

Electronic Supplementary Information

Development of a system to sensitively and specifically visualize *c-fos* mRNA in living cells using bispyrene-modified RNA probes

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Experimental procedures

OMUpy2 design and synthesis

5'-O-dimethoxytrityl-2'-O-(1-pyrenylmethyl)-uridine-3'-O-(2-cyanoethyl)-N,N'-diisopropylphosphoroamidite (5'-DMTr-Upy amidite) was synthesized according to a published method¹. The sequences of OMUpy2 and OMUpy2-S were designed *in silico* with the aid of RNAstructure Version 4.6 (5/6/2008), David H. Matthews, <http://rna.urmc.rochester.edu/rnastructure.html>. Each OMUpy2 was synthesized by phosphoramidite chemistry. Fluorescein was labeled at 5' position of OMUpy2 and OMUpy2-S using 5'-Fluorescein Phosphoramidite (Glen Research). The controlled-pore glass support was treated with 28% (v/v) ammonium hydroxide at 55 °C for 8 h. OMUpy2 was purified by reversed-phase HPLC on a CAPCELL PAK C-18 column (4.6 × 150 mm, Shiseido, Co. Ltd., Tokyo, Japan) with a linear gradient of acetonitrile in 0.1M triethylammonium acetate (TEAA, pH 7.0) at a flow rate of 0.8 mL min⁻¹ (acetonitrile gradient was shown in Table S1) and the purity of each probes was shown in Figure S3. Mass spectra of each probe purified by reversed-phase HPLC were obtained using ESI-MS (Bruker DALTONICS[®] microTOF), respectively, and the data were shown in Table S2.

Cell culture

C4II cells (cervical cancer cells) were kindly provided by Dr. Kato K., Kyushu University. HeLa cells (cervical cancer cells) were purchased from RIKEN BioResource Center (Cell Bank, Japan). C4II and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units of penicillin/streptomycin at 37 °C in 5% CO₂.

RT-PCR

The expression level of *c-fos* mRNA was analyzed by RT-PCR. Total RNA was isolated using QuickGene RNA cultured kit S (Fujifilm, Japan) from either C4II cells cultured in 10% FBS/DMEM, or quiescent HeLa cells treated with EGF(1 ng mL⁻¹) and insulin (0.5 μg mL⁻¹) in HBSS. Reverse transcription reactions were carried out at 37 °C for 1 h using Omniscript kit (Qiagen, USA) with Oligo (dT)₁₆ primer. PCR primer sequences were obtained from published sequences. The *c-fos*² forward primer was 5'-GGA TAG CCT CTC TTA CTA CCA C-3' and the reverse primer was 5'-TCC TGT CAT GGT CTT CAC AAC G-3'. For GAPDH³, the forward primer was 5'-GAA GGT GAA GGT CGG AGT C-3' and reverse primer was 5'-GAA GAT GGT GAT GGG ATT TC-3'. The expected sizes of the PCR products were 280 bp (*c-fos*) and 226 bp (GAPDH). The PCR products were analyzed by 2% agarose gel

electrophoresis and visualized with ethidium bromide. The *c-fos* mRNA expression levels were normalized to those of the expression level of GAPDH mRNA.

Detection of c-fos mRNA in fixed C4II cells

C4II cells were cultured in 10% FBS/DMEM for 1 day on collagen-coated glass bottom dishes. The cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. After being washed three times with PBS, the fixed cells were treated with OMUpy2 (5 μ M in PBS) for 30 min at room temperature prior to observation using a fluorescent microscope.

RNase A treatment to the fixed C4II cells

RNase A (100 μ g mL⁻¹ in PBS) was added to the fixed cells for 45 min at room temperature. The cells were washed three times, re-fixed with 4% paraformaldehyde for 3 min and washed three times with PBS. OMUpy2 (5 μ M in PBS) was added and the cells were examined with a fluorescence microscope.

Real-time detection of c-fos mRNA in living HeLa cells

HeLa cells were cultured in 10% FBS/DMEM on poly-L-lysine coated glass bottom dishes. After 48 h incubation, the medium was changed to 0.5% FBS/DMEM. Proliferating mammalian cells can be returned to a quiescent state by culturing in media containing 0.2-0.5% FBS. After 21 h incubation, OMUpy2-S (0.5 μ M in 0.5% FBS/DMEM without phenol red) was added and the cells were incubated for 3 h. After one washing with HBSS, the cells were treated with EGF solution (1 ng ml⁻¹ EGF and 0.5 μ g mL⁻¹ insulin in HBSS) and observed by a fluorescent microscopy.

Cell imaging

All imaging experiments were carried out using a fluorescent microscope (TE-300, Nikon, Japan) with a 75 watt xenon lamp and a Plan Fluor 100 \times /NA 1.40 oil objective lens (Nikon, Japan), equipped with Image Intensifire Units (C8600-05, Hamamatsu Photonics, Japan), a monochrome ORCA-ER cooled CCD camera (C4742-95, Hamamatsu Photonics, Japan). For fluorescence analysis of bispyrene, a 340/10-nm excitation filter (Nikon, Japan), a 380-nm dichroic mirror (Nikon, Japan), and a 480/30-nm emission filter (OMEGA Optical, USA) were used. Fluorescein emission was detected using a 450-490 nm excitation filter (Nikon, Japan), a 505-nm dichroic mirror (Nikon, Japan), and a 520-nm emission filter (Nikon, Japan). Image acquisition and analysis was performed using Aqua-Lite software (Version. 1.3, Hamamatsu Photonics, Japan) for fixed cells and NIS-Element software (Ar,

Nikon, Japan) for living cells. All images were captured in gray scale and converted into cyan or green pseudo-color using the software described above.

Fluorescent spectra analysis of the fixed cells or the living cells

Fluorescence spectra of the fixed cells and the living cells were acquired using a fluorescent microspectroscope (PMA-11, Hamamatsu Photonics, Japan) with a 75 watt xenon lamp, S Fluor 40×/NA 0.90 objective lens (Nikon, Japan), a 340/10-nm excitation filter, and a 380-nm dichroic mirror.

References

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- 2 H. Wang and Y.V. Stjernholm, *Reprod. Biol. Endocrinol.*, 2007, **5**, 3.
- 3 X. Wang, M.D. Garrick, F. Yang, L.A. Dailey, C.A. Piantadosi and A.J. Ghio, *Am. J. Physiol. Lung Cell Mol. Physiol.*, 2005, **289**, L24.

Supplementary Figures

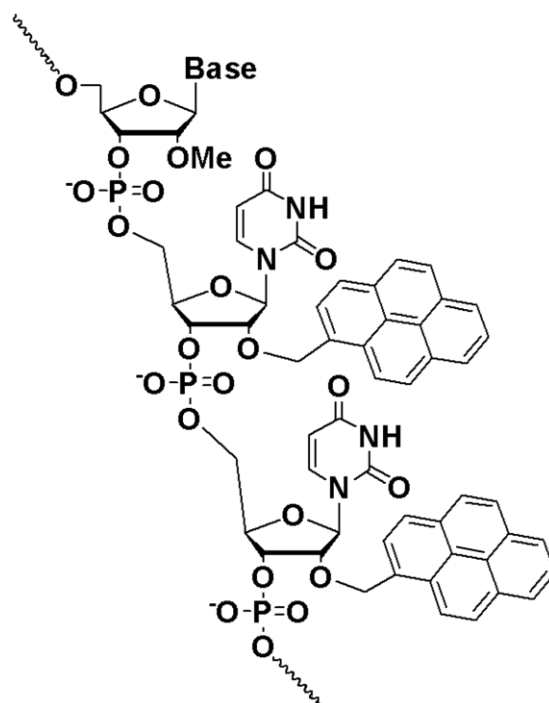


Figure S1. Structure of OMUpy2.

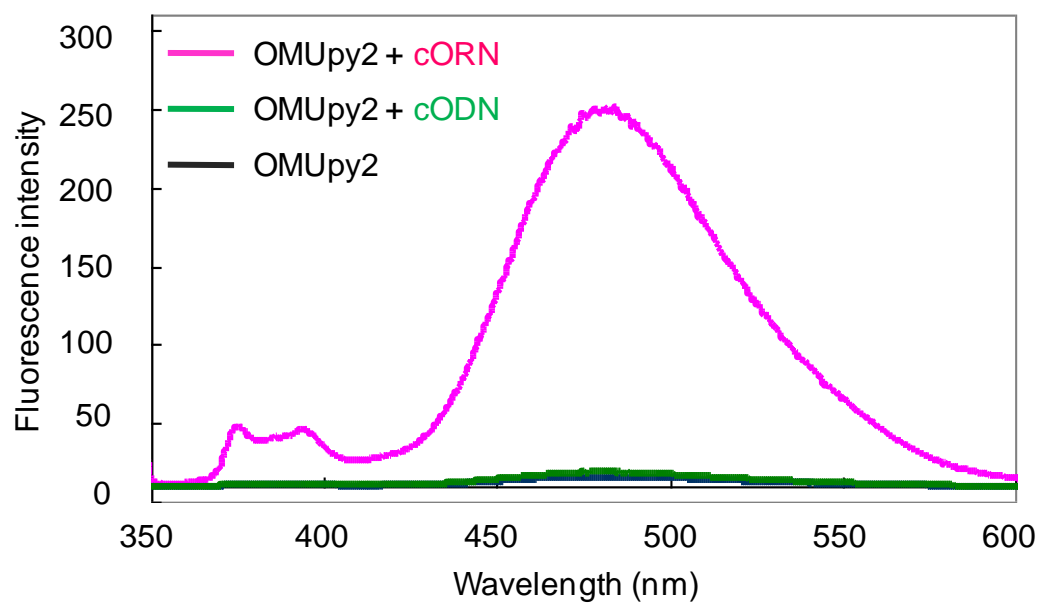


Figure S2. Fluorescence spectra of OMUpy2 and the duplex with complementary oligoribonucleotide.

Buffer: 10 mM phosphate buffer (pH 7.0), 0.1 M NaCl

Concentration: [OMUpy2]=[cORN]=[cODN]=0.5 μ M

Temperature: 11 $^{\circ}$ C

Excitation wavelength: 342 nm

Probe sequences :

OMUpy2-fos: 5'-GAUGUGUUpyUpyCUCCUC-3'

cORN: 5'-GAGGAGAAACACAUC-3'

cODN: 5'-GAGGAGAAACACATC-3'

*Upy: 2'-O-(1-pyrenylmethyl)uridine

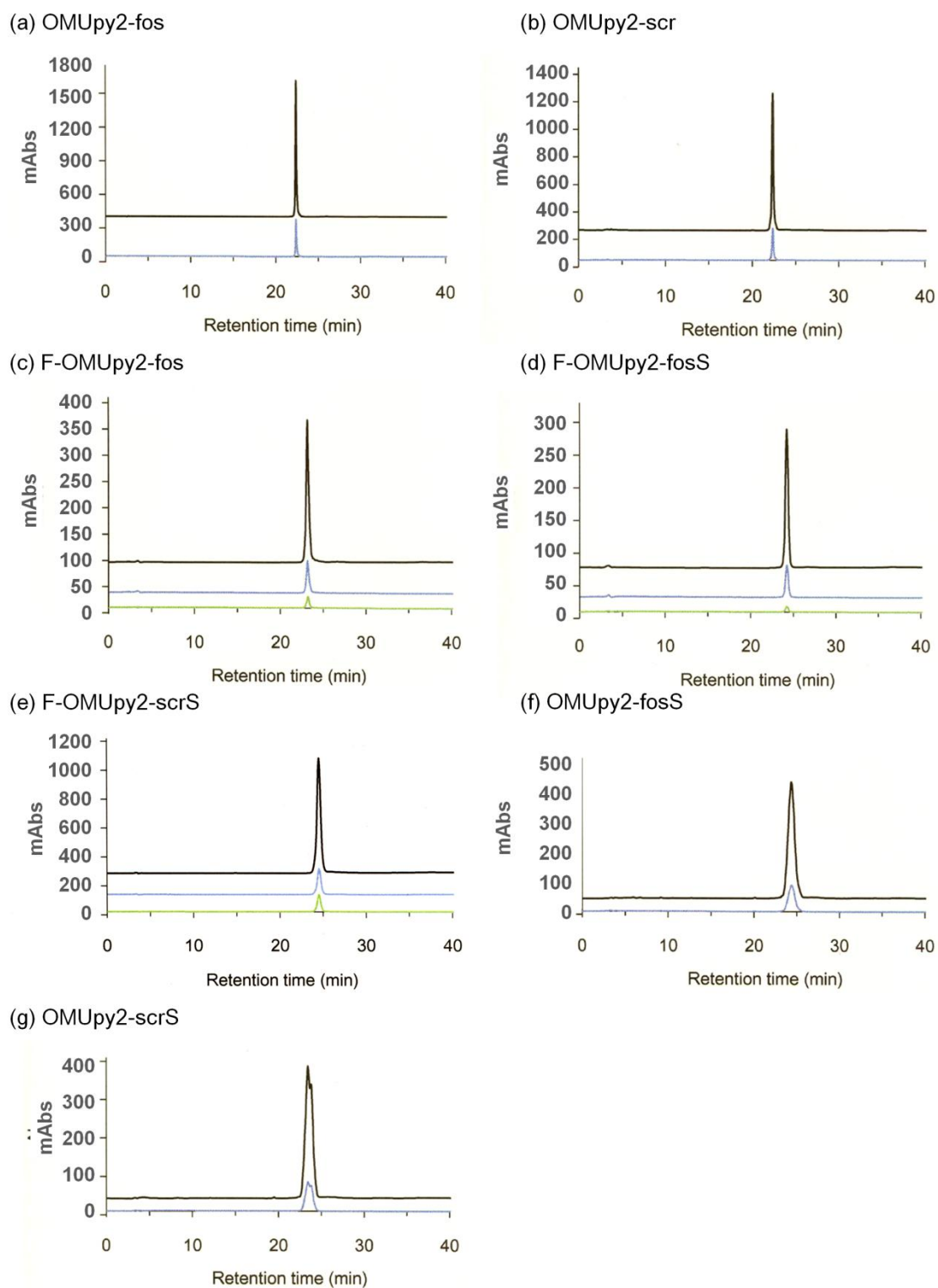


Figure S3. Reversed-phase HPLC charts of each OMUpy2.

Each chart was monitored at 260 nm (oligonucleotides, black line), at 340 nm (pyrene, blue line), and at 480 nm (fluorescein, green line). Acetonitrile gradient was shown in Table 1.

*Note: Since phosphorothioate group is a chiral P center (see Figure S3), the phosphorothioate modified oligonucleotides are mixtures of many isomers (two for each P atom). The peak of phosphorothioate modified oligonucleotides in HPLC chart is broad compared to unmodified oligonucleotides (Figure S2-f and g) and is occasionally separated into two peaks as described in Figure S2-g.

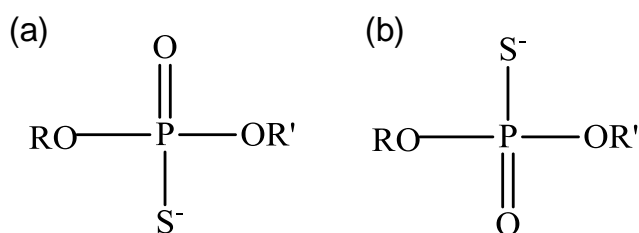


Figure S4. Structure of phosphorothioate analogs. Structures (a) and (b) represent the two stereoisomers due to the presence of the chiral P center.

Table 1. Acetonitrile gradient in 0.1M TEAA (pH 7.0)

Time (min)	0	25	30	33	35	45
Acetonitrile (%)	10	35	50	50	10	10

Table 2. ESI-MS data of each probe.

Probe	Chemical Formula	ESI-MS (<i>m/z</i>)	
		Calcd.	Found
(a) OMUpy2-fos	C ₁₈₆ H ₂₀₆ N ₄₆ O ₁₀₉ P ₁₄	([M-7H] ⁷⁻) 752.555	752.534
(b) OMUpy2-scr	C ₁₈₆ H ₂₀₇ N ₄₇ O ₁₀₆ P ₁₄	([M-8H] ⁸⁻) 654.238	654.191
(c) F-OMUpy2-fos	C ₂₁₀ H ₂₄₄ N ₄₇ O ₁₁₈ P ₁₁₅	([M-7H] ⁷⁻) 829.286	829.264
(d) F-OMUpy2-fosS	C ₂₁₃ H ₂₄₄ N ₄₇ O ₁₀₆ P ₁₅ S ₁₂	([M-7H] ⁷⁻) 856.676	856.665
(e) F-OMUpy2-scrS	C ₂₁₃ H ₂₄₅ N ₄₈ O ₁₀₃ P ₁₅ S ₁₂	([M-7H] ⁷⁻) 851.965	851.985
(f) OMUpy2-fosS	C ₁₈₆ H ₂₀₆ N ₄₆ O ₉₇ P ₁₄ S ₁₂	([M-5H] ⁶⁻) 910.103	910.137
(g) OMUpy2-scrS	C ₁₈₆ H ₂₀₇ N ₄₇ O ₉₄ P ₁₄ S ₁₂	([M-5H] ⁷⁻) 775.234	775.296