1363-D

Real-Time Image Analysis of Apoptotic to Necrotic Process in the Same Cells by Microscopy

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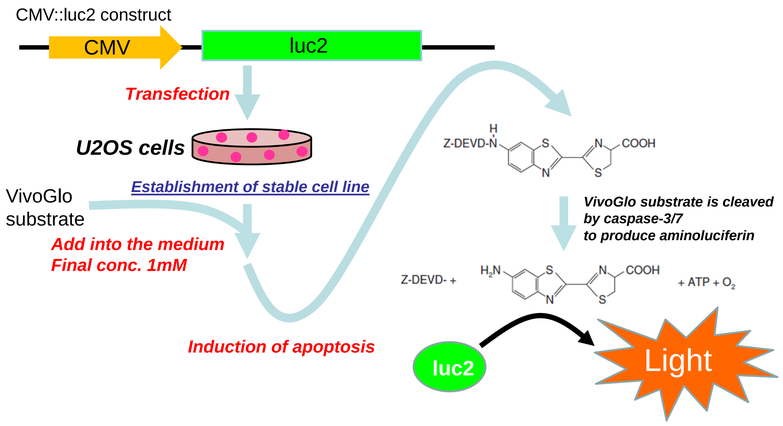
1. Introduction

Apoptosis is an indispensable process for normal tissue development and homeostasis, which allows cells to undergo timely programmed cell death. When apoptosis is induced, in most cases, caspase-3/7 is activated and the cell membrane is compromised by rearrangement of phospholipid. As activation of caspase-3/7 always leads to apoptotic death of the cells, the caspase activity is considered to be a reliable apoptosis marker. In addition, externalization of phosphatidylserine (PS) in the cell membrane is also a typical apoptosis marker. Therefore, the two markers have been assessed by enzymatic and flow cytometric endpoint assays. However, major disadvantages of these assays are (1) it obtains only a single result for each set of endpoints, and therefore (2) it is impossible for real-time measurement of live cells. In order to overcome these disadvantages, we combined bioluminescence and fluorescence microscopy and tried to detect the apoptotic to necrotic process on the same live cell samples.

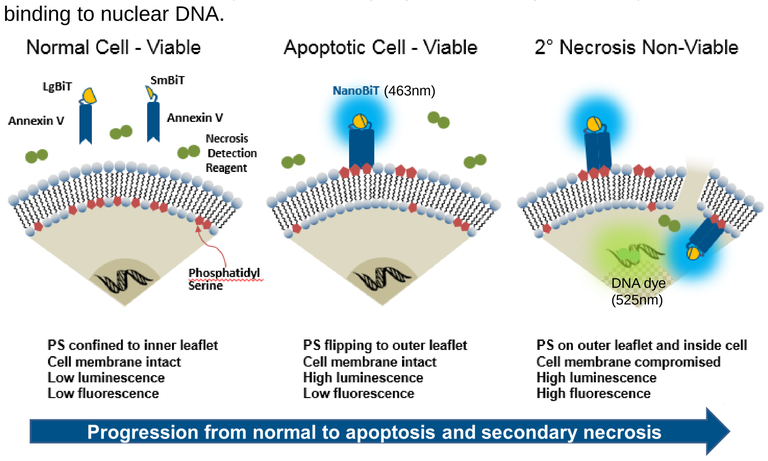
2. Real-Time Live Cell Detection of Caspase Activity

We created a stable U2OS cell line that expresses Luc2 luciferase (Promega). To detect caspase-3/7 activity, the aminoluciferin incorporating the DEVD (Asp-Glu-Val-Asp) motif recognized by the caspase-3/7 (VivoGlo™ Caspase-3/7 substrate, Promega) was added into the culture medium. When caspase-3/7 is activated, liberated luciferin reacts with Luc2 and generates bioluminescent light (609nm).



3. Real-Time Detection of Apoptosis using Annexin V Enzyme Complementation Assay

To detect externalization of PS and secondary necrosis (late apoptosis), we applied RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay Kit (Promega corp.) to the U2OS stable cell line in usage as prescribed in the kit protocol. This kit contains equal ratios of two annexin V molecules expressed as fusions with large or small subunits of NanoBiT luciferase (Promega corp.) and time-released substrate. When PS is externalized on the cell membrane, annexin V binds to PS as a dimmer on the cell surface and NanoBiT Luciferase is reconstructed, and bioluminescence light (λmax=463 nm) is generated. This kit also contains a cell-impermeant, profluorescent DNA dye, which detects necrosis by fluorescent light (λmax=525nm) with the dye binding to nuclear DNA.



4. Real-Time Imaging of Apoptotic to Necrotic Process in the Same Cells by Microscopy

We observed apoptosis and necrosis of the U2OS cells by sequential bioluminescence and fluorescence imaging using a microscope, LV200 (OLYMPUS) after induction of apoptosis by 100nM staurosporine (STS).

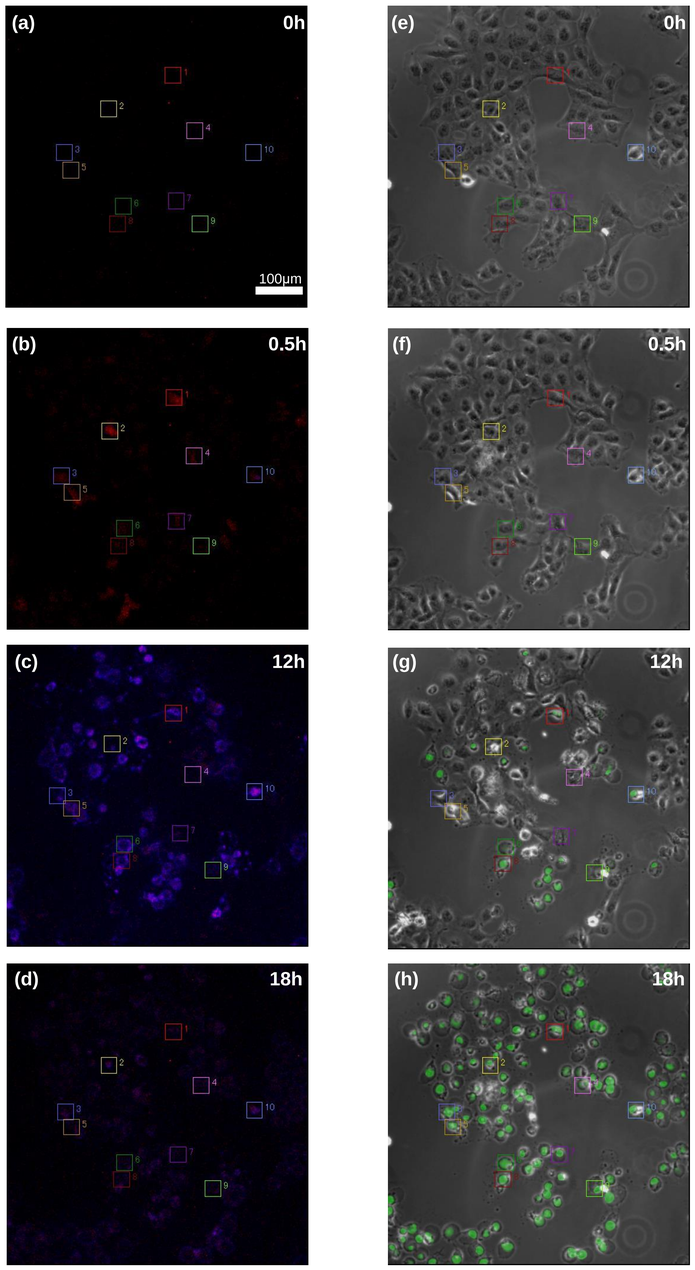


Fig. 1 (a)-(d) Bioluminescence images of caspase-3/7 activity (red) and annexin V dimmerization (blue) indicating apoptotic process from 0 to 18h.
(e)-(h) Fluorescence images of nucleus (green) indicating necrosis merged with phase contrast image from 0 to 18h.

0h: (a) No bioluminescence signals.
(e) No morphological changes of the cells.

0.5h: (b) Caspase-3/7 activity increased as apoptotic initiation.
(f) Cells were shrunk slightly.

12h: (c) Annexin V-NanoBiT dimmerization occurred.
(g) Nucleus of penetrated cells were stained as 2nd necrosis.

18h: (d) Annexin V dimmerization signal decreased as cell membrane compromise.
(h) Nucleus of all cells were stained as 2nd necrosis.

Measurement conditions:
The U2OS stable cells were seeded on a 35mm glass bottom dish. Luminescence images were acquired by the luminescence imaging system LUMINOVIEW (LV200, Olympus) attached with an electron multiplier charge-coupled device camera (ImagEM, Hamamatsu Photonics) binning 1x1 and EM-gain 1200.
Each images were taken by 40x phase contrast objective lens [numerical aperture (NA) 0.75] at 100msec exposure (Phase contrast), 100msec exposure (fluorescence, Ex: BP490-500, Em: BP515-560), 3min exposure (Annexin V-NanoBiT, BP460-510 filter), 5min exposure (luc2-caspase-3/7, 610ALP filter), 10min interval. Duration time of observation was 24 hours.
The dish was kept at 37ºC under 5% CO2 in the humidified chamber during observation.

5. Time-Course Analysis of Apoptotic to Necrotic Process

Fluorescence and bioluminescence signals from single cells were measured as an average value in a region of interest (ROI) enclosed for each cells of Fig.1 by Time-lapse Imaging Analysis software (TiLIA, Olympus). Similarly, signals in the whole visual field were measured as a plate reader-like analysis.

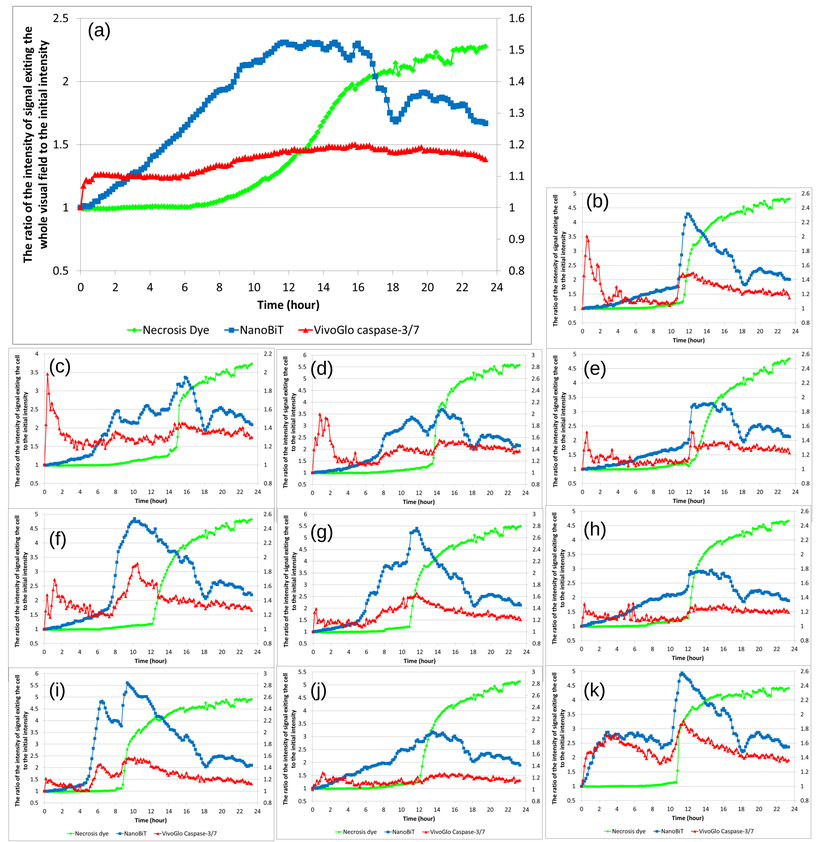


Fig. 2 (a) Time-course analysis of bioluminescence and fluorescence signals in the whole area of the image (from total cells). Necrosis Dye and NanoBiT signals were plotted in the primary axis, VivoGlo signals were shown in the secondary axis. Caspase-3/7 activity reached the first peak within 1 h after STS addition. Annexin V dimmerization signal rose in 1 h and reached the maximum in 12 h, and then decreased. Secondary necrosis signal rose in 7 h and increased for 24 h.

(b)-(k) Time-course analysis for each cell in Fig. 1 (ROI 1 to 10). Caspase-3/7 activity reached the first peak within 1 h, and annexin V dimmerization signal rose in 1 h as same as total cells analysis (a). However, signal profile of annexin V dimmerization varied among cells and the maximum peak time from 9 to 14 h. Secondary necrosis signal rose after the maximum peak time of annexin V dimmerization.

6. Conclusions

- Our imaging method makes possible real-time analysis of the apoptotic to necrotic process by visualizing the same cell samples using VivoGlo™ Caspase-3/7 Assay and RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay.
- This method revealed heterogeneous responses of apoptotic to necrotic process in individual cells, and that suggests importance of single cell analysis.
- However, this result does not conflict with total cell analysis, and that would also strengthen plate-reader based screening results. Namely, before and after screening, reliability of the assay conditions and efficacy of the candidates can be confirmed by image analysis.

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