Supplementary information

Minimal-tag polyamines for in situ probing of their intracellular distribution

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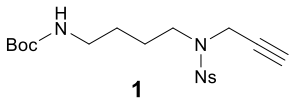
Content:

1. Methods: synthetic procedures

1.1. General

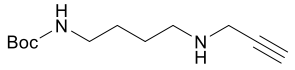
All materials were purchased from commercial suppliers and used without purification. Thin layer chromatography (TLC) was performed on Merck Silica gel 60 F_{254} precoated aluminium sheets. Components were visualized by observation under ultra violet light (254 nm) or dyed by an aqueous $KMnO_4$ reagent. Column chromatography was carried out using SiliaFlash Irregular Silica Gel P60, 40 - 63 µm, 60 A. The NMR spectra were recorded on Agilent 400 MHz spectrometers at ambient temperature. Chemical shifts are reported in parts per million (ppm) and referenced against the residual proton or carbon signal of solvent: $CDCl_3$ (^1H = 7.26 ppm, ^{13}C = 77.16 ppm), D_2O (^1H = 4.79 ppm). Coupling constants J are given in hertz (Hz). Data are reported as follows: chemical shift, multiplicity, coupling constant, integration. HRMS was collected on Q Exactive HF-X mass spectrometer.

1.2. Preparation of iPUT



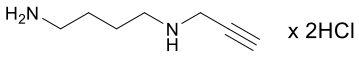
1

To a solution of N-(tert-Butoxycarbonyl)-N'-(2-nitrobenzenesulfonyl)butane-1,4-diamine (1.0 g, 2.68 mmol)[1] in anhydrous acetonitrile (20 mL), cesium carbonate (1.3 g, 3.99 mmol), tetrabutylammonium iodide (0.1 g, 0.27 mmol), and propargyl bromide (0.38 g, 3.2 mmol) were added. The reaction mixture was stirred overnight at 60 °C. After completion, the solvent was removed under reduced pressure, and the residue was partitioned between DCM and water. The aqueous layer was extracted with DCM twice. The combined organic layers were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was then purified by column chromatography (silica; 30% EtOAc/hexanes). The title compound **1** was obtained as a yellowish solid (1.0g, 91%). **TLC** (50% EtOAc/ hexanes): Rf =0.50; **^{1}H NMR (400 MHz, $CDCl_3$)** δ 8.04-8.01 (m, 1H), 7.71-7.67 (m, 2H), 7.63-7.61 (m, 1H), 4.57 (bs, 1H), 4.17 (d, J=4Hz, 2H), 3.41 (t, J=8Hz, 2H), 3.11 (dd, J_1=8Hz, J_2=12Hz, 2H), 2.16 (t, J=4Hz, 1H), 1.68-1.59 (m, 2H), 1.5-1.46 (m, 2H), 1.42 (s, 9H). **^{13}C NMR (101 MHz, $CDCl_3$)** δ 156.10, 148.38, 133.84, 132.78, 131.77, 130.95, 124.30, 79.32, 74.03, 46.64, 40.05, 36.42, 28.52, 27.16, 24.77.



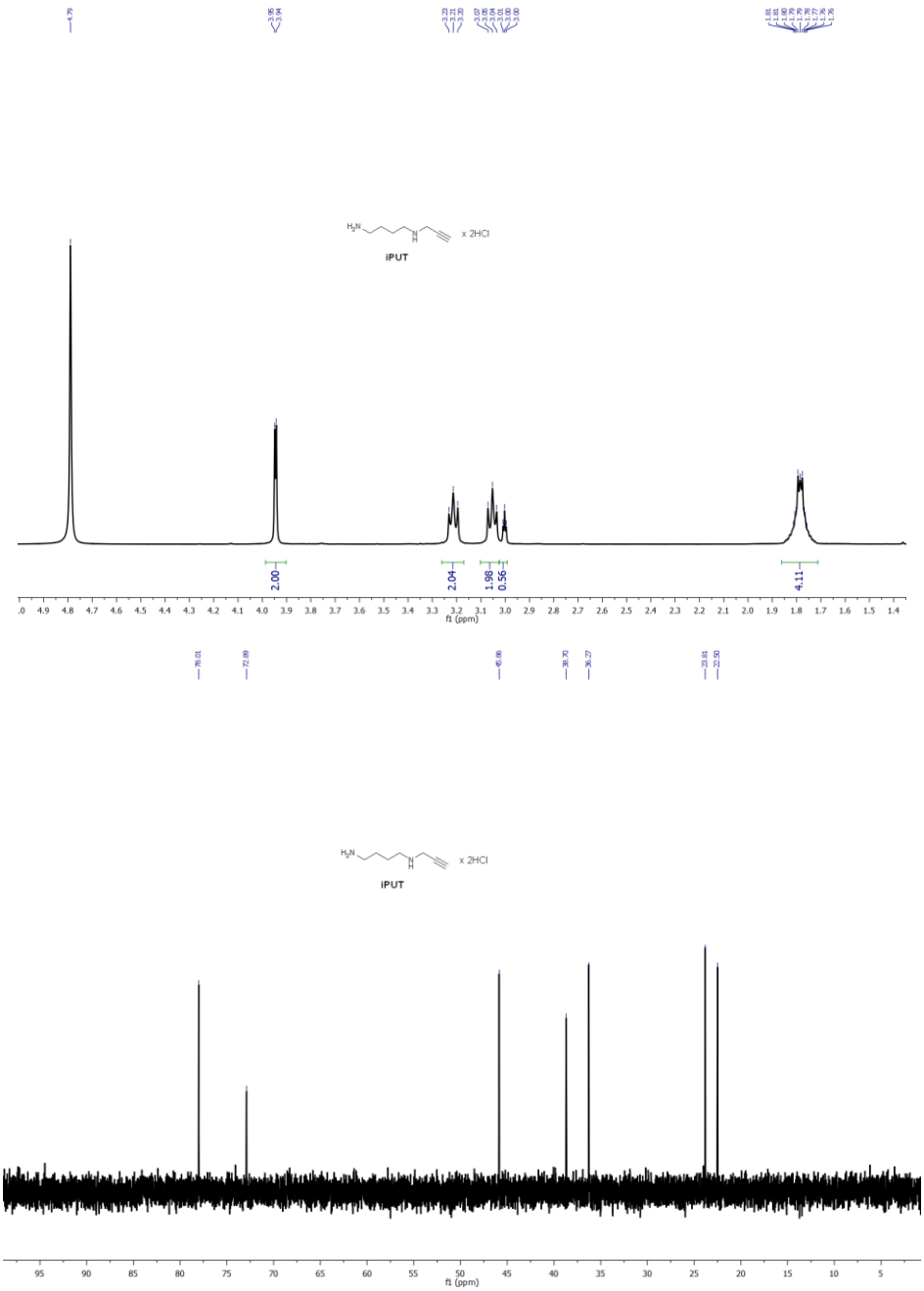
2

To a solution of **1** (1.0 g, 2.43 mmol) in anhydrous acetonitrile (20 mL), cesium carbonate (1.6 g, 4.86 mmol) and thiophenol (0.53 g, 0.5 mL, 4.86 mmol) were added. The reaction mixture was stirred at 60 °C for 2h. Then solvent was removed under reduced pressure, and the residue was partitioned between DCM and water. The aqueous layer was extracted with DCM twice. The combined organic layers were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was then purified by column chromatography (silica; 3% Ammonia-saturated methanol/DCM). The title compound **2** was obtained as a yellowish oil (0.51 g, 93%). **TLC** (5% Ammonia-saturated methanol/DCM): Rf =0.53; **^{1}H NMR (400 MHz, $CDCl_3$)** δ 4.76 (bs, 1H), 3.38 (d, J=2.4Hz, 2H), 3.11-3.06 (m, 2H), 2.66 (t, J=6.8), 2.18 (t, J=2.4Hz, 1H), 1.53-1.46 (m, 4H), 1.39 (s, 9H); **^{13}C NMR (101 MHz, $CDCl_3$)** δ 156.03, 82.20, 78.96, 71.35, 48.18, 40.42, 38.08, 28.45, 27.85, 27.08.

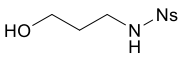


iPUT

Compound **2** (0.51 g, 2.26 mmol) was cooled in an ice-water bath. Then 5 mL of cold 4 M HCl in dioxane was added dropwise, followed by a few drops of Milli-Q water. The reaction mixture was stirred at 0 °C for 1 h and then concentrated under reduced pressure. The residue was resuspended in 3 mL of MeOH and 1 mL of $CHCl_3$ and concentrated under reduced pressure. This washing procedure was repeated three times, reducing the volume of MeOH with each repetition, to yield the dihydrochloride salt of **iPUT** as an off-white solid. The yield was quantitative. **^{1}H NMR (400 MHz, D_2O)** δ 3.95 (d, J=4Hz, 2H), 3.21 (bt, J≈7Hz, 2H), 3.05 (bt, J≈7Hz, 2H), 3.00 (bt, J≈7Hz, 1H) 1.81-1.76 (m, 4H). **^{13}C NMR (101 MHz, D_2O)** δ 78.01, 72.89, 45.86, 38.70, 36.27, 23.81, 22.50. **HRMS** (m/z): $[M+H]^+$ calculated for $C_7H_{15}N_2$ 127.1235; found 127.1241

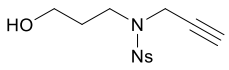


1.3. Preparation of iSPD



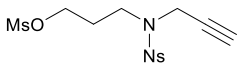
3

To a solution of 3-amino-1-propanol (2.0 g, 26.67 mmol) in DCM (100 ml), 11.0 ml (79.0 mmol) of triethylamine was added, and the mixture was cooled to 0 °C in an ice-water bath. Next, 2-nitrobenzenesylfonyl chloride (6.5 g, 29.41 mmol) was added portionwise. The reaction mixture was stirred overnight at room temperature (RT), diluted with DCM and washed twice with 1M HCl. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The compound **3** was obtained as a yellow solid (5.88 g, 98% yield); **TLC** (80% EtOAc/ hexanes): Rf=0.51; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.15-8.12 (m, 1H), 7.87-7.85 (m, 1H), 7.77-7.72 (m, 1H), 5.83 (bs, 1H), 3.76 (bt, J≈5.2Hz, 2H), 3.25(t, J_1= 12.4Hz, J_2= 6.0Hz), 1.92 (bs, 1H), 1.78 (m,2H); **^{13}C NMR (101 MHz, D_2O)** δ 148.03, 133.71, 133.43, 132.92, 131.09, 125.42, 60.31, 41.56, 31.58.



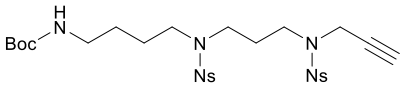
4

To a solution of **3** (6.9 g, 26.67 mmol) in anhydrous acetonitrile (100 mL), cesium carbonate (5.0 g, 15.38 mmol), tetrabutylammonium iodide (0.9 g, 2.7 mmol), and propargyl bromide 80 wt.% solution in toluene (3.2 g, 26.67 mmol) were added. The reaction mixture was stirred overnight at 60 °C. After completion, the solvent was removed under reduced pressure, and the residue was partitioned between DCM and water. The aqueous layer was extracted with DCM twice. The combined organic layers were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was then purified by column chromatography (silica; 60% EtOAc/hexanes). The title compound **4** was obtained as a yellowish solid (5.82 g, 73%). **TLC** (80% EtOAc/ hexanes): Rf =0.26; **^{1}H NMR (400 MHz, $CDCl_3$)** δ 8.06-8.03 (m, 1H), 7.72-7.67 (m, 2H), 7.66-7.64 (m, 1H), 4.22 (d, J= 2.4Hz, 2H), 3.72 (t, J= 6Hz, 2H), 3.57 (t, J= 6.4Hz, 2H), 2.19 (t, J= 2.4Hz, 1H), 1.84 (m, 2H). **^{13}C NMR (101 MHz, $CDCl_3$)** δ 148.43, 133.95, 132.65, 131.86, 130.90, 124.41, 77.48, 77.16, 76.84, 74.19, 58.99, 43.76, 36.67, 29.99.



5

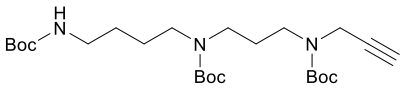
To a solution of **4** (2.97 g, 9.96 mmol) in DCM (40 ml), 2.67 ml (19.0 mmol) of triethylamine was added and the mixture was cooled to 0 °C in an ice-water bath. Next, 0.92 ml (11.8 mmol) of mesyl chloride was added. The reaction mixture was stirred overnight at room temperature (RT), diluted with DCM and washed with 1M HCl, 1M NaOH and brine. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The compound **5** was obtained as an orange oil (3.68 g, 98% yield); **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.05-8.03 (m,1H), 7.74-7.70 (m, 2H), 7.65-7.63 (m, 1H), 4.30 (t, J= 5.6Hz, 2H), 4.21 (d, J= 6.4Hz, 2H), 3.57 (t, J= 6.8Hz, 2H), 3.04 (s, 3H), 2.20 (t, J= 2.4Hz, 1H), 2.09 (m, 2H).**^{13}C NMR (101 MHz, D_2O)** δ 148.33, 134.01, 132.09, 131.76, 130.95, 124.21, 76.43, 74.38, 66.85, 43.57, 37.35, 36.89, 27.33.



6

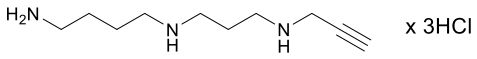
To a solution of N-(tert-Butoxycarbonyl)-N'-(2-nitrobenzenesulfonyl)butane-1,4-diamine (1.64 g, 4.4 mmol) in anhydrous acetonitrile (40 mL), cesium carbonate (1.4 g, 4.4 mmol), tetrabutylammonium iodide (160 mg, 0.44 mmol), and compound **5** (1.68 g, 4.4 mmol) were added. The reaction mixture

was stirred overnight at 60 °C. After completion, the solvent was removed under reduced pressure, and the residue was partitioned between DCM and water. The aqueous layer was extracted with DCM twice. The combined organic layers were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was then purified by column chromatography (silica; 40%→100% EtOAc/hexanes). The title compound **6** was obtained as a yellowish solid (2.54 g, 88%). **TLC** (60% EtOAc/ hexanes): Rf =0.36; **^{1}H NMR (400 MHz, $CDCl_3$)** δ 8.03-7.95 (m, 2H), 7.73-7.67 (m, 4H), 7.63-7.59 (m, 2H), 4.61 (bs, 1H), 4.18 (d, J= 2.4Hz, 2H), 3.40 (t, J= 7.2Hz, 2H), 3.32-3.27 (m, 4H), 3.07 (dd, J1= 13.2Hz, J2= 2.8Hz, 2H), 2.20 (t, J= 2.4Hz, 1H), 1.95-1.88 (m, 2H), 1.59-1.52 (m, 2H), 1.48-1.44 (m, 2H), 1.42 (bs, 9H). **^{13}C NMR (101 MHz, $CDCl_3$)** δ 156.11, 148.33, 148.10, 134.05, 133.75, 133.07, 132.29, 131.98, 131.95, 131.01, 130.77, 124.33, 124.31, 79.28, 76.70, 74.43, 47.86, 45.21, 44.72, 39.97, 36.79, 28.51, 27.26, 26.84, 25.74.



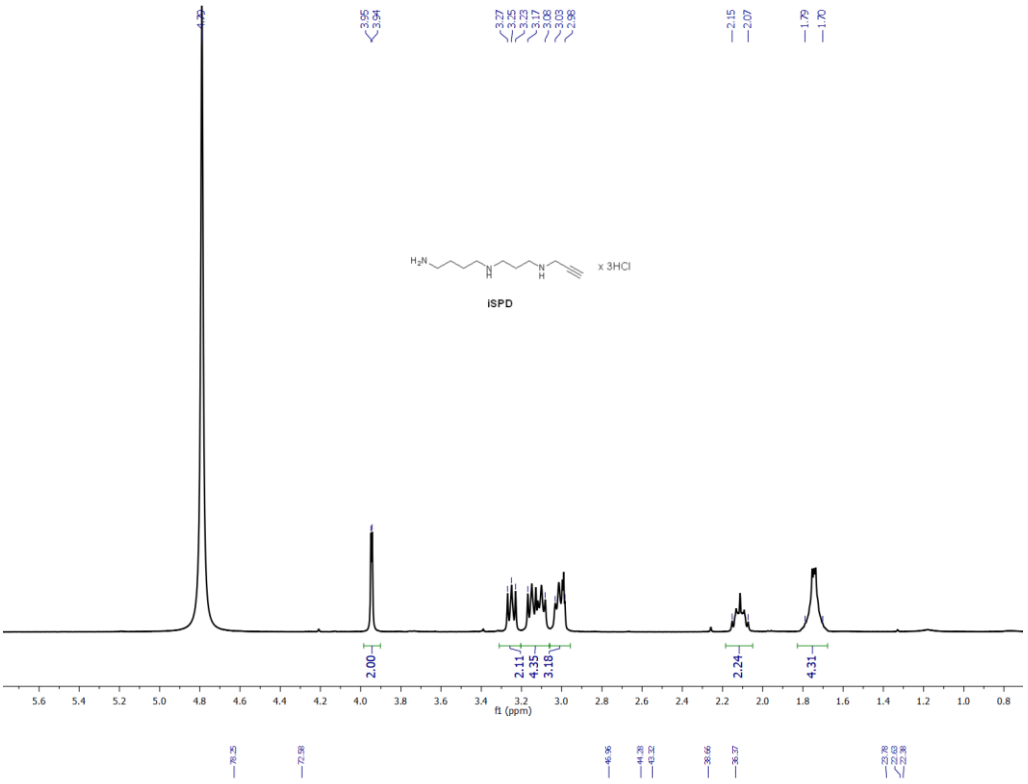
7

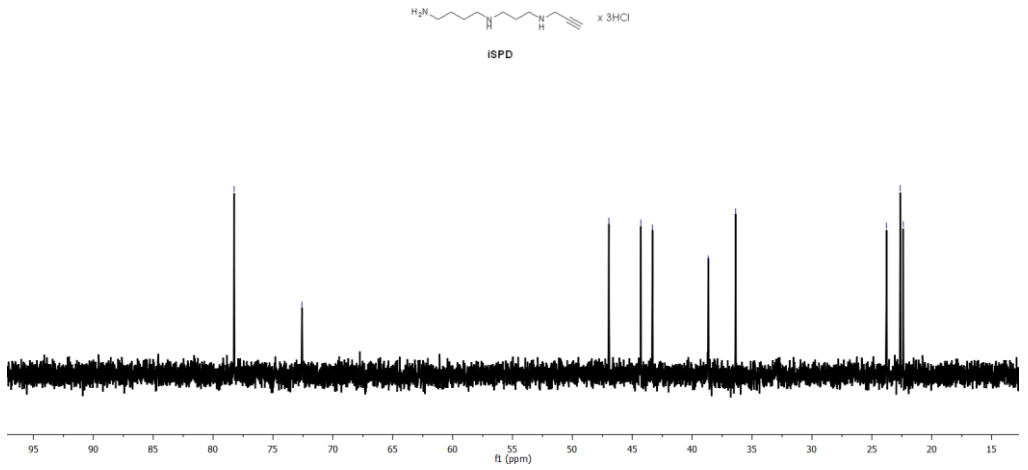
To a solution of **6** (2.54 g, 3.88 mmol) in anhydrous acetonitrile (40 mL), cesium carbonate (4.48 g, 13.76 mmol) and thiophenol (1.4 ml, 1.5 g, 13.76 mmol) were added. The reaction mixture was stirred overnight at 60 °C. After cooling to room temperature, 3.0 g (13.76 mmol) of di-tert-butyl dicarbonate in 20 mL of acetone was added, and the reaction mixture was stirred overnight at room temperature. After completion, the solvent was removed under reduced pressure, and the residue was partitioned between DCM and water. The aqueous layer was extracted with DCM twice. The combined organic layers were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was then purified by column chromatography (silica; 20% EtOAc/hexanes). The title compound **7** was obtained as a yellowish solid (1.45 g, 77%). **TLC** (20% EtOAc/ hexanes): Rf =0.2; **^{1}H NMR (400 MHz, $CDCl_3$)** δ 4.61(bs, 1H), 4.03 (bs, 2H), 3.30 (t, J= 7.2Hz), 3.17-3.10 (m, 6H), 2.20 (s, 1H), 1.82-1.75 (m, 2H), 1.54-1.52 (m, 4H), 1.46-1.43 (m, 27H); **^{13}C NMR (101 MHz, $CDCl_3$)** δ 155.95, 155.49, 154.90, 80.26, 79.37, 46.65, 44.44, 40.23, 28.52, 28.45, 28.35, 27.40.



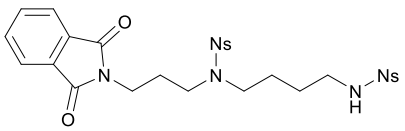
iSPD

Compound **7** (1.45 g, 3.0 mmol) was cooled in an ice-water bath. Then 10 mL of cold 4 M HCl in dioxane was added dropwise, followed by a few drops of Milli-Q water. The reaction mixture was stirred at 0 °C for 1 h and then concentrated under reduced pressure. The residue was resuspended in 3 mL of MeOH and 1 mL of $CHCl_3$ and concentrated under reduced pressure. This washing procedure was repeated three times, reducing the volume of MeOH with each repetition, to yield the dihydrochloride salt of **iSPD** as an off-white solid. The yield was quantitative. **^{1}H NMR (400 MHz, D_2O)** δ 3.94 (d, J= 2.8Hz, 2H), 3.25 (t, J= 8Hz, 2H), 3.17-3.08 (m, 4H), 3.03-2.98 (m, 3H), 2.15-2.07 (m, 2H), 1.78-1.70 (m, 4H).**^{13}C NMR (101 MHz, D_2O)** δ 78.25, 72.58, 46.93, 44.26, 43.31, 38.64, 36.35, 23.76, 22.60, 22.35. **HRMS (m/z)**: $[M+H]^+$ calculated for $C_{10}H_{22}N_3$ 184.1808; found 184.1809





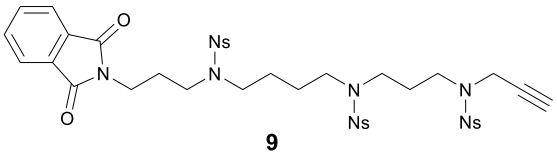
1.4. Preparation of iSPM



8

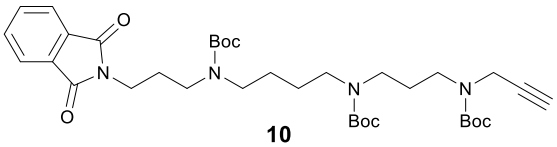
To a solution of tert-butyl (4-((N-(3-(1,3-dioxoisoindolin-2-yl)propyl)-4-nitrophenyl)sulfonamido)butyl)-carbamate[1] (3.04 g, 5.43 mmol) in 15 mL of DCM, trifluoroacetic acid

(10 mL, 15.4 g, 135.0 mmol) was added at 0 °C. The reaction mixture was stirred at room temperature (RT) for 2 h. The solvents were then removed under reduced pressure. The residue was resuspended in DCM (15 mL) and concentrated twice to ensure complete removal of volatile acids. The crude residue was then suspended in 30 mL of DCM, cooled to 0 °C, and triethylamine (5 mL, 3.65 g, 36.0 mmol) was added. After stirring for 10 minutes, 2-nitrobenzenesulfonyl chloride (1.4 g, 6.33 mmol) was added portionwise. The reaction was slowly warmed to RT and stirred overnight. The reaction mixture was then diluted with DCM and washed twice with 1 M HCl. The organic layer was dried over MgSO4, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography (silica; 65% EtOAc/hexanes). The title compound **8** was obtained as a yellowish solid (3.46 g, 98%). **TLC** (80% EtOAc/ hexanes): Rf =0.6; **^{1}H NMR (400 MHz, $CDCl_3$)** δ 8.14-8.11 (m, 1H), 7.96-7.94 (m, 1H), 7.86-7.84 (m, 3H), 7.76-7.71 (m, 4H), 7.70-7.64 (m, 2H), 7.61-7.58 (m, 1H), 5.52 (t, J= 6.4Hz, 1H), 3.68 (t, J= 7.2Hz, 2H), 3.37-3.29 (m, 4H), 3.14 (dd, J1=12.8Hz ,J2=6.4Hz, 2H), 1.93-1.86 (m,2H), 1.69-1.61 (m,2H), 1.60-1.55 (m, 2H); **^{13}C NMR (101 MHz, $CDCl_3$)** δ 168.44, 148.17, 134.25, 133.79, 133.75, 133.73, 133.10, 132.97, 132.08, 131.89, 131.21, 130.76, 125.50, 124.35, 123.53, 47.51, 45.75, 43.24, 35.54, 27.49, 26.69, 25.61.



9

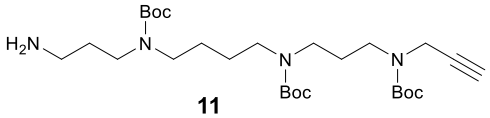
To a solution of **8** (3.48 g, 5.4 mmol) in anhydrous acetonitrile (100 mL), cesium carbonate (2.1 g, 6.44 mmol), tetrabutylammonium iodide (0.2 g, 0.54 mmol), and compound **5** (1.94 g, 5.15 mmol) were added. The reaction mixture was stirred overnight at 60 °C. After completion, the solvent was removed under reduced pressure, and the residue was partitioned between DCM and water. The aqueous layer was extracted with DCM twice. The combined organic layers were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was then purified by column chromatography (silica; 60% → 80% EtOAc/hexanes). The title compound **9** was obtained as a yellowish solid (3.02 g, 63%). **TLC** (60% EtOAc/ hexanes): Rf =0.23; **^{1}H NMR (400 MHz, $CDCl_3$)** δ 8.02-8.00 (m, 1H), 7.98-7.92 (m, 2H), 7.83-7.79 (m, 2H), 7.74-7.66 (m, 7H), 7.65-7.57 (m, 4H), 4.19 (d, J= 2.4Hz, 2H), 3.64 (t, J= 7.2Hz, 2H), 3.41-3.25 (m, 10H), 2.20 (t, J= 2.4Hz, 1H), 1.93-1.84 (m, 4H), 1.53-1.51 (m, 4H).**^{13}C NMR (101 MHz, $CDCl_3$)** δ 168.32, 148.31, 148.06, 148.02, 134.16, 134.14, 134.06, 133.82, 133.12, 133.01, 132.30, 132.07, 132.00, 131.97, 130.98, 130.79, 130.65, 124.33, 124.32, 123.40, 76.78, 74.42, 47.22, 47.03, 45.32, 44.98, 44.66, 36.79, 35.45, 27.44, 26.67, 25.16, 25.05.



10

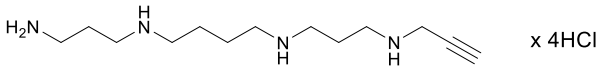
To a solution of **9** (3.02 g, 3.24 mmol) in anhydrous acetonitrile (60 mL), cesium carbonate (3.8 g, 11.66 mmol) and thiophenol (1.5 ml, 1.6 g, 14.72 mmol) were added. The reaction mixture was stirred overnight at 60 °C. After cooling to room temperature, 3.2 g (14.67 mmol) of di-tert-butyl dicarbonate in 20 mL of acetone was added, and the reaction mixture was stirred overnight at room temperature. After completion, the solvent was removed under reduced pressure, and the residue was partitioned between DCM and water. The aqueous layer was extracted with DCM twice. The combined organic layers were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was then purified by column chromatography (silica; 30% EtOAc/hexanes). The title compound **10** was obtained as a yellowish solid (1.37 g, 63%). **TLC** (50% EtOAc/ hexanes): Rf =0.7; **^{1}H NMR (400 MHz,**

$CDCl_3$) δ 7.83-7.81 (m, 2H), 7.71-7.69 (m, 2H), 4.03 (bs, 2H), 3.67(t, J= 7.2Hz, 2H), 3.31-3.17 (m, 10H), 2.20 (bs, 1H), 1.93-1.85 (m, 2H), 1.81-1.74 (m, 2H), 1.47-1.39 (m, 31H).**^{13}C NMR (101 MHz, $CDCl_3$)** δ 168.38, 155.57, 134.07, 132.21, 123.33, 80.33, 79.53, 79.39, 71.57, 46.99, 44.94, 44.59, 35.93, 29.80, 29.46, 28.58, 28.50, 28.05, 27.59, 25.84.



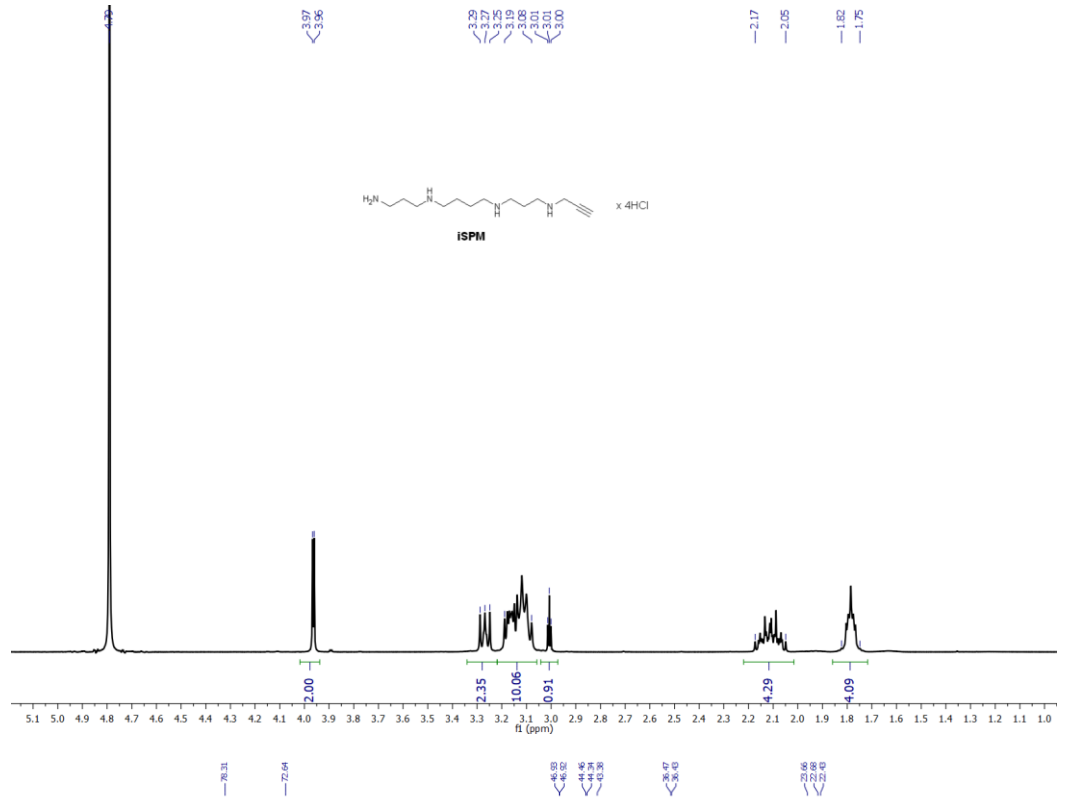
11

To a solution of **10** (1.37 g, 2.04 mmol) in ethanol (50 mL) hydrazine dihydrate (6.0 ml, 6.0 g, 48.7 mmol) was added. The reaction mixture was gently heated overnight at 80 °C in an oil bath. After cooling, the resulting white precipitate was filtered off and washed several times with DCM. The solvent was evaporated under reduced pressure. The residue was then purified by column chromatography (silica; 2% ammonia-saturated methanol/DCM). The title compound **11** was obtained as a yellowish solid (0.79 g, 72%). **TLC** (5% ammonia-saturated methanol/DCM): Rf =0.43; **^{1}H NMR (400 MHz, $CDCl_3$)** δ 4.00 (bs, 2H), 3.77 (bs, 2H), 3.30-3.13 (m, 10H), 2.65 (bs, 2H), 2.18 (bs, 1H), 1.80-1.69 (m, 2H), 1.64-1.57 (m, 2H), 1.43-1.41 (m, 29H).**^{13}C NMR (101 MHz, $CDCl_3$)** δ 155.54, 134.26, 80.31, 79.37, 71.54, 46.74, 44.53, 44.03, 39.28, 32.70, 32.01, 28.55, 28.52, 28.46, 26.09, 25.68.



iSPM

Compound **11** (0.79 g, 1.46 mmol) was cooled in an ice-water bath. Then 5 mL of cold 4 M HCl in dioxane was added dropwise, followed by a few drops of Milli-Q water. The reaction mixture was stirred at 0 °C for 1 h and then concentrated under reduced pressure. The residue was resuspended in 3 mL of MeOH and 1 mL of $CHCl_3$ and concentrated under reduced pressure. This washing procedure was repeated three times, reducing the volume of MeOH with each repetition, to yield the dihydrochloride salt of **iSPM** as an off-white solid. The yield was quantitative**^{1}H NMR (400 MHz, D_2O)** δ 3.97 (d, J= 2.8Hz, 2H), 3.27 (t, J= 8Hz, 2H), 3.19- 3.08 (m, 10H), 3.01 (t, J= 2.8 Hz,1H), 2.17-2.05 (m, 4H), 1.82-1.75 (m, 4H).**^{13}C NMR (101 MHz, D_2O)** δ 78.31, 72.64, 46.93, 46.92, 44.46, 44.34, 43.38, 36.47, 36.43, 23.66, 22.68, 22.43. **HRMS** (m/z): $[M+H]^+$ calculated for $C_{13}H_{29}N_4$ 241.2387; found 241.2386


4.79
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3.19
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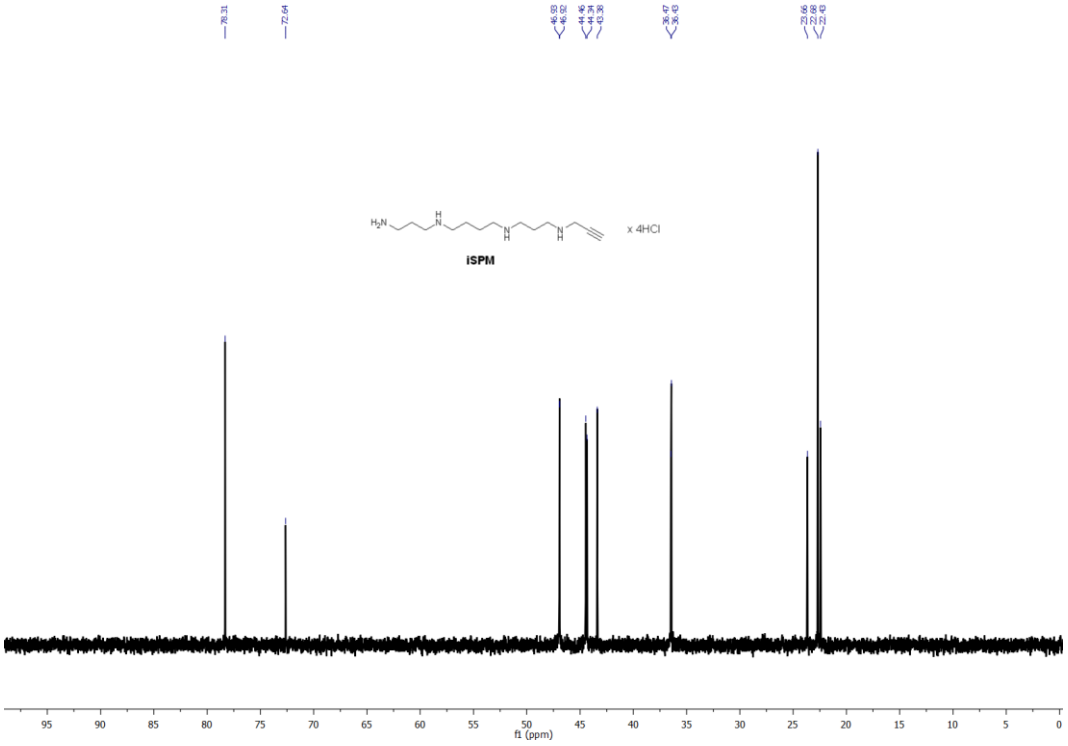
3.01
3.01
3.00
2.17
2.05
1.82
1.75
x 4HCl
iSPM
2.00
2.35
10.06
0.91
4.29
4.09
f1 (ppm)


78.31
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x 4HCl
iSPM
f1 (ppm)

2. Methods: experiments *in cellulo* and *in vivo*

2.1. General

Unless stated otherwise, all reagents were purchased from Sigma Aldrich or Thermo Fisher Scientific. MCF-7 cell line was a kind gift from CRUK Manchester Institute, HeLa were purchased from ATCC. Cells were routinely tested for mycoplasma infection (Universal Mycoplasma Detection Kit, ATCC).

2.2. MCF-7 and HeLa cell culture

MCF-7 and HeLa were cultured in Dulbecco's Modified Eagle Medium (DMEM; Capricorn) supplemented with 10% fetal bovine serum (FBS; Capricorn) and 1% penicillin/streptomycin (P/S, Gibco). The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Routine mycoplasma testing was performed, and all cultures were confirmed to be mycoplasma-free.

2.3. Treatment of cells with iPAs and preparation of samples for imaging

For the confocal-microscope analysis, MCF-7 or HeLa cells were seeded on coverslips in 12-well plate at the density of $0.1x10^5$ cells per well. The following day, complete medium was removed, cells were washed with PBS and incubated with iPAs (25-225 µM) or BODIPY-PAs (5 µM, Sigma-Aldrich) in FBS-free DMEM supplemented with 1% P/S for 0.5-2 h. Negative control cells were incubated with unmodified polyamines (Sigma-Aldrich; concentrations and time matched to iPAs and BODIPY-PAs incubations). For experiments involving inhibition of the polyamine transport system, cells were pre-treated with AMXT-1501 (2 µM, MedChemExpress) in FBS-free media for 1.5 h. After incubation with PAs-probes cells were washed with PBS and fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich) for 10 min at room temperature (RT).

For pulse/chase experiments, cells were incubated with the iPAs-probes for 2 h, followed by PBS washing and incubation in serum-free medium. Cells were then washed with PBS and fixed with 4% PFA at 30, 60, and 120 min post-chase.

For conditioned media experiments, cells were incubated with iPUT (75 µM and 750 µM) for 2 hours. The iPUT-conditioned medium was then transferred to recipient cells for an additional 2 h. Next, cells were washed with PBS and fixed with 4% PFA.

2.4. Click reaction

Following fixed with PFA, cells were permeabilized with 0.05% Triton X-100 (Sigma-Aldrich) in PBS at RT for 3 min. Subsequently, cells were incubated at RT for 1 h with a click reaction mixture containing 0.1 mM TAMRA-5-azide (MedChemExpress), 1mM $CuSO_4$, 1mM tris(2-carboxyethyl)phosphine (TCEP; Thermo Fisher Scientific), and 0.2 mM tris[(1-hydroxypropyl)-1H-1,2,3-triazol-4-yl)methyl]amine (THPTA; Ambeed) in PBS. Finally, cells were washed twice with PBS.

2.5. Immunofluorescence

Potential nonspecific antibody binding was blocked by incubating cells in 3% bovine serum albumin (BSA) in PBS for 30 min at RT. Cells were then incubated overnight at 4 °C with the primary anti-TOM20 antibody (Proteintech, #11802-1-AP; 1:250) diluted in 0.5% BSA in PBS. Subsequently, cells were washed twice with PBS and incubated with Alexa Fluor 488-conjugated secondary antibody (Invitrogen, #A-11008; 1:1000) diluted in 0.5% BSA in PBS for 1 h at RT. Cells were washed twice with PBS and samples were mounted using a fluorescence mounting medium containing DAPI for nuclear

staining (Invitrogen). The specificity of the primary antibody was verified by performing control incubations with the secondary antibody only.

2.6. Fluorescent microscopy imaging of iPAs in PFA-fixed cells

Samples were imaged using a Zeiss LSM910 confocal microscope. Fluorescence images were processed and analyzed in FIJI (ImageJ).[2] Raw image files were imported, and all channels were adjusted to identical exposure settings for both experimental and control groups to ensure consistency in fluorescence intensity comparisons. Brightness and contrast were modified uniformly across all images and channels within each dataset. Using ImageJ software, the percentage of positively associated (colocalized) pixels from analyzed polyamines in MCF-7 or HeLa cells from randomly selected fields of view were determined. A Person R-value close to 1 indicates strong colocalization of signals, close to 0 weak colocalization of signals. Each analysis was performed at least in three independent biological repeats. Statistical analyses were conducted using GraphPad Prism (GraphPad Software). Student's *t*-test was applied to determine statistical significance between groups, with differences considered significant at $p < 0.05$. Pearson correlation coefficients were calculated using the Coloc2 plugin in FIJI.

2.7. Copper-catalyzed azide-alkyne-thiol conjugation, enrichment of thio-triazole-biotin-labeled peptides, and proteomic analysis

The complete medium was removed, cells were washed with PBS and incubated with iPAs (75 µM) in FBS-free DMEM supplemented with 1% P/S for 2 h. Then cells were washed with PBS (twice), lysed in 0.5% SDS in PBS, lysates were homogenized with a probe sonicator, and protein concentration was determined by DC Protein Assay (Bio-Rad). A click reaction mixture was prepared by combining 4 reagents (volumes given per 100 µg of proteins): 5-TAMRA-azide (Jena Bioscience, 1 µl, stock 10 mM in DMSO, final conc. 0.1 mM) or azido-PEG_3-biotin (Thermo Fisher Scientific, 1 µl, stock 20 mM in water, final conc. 0.2 mM), $CuSO_4$ (2 µl, stock 50 mM in water, final conc. 1 mM), tris(2-carboxyethyl)phosphine (TCEP) (2 µl, stock 50 mM in water, final conc. 1 mM), tris((1-hydroxy-propyl-1H-1,2,3-triazol-4-yl)methyl)amine (THPTA) (1 µl, stock 20 mM in water, final conc. 0.2 mM). Click mixture (6 µl) was added to protein samples (100 µg). The samples were vortex-mixed at RT for 1 h. EDTA (final conc. 5 mM), methanol (1 vol.), and chloroform (1/4 vol.) were added to each sample. The samples were quickly vortexed and centrifuged at 21,000 *g* for 5 min to pellet precipitated proteins. The pellets were washed with methanol twice and dried. The pellet was suspended in 100 mM HEPES pH 8.0 containing 5 mM TCEP and 10 mM chloroacetamide. Sequencing Grade Modified Trypsin (Promega) was added and the samples incubated overnight at 37 °C with 1100 RPM shaking. EDTA-free Protease Inhibitor Cocktail (1x, Roche) was added to quench residual trypsin activity. NeutrAvidin agarose resin was washed with 100 mM HEPES (3×). The tryptic peptide samples were centrifuged and clear supernatants added to the beads. The enrichment was carried out for 2 h at RT with 1100 RPM shaking. The supernatants were removed and the beads were washed with 100 mM HEPES (3×), with water (2×), and with 10% acetonitrile in water (2×). Probe-modified peptides were eluted at 37 °C with 0.1% trifluoroacetic acid and 1% formic acid in water (2×15 min). Combined eluates were concentrated using a SpeedVac concentrator. The samples were pre-cleaned using C18 StageTips and resuspended in 0.1% TFA and 2% acetonitrile in water prior to the LC-MS/MS measurements. Chromatographic separation was performed on an Easy-Spray Acclaim PepMap column (50 cm length × 75 µm inner diameter, Thermo Fisher Scientific) at 55 °C by applying 90 min acetonitrile gradients in 0.1% aqueous formic acid at a flow rate of 300 nl/min. An UltiMate 3000 nano-LC system was coupled to a Q Exactive HF-X mass spectrometer via an easy-spray source (all Thermo Fisher Scientific). The Q Exactive HF-X

was operated in TMT mode with survey scans acquired at a resolution of 60,000 at m/z 200. Up to 15 of the most abundant isotope patterns with charges 3-6 from the survey scan were selected with an isolation window of 1.3 m/z and fragmented by higher-energy collision dissociation with normalized collision energies of 27, while the dynamic exclusion was set to 35 s. The maximum ion injection times for the survey scan and dual MS (MS/MS) scans (acquired with a resolution of 15,000 at m/z 200) were 45 and 150 ms, respectively. The ion target value for MS was set to 3e6 and for MS/MS was set to 1e5, and the minimum AGC target was set to 1e3. The data were processed using FragPipe platform v22.0. Modified and unmodified peptides were identified from the MS/MS spectra searched against Human UniprotKB database,[3] using the Open Search workflow of the MSFragger search engine.[4] For peak matching, precursor mass tolerance range (-150, 750) Da was selected, fixed modifications were not set, other MSFragger parameters were default. The global.modsummary output was imported into Microsoft Excel 2016 and filtered as follows: (i) mass shifts < 445 Da - the molecular weight of the biotinylated capture reagent - were removed, except for "unmodified" (None) and Formylation; (ii) within each sample, mass shifts with a peptide-spectrum match (PSM) count < 5% of the PSM count for the intact probe were removed.

2.8. Breeding of adult zebrafish and culturing embryos

The *casper* line (mitfa$^{w2/w2}$; mpv17$^{a9/a9}$) was used.[5] Adult zebrafish were bred at the Zebrafish Core Facility (International Institute of Molecular and Cell Biology, Warsaw, Poland) according to international standards. Embryos were collected and kept in E3 solution (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) in 28.5 °C on Petri dishes. Offspring of at least two parental pairs were used in each experiment. Zebrafish larvae used in this study were 5 days post-fertilization (dpf) and had no specified sex yet. Zebrafish used for experiments were up to 120 hours post-fertilization (hpf), a developmental stage prior to the onset of independent feeding at which they are not classified as protected animals under current European and Polish legislation (Directive 2010/63/EU and corresponding national regulations), and therefore did not require formal approval by an institutional animal ethics committee

2.9. Feeding of *D. rerio* larvae with iPAs and sample preparation for imaging

On 2 dpf embryos were dechorionized and equal number of embyos were placed on a 6-well plate in 4 ml of E3 solution containing 200 µM iPAs. On 5dpf each group of larvae were euthanized with Tricaine and fixed in 4% PFA overnight at 4 °C. The following day, the larvae were washed with PBS, and permeabilized by subsequent addition of: PBS + 0.2% TX100; PBS + 20% DMSO + 0.2% TX100; PBS + 20% DMSO + 0.2% TX100 + 0.1% Tween20; and PBS + 0.2% TX100. The larvae were incubated at 4 °C for 36 h with a click reaction mixture containing 0.1 mM TAMRA-5-azide, 1mM $CuSO_4$, 1mM TCEP, and 0.2 mM THPTA in PBS. The larvae were washed extensively with PBS, subjected to a glycerol gradient (30%, 50%, 70%) and mounted in 2% propyl gallate (in glycerol) and then left in -4 °C covered from light ready for imaging.

2.10. Fluorescent microscopy imaging of iPA in PFA-fixed *D. rerio* larvae

Images were acquired using a Zeiss Lightsheet Z.1 microscope (20x water immersion objective, NA = 1.0) at 1024 x 1024 pixel-resolution. Z-stacks of the images were taken with an optimal interval (approximately 0.5 µm).

3. Fig. S1-11

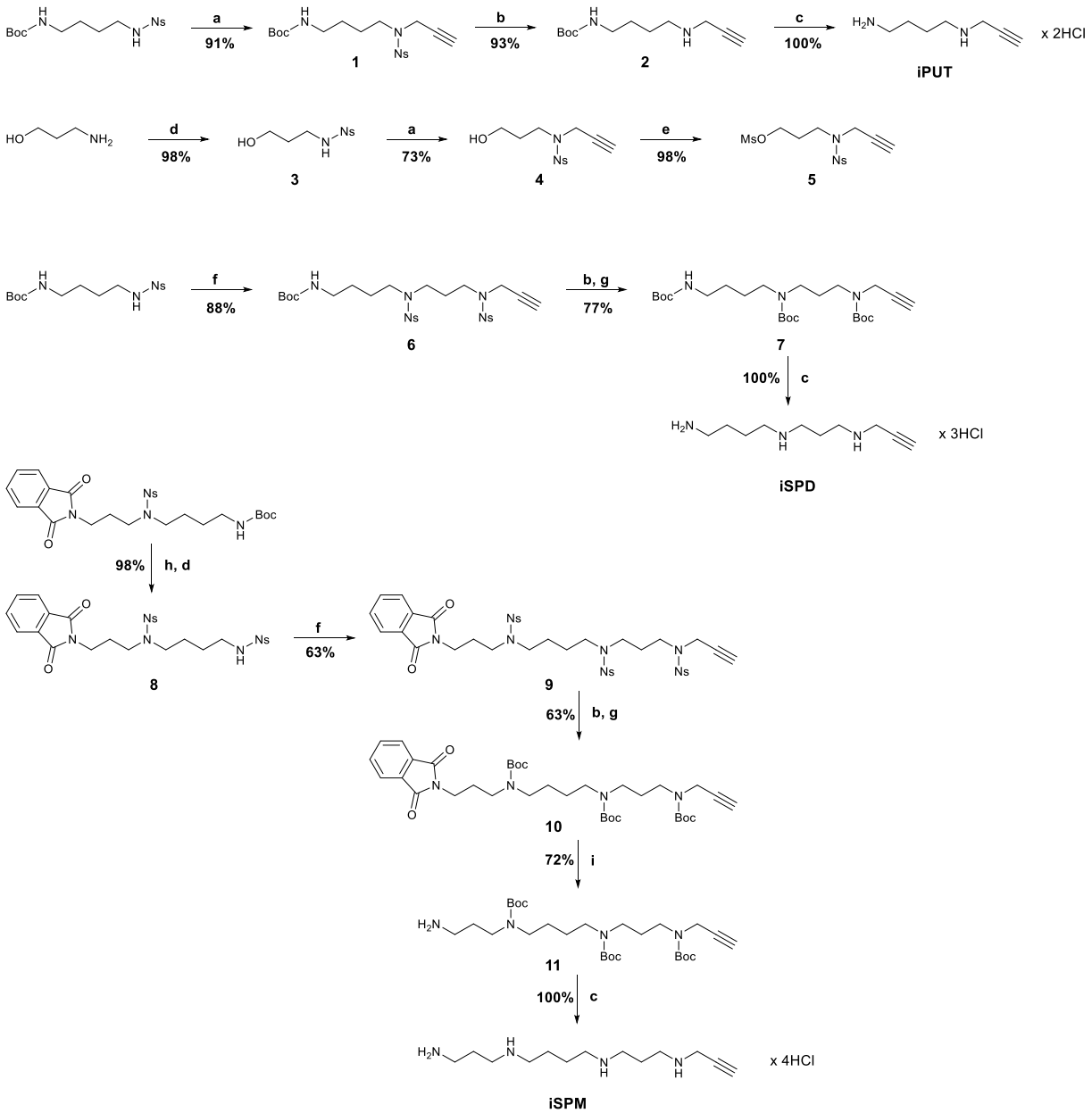


Fig. S1. Synthetic pathway to iPA. Reagents: (a) propargyl bromide, Cs_2CO_3, TBAI; (b) Cs_2CO_3, thiophenol (c) 4M HCl/Dioxane; (d) NsCl, Et_3N; (e) MsCl, Et_3N; (f) Cs_2CO_3, **5**, TBAI; (g) $(Boc)_2O$; (h) TFA; (i) N_2H_4.

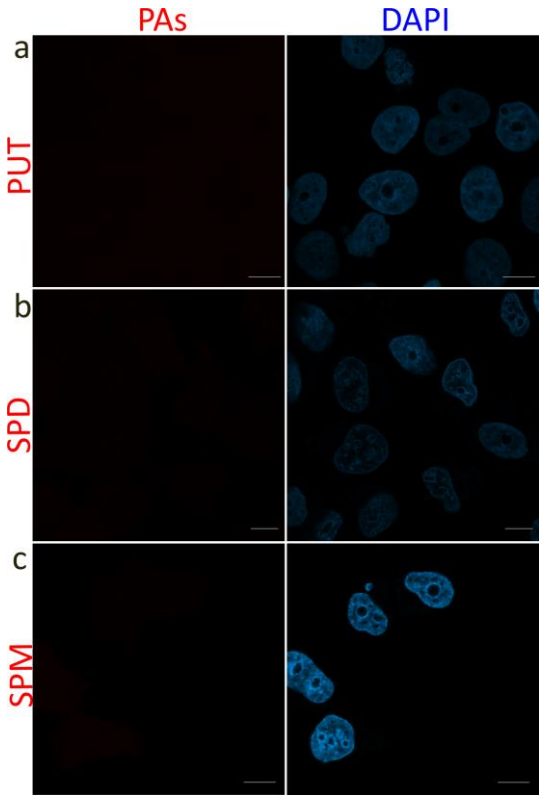


Fig. S2. Negative control for cells treated with iPAs (depicted in Fig. 2). MCF-7 cells were treated with PAs (75 µM) for 2 h, washed, fixed with PFA, and subjected to bioorthogonal ligation with 5-TAMRA-azide. Nuclei were stained with DAPI (blue) and imaged by fluorescence microscopy. No detectable fluorescence was observed in cells treated with PAs and processed for CuAAC. (a) PUT-treated cells. (b) SPD-treated cells. (c) SPM-treated cells. Scale bars: 10 µm.

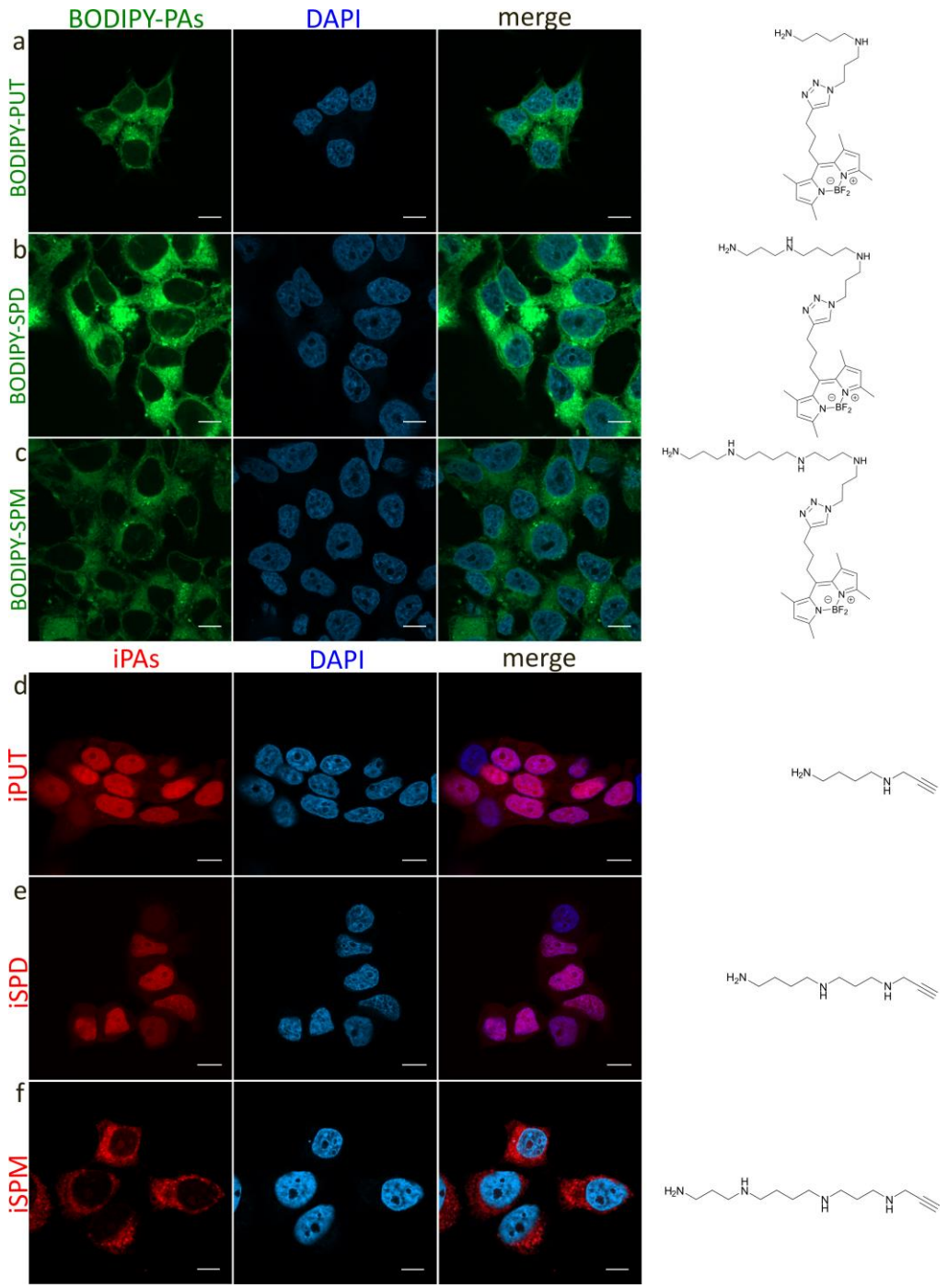


Fig. S3. Subcellular localization of BODIPY-PAs (green) and iPAs (red, images corresponding to the fields of view in Fig. 2) in MCF-7 cells. Cells were treated with BODIPY-PAs (5 µM) or with iPAs (75 µM) for 2 h, washed and PFA-fixed. Cells treated with iPAs were subsequently subjected to bioorthogonal ligation with 5-TAMRA-azide. Nuclei were stained with DAPI (blue) prior to visualization by fluorescence microscopy. (a) Cells treated with BODIPY-PUT. (b) Cells treated with BODIPY-SPD. (c) Cells treated with BODIPY-SPM. (d) Cells treated with iPUT. (e) Cells treated with iSPD. (c) Cells treated with iSPM. Scale bars: 10 µm. No detectable fluorescence signal was observed in cells incubated without BODIPY-PAs (data not shown). **Note:** The chemical structures of the commercial BODIPY-PAs were deduced from the molecular formulas provided by the supplier (who, under the applicable regulations, is not obliged to disclose the full chemical structures) and from the literature references cited by the supplier.

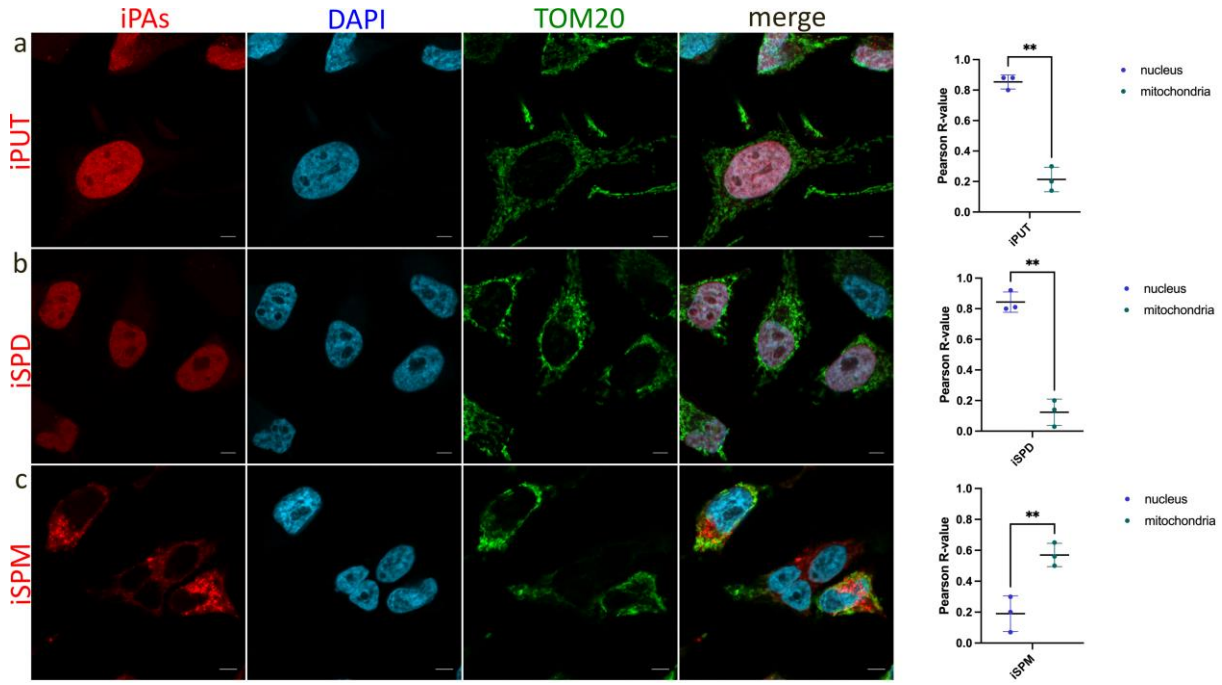
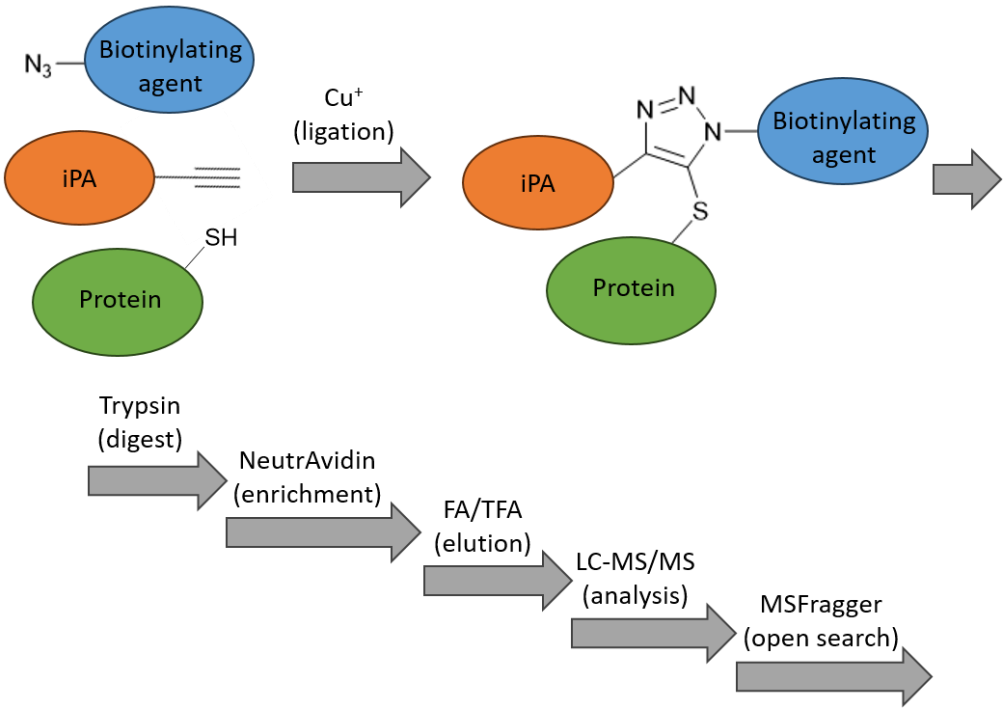


Fig. S4. Subcellular accumulation of iPAs in HeLa cells, colocalization of iPAs with the mitochondrial marker TOM20 (green) and cell nuclei (blue). Cells were treated with iPAs (75 µM) for 2 h, washed, fixed with PFA, and subjected to bioorthogonal ligation with 5-TAMRA-azide (red). Cells were immunostained with a primary anti-TOM20 antibody followed by an Alexa Fluor 488-conjugated secondary antibody. Nuclei were stained with DAPI (blue) and imaged by fluorescence microscopy. (a) Cells treated with iPUT. (b) Cells treated with iSPD. (c) Cells treated with iSPM. Scale bars, 5 µm. Pearson correlation coefficients were calculated using the Coloc2 plugin in FIJI. Student's t-test was applied to determine statistical significance between groups, with differences considered significant at $p < 0.05$. No detectable fluorescence was observed in cells incubated without iPAs and processed for CuAAC (not shown).

a



b

Modification	Mass Shift [Da]	iPUT_s1 PSMs	iPUT_s2 PSMs	iSPD_s1 PSMs	iSPD_s2 PSMs	iSPM_s1 PSMs	iSPM_s2_PSMs
iSPM	682.4322	0	0	3	1	1291	1341
iSPD + Formylation	653.3672	2	2	482	456	3	5
iSPD	625.3734	1	2	2411	2319	48	50
iPUT + Formylation	596.3108	606	550	6	11	2	1
iPUT	568.3158	2795	2579	125	128	3	2
Formylation	27.994915	69	62	61	58	71	68
None	0	1885	1871	1989	1954	2118	2216

Fig. S5. Mass spectrometry-based assay to assess iPA interconversion. (a) Workflow following incubation of MCF-7 cells with iPAs and lysis: azide-PEG3-biotin and Cu(I) were supplemented to initiate the copper-catalyzed azide-alkyne-thiol reaction; proteins were digested with trypsin; thio-triazole-biotin-labeled peptides were enriched on NeutrAvidin resin, eluted with acetonitrile/water containing formic acid (FA) and trifluoroacetic acid (TFA), analyzed by LC-MS/MS, and queried with MSFragger (open search) to detect mass shifts on cysteine-containing peptides. (b) Open-search results show cysteine mass shifts consistent with incorporation of the intact probe; no detectable iPA interconversion is evident in this assay. Observed formylated adducts are attributed to an artifact from overnight SpeedVac evaporation at 45 °C in the presence of FA. Unmodified peptides (“None”), nonspecifically bound to the resin and subsequently eluted, serve as a loading control, indicating comparable peptide input across samples.

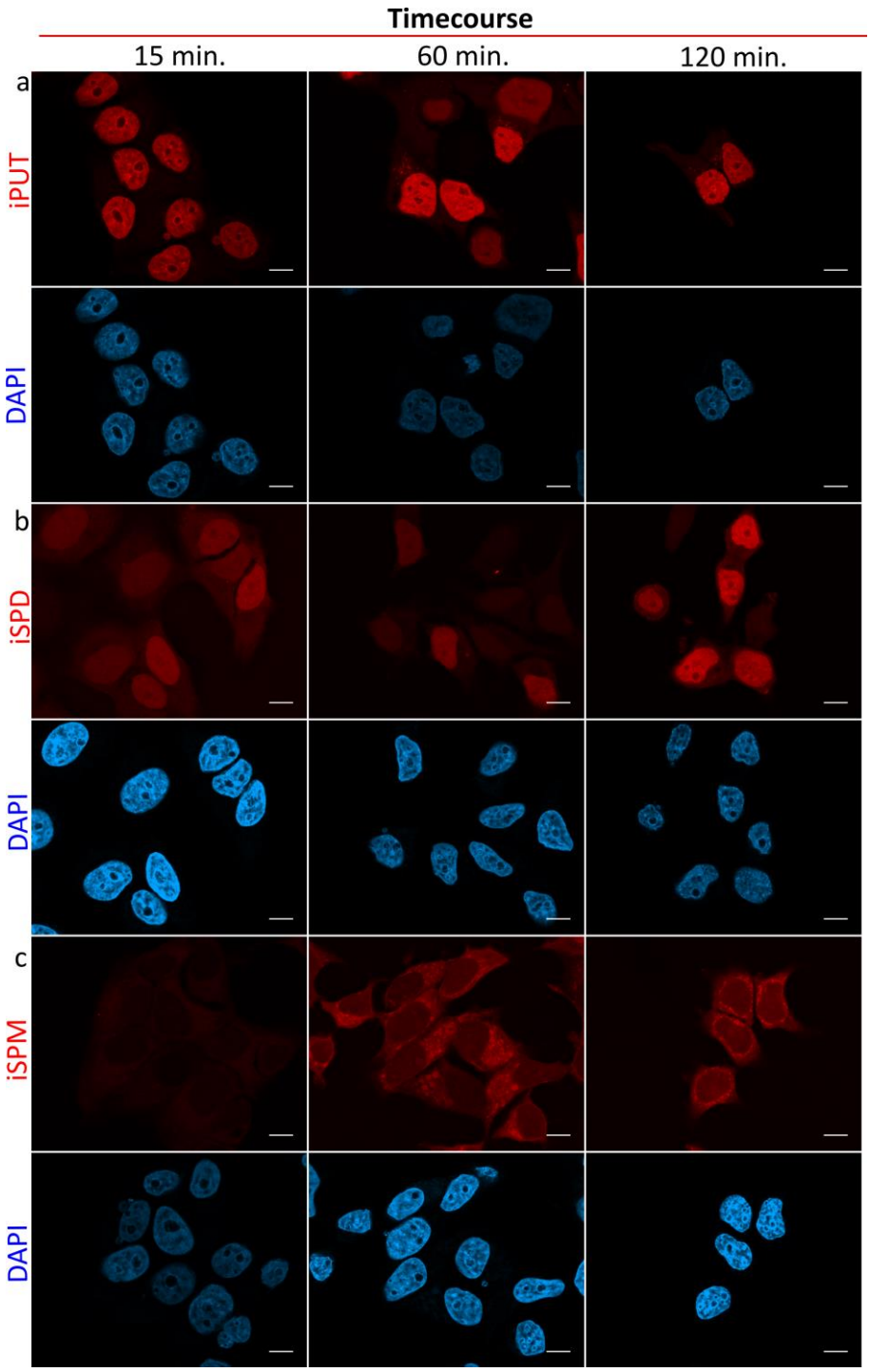


Fig. S6. Time-dependent accumulation of iPAs in MCF-7 cells. Cells were treated with iPAs (75 µM) for 15, 60, and 120 min, washed, fixed with PFA, and subjected to bioorthogonal ligation (CuAAC) with 5-TAMRA-azide (red). Nuclei were counterstained with DAPI (blue) and imaged by fluorescence microscopy. (a) iPUT-treated cells. (b) iSPD-treated cells. (c) iSPM-treated cells. Scale bars: 10 µm. No detectable fluorescence was observed in cells incubated without iPAs and processed for CuAAC (not shown).

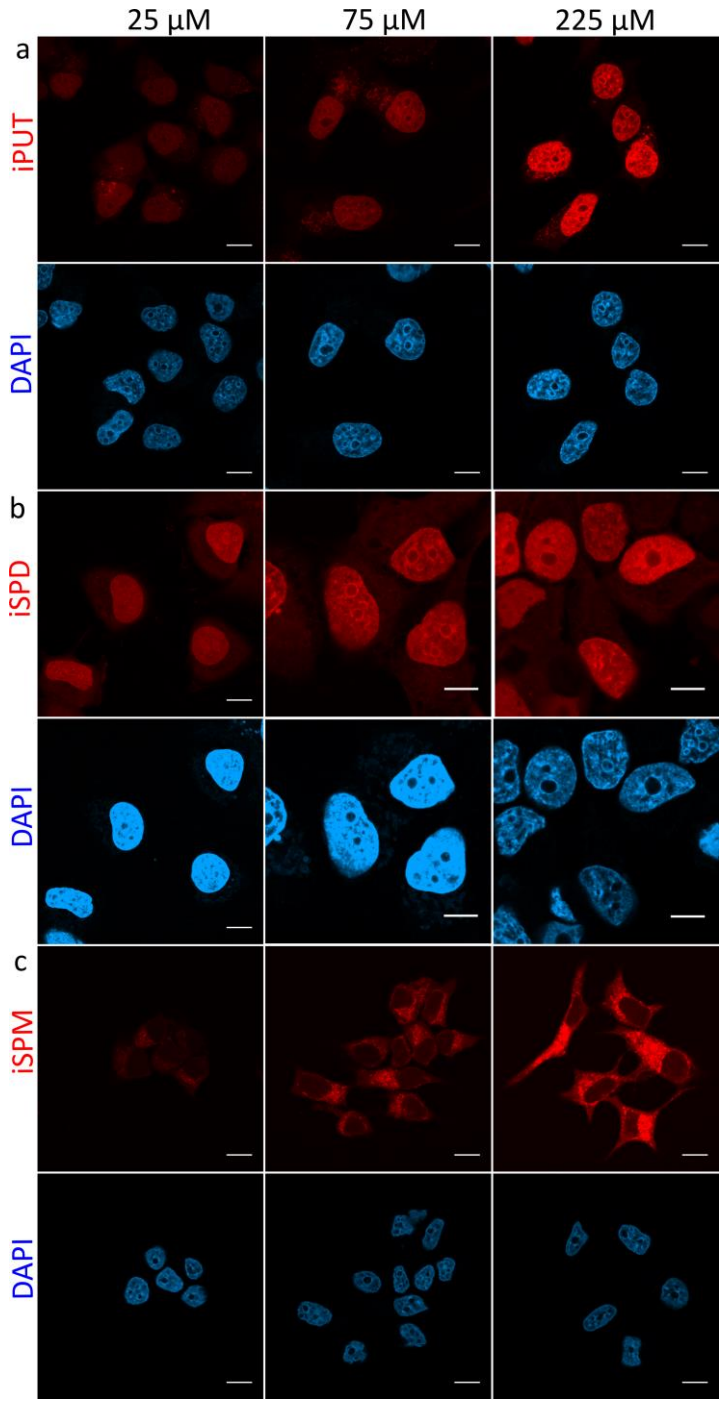


Fig. S7. Dose-dependent accumulation of iPAs in MCF7 cells. Cells were treated with iPAs (25, 75 or 225 µM) for 2 h, washed, fixed with PFA, and subjected to bioorthogonal ligation (CuAAC) with 5-TAMRA-azide (red). Nuclei were stained with DAPI (blue) and imaged by fluorescence microscopy. (a) iPUT-treated cells. (b) iSPD-treated cells. (c) iSPM-treated cells. Scale bars: 10 µm. No detectable fluorescence was observed in cells incubated without iPAs and processed for CuAAC (not shown).

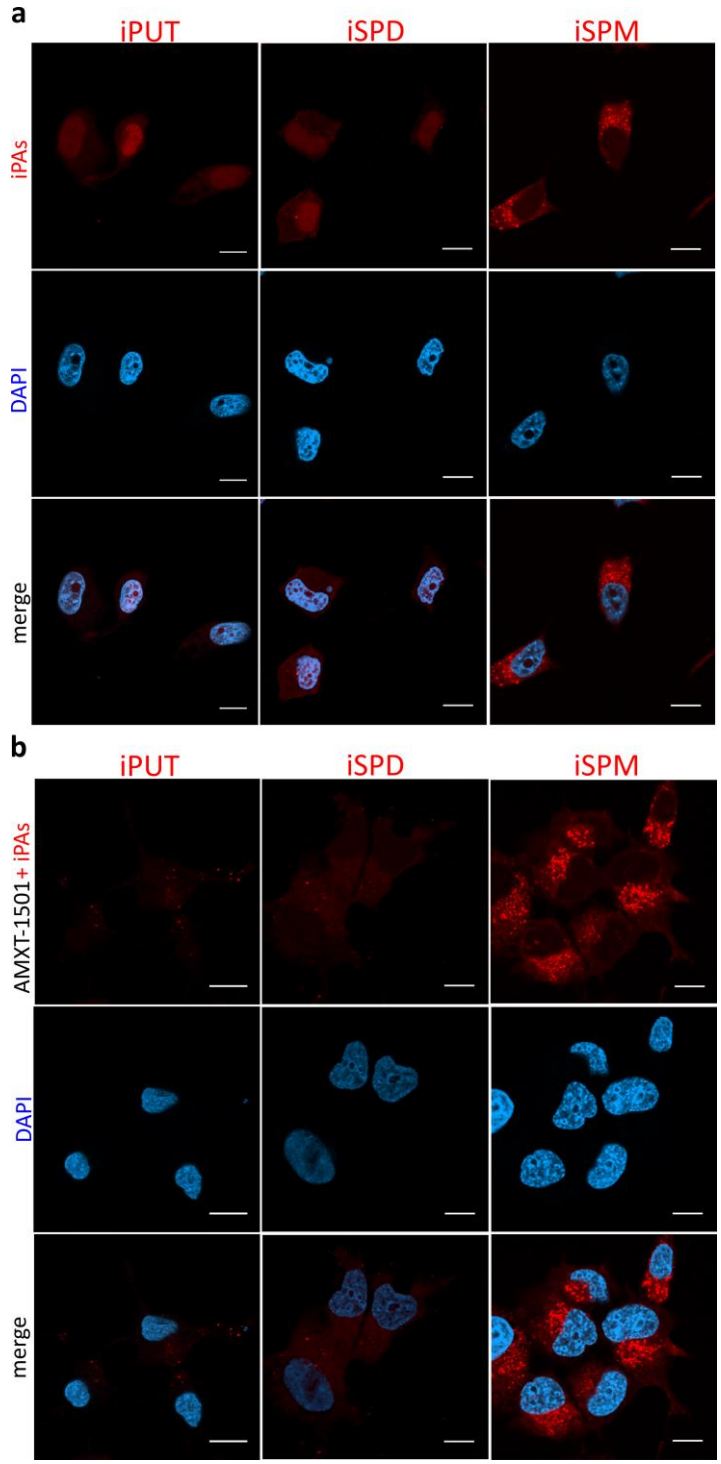


Fig. S8. Fluorescence microscopy images of DAPI-stained nuclei corresponding to the fields of view shown in Fig. 3. (a) MCF-7 cells treated with iPAs. (b) Cells pretreated with the polyamine transport inhibitor AMXT-1501 followed by incubation with iPAs. Cells were pretreated with AMXT-1501 (2 µM) or vehicle for 1.5 h, then incubated with iPAs (75 µM) for 2 h, washed, fixed with paraformaldehyde (PFA), and subjected to bioorthogonal ligation (CuAAC) with 5-TAMRA-azide (red). Nuclei were stained with DAPI (blue) and imaged by fluorescence microscopy. Scale bars: 10 µm. No detectable fluorescence was observed in cells incubated without iPAs and processed for CuAAC (not shown).

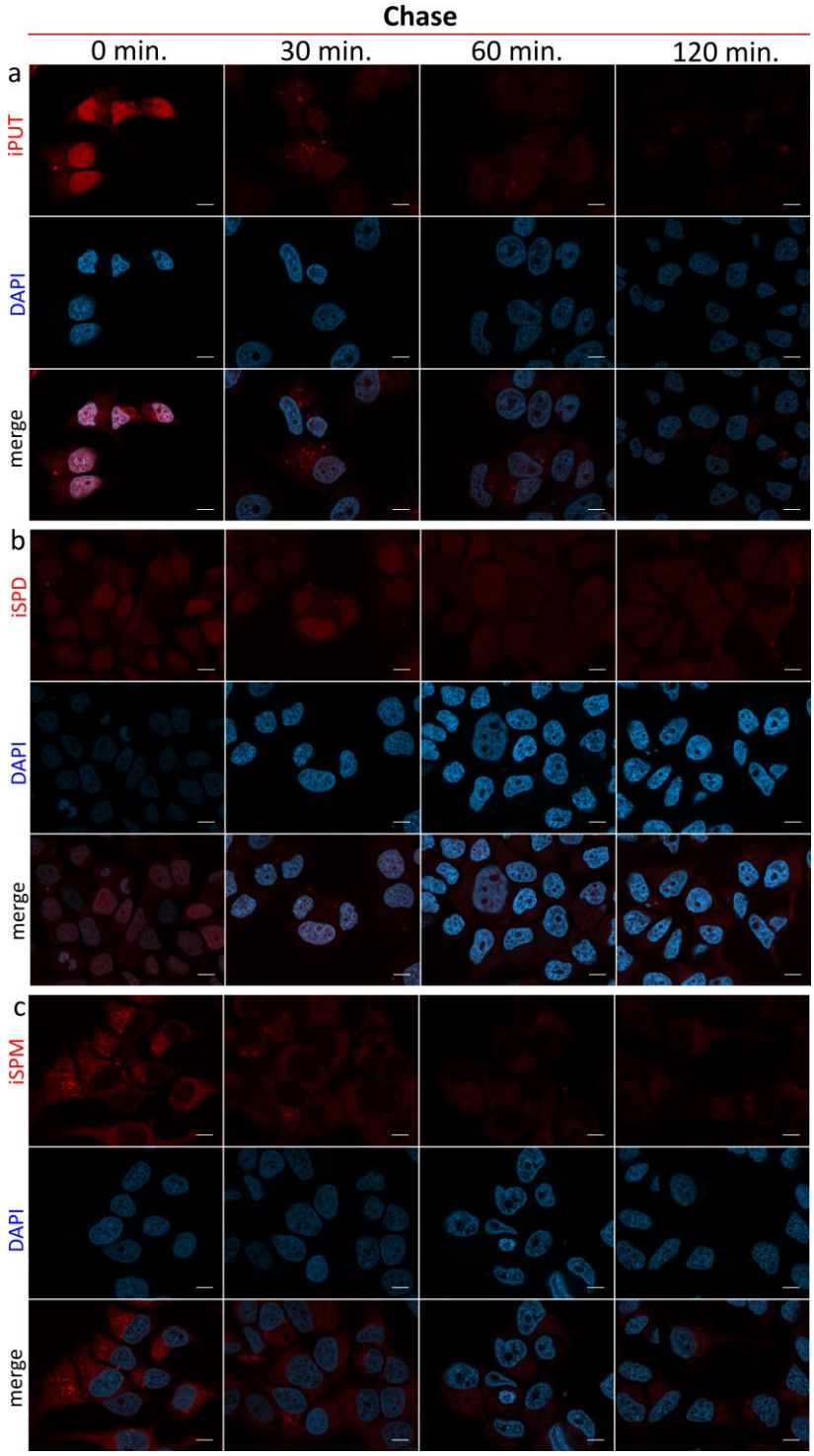


Fig. S9. Fluorescence microscopy images of DAPI-stained nuclei corresponding to the fields of view shown in Fig. 4. MCF-7 cells were incubated with iPAs (75 µM) for 2 h, washed, and then incubated in iPA-free medium for 0, 30, 60, or 120 min. Cells were washed, PFA-fixed and subjected to bioorthogonal ligation (CuAAC) with 5-TAMRA-azide (red). Nuclei were stained with DAPI (blue) and imaged by fluorescence microscopy. Panels: (a) iPUT, (b) iSPD, (c) iSPM. Scale bars: 10 µm. No detectable fluorescence was observed in cells incubated without iPAs and processed for CuAAC (not shown).

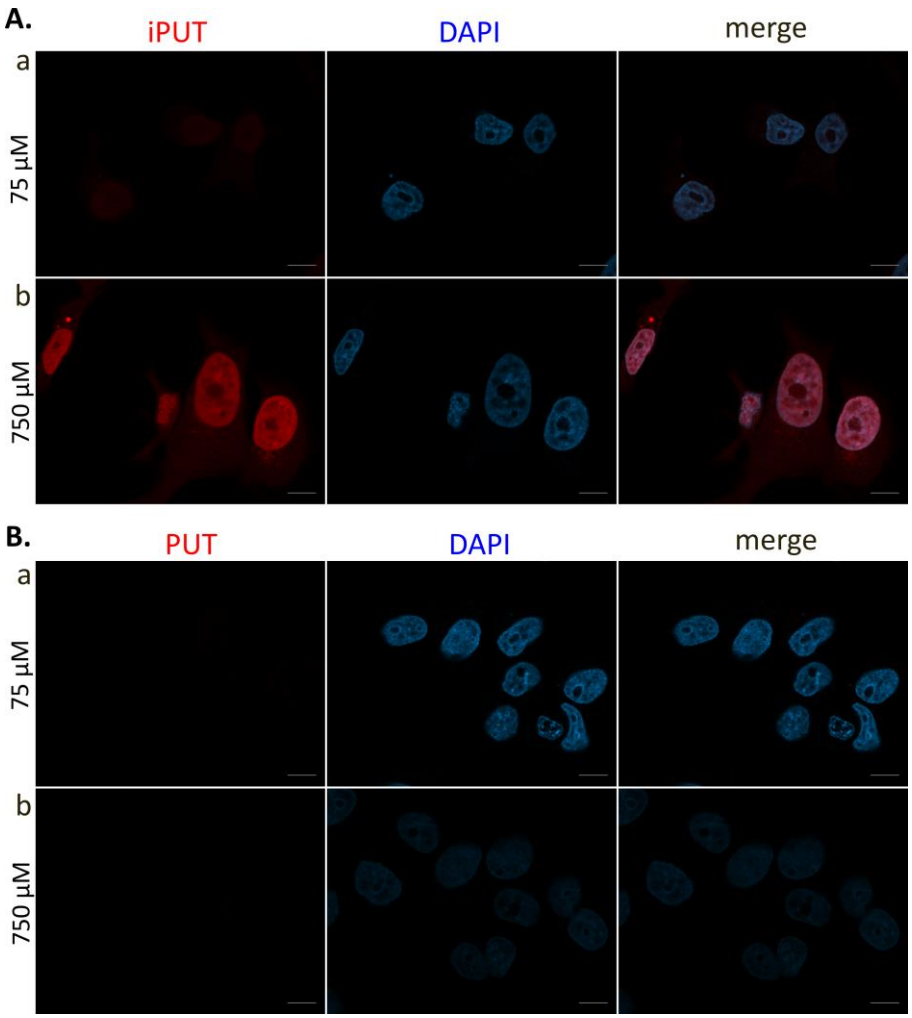


Fig. S10. Uptake of iPUT from conditioned medium in MCF-7 cells. Donor MCF-7 cells were incubated with iPUT (75 or 750 µM; A) or PUT (75 or 750 µM; B) for 2 h, washed, and then incubated for an additional 2 h in additive-free medium to generate conditioned media. The resulting iPUT- or PUT-conditioned media were transferred to recipient MCF-7 cells for 2 h. Cells were washed, PFA-fixed, and subjected to bioorthogonal ligation (CuAAC) with 5-TAMRA-azide (red). Nuclei were stained with DAPI (blue) and imaged by fluorescence microscopy. Panels: A - iPUT-conditioned medium: (a) 75 µM, (b) 750 µM; B - PUT-conditioned medium: (a) 75 µM, (b) 750 µM. Scale bars: 10 µm.

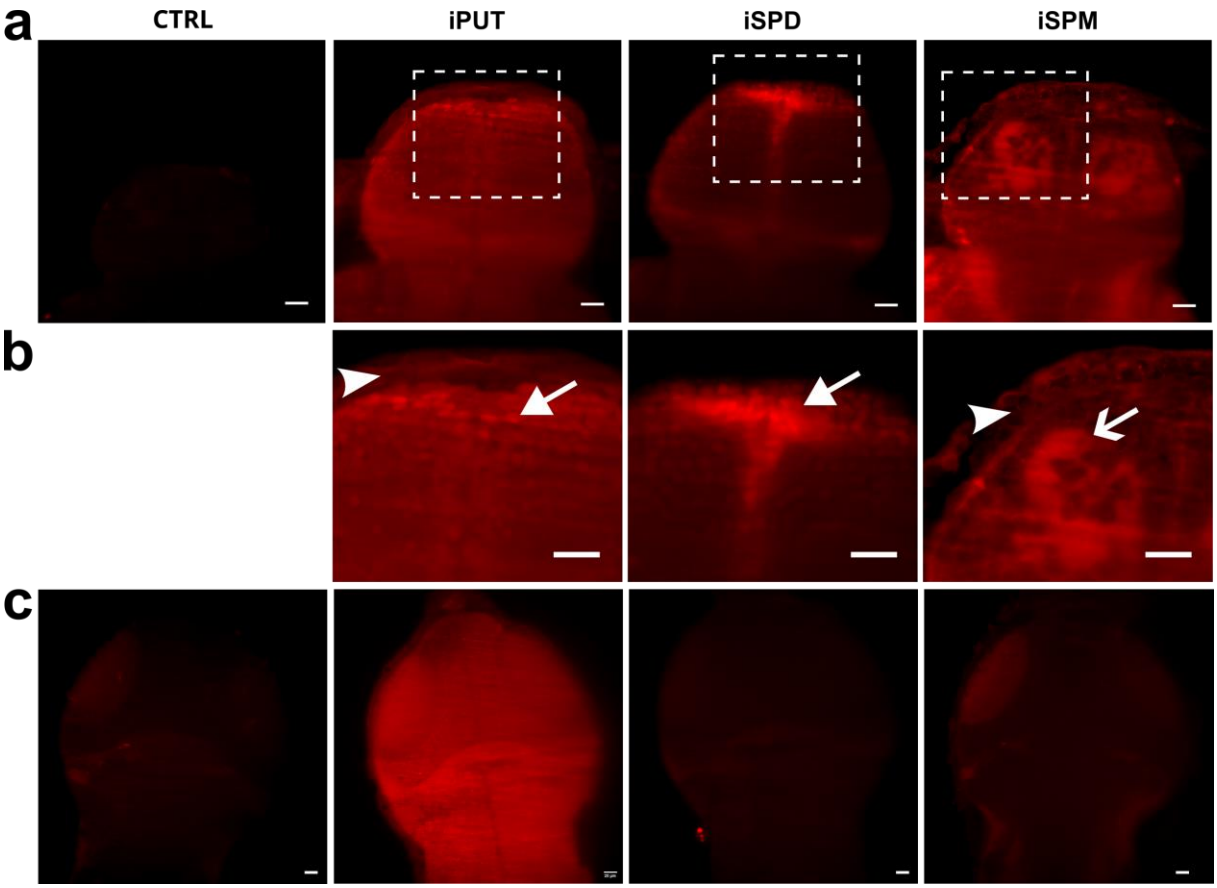


Fig. S11. Differential accumulation of iPAs in the brain of the developing D. rerio larvae. This figure shows the content of Fig. 5 extended by negative control images (CTRL) obtained for zebrafish embryos treated with vehicle control (H_2O). The embryos were treated (48-120 hpf) with the probes (iPUT, iSPD, iSPM, at 200 µM) or vehicle control (CTRL), washed, euthanized, PFA-fixed, and subjected to bioorthogonal ligation to 5-TAMRA-azide (red). (a-b) Localization of iPAs in the pallium (dorsal forebrain) and surrounding skin. iPUT localizes to the nuclei of pallial cells (arrow) and cytoplasmically in the skin cells (arrowhead). iSPD localizes to the nuclei, is strongly enriched in the dividing cells at ventricular zones (arrow), and is absent from skin cells. iSPM localizes cytoplasmically in both, skin cells (arrowhead) and brain cells and is enriched in white matter (open arrow). (c) Localization of iPAs in the optic tectum and cerebellum. iPUT localizes to the nuclei of brain cells. iSPD is absent in the tectum. iSPM localizes cytoplasmically in brain cells of the optic tectum and is enriched in white matter. Scale bars: 20 µm.

4. References

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