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Monitoring the caspase cascade in single apoptotic cells using a  
three-color fluorescent protein substrate

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## Abstract

Fluorescence cross-correlation spectroscopy (FCCS) reveals information about the spatiotemporal coincidence of two spectrally well-defined fluorescent molecules in a small observation area at the level of single-molecule sensitivity. To simultaneously evaluate the activities of caspase-3 and caspase-9, we constructed a chimeral protein that consisted of tandemly fused enhanced cyan fluorescent protein (ECFP), monomeric red fluorescent protein (mCherry) and monomeric yellow fluorescent protein (Venus). In HeLa cell lysates, a combination of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )- and cycloheximide (CHX-)-induced apoptosis was monitored. In this, decreases of cross-correlation amplitudes were observed between ECFP and mCherry and between mCherry and Venus. Moreover, time-dependent monitoring of single cells revealed decreases in the cross-correlation amplitudes between ECFP and mCherry and between mCherry and Venus before morphologic changes were observed by laser scanning fluorescence microscopy (LSM). Thus, our method could predict the fate of the cell in the early apoptotic stage.

*Keywords:*

FCCS, caspase inhibition, multi color, morphological change.

\*Abbreviations:

FCS, fluorescence correlation spectroscopy;

FCCS, fluorescence cross-correlation spectroscopy;

TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ;

CHX, cycloheximide;

LSM, laser scanning fluorescence microscopy;

RCA, relative cross-correlation amplitude.

Apoptosis, also called programmed cell death, is cellular suicide in multicellular organisms [1-3]. The caspases are a family of cysteine proteases that play a critical role in the execution phase of apoptosis. They are responsible for the biochemical and morphological changes associated with apoptosis [4]. It has been proposed that caspases can be divided into two categories: "initiator" caspases with a long prodomain, such as caspase-9, and "effector" caspases such as caspase-3 [5]. Caspase-3 has been shown to be a key component involved in the underlying mechanisms of apoptosis [6], and the activation of caspase-3 is considered to be the final step in many apoptotic pathways [7]. Most commonly, two major apoptotic pathways have been identified: the death receptor pathway [8] and the mitochondrial pathway [9]. Signal transduction and execution of apoptosis require the activation of the caspase cascade [10]. Most apoptosis depending on the caspase cascade, as well as the cascade the process itself, is obstructed by caspase-inhibitors [11-13]. The current method to monitor caspase activities includes the use of caspase substrates and inhibitors [14-21]. Previously, we constructed tandem fused fluorescent proteins with two colors for measurement of caspase-3 activity [22], and three

colors for measurement of caspase-3 and caspase-9 activities [23] by using FCCS. In this study, we carried out FCCS analysis and found that caspase-9 was activated earlier than caspase-3 in apoptotic cells. Moreover, the combination of FCCS and three colors tandem fused fluorescent proteins can predict progression of the cell to apoptosis before the morphologic changes appeared.

## **Materials and methods**

*Plasmids and chemicals.* The CRY chimera encoded ECFP, DEVD, which is a caspase-3 recognition site, mCherry [24], LEHD, which is a caspase-9 recognition site, and Venus [25] (Fig. 1A, ECFP-DEVD-mCherry-LEHD-Venus, CRY chimera). ECFP and mCherry pairs and mCherry and Venus pairs have been reported to be adequate for *in vitro* and in cell by FCCS measurements [23]. TNF- $\alpha$  (Med. & Biol. Lab) was dissolved in water and diluted into other adequate buffers. CHX (Med. & Biol. Lab), caspase-3 inhibitor II (Calbiochem), which consists of a z-DEVD-fmk inhibitor sequence and caspase-9 inhibitor I (Calbiochem), which consists of a z-LEHD-fmk inhibitor sequence were dissolved in DMSO, and diluted to final concentrations containing less than 0.1% DMSO

using Dulbecco's modified Eagle's medium (DMEM, Sigma).

*Cell culture and transfection.* HeLa cells were cultured in DMEM supplemented with  $2 \times 10^5$  U/l penicillin G, 200 mg/l streptomycin sulfate, and 10% fetal bovine serum in humidified air with 5% CO<sub>2</sub> at 37°C. Sixteen hours before transfection, HeLa cells were cultured on a 6-well chamber Nunclon™Δ surface (Nalge Nunc International) for preparation of cell lysates, or on a Lab-Tek™ 8-well chamber coverglass (Nalge Nunc International) for measurement of single living cells. HeLa cells were transfected with 0.5 µg/ml for 6-well, or 0.2 µg/ml for 8-well of plasmid DNA encoding the CRY chimera using Optifect™ (Invitrogen).

*Induction and inhibition of apoptosis.* For induction of apoptosis, the CRY chimera-expressing HeLa cells were incubated with 250 ng/ml TNF-α and 20 µg/ml CHX in humidified air with 5% CO<sub>2</sub> at 37°C. To specifically inhibit caspase-3 or caspase-9, the CRY chimera-expressing HeLa cells were treated with 5 µM caspase-3 inhibitor II or 25 µM caspase-9 inhibitor I two hours before addition of TNF-α/CHX.

*Preparation of cell lysates.* The CRY chimera-expressing HeLa cells were lysed with lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Triton X-100) [26] at 0 min, 30 min and 60 min after TNF-α/CHX treatment.

*FCCS measurement and data analysis.* FCCS measurements were performed with an LSM510-ConfoCor3 (Carl Zeiss), the setup of which followed our previous report [23]. ECFP and Venus were excited at the 458 nm laser line because the brightness of Venus was sufficient in this case for FCCS measurement, and mCherry was excited at the 594 nm laser line. These lines were via a single-band dichroic mirror for 458 nm, which reflected 2% (0.35  $\mu$ W) of the 594 nm laser line. Measurements in single living cells were performed ten times for 2 s every 5 min after TNF- $\alpha$ /CHX treatment. Data from FCCS were analyzed with the ConfoCor3 software. The fluorescence autocorrelation functions from the cyan, yellow and red channels,  $G_C(\tau)$ ,  $G_Y(\tau)$ ,  $G_R(\tau)$ , respectively, and the fluorescence cross-correlation functions,  $G(i, j, \tau)$ , were calculated according to the normalized correlation function

$$G(i, j, \tau) = \frac{\langle I_i(t) \rangle \cdot \langle I_j(t + \tau) \rangle}{\langle I_i(t) \rangle \cdot \langle I_j(t) \rangle} = \frac{\langle \delta I_i(t) \rangle \cdot \langle \delta I_j(t + \tau) \rangle}{\langle I_i(t) \rangle \cdot \langle I_j(t) \rangle} + 1 \quad (1)$$

where  $\tau$  indicates the time delay,  $I_i$  is the fluorescence intensity of the red channel ( $i = R$ ), cyan channel ( $i = C$ ), or yellow channel ( $i = Y$ ), and  $G_C(\tau)$ ,  $G_Y(\tau)$ ,  $G_R(\tau)$ , and  $G(i, j, \tau)$  denote the autocorrelation functions of cyan ( $i = j = C$ ), yellow ( $i = j = Y$ ), red ( $i = j = R$ ), and cross ( $i = R, j = C, Y$ ), respectively. The acquired  $G(i, j, \tau)$  values

were fitted with a one-component model as

$$G(\tau) = 1 + \frac{\left(1 - T + T \exp\left(\frac{-\tau}{\tau_{\text{triplet}}}\right)\right)}{N(1-T)} \times \left(1 + \frac{\tau}{\tau_D}\right)^{-1} \left(1 + s^2 \frac{\tau}{\tau_D}\right)^{-\frac{1}{2}} \quad (2)$$

where  $T$  and  $\tau_{\text{triplet}}$  indicate the average fraction and the lifetime of dye molecules in the triplet state, respectively.  $\tau_D$  is related to the translation diffusion constant  $D$  of the fluorescing species by  $\tau_D = w_0^2 / 4D$ .

$N$  is the average number of fluorescent particles in the excitation-detection volume defined by radius  $w_0$  and length  $2z_0$ , and  $s$  is the structural parameter representing the ratio  $s = z_0 / w_0$ . The average numbers of cyan fluorescent particles ( $N_C$ ), red fluorescent particles ( $N_R$ ), yellow fluorescent particles ( $N_Y$ ),

$$N_C = \frac{1}{G_C(0) - 1}, \quad N_R = \frac{1}{G_R(0) - 1}, \quad N_Y = \frac{1}{G_Y(0) - 1} \quad (3)$$

and particles that have both red and cyan fluorescence ( $N_{\text{cross}(CR)}$ ), or red and yellow fluorescence ( $N_{\text{cross}(RY)}$ ) can be calculated.

$$N_{\text{cross}(CR)} = \frac{G_{\text{cross}(CR)}(0) - 1}{(G_C(0) - 1)(G_R(0) - 1)}, \quad N_{\text{cross}(RY)} = \frac{G_{\text{cross}(RY)}(0) - 1}{(G_R(0) - 1)(G_Y(0) - 1)} \quad (4)$$

When  $N_R$ ,  $N_C$  and  $N_Y$  are constant,  $G_{\text{cross}}(0)$  is directly proportional to  $N_{\text{cross}}$ . For quantitative evaluation of cross-correlations among various samples, the relative cross-correlation amplitude (RCA) was calculated as

$$RCA = \frac{G_{cross}(0) - 1}{G_R(0) - 1} \quad (5)$$

Here, to subtract the background, the normalized RCA was also calculated as

$$NormalizedRCA = \frac{RCA_{time} - RCA_{Mix}}{RCA_0 - RCA_{Mix}} \quad (6)$$

where  $RCA_{time}$  and  $RCA_0$  indicate the RCA at the apoptosis induced at each time and 0 min, respectively.  $RCA_{Mix}$  denotes the RCA of mixture of ECFP and mCherry (or mixture of mCherry and Venus) as negative controls. Here, the  $RCA_{Mix}$  value reported previously was used for cell lysates [23].

## Results

TNF- $\alpha$ /CHX-induced time-dependent activation of caspases in HeLa cell lysates

In this study a fusion protein was constructed and named CRY chimera. As shown in Fig. 1A, the CRY chimera was formed by inserting DEVD and LEHD amino acids between ECFP and mCherry and mCherry and Venus, respectively. The CRY chimera-expressing HeLa cells are shown in Fig. 1B. DEVD sequences between ECFP and mCherry and LEHD sequences between

mCherry and Venus could be cleaved by the activated caspase-3 and caspase-9, respectively. The cross-correlation of CRY chimera was observed in fluorescent protein pairs ECFP and mCherry (C-DEVD-R) and mCherry and Venus (R-LEHD-Y) in HeLa cells (Fig. 1C and D). To evaluate the activation of caspase-3 and caspase-9, FCCS measurements were carried out in HeLa cell lysates. The CRY chimera-expressing HeLa cells were incubated with TNF- $\alpha$ /CHX for 0 min, 30 min, and 60 min, then lysed with lysis buffer. To subtract the background of various cell contents, the normalized RCA was calculated. Before addition of TNF- $\alpha$ /CHX, the highest amplitude was defined as activity of 1.0 and used as the control for cleavage. A decrease of the normalized RCA of C-DEVD-R was not observed at 30 min, but a significant decrease of the normalized RCA of C-DEVD-R was observed at 60 min after TNF- $\alpha$ /CHX treatment (Fig. 2A). On the other hand, significant decreases of the normalized RCA of R-LEHD-Y were already observed at 30 min and also 60 min after TNF- $\alpha$ /CHX treatment (Fig. 2B). These results indicate that caspase-9 could be activated earlier than caspase-3 after TNF- $\alpha$ /CHX treatment. In apoptotic cell lysates, there might be caspase-3, caspase-9, and other protease activities induced by TNF- $\alpha$ /CHX. To confirm the specificity

of caspase-3 for DEVD and caspase-9 for LEHD, inhibitor experiments were carried out. As shown in Fig. 2A, in the presence of caspase-3 inhibitor II (C3Inhi), the decreases of the normalized RCA of C-DEVD-R were not observed at 30 min and 60 min after TNF- $\alpha$ /CHX treatment. Interestingly, in the presence of caspase-9 inhibitor I (C9Inhi), only a small decrease of the normalized RCA of C-DEVD-R was observed at 60 min after TNF- $\alpha$ /CHX treatment (Fig. 2A). As shown in Fig. 2B, in the presence of C9Inhi, the decreases of the normalized RCA of R-LEHD-Y were not observed at 30 min and 60 min after TNF- $\alpha$ /CHX treatment. On the other hand, in the presence of C3Inhi, clear decreases of the normalized RCA of R-LEHD-Y were observed at 30 min and 60 min after TNF- $\alpha$ /CHX treatment. These results indicated that C3Inhi inhibited caspase-3 but not caspase-9. On the other hand, C9Inhi strongly inhibited caspase-9, but also inhibited caspase-3 indirectly, which suggested that the cascade from caspase-9 to caspase-3 was blocked.

Time-course FCCS measurement for detection of caspase-3 activation in single living cells

Next, we subjected the CRY chimera to time-course measurement using

FCCS. HeLa cells expressing the CRY chimera were analyzed every 5 min after TNF- $\alpha$ /CHX treatment (Fig. 3). For FCCS measurements, weakly fluorescent cells were selected to obtain sufficient auto- and cross-correlation amplitudes. To reduce the background effect of the live cell, the normalized RCA was also calculated. The cross-correlation signals of C-DEVD-R were detected in HeLa cells at the indicated times after TNF- $\alpha$ /CHX treatment (Fig. 3A). LSM images of C-DEVD-R were obtained at 0 min and 30 min after TNF- $\alpha$ /CHX treatment (Fig. 3B and 3C). Decreases of the normalized RCA of C-DEVD-R were observed in cells 1, 3, 4, and 5 (Fig. 3A), whereas apoptotic morphology was only observed in cells 4 and 5 at 30 min after TNF- $\alpha$ /CHX treatment (Fig. 3C). To identify whether the cells in which the normalized RCA decreased became apoptotic, long-term time-course FCCS measurement for detection of caspase-3 activation was carried out in single living cells. A decrease of the normalized RCA of C-DEVD-R was observed (Fig. 3D), but no change of cell morphology was observed in the first 90 min after TNF- $\alpha$ /CHX treatment (Fig. 3F). The apoptotic morphology appeared at 180 min after TNF- $\alpha$ /CHX treatment, and a very low value of the normalized RCA was obtained. These results demonstrated that a decrease in the normalized

RCA of C-DEVD-R showed the activation of caspase-3 and could predict progression of the cell to apoptosis before the morphologic changes appeared.

Time-course FCCS measurement for detection of caspase-9 activation in single living cells

Time-course FCCS measurement for detection of caspase-9 activation was also carried out in single living cells (Fig. 4). The cross-correlation signals of R-LEHD-Y were detected in HeLa cells at the indicated times after TNF- $\alpha$ /CHX treatment (Fig. 4A). LSM images of R-LEHD-Y were obtained at 0 min and 30 min after TNF- $\alpha$ /CHX treatment (Fig. 4B-E). Decreases of the normalized RCA of R-LEHD-Y were observed but apoptotic morphology was not observed in cells 3 and 4 at 30 min after TNF- $\alpha$ /CHX treatment. Here, long-term time-course FCCS measurement for detection of caspase-9 activation was also carried out in single living cells. Although no change of cell morphology was observed at 30 min after TNF- $\alpha$ /CHX treatment (Fig. 4H), a decrease of the normalized RCA of R-LEHD-Y was observed (Fig. 4F). A depressed value of the normalized RCA was observed when apoptotic cell morphology appeared at 240 min

after TNF- $\alpha$ /CHX treatment (Fig. 4I). Thus the decrease of the normalized RCA of R-LEHD-Y also could predict the progression of the cells to apoptosis before morphologic changes appeared. Moreover, the decrease of the normalized RCA of R-LEHD-Y started at least 15 min earlier than that of C-DEVD-R. These results suggested that caspase-9 was activated earlier than caspase-3 in apoptotic cells.

## **Discussion**

In this study, we successfully confirmed that cross-correlation measurement could clearly distinguish the difference between chimeral and unlinked fluorescent proteins. In the bulk measurement, the decreases of the normalized RCA of R-LEHD-Y were observed (Fig. 2B). On the other hand, no decrease of the normalized RCA of C-DEVD-R was observed at 30 min after TNF- $\alpha$ /CHX treatment. The results indicated that caspase-9 was activated earlier than caspase-3.

In single living cell analysis for detection of caspase-3 and caspase-9 activation (Fig. 3 and Fig. 4), we could clearly distinguish the difference between apoptotic cells and non-apoptotic cells in the early stage of apoptosis at 15 min by the cross-correlation amplitude of

R-LEHD-Y before the morphology of the cell changed. Therefore, we could judge the fate of cell by FCCS. Thus, the CRY chimera is a useful substrate for determination of two kinds of protease activities at exactly the same position in single cells by using triple-color FCCS. In the present work, it was difficult to carry out simultaneous FCCS analysis of caspase-3 and caspase-9 in single cells because our commercially available instruments had only two channels. In the future, development of an instrument that can analyze by multiple channels simultaneously will improve the time resolution for detection of caspase activities, and may clarify the initial step or the point of no return in the process of apoptosis. Moreover, by customizing the sequences of these linked amino acids, our method can be applied to simultaneously evaluate the activities of many other proteases. Using a three-color fluorescent protein substrate, we have demonstrated that the activation of caspase-3 and caspase-9 can be monitored *in vitro* and in living cells. Moreover, we found that caspase-9 was activated earlier than caspase-3 in apoptotic cells. This study provides a method to judge the early apoptotic stage before changes appear.

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## Figure legends

**Fig. 1.** Schematic diagram of CRY chimera and  $G(\tau)$  of CRY chimera-expressing HeLa cells.

(A) Encoded ECFP, mCherry and Venus fusion proteins (CRY chimera). DEVD and LEHD indicate caspase-3- and caspase-9-sensitive sequences, respectively. (B) LSM image of CRY chimera-expressing HeLa cells. Scale bars: 20  $\mu\text{m}$ . (C) and (D) show auto- and cross-correlations of C-DEVD-R and R-LEHD-Y, respectively. The blue, yellow, red and black curves represent  $G_C(\tau)$ ,  $G_Y(\tau)$ ,  $G_R(\tau)$  and  $G_{\text{cross}}(\tau)$ , respectively. Insets show the time course of fluorescence intensities in cyan, yellow, and red detection channels during FCCS measurement.

**Fig. 2.** TNF- $\alpha$ /CHX-induced time-dependent activation of caspases in HeLa cell lysates.

CRY chimera-expressing HeLa cells treated with TNF- $\alpha$ /CHX in the absence or presence of caspase-3 inhibitor II (C3Inhi) or caspase-9 inhibitor I (C9Inhi), were lysed and measured by FCCS. (A) and (B) show the normalized RCAs of C-DEVD-R and these of R-LEHD-Y at the indicated times after TNF- $\alpha$ /CHX treatment, respectively. Values are means  $\pm$  SD for four

measurements.  $*P < 0.05$ ,  $**P < 0.01$  as compared with the normalized RCA at 0 min after TNF- $\alpha$ /CHX treatment.

**Fig. 3.** Time-course FCCS measurement for detection of caspase-3 activation in single living cells.

(A) and (D) present the normalized RCAs of C-DEVD-R at the indicated times after TNF- $\alpha$ /CHX treatment. (B) and (C) show the LSM images at 0 min and 30 min, respectively, after TNF- $\alpha$ /CHX treatment shown in (A). Scale bars: 20  $\mu\text{m}$ . (E), (F) and (G) show the LSM images at 0 min, 90 min and 180 min, respectively, after TNF- $\alpha$ /CHX treatment shown in (D). Scale bars: 10  $\mu\text{m}$ .

**Fig. 4.** Time-course FCCS measurement for detection of caspase-9 activation in single living cells.

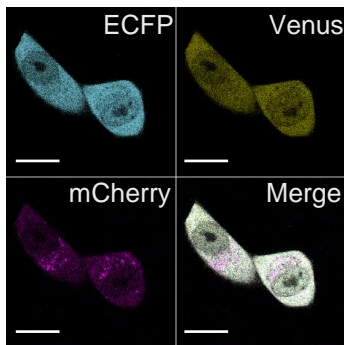
(A) and (F) present the normalized RCAs of R-LEHD-Y at the indicated times after TNF- $\alpha$ /CHX treatment. (B-E) and (G-I) show the LSM images in (A) and (F), respectively. (B), (D) and (G) were at 0 min, (C), (E) and (H) at 30 min, and (I) at 240 min after TNF- $\alpha$ /CHX treatment. Scale bars: 10  $\mu\text{m}$ .

# Figure

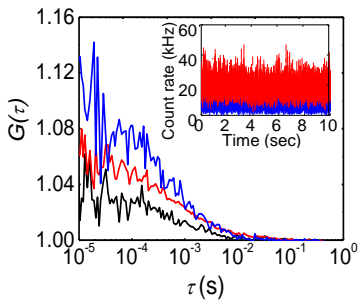
## A



## B



## C



## D

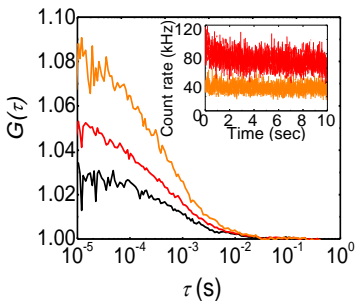


Fig. 1

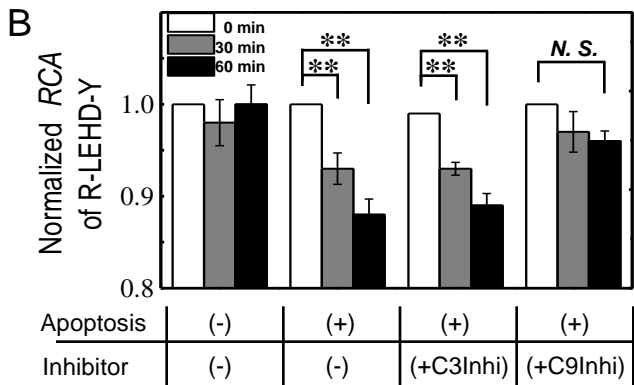
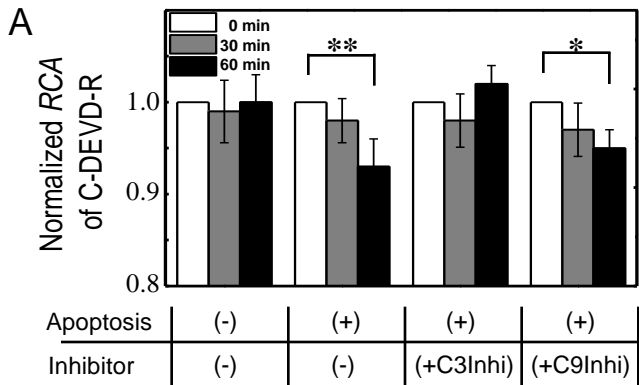


Fig. 2

Fig. 3

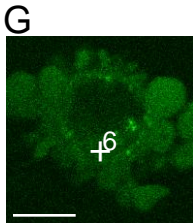
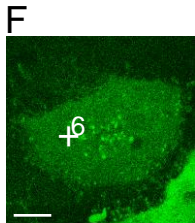
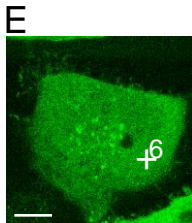
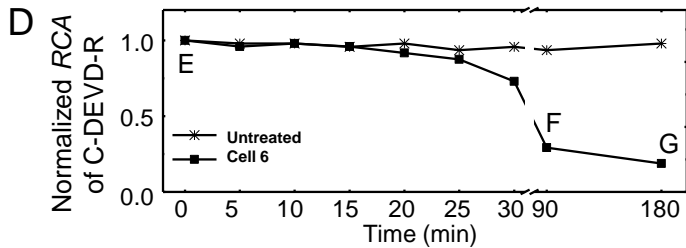
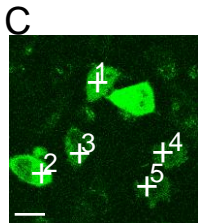
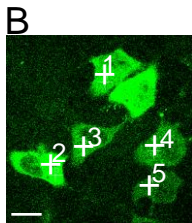
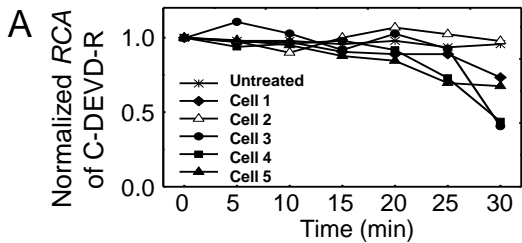


Fig. 4

