

Supporting information for

**Trimethine cyanine dyes as deep-red fluorescent indicators with high selectivity
to the internal loop of the bacterial A-site RNA**

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Japan.

Reagents: All of the RNAs were custom synthesized and HPLC purified by Gene Design Inc. (Osaka, Japan). TO-PRO-3, YO-PRO-3, TO-PRO-1, PO-PRO-1, JO-PRO-1 and YO-PRO-1 were purchased from Thermo Fisher Scientific (Waltham, MA). TO and TO3-C3 were synthesized according to the literature.^[S1] The other reagents were commercially available analytical grade and were used without further purification. Water was deionized (≥ 18.0 M Ω cm specific resistance) by an Elix 5 UV Water Purification System and a Milli-Q Synthesis A10 system (Millipore Corp., Bedford, MA), followed by filtration through a BioPak filter (Millipore Corp., Bedford, MA) in order to remove RNase.

The concentrations of RNAs were determined from the molar extinction coefficients at 260 nm, according to the literature.^[S2]

Unless otherwise mentioned, all measurements were performed in 10 mM sodium cacodylate buffer solutions (pH 7.0) containing 50 mM NaCl and 0.1 mM EDTA. Before measurements, the sample solutions were annealed as follows: heated at 85°C for 10 min, and gradually cooled to 20°C (1°C/min).

Fluorescence measurements Fluorescence spectra were measured with a JASCO model FP-6500 spectrofluorophotometer equipped with a thermoelectrically temperature-controlled cell holder (Japan Spectroscopic Co. Ltd., Tokyo, Japan) using a 3 × 3 mm quartz cell. Excitation wavelength for cyanine dyes was set at the absorption maximum wavelength in the absence of target RNAs. Fluorescence quantum yield was determined relative to cresyl violet in methanol (excitation wavelength, 600 nm).^[S3]

The binding affinity with the dissociation constant (K_d) of the ligand was determined by the fluorescence titration experiments, where the changes in fluorescence intensity of the ligand were monitored as a function of the concentration of target RNAs. The resulting titration curve was analyzed by nonlinear least-squares regression based on a 1:1 binding isotherm as previously described (ref.16 in the main text).

FID assay: The FID assay was carried out with TO-PRO-3 as a fluorescence indicator. Fluorescence response of TO-PRO-3/A-site RNA complex to test compounds was measured in a JASCO model FP-6500 spectrofluorophotometer using a 3 × 3 mm quartz cell. FID assay in the microplate format was carried out at room temperature in a 96-well black plate using a microplate reader (SpectraMax M5, Molecular Devices, CA).

Characterization of TO and TO3-C3:

TO: ¹H NMR (600 MHz, d₆-DMSO) δ 4.02 (s, 3H), 4.18 (s, 3H), 6.95 (s, 1H), 7.41 (m, 2H), 7.61 (m, 1H), 7.79 (m, 2H), 8.04 (m, 3H), 8.62 (d, $J = 10.2$ Hz, 1H), 8.81 (d, $J = 12.6$ Hz, 1H). ESI-MS for C₁₉H₁₇N₂S⁺: calcd, 305.11; found, 305.12.

TO3-C3: ¹H NMR (600 MHz, d₆-DMSO) δ 1.57 (m, 3H), 2.27 (t, $J = 7.2$ Hz, 2H), 3.73 (s, 3H), 4.55 (t, $J = 7.2$ Hz, 2H), 6.49 (d, $J = 12.4$ Hz, 1H), 7.10 (d, $J = 13.2$ Hz, 1H), 7.31 (t, $J = 8.0$ Hz, 1H), 7.49 (t, $J = 8.0$ Hz, 1H), 7.59 (d, $J = 8.0$ Hz, 1H), 7.70 (t, $J = 7.6$ Hz, 1H), 7.86 (m, 2H), 7.94 (t, $J = 7.2$ Hz, 1H), 8.07 (d, $J = 9.2$ Hz, 1H), 8.16 (t, $J = 12.8$ Hz, 1H), 8.43 (m, 2H). ESI-MS for C₂₃H₂₃N₂S⁺: calcd, 359.16; found, 359.15.

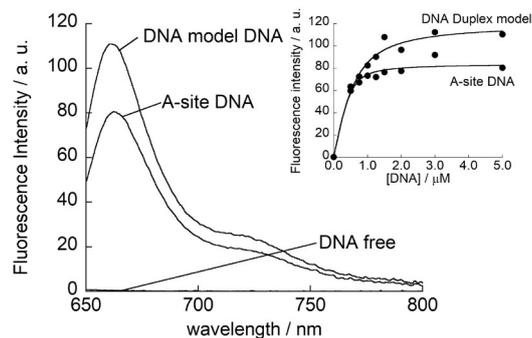


Figure S1. Fluorescence spectra of TO-PRO-3 (0.50 μM) in the absence and presence of target DNAs (5.0 μM): A-site DNA (5'-GGCGT CACAC CTTCG GGTGA AGTCG CC-3') and duplex model DNA (5'-GGCGT CACAC CTTCG GGTGT GACGC C-3'). Inset: Fluorescence titration curves for the binding to target DNAs. Solution conditions are the same as given in Figure 2A in the main text. Temperature, 20°C. Excitation, 632.5 nm. Analysis, 663 nm.

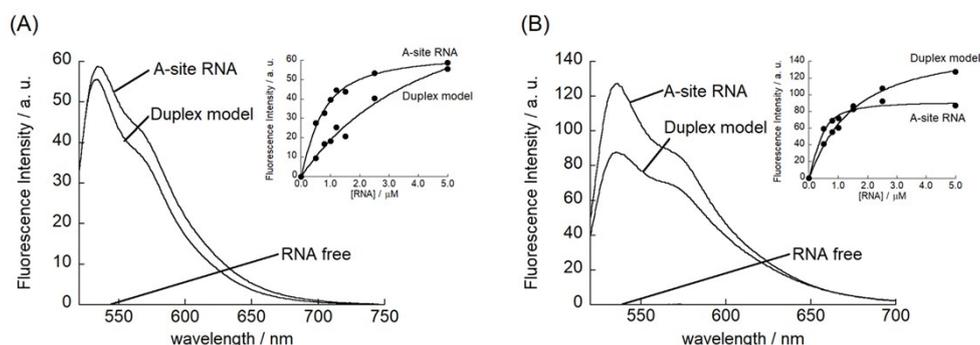


Figure S2. Fluorescence spectra of TO analogues (0.50 μM : (A) TO and (B) TO-PRO-1) in the absence and presence of target RNAs (5.0 μM : A-site RNA and duplex model). Inset: Fluorescence titration curves for binding to target RNAs. Solution conditions are the same as given in Figure 2A in the main text. Temperature, 20°C. Excitation, (A) 501 nm, (B) 508 nm. Analysis, (A) 535 nm, (B) 540.5 nm.

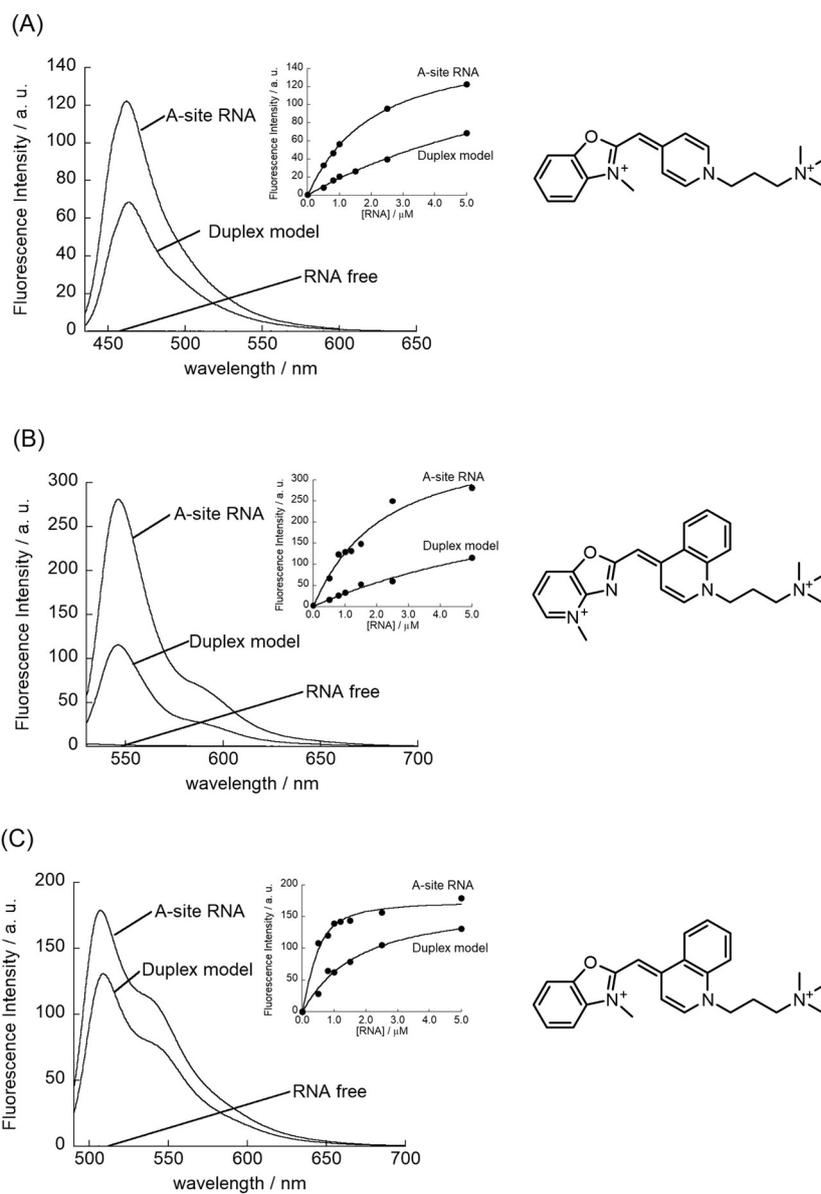


Figure S3. Fluorescence spectra of monomethine cyanine dyes ($0.50 \mu M$: (A) PO-PRO-1, (B) JO-PRO-1 and (C) YO-PRO-1) in the absence and presence of target RNAs ($5.0 \mu M$: A-site RNA and duplex model). Inset: Fluorescence titration curves for binding to target RNAs. Solution conditions are the same as given in Figure 2A in the main text. Temperature, $20^\circ C$. Excitation, (A) 424.5 nm , (B) 519.5 nm , (C) 482.5 nm . Analysis, (A) 462 nm , (B) 546 nm , (C) 507 nm .

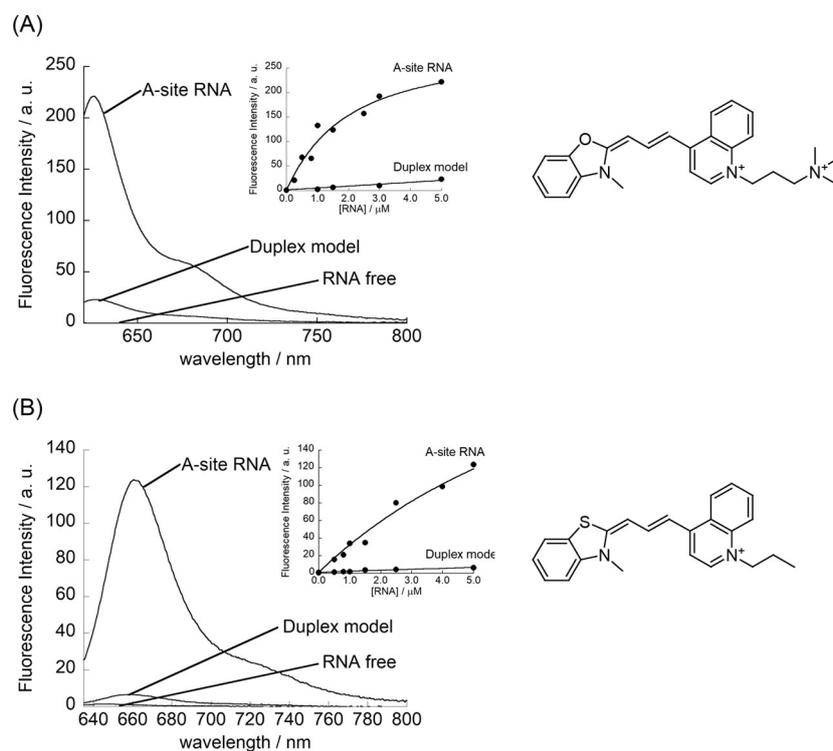


Figure S4. Fluorescence spectra of trimethine cyanine dyes ($0.50 \mu\text{M}$: (A) YO-PRO-3 and (B) TO3-C3) in the absence and presence of target RNAs ($5.0 \mu\text{M}$: A-site RNA and duplex model). Inset: Fluorescence titration curves for binding to target RNAs. Solution conditions are the same as given in Figure 2A in the main text. Temperature, 20°C . Excitation, (A) 612 nm , (B) 624 nm . Analysis, (A) 626 nm , (B) 662.5 nm .

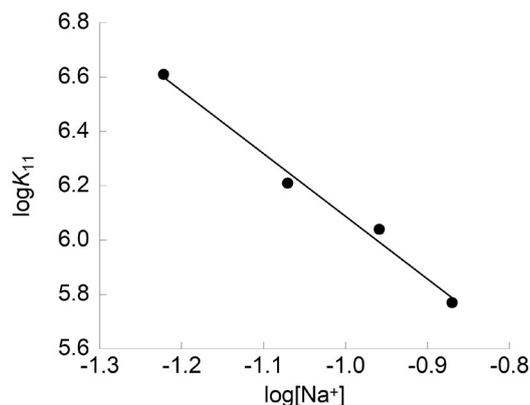


Figure S5. Salt dependence of the binding constants (K_{11}) of TO-PRO-3 for A-site RNA. Binding constants, determined at 20°C in buffer solutions (pH 7.0), were plotted as a function of Na⁺ concentration (50-125 mM).

Salt dependence of the binding affinity was examined in order to determine the apparent charge of TO-PRO-3 bound to the A-site RNA, according to the polyelectrolyte theory.^{S4} As shown in Fig. S5, the binding constants ($K_{11} = 1/K_d$) significantly decreased when the salt concentration increased. The slope (SK) of $\log K_{11}$ and $\log [Na^+]$ was used for the estimation of the apparent charge (Z) on TO-PRO-3 bound to A-site RNA by the equation: $SK = -Z\psi$ (ψ : proportion of counterions associated with each RNA phosphate group). When we assume ψ for A-site RNA to be the same as that for poly(A)-poly(U) RNA duplex ($\psi = 0.89$),^{S4} Z value was obtained as 2.5. This suggests that TO-PRO-3 binds to A-site RNA as the dicationic form. Accordingly, the cationic trimethylammonium moiety of TO-PRO-3 takes part in the binding event, as is consistent with the observed strong binding relative to TO3-C3 (cf. Figs 2A and S4B).

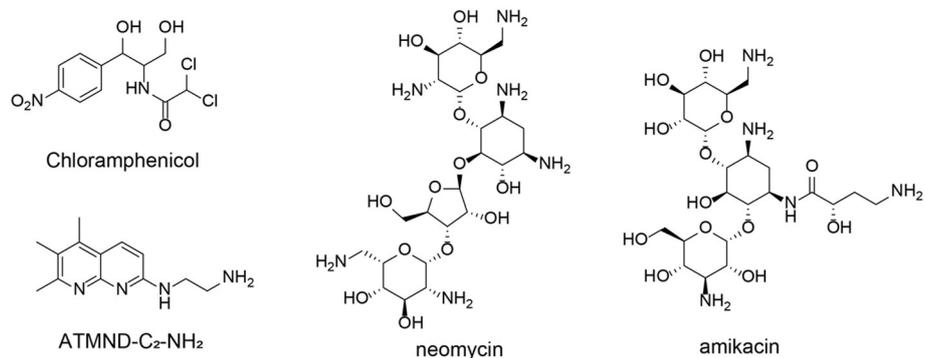


Figure S6. Chemical structures of test compounds used for FID assay. Chemical structure of JO-PRO-1 was shown in Figure S3B.

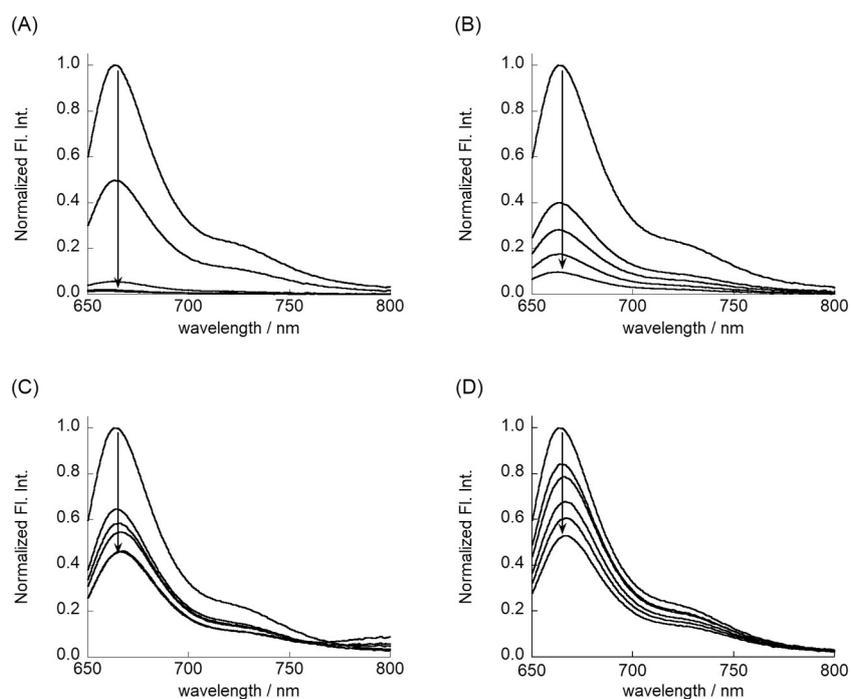


Figure S7. Fluorescence response of TO-PRO-3/A-site RNA complex to test compounds: (A) neomycin, (B) amikacin, (D) ATMND-C₂-NH₂ and (D) JO-PRO-1. [TO-PRO-3], [A-site RNA] = 0.50 μM . [test compound] = 0-50 μM . Other solution conditions are the same as given in Figure 2A in the main text. Temperature, 20°C. Excitation, 632.5 nm.

In Figure S7(C), the observed emission at ca. 800 nm would be attributed to the second order emission from ATMND-C₂-NH₂ (ref. 7 in the main text).

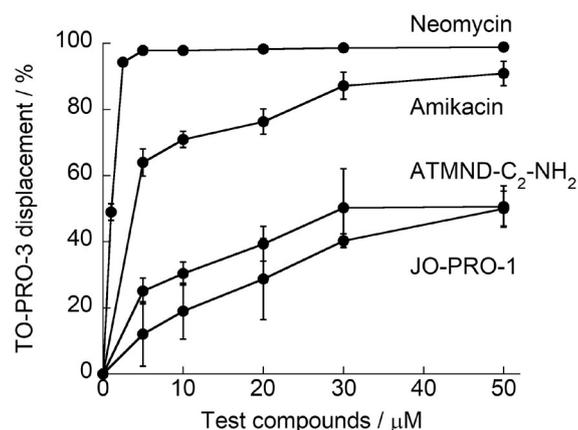


Figure S8. TO-PRO-3 displacement by test compounds in a FID assay. The percentage of TO-PRO-3 displacement was calculated from the following equation: TO-PRO-3 displacement (%) = $1 - F/F_0$ (F and F_0 denote the fluorescence intensity at 663 nm of the TO-PRO3/A-site RNA complex in the presence and absence of test compounds, respectively). Errors are the standard deviations ($N = 3$). Solution conditions are the same as given in Figure 2A in the main text. Temperature, 20°C. Excitation, 632.5 nm.

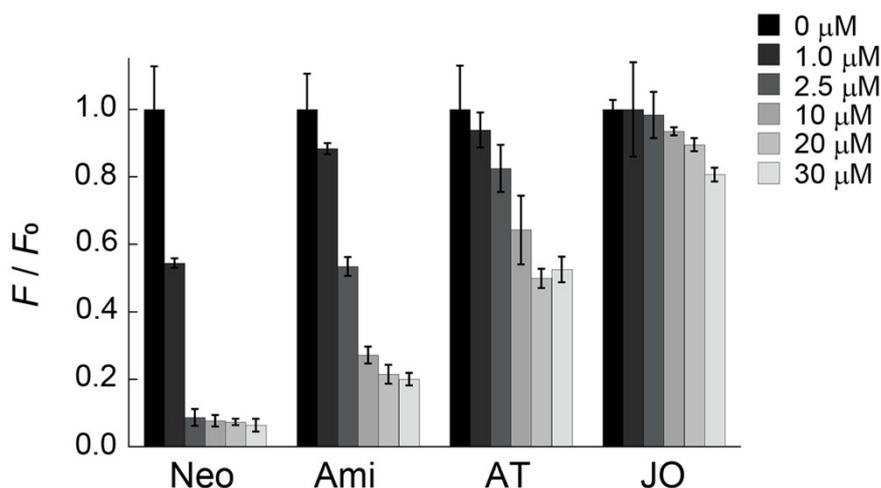


Figure S9. FID results for test compounds (Neo: neomycin, Ami: amikacin, AT: ATMND-C₂-NH₂, JO: JO-PRO-1) in microplate platform. [TO-PRO-3], [A-site RNA] = 0.50 μM . [Test compounds] = 0-30 μM . F and F_0 denote the fluorescence intensities of TO-PRO-3/A-site RNA complex in the presence and absence of test compounds, respectively. Other solution conditions are the same as given in Figure 2A in the main text. Experiments were done at room temperature. Excitation, 600 nm. Analysis, 662 nm (cut off filter: 630 nm). Errors are the standard deviations obtained from three independent experiments ($N = 3$).

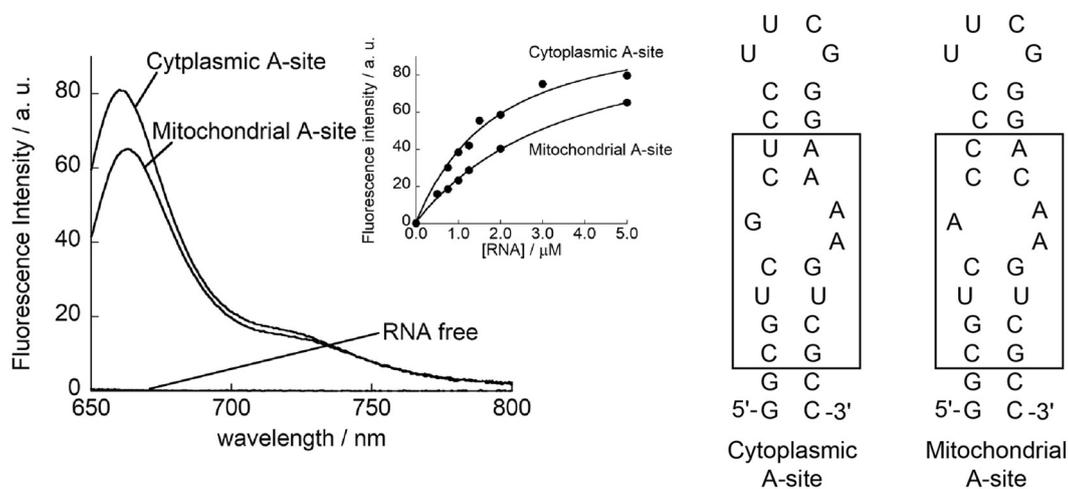


Figure S10. Fluorescence response of TO-PRO-3 (0.50 μM) for human cytoplasmic and mitochondrial A-site RNAs (5.0 μM). Inset: Fluorescence titration curves for binding to target RNAs. Solution conditions are the same as given in Figure 2A in the main text. Temperature, 20°C. Excitation, 632.5 nm. Analysis, 663 nm. Sequences of human cytoplasmic and mitochondrial A-site RNAs used here are also shown.

References

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