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Visualization of HIV Protease Inhibition Using a Novel FRET Molecular Probe

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Abstract

The *in vivo* high-throughput screening of HIV protease inhibitors is a significant challenge due to the lack of reliable assays that allow for visualization of HIV targets within living cells. In this study, we developed a new molecular probe that utilizes the principles of Förster resonance energy transfer (FRET) to visualize HIV-1 protease inhibition within living cells. The probe is constructed by linking two fluorescent proteins: AcGFP1 (a mutant green fluorescent protein) and mCherry (a red fluorescent protein) with an HIV-1 protease cleavable p2/p7 peptide. The cleavage of the linker peptide by HIV-1 protease leads to AcGFP1's separation from mCherry, quenching FRET between AcGFP1 and mCherry. Conversely, the addition of a protease inhibitor prevents the cleavage of the linker peptide by the protease, allowing FRET from AcGFP1 to mCherry. Thus, HIV-1 protease inhibition can be determined by measuring the FRET signal's change generated from the probe. Both *in vitro* and *in vivo* studies demonstrated the feasibility of applying the probe for quantitative analyses of HIV-1 protease inhibition. By co-transfecting HIV-1 protease and the probe expression plasmids into 293T cells, we showed that the inhibition of HIV-1 protease by inhibitors can be visualized or quantitatively determined within living cells through ratiometric FRET microscopy imaging measurement. It is anticipated that this new probe will allow high-content screening of new anti-HIV drugs.

Keywords

molecular probe; FRET; HIV-1 protease inhibition; AcGFP1; mCherry; high-content screening

Introduction

HIV, human immunodeficiency virus, attacks the human immune system. The treatment of HIV infection depends heavily upon a strategy referred to as highly active antiretroviral therapy (HAART),^{1, 2} in which drugs inhibiting HIV-1 protease are combined with drugs inhibiting reverse transcriptase to suppress virus replication.^{1, 2} Nevertheless, none of the existing anti-HIV drugs, even when combined, represent the ideal therapy owing to drug-related side effects, the high cost, and the emergence of drug resistant viruses.^{3, 4} Thus, it is a continuing effort to identify novel, effective drugs that exhibit improved safety and efficacy for HIV/AIDS therapy. Whereas a rational design against HIV targets can be ideal, it has proven to be a far more difficult challenge, due, largely, to the emergence of HIV mutants that are not only resistant to one drug but also to other drugs within the same class.⁵ To circumvent these problems, a high-throughput screening (HTS) approach has been proposed and widely used to discover new reagents against HIV⁶. HTS allows for selecting

or optimizing reagents and compounds against HIV from a large population of molecules or compounds in a short time period. A number of anti-HIV reagents such as HIV-1 reverse transcriptase inhibitors, integrase inhibitors, and HIV-1 protease inhibitors were discovered using this technique.^{4, 7–9} With the advent of HTS instrumentation and software, the focus of anti-HIV drug screening has evolved to the establishment of robust assays by developing new probes to follow complex cellular process and the HIV life cycle in such a manner that high content screening (HCS) can be employed to discover anti-HIV drugs. HCS allows the application of automated fluorescence microscopy for high-throughput drug discovery. With automated high resolution microscopy and robotic handling of cells in microtiter plates, HCS can be used to acquire knowledge on multiple biochemical pathways at the single-cell level in the early stage of new drug development.^{10–13} More importantly, it enables not only the spatial but also the temporal characterization of cellular processes through microscopy imaging measurement, which relies heavily on the visualization of molecular events within living cells^{14–16}. This has not yet been realized for HCS of anti-HIV drugs due, in part, to the lack of reliable molecular probes that visualize molecular events that occur during the life cycle of HIV in infected cells.

The focus of this work is to develop a novel molecular probe that enables HCS of anti-HIV drugs through an automated FRET microscopy imaging measurement. We selected HIV-1 protease as a primary target since it plays various key roles in virus replication and maturation¹⁷ and has been one of the key targets for anti-HIV drug discovery.^{18, 19} Our goal is to design a molecular probe that enables visualization or quantitative analysis of HIV-1 protease inhibitors so that the potent inhibitors can be discovered by screening a combinatorial library through HCS.

HIV-1 protease is a small 99-amino acid aspartic protease that plays a crucial role in the viral life cycle and is essential to HIV replication. This enzyme is required for the post-translational cleavage of HIV's Gag, Gag-pol, and Nef precursor polyproteins and the conformational rearrangement of immature virions, leading to the production of infectious virus particles.^{17, 20} Thus, blocking protease activity will impair the maturation of HIV and inactivate its infectivity even as it makes copies of itself. About twelve enzyme cleavage motifs have been identified and used to develop various *in vitro* HIV-1 protease assays.^{21–24} The cleavable features of these motifs provide opportunities for designing a FRET probe for visualizing enzyme inhibition within living cells. The idea is to introduce a FRET signal transduction function directly into these motifs in such a manner that the HIV-1 protease inhibition can be ascertained through ratiometric FRET measurement, as described in our previous work^{25, 26}.

On the other hand, the efficiency of a FRET probe for *in vivo* microscopy imaging measurement heavily depends on the selection of a FRET pair. CFP (cyan fluorescent protein) and YFP (yellow fluorescent protein) have been widely used as a FRET pair for microscopy imaging of molecular events within living cells.²⁷ However, crosstalk and photobleaching of CFP/YFP pair hinders the accuracy and reliability of CFP/YFP FRET probes for microscopy imaging measurement within living cells.²⁸ To circumvent these problems, we explored a new FRET pair in which AcGFP1 (an engineered, fluorescent mutant of the wild-type green fluorescent protein *Aequorea victoria*²⁹) serves as a donor and mCherry (a mutant of red fluorescent protein) acts as an acceptor for FRET microscopy imaging measurement of HIV-1 protease inhibition within living cells. Compared to CFP, AcGFP1 has a higher quantum yield ($Q_p=0.82$).³⁰ Its emission spectrum overlaps with the excitation spectrum of mCherry. The crosstalk between the AcGFP1 and mCherry is suppressed due to a relatively small overlap between the excitation spectrum of AcGFP1 and that of mCherry.³¹ Compared to CFP/YFP pair, AcGFP1 can be excited at 475 nm, matching 488 nm laser line present in most confocal laser microscope, whereas CFP needs

to be excited using UV or near UV light. Moreover, AcGFP1 is more resistant to photo bleaching, making quantitative analysis more reliable. Furthermore, the red-shift FRET pair of AcGFP1 and mCherry can reduce autofluorescence significantly, thereby raising the signal/noise ratio in live cell imaging.

Materials and Methods

Construction of a FRET probe

AcGFP1 is a mutant green fluorescent protein with an excitation wavelength of 475 nm and an emission wavelength of 505 nm. mCherry is a red fluorescent protein with an excitation wavelength of 587 nm and an emission wavelength of 610 nm. The probe was genetically engineered by fusing AcGFP1 (donor) and mCherry (acceptor) to a peptide linker that entails an HIV-1 protease cleavage site p2/p7 (ATIM*MQRG *indicates a cleavage site). mCherry was PCR amplified from the plasmid, pmCherry, (Clontech Laboratories, Inc. CA). The peptide linker containing p2/p7 was fused to the N-terminal of mCherry during PCR amplification using the following pair of PCR primers: (forward PCR primer) 5' AGCTTCGAATTCTGCTACCATAATGATGCAGAGAGGCGTGAGCAAGG GC GAGGAGGATAACATG 3' and (reverse primer) 5' GGATCCCTTGACAGCTCGTC CATGCCGCC 3'. The DNA fragments after PCR were inserted into the pLVX-AcGFP1-N1 vector (Clontech) using EcoRI and BamHI restriction enzyme sites to generate an AcGFP1-p2/p7-mCherry fusion protein. The resultant DNA plasmid was referred to as pLVX-GcC and used for probe expression in mammalian cells. For *in vitro* study, the gene encoding the FRET probe was subcloned into a pTA-GBP²⁶ with a (his)₆ tag fused at its C-terminus using forward primer: 5' AGATGCACTAGTGCTAGCGTCGCCACCATGGTGAGCAAG 3' and the reverse primer: 5' GAGCTCGGTACCTCCGGTGGATCC CTTGTACAGCTC 3'. Meanwhile, we constructed a mutated probe by fusing AcGFP1 and mCherry to a peptide linker that contains a mutated cleavage site, GTIIMQSG, making the probe uncleavable by HIV-1 protease. All expression vectors were verified by DNA sequencing analyses.

Construction of the HIV-1 protease expression vector

To express HIV-1 protease in mammalian cells for testing the probe in live cells, we constructed a HIV-1 protease expression plasmid by amplifying a gene encoding the protease from the pProtease plasmid (NIH AIDS Research and Reference Reagent Program) using the following primer set: (forward primer) 5' GGATCG AGATCTATGCCGCAAATCACTCTGTGG 3' and (reverse primer) 5' GGCCGCGAATTCTAGAAAGTTAAAGTGCACCCGAT-3'. The PCR amplified protease gene was subcloned into pMSCVpuro (Clontech) through BglII and EcoRI restriction enzyme sites. The resultant vector was referred to as pMSCV-PR.

Purification of the FRET probe

The production and purification of the probe was carried out, as described in our previous work.³² In brief, recombinant *E. coli* DH5 α harboring pTA-GcC that encodes the probe were grown at 37°C until they reached an OD₆₀₀ of 0.5. IPTG (1 mM) was then added to the culture medium to induce the expression of the probes. After induction for 4 hours, recombinant *E. coli* were harvested by centrifugation at 3,500 rpm for 10 min. The bacterial pellets were stored at -20°C until use. The pellets were lysed in a phosphate buffer in the presence of bacterial protein extraction reagent (Pierce, IL) and phenylmethanesulfonylfluoride (PMSF, 1 mM). Lysate was filtered through a 0.45 μ m filter (Millipore, MA) before it was loaded to an HiTrap Chelating HP column (GE Health, WI). The binding buffer contained 0.02 M disodium phosphate, 0.5 M NaCl, and 40 mM imidazole (pH of 7.4), and the probe was eluted from the IMAC (immobilized metal affinity

chromatography) column using a buffer containing 0.02 M disodium phosphate, 0.5 M NaCl, and 500 mM imidazole (pH of 7.4). The IMAC purified probe proteins were then dialyzed against the protease reaction buffer, which contains 0.8 M NaCl, 80 mM sodium acetate, and 1 mM EDTA (pH 6.5). SDS-PAGE assay was used to determine the purity of the probe proteins prepared as describe above.

Protein assay and Western blot

The probe concentration was determined using the PIERCE protein assay reagent, and BSA was used as a standard. Western blot analysis was performed as described in our previous work.³² The mouse anti-mCherry from Clontech [1:500] was used as primary antibody and the HRP-conjugate anti-mouse IgG (Sigma, St. Louis, MO) [1:80,000] as secondary antibody.

In Vitro characterization

The *in vitro* characterization of the FRET probe was conducted using a luminescence spectrophotometer (Perkin Elmer LS-55B). The fluorescent intensities of both AcGFP1 and mCherry were measured at 510 nm and 605 nm, respectively, by exciting the probe at 475 nm (the maximum excitation wavelength of AcGFP1). The slit width of both excitation and emission was set at 10 nm. The FRET signal was defined as a ratio of the fluorescent intensity of the acceptor mCherry over that of the donor AcGFP1 when excited at 475 nm. All of the measurements were carried out at room temperature in triplicate.

To determine the probe function *in vitro*, the HIV-1 protease (AnaSpec, Fremont, CA) was added to a reaction buffer (60 μ l, pH 6.5) containing 0.8 M NaCl, 80 mM sodium acetate, 1 mM EDTA, 1 mM DTT (added fresh), and the probe. The digestion of the peptide linker by the HIV-1 protease was performed at 37°C for 30 min, after which the reaction was stopped by the addition of 40 μ l of stop buffer (0.66 M potassium phosphate, pH of 8.0). To assess whether the probe can ascertain the inhibition of HIV-1 protease by an inhibitor, an inhibitor such as ritonavir (NIH AIDS Research & Reference Reagent Program) was added to the aforementioned reaction along with the HIV-1 protease. The FRET signals generated from the probe in the presence and absence of inhibitors were measured and compared to determine whether the inhibitory effect on HIV-1 protease by inhibitors can be detected using the probe.

In vivo characterization and FRET microscopy imaging measurement

Human embryonic kidney cell 293T (ATCC CRL-11269, Manassas, VA) were propagated in the DMEM (Fisher Scientifics, Pittsburgh, PA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (ATCC) and 2 mM L-glutamine at 37 °C in a 5% CO₂ incubator. The probe-encoding plasmids were transiently transfected into the 293T cells, as described in our previous work.³³ FRET imaging was performed at an indicated time post transfection using a live cell FRET imaging microscopy measurement system (Olympus IX-71) with Slidebook 4.2 software (Intelligent Imaging Innovations, Inc Denver, CO). To visualize the FRET signal, a 472/30 nm excitation filter (AcGFP1 donor excitation), and two different emission filters, 520/35 nm for AcGFP1 and 607/36 nm for mCherry (Semrock, Rochester, NY), were used. The excitation and emission filters were integrated into a microprocessor-controlled multi-filter wheel system (Lambda 10-3, Sutter Instrument Company, Novato, CA). Pseudo-colored FRET ratiometric images of the cells were generated by the Slidebook and the average ratio values were determined from the images by selecting randomized regions of interest.

All the protease inhibitors, including indinavir, lopinavir, nelfinavir, ritonavir, and sequinavir, are provided by NIH AIDS Research & Reference Reagent Program.

Results and Discussion

In vitro characterization of the probe

A schematic diagram of the probe is illustrated in Fig. 1A. HIV-1 protease cleavage site p2/p7 with amino acid sequence of ATIMMQRG was selected to establish a model system for visualizing HIV protease inhibition within living cells. The probe was referred to as GcC. To construct a negative control probe GcmC, we mutated the cleavage site of the p2/p7 peptide. The mutated peptide linker, GTIIMQSG, is thus not cleavable by HIV-1 protease. The probe ability of reporting HIV-1 protease inhibition in live cells was first characterized *in vitro*. First, we determined whether a FRET signal could be detected from the probe (GcC). As shown in Fig. 1B, two characteristic FRET emission peaks, one at 510 nm (the typical emission wavelength of AcGFP1; GcC on Fig. 1B) and the other at 605 nm (emission wavelength of mCherry), were detected when the probe was excited at 475 nm, whereas no sensitized mCherry emission could be measured in the presence of HIV-1 protease (GcG+PR on Fig. 1B). The vanishing of fluorescence emission from mCherry in the presence of HIV-1 protease is due to the cleavage of the peptide linker by the enzyme, leading to the separation of AcGFP1 from mCherry, which prevents the energy resonance transfer from AcGFP1 to mCherry. As the inhibition of the protease's activity will avoid the cleavage of the peptide linker, the FRET-induced emission of fluorescence from mCherry was observed when the probe GcC was incubated with the protease along with an inhibitor, ritonavir (10 μ M) (GcG+PR+Rit on Fig. 1B). As a control, we investigated whether the inhibitor had any influence on the FRET signals of the probe. As indicated in Fig. 1B, the incubation of GcC with the inhibitor did not alter the characteristics of the probe's FRET spectrum. It should be noted that the FRET signals detected in the presence and absence of inhibitors are 0.136 and 0.135, respectively, indicating no influence of inhibitors on FRET signals. The FRET signal was reduced from 0.135 to 0.030 when the protease was added to the solution and cleaved the probe. This reduction reflected an approximately 78% of reduction in FRET signals. The decrease of AcGFP1 fluorescence intensity after the addition of both protease and inhibitors to the reaction buffer can be explained by the combinatorial effect of both enzyme and inhibitors on AcGFP1. However, the FRET determined from the ratio of AcGFP1 and mCherry fluorescent intensities was 0.138, indicating no change in the FRET signal. Clearly, the presence of inhibitors in the solution prevented the probe from cleavage by the protease. These experimental results suggested that the probe developed in this work responds well to HIV-1 protease inhibition.

Next, we determined the optimal concentration of HIV-1 protease for probe characterization. The optimized HIV-1 protease concentration was examined by adjusting its concentration from 0.09 μ M to 0.55 μ M in the reaction buffer, while the concentration of GcC remained constant at 1.9 μ M. The FRET signals detected in the absence of the protease were used to normalize those measured in the presence of the protease. As illustrated in Fig. 1C, the FRET signals were reduced by 55% when six units of protease were used for the reaction. Based on these analyses, we decided to use six units of the enzyme for all subsequent experiments.

To further confirm the enzyme-specific decrease of the probe FRET signals, we determined signals by incubating the control probe (GcmC) with the enzyme and inhibitors. The GcmC probe entails a peptide linker that is not cleavable by the protease. Thus, the incubation of the probe with the HIV-1 protease will not quench the FRET, as AcGFP1 will still be linked with mCherry. As expected, no reduction of FRET signals could be observed when the GcmC probe was incubated with the protease (Fig. 2). Thus, the decrease of FRET signals of the probe GcC in the presence of HIV-protease is enzyme-specific. The separation of AcGFP1 from mCherry in the presence of protease was also validated through Western blot as described below.

Visualization of HIV-1 protease inhibition within living cells

Having verified the detective ability of the probe GcC to HIV-1 protease inhibition *in vitro*, we next investigated whether the probe can be used to visualize the HIV-1 protease inhibition within living cells. 293T cells were used as a model for the study. To this purpose, 293T cells were either transduced with the probe alone (Fig. 3A) or co-transduced with both probe and HIV-1 protease plasmids (Fig. 3B). FRET microscopy imaging measurement was performed to determine the FRET signals of the probe within living cells. The probe and protease plasmid co-transduced cells displayed reduced FRET signals at 48 h post transfection (Fig. 3B). This reduction was caused by the separation of AcGFP1 from mCherry due to the cleavage of the p2/p7 peptide linker by the HIV-1 protease. In contrast, the probe and protease co-transfected cells emitted sensitized red fluorescence when an inhibitor, ritonavir, was added to the cell culture medium (Fig. 3C). The addition of inhibitors to the cell culture medium prevented the cleavage of the peptide linker by the protease and thus retained the FRET from AcGFP1 to mCherry.

To statistically examine the changes in the FRET signals in a living cell culture system, a quantitative analysis was performed (Fig. 3D). The changes in the probe's FRET signals in response to the protease and its inhibitor were quantified using an imaging SlideBook software. FRET imaging was performed in a 6-well plate at 48 h post transfection. Eight regions in each well were randomly selected for imaging. The FRET signals determined in these regions were averaged to give a FRET signal for each well. Notably, the FRET signal detected from the probe decreased by 58% in the presence of HIV-1 protease, while the signal remained unchanged when the protease and its inhibitor were co-existent in the cell culture. Although the FRET from AcGFP1 to mCherry seems to be lower due to relatively small spectral overlap between emission of donor protein and excitation of acceptor protein, reduced crosstalk between donor and acceptor channels can be achieved. In addition, the AcGFP1 is more tolerant of photo bleaching, making quantitative analysis more reliable. Furthermore, the red-shift FRET pair can reduce auto-fluorescence significantly and thus raise the signal/noise ratio in live cell imaging. Our experimental data demonstrated that the sensitivity of the probe engineered in this work is suitable for visualizing inhibition of HIV-1 protease within living cells. Thus, it is feasible to adopt an HCS system such as the Perkin Elmer's Operetta to discover HIV protease inhibitors from chemical compounds and natural product libraries.

In the next experiment, we investigated whether the response of the probe's FRET signals to protease inhibition could be restored after removal of the inhibitors from the cell culture medium. To this purpose, we co-transfected 293T cells with both the probe and the HIV-1 protease expression vectors and cultured cells in the presence of ritonavir for 24 h (Fig. 4C). The FRET signal was detected from these cells due to the inhibition of the protease by the inhibitors. Afterwards, the inhibitors were removed from the cell culture medium, and the cells were cultured continuously for another 24 h. As shown in Fig. 4F, a significant reduction of FRET signals was observed, indicating the cleavage of the peptide linker by the protease after removal of the inhibitors. This experiment demonstrated that the response of the probe to protease inhibition is enzyme dependent.

Compared to at 24 h, more probes were synthesized in cells at 48 h post transfection, as shown in Fig. 4D and 4E. It appears that 48 h post transfection is the best time for visualizing the inhibition of HIV-1 protease within living cells. Western blot was performed to validate the cleavage of the probe by the protease. Cell samples were collected at 48 h post transfection. Mouse anti-mCherry antibodies were used as primary antibodies and rabbit anti-mouse IgG HRP conjugates were employed as secondary antibodies to detect both intact and cleaved probes. As revealed in Fig. 4G, the cleavage of the probe by the HIV-1 protease was only observed in probe and protease expression plasmid co-transfected

cells (Fig. 4G lane 3), and the presence of ritonavir in the cell culture medium prevented the cleavage of the probe (Fig. 4G lane 4). The probe band shown in lane 3 suggests that the enzyme cleavage fell behind the probe synthesis rate. As the probes were constantly synthesized by the cells, if the enzyme cleavage rate of the probe was slower than the probe synthesis rate, part of the probes would remain intact, giving rise to FRET signals. Moreover, transient transfection can give rise to intracellular protein concentrations that vary over orders of magnitude. Therefore, there is a dynamic balance between the cleaved and intact or newly synthesized probes in the cells that were co-transfected with protease and probe expression plasmids. This also explains our observation that there was variation in FRET signals (indicated by the different colors) imaged in cells co-transfected with probe and protease expression plasmids (Fig. 4B and E). No probe cleavage could be detected in either cells transfected with probe expression plasmid alone (Fig. 4G lane 2) or cells transfected with the mutated probe expression plasmid (Fig. 4G lane 5, 6, and 7). It was found that there was an extra-band appeared in western blot. The band could be the result of constitutive digestion of the peptide linker by cellular protease, as the molecular weight is slightly higher than that of mCherry. Nevertheless, our *in vivo* FRET imaging microscopy measurement and *in vitro* assay clearly demonstrated that the FRET signal of the probe responded to protease and inhibitors specifically.

Furthermore, we aimed to determine the robustness of the detection system to verify whether the probe could be used to detect the activity of other protease inhibitors. This is of critical importance for HCS discovery of anti-HIV protease inhibitors. We adopted a panel of known HIV-1 protease inhibitors, including indinavir, lopinavir, nelfinavir, ritonavir, and zidovudine. The experiment was carried out using a 96-well black plate with clear bottom, and the experiments were performed in triplicate at all culture conditions. As described above, 293T cells were co-transfected with the probe GcC and the protease expression plasmid PR. The aforementioned five inhibitors were added to the cell culture medium after co-transfection. The final concentration of these inhibitors in cell culture medium was 10 μ M. FRET microscopy imaging measurements were performed at 30 h and 52 h post transfection, respectively. As illustrated in Fig. 5, FRET signals were detected in the presence of all five inhibitors due to the inhibition of HIV-1 protease's activity by these inhibitors. Eight regions were randomly selected and values of the FRET ratio were recorded in each sample for statistical analysis. It was found that the difference in inhibitors does not seem to affect the FRET ratios. The FRET ratios detected in the presence of these five inhibitors were 0.34 ± 0.01 , 0.34 ± 0.01 , 0.33 ± 0.02 , 0.35 ± 0.02 , and 0.34 ± 0.02 , respective. Nevertheless, the FRET ratio was 0.23 ± 0.03 in the absence of these inhibitors. Thus, we concluded that all the five inhibitors have similar inhibitory effect on the protease activity. To further verify the specificity of the FRET signal's change the probe with mutated cleavage site, GcmC, was also examined. The image results were presented in Fig. 5O–5Q. Markedly, mutated probe proteins cannot be recognized by HIV-1 protease, and thus no FRET signal change could be detected. Taken together, we demonstrated that the probe developed in this work could be used to report the inhibition of HIV-1 protease by chemical compounds.

Conclusions

To develop an *in vivo* HIV-1 protease inhibition reporter suitable for the HCS of anti-HIV drugs, a peptide linker containing an HIV-1 protease cleavage site p2/p7 was fused with AcGFP1 and mCherry to form a FRET molecular probe. Our experimental results markedly demonstrate that the activity of the HIV-1 protease inhibitors can be visualized through ratiometric FRET microscopy imaging measurement of the probe in live cells. Both *in vitro* and *in vivo* experiments suggest that the response of the probe to HIV-1 protease inhibition is enzyme dependent. The quantitative analysis of FRET signals detected through FRET

microscopy imaging measurement of probes in live cells indicate that the FRET signals generated from the probe are sensitive to the activity of HIV-1 protease. Thus, the probe developed here provides a useful and sensitive tool for visualizing protease inhibition within living cells and enables one to visualize HIV targets within living cells for HCS of anti-HIV-1 protease inhibitors.

On the other hand, our experimental data suggest that the enzyme cleavage rate is relatively slower than the probe synthesis rate, which could affect the sensitivity and the dynamic measurement range of the probe. This is possibly caused by the difference in protein expression levels between the probe and the protease. As HIV-1 protease is cytotoxic to the cells, the slow expression of the protease in cells can be expected. Although the increase of protease expression level can be difficult, the increase of the probe's cleavage rate can possibly be achieved by selecting other cleavage motifs. While the p2/p7 peptide used is known to play major roles in the polyprotein processing in HIV-1 replication,^{34–36} other protease cleavage sites might also be worth trying. The use of other cleavage motifs may also improve the sensitivity of the probe, as the efficiency of the FRET between AcGFP1 and mCherry is heavily dependent upon the distance and orientation of these two fluorescent proteins. The use of different cleavage motifs may change both the distance and orientation of AcGFP1 and mCherry when they are linked by a peptide containing these motifs.

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Fig. 1. Ratiometric FRET measurement of the probe (GcC) *in vitro*

(A) Schematic diagram of the probe designed to visualize activity of HIV-1 protease inhibition within living cells. The probe is designed to link two fluorescent proteins with an HIV-1 cleavable peptide stretch. (B) Spectra of the probe under various conditions. The emission spectra of the probe were scanned after incubating the probe with protease (PR) in the presence or the absence of inhibitor (ritonavir). The reaction solution (76 mL) contains 7.5 mg of probe proteins. The HIV-1 protease (6 units) was added to the reaction solution to quench the FRET of the probe, and ritonavir (10 mM) was used to inhibit the protease in aforementioned reaction solution. The probe was excited at 475 nm and the slit width of both excitation and emission were set at 10 nm. (C) Effect of the protease activity on the FRET signal of the probe. The reaction solution (76 mL) contains 7.5 mg of probe proteins. Experiments were performed in triplicates. Error bar: $\pm$ SD. Symbols: PR, protease; and Rit, ritonavir.

Fig. 2. Detection of the inhibition of HIV-1 protease using a FRET probe

All FRET signals were normalized to those of measured with probe GcC alone. The enzyme reaction was conducted at 37°C for 30 min and stopped by addition of stop buffer to the reaction buffer. The reaction solution (76 mL) contains 7.5 mg of probe proteins and 6 units of proteases were used to quench the FRET of the probe and 10 μ M of ritonavir was employed to inhibit protease in the reaction solution. All experiments were performed at room temperature and in triplicate. Error bars represent standard deviations of the measurements. Symbols: GcC, the FRET probe; GcmC, a mutated probe; PR, protease; and Rit, ritonavir.

Fig. 3. Visualization of HIV-1 protease inhibition within living cells through FRET imaging microscopy measurement

293T cells were either transfected with probe encoding plasmid (A) or co-transfected with both probe and HIV-1 protease encoding plasmids (B). The inhibitor, ritonavir, was added to the cell culture medium after cotransfecting the cells with both probe and HIV-1 protease expressing plasmids (C). The FRET imaging was performed at 48 h post transfection using a live cell FRET microscopy imaging measurement system. The color bar is a look up table (LTB) that indicates the FRET signals shown on the FRET images. (D) Comparison of FRET signals measured under various conditions. Eight regions per well in a 6-well plate were randomly selected for taking FRET images. The average of the FRET ratios determined from these eight regions was used to represent the FRET signal of the probe in each well. Error bars show the standard deviation. Scale bars: 10 μ m.

Fig. 4. Recovery of FRET signals after removal of inhibitors from cell culture medium
 293T cells were either transfected with the probe alone (A and D) or co-transfected with both probe and HIV-1 protease expression plasmids (B, C, E, and F). Ritonavir, an HIV-1 protease inhibitor, was added to the cell culture medium (C) to prevent the cleavage of the peptide that linked AcGFP1 to mCherry. The inhibitors were removed from the cell culture medium at 24 h post transfection, allowing the cleavage of peptide by the HIV-1 protease (F). The color bar indicates FRET ratio. Scale bars in panel A–F: 10 μm. (G) Western blot analysis of the probe cleavage by the HIV-1 protease. Cells were harvested at 48 h post transfection and lysed for Western blot. Lane 1: 293T cells, lane 2: probe plasmid transfected cells; lane 3: probe and HIV-1 protease plasmid co-transfected cells; lane 4: probe and HIV-1 protease plasmid co-transfected cells in the presence of inhibitor; lane 5: mutated probe plasmid transfected cells; lane 6: mutated probe plasmid and protease plasmid co-transfected cells; lane 7: mutated probe plasmid and protease plasmid co-transfected cells in the presence of inhibitor.

Fig. 5. The response of the probe to a panel of HIV-1 protease inhibitors

293T cells were either transfected with the probe expression plasmid (A and H) or co-transfected with both probe and protease expression plasmids (B–G and I–N). Another group of cells were either transfected with mutated probe expression plasmid, GcmC, alone (O) or co-transfected with mutated probe plasmid GcmC and protease expression plasmid PR (P–Q). A panel of HIV-1 protease inhibitors indinavir, lopinavir, nelfinavir, ritonavir, and sequinavir were used to determine whether the probe can respond to all of these inhibitors. The inhibitors were added to the cell culture medium after plasmid transfection. The FRET microscopy imaging measurement was performed at 30 h (A–G) and 52 h (H–Q) post transfection, respectively. All the experiments were carried out in triplicate. Scale bars: 10 μ m. The color bar indicates the FRET signals.