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Real-Time High Throughput Detection of Annexin V Binding and Caspase-3 Activity Using a Plate Reader

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Abstract

1. Introduction

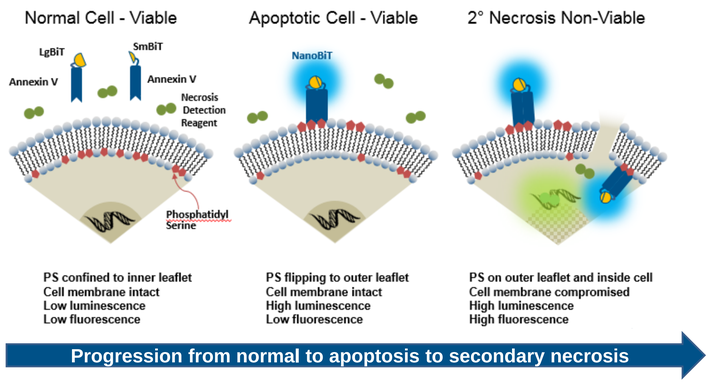
We developed two homogeneous real-time assays for detecting apoptosis that can be recorded using a plate-reading luminometer. The first assay is based on binding of annexin V to phosphatidyl serine (PS) which becomes exposed on the outer leaflet of the cell membrane during apoptosis. We engineered two fusion proteins composed of annexin V linked to a small or large subunit of NanoBiT® luciferase. When the fusion proteins bind in close proximity on the surface of apoptotic cells, the reconstituted NanoBiT generates light to report apoptosis in real-time. Multiplexing with a DNA binding dye reports 2° necrosis in real-time from the same sample.

The second approach relies on expression of a modified firefly luciferase (GloSensor™ Caspase-3/7 Biosensor) containing the DEVD amino acid sequence that is cleaved by caspase-3. GloSensor is inactive in viable cells. Upon induction of apoptosis, caspase-3 cleavage of the DEVD sequence enables GloSensor to fold into an active conformation and generate a luminescence in real-time as the population of cells undergoes apoptosis.

These real-time homogeneous apoptosis assay methods represent an improvement over endpoint assay methods by providing kinetic data from the same sample of live cells in real time using a standard plate reading luminometer.

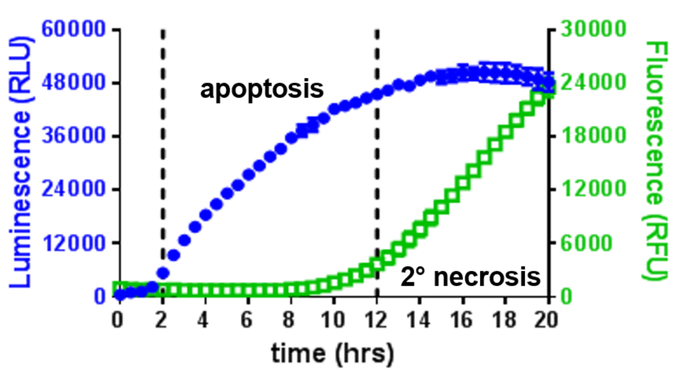
2. Real-Time Detection of Apoptosis using Annexin V Enzyme Complementation Assay

The RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay is a homogeneous method to detect the kinetics of phosphatidylserine (PS) exposure as a marker of apoptosis and DNA staining to detect necrosis.



3. Real-Time Detection of Apoptosis and Necrosis

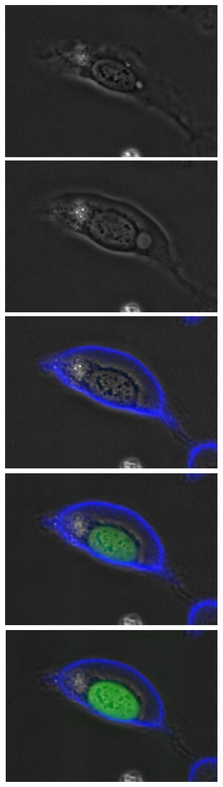
Multiplexing real-time apoptosis (annexin V binding to PS) and secondary necrosis (DNA staining dead cells) assays from the same sample of cells.



The RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay reagent was added once at time zero to DLD-1 cells treated with rhTRAIL. Luminescence from annexin V binding indicating apoptosis (blue) and fluorescence from DNA staining indicating necrosis (green) were recorded repeatedly from the same samples.

4. Real-Time Imaging of Luminescent Annexin V Binding and Fluorescent DNA Dye Staining

U2OS cells were cultured in presence of Apoptosis and Necrosis Assay reagent, exposed to 1 µM staurosporine and photographed at indicated times using fluorescent and luminescent modes with an Olympus LV200 microscope. Data provided by Olympus.



1 hr after staurosporine exposure. No staining.

3:45 hr. Note slight cell shape change.

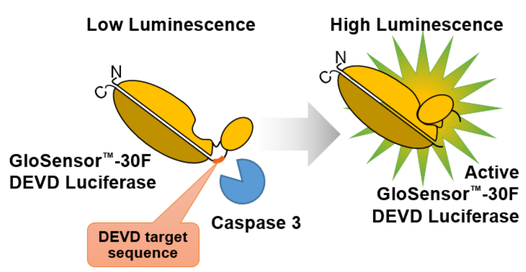
6 hr. Cell membrane is decorated with Annexin V fusion proteins enabling reconstitution of luciferase to generate light indicating apoptosis.

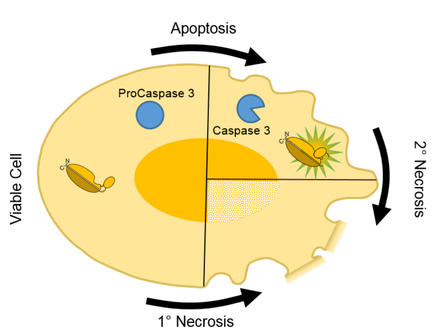
9 hr. Fluorogenic DNA dye has penetrated cell membrane to stain nucleus green indicating 2° necrosis.

12 hr. More intense staining of nucleus with DNA dye. Luminescent signal remains on outer membrane.

5. Real-Time Live Cell Detection of Caspase Activity

The GloSensor™ luciferase contains DEVD sequence that restricts the conformation of the molecule in an inactive confirmation. Caspase cleavage at DEVD activates luciferase to generate a real-time luminescent signal.

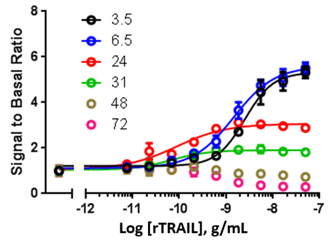




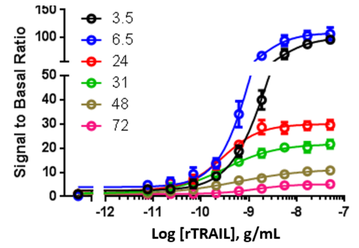
6. Comparison of Real-Time GloSensor™ 30F and Endpoint Caspase-Glo® 3/7 Assays

A plate containing U2OS cells was forward transduced for 18 hr with BacMam GloSensor™ 30F Caspase particles at a MOI of 60, then exposed to serial dilutions of rhTRAIL prepared in medium containing luciferin. Luminescence was recorded from the GloSensor™ plate at indicated times. Six parallel plates were prepared with U20S cells treated with the same rhTRAIL concentrations. Caspase-Glo® 3/7 Reagent (endpoint lytic assay) was added to one of the six parallel plates at the indicated times (3.5-72hr) and luminescence recorded.

Caspase-Glo® 3/7 Endpoint Assay

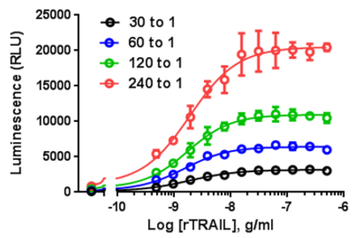


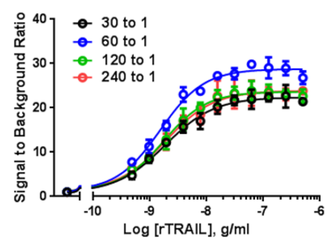
GloSensor™ Real-Time Assay



7. Performance of GloSensor™ Caspase Assay Delivered at Different MOI using BacMam

U20S were forward transduced by the addition of BacMam GloSensor™ 30F Caspase particles at ratios of 30, 60, 120 and 240 particles per cell (multiplicity of infection, MOI) for 16 h. Serial dilutions of recombinant human TRAIL were added to replicate wells to induce an apoptotic response during an 18 h exposure. Bright-Glo™ lytic reagent was added to reveal the magnitude of GloSensor™ 30F activation for each MOI.





8. Conclusions

- The RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay detects the kinetics of apoptosis and secondary necrosis in real time using a plate reader.
- The assay requires one homogenous reagent addition step and no other washes or processing steps.
- The non-lytic assay reagents enable multiplexing with other assay chemistries such as endpoint methods to measure caspase-3/7 activity as an orthogonal marker of apoptosis.
- The RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay enables luminescent time lapse imaging of cells undergoing apoptosis to illustrate heterogeneity among the population of cells and the “real-time” kinetics of apoptosis.
- The GloSensor™ Caspase-3/7 Biosensor containing the DEVD sequence is expressed in cells in an inactive form.
- During apoptosis, caspase-3/7 cleavage at the DEVD sequence enables folding of GloSensor™ luciferase to form an active enzyme to generate light and detection of apoptosis in real-time.
- Stable expression of GloSensor™ luciferase or BacMam delivery of plasmid can be used to generate cells for this real-time caspase assay.

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