

Electronic Supporting Information for the article:

Cell-based Dual *in vivo* Imaging Probes using Genetically Expressed Tags and Chemical Contrast Agents

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Cell culture

HeLa cells provided by the RIKEN CELL BANK (Tsukuba, Japan) were cultured in Minimum Essential Medium (MEM) containing 10% fetal bovine serum (FBS) and 1% antibiotic-antimycin (GIBCO) at 37 °C under 5% CO₂.

The constructed DNA of BAP-YFP-TM

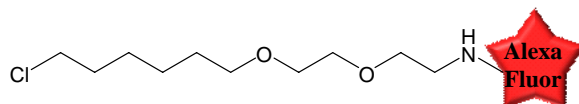
The sequence encoding BAP (216 base pairs) was amplified by PCR using a pcDNA 6 / BioEase vector (Invitrogen) as the template with primers 5'-GA TCT CCC GGG ATG GGC GCC GGC ACC CCG GT-3' (upstream) and 5'-GA TCT CCC GGG CGC CAG GGT CAT CAG GGT GTC G-3' (downstream), which introduced a *Xma I* restriction site at the 5' end. The sequence encoding YFP (720 base pairs) was also amplified by PCR using a pEYFP-N1 vector (Invitrogen) as the template with primers 5'-GGG ATC CGC GGT ATG GTG AGC AAG GGC GAG GAG-3' (upstream) and 5'-GG GAT CCG CGG CTT GAT CAG CTC GTC CAT GCC-3' (downstream), which introduced a *Sac II* restriction site at the 5' end. The PCR products were digested with enzymes and gel purified. The obtained fragments were inserted into the multiple cloning site of the pDisplay vector (Invitrogen) using T4 DNA ligase (Roche). We cloned the gene encoding BAP and YFP between the gene encoding the N-terminal murine Ig κ -chain leader and the gene encoding the C-terminal PDGFR -TM, to generate the BAP-YFP-TM.

The constructed DNA of HTP-CFP-TM

The sequence encoding HTP (890 base pairs) was amplified by PCR using a pFC14K (HaloTag 7) CMV Flexi vector (Promega) as the template with primers 5'-GA AGA TCT GGA TCC GAA ATC GGT ACT G-3' (upstream) and 5'-TCC CCG CGG ACC GGA AAT CTC CAG AGT AG-3' (downstream), which introduced a *Bgl II* restriction site at the 5' end and a *Sac II* restriction site at the 3' end. The sequence encoding CFP (720 base pairs) was also amplified by PCR using a pECFP-N1 vector (Invitrogen) as the template with primers 5'-TCC CCG CGG ATG GTG AGC AAG GGC GAG GA-3' (upstream) and 5'-CC AAT GCA TTG GTT CTG CAG CTT GTA CAG CTC GTC CAT G-3' (downstream), which introduced a *Sac II* restriction site at the 5' end and a *Pst I* restriction site at the 3' end. The PCR products were digested with enzymes and gel purified. The obtained fragments were inserted into the multiple cloning site of the pDisplay vector (Invitrogen) using T4 DNA ligase (Roche). We cloned the gene encoding HTP and CFP between the gene encoding the N-terminal murine Ig κ -chain leader and the gene encoding the C-terminal PDGFR-TM, to generate the HTP-CFP-TM.

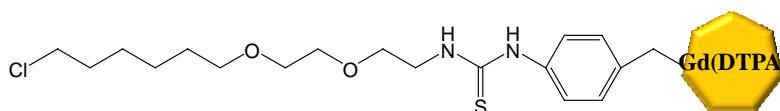
General synthetic procedure for HTL-AlexaFluor

2-(2-(6-Chlorohexyloxy)ethoxy)ethanamine (HTL), which was prepared from 6-chloro-1-iodohexane and 2-(2-aminoethoxy)ethanol by the synthetic route described,¹ and 1.0 equivalent of *N*-hydroxysuccinimide ester (NHS ester)-AlexaFluor (Invitrogen) were dissolved in *N,N*-dimethylformamide in the presence of trimethylamine (1 drop), and the solution was stirred at room temperature for 24 h. The resulting HTL-AlexaFluor was analyzed by TLC.



General synthetic procedure for HTL-Gd(DTPA)

HTL (1 eq.) and *S*-2-(4-isothiocyanatobenzyl)-diethylenetriamine pentaacetic acid (*p*-NCS-Bn-DTPA, Macrocyclics, 1 eq.) were dissolved in 0.1 M NaHCO₃ (100 μ l) and stirred at room temperature for 24 h. GdCl₃ (0.9 e.q.) was added to the reaction mixture, and stirring was continued for 24 h at room temperature (pH = 6.0). The resulting HTL-Gd(DTPA) was analyzed by TLC and MALDI-TOF MS.



Fluorescence imaging of HeLa cells expressing BAP-YFP-TM or HTP-CFP-TM labelled with SA-DL547 conjugate and HTL-AF594 conjugate (Fig. 2)

HeLa cells were transformed with BAP-YFP-TM or HTP-CFP-TM using Lipofectamine LTX (Invitrogen), and plated together on the same dish. Two identical dishes were prepared. After 48 h, the cells of the first dish were incubated with 10 μ l/ml streptavidin (SA)-Dylight547 (DL547) conjugate (Pierce) in PBS for 1 h at room temperature. After washing three times with PBS, cells were observed by a confocal fluorescence microscope (Fluoview FV1000). The fluorescence and differential interference contrast (DIC) of the labelled cells are shown in Figs. 2(a)–(d). The cells of the second dish were incubated with 10 μ M HTL-AlexaFluor594 (AF594) conjugate in the culture medium for 15 min at 37 °C, 5% CO₂. The cells were washed according to a recommended protocol² and observed. The fluorescence and DIC of the labelled cells are shown in Figs. 2(e)–(h).

Fluorescence imaging of HeLa cells coexpressing BAP-YFP-TM and HTP-CFP-TM labeled with SA-DL547 conjugate and HTL-AF594 conjugate (Fig. 3)

For the introduction of two kinds of chemical probes to the same cell, two kinds of DNA (BAP-YFP-TM and HTP-CFP-TM) were introduced to HeLa cells to obtain cells coexpressing BAP-YFP-TM and HTP-CFP-TM. Two identical dishes were prepared. In the first dish, BAP-YFP-TM and HTP-CFP-TM coexpressing cells were labelled with SA-DL547 conjugate, and observed by a confocal fluorescence microscope by the same method as described above. The fluorescence and DIC of the labelled cells are shown in Figs. 3(a)–(d). The cells of the second dish were labelled with HTL-AF594 conjugate, and observed. The fluorescence and DIC of the labelled cells are shown in Figs. 3(e)–(h).

In vitro two-colour NIR fluorescence imaging of cells

Two kinds of NIR fluorescent probes (AlexaFluors: AF 680 and AF750) were simultaneously introduced to the cells to establish two-colour NIR fluorescence imaging. HeLa cells coexpressing BAP and HTP on the surface were incubated with 50 μ M HTL-AF750 conjugate in culture medium for 15 min at 37 °C, 5% CO₂ and washed. Subsequently, they were incubated with 50 μ l/ml streptavidin (SA)-AF680 conjugate (Invitrogen) in PBS for 1 h at room temperature and washed three times. After trypsinization and washing, the cells were transferred to 1.5 mL microtubes at a concentration of about 7×10^6 cells/tube, and AF 680-AF750-labelled cell pellets (iv; Sample 4) were prepared. For comparison purposes, unlabelled cell pellets (C; Control), cell pellets labelled with SA-AF680 conjugate alone (i; Sample 1), HTL-AF680 conjugate alone (ii; Sample 2) and HTL-AF750 conjugate alone (iii; Sample 3) were prepared. The cell pellets in microtubes were imaged via optical imaging using an Optix MX2 (ART, Inc.). The AF680 fluorescence images of these cell pellets are shown in Fig. S1 (a) and the AF750 images in Fig. S1 (b). Pellets of cells labelled with AF680 via BAP (i, ii and iv) showed AF680 fluorescence (Fig. S1(a)) and pellets of cells labelled with AF750 via HTP (iii and iv) showed AF750 fluorescence (Fig. S1 (b)). iv showed both AF680 and AF750 fluorescence, which indicated that *in vitro* two-colour NIR fluorescence imaging of cell pellets could be achieved.

In vitro dual NIR fluorescence-MR imaging of cells

A NIR fluorescent probe (AF680) and a MRI probe (Gd(DTAP)) were introduced to the cells to establish dual-NIR fluorescence-MR-imaging. HeLa cells coexpressing BAP and HTP on the surface were incubated with 50 μ M HTL-Gd(DTPA) conjugate in culture medium for 15 min at 37 °C, 5% CO₂ and washed. Subsequently, they were incubated with 50 μ l/ml SA-AF680 conjugate (Invitrogen) in PBS for 1 h at room temperature and washed three times. After trypsinization and washing, the cells were transferred to 1.5 mL microtubes at a concentration of about 7×10^6 cells/tube, and AF680-Gd(DTPA)-labelled cell pellets were prepared. The cell pellets in microtubes were imaged optically with the Optix MX2 (ART, Inc.) and via MRI by a Sigma EXITE XI 1.5 T (GE healthcare). *In vitro* NIR fluorescence images and MR images of the labelled cell pellets (Sample) and the unlabelled cell pellets (Control) are shown in Figs. S2(a) and (b), respectively. While unlabelled cell pellets showed no fluorescence, labelled cell pellets showed fluorescence (Fig. S2(a)). Also, labelled cell pellets showed stronger T1 signal enhancement compared to the unlabelled cells (Fig. S2(b)). These results demonstrate that *in vitro* dual NIR fluorescence-MR imaging of cell pellets could be achieved.

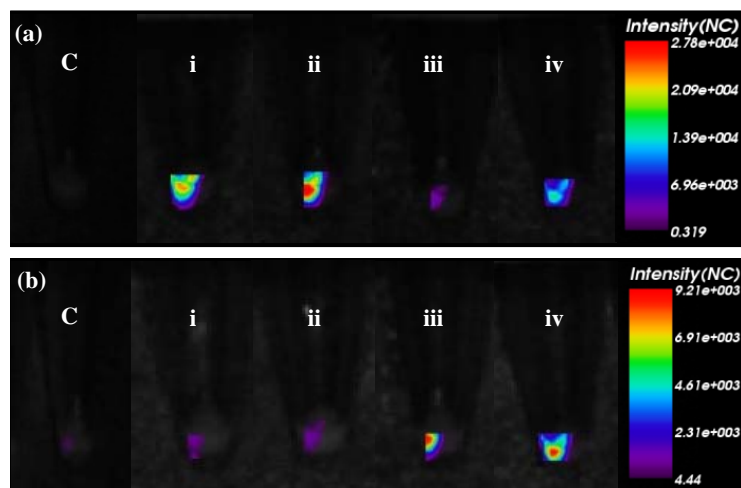


Fig. S1 *In vitro* NIR fluorescence imaging of unlabelled cell pellets (C), cell pellets labelled with SA-AF680 conjugate alone (i), HTL-AF680 conjugate alone (ii), HTL-AF750 conjugate alone (iii) and both SA-AF680 conjugate and HTL-AF750 conjugate (iv). (a) AF680 fluorescence; Exc: 670 nm, Filter: 700LP, (b) AF750 fluorescence; Exc: 730 nm, Filter: 750LP

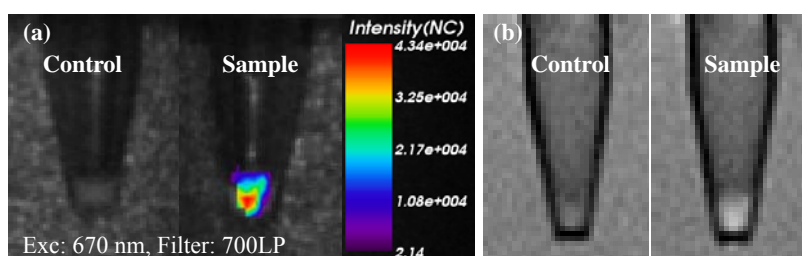


Fig. S2 *In vitro* NIR fluorescence imaging (a) and T1-weighted MR imaging (b) of AF680-Gd (DTPA)-labelled cell pellets (Sample) and unlabelled cell pellets (Control).

***In vivo* NIR fluorescence imaging of a mouse subcutaneously injected with cells (Fig. 4)**

Two kinds of NIR fluorescent probes (AF680 and AF750) were simultaneously introduced to the cells to establish *in vivo* two-colour NIR fluorescence imaging in a first application example. HeLa cells coexpressing BAP and HTP on the surface were labelled with SA-AF680 conjugate (Invitrogen) and HTL-AF750 conjugate. After trypsinization and washing, AF680-AF750-labeled cells (iv; Sample 4) were prepared. For comparison purposes, unlabelled cells (C; Control), cells labelled with SA-AF680 conjugate alone (i; Sample 1), HTL-AF680 conjugate alone (ii; Sample 2) and HTL-AF750 conjugate alone (iii; Sample 3) were prepared. Labelled cells (i-iv) and unlabelled cells (C) were suspended in PBS at about 3.5×10^6 cells / 100 μ l and injected subcutaneously into the corresponding position of a mouse previously anesthetized by injection of 10% nembutal and inhalation of isoflurane (shown in Fig. 4). Then, NIR fluorescence imaging of the mouse was performed using an Optix MX2 (ART, Inc.). The resulting AF680 fluorescence image of the mouse is shown in Fig. 4(a) and the AF750 fluorescence image in Fig. 4(b).

***In vivo* NIR fluorescence imaging and T1-weighted MR imaging of mouse (Fig. 5)**

A NIR fluorescent probe (AF680) and a MR imaging probe (Gd(DTPA)) were introduced to the cells to establish *in vivo* dual-NIR fluorescence-MR-imaging. HeLa cells coexpressing BAP and HTP on the surface were labelled with HTL-Gd(DTPA) conjugate and SA-AF680 conjugate (Invitrogen) to obtain doubly AF680-Gd(DTPA)-labelled cells (Sample). Labelled cells (Sample) and unlabelled cells (Control) were suspended in PBS at about 3.5×10^6 cells / 100 μ l, and injected subcutaneously in the hind limb of a nude mouse (BALB/C Slc-nu/nu 8w male) anesthetized by injection of 10% nembutal and inhalation of isoflurane. The mouse was imaged optically with the Optix MX2 (ART, Inc.) and by MR with a Sigma EXITE XI 1.5 T (GE healthcare). *In vivo* NIR fluorescence images and MR images of the mouse are shown in Figs. 5(a) and (b).

Reference

- 1 Yan Zhang and Min-kyung, *Angew. Chem. Int. Ed.*, 2006, **45**, 4936
- 2 Technical Manual, Promega, "HaloTag Interchangeable Labeling Technology"; <http://www.promega.com>, 2006.