Electronic Supplementary Information

A redox-responsive folate-fluorophore conjugate as a new target-cell-specific imaging probe

Jisu Kim‡, Hyunjin Kim‡, and Yongdoo Choi*

Research Institute, National Cancer Center, 323 Ilsan-ro, Ilsandong-gu, Goyang-si, Gyeonggi-do 10408, Republic of Korea.

‡ These authors contributed equally to this work

[* Corresponding author]
Yongdoo Choi, Ph. D.
Tel: +82-31-920-2512
Fax: +82-31-920-2630
E-mail: ydchoi@ncc.re.kr

Materials and methods

Materials

ATTO655-COOH and ATTO655-NHS ester were obtained from ATTO-TEC GmbH (Siegen, Germany). Dithiothreitol (DTT), glutathione (GSH), *N*-ethylmaleimide (NEM), and folate (FA) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Methods

Synthesis of redox-responsive folate-fluorophore conjugates (RFC)

RFC was synthesized by 9-fluorenylmethoxycarbonyl solid phase peptide synthesis (Fmoc-SPPS) using ASP48S (Peptron Inc. Daejeon, Korea): 8 eq. Fmoc-Glu(OtBu) and 8 eq. 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/8 eq. N-Hydroxybenzotriazole (HOBt)/16 eq. 4-Methylmorpholine (NMM) in dimethylformamide (DMF) were added to Cysteamine-chloro-Trityl resin (Anaspec, CA, USA). The mixture was reacted for 2 h at room temperature and washed with DMF, MeOH, and DMF. For Fmoc deprotection, 20 % piperidine in DMF was added to the reaction mixture and incubated for 5 min. The previous step was repeated once more and then the product was washed with DMF, MeOH, and DMF. The peptide backbone-attached resin (NH_2-gamma-Glu(OtBu)-Glu(OtBu)-Glu(OtBu)-cysteamide-2-chloro-Trityl Resin) was prepared by repeating the above procedure. Pteroic acid (4 eq.) and HBTU (4 eq.)/HOBt (4 eq.)/NMM (8 eq.) in dimethyl sulfoxide (DMSO) were added to the prepared peptide resin. The mixture was incubated for 12 h, suctioned, and washed with DMF, MeOH, and DMF. Afterwards, the peptide (Pteroyl-gamma-Glu(OtBu)-Glu(OtBu)-Glu(OtBu)-cysteamide) was cleaved from the resin by treatment with trifluoroacetic acid (TFA)/EDT/thioanisole/triisopropyl-silane (TIS)/H_2O (90/2.5/2.5/2.5/2.5). The reaction mixture was added to 10-fold cold diethyl ether for precipitation and centrifuged at 3000 rpm for 10 min. After removing the supernatant, the collected peptide was purified by prep-LC and then lyophilized.

FA-Glu-Glu-cysteamide and Aldrithiol™-2 (5 eq.) were reacted in acetic acid for 12 h. The reaction mixture was added to 10-fold cold diethyl ether for precipitation and centrifuged at 3000 rpm for 10 min. After removing the supernatant, the collected peptide was purified by prep-LC and then lyophilized. Prepared FA-Glu-Glu-cysteamide-Pys and cysteine (2 eq.) were reacted in acetic acid for 12 h. The reaction mixture was added to 10-fold cold diethyl ether for precipitation and then centrifuged at 3000 rpm for 10 min. After removing the supernatant, the collected peptide was purified by prep-LC and lyophilized.

FA-Glu-Glu-cysteamide-Cys and ATTO655-NHS ester were dissolved in DMF, added to N,N-diisopropylethylamine (DIPEA), and reacted for 12 h at room temperature in the dark. When it was confirmed that the reaction was completed, then the synthesized FA-s-s-ATTO655 was purified by prep-LC, lyophilized, and preserved at -24 °C before use.

Characterization of RFC

The molecular weight of the purified RFC was confirmed by LC/MSD (Agilent Hewlett Packard 1100 series, California, USA). The UV/Vis spectra of RFC and free ATTO655-COOH were measured using an UV/Vis spectrometer (DU730, Beckman Coulter, California, USA).

Fluorescence quenching of the fluorophore by folate

Fluorescence quenching of the ATTO655 dye was tested in the presence of various concentration of FA in an aqueous solution. ATTO655-COOH was diluted in phosphate-buffered saline (PBS; 6.7 mM; pH 7.4; 154 mM NaCl) to a final concentration of 1 μM, and various concentrations of folate were added. Folate was prepared as a 100 mM stock in 1 M NaOH and serially diluted in PBS buffer (pH 7.4). The fluorescence intensity was measured using a microplate reader (λ_{ex} 600 nm, λ_{em} 610-850 nm).

Redox-responsive recovery of near-infrared (NIR) fluorescence

RFC was dissolved in PBS to a final concentration of 2 μM to analyze fluorescence quenching of RFC. Then, their fluorescence spectra ($\lambda_{ex.}$ 610 nm) were obtained using a multifunctional microplate reader (Tecan, Männedorf, Switzerland) and compared.

The redox-responsive increase in NIR fluorescence was evaluated by treating RFC (2 μM) with and without GSH (final concentration of GSH: 2 μM and 10 mM) for 5.5 h at 37 °C. After that, the fluorescence spectra of these samples were measured and compared. For the analysis of the time-dependent fluorescence changes, RFC was treated with and without GSH, and thereafter the fluorescence intensities ($\lambda_{ex.}$ 610 nm and $\lambda_{em.}$ 680 nm) of the samples were measured every 10 min for 5.5 h at 37 °C. All experiments were performed in quadruplicate. Data are expressed as the mean (SD).

Stability of RFC fluorescence in serum conditions

Prior to *in vitro* cell culture experiments, the stability of the RFC fluorescence was analyzed in various serum conditions. RFC was dissolved in PBS, PBS containing 10 % fetal bovine serum (FBS; Gibco, OR, USA), 100 % FBS, and Dulbecco's Modified Eagle's medium (DMEM, Gibco, OR, USA). The final concentration of RFC in the solution was adjusted to 1 μM. The solution was maintained at room temperature, and their fluorescence spectra ($\lambda_{ex.}$ 610 nm) were measured at 0 min, 15 min, 1 h, and 4 h.

Cell culture

Folate receptor-negative A549 cells and folate receptor-positive HeLa cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). A549 cells were cultured in RPMI1640 (Gibco, OR, USA) supplemented with 10 % FBS and 1 % antibiotic/antimycotic at 37 °C, in a 5 % CO_2 atmosphere. HeLa cells were maintained in DMEM supplemented with 1 % antibiotic/antimycotic and 10 % FBS.

Confocal fluorescence microscopy for cell-targeting

To analyze folate receptor-mediated internalization and subsequent fluorescence activation, HeLa cells and A549 cells were plated on 8-well Lab-Tek chamber slides (Nalgene Nunc International, USA) with a density of 5×10^4 cells per well. Then they were incubated for 24 h to allow the cell to attach. RFC was dissolved and diluted with cell culture medium. The existing culture medium was replaced with 200 μL of fresh medium containing RFC (1 μM), after which the cells were incubated for 2 h. For the competition assay, HeLa cells were incubated with an excess of free FA (1 mM) for 30 min, and then co-incubated with RFC for an additional 2 h. Afterwards, all the cells were washed 3 times with cell culture media, and the NIR fluorescence images ($\lambda_{ex.}$ 633 nm, $\lambda_{em.}$ 646–753 nm) were acquired using a

confocal laser scanning microscope (CLSM, ZEISS LSM 510 META).

Inhibition test of redox-responsive RFC activation

HeLa cells were seeded in 96-well plates (Corning, NY, USA) at a density of 1×10^4 cells per well and allowed to attach for 24 h. The cells were then treated with fresh culture medium containing 1 μM RFC for 2 h. For inhibition of intracellular GSH, HeLa cells were pre-treated with 1 mM N-ethylmaleimide (NEM) for 20 min, and then treated with 1 μM RFC for 2 h. After washing the cells, fresh cell culture medium was added, and NIR fluorescence images ($\lambda_{ex.}$ 655 ± 20 nm and $\lambda_{em.}$ 716 ± 20 nm) were obtained by using Olympus fluorescence microscopy (IX73P2F, Japan) with an NIR camera (Hamamatsu digital camera, C11440). All images were captured using the same microscope settings in an effort to ensure reproducibility.

Evaluation of NIR fluorescence activation inside the target cells

The fluorescence turn-on of the quenched RFC conjugate inside the target cells was demonstrated by obtaining confocal fluorescence images of either free dye-treated or RFC-treated cells without washing process. Briefly, folate receptor-positive HeLa cells were plated on 8-well Labtek chamber slides with a density of 5×10^4 cells per well. Then they were incubated for 24 h to allow the cell to attach. RFC and free ATTO655-COOH were dissolved and diluted with cell culture medium. The existing culture medium was replaced with 200 μL of fresh medium containing RFC or free ATTO655-COOH (2 μM). Then, without washing the cells, NIR fluorescence images ($\lambda_{ex.}$ 633 nm, $\lambda_{em.}$ 646–753 nm) of the cells were acquired at 15 min, 1 h, and 2 h using CLSM. Afterwards, the cells were washed twice to remove the dyes in the extracellular space, and NIR fluorescence images were obtained again to analyze whether the free dyes were adsorbed by the cell's surface or taken up into the cells during the test period. Profiling of fluorescence intensities in the confocal fluorescence images was performed using the Zen software (Carl ZEISS).

Real-time NIR fluorescence imaging of cancer cells

The potential utility of RFC in the real-time NIR fluorescence imaging was evaluated using a live cell imaging system (Axio Observer Z1, Carl Zeiss, Oberkochen, Germany). HeLa cells were seeded in 12-well plates (BD Biosciences) with a density of 5×10^5 cells per well and allowed to attach for 24 h. RFC and free ATTO655-COOH were dissolved in DMSO and diluted with cell culture medium to a final concentration of 1 μM. The existing culture medium was replaced with 1 mL of fresh culture medium containing RFC or free ATTO655-COOH. Without washing the cells, NIR fluorescence images were obtained every 10 min for 2 h ($\lambda_{ex.}$ 640 ± 15 nm and $\lambda_{em.}$ 690 ± 25 nm) by live cell imaging. All images were acquired using the same microscope settings in order to ensure reproducibility.

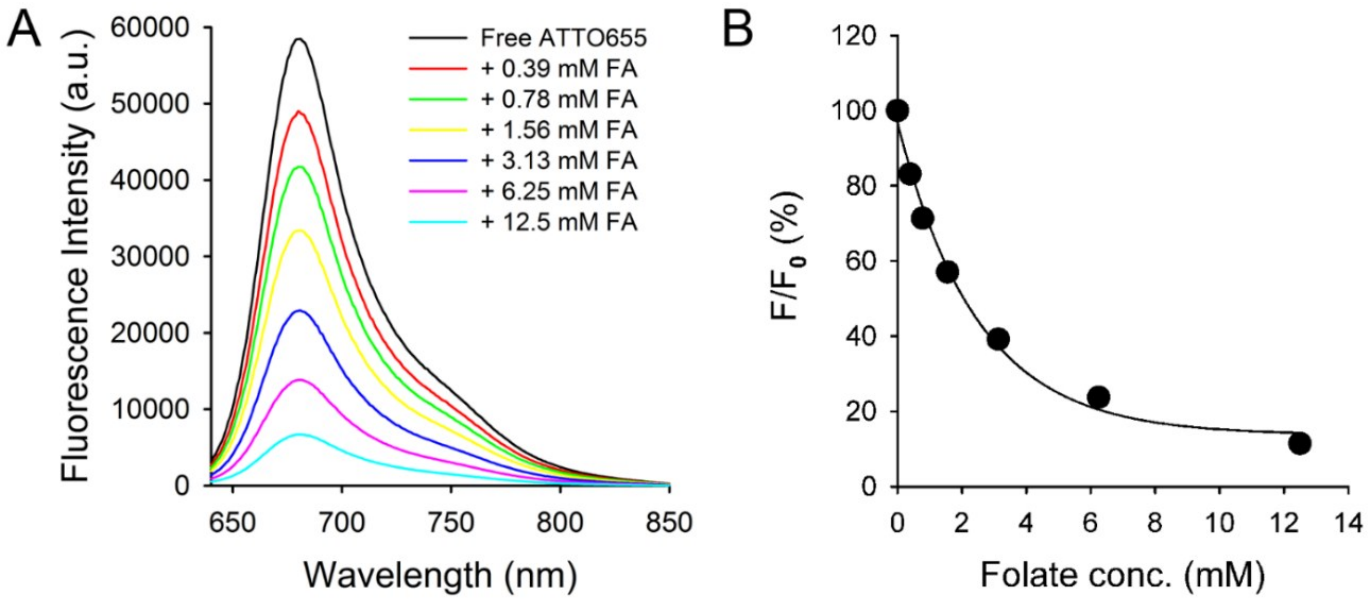

Figure S1 Fluorescence quenching of free ATTO655 dye by FA. (A) The fluorescence spectra ($\lambda_{ex.}$ 600 nm, λ_{em} 610-850 nm) of free ATTO655-COOH (1 μM) in PBS solution were measured in the absence (F_0) and presence (F) of FA at various concentrations (0, 0.39, 0.78, 1.56, 3.13, 6.25, and 12.5 mM). (B) The plot of fluorescence decrease of free ATTO655-COOH ($\lambda_{ex.}$ 600 nm, λ_{em} 684 nm) *versus* FA concentration. As expected, effective fluorescence quenching of the dye could be obtained by increasing the concentration of FA in an aqueous solution, because of the increased chance of photo-induced electron transfer between FA and the dye.

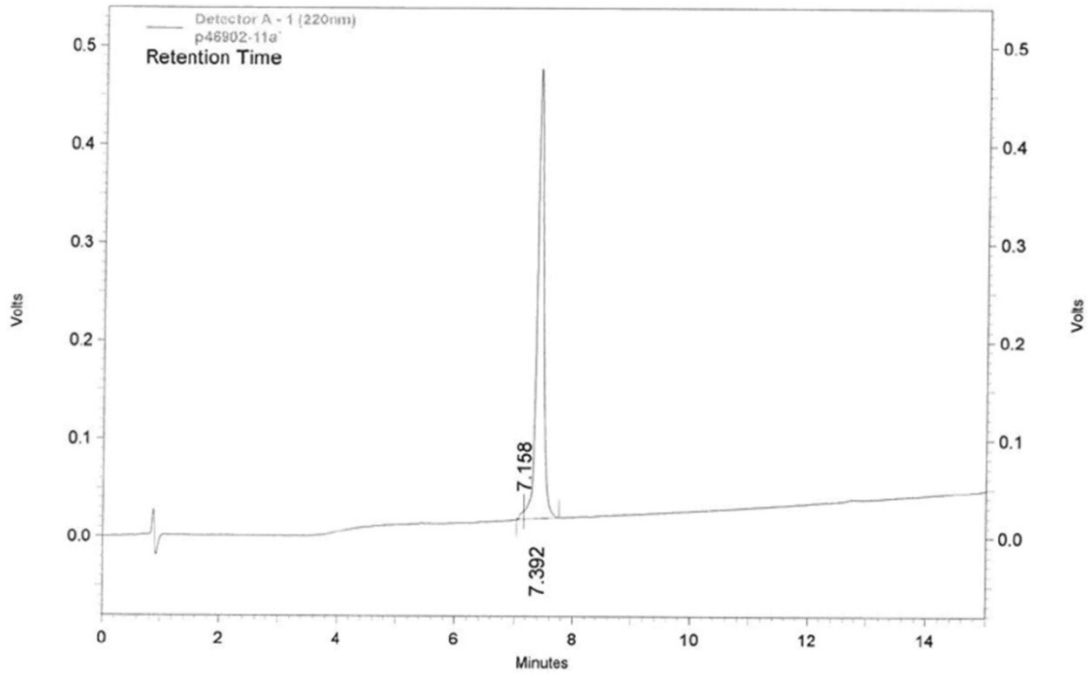

Figure S2 HPLC chromatogram of synthesized RFC. Purified FA-s-s-ATTO655 (i.e., RFC) was analyzed by reversed-phase HPLC (Shimadzu Prominence HPLC, Kyoto, Japan) using a Shiseido capcell pak C18 column. The elution was carried out with a water–acetonitrile linear gradient containing 0.1 % (v/v) TFA. The flow rate was 1 mL/min. The composition of the mobile phase was as follows: 100 % water and 0 % acetonitrile at 0 min, 80 % water and 20 % acetonitrile at 2 min, 50 % water and 50 % acetonitrile at 12 min, and 20 % water and 80 % acetonitrile at 14 min. RFC was detected at 220 nm with a retention time of 7.392 min and a measured purity of 99.08 %.

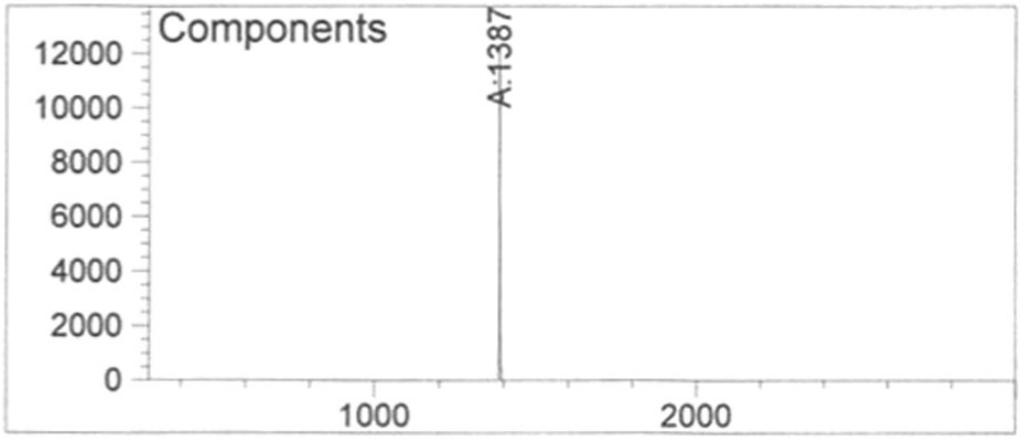

Figure S3 Mass spectrum of RFC (the theoretical molar mass of RFC is 1386.56 g/mol).

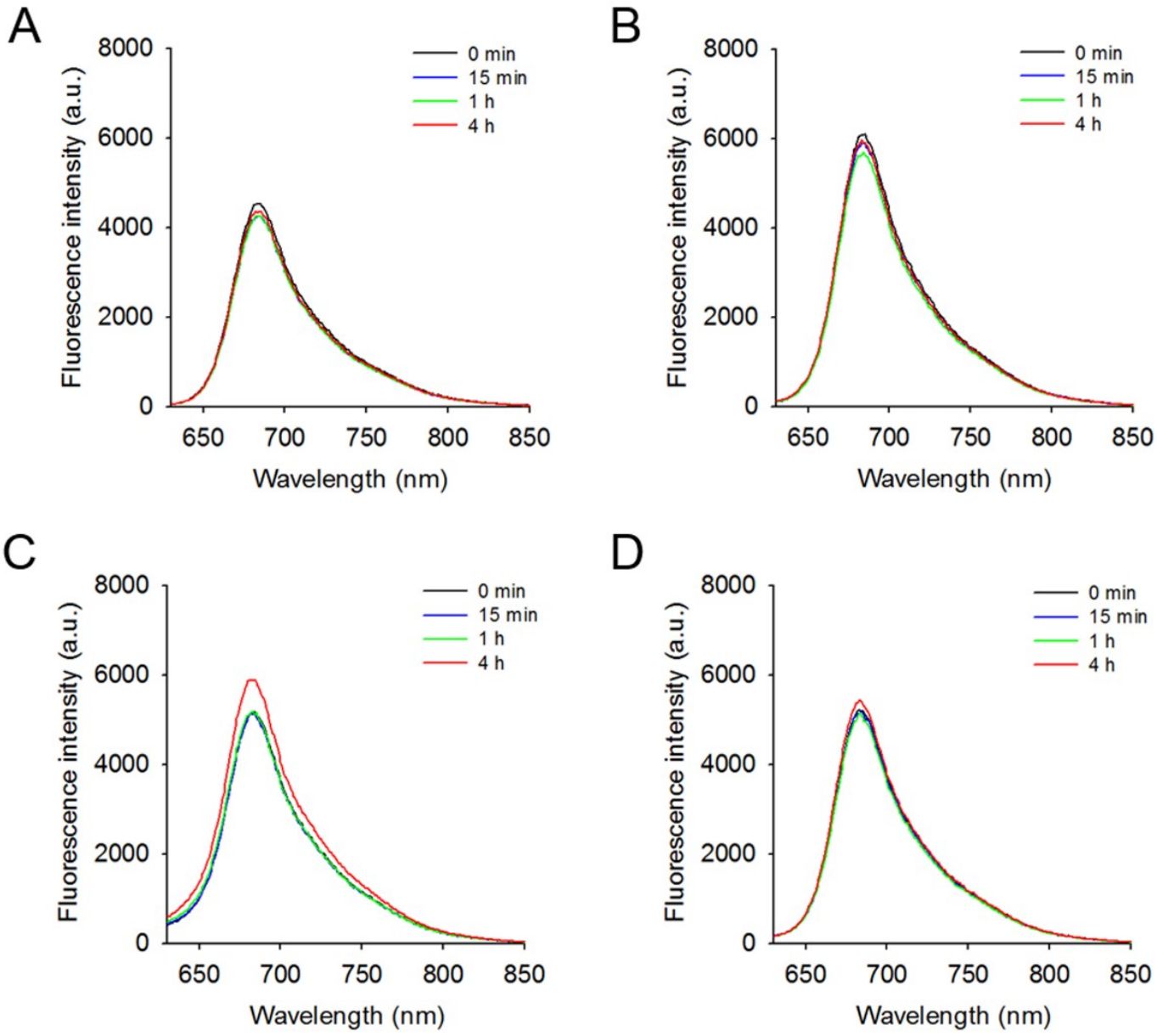

Figure S4 Stability test of the quenched state of RFC fluorescence under serum conditions. RFC was dissolved in (A) PBS solution (pH 7.4), (B) PBS containing 10 % FBS, (C) 100 % FBS, and (D) DMEM culture media. Then the fluorescence spectra of the solutions were monitored for 4 h ($\lambda_{ex.}$ 610 nm, $\lambda_{em.}$ 630–850 nm).

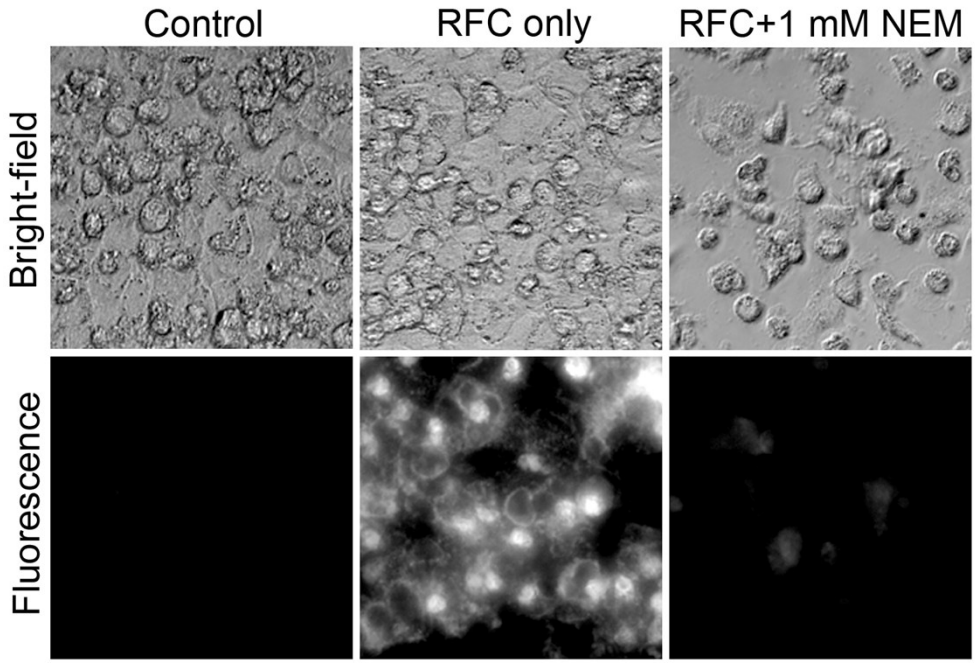

Figure S5 Fluorescence images of live HeLa cells following incubation with RFC in the presence or absence of NEM pre-treatment. *Left column*: HeLa cells in the control group were treated with cell culture medium alone. *Middle column*: HeLa cells in the RFC-treated group were incubated with 1 μM RFC for 2 h, washed, and imaged for NIR fluorescence ($\lambda_{ex.}$ 655 ± 20 nm and $\lambda_{em.}$ 716 ± 20 nm). *Right column*: HeLa cells in the inhibition group were pre-treated with 1 mM NEM for 20 min and then incubated with 1 μM RFC for 2 h.

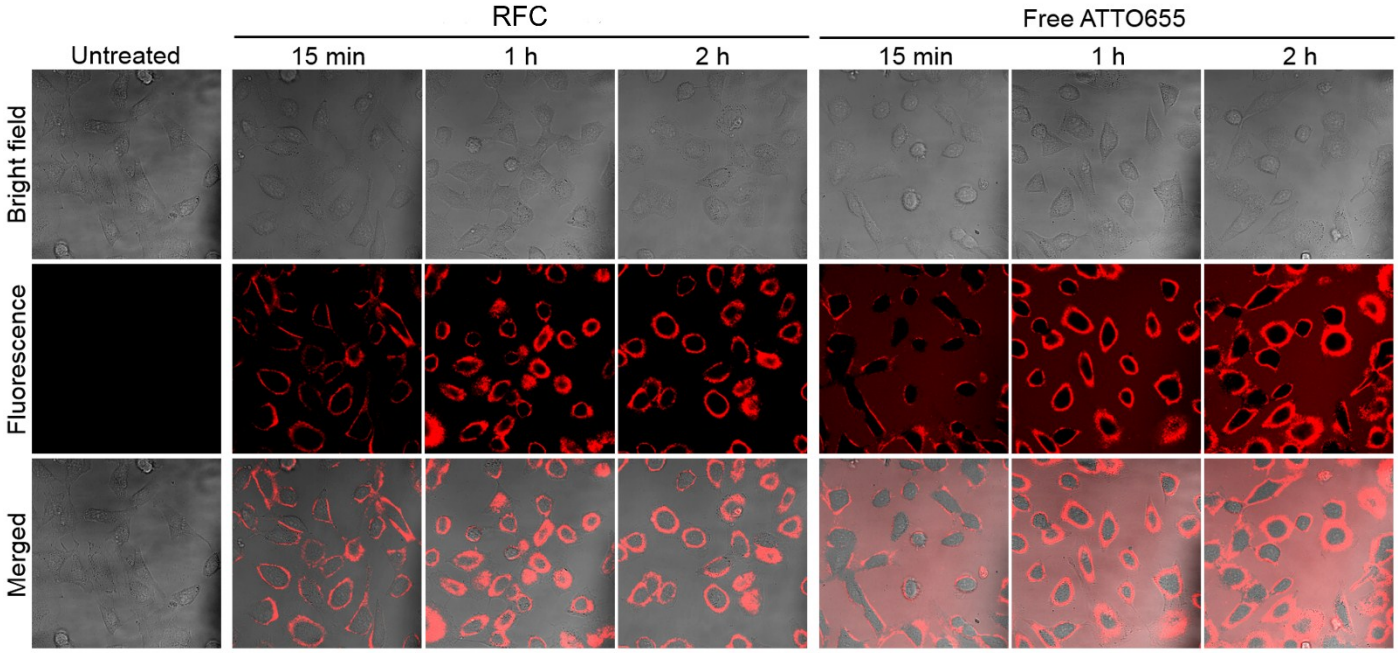

Figure S6 Confocal microscopy images of HeLa cells that were treated with FRC or free ATTO dye. NIR fluorescence images were obtained at 15 min, 1 h, and 2 h without washing the cells.