DOI: 10.1002/anie.200700538

In Vivo Imaging

Cyclic Luciferase for Real-Time Sensing of Caspase-3 Activities in Living Mammals**

*Akira Kanno, Yuko Yamanaka, Hisashi Hirano, Yoshio Umezawa, and Takeaki Ozawa**

Eukaryotic cells contain many proteases involved in cell growth, division, differentiation, migration, and intra- or extracellular signaling. One of the complicated proteolytic signaling processes is used to decide programmed cell death or apoptosis. Improper apoptosis causes many diseases including Alzheimer's disease, Huntington's disease, ischaemia, autoimmune disorders, and immortality of cancer cells.[1,2] As cytosolic caspases play a central role in mediating the initiation and propagation of apoptosis, chemical compounds that can either inhibit or accelerate caspase activity are a major concern. Also, an understanding of the physiological proteolytic processes inside living organisms is of crucial importance for assessing the roles of proteases in normal states and diseases. Hence, the development of a rapid screening system to detect caspase activity and a noninvasive method to image apoptosis is essential for the discovery of novel compounds that are potential therapeutic chemicals.[3] These developments would provide new insights into the mechanism of apoptosis.

Several methods for monitoring protease activities have been developed; the use of peptide substrates containing a fluorescence resonance energy transfer (FRET) pair, such as fluorescein and tetramethylrhodamine, is a simple strategy for the detection of proteolytic activities.[4] D-Luciferin connected with a peptide substrate for caspase enables highly sensitive detection of caspase activity in vitro. However, these chemical probes do not diffuse across membranes. These methods therefore require complex assay procedures like preparation of cell lysates and elimination of sediments upon analysis of intracellular protease. Genetically encoded fluorescent indicators that include green-fluorescent protein (GFP) derivatives overcome this drawback.[5–8] These indicators became valuable tools for studying temporal caspase activities in single living cells; however, the changes in the fluorescence signals are very small and the number of cells that can be analyzed is limited. In addition, it is difficult to apply the indicators to living animals because the light for excitation of the fluorophores is mostly absorbed by their tissues.[9] A recombinant bioluminescent indicator for monitoring caspase activities has been developed by using *Photinus pyralis* luciferase (firefly luciferase, Fluc).[10] The indicator is composed of Fluc sandwiched between estrogen-receptor regulatory domains with an Asp–Glu–Val–Asp (DEVD) sequence that can be digested by caspase-3. The activity of caspase-3 was monitored noninvasively over time in living animals. However, the molecular size of the indicator is quite large and the intensity of the luminescence signals is relatively low because of insufficient digestion of the DEVD sequence during apotosis.

Here we describe a genetically encoded cyclic luciferase with general applicability to the detection of protease activities in living cells and animals. Two fragments of DnaE intein are fused to neighboring ends of Fluc connected with a substrate sequence for the protease (Figure 1). After translation into a single polypeptide in living cells, the amino (N) and carboxy (C) terminals of the luciferase are ligated by protein splicing, which results in a closed circular polypeptide chain. Since the structure of the cyclic luciferase is distorted, the luciferase loses its bioluminescence activity. If the substrate sequence is digested by a protease, the luciferase changes into an active form and restores its activity. To prove the usefulness of the cyclic luciferase, we selected a well-known protease, caspase-3. We report a method for quantitative sensing of caspase-3 activity in living cells upon extracellular stimuli and for noninvasive imaging of time-dependent caspase activity in living mice.

The crystal structure of Fluc indicates that the luciferase folds into two compact domains (Figure 1a),[11] a large N-terminal domain (Fluc-N) and a small C-terminal one (Fluc-C). These domains are separated by a wide cleft, in which the active center of Fluc is located. The N- and C-terminal ends of Fluc, which are visible in the crystal structure, are not in close proximity ($\approx$4 nm between residue Asp3 and Lys544). They are located on the same side behind the active center. On the basis of the structural information about Fluc, we suppose that linkage of the N- and C-terminal ends of Fluc by the substrate sequence for caspase-3, DEVD, may change the

[*] Dr. T. Ozawa
Department of Molecular Structure
Institute for Molecular Science and
PRESTO, Japan Science and Technology Agency
38 Nishigonaka, Myodaiji, Okazaki, Aichi 444-8585 (Japan)
Fax: (+81) 564-55-4639
E-mail: ozawa@ims.ac.jp
Dr. A. Kanno, Prof. Dr. Y. Umezawa, Dr. T. Ozawa
Department of Chemistry, School of Science
The University of Tokyo and
Japan Science and Technology Agency
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033 (Japan)
Y. Yamanaka, Prof. Dr. H. Hirano
Supramolecular Biology
International Graduate School of Arts and Sciences
Yokohama City University
1-7-29 Suehiro-cho, Tsurumi-ku
Yokohama, Kanagawa 244-0813 (Japan)

[**] This work was supported by grants from the Japan Science and Technology Agency (JST), the Japan Society for the Promotion of Science (JSPS), and the New Energy and Industrial Technology Development Organization (NEDO) of Japan.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

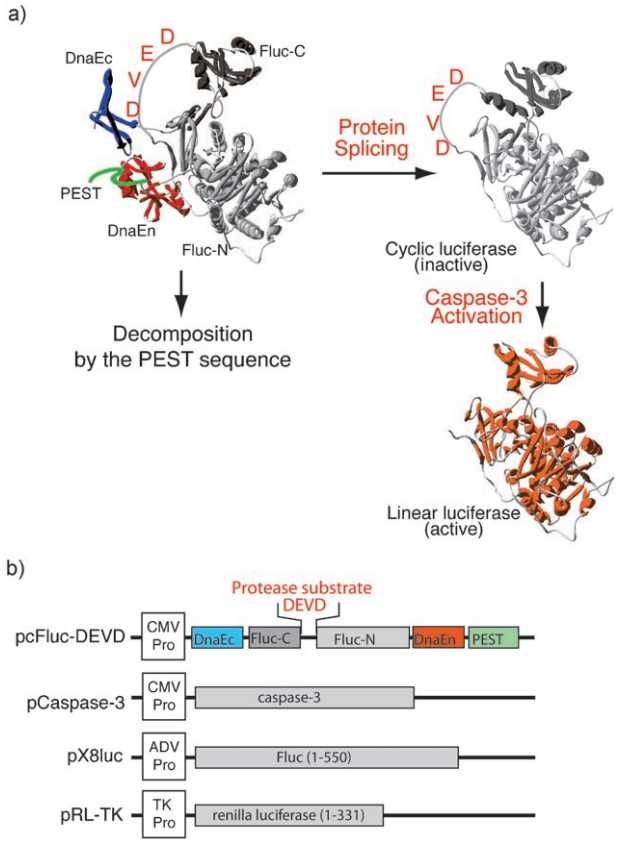

Figure 1. Strategy for the detection of caspase-3 activity. a) Principle for monitoring the activity of caspase-3 by using cyclic firefly luciferase (Fluc). b) Schematic structures of the cDNA constructs. For an efficient protein splicing reaction, CFNIS and KFAEYC sequences were inserted between DnaEc and Fluc-C and between Fluc-N and DnaEn, respectively (see the Supporting Information). "Pro" means a promoter, other abbreviations are defined in the text. All text in the colored and shaded boxes refer to the genes of the corresponding proteins.

overall structure and this change results in great attenuation of the Fluc activity. In order to connect the N-terminal end with the C-terminal one, we chose a naturally split DnaE intein derived from *Synechocystis* sp. PCC6803,[12] because previous reports have indicated that this DnaE intein gives cyclic peptides or proteins with a high yield.[13–16] The C- and N-terminal fragments of DnaE (DnaEc and DnaEn, respectively) are connected with the N- and C-terminal ends of the circularly permuted luciferase, respectively (Figure 1). In addition, a PEST sequence, which is known to accelerate degradation of a protein in 4 h (half-life time),[17] was attached to the C-terminal end of the fusion construct. The PEST sequence results in the degradation of only unspliced products because cyclic Fluc does not possess the PEST sequence. Consequently, only cyclic Fluc accumulates inside the cells. If caspase-3 is activated in cells expressing the cyclic Fluc, the Fluc changes into an active form and its luminescence activity is restored (Figure 1). Thus, cells expressing the cyclic Fluc allow monitoring of caspase-3 activity with luminescence signals.

To assess whether the fusion protein expressed by pcFluc-DEVD produces cyclic Fluc by protein splicing, we transiently transfected COS-7 cells with pcFluc-DEVD for 48 h and examined the spliced products by Western blot (Figure 2a). As a molecular-size marker for linear Fluc, we analyzed samples from COS-7 cells transfected with pFluc-DEVD, which expresses the fusion protein Fluc-N-Fluc-C-DEVD (64 kDa), by electrophoresis (see the Supporting Information). For inducing the apoptosis, we used an apoptosis-inducing reagent, staurosporine (STS), which triggers the digestion of the DEVD amino acid sequence by caspase-3. Caspase-3 activity in the cells was confirmed by blotting a poly(adenosine 5′-diphosphate–ribose) polymerase (PARP) that contains a DEVD sequence. In lysates of the cells in the absence of STS, the luciferase antibody recognized two components near to 60 kDa; the molecular weight of the major product, corresponding to cyclic Fluc, is higher than that of the other product. The luciferase antibody strongly recognized the lower product, which corresponds to a linear form of Fluc, in the presence of STS, whereas the intensity of the product with higher molecular weight was very weak in this case. Formation of the linear form of Fluc was dependent on the concentration of STS. The band emerging after stimulation with STS is neither a degradation product nor a product from an intermolecular protein splicing because the molecular weights of the expected byproducts are significantly higher than those of cyclic or linear Fluc (see the Supporting Information). To confirm cleavage of the DEVD sequence by caspase-3, a mass spectrometric (MS) analysis was performed. COS-7 cells transfected with pcFluc-DEVD were stimulated with STS and the luciferases were pulled down with anti-luciferase antibody. The samples were electrophoretically separated and a band that appeared at $\approx$60 kDa was obtained. The MS analysis revealed that the band was a splicing product that had been cleaved at the DEVD sequence (see the Supporting Information). We next performed a Western blot analysis by using a mutated luciferase; the DNA sequence encoding DEVD was changed into one encoding DEVG, which is not digested by active caspase-3.[6] The plasmid was named as pcFluc-DEVG. In the presence and absence of STS, Western blot analysis of COS-7 cells transfected with pcFluc-DEVG showed only single bands (see the Supporting Information), thereby demonstrating that the band around 60 kDa which disappears after STS treatment is a cyclic form of Fluc.

We next examined whether the activity of the cyclic Fluc was restored after stimulation with STS. COS-7 cells transfected with pcFluc-DEVD were stimulated with STS or a caspase inhibitor (*N*-benzyloxycarbonyl–Val–Ala–Asp(O-Me) fluoromethyl ketone, Z-VAD-FMK), or with both STS and Z-VAD-FMK. The cells were harvested and their lysates were mixed with a Fluc substrate, D-luciferin. The luminescence signals increased with increased concentrations of STS and the maximum response was strong enough to be discriminated from the signal in the absence of STS (Figure 2b). The cells with 100 μM Z-VAD-FMK showed no increase in luminescence in the presence or absence of 1 μM STS. The luminescence signals in the presence of Z-VAD-FMK were smaller than the signals that originated from the cells in the absence of STS and Z-VAD-FMK. This indicates that the unstimulated cells had slight caspase-3 activity.

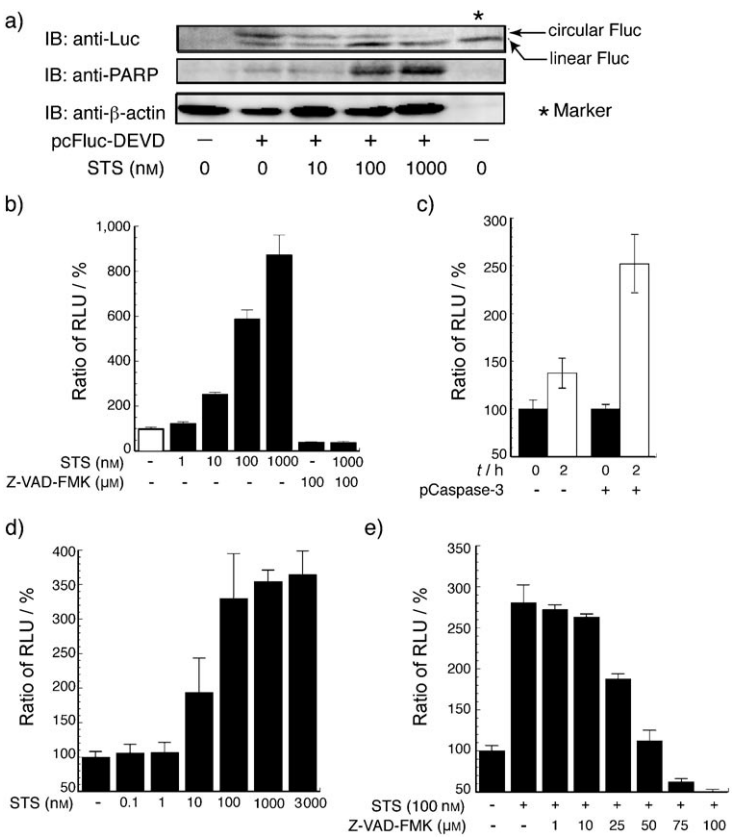

Figure 2. In vitro characterization of cyclic Fluc. a) Western blot analysis of COS-7 cells transfected with pcFluc-DEVD for 48 h. The cells were treated with different concentrations of staurosporine (STS) for 2 h and harvested. The activity of caspase-3 was evaluated from the digested poly(adenosine 5′-diphosphate–ribose) polymerase (PARP) with an anti-PARP antibody. As a reference for the amounts of the proteins in the electrophoresis, β-actin was stained with its specific antibody. The asterisk (*) is a molecular-size marker of a linear Fluc. b) Quantitative analysis of the Fluc activity with STS and *N*-benzyloxycarbonyl-Val–Ala–Asp(O-Me) fluoromethyl ketone (Z-VAD-FMK). After transfection of COS-7 cells with pcFluc-DEVD, the cells were treated with 100 μM Z-VAD-FMK or vehicle (0.1% dimethylsulfoxide (DMSO)) for 1 h and then stimulated with various concentrations of STS for 2 h. c) Analysis of the Fluc activity in the caspase-3-deficient MCF-7 cells. MCF-7 cells were transfected with only pcFluc-DEVD (left) or with both pcFluc-DEVD and pCaspase-3 (right). The cells were incubated for 48 h and the luminescence intensities were evaluated at the indicated times after stimulation with 100 nM STS. d) Quantitative analysis of the Fluc activity with STS. After transfection of HeLa cells with pcFluc-DEVD, the cells were treated with various concentrations of STS for 2 h. e) Quantitative analysis of the Fluc activity with Z-VAD-FMK. The HeLa cells were treated with different concentrations of Z-VAD-FMK or vehicle (0.1% DMSO) for 1 h and stimulated with various concentrations of STS for 2 h.

To provide evidence that digestion of the cyclic Fluc was indeed mediated by caspase-3, we used caspase-3-deficient MCF-7 cells.[18] When cells transfected with pcFluc-DEVD were stimulated with 100 nM STS for 2 h, little change in the bioluminescence intensity was observed (Figure 2c). By contrast, when MCF-7 cells were cotransfected with pcFluc-DEVD and pCaspase-3, the luminescence from the cells significantly increased upon stimulation with STS. From these results, we conclude that the inactive cyclic Fluc was created by protein splicing inside the cells and the activity of Fluc was restored when caspase-3 digested the DEVD sequence.

To show the feasibility of cyclic Fluc for characterizing apoptotic reagents, we examined the sensitivity of cyclic Fluc with another cell line, HeLa cells. The cells were transiently transfected with pcFluc-DEVD and stimulated with different concentrations of STS for 2 h. We found that STS induced an increase in the bioluminescence intensity at concentrations of 1.0×10^{-8}–1.0×10^{-6} M (Figure 2d). Next, we examined inhibitory effects on the caspase-3 activity with a model caspase-3 inhibitor, Z-VAD-FMK (Figure 2e). The inhibitory effect of Z-VAD-FMK on the cleavage of cyclic Fluc was observed at the concentrations of Z-VAD-FMK higher than 10 μM. With 100 μM Z-VAD-FMK, the cleavage of cyclic Fluc was completely suppressed. These results indicate that the cells expressing cyclic Fluc can be used for quantitative analysis of apoptosis-inducing and -inhibiting chemical compounds in living cells.

It is known that the time required for caspase-3 activation in response to extracellular stimuli varies with the type of chemical compound and its concentration. In order to decide upon a screening time, it is crucial to know when the activity of caspase-3 gives a maximum response after stimulation with apoptotic reagents. In the next set of experiments, we performed real-time analysis of caspase-3 activity in living cells. We transiently expressed the cyclic Fluc indicator in HeLa cells and monitored time-course luminescence intensities every 5 min upon stimulation with various concentrations of STS (Figure 3a). The cells initially showed a slow increase in the intensity of the luminescence signals for 25 min. Thereafter, a rapid increase in the luminescence signal intensity was observed. The rate of increase in the luminescence intensity depended on the concentration of STS. The luminescence signals were found to reach a maximum 100 min after stimulation with STS, and the intensity of the signals then gradually decreased. The decrease in the luminescence signal intensity indicates attenuation of active luciferase by proteolysis in the HeLa cells. When HeLa cells including the cyclic Fluc were stimulated with a potent antitumor reagent, actinomycin D (ActD), the luminescence signal intensity similarly increased over the time period of 25–120 min and then gradually decreased (Figure 3b). These data indicate that the time to obtain maximal luminescence is approximately 120 min after stimulation with the chemical compounds and this is the most suitable time for high-throughput screening of chemical compounds.

To demonstrate a further application of cyclic Fluc, we applied this reporter to the investigation of the distribution of chemical compounds in organs of living mice and to the quantification of their effects on caspase-3 activity. We implanted HeLa cells expressing cyclic Fluc in the right side of the back of mice (Figure 4a). As the control experiment, HeLa cells expressing full-length Fluc were implanted on the left side. When the mice were injected intraperitoneally with STS (100 μg kg^{-1} of body weight), the observed images of the

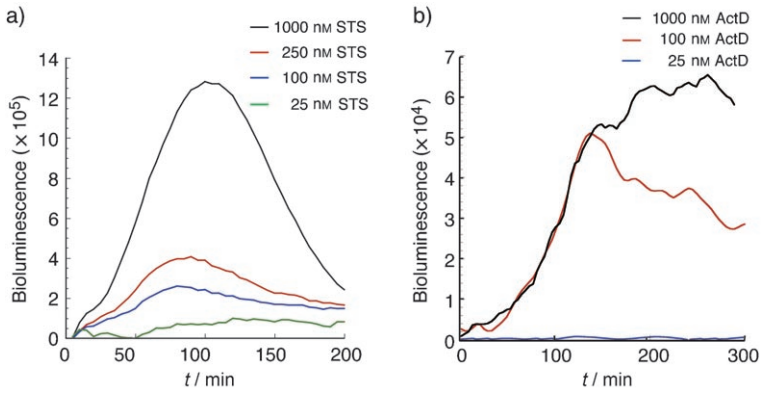

Figure 3. Real-time in vitro analysis of caspase-3 activity. HeLa cells transfected with pcFluc-DEVD were treated with different concentrations of a) STS or b) actinomycin D (ActD) and then the bioluminescence intensities were measured every 5 min. The indicated time represents the time after the addition of each agent.

mice showed a significant increase in the luminescence signal intensity only from the right side, which was implanted with the cyclic Fluc. Three hours after the injection, the increase in the luminescence intensity was rapid; the luminescence gradually decreased thereafter. In the left side of the back of the mice, there is no significant change in the luminescence signal after the injection of STS. This indicates that the concentrations of D-luciferin in the bodies of the mice were kept constant during the data acquisition.

To quantitatively evaluate the effect of STS on the caspase-3 activity, we defined a relative luminescence unit (RLU), where the luminescence intensity from the right side (LumR) was divided by that from the left side (LumL), as RLU = LumR/LumL. This calculation eliminates variations in the experimental conditions such as transfection efficiency, decomposition of the implanted cells, and the amount of injected D-luciferin circulating in the mice. Figure 4b shows the temporal changes in the RLU, which was evaluated from the bioluminescence images at the indicated times. Within 3 h of the intraperitoneal injection of STS, the RLU has reached a maximum ratio in comparison to the RLU before stimulation with STS. Thereafter, the RLU ratio decreased gradually. The agreement between this and the results in Figure 3a suggests that the present system is applicable to in vivo sensing of caspase-3 activities in real time upon stimulation with exogenous chemical compounds.

In this study we demonstrated a method for the detection of caspase-3 activity in vitro and in vivo with a genetically encoded de novo reporter of cyclic Fluc generated by protein splicing. The method of cell-based sensing with the reporter provided quantitative and real-time measurements of the extent of caspase-3 activity in response to extracellular stimuli. The cyclic Fluc reporter further allowed noninvasive imaging of caspase-3 activity in living mice in a time-dependent manner. In the existing methods, analyses of caspase-3 activity have mostly depended upon FRET-based indicators. The FRET-based methods have several limitations in the screening and in vivo imaging of chemicals. Among the apoptotic reagents or drug candidates, there are fluorescent or light-absorbing compounds, such as apoptosis-inducing ActD and wortmannin catabolites. These may disturb the observed fluorescence signals. The precision of the fluorescence observed by fluorescence microscopy is not very high, because the number of analyzed cells is quite limited. When applied to living mice, the low-wavelength excitation light for FRET is scattered by the organs, which hamper the detection of emitted fluorescence. These limitations can be overcome by the present method with cyclic Fluc, due to the background-free luminescence signals generated during apoptosis. The activity of the cyclic Fluc was found to be quite low and it therefore enabled highly sensitive detection of the caspase-3 activity. Moreover, the number of cells analyzed in a single well was 10^4–10^6 cells, which was enough to precisely evaluate the extent of the caspase-3 activity.

Intein-mediated cyclization of peptide backbones has been demonstrated for analysis of the protein folding of dihydrofolate reductase (DHFR) and GFP.[13,19] A method of short-peptide cyclization with randomly variable amino acids has been shown to yield a potential inhibitor of intracellular signaling.[20] Compared to these previous cyclization techniques, the most unique feature of the present cyclic Fluc method is that there is a large difference in the luminescence signals between the cyclic and linear forms, regardless of the similarity in molecular weights and amino acid sequences. We

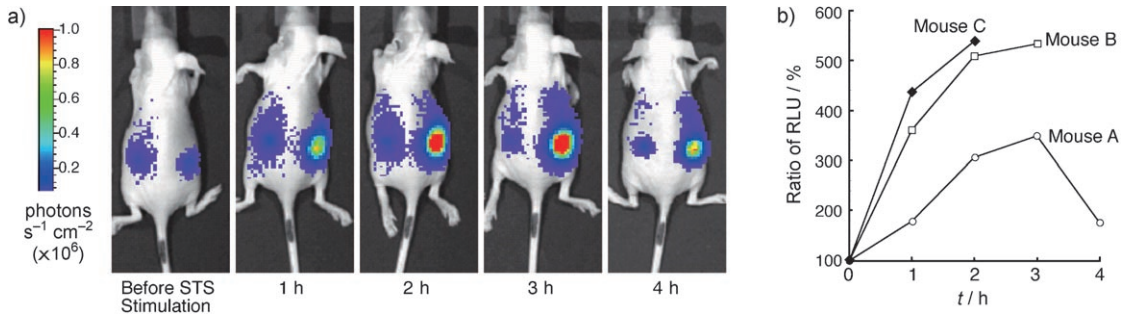

Figure 4. Time-dependent caspase-3 activity in living mice. a) In vivo optical CCD imaging of mice carrying transiently transfected HeLa cells. The mice were subcutaneously implanted with HeLa cells expressing cyclic Fluc (right side) and full-length Fluc (left side). Images of the mice were taken at the indicated times after intraperitoneal injection of STS. A representative of three mice is shown. b) Time-dependent changes in the photon count from the implanted site in (a). The results of three mice are shown.

demonstrated that the connection of the N- and C-terminal ends of Fluc with the substrate peptide of caspase-3 resulted in a decrease in the enzymatic activity, which was restored rapidly after digestion of the substrate by caspase-3. A C-terminal PEST sequence worked for the decomposition of unspliced precursors. The presence of the PEST sequence is crucial for highly sensitive detection, because the unspliced precursor gives a high background luminescence. These unique properties of the circular-luciferase-based indicator are entirely different from the engineered luciferase indicators reported previously; luciferase has been used for the analysis of protein–protein interactions and protein translocation into the nucleus on the basis of complementation or reconstitution of split luciferases.[21–25]

We showed quantitative effects of both apoptosis-inducing and -inhibiting chemicals on caspase-3 activity. Unexpectedly, the luminescence signals generated from cells treated with 100 μM Z-VAD-FMK were reduced to 50% of the signals from vehicle-treated cells (Figure 2b). This result indicates that the mild ischemic conditions of cells upon transfection induced a basal activation of caspase-3 and that Z-VAD-FMK completely inhibited the basal caspase-3 activities. Although such unwanted effects were present, cyclic Fluc provided information concerning the spatial and temporal patterns of caspase-3 activity in living mice. Thus, rapid assessment of potential therapeutic chemicals in a fully quantitative manner is possible.

In summary, we demonstrated the usefulness of cyclic Fluc for quantitative detection of caspase-3 activity in living cells and animals. Cell-based analysis by using the presented assay with cyclic Fluc allows for precise and quantitative measurement of caspase-3 activity because a statistically significant number of cells can be analyzed. The response of cyclic Fluc upon caspase-3 activation is very fast, which enables high-throughput screening and characterization of therapeutic anticancer drugs and caspase inhibitors. Since the amounts of probes, in general, are controllable with the conventional stable expression or viral infection techniques, the use of such transfecting techniques may improve the specification of the present method. We showed in vivo real-time imaging of caspase-3 activity in living mice. Chemical compounds, in many cases, are metabolized or chemically modified in living mice. Effective concentrations of the compounds can be estimated noninvasively by using the present method. Therefore, the availability of this genetically encoded reporter facilitates the development of transgenic animals that express the cyclic Fluc in a specific tissue with controllable promoters. Although we have demonstrated the feasibility for only caspase-3 activity, modifications in the substrate region of this cyclic Fluc reporter will enable the monitoring of other protease activities. This basic concept is also applicable to other luciferases emitting light of different wavelengths with D-luciferin.

Received: February 6, 2007
Revised: June 20, 2007
Published online: August 27, 2007

Keywords: apoptosis · caspases · imaging · luciferases · luminescence

[1] D. Nijhawan, N. Honarpour, X. D. Wang, *Annu. Rev. Neurosci.* **2000**, *23*, 73–87.
[2] H. J. Riedout, L. Stefanis, *Histol. Histopathol.* **2001**, *16*, 895–908.
[3] J. Neefjes, N. P. Dantuma, *Nat. Rev. Drug Discovery* **2004**, *3*, 58–69.
[4] E. Reits, A. Griekspoor, J. Neijssen, T. Groothuis, K. Jalink, P. van Veelen, H. Janssen, J. Calafat, J. W. Drijfhout, J. Neefjes, *Immunity* **2003**, *18*, 97–108.
[5] T. Nagai, A. Miyawaki, *Biochem. Biophys. Res. Commun.* **2004**, *319*, 72–77.
[6] K. Takemoto, T. Nagai, A. Miyawaki, M. Miura, *J. Cell Biol.* **2003**, *160*, 235–243.
[7] X. Xu, A. L. V. Gerard, B. C. B. Huang, D. C. Anderson, D. G. Payan, Y. Luo, *Nucleic Acids Res.* **1998**, *26*, 2034–2035.
[8] N. P. Mahajan, D. C. Harrison-Shostak, J. Michaux, B. Herman, *Chem. Biol.* **1999**, *6*, 401–409.
[9] R. Weissleder, V. Ntziachristos, *Nat. Med.* **2003**, *9*, 123–128.
[10] B. Laxman, D. E. Hall, M. S. Bhojani, D. A. Hamstra, T. L. Chenevert, B. D. Ross, A. Rehemtulla, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 16551–16555.
[11] E. Conti, N. P. Franks, P. Brick, *Structure* **1996**, *4*, 287–298.
[12] H. Wu, Z. M. Hu, X. Q. Liu, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 9226–9231.
[13] C. P. Scott, E. Abel-Santos, M. Wall, D. C. Wahnon, S. J. Benkovic, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 13638–13643.
[14] C. P. Scott, E. Abel-Santos, A. D. Jones, S. J. Benkovic, *Chem. Biol.* **2001**, *8*, 801–815.
[15] N. M. Nichols, T. C. Evans, *Biochemistry* **2004**, *43*, 10265–10276.
[16] T. C. Evans, D. Martin, R. Kolly, D. Panne, L. Sun, I. Ghosh, L. X. Chen, J. Benner, X. Q. Liu, M. Q. Xu, *J. Biol. Chem.* **2000**, *275*, 9091–9094.
[17] X. Q. Li, X. N. Zhao, Y. Fang, X. Jiang, T. Duong, C. Fan, C. C. Huang, S. R. Kain, *J. Biol. Chem.* **1998**, *273*, 34970–34975.
[18] R. U. Janicke, M. L. Sprengart, M. R. Wati, A. G. Porter, *J. Biol. Chem.* **1998**, *273*, 9357–9360.
[19] H. Iwai, A. Lingel, A. Pluckthun, *J. Biol. Chem.* **2001**, *276*, 16548–16554.
[20] T. M. Kinsella, C. T. Ohashi, A. G. Harder, G. C. Yam, W. Q. Li, B. Peelle, E. S. Pali, M. K. Bennett, S. M. Molineaux, D. A. Anderson, E. S. Masuda, D. G. Payan, *J. Biol. Chem.* **2002**, *277*, 37512–37518.
[21] R. Paulmurugan, Y. Umezawa, S. S. Gambhir, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 15608–15613.
[22] T. Ozawa, A. Kaihara, M. Sato, K. Tachihara, Y. Umezawa, *Anal. Chem.* **2001**, *73*, 2516–2521.
[23] S. B. Kim, T. Ozawa, S. Watanabe, Y. Umezawa, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 11542–11547.
[24] K. E. Luker, M. C. P. Smith, G. D. Luker, S. T. Gammon, H. Piwnica-Worms, D. P. Piwnica-Worms, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 12288–12293.
[25] I. Remy, S. W. Michnick, *Nat. Methods* **2006**, *3*, 977–979.

