A Real-Time, Bioluminescent Annexin V Assay for the Assessment of Apoptosis

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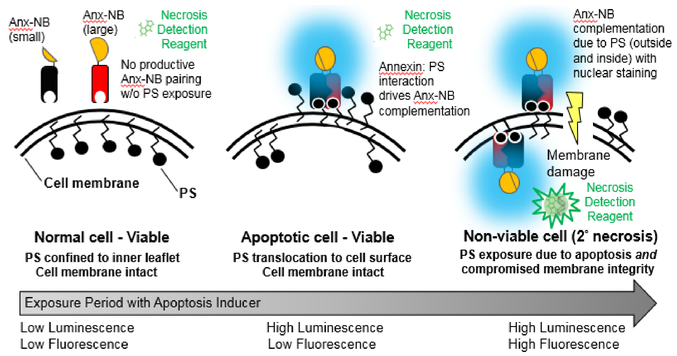
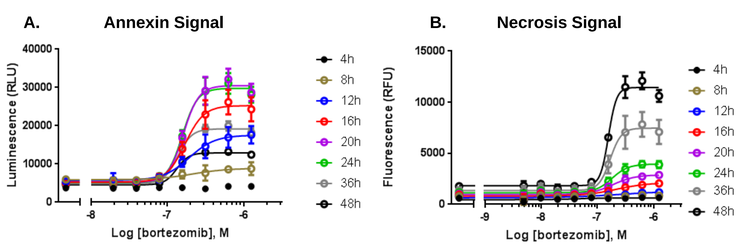
1. Introduction

We have developed a real-time, live cell assay method that utilizes a fully homogeneous, bioluminescent annexin V reagent. The method obviates laborious washing and sample preparation steps associated with traditional annexin methods and is fully compatible with either plate-based multimodal signal detection or bioluminescent imaging systems. The reagent contains two annexin proteins which have been engineered to contain separate and distinct complementing domains of a binary luciferase (NanoBiT). Additionally, the reagent contains a novel time-released luciferase substrate and a cell impermeable, fluorogenic DNA dye for monitoring cell membrane integrity. Because the annexin-luciferase fusion pairs have only modest affinity for each other, luminescence remains low until phosphatidylserine exposure, a hallmark of the apoptotic phenotype, brings annexin monomers into close proximity with each other permitting complementation of the NanoBiT sensor. The reagent can be applied at dosing for real-time measurement of the dose-dependency and magnitude of apoptotic progression. Here we demonstrate the robustness and scalability of the reagent with kinetically divergent inducers of apoptosis in real-time and in repeated, scheduled intervals by the qHTS method in a high density microplate format. Additionally, we examine the concordance of the annexin-based assay data with caspase activation data collected over a 48hr time course using an endpoint method. Last, we provide an example of the utility of the reagent for analysis of apoptotic events at the single cell level by bioluminescence imaging.

2. Bioluminescent Annexin Assay Principle



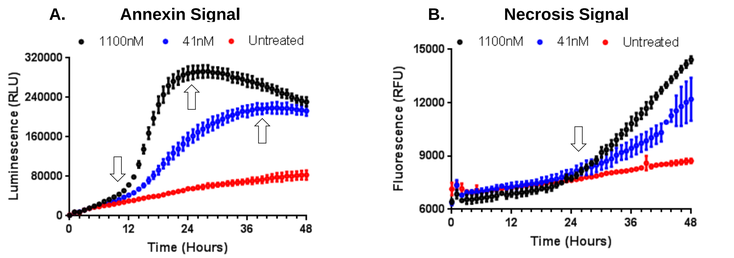
3. Real-Time Potency and Response Magnitude(s)



K562 (2000 cells/well) were dosed with serial dilutions of bortezomib in the presence of the RealTime-Glo™ Annexin V Apoptosis reagent in a 384 well format (qHTS). Luminescence and fluorescence were measured every 15 minutes for 48h (all data not shown) using a BMG CLARIOstar™ equipped with an atmospheric control unit (ACU).

Panel A. Dose- and time-dependent increases in PS exposure due to apoptosis. Note kinetic divergence in apoptotic response vs. paclitaxel (Pane 7, Panel B). **Panel B.** Dose- and time-dependent losses in membrane integrity occur as a result of the progression of apoptosis.

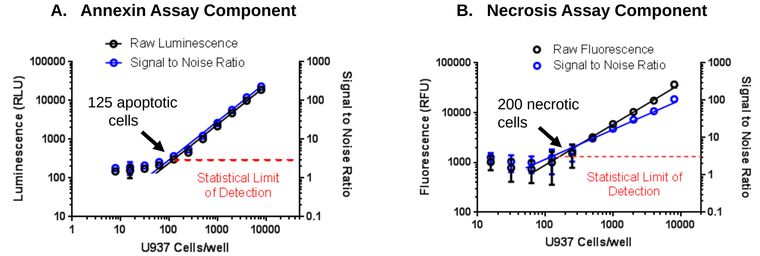
4. Temporal Resolution of Dose-Dependency



K562 cells were dosed with bortezomib in the presence of the RealTime-Glo™ Annexin V Apoptosis reagent and luminescence and fluorescence measured every hour for 48h using a BMG CLARIOstar™ equipped with an ACU.

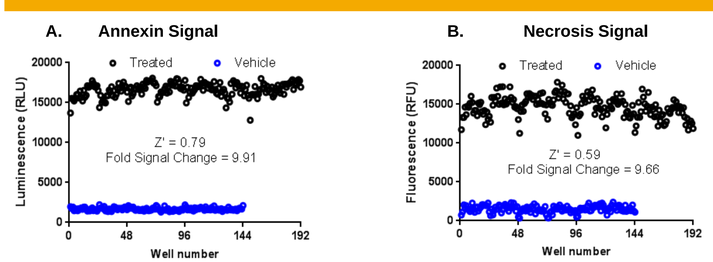
Panel A. PS exposure resulting from the induction of apoptosis begins after 10h exposure in both doses of test agent, but the maximal effect is observed at 24h at the high dose and ~40h with the low dose. **Panel B.** Changes in membrane integrity lag behind the annexin signal and begin at 26h. The necrotic magnitude mirrors the apoptotic magnitude.

5. Practical Sensitivities of Assay Measures



Panel A. U937 were serially diluted from 8000 cells/well and treated with 5µm bortezomib (a concentration known to induce near 100% apoptosis in 24h) in the presence of the RealTime-Glo™ Annexin V Apoptosis reagent. Luminescence was collected at 24h. **Panel B.** U937 were serially diluted from 8000 cells/well in the presence of the Necrosis Detection Reagent and treated with 30µg/ml digitonin to create necrotic cells. Fluorescence was collected after 15min.

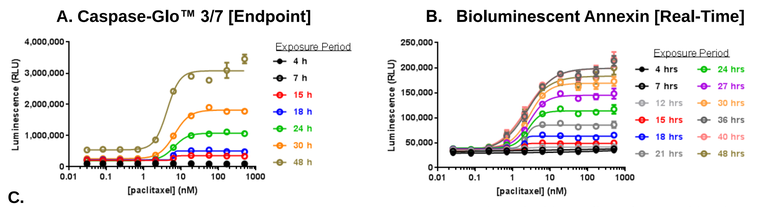
6. High Throughput Fitness: Z' Values



K562 cells were dispensed into a 384 well plate at a density of 2000 cells/well with a ThermoFisher MultiDrop™ Combi nL and dosed with either 2µM bortezomib or vehicle control in the presence of RealTime-Glo™ Annexin V Apoptosis reagent. Luminescence and fluorescence measurements were collected with a Tecan Spark® 20M.

Panel A. The bioluminescent annexin component produced a Z' of 0.79 after 24h of incubation with bortezomib. This Z' and fold signal change was largely maintained at 48h (data not shown). **Panel B.** The necrosis detection component produced a Z' of 0.59 at 24h and improved to 0.69 at 48h as cells progressed from apoptosis to secondary necrosis (data not shown).

7. Caspase Activity vs. Annexin Method



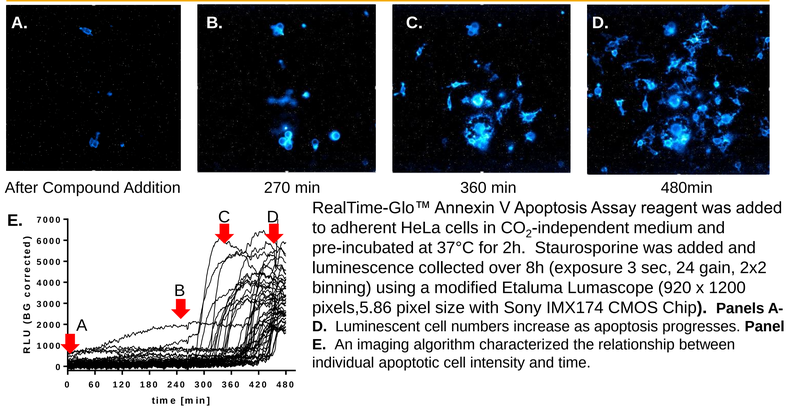
C.

Exposure	Caspase EC_{50}	Annexin EC_{50}
15h	~ 6nM	~ 2nM
18h	~ 6nM	~ 2nM
24h	6.5nM	2.5nM
30h	6.6nM	2.7nM
48h	4.2nM	1.9nM

HepG2 were dosed with paclitaxel. Note kinetic divergence in apoptotic induction kinetics vs. bortezomib (Pane 3).

Panel A. Caspase 3/7 activity data were collected in parallel plates at various time points using the Caspase-Glo™ 3/7 Assay in endpoint format using seven separate plates. **Panel B**. Bioluminescent annexin data were collected in real-time using one plate with one addition. **Panel C**. Caspase activity and bioluminescence annexin EC_{50} values are concordant.

8. Bioluminescence Imaging



RealTime-Glo™ Annexin V Apoptosis Assay reagent was added to adherent HeLa cells in CO_2-independent medium and pre-incubated at 37°C for 2h. Staurosporine was added and luminescence collected over 8h (exposure 3 sec, 24 gain, 2x2 binning) using a modified Etaluma Lumascope (920 x 1200 pixels,5.86 pixel size with Sony IMX174 CMOS Chip). **Panels A-D.** Luminescent cell numbers increase as apoptosis progresses. **Panel E.** An imaging algorithm characterized the relationship between individual apoptotic cell intensity and time.

9. Conclusions

The RealTime-Glo™ Annexin V Apoptosis Assay:

- **Is fully homogeneous**
  - Utilizes the "Add-Mix-Measure" format
  - No washes or sample preparation is required
- **Can be employed in real-time for up to 48h**
  - Defines apoptosis induction kinetics and magnitude of response
  - Defines the kinetics of cell death as a result of the apoptotic program
- **Is scalable for high throughput formats**
  - Is suitably sensitive and robust for high density formats
  - Produces excellent Z' values in 384-well format
- **Produces data comparable to orthogonal, endpoint methods**
  - Excellent concordance with an endpoint caspase activity method
- **Exhibits utility for bioluminescent imaging**
  - Links changes in morphology to the apoptotic phenotype
  - Allows for analysis of apoptosis at the single cell level

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