Supplementary Information for

Classical thiazole orange and its regioisomer as fluorogenic probes for nucleolar RNA imaging in living cells

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Experimental

General

All of the synthetic RNAs and DNAs were custom synthesized and HPLC purified by GeneDesign Inc. (Osaka, Japan) and Nihon Gene Research Laboratories Inc. (Miyagi, Japan), respectively. Calf thymus DNA and *E. coli* total RNA were purchased from Sigma-Aldrich (Darmstadt, Germany) and Thermo Fisher Scientific (Tokyo, Japan), respectively. The other reagents were commercially available and of analytical grade. NMR spectra were recorded on a Bruker Avance III 500 spectrometer. High-resolution mass spectra (HRMS) were recorded on a JEOL JMS-T100CS or JMS-T100GCV instrument. The concentrations of calf thymus DNA and *E. coli* total RNA were determined from the absorbance at 260 nm at 25°C using a molar absorption coefficient of 6600 $M^{-1}cm^{-1}$ and 9250 $M^{-1}cm^{-1}$ (M = mol/L per nucleotide), respectively.[S1] The concentrations of synthetic RNAs were determined from the absorbance at 260 nm measured at 80˚C, using the molar extinction coefficient provided by the manufacturer. The concentrations of synthetic DNAs were determined according to the literature.[S2] Water was deionized (≥18.0 MΩ cm specific resistance) by an Elix 5 UV water purification system and a Milli-Q Synthesis A10 system (Millipore Corp., Bedford, MA, USA), followed by filtration through a BioPak filter (Millipore Corp., Bedford, MA, USA) in order to remove RNase.

Unless otherwise mentioned, TO derivatives were dissolved in DMSO to obtain the stock solutions. The stock solutions were kept at 4°C in the dark before their use. Final DMSO concentration in sample solutions containing the probes was below 0.1 % (v/v).

UV-visible and fluorescence spectra measurements

Absorption and fluorescence spectra were measured using a JASCO model V-570 UV–vis sectrophotometer and FP-6500 spectrofluorophotometer (Japan Spectroscopic Co. Ltd., Tokyo, Japan), respectively. Both instruments were equipped with thermoelectrically temperature-controlled cell holders. Measurements of absorption and fluorescence spectra were done using a 2 × 10 mm quartz cuvette (optical path length: 10 mm) and a 3 × 3 mm quartz cuvette, respectively.

Fluorescence live-cell imaging

MCF7 cells were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum and 2% penicillin/streptomycin at 37°C in a 5% CO_2 incubator. For the fluorescence imaging experiments, MCF7 cells were seeded in a 8-well glass plate (Iwaki, Tokyo, Japan) at a density of about 0.5×10^4

cells/well and maintained for 24 h. The cells were then incubated in media containing appropriate concentration of TO derivatives for 20 min or 24 h at 37˚C in a 5% CO_2 incubator. After washing with HBSS buffer twice, the cells were imaged in HBSS buffer using a Deltavision Elite microscopy system (GE Healthcare Japan, Tokyo, Japan). In the co-staining experiments, the cells stained with TO derivatives were incubated in media containing co-staining probe (100 nM MitoBrigtht LT Deep red, 1 μM LysoBrite™ NIR or 176 nM Hoechst 33342) for 20 min according to the manufacturer's protocol, respectively. The following filter sets were used: DAPI filter set (Ex 390/18; Em 435/48) for Hoechst 33342; FITC filter set (Ex 475/28; Em 545/48) for TO derivatives; Cy5 filter set (Ex 632/22; Em 679/34) for MitoBrigtht LT Deep Red. The obtained images were processed with the softWoRx software.

DNase and RNase digestion experiments

MCF-7 cells were fixed in pre-chilled methanol at -20°C for 1 min. The cell membrane was then permeablized with 1% Triton X-100 in D-PBS for 2 min at room temperature. After rinsing with D-PBS twice, the cells were incubated with 1.0 μM TO derivatives in D-PBS solution for 20 min at 37°C in a 5% CO_2 atmosphere, followed by washing with D-PBS buffer twice. DNase (100 U/mL), RNase (20 μg/mL), or D-PBS (control) were added into each of the three cells, which was then incubated at 37˚C in a 5% CO_2 atmosphere for 3 h. Cells were rinsed again by D-PBS buffer twice before imaging. The fluorescent imaging pictures were obtained in D-PBS buffer by using an equal exposure time for control, DNase, and RNase experiments.

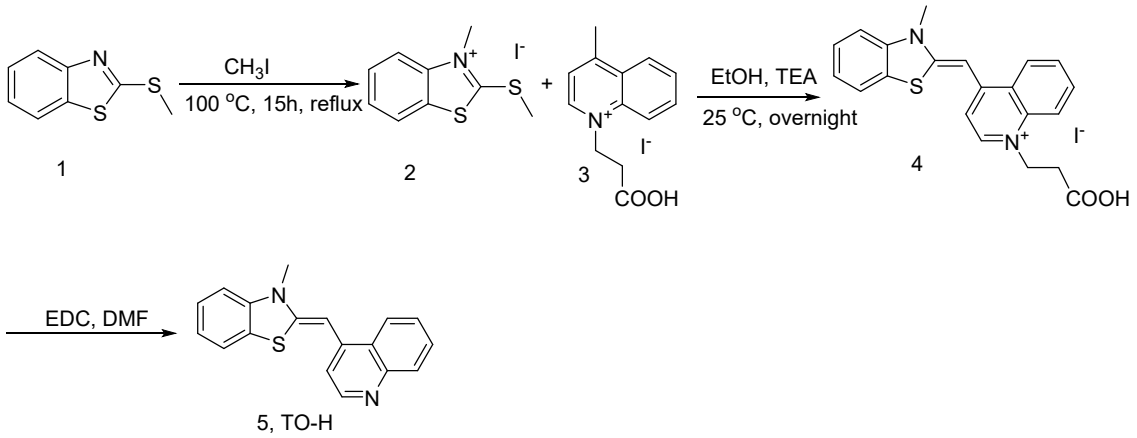
Cell toxicity Test (Alamarblue assay)

Stock solution of TO derivatives was diluted by fresh medium into four desired concentration (0.5, 2, 5, 10 μM). MCF7 cells were seeded in 96-well microplates (Thermo Fisher Scientific, Tokyo, Japan) at a density of 5.0×10^4 cells/well in medium. After 24 h of cell attachment, the cell medium was exchanged by different concentrations of TO derivatives-containing medium solutions. They were then incubated at 37 °C in 5% CO_2 for 20 min. After rinsing with D-PBS twice, fresh medium (100 μL) was added to each well, followed by the addition of 10 μL alamarBlue stock solution (Invitrogen, Carlsbad, CA) to each well. After incubation for 3h where the microplates were covered with aluminum foil, the fluorescence intensity of each cell plate at 610 nm (Excitation 540 nm, cut off: 590 nm) was measured with a microplate reader (SpectraMax M5, Molecular Devices, CA).

Synthesis of TO (4TO), 2TO, and TO derivatives

(Z)-1-methyl-4-((3-methylbenzo[*d*]thiazol-2(3*H*)-ylidene)methyl)quinolin-1-ium iodide (4TO): This compound was prepared according to the literature.[S3]

^{1}H-NMR (500 MHz, DMSO-d_6): δ 8.81 (d, J = 8.5 Hz, 1H), 8.62 (d, J = 7.3 Hz, 1H), 8.08-8.01 (m, 3H), 7.80-7.77 (m, 2H), 7.62 (t, J = 7.8 Hz, 1H), 7.44-7.38 (m, 2H), 6.95 (s, 1H), 4.18 (s, 3H), 4.02 (s, 3H); ^{13}C-NMR (500 MHz, DMSO-d_6): δ 159.6, 148.3, 144.9, 140.3, 137.9, 133.1, 128.0, 126.8, 125.4, 124.3, 123.9, 123.7, 122.7, 118.1, 112.8, 107.7, 87.8, 42.3, 33.8; ESI-MS for $C_{19}H_{17}N_2S$ ($[M]^+$): calcd, 305.11; found, 305.11.



Scheme S1 Synthetic scheme for the preparation of TO-H.

3-methyl-2-(methylthio)benzo[*d*]thiazol-3-ium iodide (2):[S4] To a dry 50 mL round bottom flask 2-(methylthio)benzothiazole (**1**) (4.0 g, 22 mmol) and iodomethane (29.79 mL, 45 mmol) were added. The flask was heated at 50 °C for 4 h. The reaction mixture became a white solid. After cooling to room temperature, MeOH (150 mL) and Et_2O (120 mL) were added to ensure that the product had fully precipitated. The precipitate was collected via vacuum filtration, washed with Et_2O (3×20 mL), and dried under reduced pressure to yield a white solid (0.42 g, 6%).

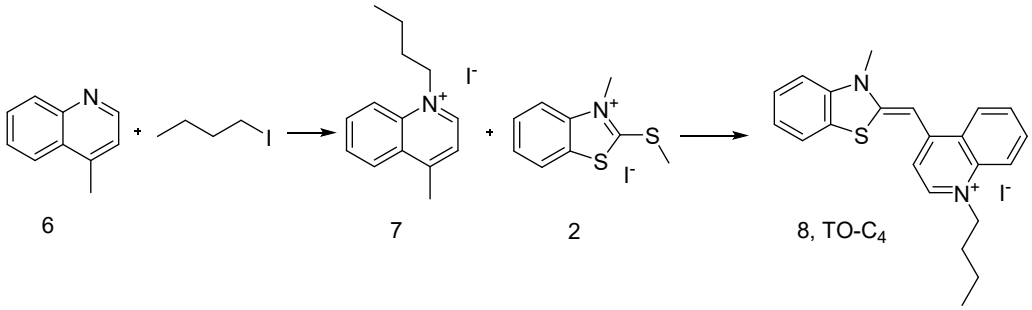
^{1}H-NMR (500 MHz, MeOH-d_4): δ 8.16-8.14 (m, 1H), 8.01 (d, J = 8.5 Hz, 1H), 7.79-7.75 (m, 1H), 7.65 (dd, J = 8.2, 7.3 Hz, 1H), 4.08 (s, 3H), 3.06 (s, 3H).

***(Z)*-1-(2-carboxyethyl)-4-((3-methylbenzo[d]thiazol-2(3*H*)-ylidene)methyl)quinolin-1-ium iodide (4):**[S5] The crude compound **3** (1.48 g, 5.0 mmol) and **2** (1.62 g, 5.0 mmol) were suspended in ethanol (50 mL). Triethylamine (7.0 mL, 50 mmol) was added to the suspension, and the resultant red solution was stirred at 25 °C overnight. Diethyl ether (100 mL) was added, and the resultant suspension was stirred for 10 min. The precipitate was filtered, washed with diethyl ether, and dried under reduced pressure. Water (50 mL) was added to the resultant red solid and the suspension was stirred for 10 min. The precipitate was filtered, washed with water, and dried under reduced pressure. The product **4** was obtained as a red solid (1.92 g, 78%).

^{1}H-NMR (500 MHz, DMSO-d_6): δ 8.87 (d, J = 8.9 Hz, 1H), 8.68 (d, J = 7.3 Hz, 1H), 8.20 (d, J = 8.9 Hz, 1H), 8.12 (d, J = 7.3 Hz, 1H), 8.05 (t, J = 7.8 Hz, 1H), 7.87 (d, J = 8.2 Hz, 1H), 7.82 (t, J = 7.8 Hz, 1H), 7.69 (t, J = 7.8 Hz, 1H), 7.50 (t, J = 7.6 Hz, 1H), 7.42 (d, J = 7.3 Hz, 1H), 7.01 (s, 1H), 4.86 (t, J = 6.9 Hz, 2H), 3.36 (t, J = 7.0 Hz, 2H), 2.75 (s, 3H); ESI-MS for $C_{21}H_{19}N_2O_2S$ ($[M]^+$): calcd, 363.12; found, 363.15.

(Z)-3-methyl-2-(quinolin-4-ylmethylene)-2,3-dihydrobenzo[*d*]thiazole (5, TO-H):[S5] **4** (443 mg, 1.0 mmol) and 1-ethyl-3- (3-dimethylaminopropyl)carbodiimide hydrochloride (383 mg, 2.0 mmol) were suspended in DMF (10 mL). The suspension was stirred at 25 °C for 24 h. After addition of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (192 mg, 1.0 mmol), it was further stirred at 25 °C overnight. The reaction mixture was diluted with chloroform (100 mL) and washed with water (100 mL). The aqueous layer was extracted with chloroform (50 mL) twice. The combined organic layer was dried with Na_2SO_4 and evaporated in vacuo. The product was purified by silica gel column chromatography (5% MeOH–CH_2Cl_2), giving the product **5** as an orange powder (70 mg, 48%).

^{1}H-NMR (500 MHz, DMSO-d_6): δ 8.73 (d, J = 4.9 Hz, 1H), 8.42 (d, J = 8.2 Hz, 1H), 7.93 (d, J = 8.5 Hz, 1H), 7.73-7.70 (m, 1H), 7.65 (d, J = 7.0 Hz, 1H), 7.57 (t, J = 7.0 Hz, 1H), 7.41 (d, J = 5.2 Hz, 1H), 7.36-7.28 (m, 2H), 7.07 (t, J = 7.5 Hz, 1H), 6.46 (s, 1H), 3.65 (s, 3H); ^{13}C-NMR (500 MHz, DMSO-d_6): δ 149.6, 149.4, 147.9, 142.4, 141.5, 129.2, 128.9, 126.9, 125.3, 125.2, 123.9, 122.4, 121.8, 121.3, 112.9, 109.5, 84.9, 32.0; ESI-MS for $C_{18}H_{15}N_2S$ ($[M+H]^+$): calcd, 291.0955; found, 291.0940.



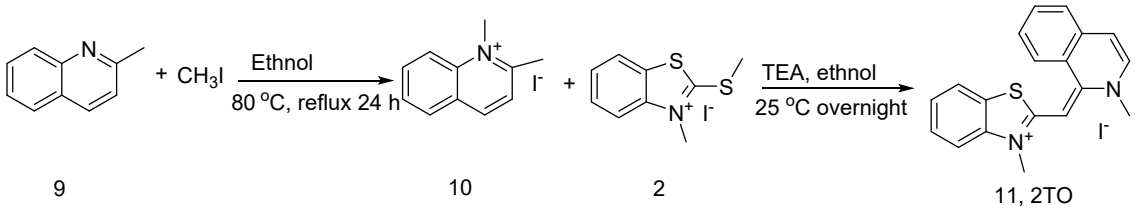
Scheme S2 Synthetic scheme for the preparation of TO-C_4.

1-butyl-4-methylquinolin-1-ium iodide (7):[S6] Equimolar amounts of the compound **6** and the alkyl iodide (10 mmol each) were heated with stirring for 3 h at 85 °C. After cooling to room temperature, the solidified crude product was suspended in tetrahydrofuran for 10 min. Precipitation of quaternary ammonium salt was improved by adding diethyl ether. The crude product was collected, washed several times with diethyl ether, and dried in a vacuum. The compound **7** was finally obtained as yellow oils (1.8g, 55%).

^{1}H-NMR (500 MHz, DMSO-d_6): δ 9.41 (d, J = 6.1 Hz, 1H), 8.60-8.54 (m, 2H), 8.26 (t, J = 7.3 Hz, 1H), 8.08-8.04 (m, 2H), 5.00 (t, J = 7.6 Hz, 2H), 2.98-3.03 (3H), 1.95-1.89 (m, 2H), 1.39 (td, J = 15.1, 7.4 Hz, 2H), 0.92 (t, J = 7.3 Hz, 3H).

(Z) **-1-butyl-4-((3-methylbenzo[*d*]thiazol-2(3H)-ylidene)methyl)quinolin-1-ium iodide (8, TO-C_4):**[S5] The crude compound **7** (832.80mg, 2.5 mmol) and compound **2** (802.62 mg, 2.5 mmol) were suspended in ethanol (25 mL). Triethylamine (3.5 mL, 25 mmol) was added to the suspension, and the resultant red solution was stirred at 25 °C overnight. Diethyl ether (50 mL) was added, and the resultant suspension was stirred for 10 min. The precipitate was filtered, washed with diethyl ether, and dried under reduced pressure. Water (25 mL) was added to the resultant red solid and the suspension was stirred for 10 min. The precipitate was filtered, washed with water, and dried under reduced pressure. The product **8** was obtained as a red solid (1.92 g, 78%).

^{1}H-NMR (500 MHz, DMSO-d_6): δ 8.81 (d, J = 8.2 Hz, 1H), 8.64 (d, J = 7.0 Hz, 1H), 8.15 (d, J = 8.5 Hz, 1H), 8.06 (d, J = 7.3 Hz, 1H), 8.00 (t, J = 7.8 Hz, 1H), 7.81-7.75 (m, 2H), 7.63 (t, J = 7.8 Hz, 1H), 7.43 (t, J = 7.3 Hz, 1H), 7.38 (d, J = 7.0 Hz, 1H), 6.94 (s, 1H), 4.61 (t, J = 7.3 Hz, 2H), 4.03 (s, 3H), 1.87-1.81 (m, 2H), 1.38 (td, J = 15.0, 7.5 Hz, 2H), 0.93 (t, J = 7.5 Hz, 3H); ^{13}C-NMR (500 MHz, DMSO-d_6): δ 160.0, 148.5, 144.3, 140.4, 137.0, 133.2, 128.1, 126.7, 125.8, 124.4, 124.2, 123.8, 122.8, 118.1, 112.9, 107.8, 88.1, 53.9, 33.8, 30.8, 19.2, 13.5; ESI-MS for $C_{22}H_{23}N_2S$ ($[M]^+$): calcd, 347.16; found, 347.16.



Scheme S3 Synthetic scheme for the preparation of 2TO.

1,2-dimethylquinolin-1-ium iodide (10):[S7] Toward a round-bottom flask with 5 mL ethanol, 1.0 mL (7.4 mmol) of 2-methylquinoline and 0.46 mL (7.4 mmol) iodomethane were added. The mixture was stirred evenly and heated to reflux at 80 °C for 24 h. The system was then cooled down to room temperature, and dark yellow powder was precipitated. The product was filtered and washed with ethanol three times, and was used for the next step without further purification (0.61 g, 28%).
^{1}H-NMR (500 MHz, DMSO-d_6): δ 9.10 (d, J = 8.5 Hz, 1H), 8.59 (d, J = 8.9 Hz, 1H), 8.40 (d, J = 8.2 Hz, 1H), 8.23 (t, J = 7.2 Hz, 1H), 8.12 (d, J = 8.5 Hz, 1H), 7.99 (t, J = 7.5 Hz, 1H), 4.45 (s, 3H), 3.08 (s, 3H).

(*E*)-3-methyl-2-((2-methylisoquinolin-1(2*H*)-ylidene)methyl)benzo[*d*]thiazol-3-ium iodide (11, 2TO):[S5] The crude 1,2-dimethylquinolin-1-ium (148 mg, 0.5 mmol) and 2-methylmercapto-3-methyl benzothiazolium iodide (162 mg, 0.5 mmol) were suspended in ethanol (5 mL). Triethylamine (0.7 mL, 5 mmol) was added to the suspension, and the resultant red solution was stirred at 25 °C overnight. Diethyl ether (10 mL) was added, and the resultant suspension was stirred for 10 min. The precipitate was filtered, washed with diethyl ether, and dried under reduced pressure. Water (5 mL) was added to the resultant red solid and the suspension was stirred for 10 min. The precipitate was filtered, washed with water, and dried under reduced pressure. The product **11** was obtained as a red solid (40 mg, 16%).
^{1}H-NMR (500 MHz, DMSO-d_6): δ 8.45 (d, J = 9.2 Hz, 1H), 8.14 (d, J = 8.9 Hz, 1H), 8.09 (d, J = 9.5 Hz, 1H), 8.03 (q, J = 3.8 Hz, 2H), 7.93-7.90 (m, 1H), 7.79 (d, J = 8.2 Hz, 1H), 7.63-7.60 (m, 2H), 7.43 (t, J = 7.6 Hz, 1H), 6.17 (s, 1H), 4.13 (s, 3H), 3.94 (s, 3H); ^{13}C-NMR (500 MHz, DMSO-d_6): δ 161.6, 153.4, 140.7, 139.8, 133.4, 129.4, 128.1, 125.6, 124.8, 124.0, 123.4, 122.8, 118.5, 117.3, 113.1, 86.6, 37.7, 33.9; ESI-MS for for $C_{19}H_{17}N_2S$ ($[M]^+$): calcd, 305.11; found, 305.11.

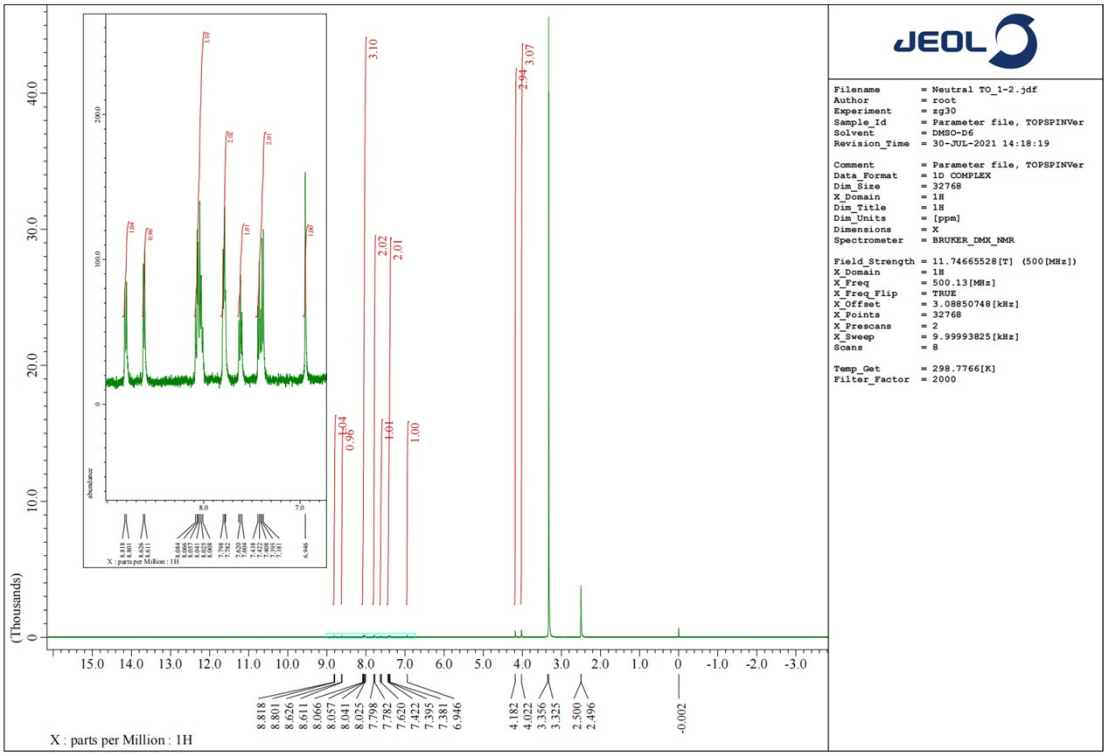


Fig. S1 ^{1}H NMR of common TO (4TO) in DMSO-d_6.

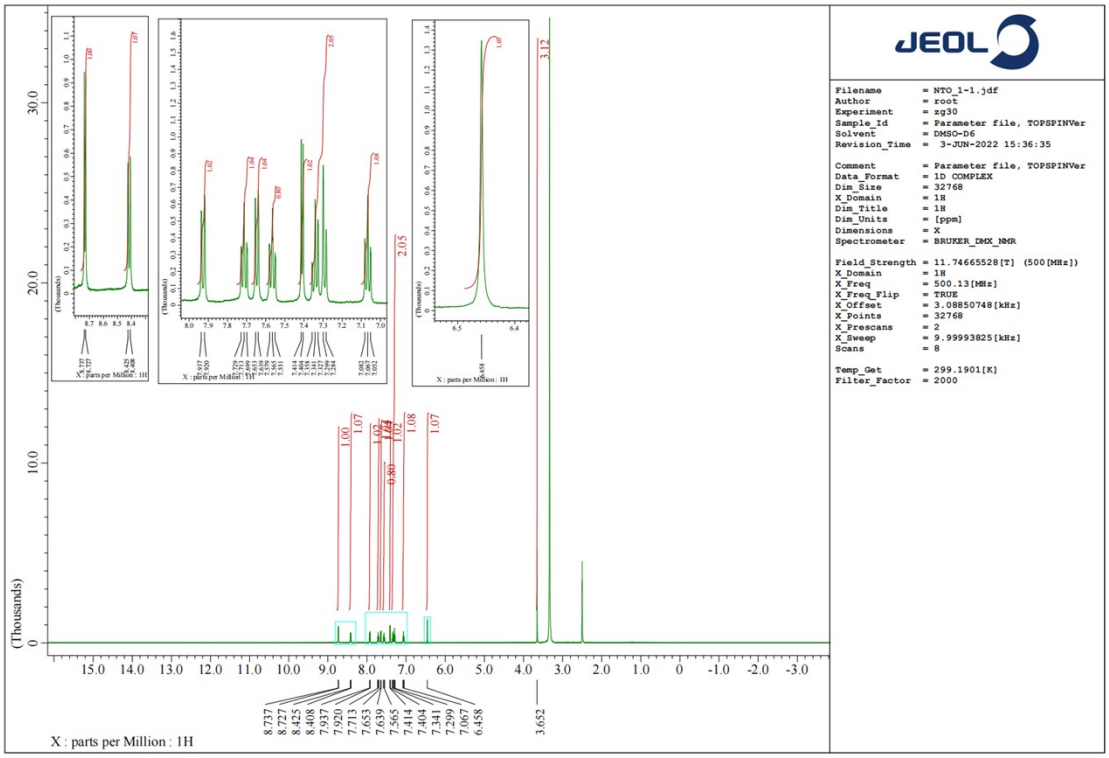


Fig. S2 ^{1}H NMR of TO-H in DMSO-d_6.

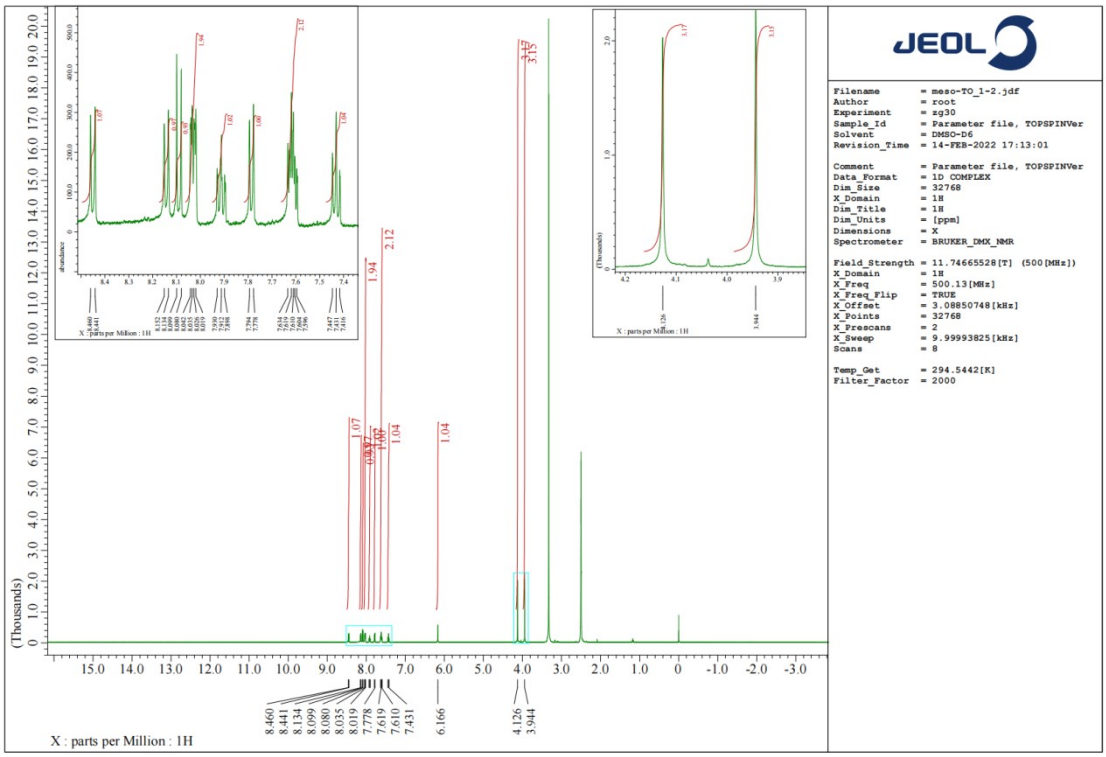


Fig. S3 ^{1}H NMR of 2TO in DMSO-d_6.

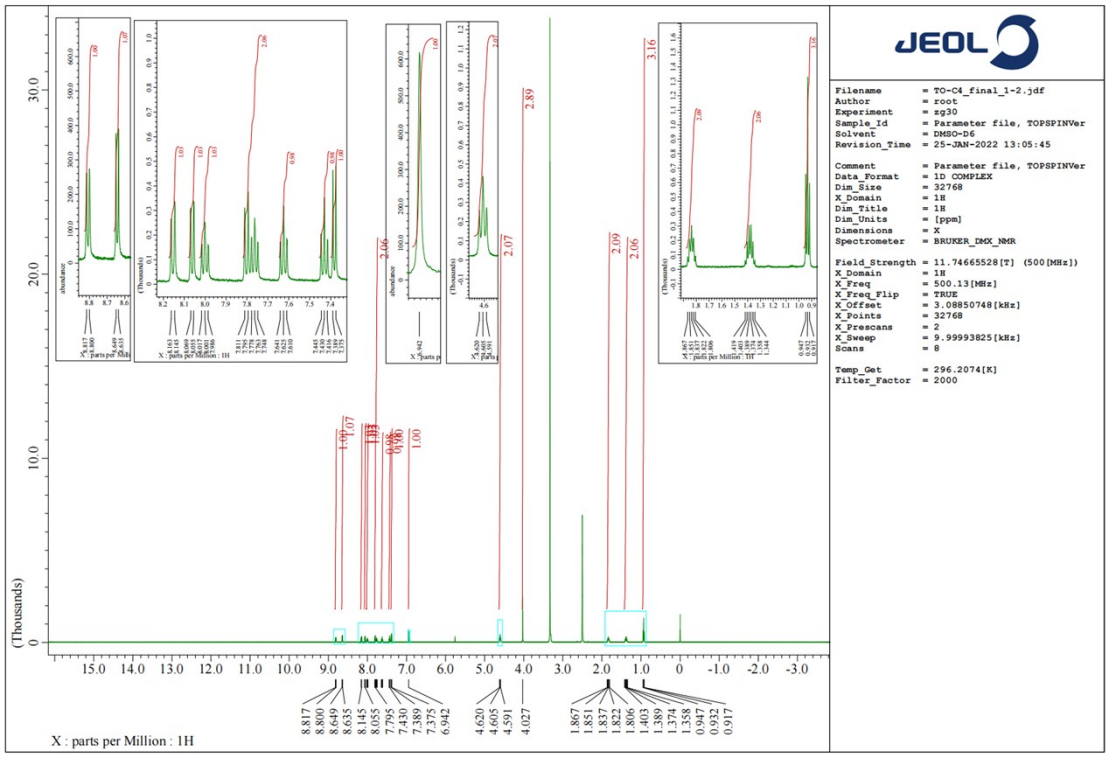


Fig. S4 ^{1}H NMR of TO-C_4 in DMSO-d_6.

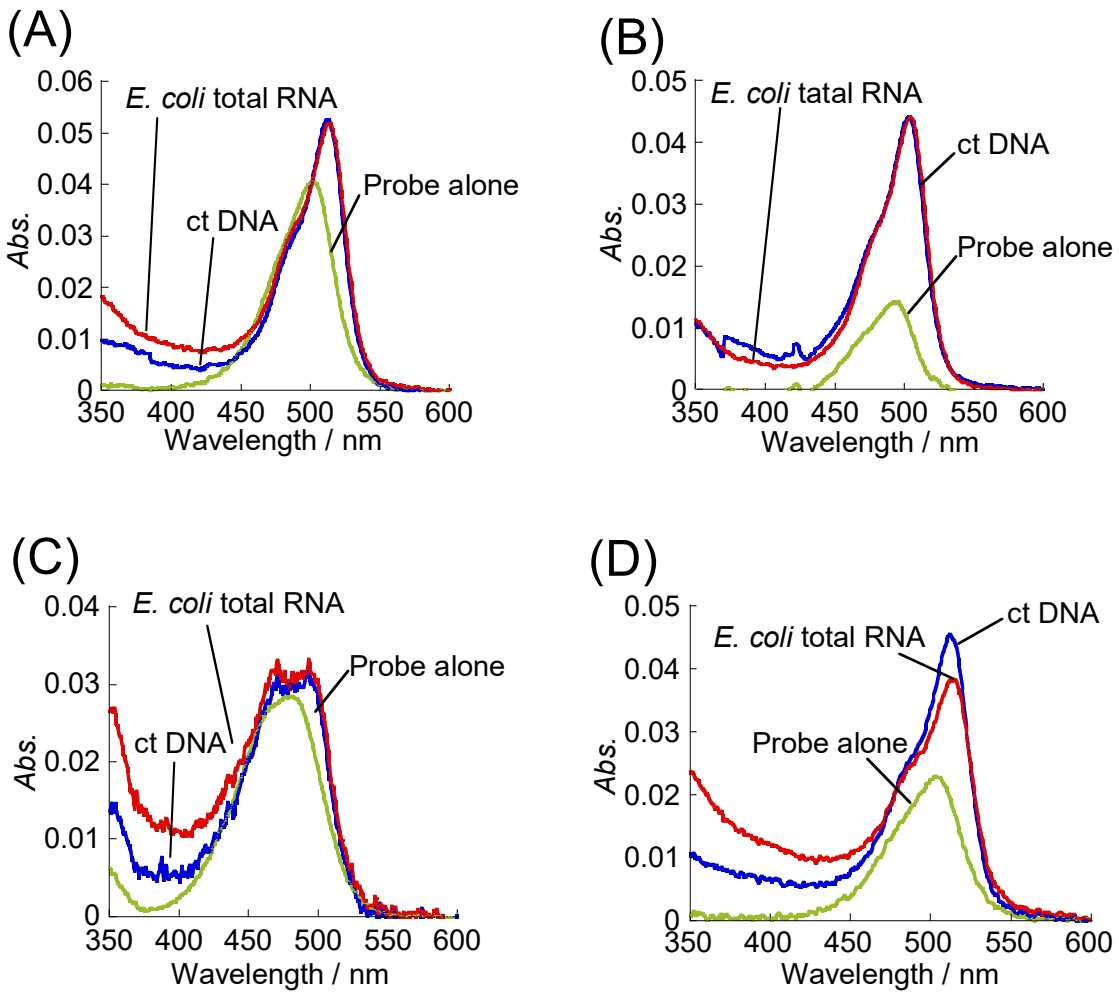
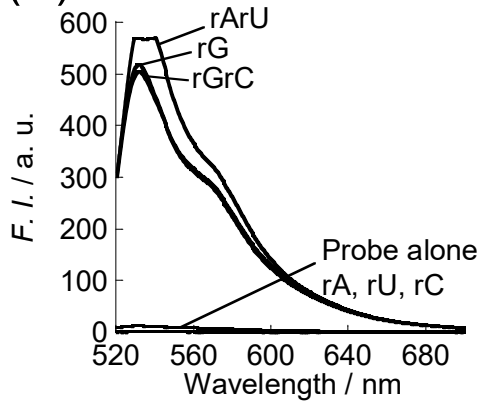


Fig. S5 Absorption spectra of TOs (1.0 μM) in the absence and presence of 1.0 mM calf thymus DNA or 1.0 mM *E. coli* total RNA : (A) TO, (B) TO-H, (C) 2TO and (D) TO-C_4. Measurements were done in 10 mM sodium phosphate buffer solution (pH 7.0) containing 100 mM NaCl and 1.0 mM EDTA. Temperature: 25 ˚C.

(A)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200
100
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F. I. / a. u.
rArU
rG
rGrC
Probe alone
rA, rU, rC
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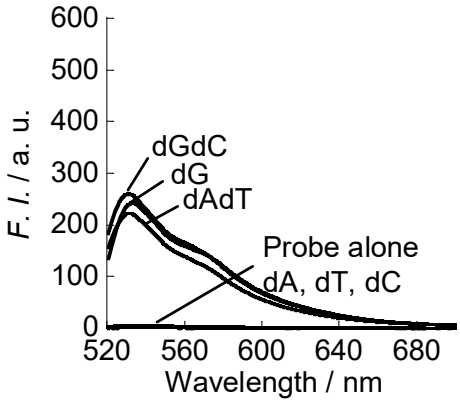
560
600
640
680
Wavelength / nm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dG
dAdT
Probe alone
dA, dT, dC
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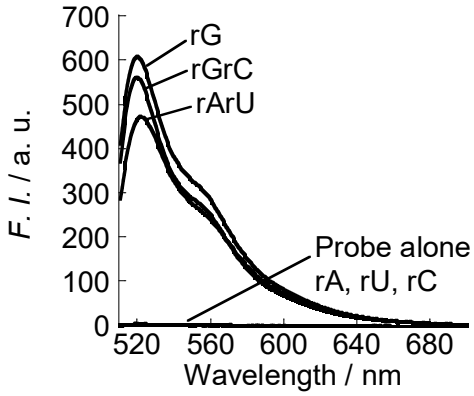
600
640
680
Wavelength / nm

(B)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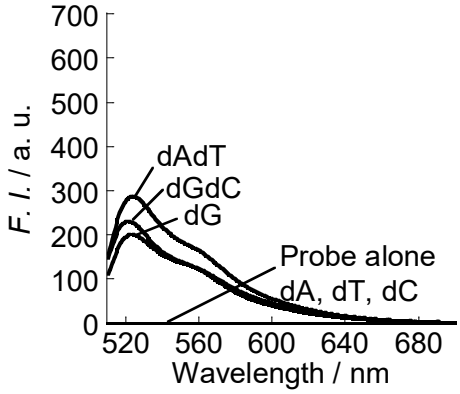
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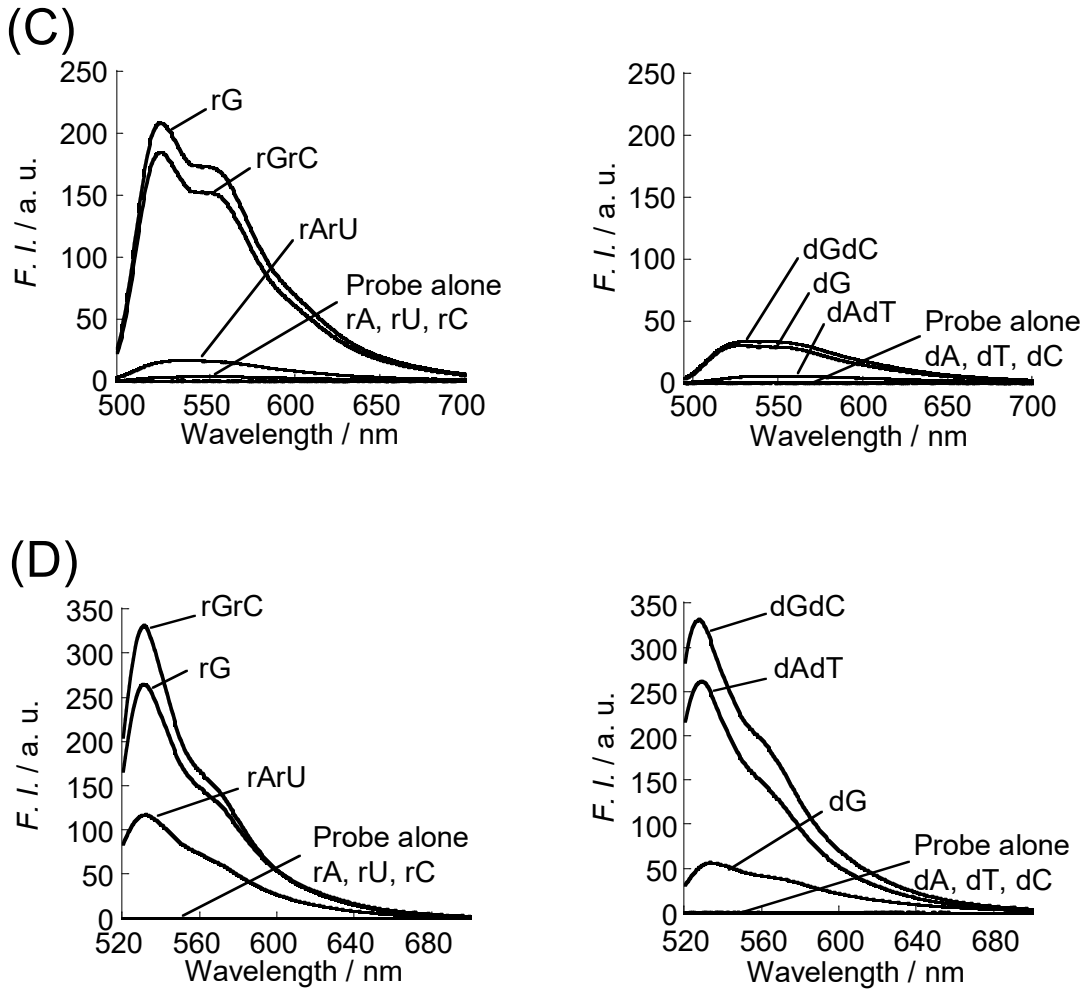

rG: 5'- r(GGG GGG GGG GGG G)-3'

rC: 5'- r(CCC CCC CCC CCC C)-3'

rA: 5'- r(AAA AAA AAA AAA A)-3'

rU: 5'- r(UUU UUU UUU UUU U)-3'

dG: 5'- d(GGG GGG GGG GGG G)-3'

dC: 5'- d(CCC CCC CCC CCC C)-3'

dA: 5'- d(AAA AAA AAA AAA A)-3'

dT: 5'- d(TTT TTT TTT TTT T)-3'

Fig. S6 Fluorescence response of TOs (1.0 μM) to synthetic nucleic acids (20 μM): (A) TO, (B) TO-H, (C) 2TO and (D) TO-C_4. Data were obtained after mixing TOs with nucleic acid solutions and subsequent incubation for 30 min. Other solutions conditions were same as Fig. 2 in the main text. Excitation: (A) 503 nm, (B) 493.5 nm, (C) 479 nm, (D) 503 nm. Temperature: 25 °C. The nucleic acid sequences used here were also shown.

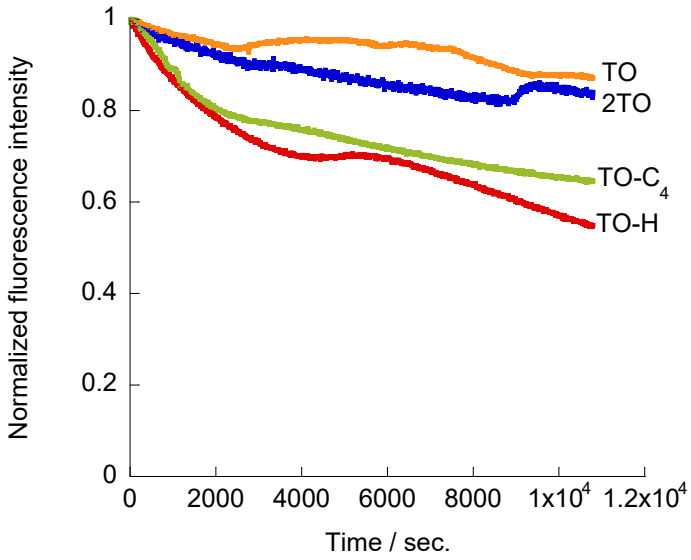

Fig. S7 Changes in fluorescence intensity of four TOs when bound to *E. coli* total RNA under continuous irradiation (3 h). [Probe] = 1.0 μM, [*E. coli* total RNA] = 1.0 mM in solutions buffered at pH 7.0 (10 mM sodium phosphate) containing 100 mM NaCl and 1.0 mM EDTA. Before the measurement, sample solutions were incubated for 30 min. Excitation: 503 nm (TO), 493.5 nm (TO-H), 479 nm (2TO), 503 nm (TO-C_4). Temperature: 25 °C. The measurements were performed using FP-6500 spectrofluorophotometer.

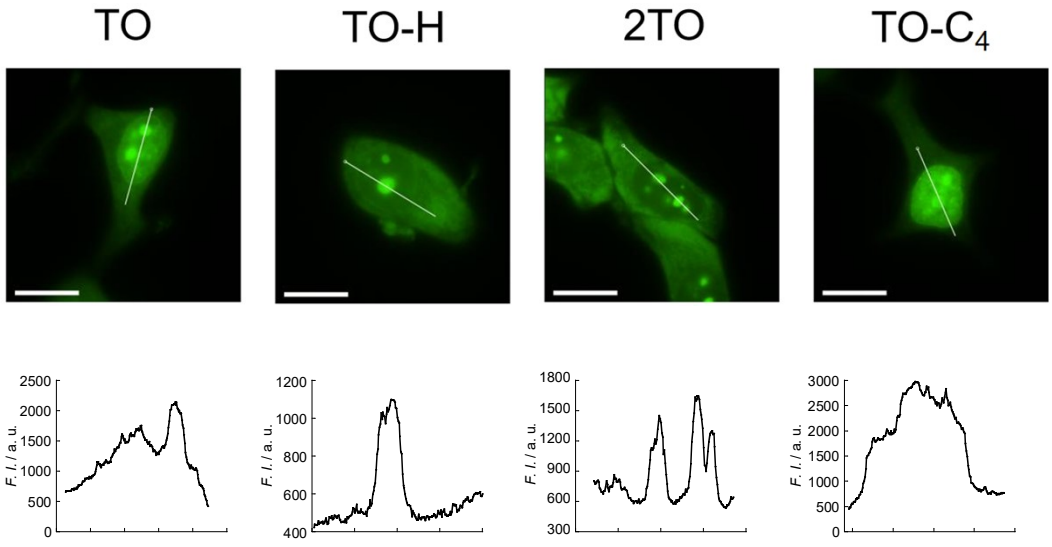

Fig. S8 *No-wash* fluorescence imaging of living MCF7 cells stained by TO (0.5 μM), TO-H (2 μM), 2TO (0.5 μM) or TO-C_4 (0.3 μM) after 20 min incubation. Fluorescence intensity profiles along the white line are also shown. Scale bar: 15 μm. The cells were imaged in HBSS buffer.

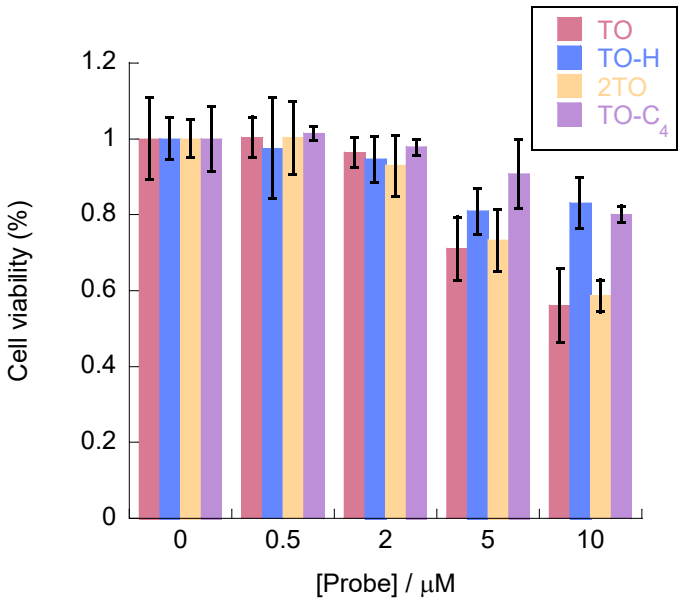

Fig. S9 Evaluation of probe cytotoxicity after 20 min incubation. Buffer treatment served as a positive control. Values are means ± standard deviation of six independent experiments ($N = 6$).

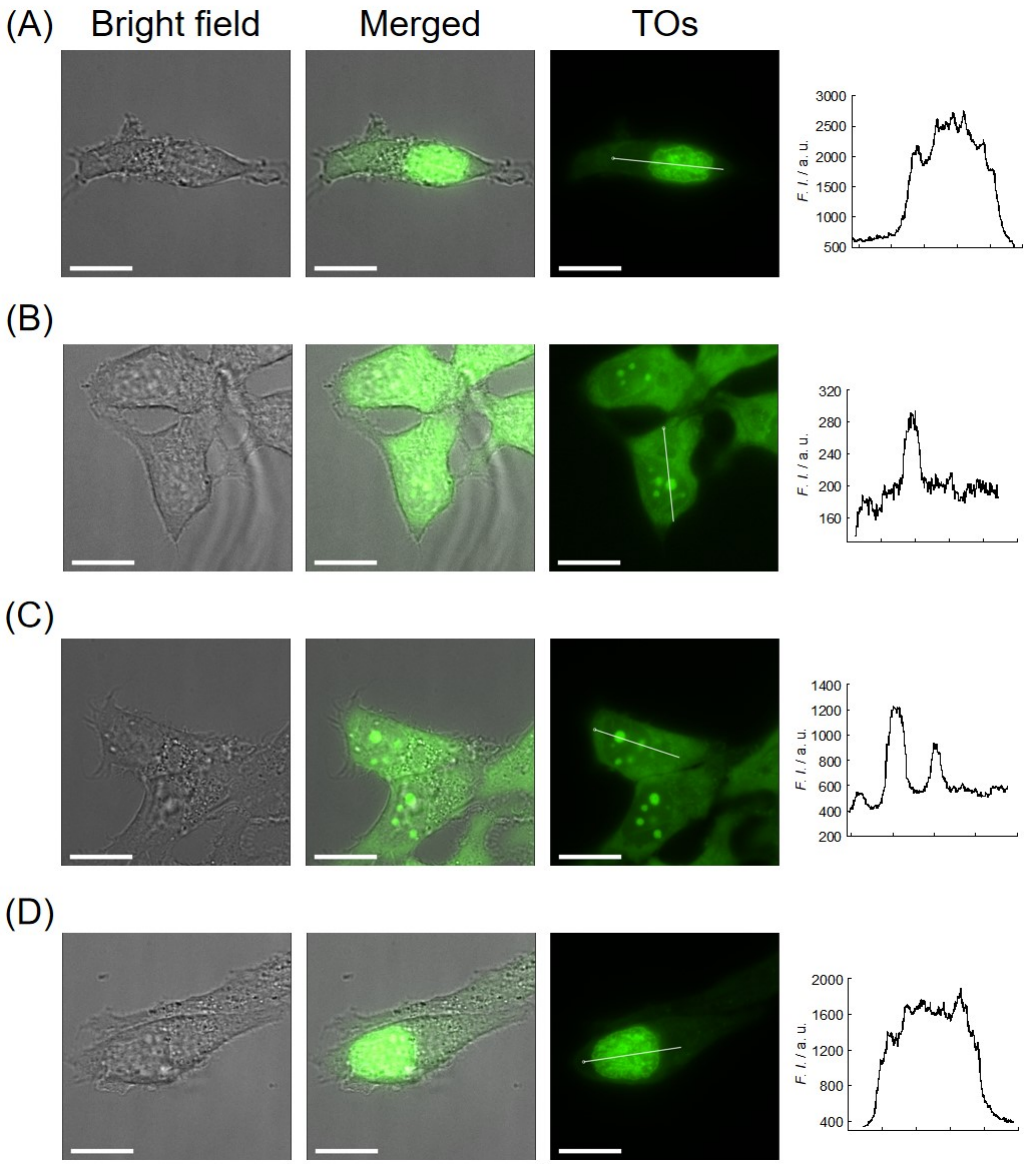

Fig. S10 Images of living MCF7 cells stained by (A) TO (0.5 μM), (B) TO-H (2 μM), (C) 2TO (0.5 μM), or (D) TO-C_4 (0.3 μM) after 24 h incubation. Fluorescence intensity profiles along the white line are also shown. Scale bar: 15 μm. After washing with HBSS buffer twice, the cells were imaged in HBSS buffer.

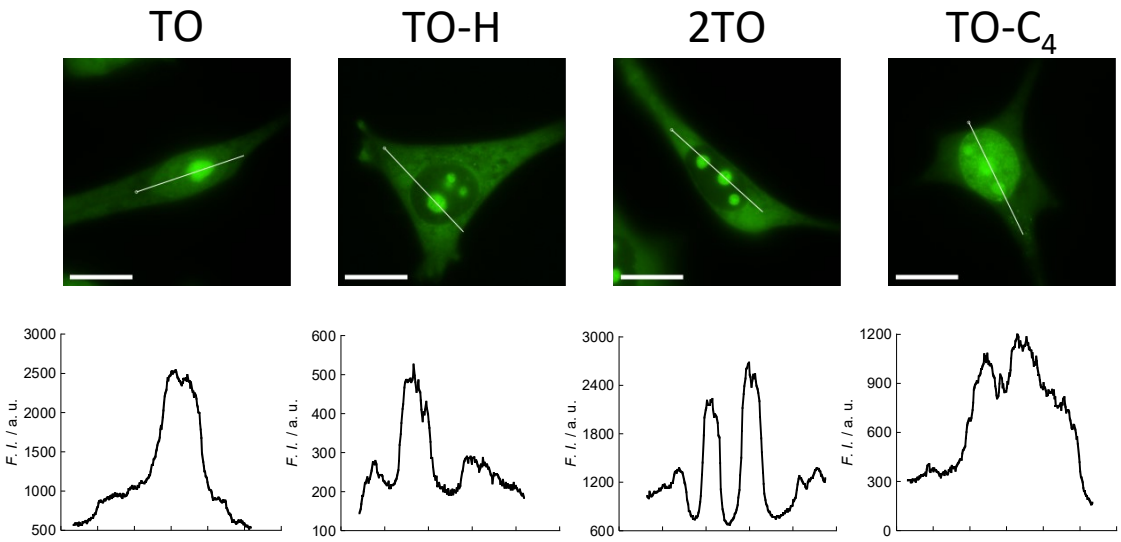

Fig. S11 Fluorescence imaging of living HeLa cells stained by TO (0.5 μM), TO-H (2 μM), 2TO (0.5 μM) or TO-C_4 (0.3 μM) after 20 min incubation. Fluorescence intensity profiles along the white line are also shown. Scale bar: 15 μm. After washing with HBSS buffer twice, the cells were imaged in HBSS buffer.

As shown above, TO, TO-H and 2TO were also applicable to living HeLa cells, revealing the versatility of these dyes as a useful imaging probe for necleolar RNA in living cells. Significantly, 2TO worked best among these probes, where the green emission was clearly observed in the nucleolus with the highest contrast to the emission from the nucleus (nucleoplasm).

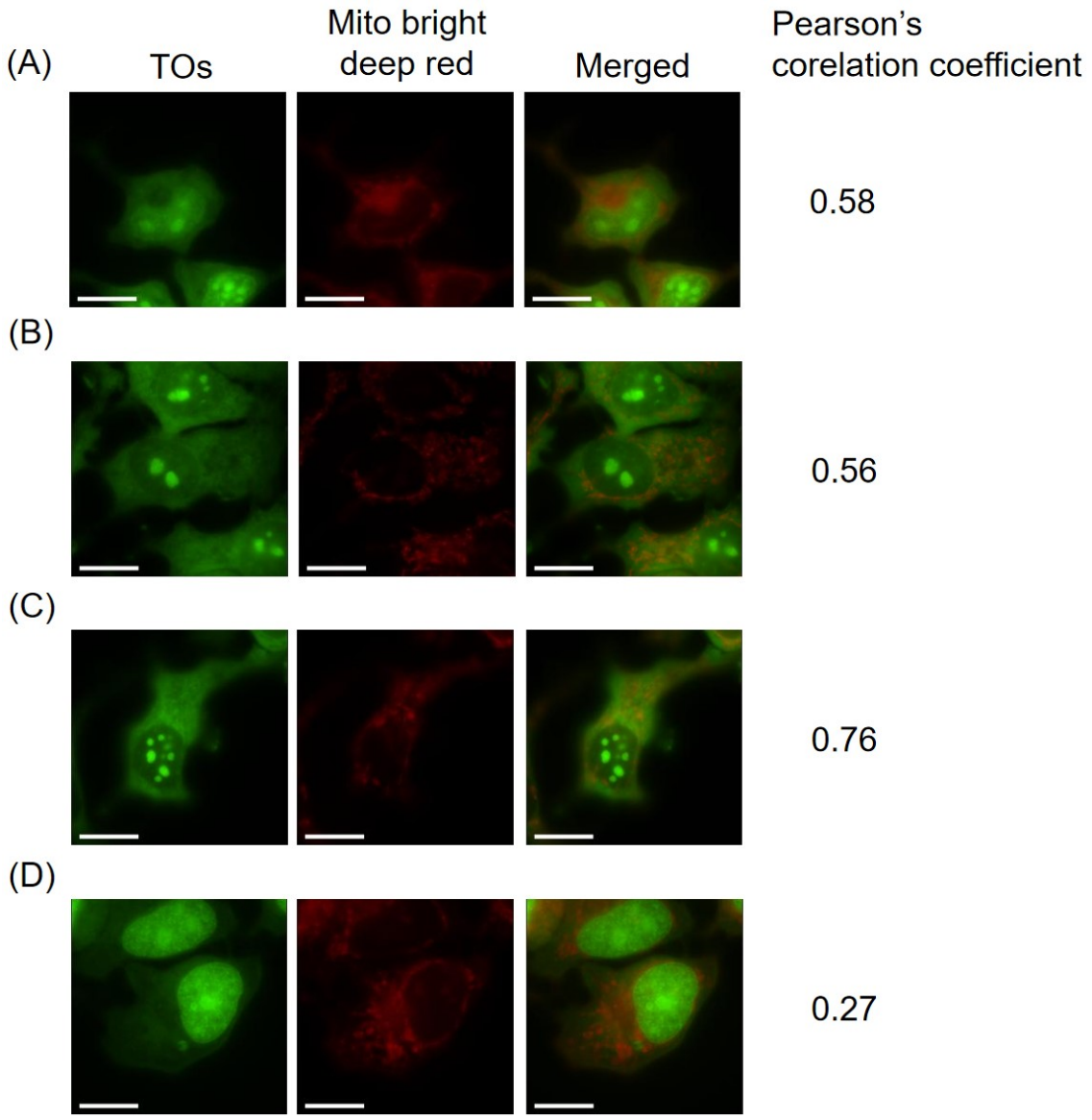

Fig. S12 Co-staining of living MCF7 cells with each probe (TO, 2TO and TO derivatives) and mitochondria-staining probe (MitoBrigtht LT Deep Red). (A) [TO] = 0.5 μM, (B) [TO-H] = 2 μM, (C) [2TO] = 0.5 μM, and (D) [TO-C_4] = 0.3 μM. [MitoBrigtht LT Deep Red] = 100 nM. Scale bar: 15 μm. The cells stained with TO derivatives (20 min) were further incubated in media containing co-staining probe for 20 min. After washing with HBSS buffer twice, the cells were imaged in HBSS buffer.

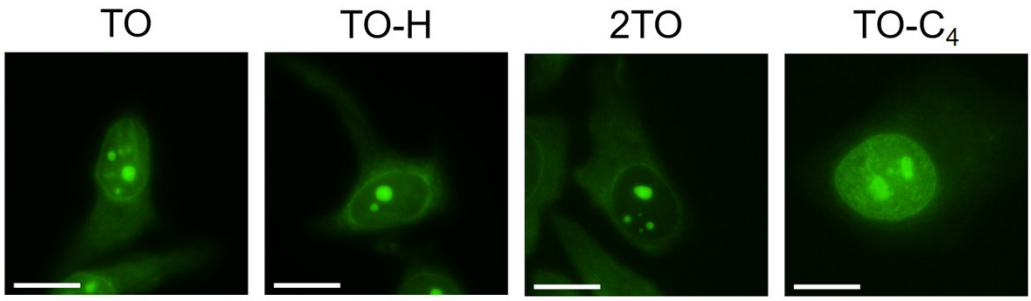

Fig. S13 Fluorescence images of fixed-permeabilized MCF7 cells stained by TO, 2TO or TO derivatives after 20 min incubation. [TO] = 0.5 μM, [TO-H] = 2 μM, [2TO] = 0.5 μM, [TO-C_4] = 0.3 μM. Scale bar: 15 μM. After washing with D-PBS buffer twice, the cells were imaged in D-PBS buffer.

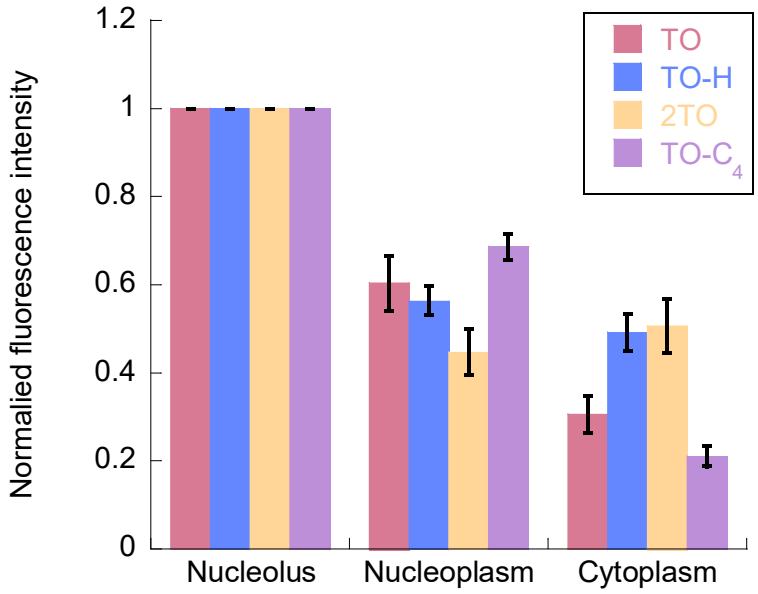

Fig. S14 Relative fluorescence intensity in each organelle in living MCF7 cells stained by TO, 2TO or TO derivatives after 20 min incubation. Values are means ± SEM (standard error of the mean) of nine cells. Error bars are standard deviations obtained from 9 independent experiments (*N* = 9). [TO] = 0.5 μM, [TO-H] = 2 μM, [2TO] = 0.5 μM, [TO-C_4] = 0.3 μM.

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