Electronic Supplementary Information (ESI)

Cascade imaging of proteolytic pathway in cancer cell using fluorescent protein-conjugated gold nanoquenchers

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Experimental details

Materials. Gold chloride trihydrate (99.9%), trisodium citrate, cisplatinum, Staurosporine (STS), and recombinant human caspase-9 were all purchased Sigma-Aldrich (St. Louis, MO). PEG_{5000}-SH was purchased from SunBio Co. (Anyang, Korea) and TRAIL was acquired from Genenmed Inc. (Seoul, Korea). Recombinant human Caspase-3, -8, caspase inhibitors (Z-DEVD-FMK, Z-IETD-FMK, and Z-LEHD-FMK for caspase-3, caspase-8, and caspase-9), and caspase colorimetric assay kits were obtained from R&D systems (Minneapolis, MN). All the other chemicals were of analytical grade, and purified water was produced by a Millipore water purification system.

Gene cloning, expression, and purification of recombinant chimeric proteins. *Escherichia coli* strain DH 5α was utilized as a host for subcloning and *E. coli* BL21 (DE3) (Novagen, WI) was used for gene expression. The *E. coli* strain was grown in LB medium at 37°C and 50 mg/mL of ampicillin was added for the plasmid-harboring strains. pET 21a (Novagen, WI) was utilized as a vector for the subcloning and expression. The restriction enzymes and modifying enzymes were obtained from Roche Applied Science (Germany) and utilized in accordance with the supplier's recommendations. Vector DNA was prepared using a QIAEX II gel extraction kit (Qiagen, Germany). In order to clone the cysteine-tagged glutathione-S-transferase (cysGST)-linker-FPs chimeric gene, the cysteine-tagged GST gene was amplified with the 5_ primer 1 and the 3_ primer 2 *via* the polymerase chain reaction (PCR). The 5_ and 3_ termini were designed to harbor the BglII and BamHI restriction enzyme cleavage sites, respectively. The full-length gene encoding for the linker: FP was amplified with the 5_ primer (primer 3 for eBFP, primer 4 for eYFP, and primer 5 for DsRed-monomer) and the 3_ primer (primer 6 for eBFP and eYFP, primer 7 for DsRed-monomer), *via* the polymerase chain reaction (PCR). These linkers all harbored flanking regions on both sides of the caspase cleavage site. The flanking regions located at the N-terminus or C-terminus of cleavage sequence were artificial sequences that consisted of two glycine residues. The 5_ and 3_ termini were designed to harbor the BamHI and XhoI restriction enzyme cleavage sites, respectively. All primer sequences were summarized in Table S1. The PCR products were then purified using a DNA purification kit (Qiagen), and digested with the restriction enzymes. The resultant DNA fragments were then inserted into the pET 21a

plasmid to generate the in-frame fusion of cysGST:linker:FPs. The cysGST:linker:FPs chimeric gene was then verified *via* DNA sequencing, and transformed into *E.coli* BL21 (DE3) for the expression of the recombinant protein. The cells were grown at 37°C with shaking to an OD_{600} of 0.6~0.8. The cells were induced with 1 mM isopropyl-2-D-thiogalactopyranoside (IPTG) (Gibco-BRL, USA) and grown for an additional 16 h at 25°C. The cells were then harvested *via* 10 min of centrifugation at 6000 g at 4°C. In order to purify the proteins, Cell lysate was loaded onto a IDA-mini excellose affinity column (Bioprogen, Korea) and washed three times with 20 mL washing buffer (50 mM Tris-HCl, 0.5 N NaCl, pH 8.0, 50 mM imidazole), respectively. The recombinant proteins were then eluted with 10 mL of 0.5 M imidazole in the same buffer (50 mM Tris-HCl, 0.5 N NaCl, pH 8.0) and dialyzed against phosphate-buffered saline (PBS, pH 7.4) buffer. The purified recombinant cysGST:linker:FPs proteins were resolved on 12% SDS-PAGE gel and gels were stained with Coomassie brilliant blue R250. The protein concentrations were determined *via* the Bradford method, using bovine serum albumin as a standard.

Preparation of fluorescent protein-conjugated gold nanoparticle. AuNPs were synthesized by reducing gold (III) chloride with citrate. Sixty mg of $HAuCl_4$ was dissolved in 125 mL of boiling water before adding 25 mL of 1% sodium citrate with stirring and boiling for another 20 min until the solution turned a red color. After cooling, 40 μg of each FPs was individually added to 1 mL of a AuNP solution (0.1 mg/mL) and the mixed solution was incubated for 1 h at room temperature before adding 20 μL of 0.5 mM thiol-terminated PEG (5 kDa) solution and incubating for another 4 h to stabilize the conjugate. Remaining unbound proteins and PEG were removed by centrifugation (15,000 rpm, 15 min) three times with 0.1x PBS (pH 7.4) containing 0.05% Tween-20™. Finally, the conjugate was dissolved in 1x PBS containing 0.05% Tween-20™ for use.

Characterization of fluorescent protein-conjugated gold nanoparticle. The size and shape of AuNPs were observed by transmission electron microscopy using JEOL 2100 UHR-TEM instrument operating at an accelerating voltage of 120 KeV. The hydrodynamic diameter of AuNPs and protein-conjugated AuNPs (AuNP-FPs) were obtained from dynamic light scattering (ELS-Z, Otzka, Japan) by averaging three measurements. Absorption spectra were measured by UV/Visible spectroscopy (DU800, Beckman Coulter, UK) which scanned over the range of 300 ~ 700 nm. The fluorescence of various FPs was measured using fluorescence spectroscopy (LS55, PerkinElmer, UK). The excitation wavelength was fixed 380 nm for eBFP and emission spectra recorded from 400 ~ 600 nm. The excitation wavelength of eYFP was 500 nm and emission spectra was from 520 ~ 600 nm. For DsRed-monomer, the excitation wavelength was 560 nm and emission spectra was measured from 580 ~ 650 nm. The measurements were carried out under ambient conditions. To investigate the effect of various conditions such as pH, salt concentration, serum-containing media and reduced glutathione (GSH) solution, 0.1 mg/mL of AuNP-3Y were incubated with acetic acid buffers with pH 4.0, 100 mM NaCl solution, 10 % FBS containing DMEM, and 20 mM concentrations of GSH solution. After incubation, the size of AuNP-3Y in various conditions was measured by DLS. The number of FPs conjugated with a single AuNP was briefly calculated based on the assumptions that all FPs (40 μg) added were conjugated on AuNPs (0.1mg) since the fluorescence of FPs was totally quenched after AuNP conjugation at this

condition.

$$\text{the number of FPs in 0.4 mg protein solution} = \frac{\text{conjugated protein weight x FP purity x avogadro's number}}{\text{molecular weight of FP}}$$

$$= \frac{0.4 \times 10^{-3}\,(\text{g}) \times 0.8 \times 6.022 \times 10^{23}\,(/\text{mol})}{52 \times 10^{3}\,(\text{g/mol})} = 3.7 \times 10^{15}$$

$$\text{the number of AuNPs in 1 mg Au solution} = \frac{\text{Au weight}}{\text{the volume of a AuNP x density of Au}}$$

$$= \frac{1 \times 10^{-3}\,(\text{g})}{4\pi/3 \times (5 \times 10^{-9}\,\text{m})^3 \times 19.6\,(\text{g/cm}^3)} = 9.7 \times 10^{13}$$

$$\text{the number of conjugated FPs on a AuNP} = \frac{\text{the number of FPs in 0.4 mg protein solution}}{\text{the number of AuNPs in 1 mg Au solution}}$$

$$= \frac{3.7 \times 10^{15}}{9.7 \times 10^{13}} = 38.14$$

***In vitro* assay of caspase activity using AuNP-FPs.** *In vitro* caspase activities were measured fluorescence of releasing FPs from AuNP-FPs (0.1 mg) by adding various caspases in different concentrations. After incubation for 1 h, the reacted AuNPs were removed by centrifugation (15,000 rpm, 15 min) and the supernatant was measured fluorescence intensity using by fluorescence spectroscopy. The emission spectra of each FPs were recorded under the excitation at 380 nm for eBFP, 500 nm for eYFP, and 560 nm for DsRed-monomer. The caspase selectivity of the AuNP-FPs was evaluated by incubation in a tube containing 0.5 mL of AuNP-FP (0.1 mg/mL) with different caspases (400 U/mL). After incubation at RT for 1 h, reacted AuNPs were removed by centrifugation (15,000 rpm, 15 min) and the supernatant was measured fluorescence intensity under excitation/emission set at 380/445, 500/528, 560/592 nm for AuNP-3Y, AuNP-8B, and AuNP-9R, respectively.

Cell culture. Human adenocarcinoma HeLa cells (ATCC, USA) were grown in Dulbecco's modified Eagle medium (DMEM, GIBCO, NY) supplemented with 10% (v/v) fetal bovine serum (FBS, GIBCO, NY), 1% (w/v) penicillin/streptomycin. Cells were cultured in incubators maintained at 37°C under a humidified atmosphere containing 5% CO_2.

Real-time monitoring of caspase activity using AuNP-FPs. HeLa cells were plated at a density of 5000 cells/well in a μ-slide 8 well plate (Ibidi, Germany). After the overnight incubation with 3 μg AuNP-FPs, the cells were washed three times with PBS and then treated with anticancer drugs in 37°C for induction of apoptosis. Staurosporine (STS) was dissolved in DMSO. STS was treated to cells at final concentration of 5 μM. For the inhibitor studies, cells were incubated with 100 μM caspase inhibitors (Z-DEVD-FMK, Z-IETD-FMK, and Z-LEHD-FMK for caspase-3, caspase-8, and caspase-9, respectively) 2 h before STS treatment. After incubation of anticancer drug in AuNP-FPs uptake cells, the caspase activities were monitored by observation of fluorescence from released FPs using Laser Scanning Microscope (LSM) 510 META confocal microscope system (Carl Zeiss., Germany) using the c-Apochromat 40x1.2 w objective. The images were taken using excitation/emission band

pass filter (or long pass filter) set at 405/420-480 nm, 514/530-600 nm, and 542/560 nm for eBFP, eYFP, and DsRed-monomer, respectively.

Cell viability assay. HeLa cells were used to evaluate the cytotoxicity of AuNP-3Y. Cell viabilities were determined by a quantitative colorimetric assay using Cell Counting Kit-8 (Dojindo, Japan). Briefly, cells were seeded in 96-well plates with a density of 5000 cells/well, and allowed to adhere for 24 h prior to assay. Cells were incubated with various concentrations (0, 0.012, 0.025, 0.05, and 0.1 mg/mL) of AuNP-3Y for 24 h. After treatment of AuNP-3Y, 10 μL of the CCK-8 solution was added into the each well and incubated for 1 h at 37°C. The absorbance at 450 nm was read using a microplate reader (SpectraMax M2e, Molecular Devices, USA). The absorption from the control cells was set as 100% cell viability.

Colorimteric caspase activity assay in cell extracts. The activity of caspase was determined using a colorimteric assay (R&D Systems, Inc.) according to the manufacturer's instructions. HeLa cells were exposed to STS (5 μM) for up to 4 h. At every hour, cells were collected by centrifugation (250 g for 10 minutes). The supernatants were discarded and the cells were lysed by the addition of the lysis buffer. The cell lysate was centrifuged at 10,000 *g* for 1 min, supernatant was collected. Enzymatic reactions for caspase activity were carried out in 96-well flat bottom microplates. Caspase-3 activity in the lysates was measured using DEVD-p-nitroalanine (DEVD-pNA) as caspase-3 substrate. For each sample, 50 μL of the cell lysate, 5 μL of the DEVD-pNA, and 50 μL of reaction buffer were transferred to a 96-well plate, mixed, and incubated at 37°C for up to 2 h. The assay is based on spectrophotometric detection of the chromophore pNA after cleavage from the labeled substrate. Release of free pNA chromophores was monitored by tracking changes in absorbance at 405 nm on microplate reader (SpectraMax M2e, Molecular Devices, USA).Caspase-8- and caspase-9 activities in the lysates were measured in the same way as caspases-3 activity except using IETD-pNA as the substrate of caspase-8 and LEHD-pNA as the substrate of caspase-9.

Table S1. Primer sequences

Primer	Sequence
1	GA AGA TCT ATG TGC TCC CCT ATA CTA GG
2	CG GGA TCC ACG CGG AAC CAG ATC CGA
3	CG GGA TCC GGA GGG ATC GAG ACC GAT GGG GGC ATG GTG AGC AAG GGC GAG
4	CG GGA TCC GGA GGG GAT GAG GTG GAT GGG GGC ATG GTG AGC AAG GGC GAG
5	CG GGA TCC GGA GGG TTA GAG CAC GAT GGG GGC ATG GAC AAC ACC GAG G
6	CCG CTC GAG CTT GTA CAG CTC GTC CAT GC
7	CCG CTC GAG CTG GGA GCC GGA GTG G

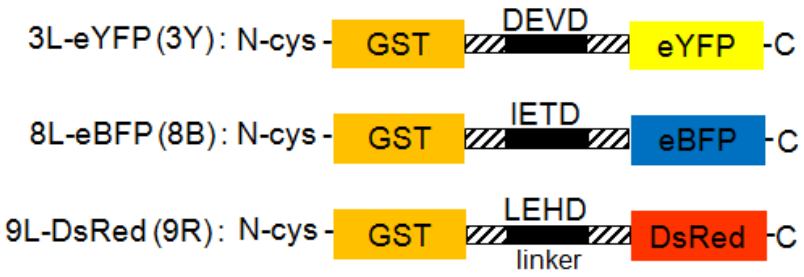

Fig. S1 Design of caspase-activated linker-conjugated various fluorescent proteins having cysteine-tagged glutathione-S-transferase (GST) in N-termini.

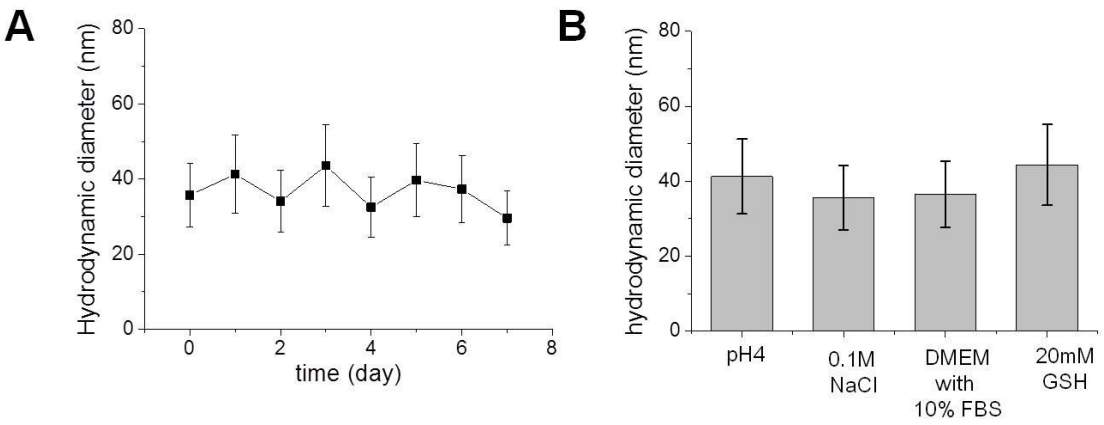

Fig. S2 Stability analysis of FP-conjugated AuNPs with A) time-dependent and B) various conditions by measuring hydrodynamic size.

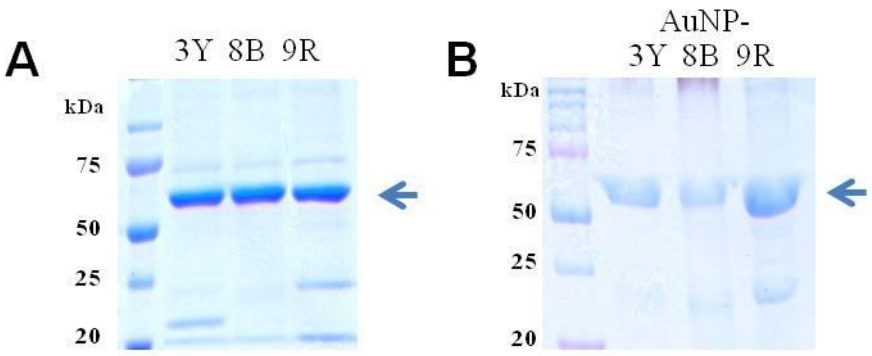

Fig. S3 SDS-PAGE of A) purified recombinant fluorescent proteins and B) conjugated proteins on AuNPs. These proteins were visualized via coomassie staining.

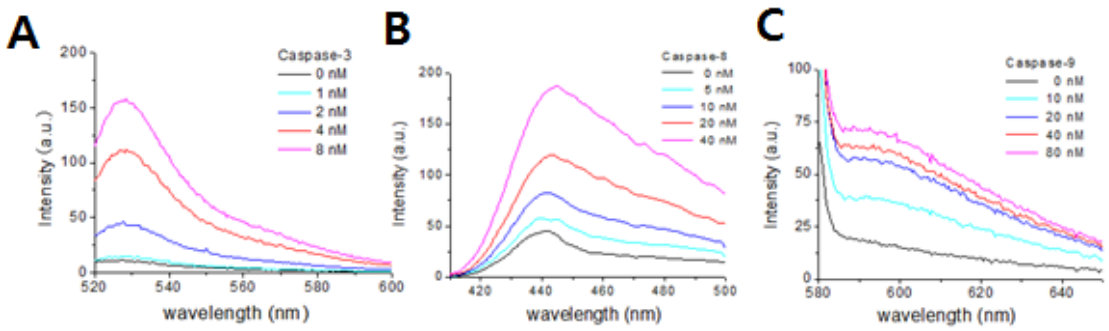

Fig. S4 *In vitro* assay of various caspase activities using (a) AuNP-3Y for caspase-3, (b) AuNP-8B for caspase-8, and (c) AuNP-9R for caspase-9 in PBS (pH 7.4) after incubation 1 h.

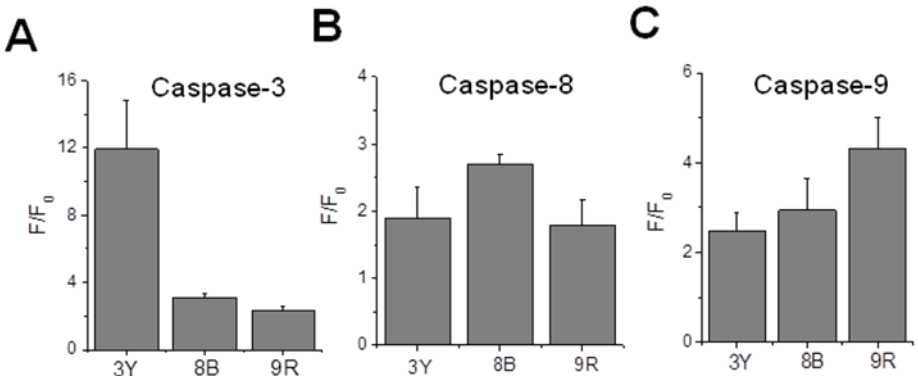

Fig. S5 Selectivity assay of AuNP- 3Y, AuNP-8B, and AuNP-9R in caspase-3 (A), caspase-8 (B), and caspase-9 (C), respectively. Each of the AuNP-FPs was separately incubated without or with caspase-3, -8, and -9 for 1 h and fluorescence intensity ratio of F_0 and F was measured in the absence and the presence of caspase, respectively. All bars represent mean ± standard deviation (SD) for n = 3.

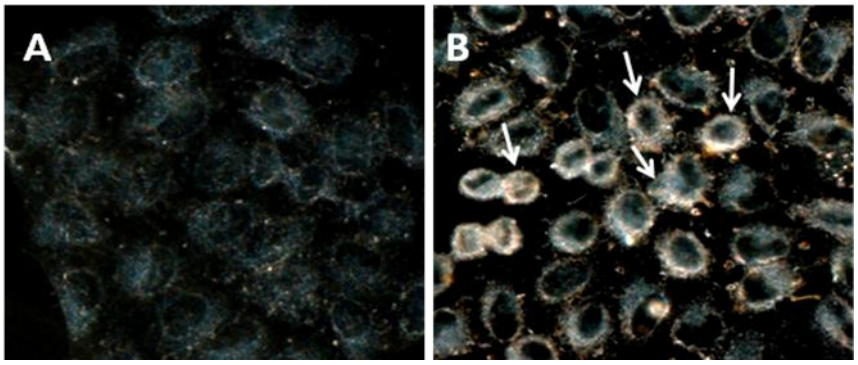

Fig. S6 Dark-field microscopic images of HeLa cells without (A) and with AuNP-FP (B) treatment. The bright colors pointed by white arrows indicates strong scattering light of AuNPs in cells compared to the paint scattering in non-treated HeLa cells as a control.

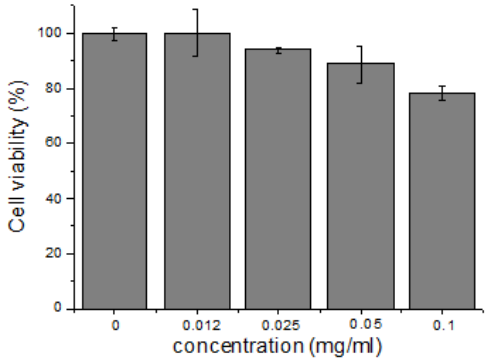

Fig. S7 Cell viability of various concentrations of AuNP-3Y in HeLa cells. Data represent mean ± SD for $n = 3$.

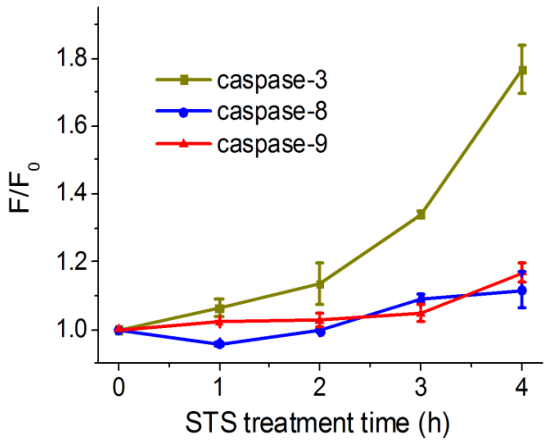

Fig. S8 Time-dependent caspase cascade assay using conventional colorimetric assay kit. Data represent mean ± SD for $n = 3$.