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Supplementary Information

Fluorescent nanobiosensor for the facile analysis of m^6A RNA demethylase activity

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Reagents

Graphite was purchased from Graphite Kropfmühl AG, Germany. Sodium nitrate was purchased from Junsei, Japan. Sulfuric acid was purchased from Samchun Chemical, Korea. Hydrogen peroxide (30 % in water) and Boric acid were purchased from DaeJung Chemicals & Metals, Korea. Terrific Broth (TB) was purchased from Merck, Germany. Isopropyl β-D-thiogalactopyranoside (IPTG), glycerol, guanidine hydrochloride (GdnHCl), and imidazole were purchased from Amresco, USA. Phenylmethylsulfonyl fluoride (PMSF) was purchased from Roche, Switzerland. Protease inhibitor cocktail and Tris base were purchased from Thermo Fisher Scientific, USA. Dithiothreitol (DTT) was purchased from Promega, USA. HEPES free acid was purchased from Gold Biotechnology, USA. RNase inhibitor was purchased form Cosmogenetech, Korea. Acrylamide/Bis solution (30 %, 29:1) and Ethylenediaminetetraacetic acid (EDTA) were purchased from Bio-rad, USA. SYBR ® Gold nucleic acid gel staining solution was purchased from Life Technologies, USA. Other chemicals were purchased from Sigma-Aldrich, USA, unless otherwise noted.

Oligonucleotides

DNA, RNA oligonucleotides showing below were purchased from Bioneer, Korea.

DNA probe: 5'-Cy5-ACT ATA CAT GTC CAA ACC AA-3'

(m^6A)CA RNA oligo: 5'-UUG GUU UGG (m^6A)CA UGU AUA GU-3'

ACA RNA oligo: 5'-UUG GUU UGG ACA UGU AUA GU-3'

Fragment RNAs (expected products of MazF cleavage): 1) 5'-UUG GUU UGG-3' 2) 5'-ACA UGU AUA GU-3'

Preparation of Nano Graphene Oxide (NGO)

NGO was synthesized from graphite, using a modified Hummer's method.[1] 500 mg of graphite, 500 mg of sodium nitrate and 23 mL of sulfuric acid were mixed in an ice bath with vigorous stirring. 3 g of potassium permanganate was added cautiously in a 35 °C water bath and stirred for 1 h. 40 mL of distilled water added slowly and the reaction temperature was increased to 95 °C with stirring for 30 min. After cooling to the room temperature, the reaction mixture was poured to 200 mL of distilled water with stirring. The reaction was terminated using 5 mL of hydrogen peroxide, then the reaction

mixture was filtered by the Buchner funnel and washed with distilled water. The filter cake was dried and redispersed into distilled water to a final concentration of 1 mg/mL. The graphene oxide (GO) solution was tip-sonicated using tip sonicator 102C(CE) (BRANSON, USA) to prepare NGO.

Characterisation of NGO

The atomic force microscopy (AFM) image and line profile of NGO were obtained by an atomic force microscope NX-10 (Park Systems, Korea). Raman analysis was carried out with LabRAM HR UV/vis/NIR (Horiba Jobin Yvon, France), using an Ar ion CW laser (514.5 nm) as an excitation source focused through a BXFM confocal microscope with an objective lens (50x, numerical aperture= 0.50). Fourier Transform Infrared (FT-IR) measurement of NGO powder was conducted by FT-IR spectrometer NICOLET iS10 (Thermo Scientific, USA). Hydrodynamic diameter and zeta potential were measured by Zetasizer NS90 (Malvern, UK). UV-vis spectrum was obtained by UV/vis spectrophotometer S-3100 (Scinco, Korea).

Cloning, expression, and purification of MazF

Each full-length coding sequence of *Escherichia coli* (*E. coli*) MazF and *E. coli* MazE was synthesized by Integrated DNA Technologies (IDT). The coding sequence of MazF fused to the C-terminal 10×His tag and the coding sequence of MazE were inserted into first multiple cloning site (MCS) and second MCS in pRSFDeut-1 expression vector (Novagen, Germany), respectively. The expression vector was transformed into *E. coli* BL21(DE3) (Novagen, Germany) strains and the *E. coli* cells were grown in TB media at 37 °C until the OD_{600} value reached about 1.2. After induction by adding 0.5 mM IPTG, the cells were further cultured overnight at 18 °C. The harvested cells were lysed by sonication in buffer A (20 mM Tris [pH 7.5], 150 mM NaCl, and 10 % glycerol) supplemented with 2 μg/ml ribonuclease A (RNase A), 2 μg/ml Staphylococcal nuclease,[2] 5 mM $CaCl_2$, 0.1 mM PMSF, and protease inhibitor cocktail. The lysate was clarified by centrifugation at 35,000 g for 1 h. The supernatant was applied onto Ni-NTA resin (Novagen, Germany) equilibrated with Buffer B (20 mM Tris [pH 7.5] and 500 mM NaCl). The resin was washed with buffer C (20 mM Tris [pH 7.5], 500 mM NaCl, and 10 % glycerol). To release the MazE proteins from MazF, the buffer B supplemented with 5 M GdnHCl was added into the Ni-NTA column. Then the resin-bound MazF proteins were refolded by adding the following buffers sequentially: buffer C and buffer B. The resin-bound MazF proteins were eluted using buffer D (20 mM Tris [pH 7.5], 150 mM NaCl, and 0.9 M imidazole). The eluted proteins were further purified using a

Superdex 75 10/300 column (GE Healthcare, USA) equilibrated with buffer A. The fractions containing MazF proteins were pooled, flash-frozen in liquid nitrogen and stored at −80 °C prior to the enzymatic assay.

Cloning, expression, and purification of ALKBH5

The coding sequence of human ALKBH5 (residues 66-292) was amplified by PCR using the full-length sequence of ALKHB5 from Addgene (plasmid no. 38073). The fragment was fused with the coding sequences of an N-terminal His-tagged superfolder GFP (sfGFP) and a human rhinovirus (HRV) 3C cleavage site and inserted into pET28a vector (Novagen, Germany). The expression vector was also transformed into BL21(DE3) cells. Like in the case of MazF, the expression of ALKBH5 was induced by IPTG in TB media. The harvested cells were lysed by sonication in a buffer (20 mM Tris [pH 7.5], 500 mM NaCl, and 10 % glycerol) supplemented with RNase A, Staphylococcal nuclease, $CaCl_2$, PMSF, and protease inhibitor cocktail. After centrifugation, the supernatant was loaded onto the Ni-NTA resin equilibrated with buffer E (20 mM Tris [pH 7.5], 500 mM NaCl, and 2 mM beta-mercaptoethanol). The resin was washed with buffer E supplemented with 40 mM imidazole. And the resin-bound proteins were eluted with buffer E supplemented with 200 mM imidazole. After the HRV 3C protease treatment, the cleaved proteins were loaded on a HiTrap Q column (GE Healthcare, USA) with a salt gradient (100 mM to 1 M NaCl). The fractions containing ALKBH5 proteins were pooled and stored at −80 °C prior to use in the further assay.

MazF activity test

50 pmol of (m^6A)CA or ACA RNA oligo was mixed with 20 pmol of MazF protein in the MazF reaction buffer (20 mM Tris [pH 7.5], 1 mM DTT, and 0.01 % Triton™ X-100). The mixture was incubated 30 min at 37 °C and heat-inactivated at 90 °C for 10 min, using T100™ Thermal Cycler (Bio-rad, USA). The samples were loaded on a 15 % native polyacrylamide gel and electrophoresed in 1X TBE buffer. The gel was stained using SYBR ® Gold nucleic acid gel staining solution and then analysed with ChemiDoc™ MP Imaging System (Bio-rad, USA).

ALKBH5 activity test

50 pmol of (m^6A)CA or ACA RNA oligo was incubated with 200 pmol of ALKBH5 protein in the ALKBH5

reaction buffer (50 mM HEPES [pH 7.4], 150 μM $(NH_4)_2Fe(SO_4)_2{\cdot}6H_2O$, 2 mM L-ascorbate, 300 μM α-ketoglutarate, and 1 U/μL RNase inhibitor) at 37 °C for 1 h and heat-inactivated at 90 °C for 10 min. After the demethylase reaction, 20 pmol of MazF protein and MazF reaction buffer were added to the reaction mixture. The mixture was incubated 30 min at 37 °C and heat-inactivated at 90 °C for 10 min. The samples were loaded on a 15 % native polyacrylamide gel and electrophoresed in 1X TBE buffer. The gel was stained using SYBR ® Gold nucleic acid gel staining solution and then analysed with ChemiDoc™ MP Imaging System.

Fluorescence analysis of RNA oligonucleotides

10 pmol of DNA probe was mixed with 50 pmol of (m^6A)CA RNA or ACA RNA or fragment RNAs and incubated 10 min. After 0.2 mg/mL NGO solution was added and incubated 20 min (total volume= 100 μL), the fluorescence intensity corresponding to Cy5 dye (Ex/Em= 649/666 nm) was measured using a platereader SYNERGY Mx (BioTek Instruments, USA). The fluorescent image of the well plate at the wavelength of Cy5 dye was obtained using ChemiDoc™ MP Imaging System for visualization.

Fluorescence analysis of MazF reaction

50 pmol of (m^6A)CA or ACA RNA oligo was mixed with 20 pmol of MazF protein in the MazF reaction buffer. The mixture was incubated 30 min at 37 °C and heat-inactivated at 90 °C for 10 min. Then 10 pmol of DNA probe was mixed with the reaction mixtures and incubated 10 min. After 0.2 mg/mL NGO solution was added and incubated 20 min (total volume= 100 μL), the fluorescence intensity corresponding to Cy5 dye (Ex/Em= 649/666 nm) was measured using the platereader. The fluorescent image of the well plate at the wavelength of Cy5 dye was obtained using ChemiDoc™ MP Imaging System for visualization.

Fluorescence analysis of ALKBH5 reaction

50 pmol of (m^6A)CA RNA oligo was incubated with 200 pmol of ALKBH5 protein or dead (heat-inactivated) ALKBH5 in the ALKBH5 reaction buffer at 37 °C for 1 h and heat-inactivated at 90 °C for 10 min. After the demethylase reaction, 20 pmol of MazF protein and MazF reaction buffer were added to the reaction mixture. The mixture was incubated 30 min at 37 °C and heat-inactivated at 90 °C for 10 min. Then 10 pmol of DNA probe was mixed with the reaction mixtures and incubated 10 min. After

0.2 mg/mL NGO solution was added and incubated 20 min (total volume= 100 μL), the fluorescence intensity corresponding to Cy5 dye (Ex/Em= 649/666 nm) was measured using the platereader. The fluorescent image of the well plate at the wavelength of Cy5 dye was obtained using ChemiDoc™ MP Imaging System for visualization.

Z' factor calculation

Fluorescence intensities of 20 positive and negative controls were investigated. As a positive control, heat-inactivated ALKBH5 was used, assuming the situation that ALKBH5 completely lost its activity. The sequential ALKBH5 and MazF reaction, followed by measurement of fluorescence intensity, were conducted in the same way with the previous fluorescence analysis experiment. Based on the measured fluorescence intensity, Z' factor was calculated by the following equation, where μ is mean, σ is a standard deviation, and subscript p and n refer to the positive and negative control, respectively.

$$\mathrm{Z}'factor = 1 - \frac{3\left(\sigma_p + \sigma_n\right)}{\left|\mu_p - \mu_n\right|}$$

For positive control, the mean and standard deviation were 4450 and 129, respectively. For negative control, the mean and standard deviation were 618 and 70, respectively. Using the above equation, Z' factor was calculated as 0.84.

Inhibition assay

Serial concentrations of EDTA (0 to 20 mM) were treated to ALKBH5 in its reaction buffer for 30 min before the RNA substrate was added. After sequential ALKBH5 and MazF enzyme reaction, the fluorescence signals were measured and the half-maximal inhibitory concentration (IC_{50}) value was calculated based on the measured fluorescence intensities. The fluorescent image of the well plate of each sample, at the wavelength of Cy5 dye, was obtained using ChemiDoc™ MP Imaging System for visualization.

References

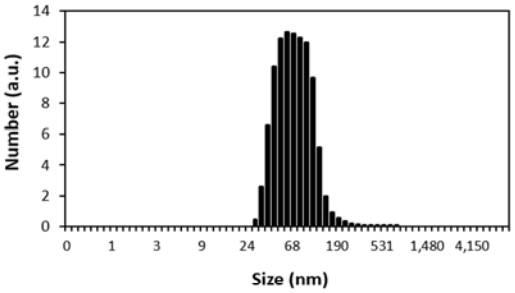
1. W. S. Hummers Jr and R. E. Offeman, *J. Am. Chem. Soc.*, 1958, **80**, 1339-1339.

2. P. Cuatrecasas, S. Fuchs and C. B. Anfinsen, *J. Biol. Chem.*, 1967, **242**, 1541-1547.

a)

	Hydrodynamic diameter (nm)	Zeta potential (mV)
Average	137	-44.1
Standard deviation	0.3	0.4

b)



c)

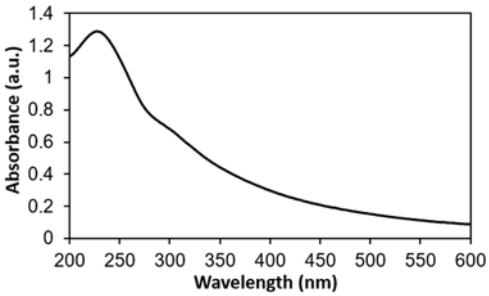


Figure S1 Characterization of NGO. a) Results of DLS and zeta potential analysis. b) The size distribution of NGO. c) UV-vis spectrum of NGO.

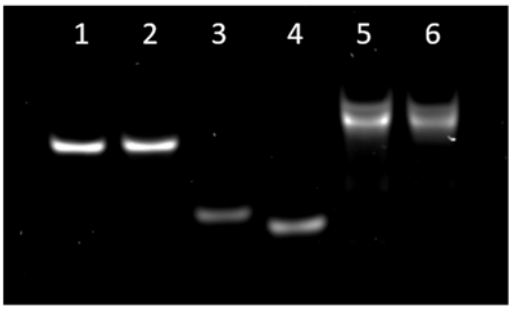


Figure S2 Polyacrylamide gel image of RNA oligonucleotides. 1: (m^6A)CA RNA oligo, 2: ACA RNA oligo, 3: fragment RNA 1), 4: fragment RNA 2), 5: DNA/RNA hybrid (DNA probe + (m^6A)CA RNA oligo), 6: DNA/RNA hybrid (DNA probe + ACA RNA oligo). The DNA/RNA hybrids were obtained by heating of the two oligonucleotides at 95 °C for 5 min, followed by slow cooling to room temperature.

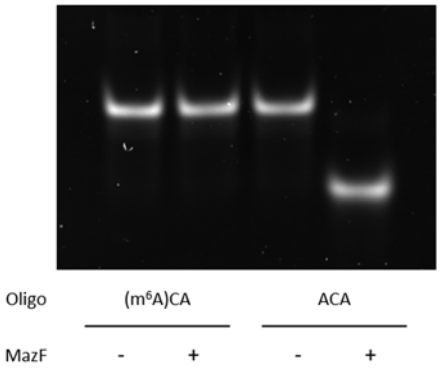


Figure S3 MazF activity test. The selective cleavage ability of MazF was confirmed by polyacrylamide gel analysis. The (m^6A)CA or ACA RNA oligo was incubated with or without MazF.

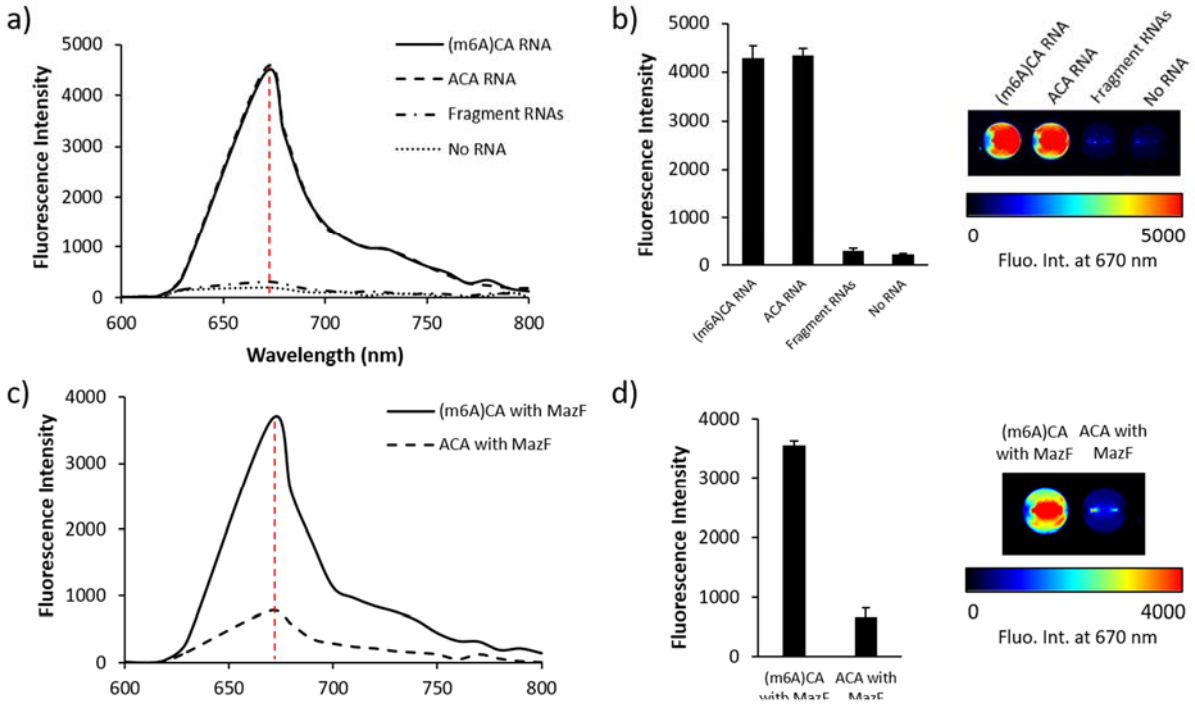


Figure S4 Fluorescence analysis. a) Fluorescence spectra of various RNAs incubated with DNA probe and NGO. b) A bar graph and a fluorescent image showing the difference between intensities of samples, at the wavelength of 670 nm (the red dotted line of a)) c) Fluorescence spectra of MazF reaction products incubated with DNA probe and NGO. D) A bar graph and a fluorescent image showing the difference between intensities of samples, at the wavelength of 670 nm (the red dotted line of c))

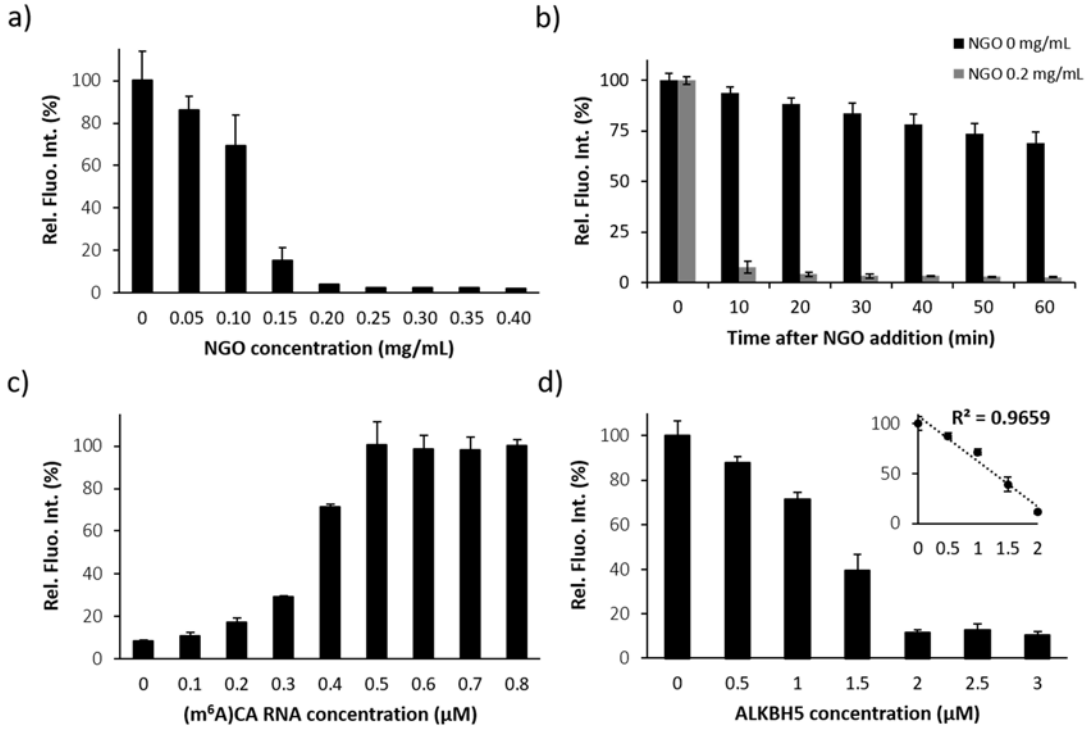


Figure S5 Optimization of assay condition a) A bar graph showing the fluorescence intensities according to the NGO concentration. The concentration of the DNA probe was fixed as 0.1 μM. b) A bar graph showing the change of fluorescence intensities after NGO addition. c) A bar graph showing the fluorescence intensities according to the (m^6A)CA RNA concentration. The concentration of the DNA probe and the NGO were fixed to 0.1 μM and 0.2 mg/mL, respectively. d) A bar graph showing the fluorescence intensities according to the ALKBH5 concentration. The concentration of the DNA probe, the NGO, and (m^6A)CA RNA were fixed to 0.1 μM, 0.2 mg/mL, and 0.5 μM.