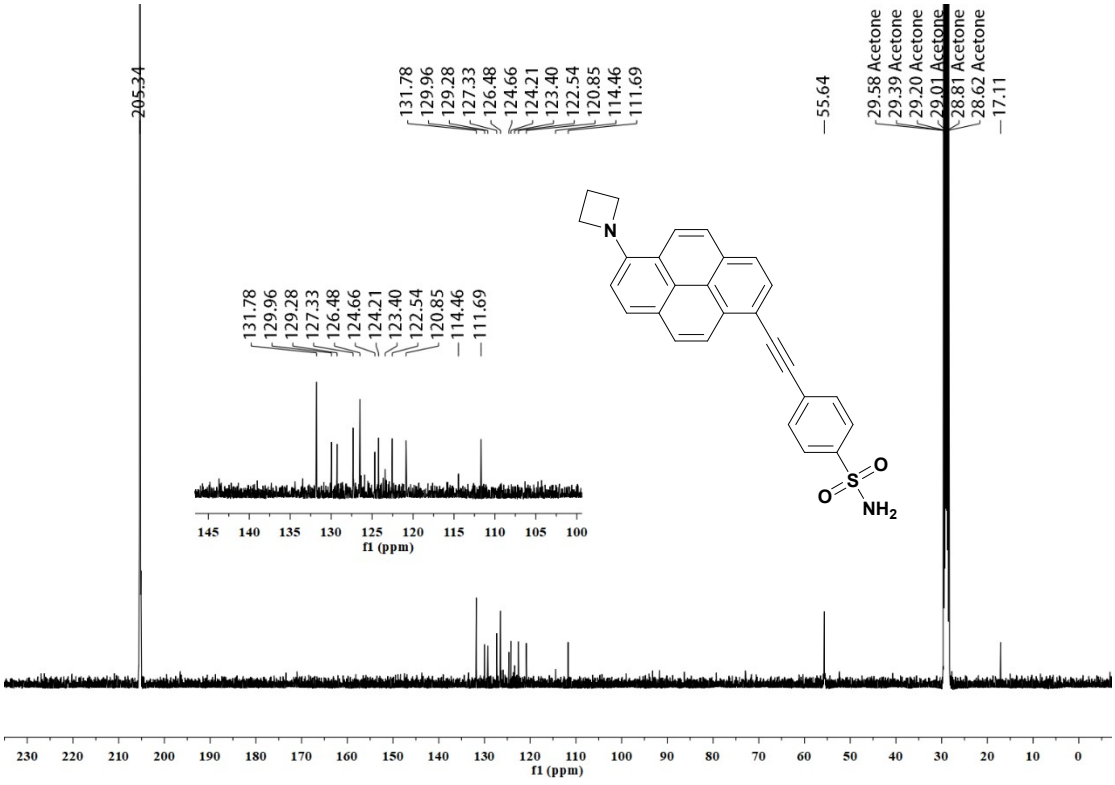


Fig. S18 [13]C-NMR spectra of compound PAS-2 (Acetone-d6).

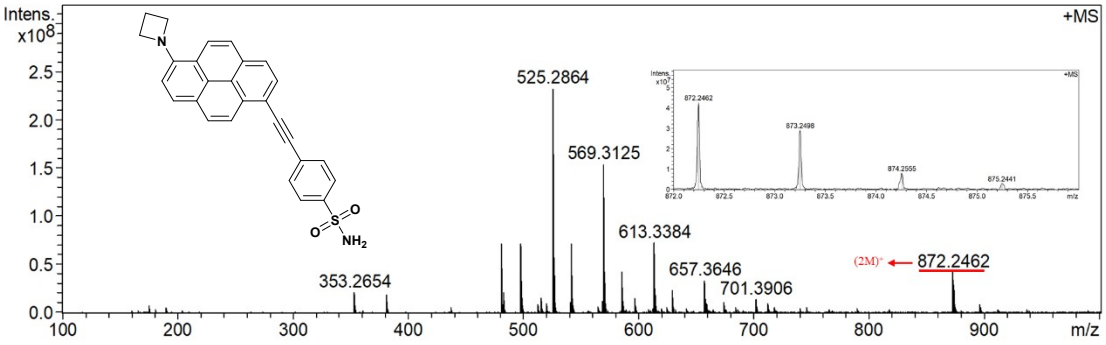


Fig. S19 HRMS spectra of compound PAS-2.

([2M]$^+$: Calcd. for $C_{27}H_{20}N_2O_2S$: 872.2490 found: 872.2462)

7. Reference

1 Y. Niko, P. Didier, Y. Mely, G. Konishi and A. S. Klymchenko, *Sci. Rep.*, 2016, **6**, 18870.

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