

Electronic supplementary information (ESI)

Fluorescence imaging of potassium ion in living cells using a fluorescent probe based on a
thrombin binding aptamer-peptide conjugate

**Keiichi Ohtsuka,^a Shinobu Sato,^a Yusuke Sato,^a Kojiro Sota,^a Shinsuke Ohzawa,^a
Tomoki Matsuda,^b Kiwamu Takemoto,^b Nobutoki Takamune,^c Bernard Juskowiak,^d
Takeharu Nagai,^b and Shigeori Takenaka^{a*}**

^a *Department of Applied Chemistry, Graduate School of Engineering, Kyushu Institute of
Technology, Fukuoka 804-8550 (Japan)*

^b *Research Institute for Electronic Science, Hokkaido University, Hokkaido 001-0020 (Japan)*

^c *Department of Pharmaceutical Biochemistry, Kumamoto University, Kumamoto 862-0973 (Japan)*

^d *Faculty of Chemistry, A. Mickiewicz University, Poznan 60-780 (Poland)*

1. Materials and apparatus

Reagents for peptide synthesis were purchased from Watanabe Chemical Industries Ltd. (Hiroshima, Japan). 3'-FAM modified oligonucleotide was custom synthesized by Genosys (Sigma-Aldrich, Louis, MO). Sulfosuccinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (sulfo-SMCC) was purchased from Thermo Fisher Scientific Inc. (Rockford, IL). 5(6)-carboxytetramethyl-rhodamine succinimidyl ester was purchased from Invitrogen (Carlsbad, CA).

Peptide was synthesized by Fmoc chemistry using a Model 433A peptide synthesizer (Applied Biosystems, Foster City, CA). A fluorescence spectrum was measured in LS-50 Luminescence spectrometer (Perkin Elmer, Waltham, MA). Matrix-assisted laser desorption ionization-TOF MS (MALDI-TOF MS) was measured by Voyager Linear-SA mass spectrometer (PerSeptive Biosystems, Framingham, MA) with α -cyano-4-hydroxycinnamic acid (α CHCA) or 3-hydroxy propionic acid as a matrix for peptide or oligonucleotide, respectively. Analysis and purification by reversed phase HPLC were carried out using a Hitachi HPLC system composed of an L-4200 UV-VIS detector, an L-6200 intelligent pump, an L-6000 pump, and a D-2600 chromato-integrator. Inertsil ODS-3 column (4.6 x 250 mm) (GL Science Inc., Tokyo, Japan) or Mightysil RP-18 column (4.6 x 250 mm) (Kanto Chemical Co., Inc., Japan) were used for peptide or oligonucleotide separation, respectively. Circular dichroism spectra were measured by a Jasco J-820 Spectropolarimeter (JASCO Corp. Tokyo, Japan).

2. Preparation of the peptide-oligonucleotide conjugate (PSO-5, 5)

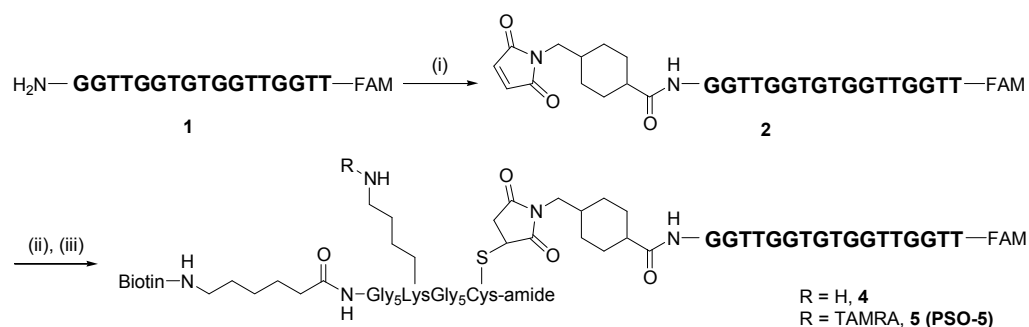


Figure S1. Synthetic route of PSO-5. (i): sulfo-SMCC/PBS buffer (pH 7.4), (ii): Spacer peptide **3** (Biotin-AcpGly₅LysGly₅Cys-amide)/10 mM potassium phosphate buffer (pH 7.0), (iii): Tetramethylrhodamine (TAMRA) succinimidyl ester/PBS buffer (pH 7.4).

PSO-5 was synthesized according to the route shown in Figure S1. The reaction

conditions used were similar to those described by Balasubramanian et al.¹⁾ Oligonucleotide **2** carrying maleimide and FAM at 5'- and 3'-termini, respectively, and a spacer peptide **3** carrying biotin and cysteine at C- and N-termini, respectively, were synthesized separately. After the maleimide and cysteine parts were linked, TAMRA was attached to ϵ -amino moiety of lysine to obtain PSO-5 probe (**5**).

2-1. Synthesis of spacer peptide (3)

Spacer peptide (**3**) was designed to keep the proper distance between FAM as donor attached to oligonucleotide and TAMRA as acceptor linked with peptide segment. This spacer peptide was additionally biotinylated at N-terminal. The peptide synthesis was carried out using the peptide synthesizer and 50 mg of the obtained peptide-elongated resin was dissolved in 1 mL of the solution containing 92.5 % trifluoroacetic acid, 2.5 % water, 2.5 % triisopropylsilane, and 2.5 % ethanedithiol and kept for 1.5 h at room temperature to cleave the synthesized peptide from the resin and remove the protection group. The reaction mixture was filtrated through glass filter and the filtrate was mixed with 150 mL diethyl ether. The obtained white powder was collected by centrifugation. The peptide was purified by the reversed phase HPLC with the Inertsil ODS-3 column under gradient elution from 7 % to 21 % of acetonitrile in water containing 0.1 % TFA, at the flow rate of 1 mL/min. The elution was monitored at 210 nm and a fraction containing target peptide was collected and lyophilized. MALDI-TOF mass spectrum of the obtained white powder was measured with α CHCA as a matrix and obtained peak was identified as $[M+H]^+ = 1159.74$, which is in good agreement with the theoretical value of $[M+H]^+ = 1159.32$.

2-2. Preparation of oligonucleotide (2) carrying maleimide and FAM at 5'- and 3'-termini, respectively.

A 17-meric oligonucleotide with thrombin binding aptamer sequence (**1**) carrying amino moiety and FAM at 5'- and 3'-termini (53.1 nmol) was dissolved in 106.2 μ L of PBS buffer (pH 7.4) and 2 mg of sulfo-SMCC was added to this solution and mixed with a Vortex mixer for 1 h. The target oligonucleotide (**2**) was purified with NAP-5 column (GE Healthcare Life Science, Japan) by elution with 600 μ L water.

2-3. Linkage reaction between the spacer peptide (3) and the modified oligonucleotide (2)

The aqueous eluate from the NAP-5 column containing oligonucleotide (**2**) carrying maleimide and FAM at 5'- and 3'-termini, respectively (600 μ L) was mixed with 60 μ L of 1 M potassium phosphate buffer (pH 7.0) and 2 mg of the spacer peptide (**3**) was added

to this solution and vortexed for 4 h. The target conjugate was chromatographed by the reversed phase HPLC with the Mightysil RP-18 column using gradient elution from 10 % to 70 % acetonitrile in aqueous 0.1 M TEAA buffer (pH 7.0) for 30 min, monitored at 260 nm. The fraction containing the conjugate (4) was lyophilized.

2-4. Labeling the conjugate (4) with TAMRA

The DMSO solution (20 μ L) containing 0.4 mg of 5(6)-carboxytetramethyl-rhodamine succinimidyl ester was mixed with PBS buffer (pH 7.4) (500 μ L) containing 25.3 nmole of the conjugate (4) and the mixture was vortexed for 4 h at room temperature. The target conjugate was chromatographed by the reversed phase HPLC with the Mightysil RP-18 column using gradient elution from 10 % to 70 % acetonitrile in 0.1 M TEAA buffer (pH 7.0) for 30 min, monitored at 260 nm. The fraction containing the target conjugate (5) was lyophilized. MALDI-TOF mass spectrum was measured by the negative mode with 3-hydroxypropionic acid as a matrix and obtained peak at $m/z = 7956.28$ was in agreement with the theoretical value of $[M+Na-H]^- = 7956.52$.

2-5. Reversed phase HPLC of PSO-5 (5) and its intermediates, 1, 2, and 4.

PSO-5 and its intermediates were analyzed by reversed phase HPLC equipped with the Mightysil RP-18 column (Kanto Chemical Co., Inc.) using linear gradient from 0 % to 100 % of Eluent B, where Eluent A or B were 0.1 M TEAA buffer containing 0% or 70 % CH_3CN , respectively. The collected chromatograms are shown in Fig. S3.

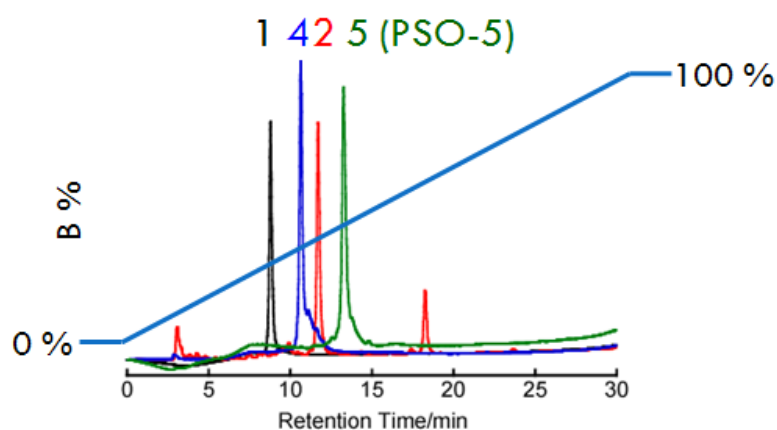


Figure S2. Reversed phase HPLC of PSO-5 (5) and its intermediates, 1, 2, and 4. HPLC separations were conducted with the Mightysil RP-18 column using linear gradient from 0 % to 100 % of Eluent B, where Eluent A or B were 0.1 M TEAA buffer containing 0% or 70 % CH_3CN , respectively.

2-6. Native gel electropherogram of PSO-5 (5) and its intermediates, 1, 2, and 4.

PSO-5 and its intermediates were also analyzed by 12.5% polyacrylamide gel (19/1). Electrophoresis was carried out in 0.7 x TBE buffer and results are shown in Fig. S3. The L lane denotes DNA ladder (20 bp, TaKaRa, Japan). Gel was stained with GelStar. GelStar is known to show emission at 527 nm under excitation at 493 nm, which overlapped the FAM emission band. Color of Lanes 1, 2, and 4 was different from that for lane 5 and this may result from direct excitation of FAM in intermediates 1, 2, and 4. Lane 5 showed the band with an orange color suggesting the contribution from TAMRA emission generated by FRET from FAM.

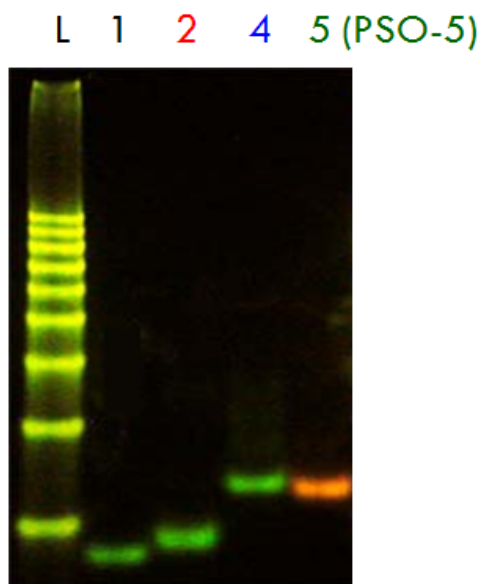


Figure S3. Native polyacrylamide gel electropherogram of PSO-5 (5) and its intermediates, 1, 2, and 4. Electrophoresis was carried out in 0.7 x TBE buffer. Lane L refers to DNA ladder (20 bp, TaKaRa, Japan)

3. Synthesis of biotinylated nuclear export signal peptide (B-NES₂)

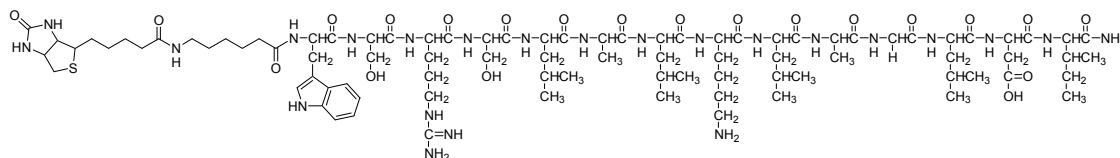


Figure S4. Chemical structure of biotinylated nuclear export signal peptide (B-NES₂).

Chemical structure of biotinylated nuclear export signal peptide (B-NES₂) was shown in

Fig. S4. This peptide contained amino acid sequence of Nuclear Export Signal of Protein Kinase Inhibitor²⁾ and was sequentially synthesized on an Fmoc-NH-SAL resin (Watanabe Chemical Ind., Ltd., Japan) with Fmoc chemistry. The obtained peptide resin conjugate (50 mg) was incubated in 1 mL of solution containing 95% trifluoroacetic acid, 2.5% water, and 2.5% triisopropylsilan for 1.5 h at room temperature to cleave the synthesized peptide from the resin and to remove the protecting group. The reaction mixture was filtrated and the filtrate was concentrated. The residue was dissolved in 20 mL diethyl ether and the obtained powder was collected by centrifugation. The peptide was purified by reversed phase HPLC with Inertsil ODS-3 column under flow of 1 mL/min with linear gradient from 21% to 35% acetonitrile in water containing 0.1% TFA for 20 min. After lyophilization, MALDI-TOF mass spectrum of the obtained white powder was measured with α CHCA as a matrix and the observed signal of $[M+H]^+ = 1888.5$ was in good agreement with the theoretical value of $[M+H]^+ = 1882.30$.

4. Fluorescence titration of PSO-5 with potassium or sodium ion

The 2 mL volume of 0.2 μ M PSO-5 in 20 mM Tris-HCl buffer (pH 7.4) was placed in a quartz cell (1 cm light path) and fluorescence spectra were measured in the 500 – 700 nm spectral range (excitation wavelength at 495 nm, PMV = 700 V, and 10 nm slits) upon addition of KCl or NaCl solution (volume change was neglected).

5. CD titration of PSO-5 with sodium or potassium ion

The 0.7 mL volume of 0.2 μ M PSO-5 in 20 mM Tris-HCl buffer (pH 7.4) was placed in a quartz cell with 1 cm light path length and CD spectra were measured upon addition of KCl or NaCl solution using following conditions: sensitivity, 100 mdeg; scan rate, 50 nm/min; response, 4 s; data collecting interval, 0.2 nm; band width, 2 nm; accumulated time, 4 times.

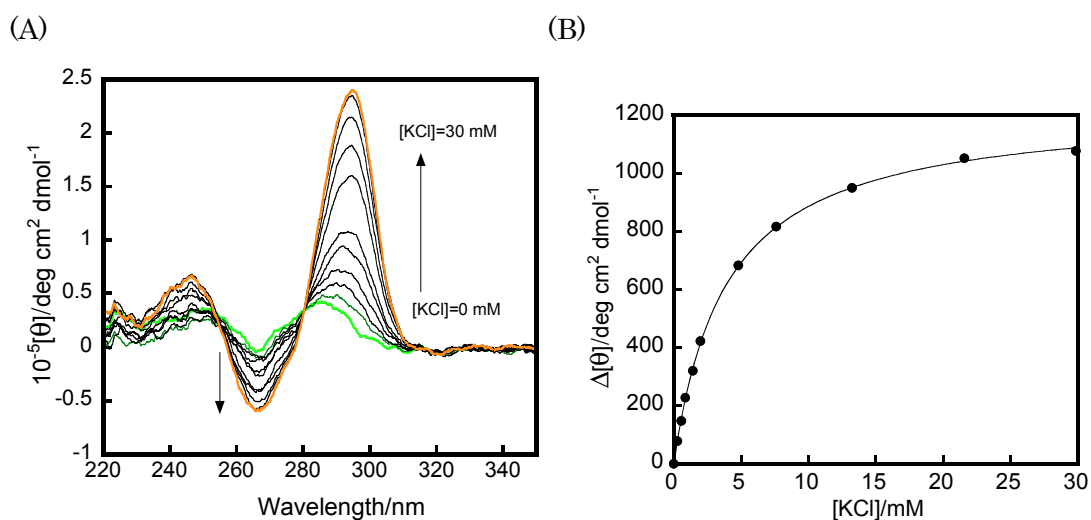


Figure S5. (A) CD spectra of PSO-5 titrated with K^+ in 20 mM Tris-HCl (pH 7.4) at 25 °C. These spectra show changes typical for the two-state system of free and K^+ -bound probe. (B) Plot of CD signal against K^+ concentration. Using the equation of $\Delta[\theta] = A \times [\text{K}^+] / (K_d + [\text{K}^+])$, the dissociation constant of PSO-5 with K^+ was obtained as 2.5 mM, which is slightly lower than that obtained from FRET changes.

6. Introduction of the probe into living cell for fluorescence imaging experiment

HeLa cell was cultured with DMEM and FBS medium in a glass-bottom dish (35 mm × 10 mm, polystyrene, Corning, Japan) at 37°C under 10 % CO_2 incubation (80% confluent). Introduction of the probe into the cell was achieved according to the following procedure. Medium was removed and the dish was washed with 2 mL of PBS buffer. The solution containing 7.5 μM PSO-5, 7.5 μM streptavidin, and 22.5 μM B-NES was placed onto the cell and 106 μM silanized glass beads was subsequently placed on the cell and the dish was gently swung. Imminently beads and excess of the probe were removed by the washing with 2 mL of PBS buffer twice. Finally, the cell was cultured with 1.5 mL DMEM, 150 μL 10% FBS, and no Phenol Red at 37°C for 4 h under 10% CO_2 .

7. Fluorescence imaging experiments

Confocal laser fluorescence inverted microscopy was carried out using Spectral Confocal Microscopy System A1R (Nikon, Japan) with Ti-E [Inverted Microscopy Ti-E (Nikon)]. Oil immersion lens at 60-fold magnification was used as an object glass and the imaging was carried out on an incubation stage for living cell (Stage Incubation System

INUG2-TIZ (Tokai HIT)) at 37°C under 10% CO₂ atmosphere. HeLa cell cultured on the glass bottom dish was placed on the stage after introduction of the probe. To change the potassium concentration in the cell, the following drugs were added to the culturing medium (i) Amphotericin B solution in DMSO as an accelerator of K⁺ efflux from cell, (ii) Ouabain as an inhibitor of K⁺ influx to cell. Drugs were mixed with 500 μL DMEM to obtain final concentration of 10 μM for each compound. After addition of this solution, fluorescence imaging was achieved with the Ar laser source as excitation light and the images were collected every 10 min. The obtained images were analyzed by the software (NIS-Elements) and obtained changes in the fluorescence spectra were exploited to plot FRET efficiency (F_{585}/F_{517} ratio) against time.

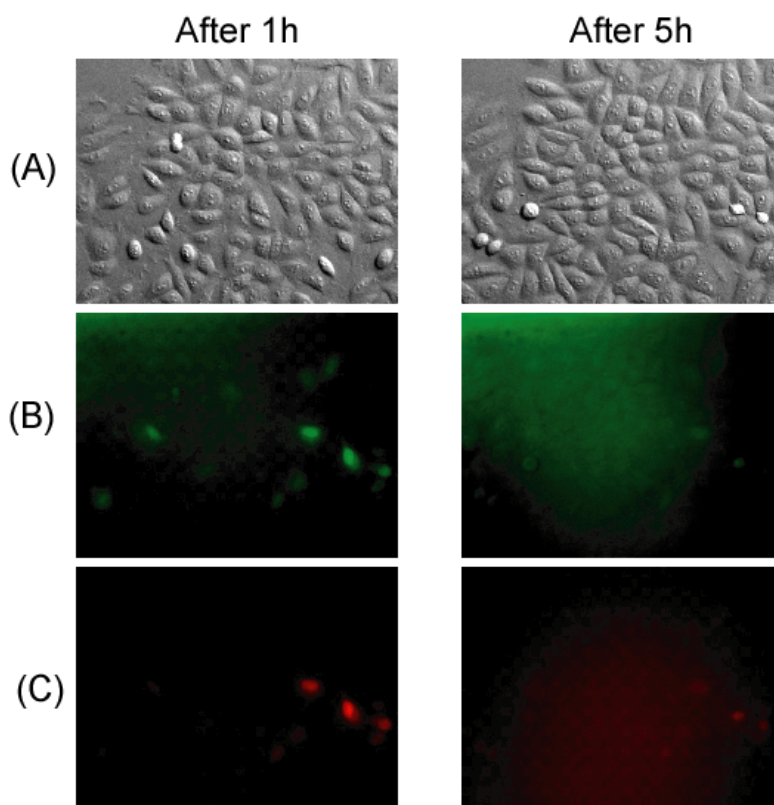


Figure S6. (A) DIC and (B and C) fluorescence images of HeLa cell loaded with PSO-5. PSO-5 was localized in the nuclei of the cells. Fluorescence emission filter: 510-560 nm (B) and 573-613 nm (C); excitation filter: 460-500 nm.

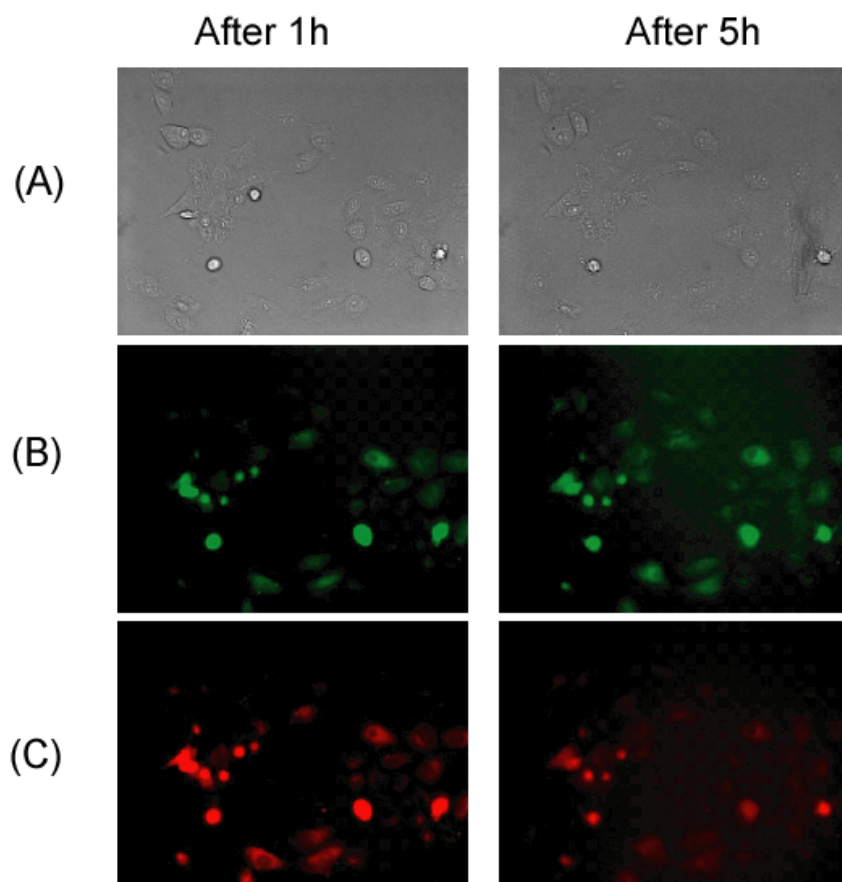


Figure S7. (A) DIC and (B and C) fluorescence images of HeLa cells loaded with the complex of PSO-5 with streptavidin. PSO-5 was localized in the nuclei of the cells. Fluorescence emission filter: 510-560 nm (B) and 573-613 nm (C); excitation filter: 460-500 nm.

Table S1. Percentage of cell death within 5 h after loaded with PSO-5.

	Rate of cell death(5hr)/%	N
None	0	100
PSO-5 alone	18	85
PSO-5 + streptavidin	19	152
PSO-5 + streptavidin + B-NES	14	177

8. Fluoresce imaging experiments using PBF1 AM as a commercially-available K⁺ sensitive probe

The fluorescence images of K⁺ in cell and its time dependent concentration profiles have not been reported before. Similar experiments were carried out with PBF1 AM (Invitrogen), a commercially-available K⁺ sensitive probe. This probe was also introduced into a HeLa cell and fluorescence images were collected after addition of Amphotericin B and Ouabain. However, fluorescence changes resulted from variation in intracellular K⁺ concentration could not be monitored because of photodegradation of the probe during the experiment.

9. ESI mass spectra of the ternary system composed of streptavidin, PSO-5 and B-NES.

Ten mM ammonium acetate (pH 6.9) containing the following components: (A) 1.0 μM streptavidin, (B) 1.0 μM streptavidin and 3.0 μM B-NES, (C) 1.0 μM streptavidin and 1.4 μM PSO-5, or (D) 1.0 μM streptavidin, 3.0 μM B-NES, and 1.4 μM PSO-5. Thirty μL of each sample solution were introduced to electrospray ionization quadrupole time-of-flight mass spectrometer (ESI Q-TOF-MS, model: Bio Tof Q; Bruker Daltonics Inc., Billerica, MA) through an infusion pump at a flow rate of 8 μL/min. Measurement conditions were as follows: dry N₂ gas temperature, 100°C; capillary voltage, 3500 V. The obtained mass spectra were analyzed by the Data analysis software Version 3.1 (Bruker Daltonics Inc.).

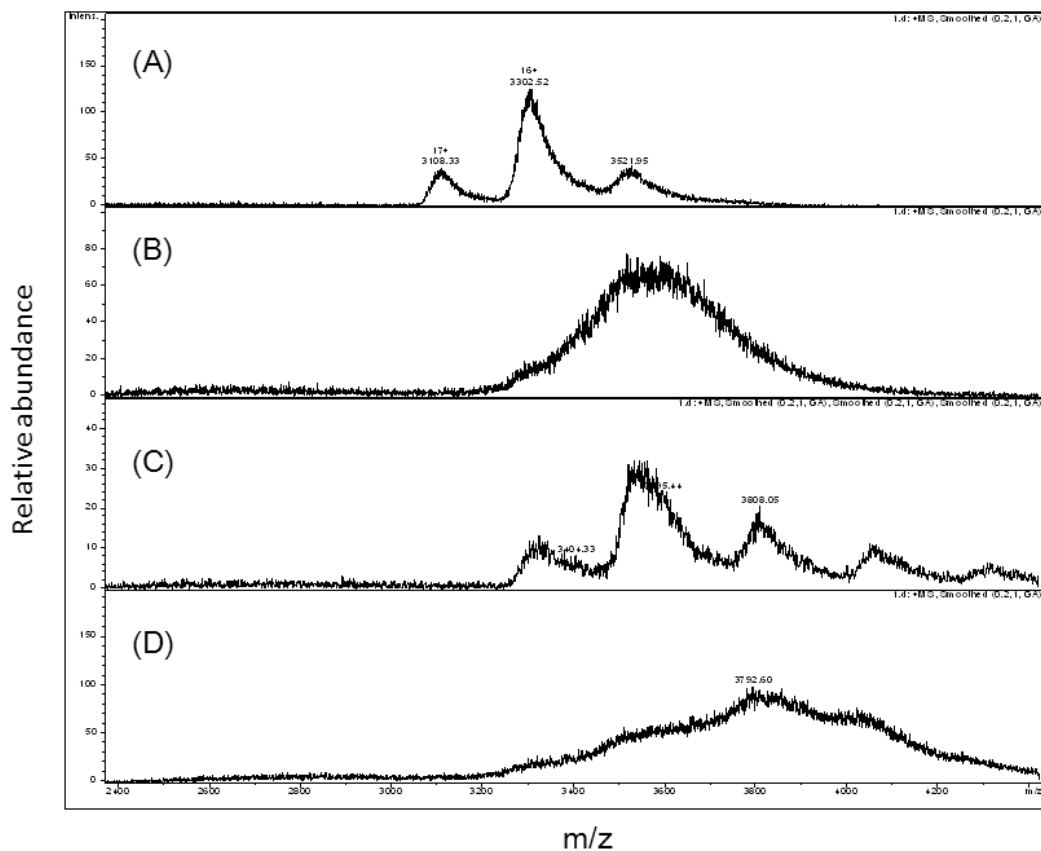


Figure S8. ESI mass spectra with peaks corresponding to the following samples: (A) 1.0 μM streptavidin, (B) 1.0 μM streptavidin and 3.0 μM B-NES, (C) 1.0 μM streptavidin and 1.4 μM PSO-5, or (D) 1.0 μM streptavidin, 3.0 μM B-NES, and 1.4 μM PSO-5.

Figure S8(A) shows the peaks corresponding to a macromolecule with MW of ca. 53000 as 17+, 16+, and 15+ charged species, which is in agreement with MW of the tetraplex of streptavidin. Figure S8(B) shows peaks with low resolution in the case of the mixture of 1 μM streptavidin and 3 μM B-NES. These peaks are distributed in higher molecular weight range than in the case of streptavidin, which suggests complexation of B NES by streptavidin. Stoichiometry of the complex cannot be precisely determined.

Figure S8(C) shows the MS spectrum for the mixture of 1 μM streptavidin and 1.4 μM PSO-5. In the case of this system, the peaks corresponding to the 1 : 1 assembly with molecular weight of ca. 61000 at 16+ and 15+ charged states and that of 1:2 stoichiometry with MW of 69000 as 21+ and 20+ charged states can be assigned. Figure S8(D) shows the MS spectrum for the mixture of 1 μM streptavidin, 3 μM B-NES, and

1.4 μM PSO-5. The obtained peaks exhibit low resolution, but are shifted to higher m/z values than in the case of the complex between streptavidin and PSO-5 alone. The molecular weight of a species represented by the MS peaks was estimated as about 65000, which is consistent with the existence of the ternary 1 : 1 : 3 complex of streptavidin, PSO-5, and B-NES, respectively.

10. MALDI TOF MS spectra of the ternary system composed of streptavidin, PSO-5 and B-NES.

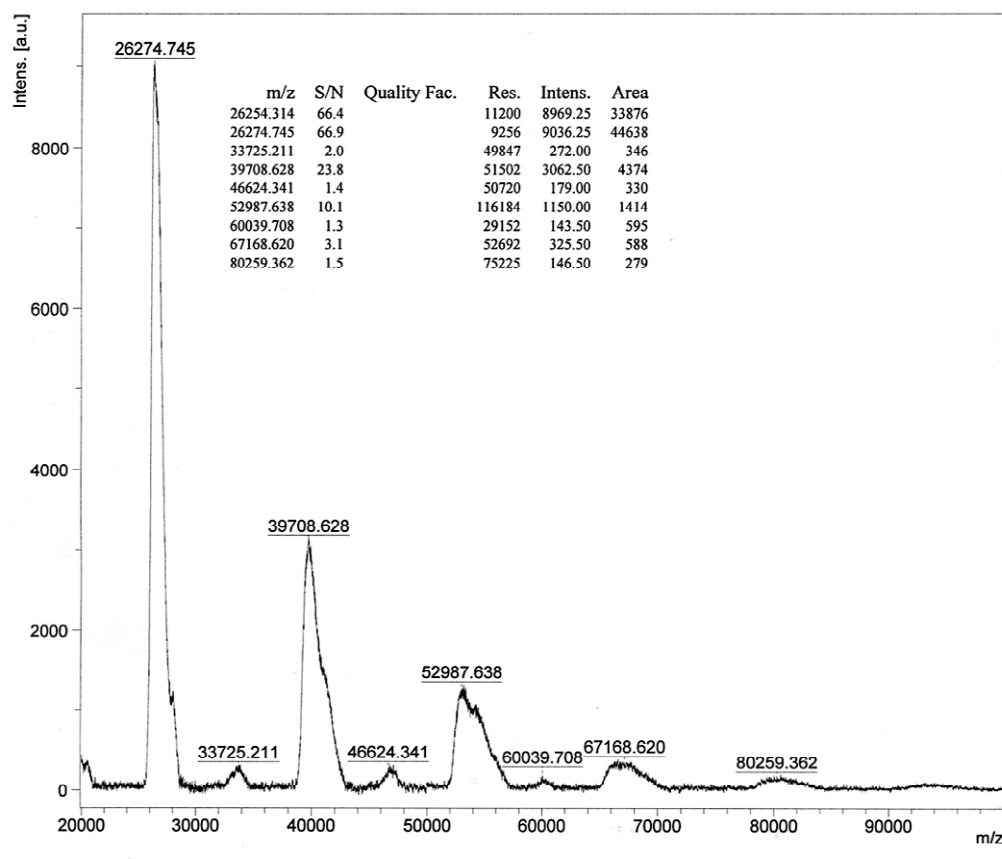


Figure S9. MALDI-TOF-MS spectrum (microflex, Bruker Daltonics Inc., Billerica, MA) of the mixture of B-NES, PSO-5, and streptavidin at 3 : 1 : 1. Peaks around 33725.211 and 67168.620 m/z were in agreement with 2- (33551.5) and 1- (67103) charged species of the 3 : 1 : 1 complex expected. Peaks around 26274.745 and 52987.638 were assigned as 2- (2650) and 1- (5300) charged species of streptavidin.

11. Native PAGE of the ternary complex of PSO-5 with streptavidin and B-NES

To estimate the stoichiometry of PSO-5/B-NES/streptavidin ternary complex, the native PAGE was carried out under the several mixing ratios. A 12.5 % polyacrylamide gel (acrylamide : bisacrylamide = 19 : 1) was prepared and 10 μL of the sample (1 μM PSO-5 (lane 1); 1.0 μM PSO-5 and 1.0 μM streptavidin in the absence (lane 2) or the presence of 3.0 μM B-NES (lane 3)) was applied to each well and electrophoresis was conducted with 0.7 x TBE buffer at 200 V for 5 min and at 120 V for 150 min. After electrophoresis, the gel was stained with GelStar and photography was collected under UV transilluminator.

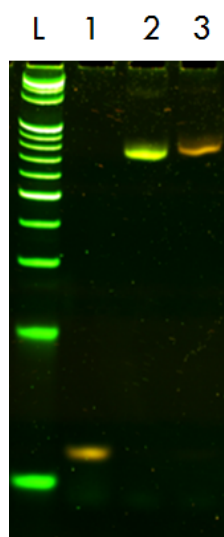


Figure S10. A 12.5% polyacrylamide gel electropherogram of 1 μM PSO-5 (**5**) (lane 1) and the mixture between 1.0 μM PSO-5 and 1.0 μM streptavidin in the absence (lane 2) or presence of 3.0 μM B-NES (lane 3). Electrophoresis was carried out in 0.7 x TBE buffer. Lane L refers to the DNA ladder (20 bp, TaKaRa, Japan).

In Lane 2, a 1 : 1 complex between PSO-5 and streptavidin should be observed because streptavidin alone could not be stained with GelStar. This shows the large retardation resulting from the complex formation of PSO-5 with streptavidin. After addition of B-NES with the amount of three times per streptavidin (lane 3), further retardation was observed. This is result of the increasing in molecular weight of the complex. After complexation between PSO-5 and streptavidin, the fluorescence was changed. This may come from the quenching of FAM or TAMRA with streptavidin. However, this fluorescence was recovered after addition of B-NES. This also suggests that there are no

interactions of FAM or TAMRA with streptavidin after saturation the biotin binding sites of streptavidin by B-NES (see 2.6).

12. Directly conjugated NES-PSO-5 probe

A probe NES-PSO-5 designed by direct conjugation potassium sensing oligonucleotide PSO-5 to the nuclear export signal peptide (NES) as shown below was synthesized and introduced into HeLa cell using bead loading method. This probe was partially localized in cytoplasm, but unfortunately, it was also transported into the nuclei of cells.

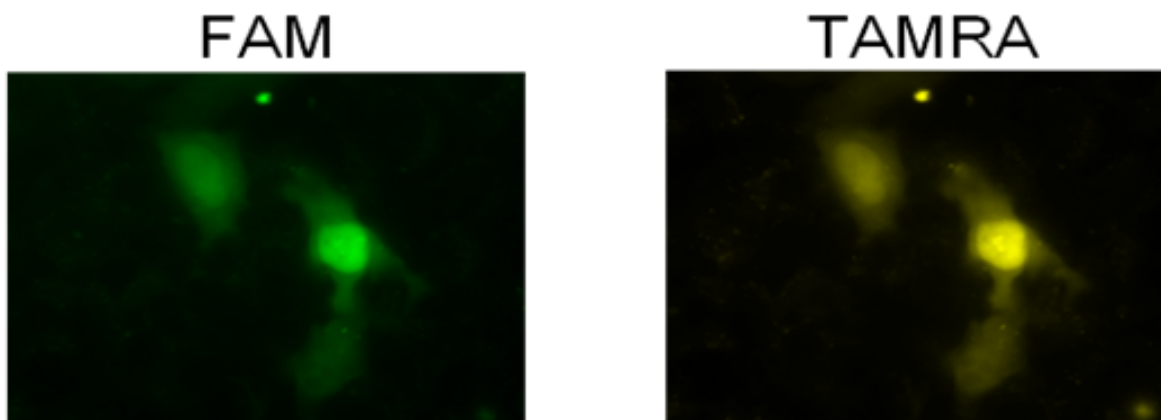
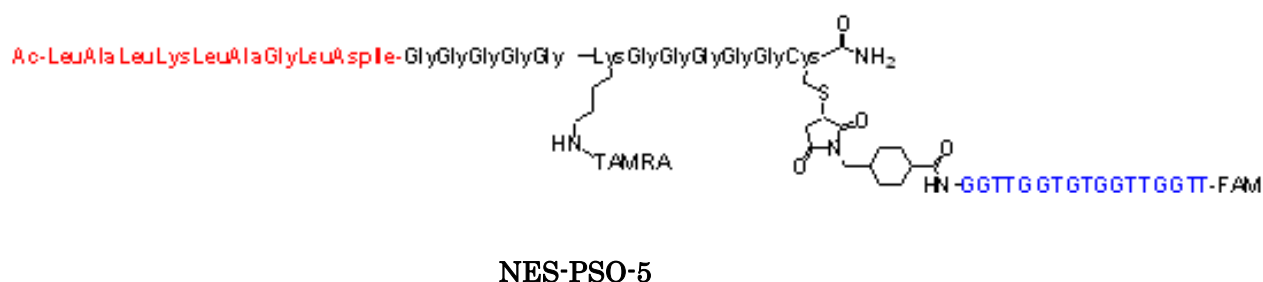


Figure S11. Fluorescence images of HeLa cells loaded with the NES-PSO-5 conjugate was localized in both the nucleus and cytoplasm of the cell.. Fluorescence emission filter: 510-560 nm (FAM) and 573-613 nm (TAMRA); excitation filter: 460-500 nm..

13. Calculation of the distance between FAM and TAMRA in PSO-5

From the Förster radius of $R_0 = 55 \text{ \AA}$ for the combination of FAM and TAMRA fluorophores³⁾ and the expected distance between these dyes far from the critical radius (62 \AA), one can conclude that the two dyes in PSO-5 are located in a shorter distance than expected. The distance between these dyes was estimated as 46 \AA using as a

reference, the oligonucleotide carrying the same sequence of PSO-5 and a single FAM label only as follows.

FRET efficiency (E) and the distance (r) between FAM and TAMRA in PSO-5 were calculated with the following equations.

$$E = 1 - \frac{F_{DA}}{F_D}$$
$$E = \frac{1}{1 + \left(\frac{r}{R_0}\right)^6}$$

where F_D or F_{DA} denote the fluorescence intensity of FAM donor for the probe without TAMRA label and the probe containing both labels, respectively; and R_0 is Forster distance. F_D and F_{DA} were measured using the same experimental conditions. $\lambda_{ex} = 495$ nm.

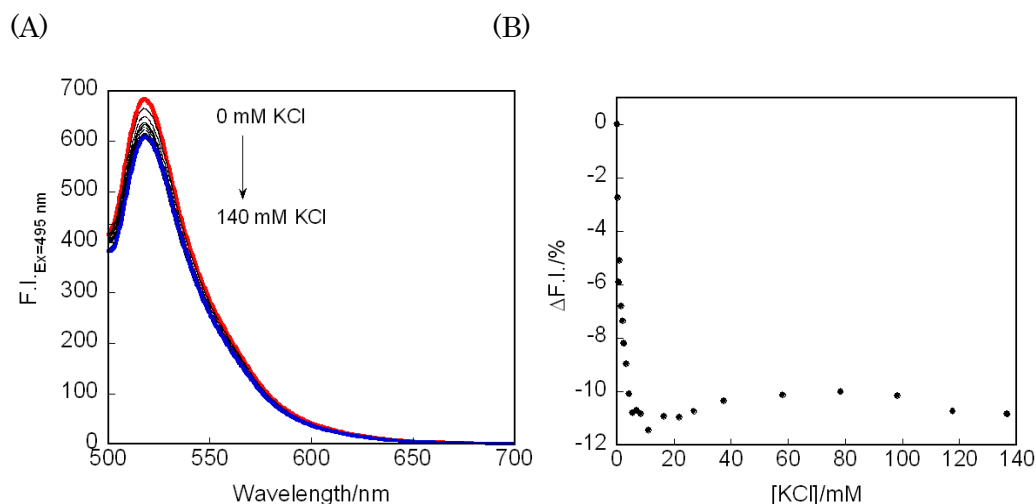


Figure S12. (A) Fluorescence spectra of 0.2 μM PSO-5 derivative having only FAM label and (B) the plot of relative fluorescence intensity against K^+ . $\lambda_{ex} = 495$ nm.

References

- 1) J. G. Harrison, J. G.; Balasubramanian, S. *Nucleic Acids Res.* **1998**, *26*, 3136-3145.
- 2) Wen, W.; Meinkoth, J. L.; Tsien, R. Y.; Taylor, S. S. *Cell* **1995**, *82*, 463-473.
- 3) F. He, Y. Tang, S. Wang, Y. Li and D. Zhu, *J. Am. Chem. Soc.*, **2005**, *127*, 12343.