Supporting Information

Fluorogenic Assay and Live Cell Imaging of HIV-1 Protease Activity Using FRET-quenched Quantum Dots-Peptide Complex

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Materials

QD495-TOPO (Evidot®, maximum emission peak at 495 nm) was purchased from Evident Technologies (Troy, USA). Fluorescamine, acetic acid, sodium acetate dihydrate, sodium chloride, dithiothreitol (DTT), bovine serum albumin (BSA), peptstain A, thioctic acid, pentafluorophenol, dichlrohexylacarbodiimide (DCC), methylene chloride (CH_2Cl_2), dimethylformamide (DMF), dimethylsulfoxide (DMSO), triethylamine (Et_3N), $NaBH_4$, $NaHCO_3$, trifluoroacetic acid (TFA), Na_2SO_4 were all purchased from Sigma-Aldrich (USA). A human recombinant HIV-1 protease (10 ~ 12 kDa) was purchased from Anaspec (Fremont, USA). Anti AIDS drug, saquinavir (SQ) was kindly provided by Medicinal Chemistry Group in Institute Pasteur Korea. Transferrin-AF634 conjugate, dulbecco's modified eagle's medium (DMEM), 10% fetal bovine serum (FBS), penicillin, streptomycin were obtained from Invitrogen (USA). HIV-1 plasmid (pCMV-dR8.2 which contains Gag/Pol, Tat and Rev components of HIV-1) was

purchased from Addgene (Cambridge,USA). Fugene 6® transfection reagent was obtained from Roche (Meylan, France) and p24 ELISA assay kit was from PerkinElmer (Boston, USA). All the chemicals were used without further purification. All the solutions were prepared using Nanopure water (18 MΩ) or autoclaved water.

Characterization methods

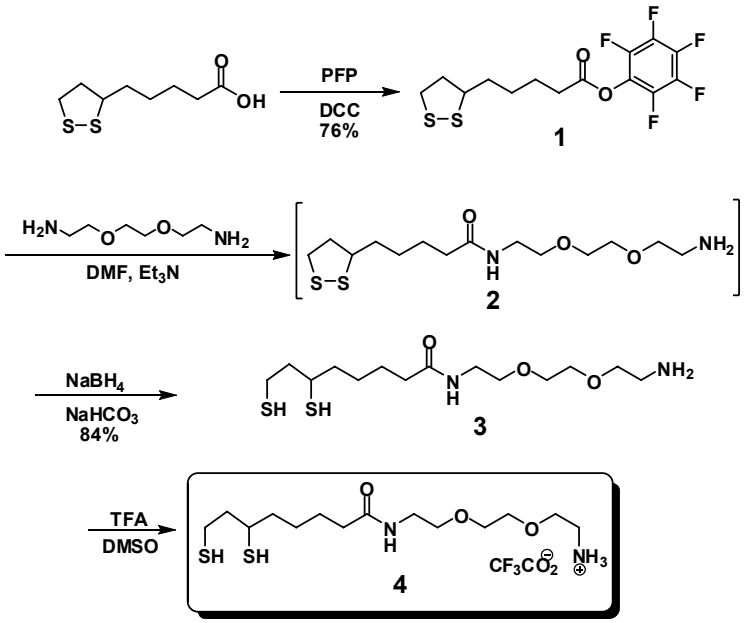
All absorption spectra of QDs were obtained in a quartz cuvette using Cary 5000 UV-Vis spectrometer (Varian Inc., CA, USA) with a spectral range (200 - 800 nm), a scan rate (600 nm min^{-1}) and a slit width (2 nm). Photoluminescence was measured by Fluorolog®-3 spectrofluorimeter (HORIBA, Jobin Yvon, UK) with excitation at 300 nm at an integration time (0.3 s) and an excitation and emission slit width (3 nm). Dynamic light scattering (DLS) and zeta potential measurements were performed at room temperature using Nano-ZS (Malvern, UK) in triplicate (n=3). The samples for DLS and zeta potential were prepared in DI water at a concentration of 1 μM. Transmission electron microscope (TEM) and high-resolution TEM image was obtained after air-drying QD solution in water on carbon-coated copper grid using JEM-2100 (JEOL Ltd.) at the accelerating voltage (200 kV). Confocal fluorescence microscope imaging was performed using LSM5 live confocal microscope (Zeiss, Germany) equipped with live incubation chamber set at 37^{o}C. For excitation of QD, 488 nm laser was used with bandpass filter (500-525 nm). Cell images were obtained using a 63X oil immersion lense (N/A 1.4) in 1024x1024 pixel format, and processed with Zen® image analysis software (Carl Zeiss).

Peptide sequence design

The HIV-1 PR substrate peptides for this electrostatic assembly were designed by adapting a modular approach as successfully utilized by the group of Mattoussi[S1,S2]. The **Pep1** peptide sequence includes (i) N-terminal six glutamic acid residues (6E) for negative charges to drive the electrostatic complex formation with positively charged amine-terminated QD-DPA, (ii) a helix linker spacer region (G-L-Aib-2G), and (iii) the recognition sequence for the HIV-1 aspartic protease (S-Q-N-Y-P-I-V-Q), and finally with K residue for dabcyl modification. For the control peptide (non-binding peptide, **Npep1**), only the recognition sequence was replaced with V-N-C-A-K-K-I-V. In addition, the N-terminal and C-terminal of each peptide was protected with acetyl and amide group, respectively. The peptides were synthesized and characterized (mass [m/z] found; 2512 for **Pep1**, 2486 for **Npep1**) by Peptron Inc. (Daejon, South Korea).

Synthesis of DPA ligand

The capping ligand **3** (DPA ; DHLA-2,2'-(ethylenedioxy) bis(ethylamine)) was synthesized according to the literature method.[S1] Briefly, the pentafluorophenol-activated thioctic acid **1** was converted to compound **2** using 2,2'-(ethylenedioxy)bis(ethylamine) as a hydrophilic linker bearing the amine functionality (**Scheme S1**). This ligand **3** was made as a trifluoroacetate (TFA) salt **4** to confer water-solubility.



Scheme S1. Synthetic scheme of DPA ligand.

Compound 2 (400 mg, 77%) as a yellow oil; ^{1}H NMR(400 MHz, $CDCl_3$) δ 6.42 (bs, 1H), δ 3.60 – 3.73 (m, 14H), δ 3.54 (t, *J* = 2.4 Hz, 2H), δ 3.45 (t, *J* = 5.2 Hz, 2H), δ 3.84 (t, *J* = 5.2 Hz, 2H), δ 3.08 – 3.21 (m, 2H), δ 2.42 – 2.49 (m, 1H), δ 2.19 (t, *J* = 7.2 Hz, 2H), δ 1.88 – 1.98 (m, 1H), 1.60 – 1.78 (m 4H), δ 1.40 – 1.53 (m, 2H)

Compound 3 (635 mg, 95%) To a solution of compound 2 (665.8 mg, 1.43 mmol, 10 mL EtOH) was added $NaBH_4$ (215.6 mg, 5.70 mmol, 4 mL H_2O) dropwise over 30 minute using a syringe pump at 0^{o}C. After stirred for 3-4 h at room temperature, evaporated EtOH, diluted with brine (20 mL), extracted with $CHCl_3$, dried over $MgSO_4$, filtered and concentrated in *vacuo* to give compound **8** (635 mg, 95%) as a yellow oil; ^{1}H NMR(400 MHz, $CDCl_3$) δ 6.68 (bs, 1H), δ 3.62 – 3.70 (m, 16H), δ 3.56 – 3.60 (m, 4H), δ 3.42 – 3.45 (m, 2H), δ 2.91 – 2.95 (m, 1H), δ 2.89 (t, *J* = 5.2 Hz, 2H), δ 2.62 – 2.76 (m, 2H), δ 2.22 (t, *J* = 7.4 Hz, 2H), δ 1.86 – 1.96 (m, 1H), 1.40 – 1.78 (m, 7H)

Compound 4. To a solution of compound 3 (635 mg, 1.35 mmol) in DMSO (1.7 mL) was treated with trifluoroacetic acid (130 μL, 1.63 mmol) at room temperature. This 800 mM solution was kept in the refrigerator for future use.

Synthesis and characterization of QD-DPA.

In order to prepare small sized QDs with stability in acidic pH, we employed ligand exchange method of QD-TOPO (emission at 495 nm; Evidot®) with DPA. The DPA ligand in DMSO (5000 equiv) was added to QD-TOPO solution, and stirred for 2 days at room temperature. The final precipitates were transferred to water by adding 2 mL DI water. The concentration of the QDs was measured by using extinction coefficient of first exciton peak of QD (490 nm, 138000 $M^{-1}cm^{-1}$). The QD-DPA shows the quantum yield (QY) of ~10% and the maximum peak at 500 nm with a slight red-shift (~ 5 nm) compared with QD495-TOPO. TEM image is shown **Figure S1**, indicating the average size of ~ 3.5 nm. The hydrodynamic size of the QD-DPA was 9.2 ± 1.4 nm (n=3) in water at room temperature and the zeta potential was +30.6 ± 5.8 mV (n=3). The number of DPA ligand per QD (~ 66 ± 10 NH_2 groups per QD, n=3) was calculated based on the fluorescamine assay to determine the amount of primary amines with slight modification of the literature method.[S4] Briefly, 100 μL of fluorescamine (1mg/mL in acetone) was mixed with 10 μL of standard DPA molecule for 10 min to obtain the calibration curve of PL intensity at 474 nm versus DPA concentration (Ex = 390 nm). Then the QD-DPA (1 μM) was reacted with the same condition and the PL intensity at 475 nm of the QD-DPA was converted to the number of DPA molecules per QD (~ 66 DPA).

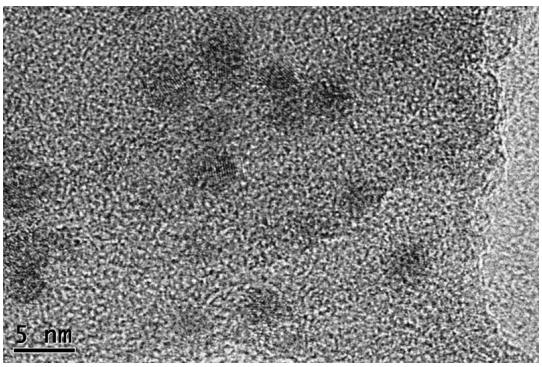


Figure S1. High-resolution transmission electron microscope (TEM) image of QD-DPA. The size in diameter is ~ 3.5 nm on average (n = 15).

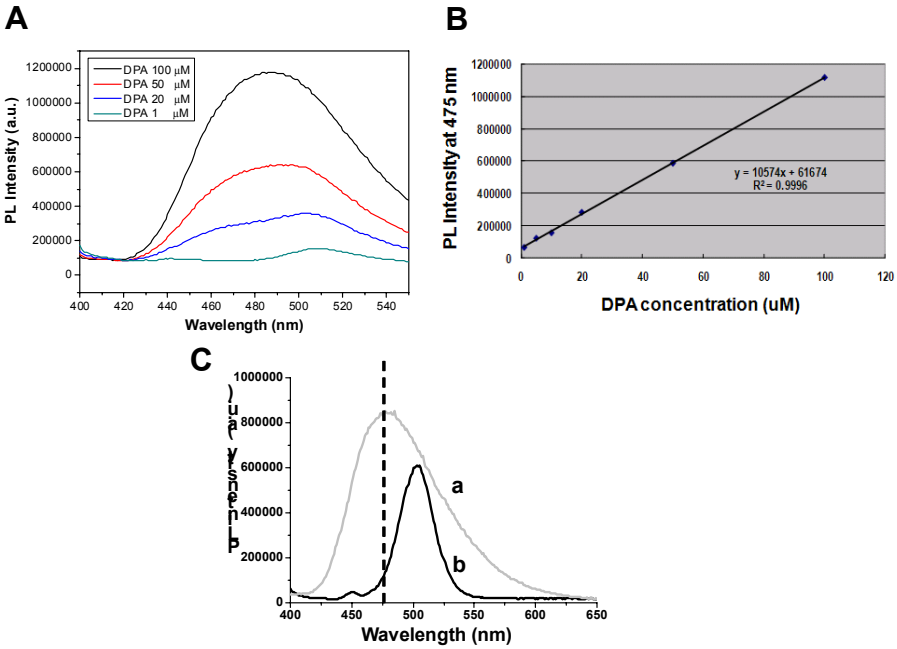


Figure S2. Fluorometric assay using fluorescamine to determine the number of DPA ligand per QD-DPA. (A) PL spectra of fluorescamine reaction product depending on the concentration of DPA ligand (1~ 100 μM), (B) the resulting calibration curve for PL intensity at 475 nm versus the concentration of DPA ligand. (C) The PL spectra of the fluorescent product of fluorescamine reacted with 1 μM QD-DPA (a) and QD-DPA itself (b). In order to accurately obtain the fluorescence only arising from the fluorescamine reaction with QD-DPA at 475 nm, the PL intensity of QD-DPA was subtracted from that of the fluorescent product of fluorescamine reacted with QD-DPA.

Stability test of QD-DPA in various pHs.

To test the feasibility of the QD-DPA for this fluorogenic assay as well as live cell imaging applications, we first tested the stability of QDs in various buffer conditions such as pHs (4.5, 7.0, and 9.0) at different time intervals (0h, 3h), where the QD-DPA showed good stability at acidic pH with the range of 4.5 ~ 6.5 (**Figure S3**). However, the QD-DPA was aggregated or precipitated at neutral to basic pHs (pH 7 ~9) mainly due to the deprotonation of ammonium moiety of the DPA ligand.

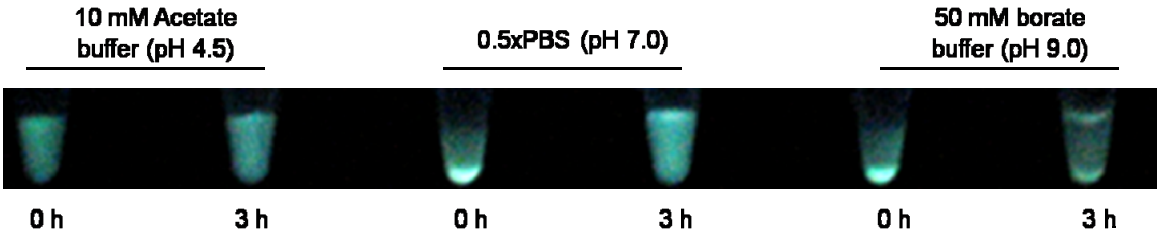


Figure S3. Stability test of QD-DPA in various pHs. The QD-DPA (1 μM) was aliquoted in 50 μL each and the pictures taken under UV-lamp (340 nm excitation)

Complex formation between QD-DPA and Pep1.

To confirm the charge-based complex formation, we tested the effect of salt and pH in the complex formation. As shown in **Figure S4**, the interaction between QD-DPA and the peptide was inhibited in the presence of 100 mM NaCl solution (pH 6.5), resulting in non-significant quenching at the molar ratios up to 5 when compared with the reaction in deionized water (DW) alone. However, at pH 4.5 in acetate buffer, the quenching was significant (= complex formation) even in the presence of 100 mM NaCl, indicating the pH dependence of the complex formation. These results suggest that the interaction between the QD-DPA and **Pep1** depends on salt and pH, confirming the binding is based upon the electrostatic interaction.

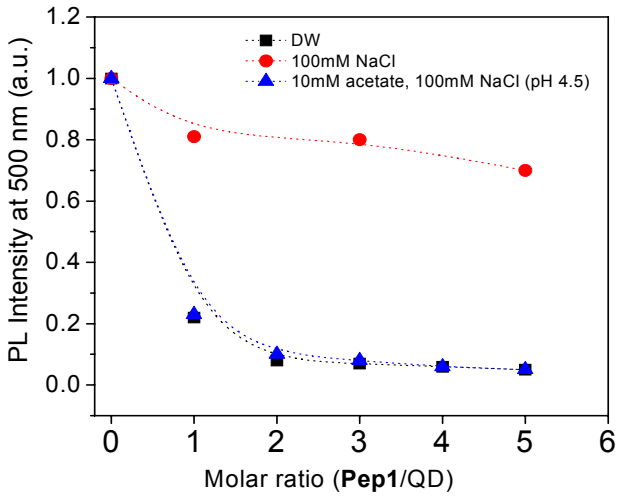


Figure S4. Effect of salt and pH on the complex formation of QD-DPA and **Pep1**.

Estimation of the distance between QD and acceptor dabcyl dye via FRET calculation.

Figure S5 displays the spectral overlap of the absorption spectrum of the dabcyl dye in the **Pep1** peptide (blue) with the emission spectrum of the QD-DPA (green), indicating the efficient FRET between the donor QD and acceptor dye dabcyl in the peptide.

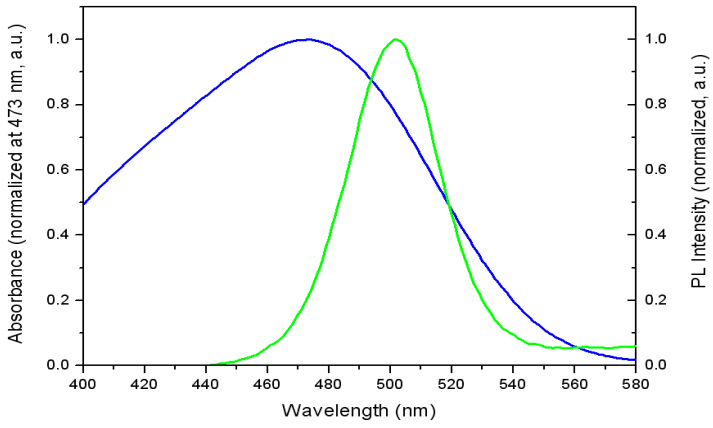


Figure S5. Spectral overlap of the absorption spectrum of the dabcyl dye in the **Pep1** peptide (blue) and the emission spectrum of the QD-DPA (green).

Spectral overlap integral (J) for the dabcyl dye (extinction coefficient at 454 nm = 32000 $M^{-1}cm^{-1}$) and the QD was obtained to be 1.519 x 10^{-13} $M^{-1}cm^{-6}$ using *PhotoChemCAD* software (version 2.1) according to the following equation (S1).

$$J(\lambda) = \frac{\int_0^\infty F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda}{\int_0^\infty F_D(\lambda)d\lambda} \quad \text{(Eq. S1)}$$

The resulting Förster radius, which is the distance between the donor and acceptor when the 50% FRET efficiency occurs, was calculated to be 3.7 nm using the following equation (S2)

$$R_0 = 9.78\times10^3(\kappa^2 n^{-4} Q_d J)^{1/6} \quad \text{(Eq. S2)}$$

, where k = orientation factor ($k^2 = 2/3$ for random dipole moments which is suitable for this QD-based nanoprobe system), n = refractive index in aq. solution (~1.33), Q_d = quantum yield of QD-DPA (0.1). Given the R_o value, we can estimate the separation distance between the QD core and the dabcyl dye in the **Pep1** according to the following equation (S3)

$$E = \frac{nR_0^6}{nR_0^6 + r^6} \quad \text{(Eq. S3)}$$

, where *n* is the number of dye acceptors associated with QD-DPA surface, *r* is the distance between the QD core and the dabcyl dye in the **Pep1**. Therefore, using the FRET efficiency data from **Figure 1** and Eq. S3, *r* values were obtained in the range of 2.4 nm to 3.0 nm depending on the number of **Pep1** (1 ~ 5) per QD-DPA. This calculated range of *r* values were found to be close to the predicted values (3.5 nm ~ 4.5 nm) from the structural estimation using ChemDraw 3D calculation (**Figure S6**). With the three **Pep1** molecules per QD, the separating distance (*r*) between the dabcyl dye and QD was calculated to be 3.0 nm.

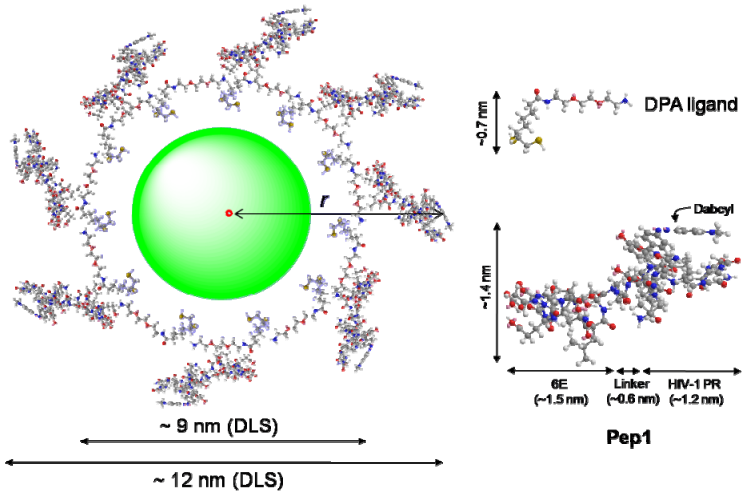


Figure S6. Overall dimension of the QD-**Pep1** complex structure. Each dimension of the DPA ligand and **Pep1** structure was measured after energy minimizing step using ChemDraw 3D MM protocol. The CdSe (green core) /ZnS (blue shell) core/shell structure was schematically drawn. The size of the QD-DPA (~9 nm) and the QD-**Pep1** complex (~ 12 nm) was derived from the hydrodynamic diameter from DLS measurement. The separation distance (*r*) between the donor QD center (red dot) and the acceptor dabcyl dye can range from 3.5 nm to 5.6 nm when the **Pep1** is at its fully extended conformation according to the model structure.

Quenching efficiency of QD-DPA-Pep1 complex depending on the QD donor emission peak (500 nm vs. 530 nm)

To prove the quenching phenomenon is based on FRET, rather than non-specific aggregation, we tested the QD530-DPA for complexation with dabcyl-modified peptide (**Pep1**). As expected, due to the lack of spectral overlapping of QD emission (530 nm) and dabcyl absorption, there was negligible quenching observed (**Figure S7**).

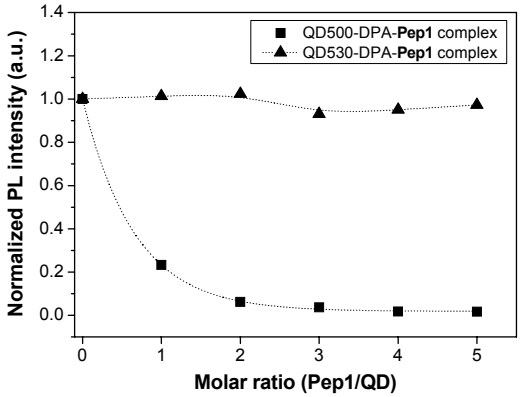


Figure S7. Quenching of QD-DPA with dabcyl-modified peptide (Pep1) depends on the spectral overlapping of QD emission (500 nm versus 530 nm) and dabcyl absorption, indicating that the quenching phenomenon is based upon FRET, rather than non-specific aggregation.

Optimization of HIV-1 PR reaction with QD-Pep1 complexes

Effect of buffer conditions

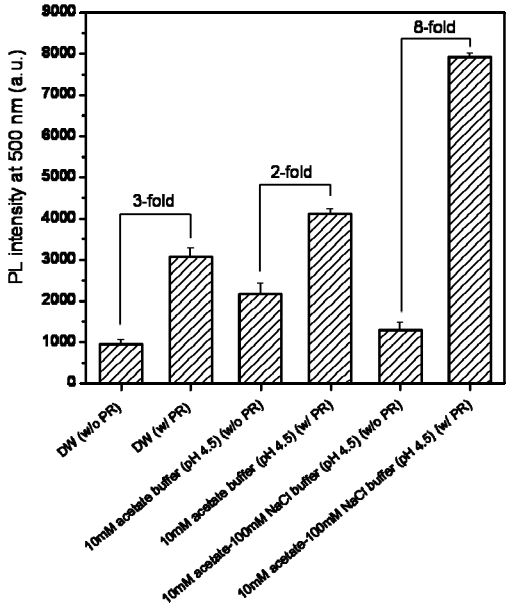


Figure S8. Effect of buffer conditions on HIV-1 PR digestion of QD-**Pep1** complex (0.18 μM). The presence of acetate buffer (10mM, pH 4.5) together with 100 mM NaCl yielded 8-fold PL increase of the pre-quenched QD-**Pep1** after 1h incubation at 37^{o}C when compared with conditions (DI water or acetate buffer alone) showing only 2~3 fold increase.

Effect of HIV-PR concentration and substrate peptide sequence

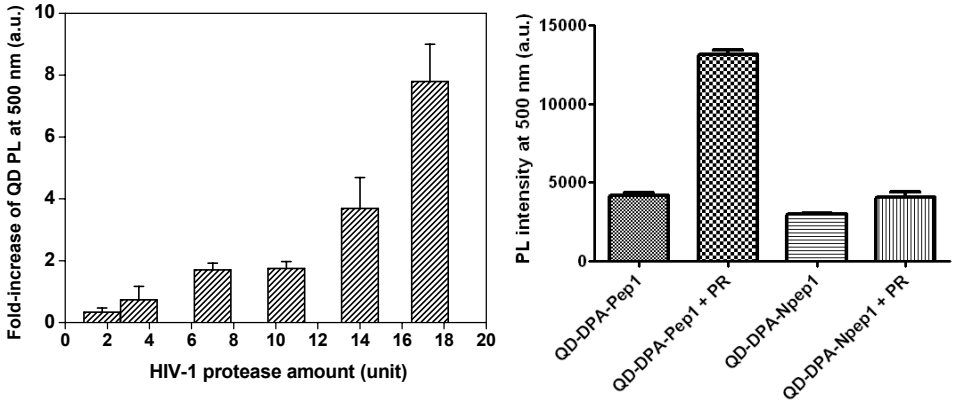


Figure S9. (left) HIV-1 PR concentration effect on the fold-increase of QD PL recovery at 500 nm after 1h incubation of QD-**Pep1** (180 nM) at 37°C. (right) Sequence specificity of the QD probe by measuring PL recovery. The 100 nM QD-peptide complexes, QD-**Pep1** (HIV-1 PR binding peptide) and QD-**Npep1** (non-binding peptide), were incubated with HIV-1 PR (14 unit) for 1h at 37°C. The PL at 500 nm was measured with 300 nm of excitation. The PL recovery of QD-**NPep1** probe was negligible compared with the QD-**Pep1**. Data were the average values of two separate experiments.

Effect of BSA on the PL recovery

We first compared the PL characteristics of QD-DPA in the presence of BSA since the BSA has been widely used to help minimize non-specific binding of proteins in in vitro assays. PL intensity of QD-DPA in the presence of BSA were not significantly affected by the presence of BSA at varying concentrations (0 ~ 25 μg/mL) (**Figure S10**, left). However, at above this concentration range up to 200 μg/mL, we observed a 2 ~ 3 fold increase of PL, which may be due to photobrightening effect from non-specific binding of BSA to the surface of QD-DPA. This effect has been reported by Mattoussi *et al.*, which has been attributed to the charge neutralization of the QD surface.[S5] Therefore, in our fluorogenic assay we used the 10ug/mL BSA to minimize the photobrightening effect, while resulting in enhancing the recovery ratio up to 2 fold upon HIV-1 protease digestion (**Figure S10**, right).

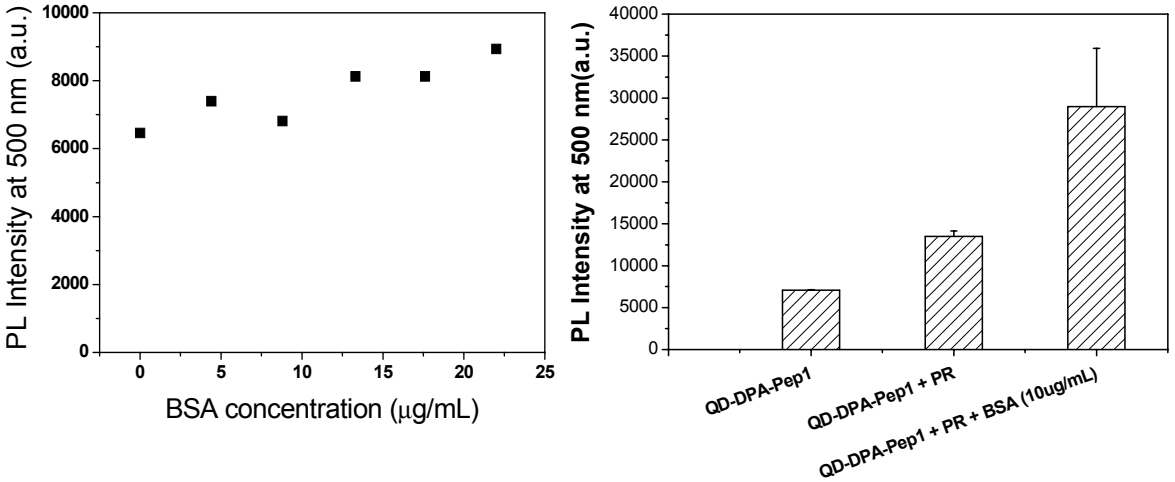


Figure S10. (left) PL characteristics of QD-DPA in the presence of BSA at varying concentrations (0 ~ 25 μg/mL). (right) In our fluorogenic assay we used the 10 μg/mL BSA , resulting in enhanced recovery ratio up to 2 fold upon HIV-1 protease (10 unit) digestion for 1 hour when compared with the control without BSA.

Production of HIV-1 virus-like particles.

HeLa cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 IU/mL streptomycin. Media were changed 2 to 3 times per week. All reagents were purchased from Invitrogen (California, U.S.A).

For HIV-1 plasmid DNA transfection, the HeLa cells were trypsinized, and then seeded in 24 well plate at a density of 1×10^4 cells per well, allowed to attach overnight, and placed in a growth medium consisting of DMEM with 10% FBS, 100 IU/mL penicillin, and 100 IU/mL streptomycin. The cells were then transfected with a pCMV-dR8.2 which contains Gag/Pol, Tat and Rev components of HIV-1 (Addgene, Cambridge, MA) using a Fugene 6® transfection reagent (Roche, Meylan, France) according to the manufacturer's instructions: DNA/lipid complexes were prepared by mixing 0.2 μg of plasmid with 0.6 μl of Fugene 6® diluted in DMEM serum-free medium and added to cells. The supernatants were harvested after 24hr, 48hr and 72hr, respectively.

To confirm that HIV-1 pseudo-particles were formed in HeLa cells, the expression level of HIV-1 p24 antigen was measured. The concentration of produced virus-like particles was determined by p24 ELISA Assay Kit (PerkinElmer, Boston, MA) according to the manufacturer's instructions. Briefly, the supernatants obtained were serially diluted, and added to 96-well microplate which is coated with a highly specific mouse monoclonal antibody to HIV-1 p24 antigen. The p24 values were calculated based on a standard p24 curve (**Figure S11**). Figure

S2 showed the p24 level measured by the ELISA assay kit. With increasing time of post-infection, the p24 level increased up to 40 ng/mL at 72 hour in the case of HeLa cells. To maximize the virus production in HeLa cells, we used 72 hour incubation time for our transfection protocol. Comparably, HEK293T cells were also tested in parallel to confirm the successful production of HIV-1 virus pseudo-particles.

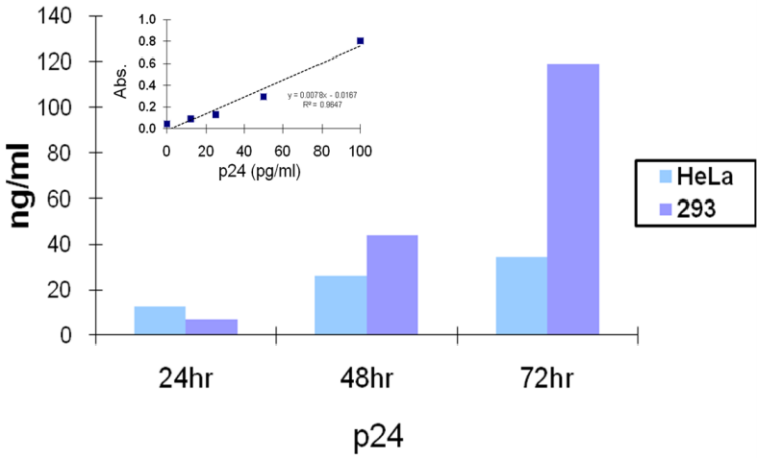


Figure S11. p24 ELISA assay for confirming HIV-1 pseudo-particles production in HeLa cells. The p24 level was measured using the standard curve for p24 ELISA assay.

Live cell imaging of HIV-1 protease activity in HIV-1 plasmid transfected HeLa cells using FRET-quenched QD-Pep1 probe.

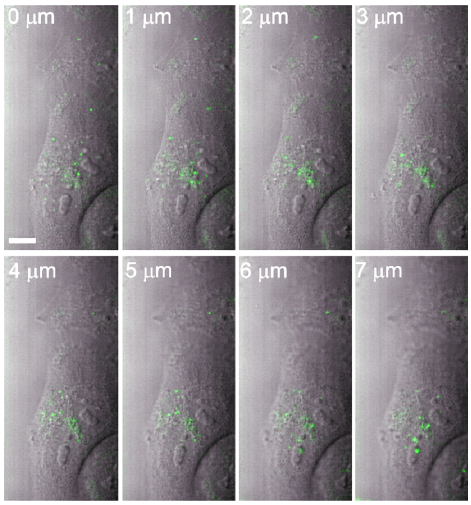


Figure S12. Z-section confocal images of HIV-1 plasmid-infected HeLa cells incubated with FRET-quenched QD-**Pep1** probe. The images are overlay of DIC and green channel filter.

Live cell imaging of HIV-1 PR inhibitor Saquinavir (SQ) effect on the HIV-1 *gag/pol* plasmid transfected HeLa cells.

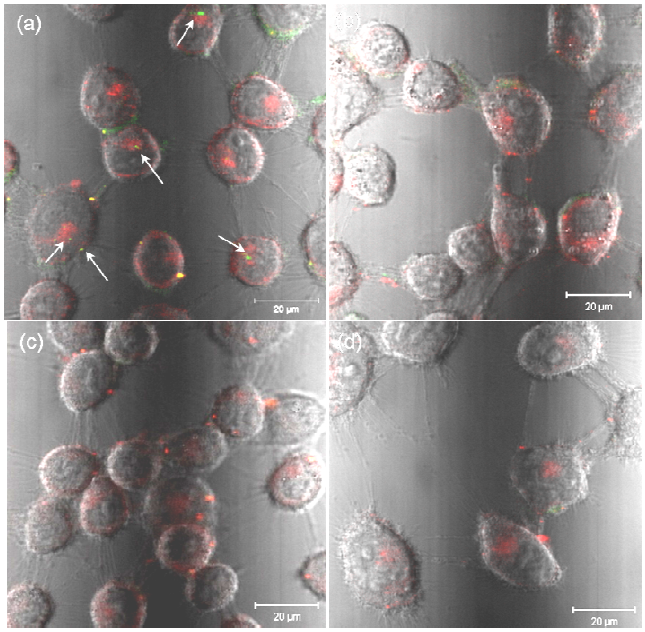


Figure S13. Visualization of anti-AIDS drug Saquinavir® effect (a, 0.025 μM, b, 0.25 μM; c, 5 μM; d, 10 μM) on the HIV-1 infected cell model (HeLa cells). QD PL (green channel) was recovered at 0.025 μM of SQ treatment, indicating the HIV-1 protease is still active in the cells at this concentration of drug treatment. However, the PL recovery was not observed at over 0.25 μM of SQ up to 10 μM, indicating that HIV-1 protease activity was totally inhibited. These cell-based screening results corroborate the in vitro enzymatic assay (IC_{50} = 0.6 μM). The endosomes were labeled with AF634-transferrin conjugate to confirm that the most of the QD probe was not sequestered in the endosomes. Data was reproduced in two separate experiments.

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