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Biochemical and Biophysical Research Communications 319 (2004) 72–77
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A high-throughput method for development of FRET-based indicators for proteolysis

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Received 6 March 2004
Available online 14 May 2004

Abstract

SCAT3 is a fluorescence resonance energy transfer (FRET)-based indicator for activity of caspase-3, which is composed of an enhanced cyan fluorescent protein, a caspase-3-sensitive linker, and an enhanced yellow fluorescent protein with efficient maturation property (Venus). Despite its considerable promise, however, greater responsivity of fluorescence to the proteolysis has been desired for better understanding of spatio-temporal pattern of the activation of caspase-3 during apoptosis. In the present study, the length of linker regions of SCAT3 has been thoroughly optimized by use of a PCR technique. The bacterial colonies expressing the constructs were screened for high FRET efficiency using our home-made fluorescence image analyzer. The FRET signal of an improved SCAT3 changed by about tenfold during apoptotic events in mammalian cells, enabling visualization of caspase-3 activation with better spatial resolution than before. This new high-throughput method will be applicable to development and improvement of FRET-based indicators for proteolysis.

Keywords: Fluorescence resonance energy transfer; Green fluorescent protein; High-throughput screening; Imaging; Apoptosis; Protease

Green fluorescent protein (GFP) from the bioluminescent jelly fish *Aequorea victoria* (*Aequorea* GFP) and its color variants have revolutionized our ability to uncover complicated details of protein dynamics and gene activation [1]. In addition, combination of GFPs with fluorescent resonance energy transfer (FRET) technique enables us to develop fluorescent indicators that allow us to visualize localized molecular events in their natural environment within living cells. To date, tens of biosensors, including those for free Ca^{2+} concentrations, protein phosphorylation, and protease activity, have been developed and successfully used in various cell types [2]. While most indicators have cyan- and yellow-emitting fluorescent proteins (CFP and YFP) as FRET donor and acceptor, their poor dynamic range often prevents detection of subtle but significant signals.

FRET is the radiationless transfer of excited-state energy from an initially excited donor to an acceptor [3]. It depends on the proper spectral overlap of the donor and acceptor, their distance from each other, and the relative orientation of the chromophore's transition dipoles. Within constructs that contain CFP and YFP, therefore, small structural alterations caused by insertion or deletion of single amino acids in linker regions may greatly change the efficiency of FRET from CFP to YFP [2]. Sensors for proteolysis carry CFPs and YFPs, which are connected with linkers containing sequences that are cleavable by proteases. Proteolysis of such linkers disrupts FRET completely. Thus, the constructs quantified in bacterial colonies to have high FRET efficiency will show large dynamic range in proteolysis experiments. In this study, we describe an efficient method for varying the length of the linker of SCAT3, a sensor for caspase-3 activation [4], and for screening those constructs in colonies for high FRET efficiency.

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doi:10.1016/j.bbrc.2004.04.147

Materials and methods

Gene construction. The cDNAs of C-terminally deleted ECFP mutants fused with DEVD sequence were amplified by PCR using $pRSET_{B\text{-}ECFP}$ as a template. They were digested with *Bam*HI and *Kpn*I. The used primers are as follows: forward, 5′-TAATACGACTCACTA TAGGG-3′; reverse, 5′-GCTGGT ACCATCGACCTCATCC TTGT ACAGCTCGTCCATGCCGAG-3′, 5′-GCTGGTACCATCGACCT CATCGTACAGCTCGTCCATGCCGAGAGT-3′, 5′-GCTGGTAC CATCGACCTCATCCAGCTCGTCCATGCCGAGAGTGAT-3′, 5′-GCTGGTACCATCGACCTCATCCTCGTCCATGCCGAGAGTG ATCCC-3′, 5′-GCTGGTACCATCGACCTCATCGTCCATGCCG AGAGTGATCCCGGC-3′, 5′-GCTGGTA CCATCGACCTCATC CATGCCGAGAGTGATCCCGGCGGC-3′, 5′-GCTGGTACCAT CGACCTCATCGCCGAGAGTGATCCCGGCGGCGGT-3′, 5′-GC TGGTACCATCGACCTCATCGAGAGTGATCCCGGCGGCGGT CAC-3′, 5′-GCTGGTACCATCGACCTCATCAGTGATCCCGGC GGCGGTCACGAA-3′, 5′-GCTGGTACCATCGACCTCATCGAT CCCGGCGGCGGTCACGAACTC-3′, and 5′-GCTGGTACCATC-GA CCTCATCCCCGGCGGCGGTCACGAACTCCAG-3′.

The cDNAs of Venus [5] fused with various linker sequences at N-terminus were amplified by PCR. They were digested with *Kpn*I and *Eco*RI. The used primers are as follows: forward, 5′-GCTGGTAC CATGGTGAGCAAGGGCGAGG-3′, 5′-GCTGGTACCGGCGGC ATGGTGAGCAAGGGCGAGG-3′, 5′-GCTGGTACCGGCGGCG GCATGGTGAGCAAGGGCGAGG-3′, 5′-GCTGGTACCGGCGG CGGCAGCATGGTGAGCAAGGGCGAGG-3′, 5′-GCTGGTACC GGCGGCGGCAGCGGCATGGTGAGCAAGGGCGAGG-3′, 5′-G CTGGTACCGGCGGCGGCAGCGGCGGCATGGTGAGCAAGG GCGAGG-3′, 5′-GCTGGTACCGGCGGCGGCAGCGGCGGCAG CATGGTGAGCAAGGGCGAGG-3′, 5′-GCTGGTACCGGCGGC GGCAGCGGCGGCAGCGGCATGGTGAGCAAGGGCGAGG-3′, reverse, 5′-GCAGAATTCTTACTTGTACAGCTCGTCCATGCC-3′.

The digested PCR fragments were gel-purified, mixed together, and cloned in-frame into the *Bam*HI/*Eco*RI sites of $pRSET_B$ (Invitrogen) for bacterial expression. The *Bam*HI/*Eco*RI fragment of a selected construct was subcloned into *Bam*HI/*Eco*RI sites of pcDNA4-His-MaxB (Invitrogen) for mammalian expression.

Colony selection. After transformation of *Escherichia coli* [JM109(DE3)] with the ligated DNAs, the bacteria were grown up on agar plates (9 cm in diameter) at 37 °C for 12 h, left for 3 days at room temperature to allow the fluorescence develop, and then analyzed for the FRET efficiency using the fluorescence image analyzing system described previously [6]. The light emitted from the colonies was passed sequentially through two interference band-pass filters (480AF30 and 535AF25), and collected with a lens (AF NIKKOR, Nikon, Japan).

Recombinant protein preparation. Recombinant proteins with N-terminal polyhistidine tags were expressed in *E. coli* [JM109(DE3)] and purified by using Ni–NTA column chromatography as per manufacturer's recommendation (Qiagen).

Spectral analysis. Recombinant proteins were incubated with or without active-caspase-3 (1 U; Medical Biological Laboratory, Japan) at 37 °C for 2 h. The reaction buffer contained 20 mM Hepes–KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 20 mg/ml aprotinin, and 0.1 mM PMSF. After the incubation, emission spectrum was measured with an excitation wavelength at 435 nm using a fluorescence spectrophotometer (F2500; Hitachi, Japan).

Cell culture and transfection. HeLa cells were transfected with cDNAs using Superfect (Qiagen). To induce apoptosis, cells were treated with 100 ng/ml of anti-Fas monoclonal antibody (CH-11, Medical Biological Laboratory, Japan) and 10 μg/ml cyclohexamide in the growth medium.

Imaging. Two days after transfection, HeLa cells in Hanks' balanced salt solution buffer (Gibco) were subjected to imaging. During imaging, the cells were plated in a heated chamber at 37 °C. Confocal FRET images were acquired using an IX-71 equipped with a PlanApo 60×, 1.4 NA oil-immersion objective (Olympus, Japan), a spinning disc-type confocal unit (CSU21, Yokogawa, Japan), a diode-pumped solid-state laser (430 nm) (85BTL, Melles Griot), and a 3CCD color camera (ORCA-3CCD, Hamamatsu Photonics, Japan). Image acquisition and analysis were performed using AquaCosmos/Ashura software (Hamamatsu Photonics, Japan).

Results

SCAT3 was originally generated by connecting ECFP and Venus with a linker composed of 18 amino acids including DEVD, the sequence for caspase-3 cleavage [4]. In the present study, 88 different constructs were prepared by means of a PCR technique. They all contained floppy linkers at both sides of the DEVD sequence. The linker at the N-terminus of DEVD was the C-terminal tail of ECFP; *Aequorea* GFP has a floppy C-terminal tail of approximately 10 amino acids [7]. On the other hand, the linker at the C-terminus of DEVD was an artificial sequence consisting of glycine and serine stretches, which is the most widely used linker design; serine residues are interspersed to improve the solubility of a poly-glycine stretch. Variation was introduced into the length of both the linkers, while the two cDNA fragments were fused using *Kpn*I site sequence encoding GT (Fig. 1A). The 88 different constructs were introduced to *E. coli*, which were grown on an agar plate. When illuminated at 435 nm, emission ratios of 535–480 nm of the colonies varied from 1.7 to 14.9, indicating great variation in FRET efficiency from ECFP to Venus (Fig. 2).

Recombinant proteins were extracted from 10 colonies that showed large emission ratio values ($\geqslant$1.42) and their emission spectra were measured (Fig. 3A). Fig. 3A shows that the FRET efficiency observed in colonies was well correlated with that obtained through in vitro measurements.

The construct that gave the best FRET efficiency was further analyzed. From sequence analysis it was revealed that its structure was ECFP-ΔC7-DEVD-GT-Venus. The construct was named SCAT3.1. Incubation of SCAT3.1 with 1 U of active caspase-3 completely abolished the FRET signal (Fig. 3B), indicating that the linker sequence was sensitive to active caspase-3. The emission ratio of 530/480 nm changed from 3.50 to 0.52. When excited at 435 nm, the proteolysis-dependent change in color of SCAT3.1 solution was easily recognized by eye through a Wratten film passing light of longer than 460 nm (data not shown). Other enzymes including caspase-9 did not alter the emission spectrum (data not shown).

Then the FRET efficiency of non-cleaved SCAT3.1 was calculated in HeLa cells by measuring donor

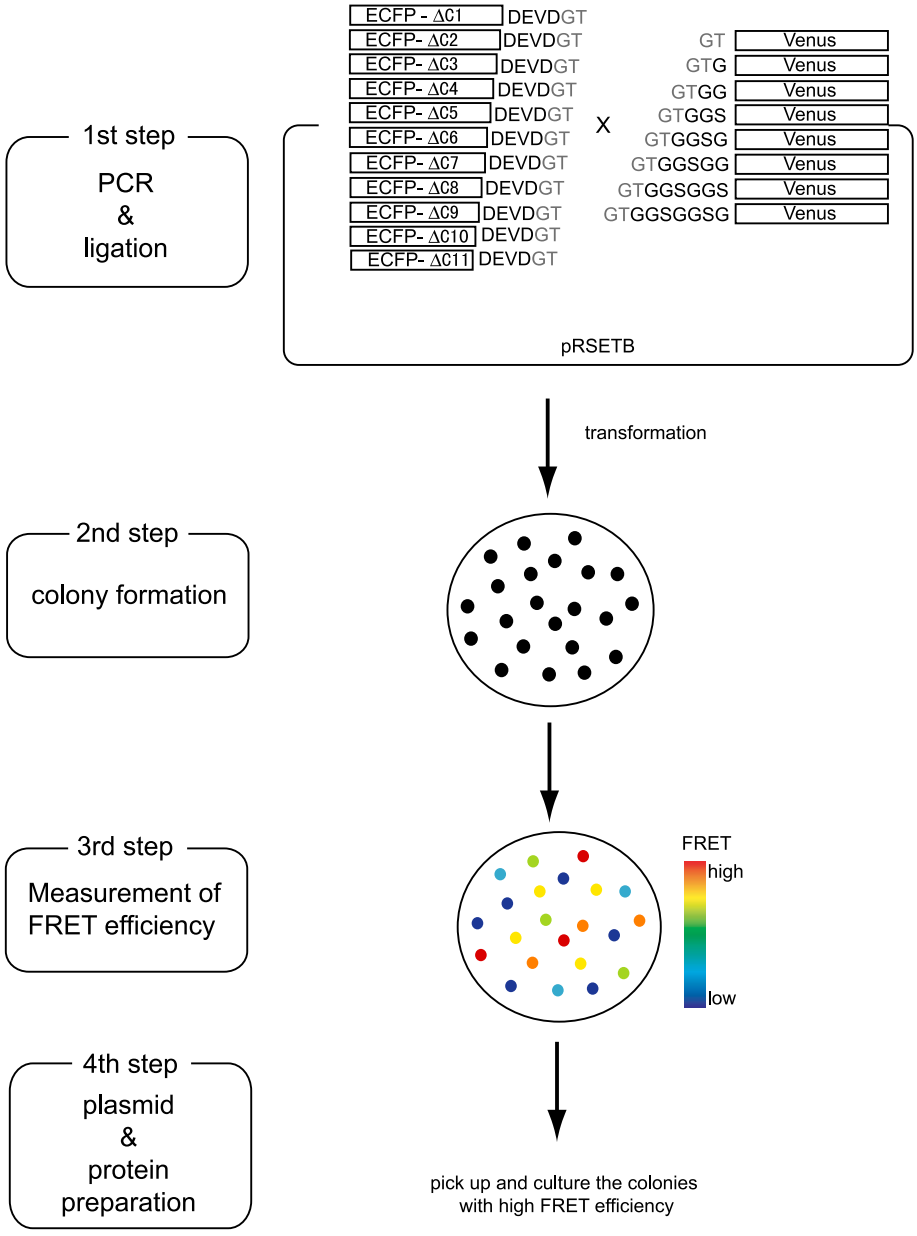

Fig. 1. An outline of the high-throughput screening method for obtaining constructs that show high efficiency of FRET from ECFP to Venus.

dequenching after acceptor photobleaching [8]. Fluorescence intensity of ECFP became 2.8 times brighter after photobleaching of Venus (Fig. 4), which gave 64% FRET efficiency. By contrast, intact SCAT3 showed 35% efficiency.

Next, HeLa cells producing SCAT3.1 or SCAT3 were treated with anti-Fas antibody and cyclohexamide. The two signals from ECFP and Venus were simultaneously captured using a color CCD camera containing three CCD chips. In addition, a spinning disc confocal unit was used to improve spatial resolution along *z*-axis at the expense of signal intensity. SCAT3.1 reported the change in FRET, i.e., caspase-3 activation more effectively than SCAT3 (Fig. 5). A significant change in color of SCAT3.1 during apoptosis was detected (Fig. 5A). Consistent with previous observations [4], caspase-3 activation was initiated in the cytosol and spread to the nucleus (Fig. 5A). Emission ratio value of 530/480 nm dropped progressively from 10 to 1 prior to cell shrinking (Fig. 5B). The value of SCAT3 decreased in the same time course from 1.6 to 1.0 (Fig. 5C). Thus, superiority of SCAT3.1 to SCAT3 in terms of dynamic range is more prominent in mammalian cells than in vitro. In HeLa cells expressing SCAT3.1, we were able to observe an emerging spot of caspase-3 activation in the cytosolic compartment (Fig. 5D, indicated by an arrowhead).

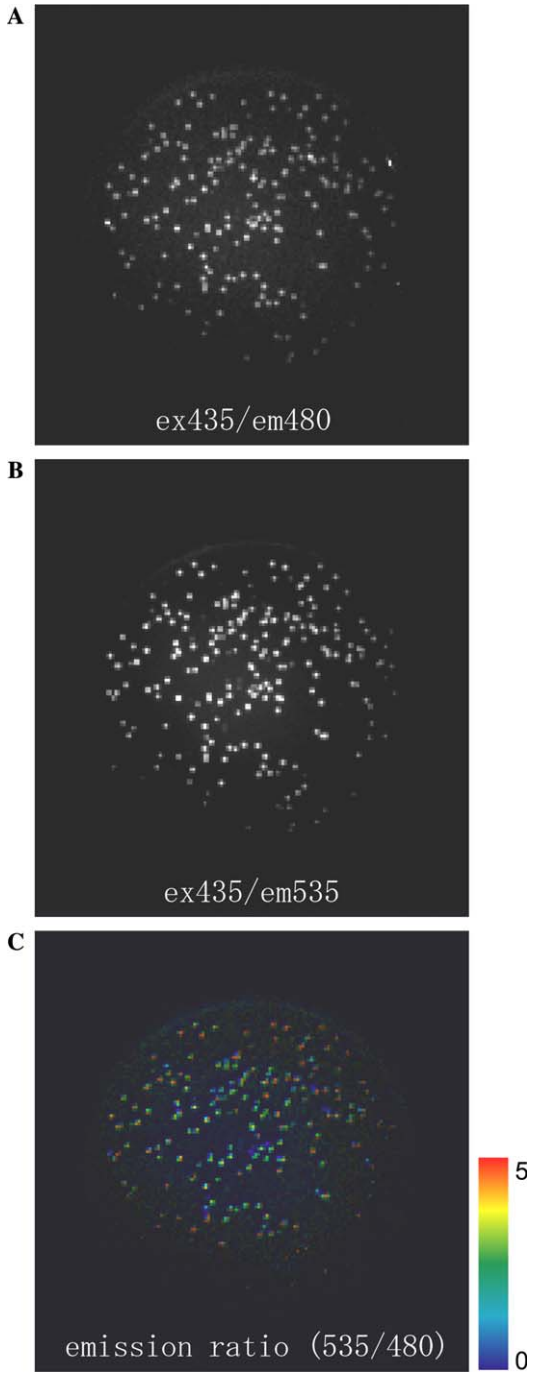

Fig. 2. Typical fluorescence images of bacterial colonies on an agar plate. (A) The excitation wavelength was 435 nm and the emission filter was 480 ± 15 nm. (B) The excitation wavelength was 435 nm and the emission filter was 535 ± 12.5 nm. (C) The ratio image of B/A.

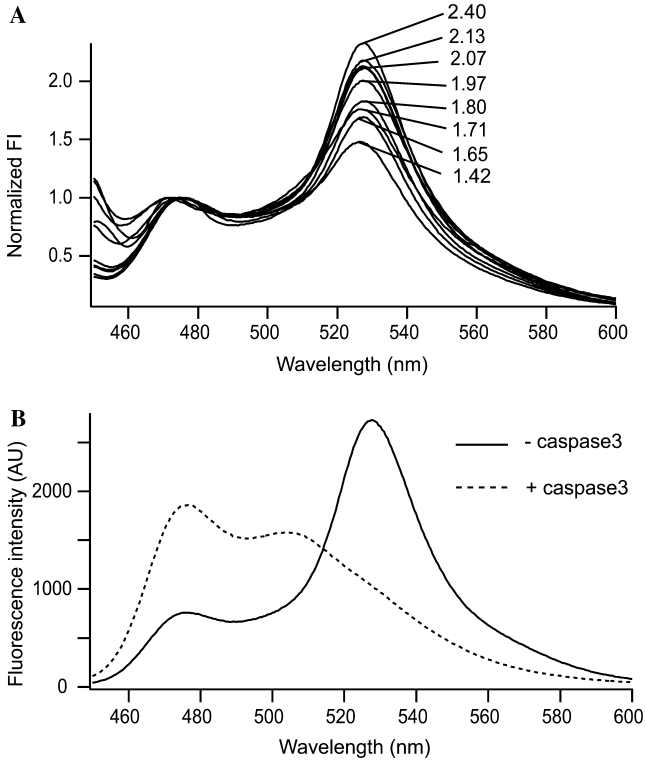

Fig. 3. Fluorescence emission spectra of selected constructs. (A) Spectra of suspensions of the colonies that showed high ratio values of 535/480 nm in the image analysis. The excitation wavelength was 435 nm. The spectra were normalized to the peaks of ECFP's emission intensity. (B) Emission spectra of SCAT3.1 before (solid line) and after (broken line) the 2 h incubation with active caspase-3.

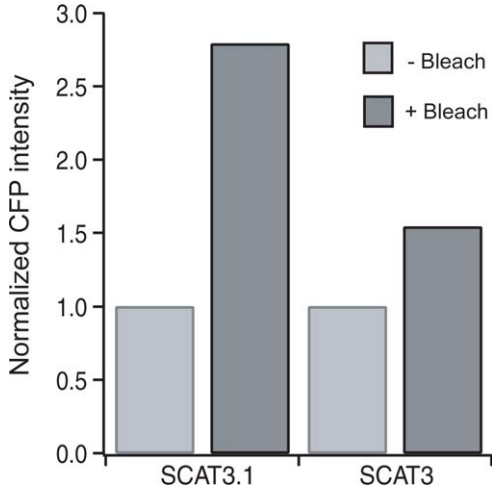

Fig. 4. Dequenching of ECFP after Venus photobleaching in SCAT3.1 and SCAT3. Photobleaching of Venus was done under microscope by intense illumination of green light (545DF40) from 75 W Xenon arc lamp. Fluorescence intensity was normalized to that of ECFP in intact sensors.

Discussion

In this study, we optimized the length of linkers of a sensor for caspase-3 activation to obtain SCAT3.1, which shows approximately 900% change in ratio of 530/480 nm in an apoptotic process. Such an increased dynamic range of SCAT3.1 will enable us to observe caspase-3 activation with better spatial and temporal resolutions. It has been noticed that constitutive expression of SCAT3 in cells or organisms results in a

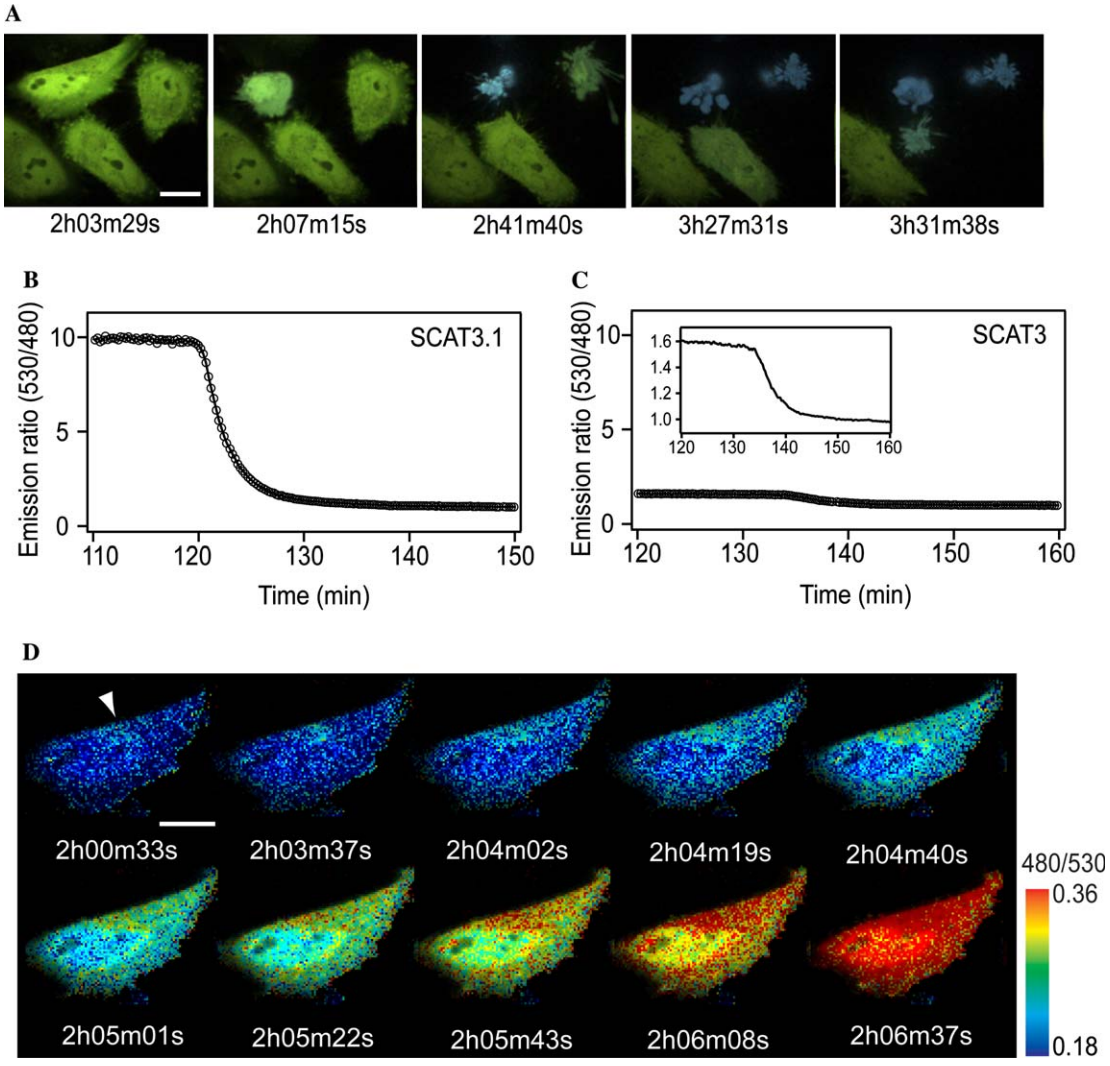

Fig. 5. Visualization of caspase-3 activation in HeLa cells expressing SCAT3.1 and SCAT3. (A) A series of confocal real color images of HeLa cells that expressed SCAT3.1 showing caspase-3 activation followed by cell-death. Time after the addition of anti-Fas monoclonal antibody and cyclohexamide is indicated below each image. Scale bar, 10 μm. (B) A time course of 530/480 nm ratio of SCAT3.1 in the experiment of (A). (C) A time course of 530/480 nm ratio of SCAT3 in a similar apoptosis experiment using SCAT3-expressing HeLa cells. (D) A series of confocal images of a HeLa cell expressing SCAT3.1 showing an emerging spot of caspase-3 activation in the cytosolic compartment (arrowhead). The 480/530 nm ratio value is shown in pseudo-color. Scale bar, 10 μm.

decrease in its responsivity during apoptosis experiments. Due to slight but persistent cleavage, prestimulus level of the ratio value is gradually decreased, resulting in a poor dynamic range. Thus, investigation of caspase-3 activation in more physiological situations will need the use of SCAT3.1.

FRET is highly sensitive to the relative orientation and distance between the two fluorophores. While the entire linker of SCAT3 is composed of 25 amino acids, it has been greatly shortened in SCAT3.1 (6 amino acids). Considering the propensity of ECFP and Venus to weakly dimerize [9], however, it remains uncertain whether the distance or relative angle between the chromophores of the two GFP variants is responsible for the improvement of FRET efficiency in SCAT3.1. The uncertainty will imply the importance of such high-throughput approaches to development and improvement of FRET-based indicators for proteases. This method can be applied to screening colonies for the constructs that monitor reversible protein–protein interactions or conformational equilibria, although the FRET signals obtained from colonies depend on many factors, such as degree of misfolding and/or proteolysis and stoichiometry of donor and acceptor.

Acknowledgments

We thank Katsuya Kominami and Kiwamu Takemoto for valuable advice. This work was partially supported by grants from PRESTO of

JST (Japan Science and Technology Agency) to T.N., and CREST of JST, the Japanese Ministry of Education, Science and Technology, and HFSP (Human Frontier Science Program) to A.M.

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