Supplementary Information

Hoechst tagging: a modular strategy to design synthetic fluorescent probes for live-cell nucleus imaging

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Supplementary Figures

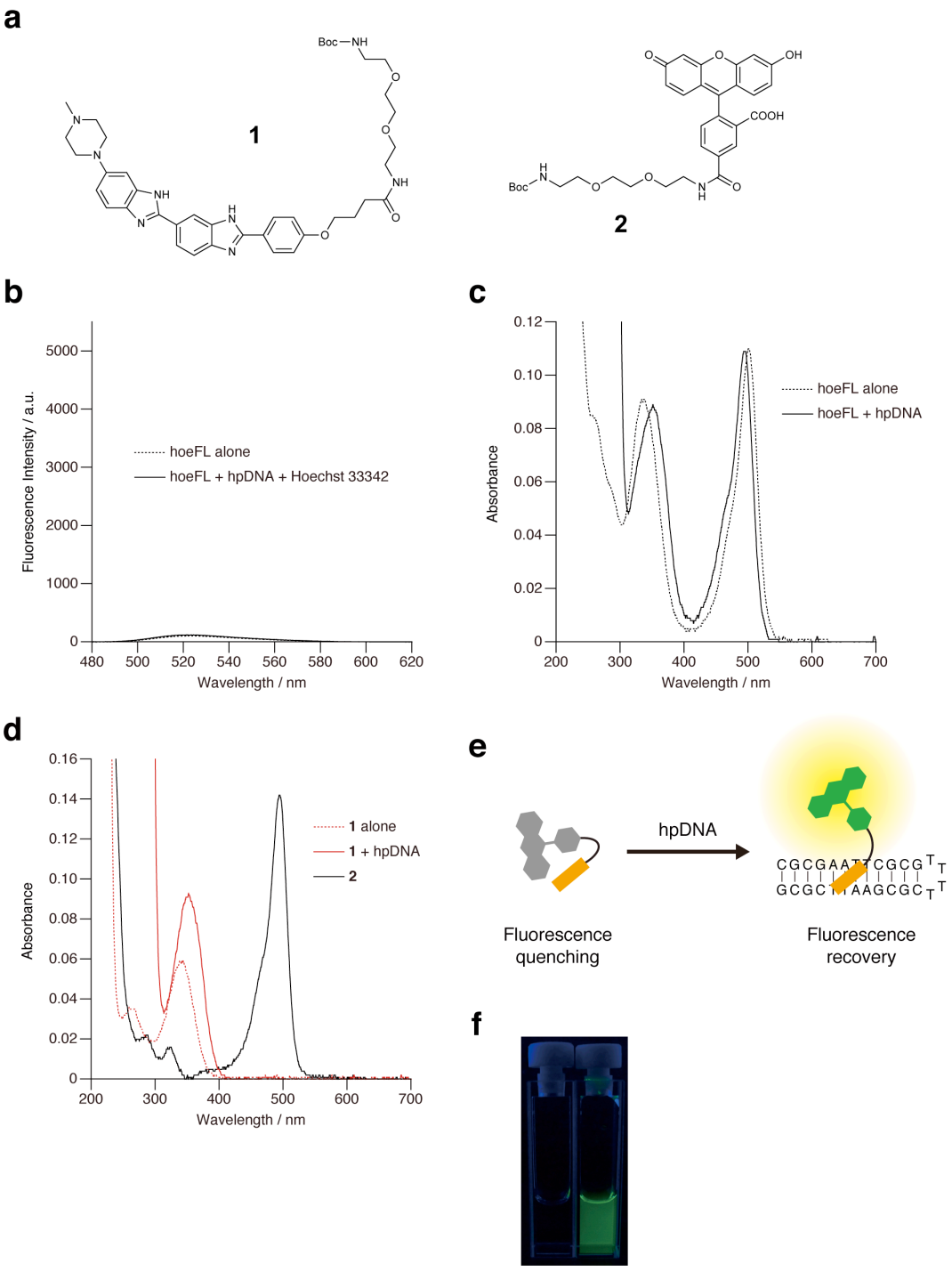


Fig. S1 (a) Chemical structures of control compounds **1** and **2**. (b) Fluorescence spectra of **hoeFL** (2 μM) in the absence (dashed line) and presence (solid line) of hpDNA (20 μM) and Hoechst 33342 (40 μM). Measurement conditions are the same as

in Fig. 2a. (c) UV-visible absorption spectra of **hoeFL** (2 μM) in the absence (dashed line) and presence (solid line) of hpDNA (20 μM) in 50 mM Tris-HCl, 100 mM NaCl, pH 7.4, 20 °C. (d) UV-visible absorption spectra of **1** (2 μM) (red dashed line), a mixture of **1** (2 μM) and hpDNA (20 μM) (red solid line), and **2** (2 μM) (black line) in the same buffer as in (c). (e) Proposed mechanism of the turn-on fluorescence response of **hoeFL**. (f) Photograph of **hoeFL** solutions (2 μM) without (left) and with (right) hpDNA (20 μM) under irradiation with a UV lamp (365 nm).

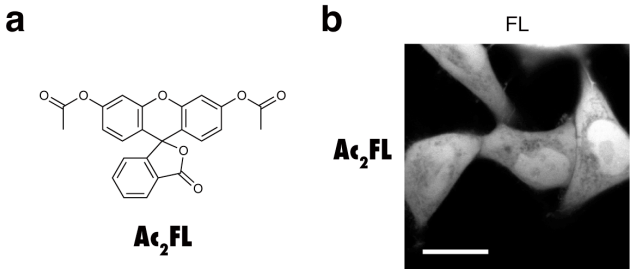


Fig. S2 (a) Chemical structure of fluorescein diacetate (**Ac_2FL**). (b) CLSM image of HeLa cells stained with **Ac_2FL**. Cells were incubated with **Ac_2FL** (2 μM) for 15 min, washed, and then observed by CLSM. Scale bar, 20 μm.

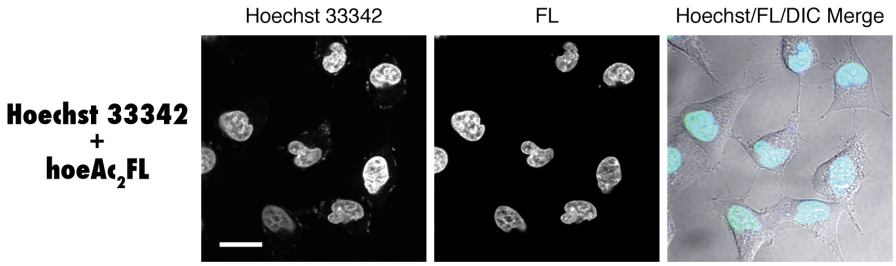


Fig. S3 CLSM images of HeLa cells co-stained with Hoechst 33342 and **hoeAc$_2$FL**. Cells were incubated with a mixture of **hoeAc$_2$FL** (5 μM) and Hoechst 33342 (1 μM) for 15 min, washed, and then observed by CLSM. Left, Hoechst fluorescence image; center, FL fluorescence image; right, DIC image merged with the Hoechst and FL fluorescence images. Scale bar, 20 μm.

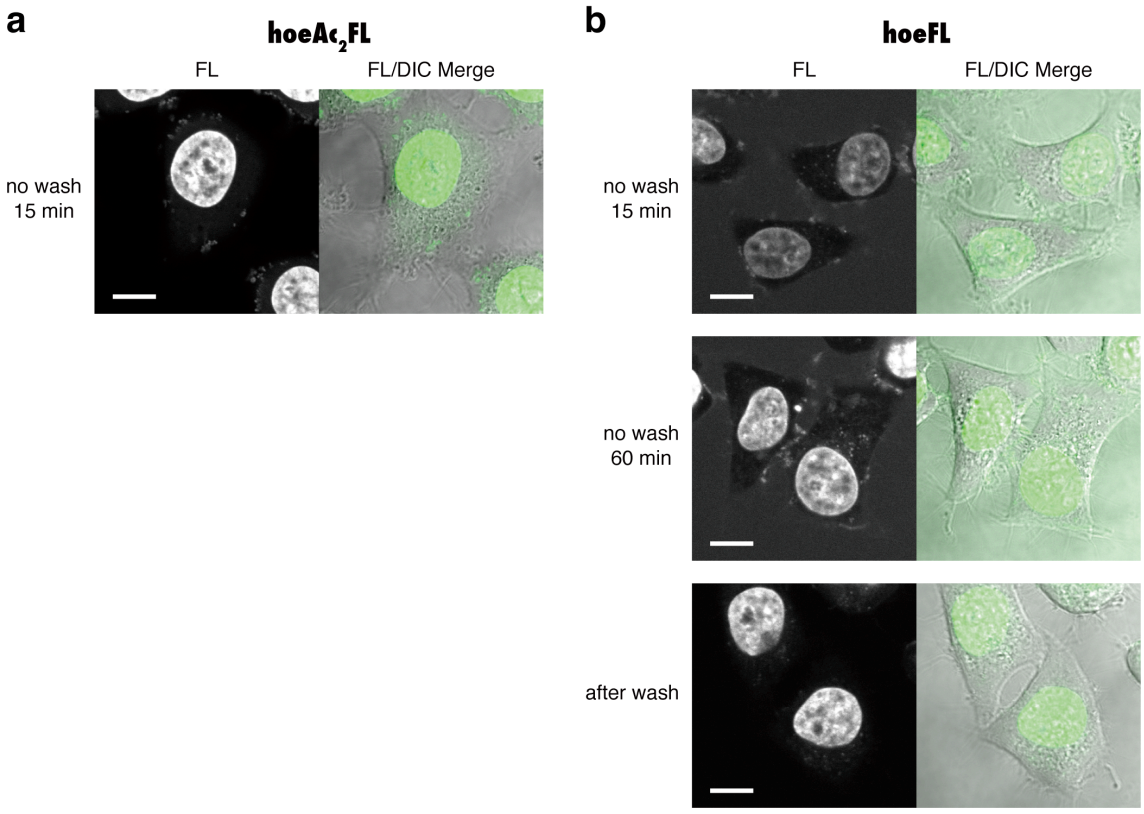


Fig. S4 (a) No-wash CLSM imaging of HeLa cells stained with **hoeAc$_2$FL**. Cells were incubated with **hoeAc$_2$FL** (5 μM) for 15 min and directly observed by CLSM without a washing procedure. The nuclei were clearly visualized with very low background (extracellular) signals. (b) No-wash CLSM imaging of HeLa cells stained with **hoeFL**. Cells were incubated with **hoeFL** (5 μM) for the indicated times and directly observed by CLSM without a washing procedure (top and middle). In this case, the nuclei were observed with high background signals. However, nucleus-specific imaging was readily achieved after washing the cells (bottom). Left, FL fluorescence image; right, DIC image merged with the fluorescence image. Scale bars, 10 μm.

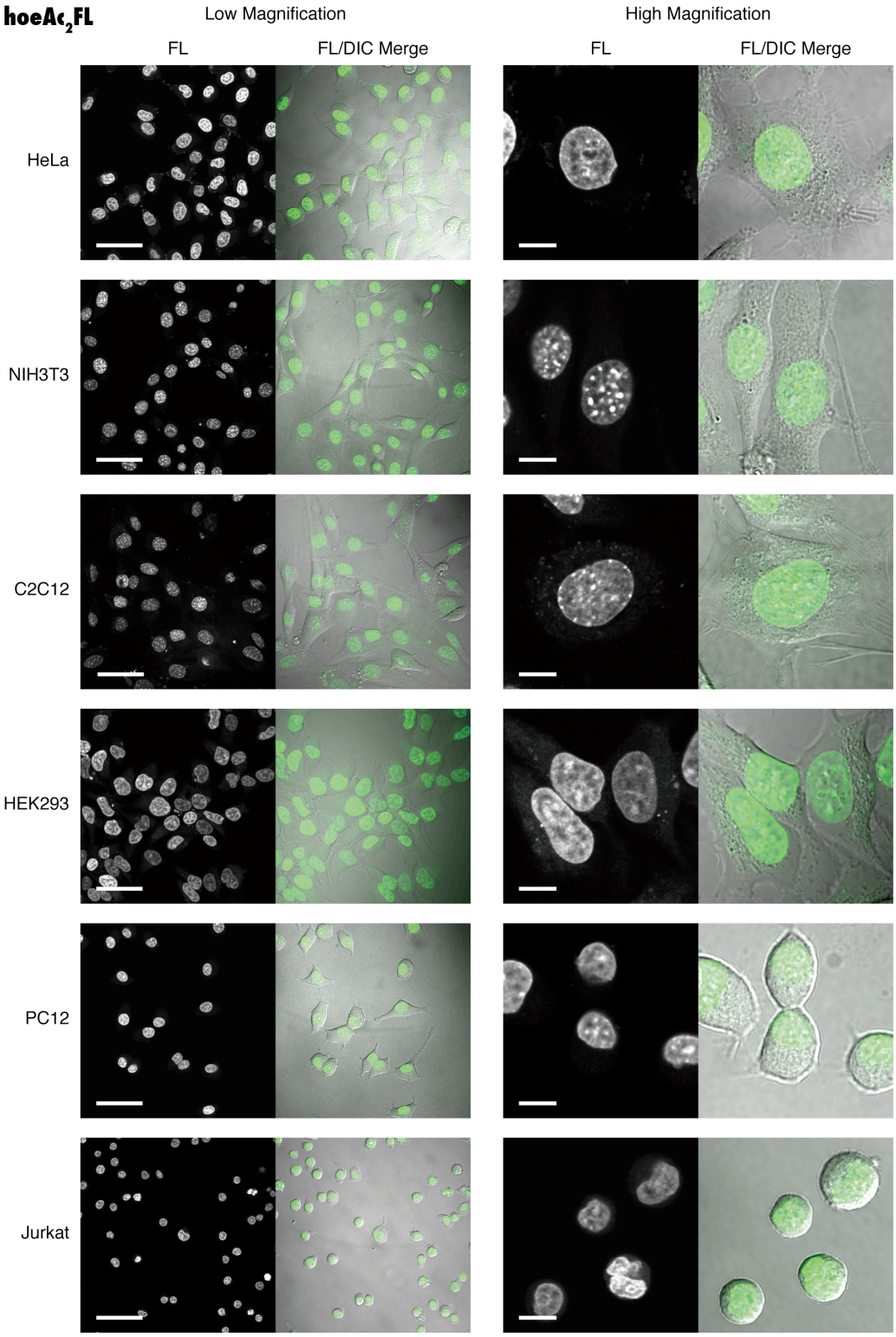


Fig. S5 Staining of various cell lines with **hoeAc$_2$FL**. Cells were incubated with **hoeAc$_2$FL** (1 μM) for 15 min, washed, and then observed by CLSM. Scale bars, 50 μm (low magnification) and 10 μm (high magnification).

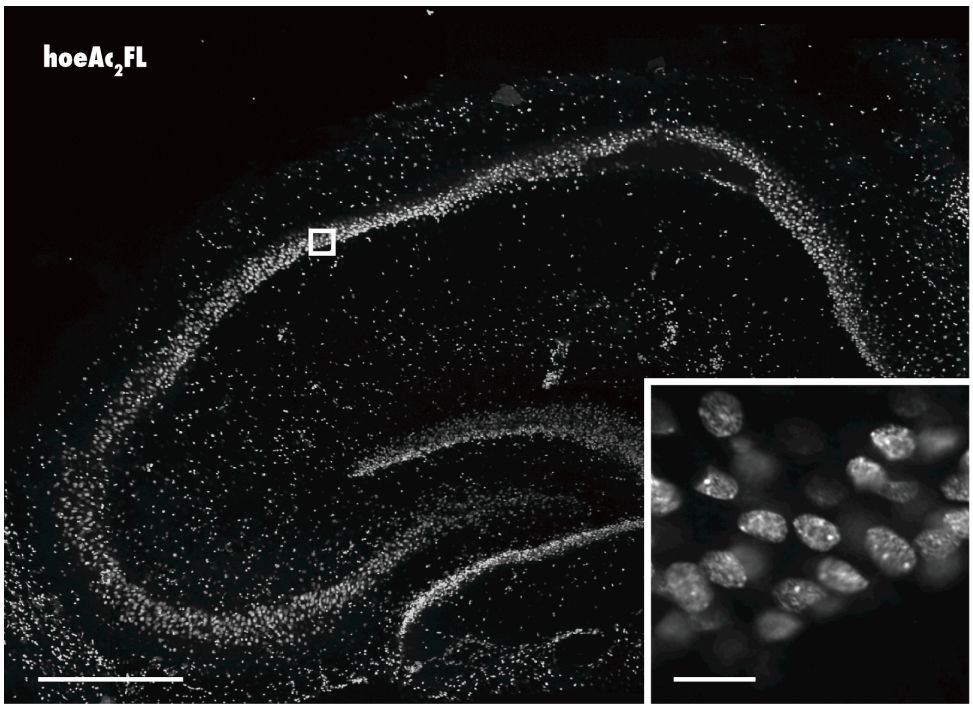


Fig. S6 Staining of live rat hippocampal tissue slice with **hoeAc$_2$FL**. A non-fixed hippocampal slice was incubated with **hoeAc$_2$FL** (20 μM) for 90 min, washed, and then observed by CLSM. The inset shows an enlarged view of the region indicated by the white square in the main image. Scale bars, 500 μm (main image) and 20 μm (inset).

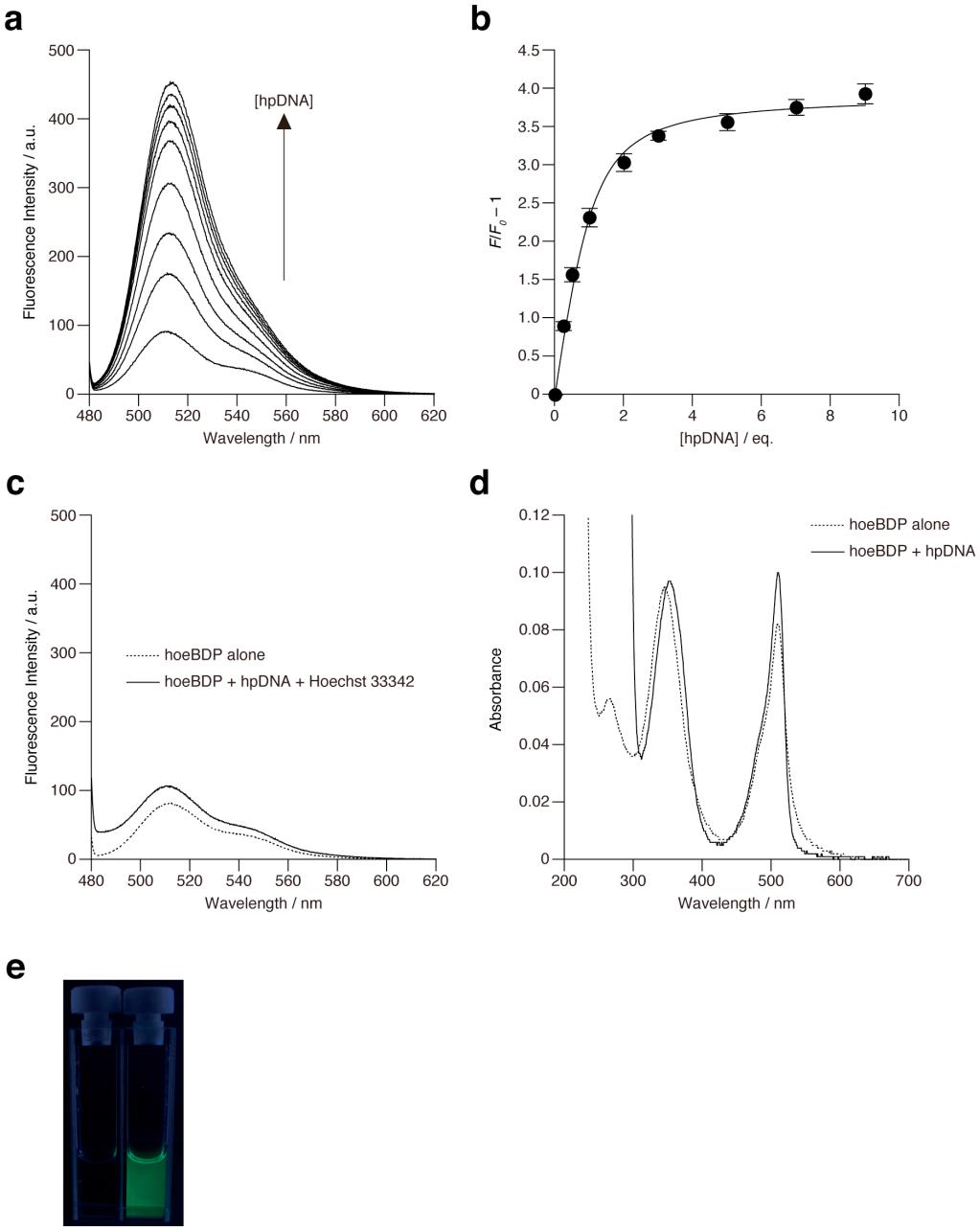


Fig. S7 (a) Fluorescence spectral changes of **hoeBDP** (0.1 μM) upon addition of hpDNA (0–0.9 μM). Measurement conditions: 50 mM Tris-HCl, 100 mM NaCl, pH 7.4, 20 °C, λ_{ex} = 460 nm. (b) Curve-fitting analysis of the fluorescence change at 514 nm. Data are represented as mean ± s.d. of three independent experiments. (c) Fluorescence spectra of **hoeBDP** (0.1 μM) in the absence (dashed line) and presence (solid line) of hpDNA (0.9 μM) and Hoechst 33342 (10 μM). Measurement conditions are the same as in (a). (d) UV-visible absorption spectra of **hoeBDP** (2 μM) in the absence (dashed line) and presence (solid line) of hpDNA (8 μM) in the same buffer as in (a). (e) Photograph

of **hoeBDP** solutions (2 μM) without (left) and with (right) hpDNA (8 μM) under irradiation with a UV lamp (365 nm).

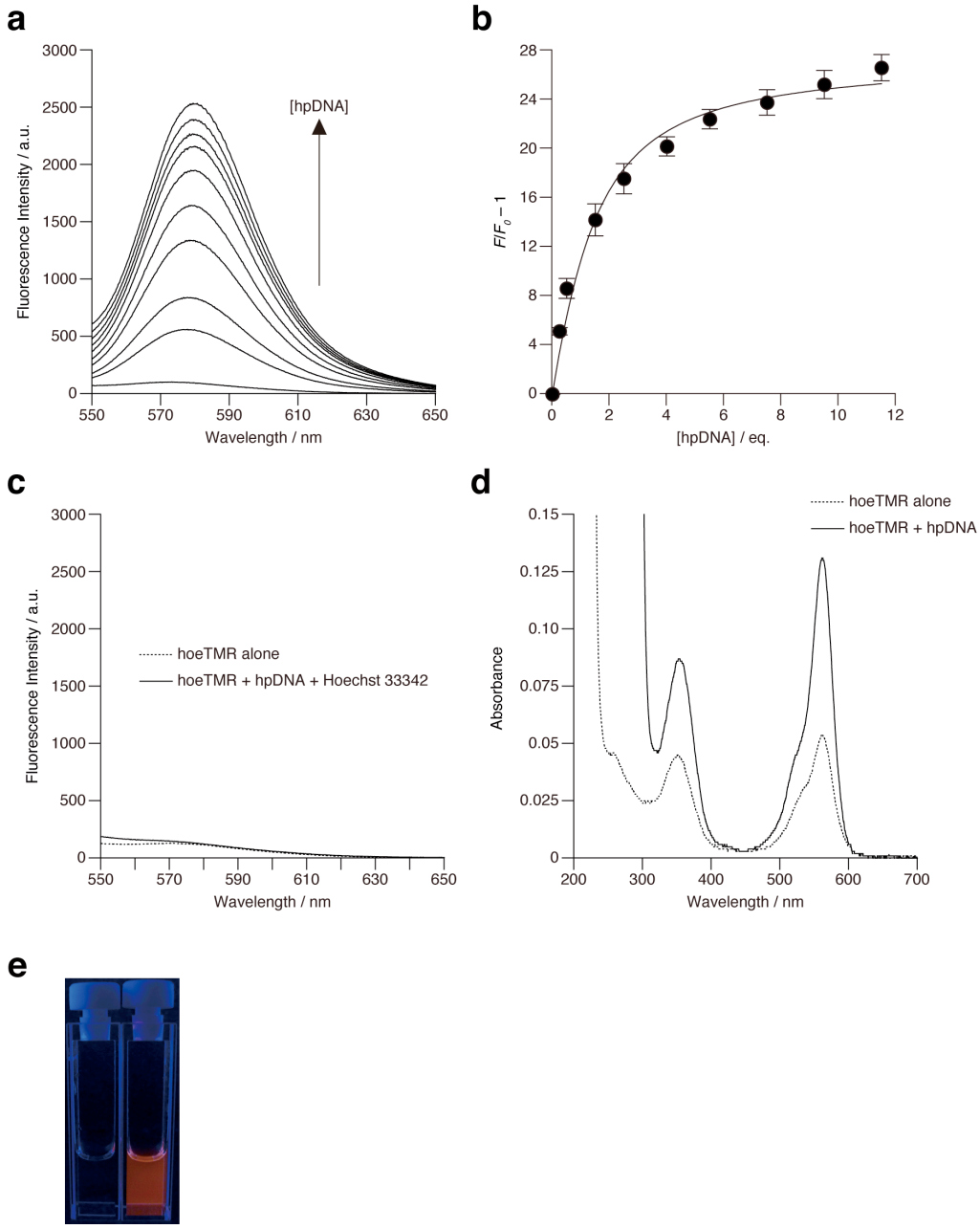


Fig. S8 (a) Fluorescence spectral changes of **hoeTMR** (2 μM) upon addition of hpDNA (0–23 μM). Measurement conditions: 50 mM Tris-HCl, 100 mM NaCl, pH 7.4, 20 °C, λ_{ex} = 500 nm. (b) Curve-fitting analysis of the fluorescence change at 580 nm. Data are represented as mean ± s.d. of three independent experiments. (c) Fluorescence spectra of **hoeTMR** (2 μM) in the absence (dashed line) and presence (solid line) of hpDNA (23 μM) and Hoechst 33342 (40 μM). Measurement conditions are the same as in (a). (d) UV-visible absorption spectra of **hoeTMR** (2 μM) in the absence (dashed line) and presence (solid line) of hpDNA (27 μM) in the same buffer as in (a). (e)

Photograph of **hoeTMR** solutions (2 μM) without (left) and with (right) hpDNA (27 μM) under irradiation with a UV lamp (365 nm).

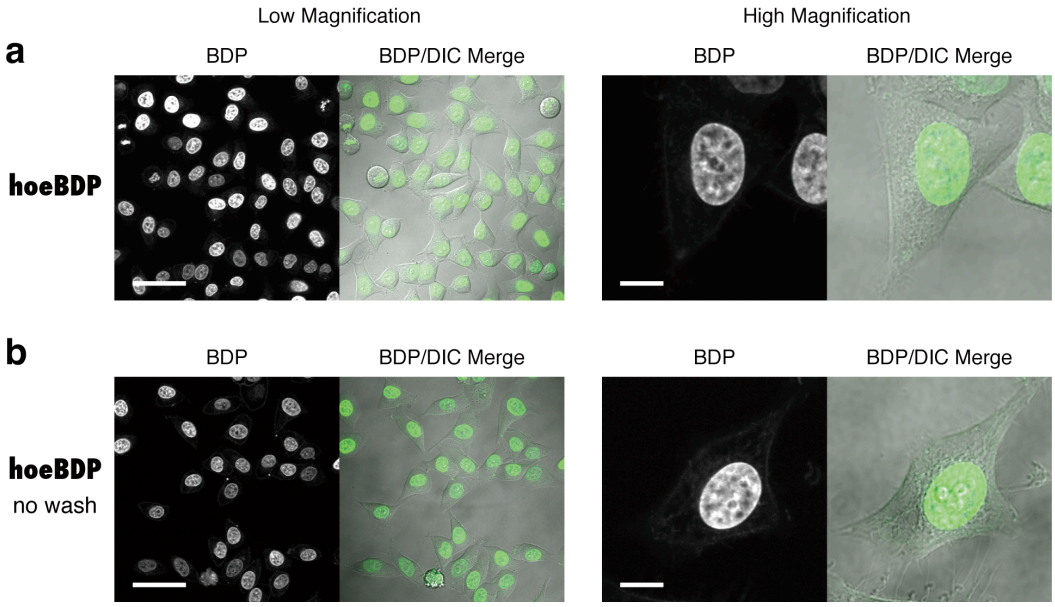


Fig. S9 (a) CLSM images of HeLa cells stained with **hoeBDP**. Cells were incubated with **hoeBDP** (5 μM) for 15 min, washed, and then observed by CLSM. (b) No-wash CLSM imaging of HeLa cells stained with **hoeBDP**. Cells were incubated with **hoeBDP** (5 μM) for 15 min and directly observed by CLSM without a washing procedure. The nuclei were clearly visualized with very low background (extracellular) signals. Left, BODIPY fluorescence image; right, DIC image merged with the fluorescence image. Scale bars, 50 μm (low magnification) and 10 μm (high magnification).

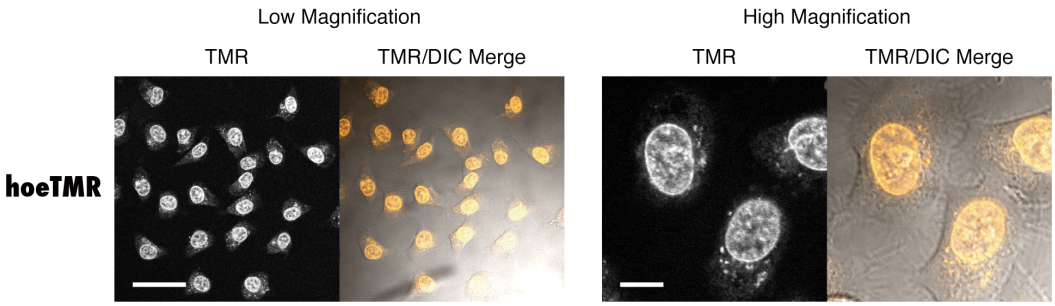


Fig. S10 CLSM images of HeLa cells stained with **hoeTMR**. Cells were incubated with **hoeTMR** (5 μM) in the presence of 0.02% Pluronic F-127 for 1 h, washed, and then observed by CLSM. Left, TMR fluorescence image; right, DIC image merged with the fluorescence image. Scale bars, 50 μm (low magnification) and 10 μm (high magnification).

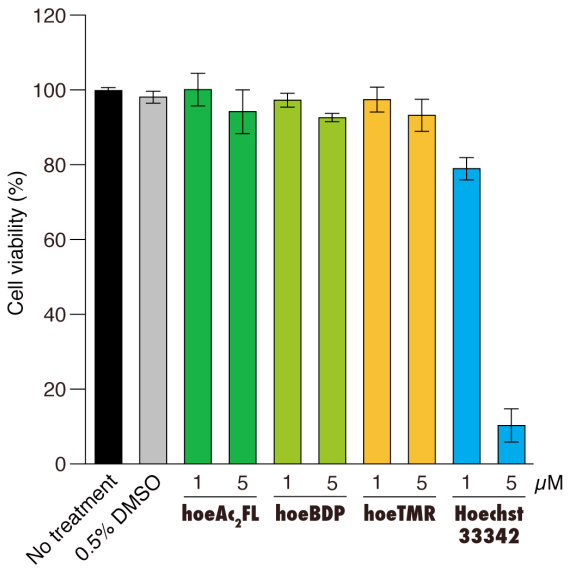


Fig. S11 Cytotoxity assays. HeLa cells were incubated with none, DMSO (vehicle), **hoeAc$_2$FL**, **hoeBDP**, **hoeTMR**, or Hoechst 33342 at the indicated concentrations for 2 h. The cells were washed, cultured for 24 h, and then assayed for viability using Cell Counting Kit-8. Data are represented as mean ± s.d. of at least three independent experiments.

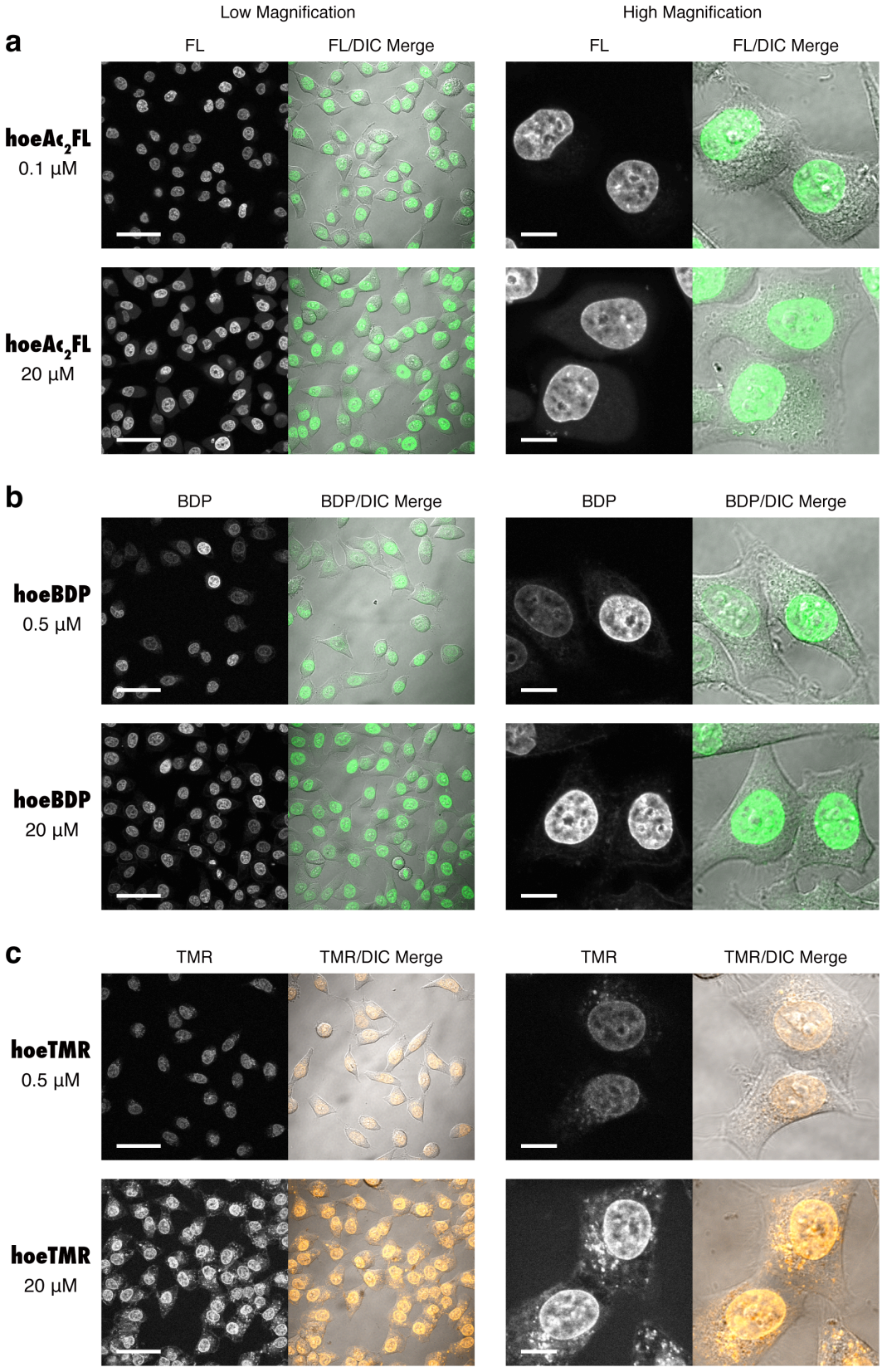


Fig. S12 Concentration-dependence of live-cell nucleus staining (HeLa cells) by

Hoechst-tagged fluorophores. (a) Cells were incubated with **hoeAc$_2$FL** (0.1 or 20 μM) for 15 min, washed, and then observed by CLSM. (b) Cells were incubated with **hoeBDP** (0.5 or 20 μM) for 15 min, washed, and then observed by CLSM. (c) Cells were incubated with **hoeTMR** (0.5 or 20 μM) in the presence of 0.02% Pluronic F-127 for 1 h, washed, and then observed by CLSM. Scale bars, 50 μm (low magnification) and 10 μm (high magnification).

Supplementary Methods: Chemical Synthesis

General materials and methods

All chemicals and solvents were purchased from standard commercial sources. Reversed-phase HPLC was performed on a Hitachi LaChrom Elite system with UV detection at 220 nm using a YMC-Pack ODS-A column (20 × 250 mm). ^{1}H NMR spectra were recorded on a JEOL AL-400 (400 MHz) spectrometer. ^{1}H NMR spectra are represented as follows: chemical shift, integration, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet), coupling constant (*J*) in Hertz (Hz). ^{1}H NMR chemical shifts were referenced to tetramethylsilane (0 ppm). High-resolution mass spectra were recorded on a Thermo Scientific Exactive Plus LC/MS system (ESI) at the ITbM (Nagoya University).

Reagent abbreviations

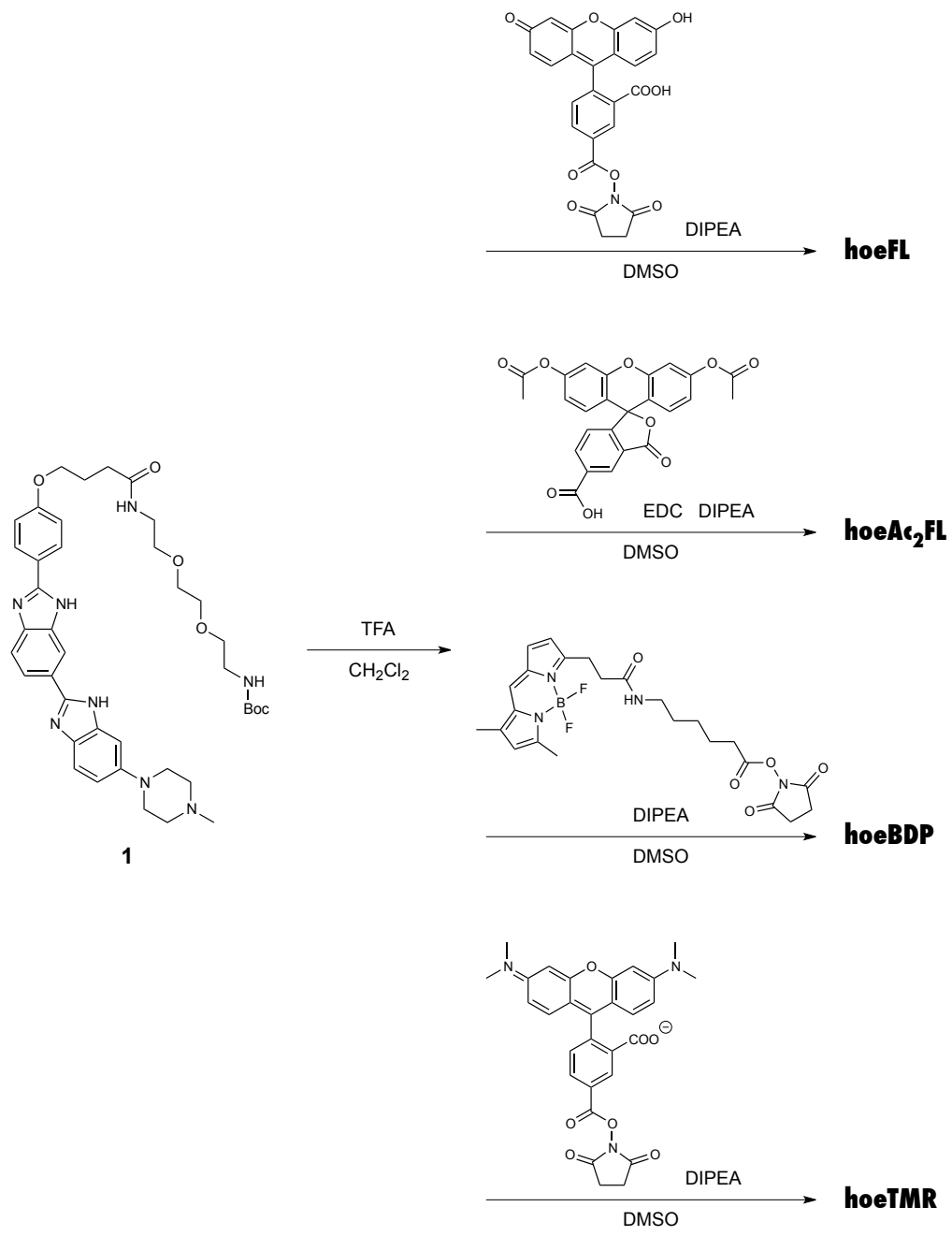
DIPEA: *N*,*N*-diisopropylethylamine
DMF: *N*,*N*-dimethylformamide
DMSO: dimethyl sulfoxide
EDC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
TFA: trifluoroacetic acid

Boc deprotection of compound 1 (see Scheme S1 on the next page)

Compound **1** was synthesized as described previously.[S1] To a solution of **1** (74 mg, 0.10 mmol) in CH_2Cl_2 (2 mL) at room temperature was added TFA (1 mL). After stirring for 1 h, the mixture was co-evaporated with toluene (5 mL × 2). The residue was dissolved in anhydrous DMSO (2.5 mL) to prepare a 40 mM DMSO solution of deprotected **1**, which was used for subsequent reactions without further purification.

Synthesis of hoeFL

5-Carboxyfluorescein succinimidyl ester (3 mg, 6.3 μmol) was dissolved in the above DMSO solution of deprotected **1** (190 μL, 7.6 μmol). To this was added DIPEA (10.3 μL, 60.6 μmol), and the mixture was incubated for 2 h at room temperature. The resulting mixture was purified by reversed-phase HPLC using a semi-preparative C18 column with a linear gradient of MeCN containing 0.1% TFA and 0.1% aqueous TFA.



Scheme S1 Synthetic route of Hoechst-tagged fluorescent probes

The collected fractions were concentrated and triturated with Et_2O to afford **hoeFL** as an orange solid (7.5 mg, 89% as a 3TFA salt).

^{1}H NMR (400 MHz, d_5-pyridine): δ 9.72 (1H, m), 9.06 (1H, s), 8.97 (1H, s), 8.75 (1H, m, overlapped with the solvent peak), 8.62 (2H, m), 8.48 (2H, d, *J* = 8.4), 7.97 (1H, d, *J* = 8.8), 7.82, (1H, d, *J* = 8.4), 7.43 (1H, s), 7.41 (1H, d, *J* = 7.6), 7.15 (1H, d, *J* = 7.6),

7.10 (4H, m), 6.93 (2H, d, *J* = 8.8), 6.86 (2H, d, *J* = 8.0), 4.08 (2H, t, *J* = 6.4), 3.93 (2H, m), 3.83 (2H, m), 3.67–3.58 (8H, m), 3.41 (4H, m), 2.95 (4H, m), 2.60 (2H, t, *J* = 6.8), 2.55 (3H, s), 2.28 (2H, m).

HRMS: calcd for $[M+H]^+$, 999.4036; found, 999.4006.

Synthesis of hoeAc$_2$FL

5-Carboxyfluorescein diacetate (5 mg, 10.9 μmol) was dissolved in the above DMSO solution of deprotected **1** (328 μL, 13.1 μmol). To this were added EDC (2.5 mg, 13.0 μmol) and DIPEA (17.8 μL, 104.7 μmol), and the mixture was incubated for 2 h at room temperature. The resulting mixture was purified by reversed-phase HPLC using a semi-preparative C18 column with a linear gradient of MeCN containing 0.1% TFA and 0.1% aqueous TFA. The collected fractions were concentrated and triturated with Et_2O to afford **hoeAc$_2$FL** as a yellow solid (6.5 mg, 42% as a 3TFA salt).

^{1}H NMR (400 MHz, d_5-pyridine): δ 9.70 (1H, m), 8.98 (2H, s), 8.77 (1H, m), 8.63 (1H, d, *J* = 8.4), 8.54 (1H, d, *J* = 8.0), 8.49 (2H, d, *J* = 8.4), 7.98 (1H, d, *J* = 8.0), 7.82 (1H, d, *J* = 8.8), 7.44 (1H, s), 7.32 (2H, d, *J* = 2.4), 7.27 (1H, d, *J* = 7.6), 7.14 (1H, d, *J* = 7.6), 7.10 (4H, m), 7.03 (2H, d, *J* = 8.8), 4.08 (2H, t, *J* = 6.0), 3.93 (2H, m), 3.83 (2H, m), 3.68–3.59 (8H, m), 3.43 (4H, m), 3.00 (4H, m), 2.60 (2H, t, *J* = 7.6), 2.59 (3H, s), 2.28 (2H, m), 2.22 (6H, s).

HRMS: calcd for $[M+H]^+$, 1083.4247; found, 1083.4240.

Synthesis of hoeBDP

BODIPY FL-X succinimidyl ester (5 mg, 10.0 μmol) was dissolved in the above DMSO solution of deprotected **1** (299 μL, 12.0 μmol). To this was added DIPEA (16.3 μL, 95.9 μmol), and the mixture was incubated for 2 h at room temperature. The resulting mixture was purified by reversed-phase HPLC using a semi-preparative C18 column with a linear gradient of MeCN containing 0.1% TFA and 0.1% aqueous TFA. The collected fractions were concentrated and triturated with Et_2O to afford **hoeBDP** as an orange-brown solid (8.2 mg, 60% as a 3TFA salt).

^{1}H NMR (400 MHz, d_5-pyridine): δ 8.98 (1H, s), 8.62 (1H, d, *J* = 8.8), 8.49 (2H, d, *J* = 8.4), 8.49 (2H, m), 7.98 (1H, d, *J* = 8.0), 7.81 (1H, d, *J* = 8.8), 7.43 (1H, s), 7.36 (1H, s), 7.12 (3H, m), 7.01 (1H, d, *J* = 3.6), 6.51 (1H, d, *J* = 3.6), 6.02 (1H, s), 4.10 (2H, t, *J* = 6.0), 3.77 (2H, t, *J* = 7.6), 3.63 (8H, m), 3.55 (4H, s), 3.41 (6H, m), 2.94 (6H, m), 2.61

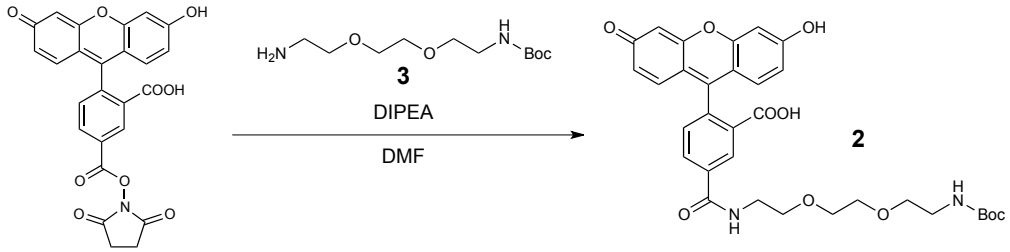
(2H, t, J = 7.2), 2.59 (3H, s), 2.55 (3H, s), 2.36–2.27 (4H, m), 2.09 (3H, s), 1.77 (2H, m), 1.57 (2H, m), 1.39 (2H, m).
HRMS: calcd for $[M+H]^+$, 1028.5488; found, 1028.5479.

Synthesis of hoeTMR

5-Carboxytetramethylrhodamine succinimidyl ester (5 mg, 9.5 μmol) was dissolved in the above DMSO solution of deprotected **1** (284 μL, 11.4 μmol). To this was added DIPEA (15.5 μL, 91.1 μmol), and the mixture was incubated for 2 h at room temperature. The resulting mixture was purified by reversed-phase HPLC using a semi-preparative C18 column with a linear gradient of MeCN containing 0.1% TFA and 0.1% aqueous TFA. The collected fractions were concentrated and triturated with Et_2O to afford **hoeTMR** as a dark red-brown solid (8.3 mg, 63% as a 3TFA salt).
^{1}H NMR (400 MHz, d_5-pyridine): δ 9.79 (1H, m), 9.17 (1H, s), 8.98 (1H, s), 8.79 (1H, m), 8.66 (1H, d, J = 7.6), 8.62 (1H, d, J = 8.4), 8.49 (2H, d, J = 8.4), 7.98 (1H, d, J = 8.4), 7.82 (1H, d, J = 8.8), 7.47 (1H, s), 7.46 (1H, d, J = 7.2), 7.13 (1H, d, J = 8.4), 7.08 (2H, d, J = 8.8), 6.94 (2H, d, J = 8.8), 6.65 (2H, s), 6.59 (2H, d, J = 9.2), 4.07 (2H, t, J = 6.4), 3.98 (2H, m), 3.87 (2H, m), 3.69–3.60 (8H, m), 3.46 (4H, m), 3.07 (4H, m), 2.87 (12H, s), 2.64 (3H, s), 2.61 (2H, t, J = 6.8), 2.28 (2H, m).
HRMS: calcd for $[M+H]^+$, 1053.4982; found, 1053.4965.

Synthesis of Compound 2 (see Scheme S2 on the next page)

Compound **3** was synthesized as described previously.[S1] 5-Carboxyfluorescein succinimidyl ester (10 mg, 21.1 μmol) was dissolved in an anhydrous DMF solution of **3** (7.4 mg, 29.8 μmol) and DIPEA (8.8 μL, 51.7μmol). The mixture was incubated for 2 h at room temperature. The resulting mixture was purified by reversed-phase HPLC using a semi-preparative C18 column with a linear gradient of MeCN containing 0.1% TFA and 0.1% aqueous TFA. The collected fractions were concentrated and triturated with Et_2O to afford compound **2** as a yellow solid (8.7 mg, 68%)
^{1}H NMR (400 MHz, CD_3OD): δ 8.54 (1H, s), 8.24 (1H, d, J = 7.2), 7.38 (1H, d, J = 8.0), 6.86 (2H, s), 6.83 (2H, d, J = 8.8), 6.71 (2H, d, J = 7.2), 3.72–3.66 (8H, m), 3.53 (2H, t, J = 5.4), 3.21 (2H, t, J = 5.4), 1.40 (9H, s).
HRMS: calcd for $[M+H]^+$, 607.2287; found, 607.2284.



Scheme S2 Synthetic route of compound **2**

Supplementary Methods: Evaluation of *In Vitro* DNA-Binding and Photochemical Properties

General materials and instruments

All reagents and buffer components were of the highest grade available from commercial sources. The 28mer hpDNA oligonucleotide (HPLC grade) was purchased from Operon Biotechnologies. Water purified with a Sartorius Arium Pro DI ultrapure water system was used for all aqueous solutions. UV-visible absorption spectra were recorded on a Hitachi U-3900H spectrophotometer. Fluorescence spectra were measured with a Hitachi F-7000 fluorescence spectrophotometer. Photographs of Hoechst-tagged fluorophore solutions were taken using a UVP benchtop UV transilluminator as a light source (365 nm).

Fluorescence titration with hpDNA

Hoechst-tagged fluorophores were dissolved in DMSO to a stock concentration of 1 mM. These stock solutions were diluted with Tris buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.4) for fluorescence assays. Lyophilized hpDNA was dissolved in H_2O to a stock concentration of 1 mM. Fluorescence titration experiments were performed by adding the hpDNA solution to the Hoechst-tagged fluorophore solution at 20 °C. Fluorescence titration curves were analyzed by a nonlinear least-square curve-fitting procedure based on a 1:1 binding model to estimate dissociation constants (K_D). Absorption spectra measurements were performed in the same manner.

Determination of fluorescence quantum yields

For the determination of fluorescence quantum yields of Hoechst-tagged fluorophores in the absence and presence of hpDNA, absorption and fluorescence spectra were measured in the following conditions: 2 μM **hoeFL** (± 20 μM hpDNA), 2 μM **hoeBDP** (± 8 μM hpDNA), or 2 μM **hoeTMR** (± 27 μM hpDNA) in 50 mM Tris-HCl, 100 mM NaCl, pH 7.4, 20 °C. The fluorescence quantum yields were determined using the equation shown below, where Φ is the quantum yield, Abs is the absorbance at the excitation wavelength (λ_{ex} = 460 nm for **hoeFL** and **hoeBDP**, and 500 nm for **hoeTMR**), F is the fluorescence intensity at each wavelength, and Σ[F] was calculated by summation of fluorescence intensity. For **hoeFL** and **hoeBDP**, fluorescein

in 0.1 M NaOH aqueous solution was used as a standard ($\Phi_{standard}$ = 0.85).[S2] For **hoeTMR**, rhodamine B in EtOH at 25 °C was used as a standard ($\Phi_{standard}$ = 0.65).[S3]

$$\Phi_{sample} = \Phi_{standard}\, Abs_{standard}\, \Sigma[F_{sample}]/Abs_{sample}\, \Sigma[F_{standard}]$$

Supplementary Methods: Cell Biological and Imaging Experiments

Cell culture and mediums

HeLa, NIH3T3, C2C12, and HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C under 5% CO_2 atmosphere. PC12 cells were cultured in DMEM supplemented with 5% FBS, 5% horse serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Jurkat cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin.

For cell staining and imaging experiments, serum- and phenol red-free DMEM ($DMEM_i$) and RPMI-1640 ($RPMI_i$) (both supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin) were used.

Microscopy

All imaging experiments were performed on an Olympus IX81-ZDC/FV1000 laser scanning confocal microscope. Fluorescence and DIC images were acquired using UPlanSApo 10×/0.40 NA, UPlanSApo 40×/0.90 NA, and PlanApo N 60×/1.42 NA (oil) objectives and lasers at 405 nm for Hoechst 33342, 488 nm for **hoeAc$_2$FL/hoeFL** and **hoeBDP**, and 543 nm for **hoeTMR**. Fluorescence images were analyzed using the Olympus FLUOVIEW viewer software.

Fluorescent staining and imaging of culture cells

Cells were plated in 35 mm glass-bottomed dishes (AGC Techno Glass) and cultured for 24 h. The cells were washed twice with $DMEM_i$ (or $RPMI_i$ for Jurkat cells) and incubated with compounds at the concentrations and times indicated in the Figure legends at 37 °C. The cells were washed twice with $DMEM_i$ (or $RPMI_i$ for Jurkat cells) and then observed by CLSM. For no-wash imaging, the incubated cells were directly observed by CLSM.

Hippocampal slice preparation and staining

Animal experiments were performed at the Shimoda Laboratory (Department of Bioengineering, Nagaoka University of Technology) in accordance with the Guidelines

for Animal Experiments of Nagaoka University of Technology. Rat hippocampal slices were prepared as previously described.[S4] A postnatal day 19 Wistar rat (Japan SLC) was anesthetized with ethyl ether and decapitated. The brain was quickly removed and immersed in ice-cold artificial cerebrospinal fluid (ACSF) containing 124 mM NaCl, 25 mM $NaHCO_3$, 3 mM KCl, 1.24 mM KH_2PO_4, 1.4 mM $MgSO_4$, 2.2 mM $CaCl_2$, and 10 mM glucose. Hippocampal slices were prepared by cutting the whole hippocampus. The live (non-fixed) hippocampal slice was incubated with 20 μM **hoeAc$_2$FL** in ACSF for 90 min. The slice was washed twice with ACSF and then observed by CLSM.

Cytotoxity assays

HeLa cells were cultured in 96-well plates (TPP) for 24 h. The cells were washed twice with $DMEM_i$ and incubated with none, 0.5% DMSO, **hoeAc$_2$FL** (1 or 5 μM), **hoeBDP** (1 or 5 μM), **hoeTMR** (1 or 5 μM), or Hoechst 33342 (1 or 5 μM) for 2 h. The cells were washed with serum-containing DMEM and cultured for 24 h. The cells were then assayed for viability using Cell Counting Kit-8 (Dojindo) according to the manufacture's protocol. Absorbance at 450 nm was measured using a Model 680 Microplate Reader (Bio-Rad Laboratories).

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