New Techniques for Continuous Real Time Monitoring of Viability and Cytotoxicity & Multiplexing with Other Assays

SLAS Tutorial
February 10, 2015

Promega Corporation

Abstract

New homogeneous multi-well plate assay technologies have been developed for repeated “real time” measurement of live and dead cells for three days in culture. NanoLuc® luciferase is used to detect conversion of a pro-substrate into a substrate for luciferase only by viable cells. Dead cells are measured using a new fluorogenic DNA binding dye that only stains cells with a compromised membrane. Both reagents are non-lytic and non-toxic leaving cells available for secondary multiplexed assays.

Presentation Outline

What's wrong with existing assay technologies?

Measuring viable cell number in real time

- How the assay works
- Multiplexing
- Advantages & Disadvantages

Measuring accumulation of dead cells in real time

- How the assay works
- Multiplexing
- Advantages & Disadvantages

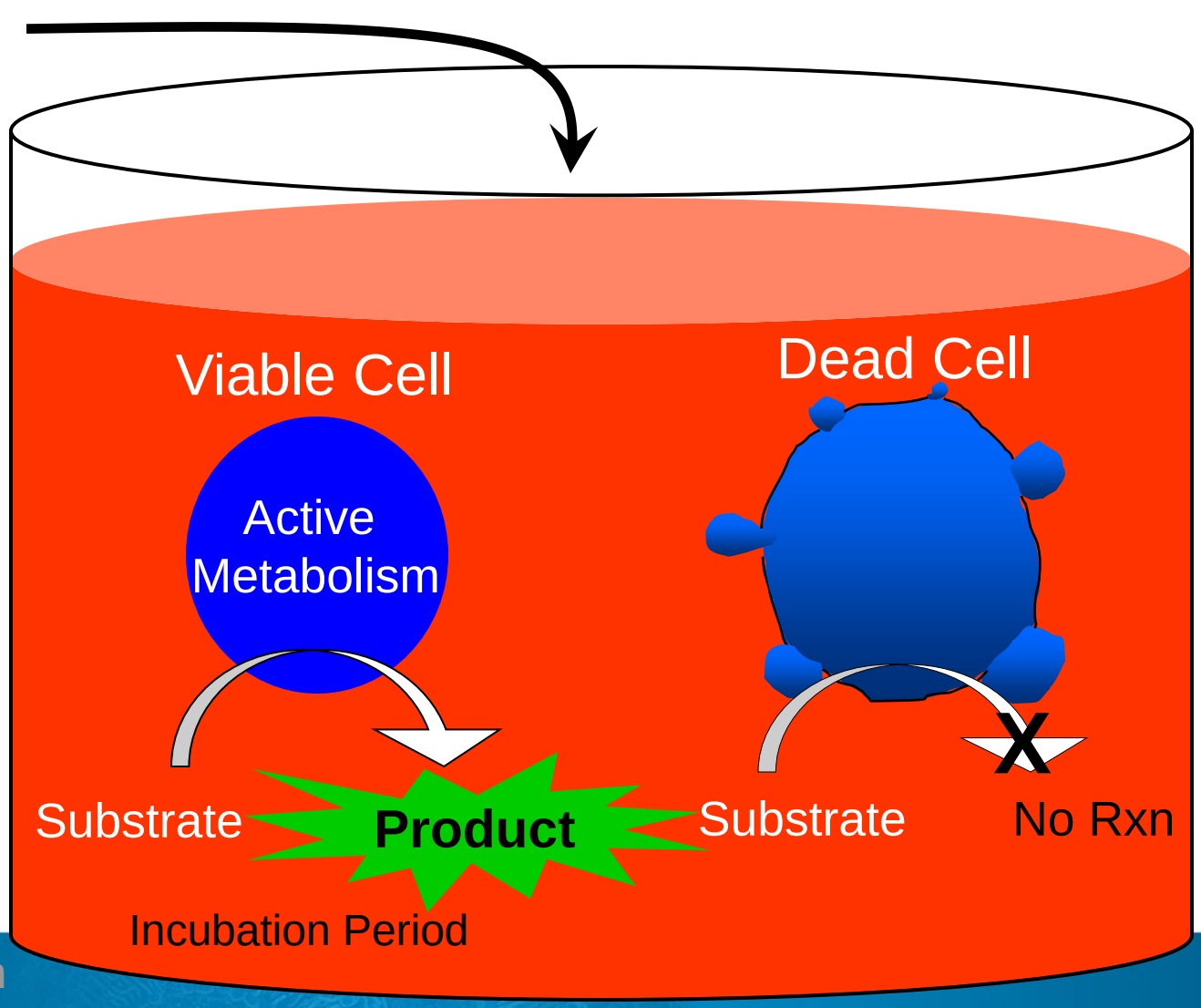
Summary

Metabolic Indicators of Cell Viability

Tetrazolium Reagents
- **MTT, MTS, XTT**

Redox Indicators
- **Resazurin**

Enzyme Substrates
- **Protease Substrates**



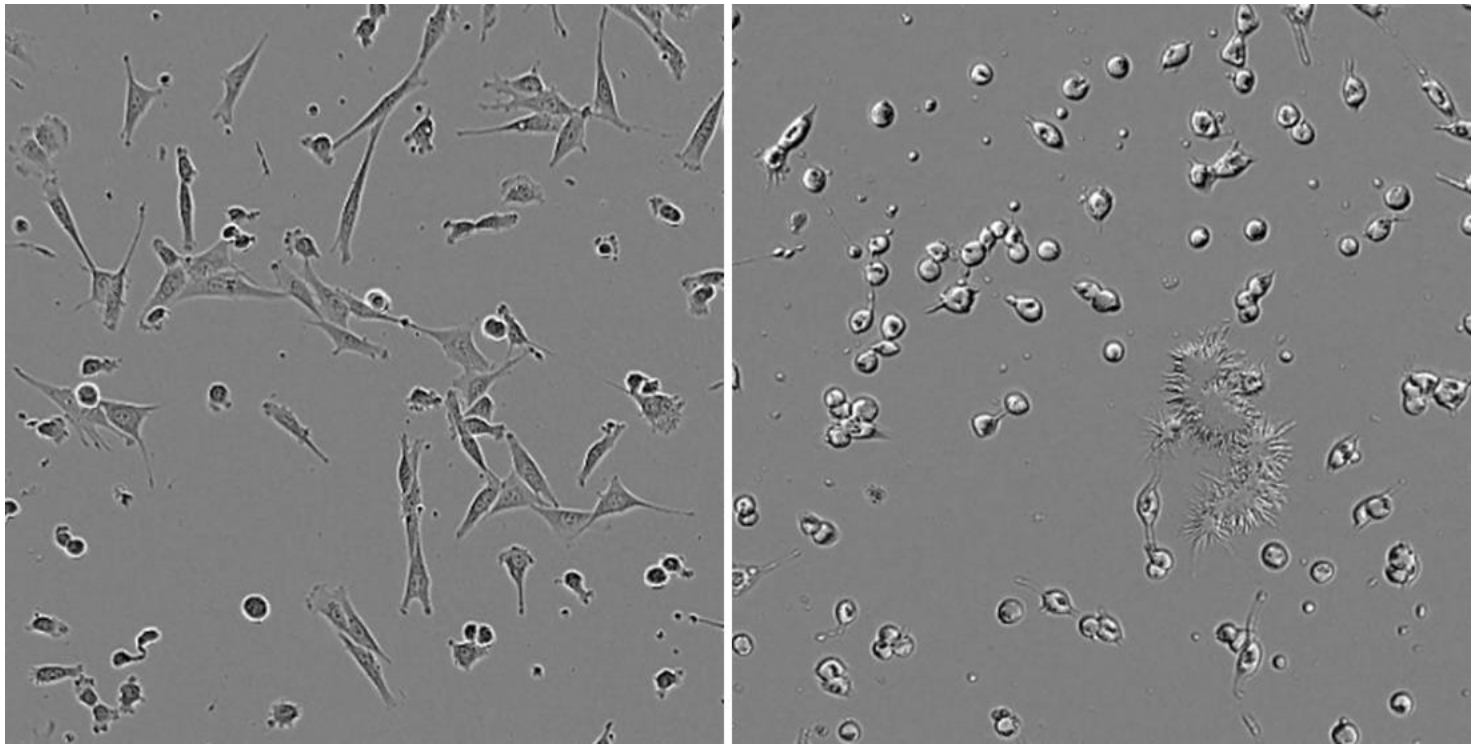
Why Not Use MTT or Resazurin Assays?

- MTT and resazurin assays have been used for some 3D culture models (e.g. MTT used in MatTek's multilayered keratinocyte EpiDerm™ System)
- Little information is available on penetration of reagents
- MTT and resazurin have been observed to be toxic to cells (Assay Guidance Manual: http://www.ncbi.nlm.nih.gov/books/NBK144065/)
- Sensitivity of MTT assay limits signal to background ratio
- Interfering compounds https://www.promega.com/resources/pubhub/is-your-mtt-assay-really-the-best-choice/

Balb 3T3 Cells Treated with MTT for 4 Hours

Time Zero

4 hours

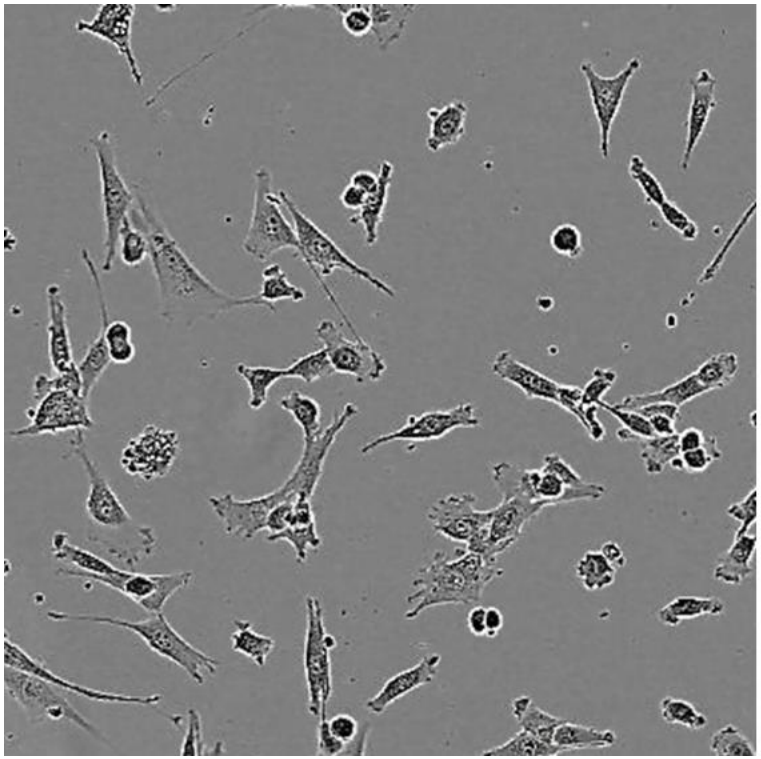


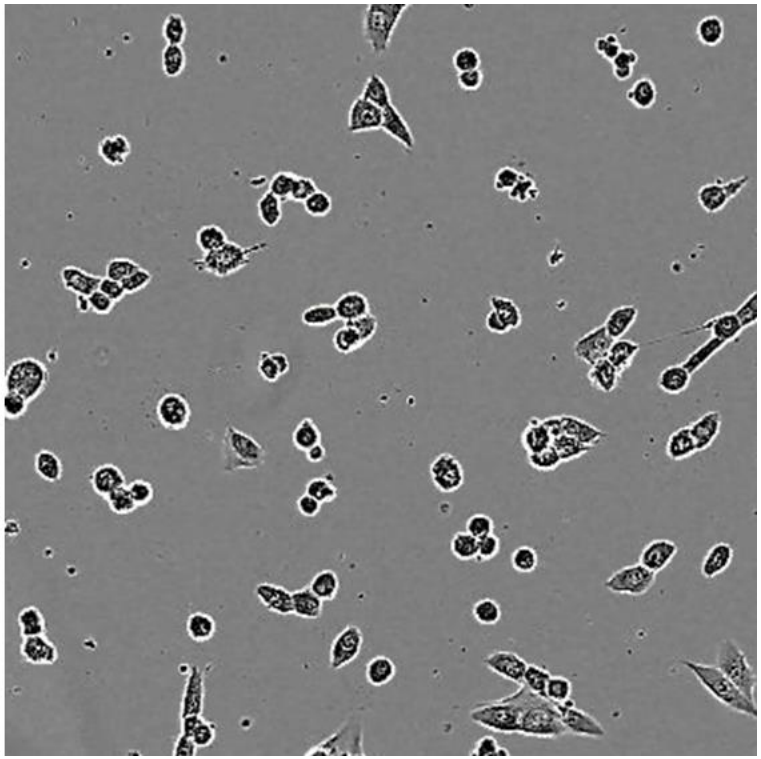
Images captured by Tracy Worzella using Incucyte instrument from Essen Biosciences
Assay Guidance Manual: http://www.ncbi.nlm.nih.gov/books/NBK144065/

Balb 3T3 Cells Treated with Resazurin for 4 Hours

Time Zero

4 hours





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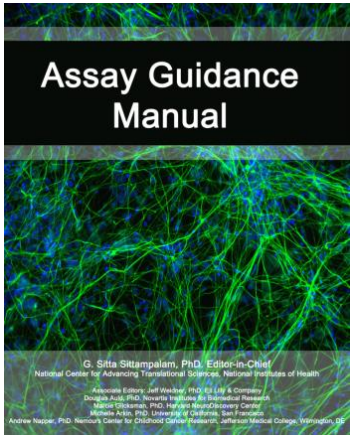
The Assay Guidance Manual e-Book



Originally Developed as Quantitative Biology Manual for HTS and Lead Optimization at Eli Lilly & Co.

- **A significant public resource for the entire Drug Discovery community.**
- **Represents the "tribal knowledge" of over 100 scientists at Lilly.**
- **Current version is edited by ~ 15 experts from Pharma, Academia and Life science companies.**
- **e-Book contributions are sought from international experts .**
- **e-Book is easily updated.**
- **Fits with NCATS Mission.**

www.ncbi.nlm.nih.gov/books/NBK53196/

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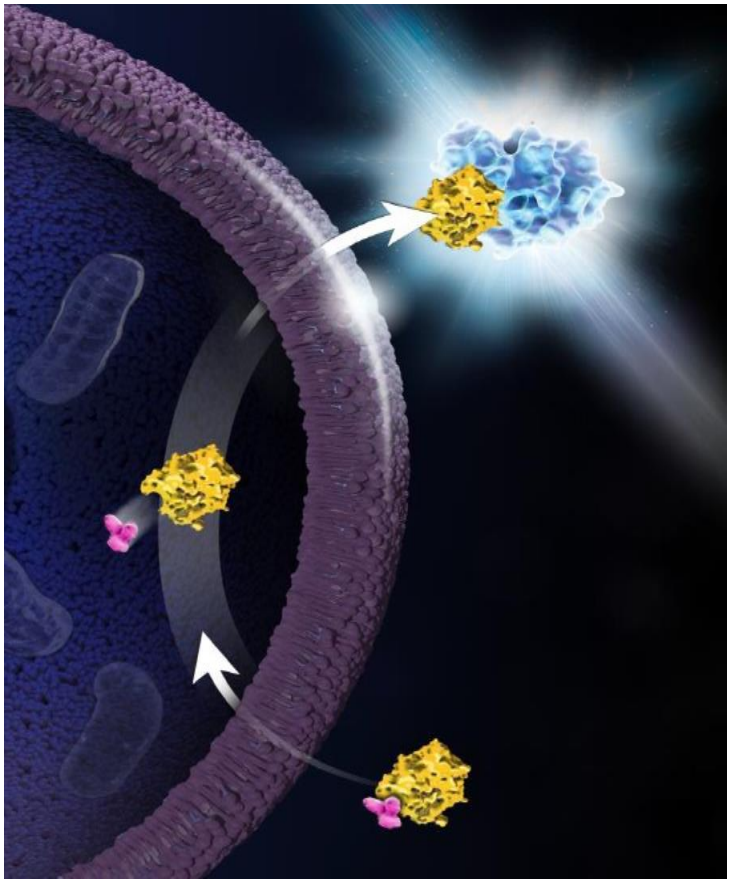
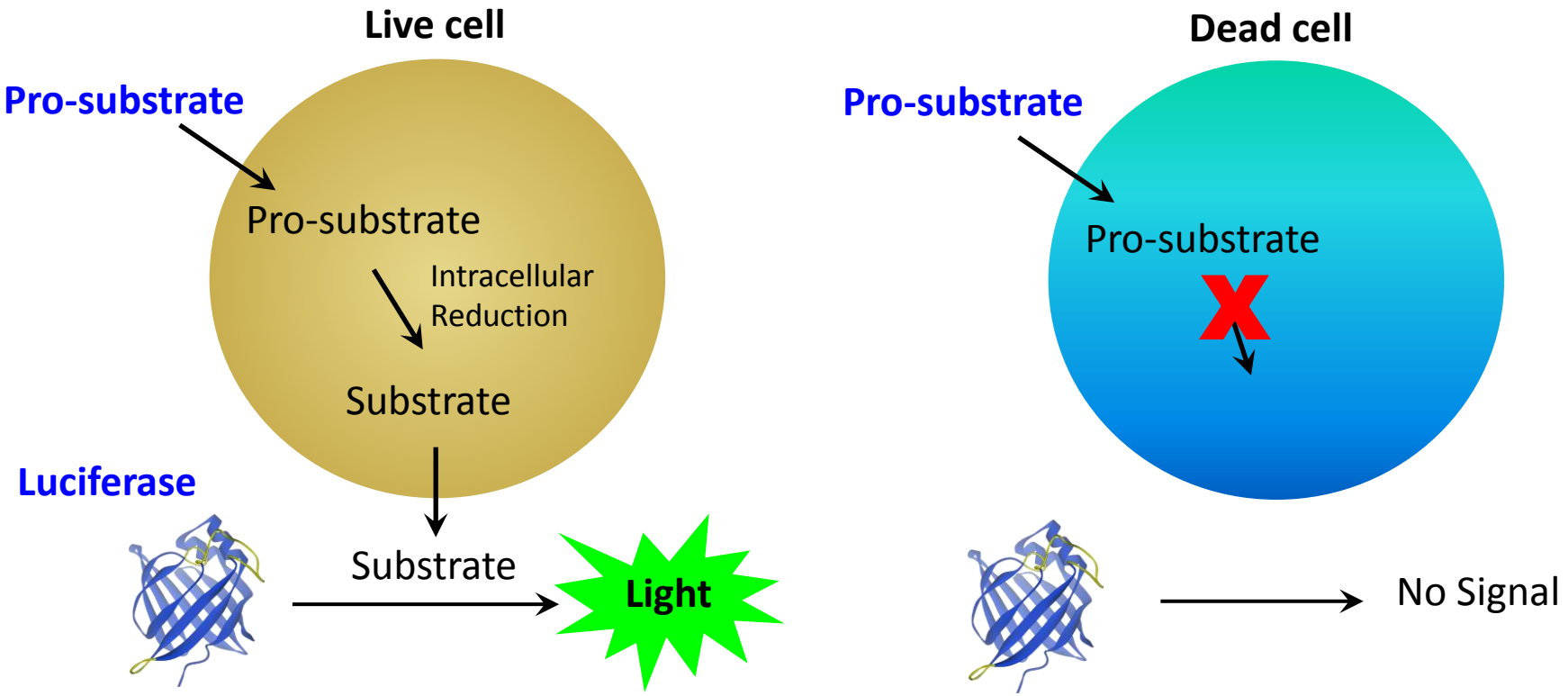
“Real Time” Cell Viability Assay

RealTime-Glo™ MT Cell Viability Assay

- Monitor viable cells continuously over 72 hours, saving time, cell samples, culture and reagent costs
- Option to add reagent at seeding, dosing of compound, or at the end
- Sensitivity is greater than colorimetric or fluorometric cell viability assays
- Multiplex with other assays and downstream applications



RealTime-Glo™ MT Cell Viability Assay Measures Reducing Potential of Cells



- **Luciferase** and **Pro-substrate** are added as reagents to culture medium
- Pro-substrate enters the cell and is reduced to form a substrate for luciferase
- Substrate diffuses from the cell and is used by luciferasse to produce light

RealTime-Glo™ Assay Protocol

Seed cells in medium containing RealTime-Glo™ Reagent

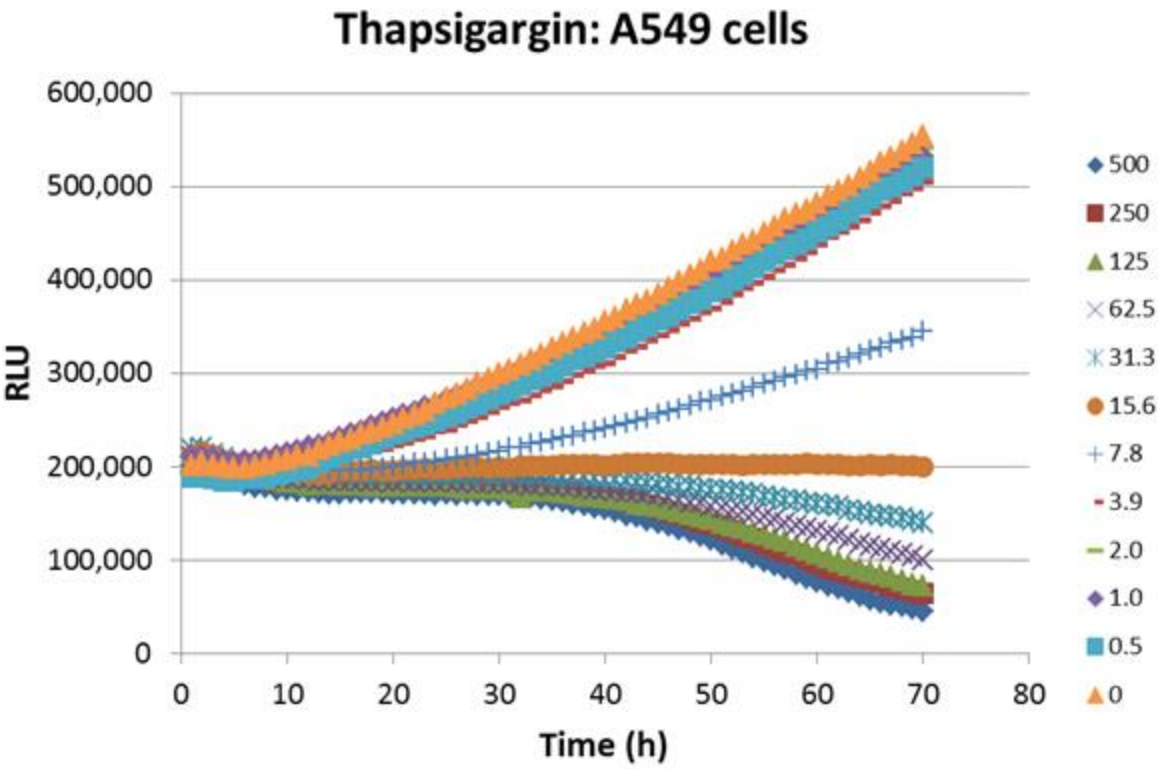
↓

Add test compound

↓

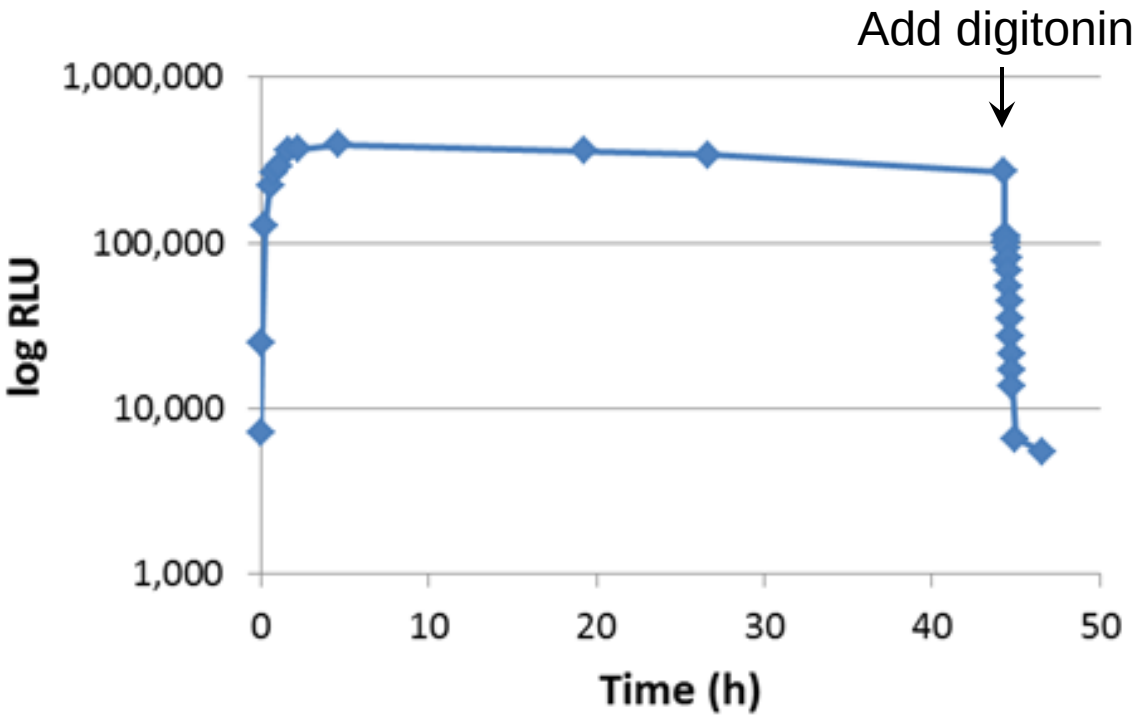
Record luminescence (continually for up to 3 days)

Measure Changes in Viability Over Time



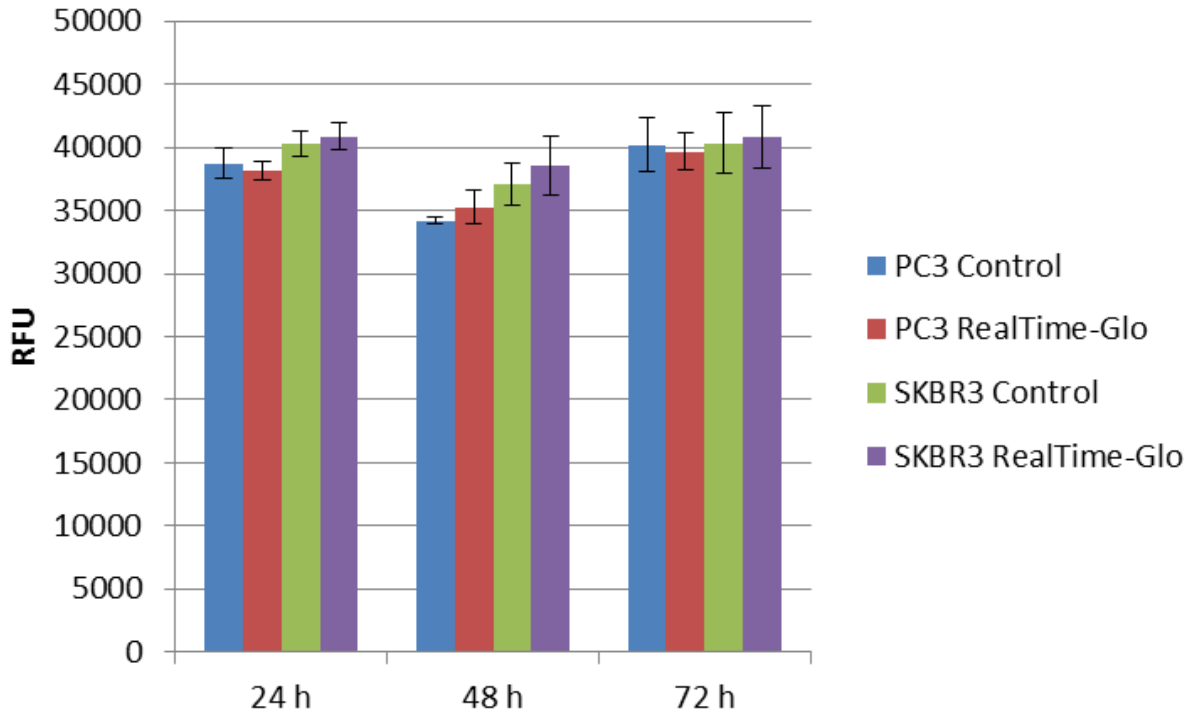
The luminescence signal was determined every hour for 72 h in a Tecan M200 plate reader with gas control module (37C/5%CO_2).

Luminescent Signal Drops Immediately Upon Cell Death



iCell cardiomyocytes were plated and grown in medium containing pro-substrate and NanoLuc luciferase. After 2 days, digitonin was added to a final concentration of 200 µg/ml.

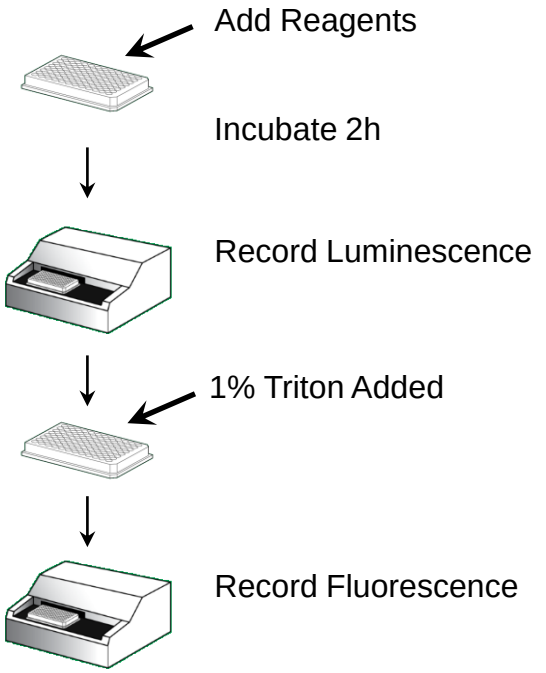
RealTime-Glo™ Reagents are Not Toxic

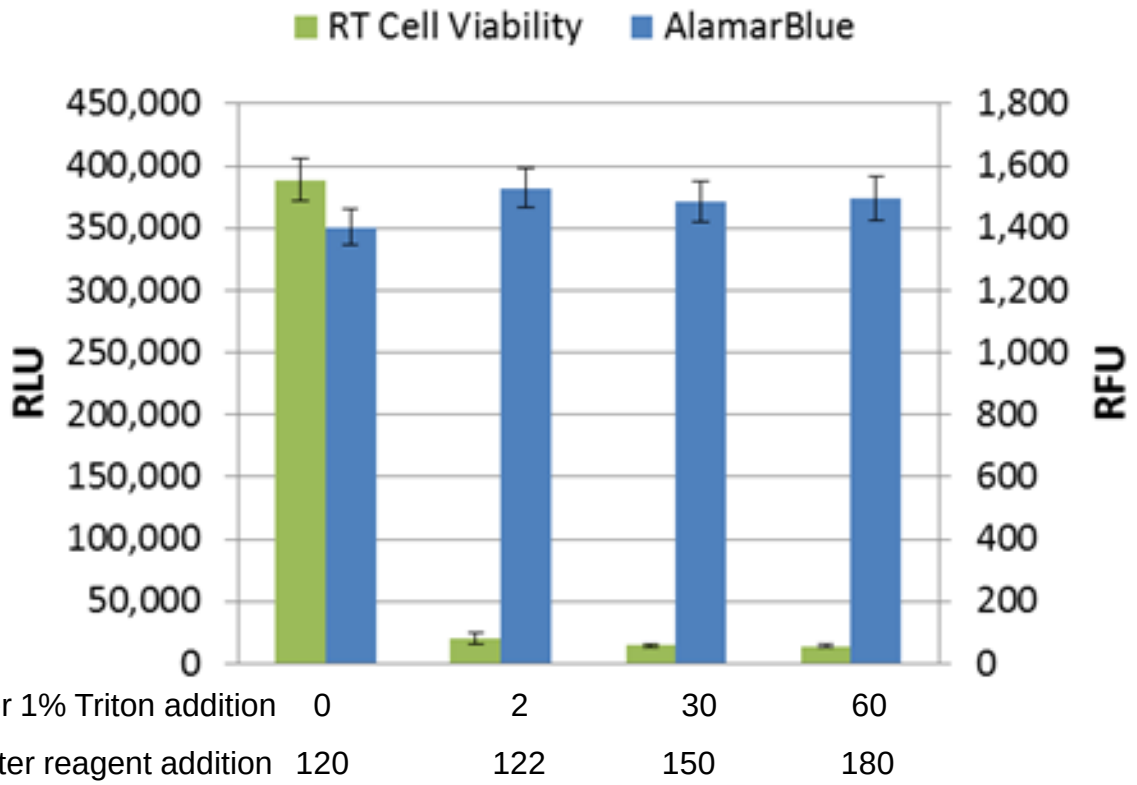


PC3 or SKBR3 cells cultured in the presence or absence or RealTime-Glo™ Reagent for 3 days. Samples were tested for membrane integrity using CytoTox-Fluor™ Cytotoxicity Assay.

Comparison of RealTime-Glo™ Assay to alamarBlue Endpoint Approach

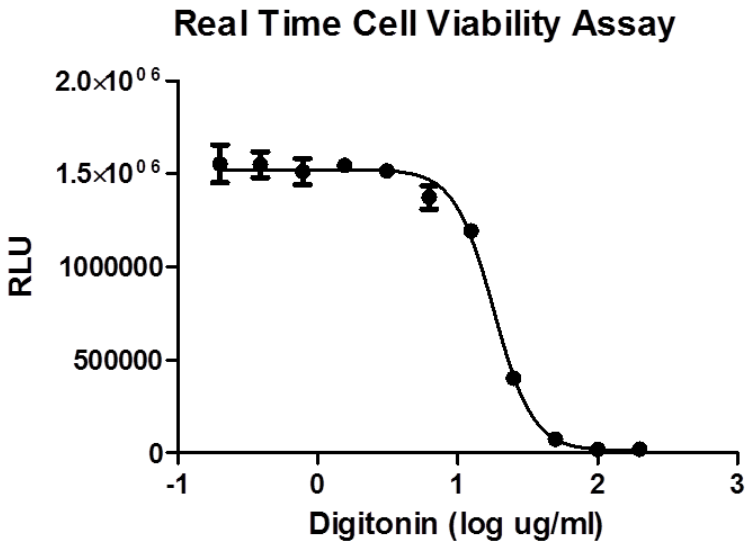
Resorufin from alamarBlue assay accumulates in medium so signal remains after cell death. Substrate from real time assay is rapidly used by NanoLuc, thus no accumulation.

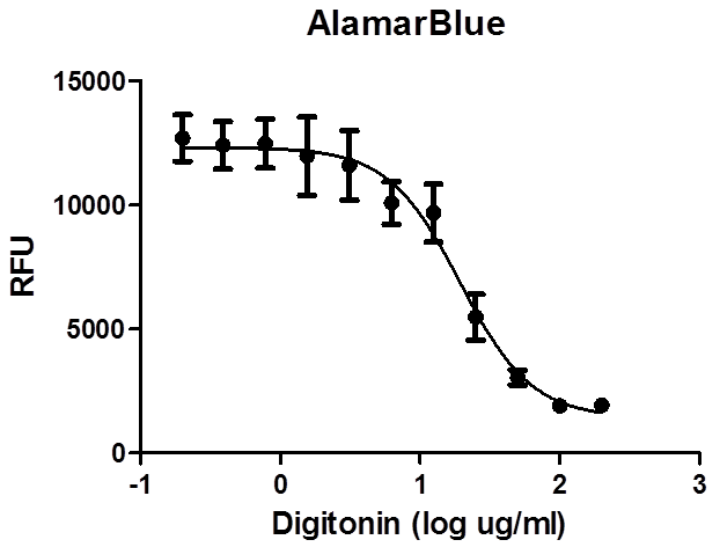




Comparison of RealTime-Glo™ Assay to alamarBlue Endpoint Approach

RealTime-Glo™ Assay is more sensitive, rapid, and robust than resazurin





Assay	IC50 (uM)	S/B	Time post-viability reagent addition
Real Time	21	65.9	30 min
AlamarBlue	19	5.9	4 hour

A549 cells were treated with digitonin for 24 h. Viability reagents were added and signal read at 30 min post-addition (RealTime-Glo™ Assay) or 4 hours post-addition (alamarBlue).

Multiplexing Real Time Cell Viability Assays

Multiplexing Viability and Luciferase Reporter Assays

Seed HEK293 cells expressing luciferase in 384 well plate

↓

Incubate overnight

↓

Add RealTime-Glo™ Reagent

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Incubate 2 hours

↓

Record Luminescence

↓

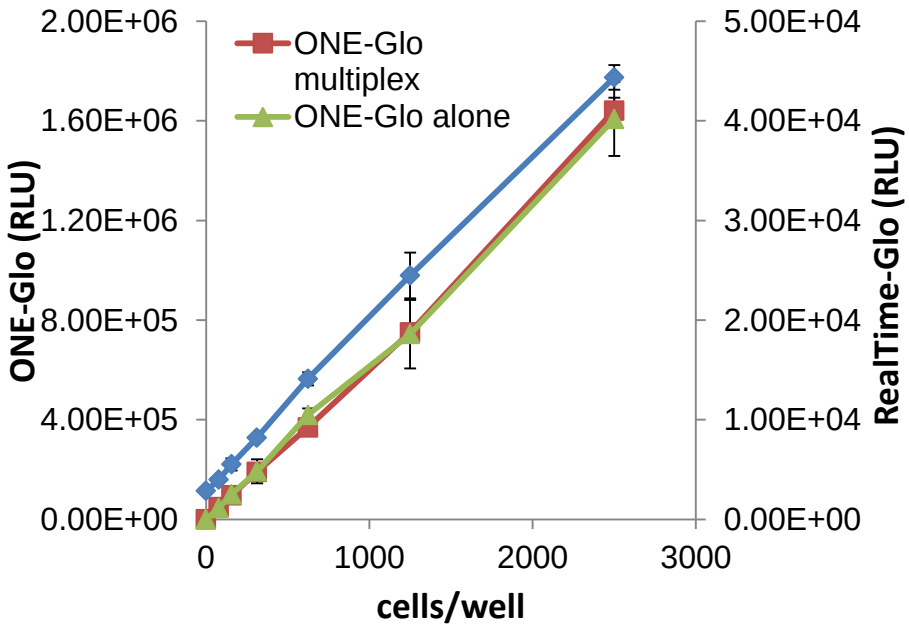
Add firefly luciferase reagent

↓

Incubate 10min

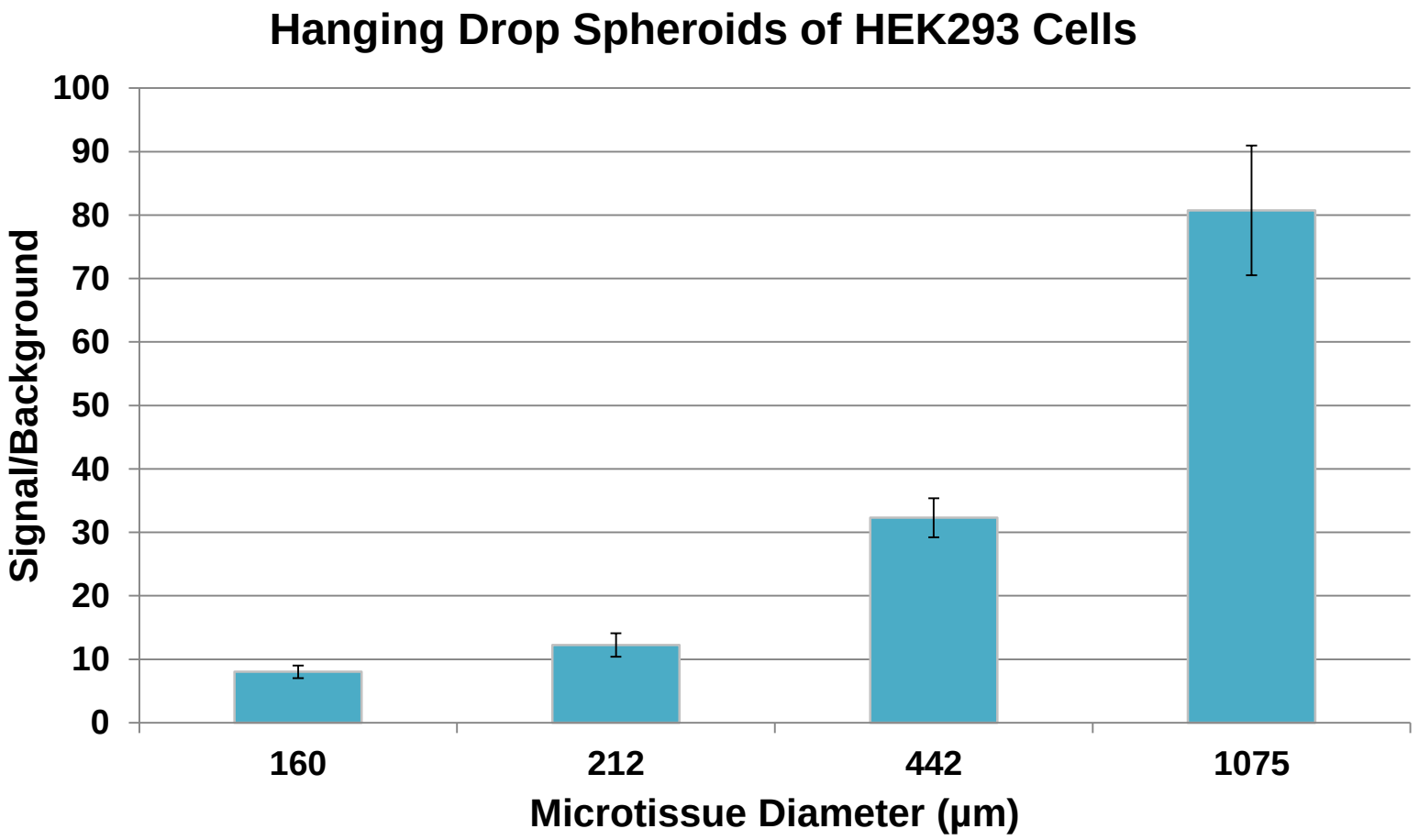
↓

Record luminescence

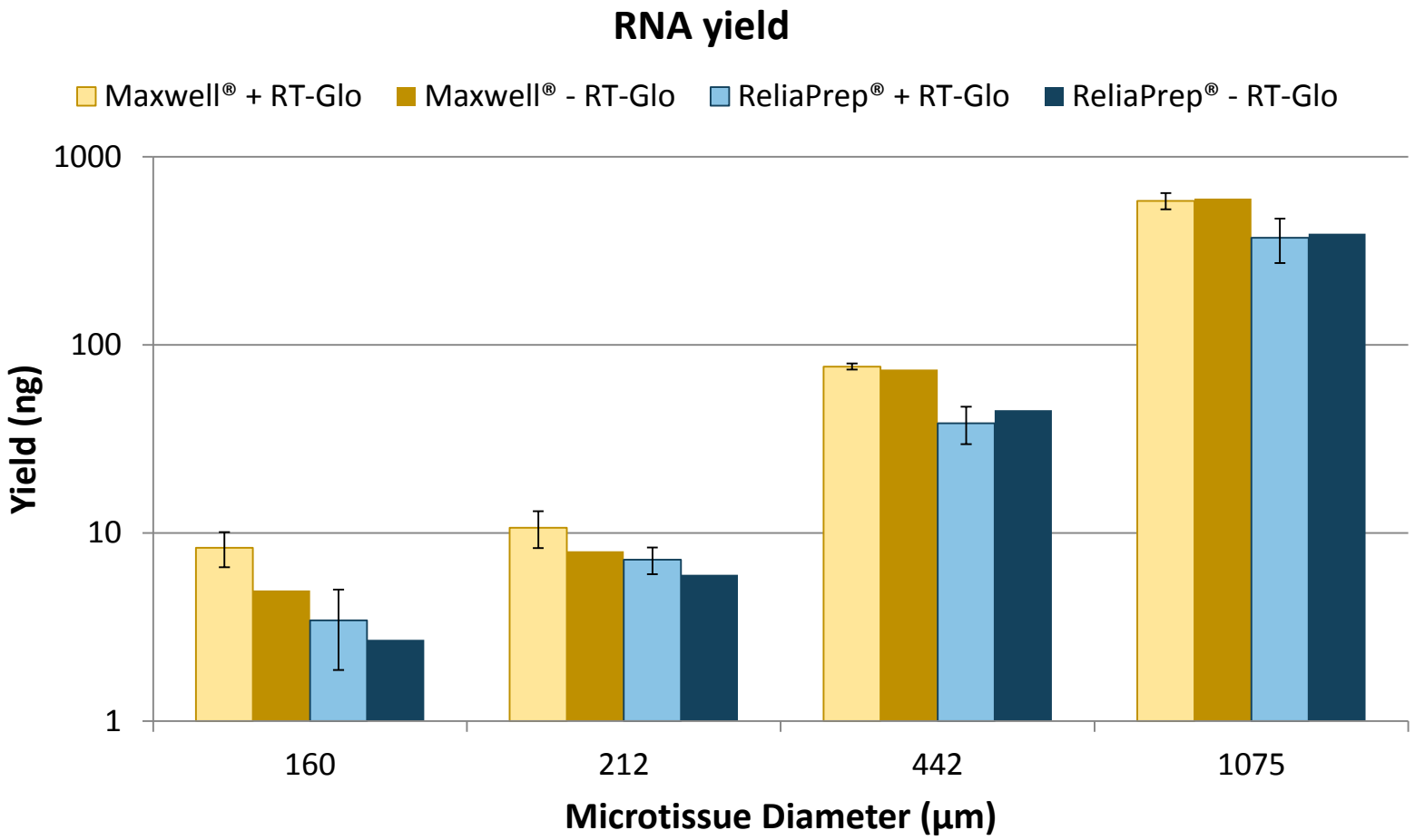


Firefly luciferase reporter assay signal is not affected by the presence (red squares) or absence (green triangles) of RealTime-Glo™ Reagent

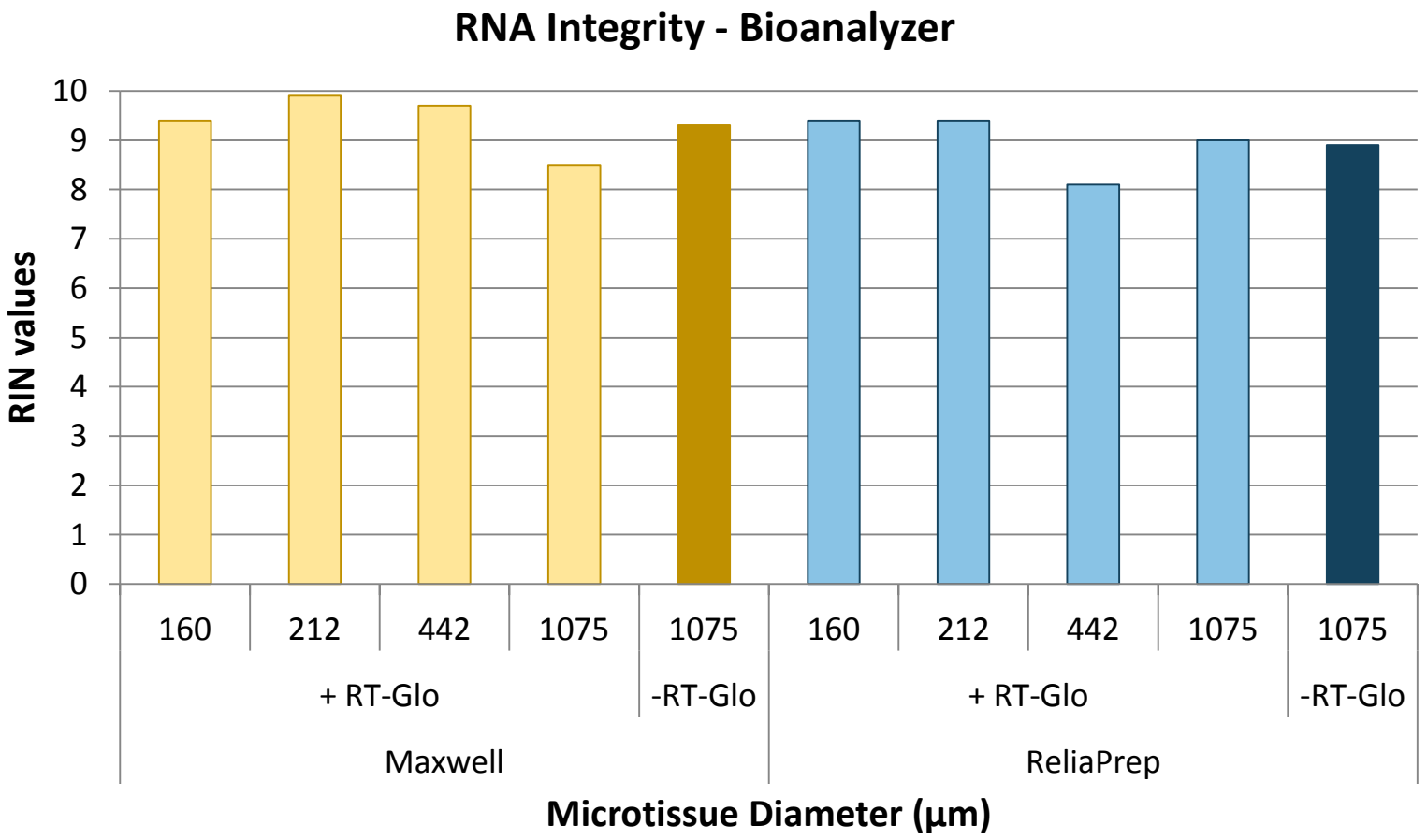
RealTime-Glo™ MT Cell Viability Assay Applied to 3D Microtissues



RealTime-Glo™ Reagents Do Not Effect RNA Yield using QuantiFluor® RNA System



RNA Integrity is Not Affected by Presence of RealTime-Glo™ Reagent



RealTime-Glo™ Reagent Does Not Affect Cycle Threshold of Extracted RNA

Table 2. C_t Values Generated from RNA Isolated from Different Cell Types With or Without the RealTime-Glo ™ Reagent.

	C_t Value	
Cell Type	With Medium Only	With RealTime-Glo ™ Reagent
A549	30.9	30.3
K562	31.3	30.2
MCF7	30.9	30.1
THP-1	29.9	29.9

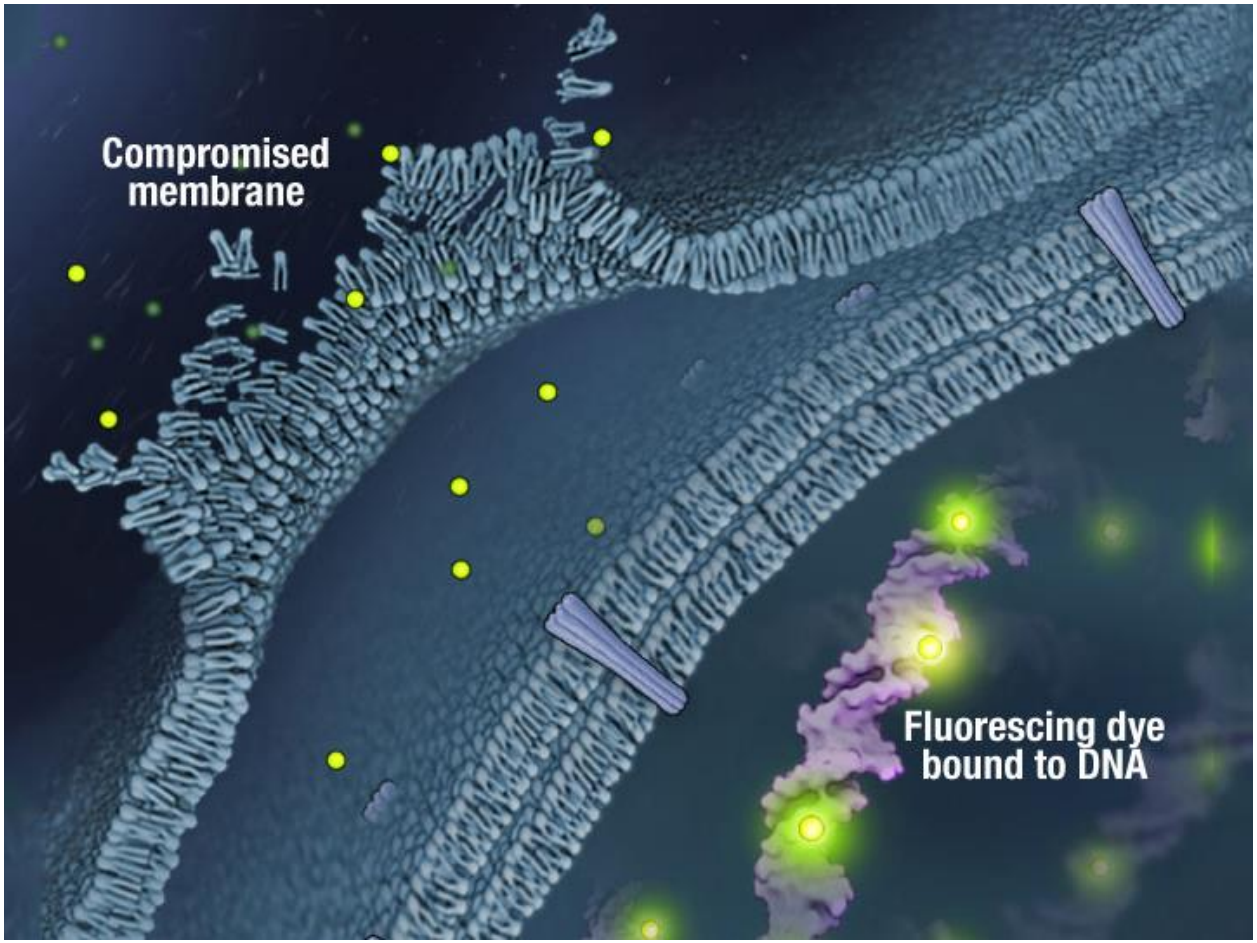
Advantages of RealTime-Glo™ Assay

- Provides kinetic information on viable cell number during the course of experiments that enables “on the fly” decision making
- Viable cells remain after applying RealTime-Glo™ Reagent (i.e. the reagent is not toxic)
- Remaining viable cells enable a variety of opportunities for multiplexing with other assay chemistries
- Optional protocols enables reagent to be added when cells are plated, when test compound is added, or at any time point when cell viability measurements are needed.
- Sensitivity is better than colorimetric or fluorometric viability assays that measure reducing potential of cells.

Real Time Detection of Dead Cells

CellTox™ Green Cytotoxicity Assay

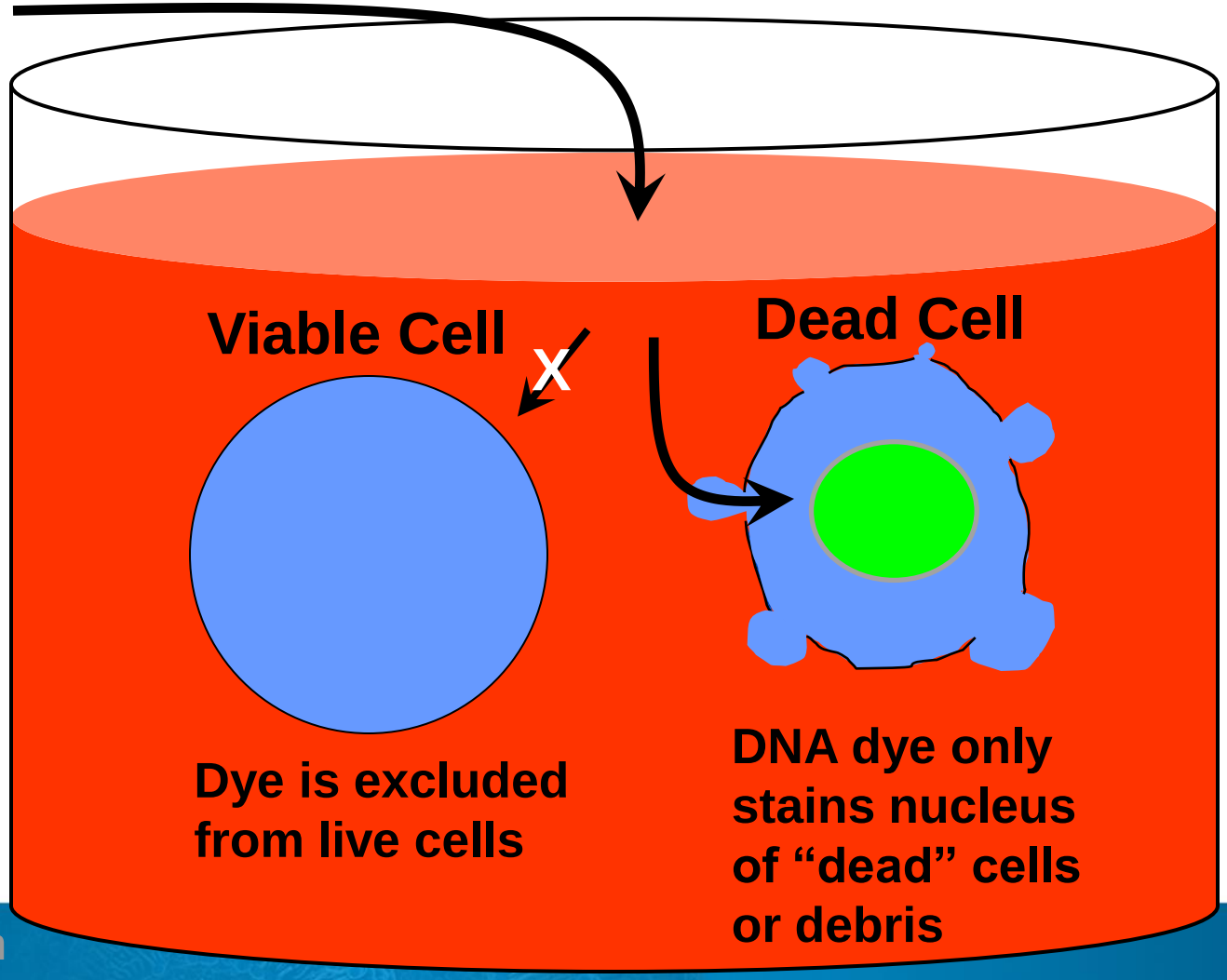
"Real Time" Method to Detect Dead Cells



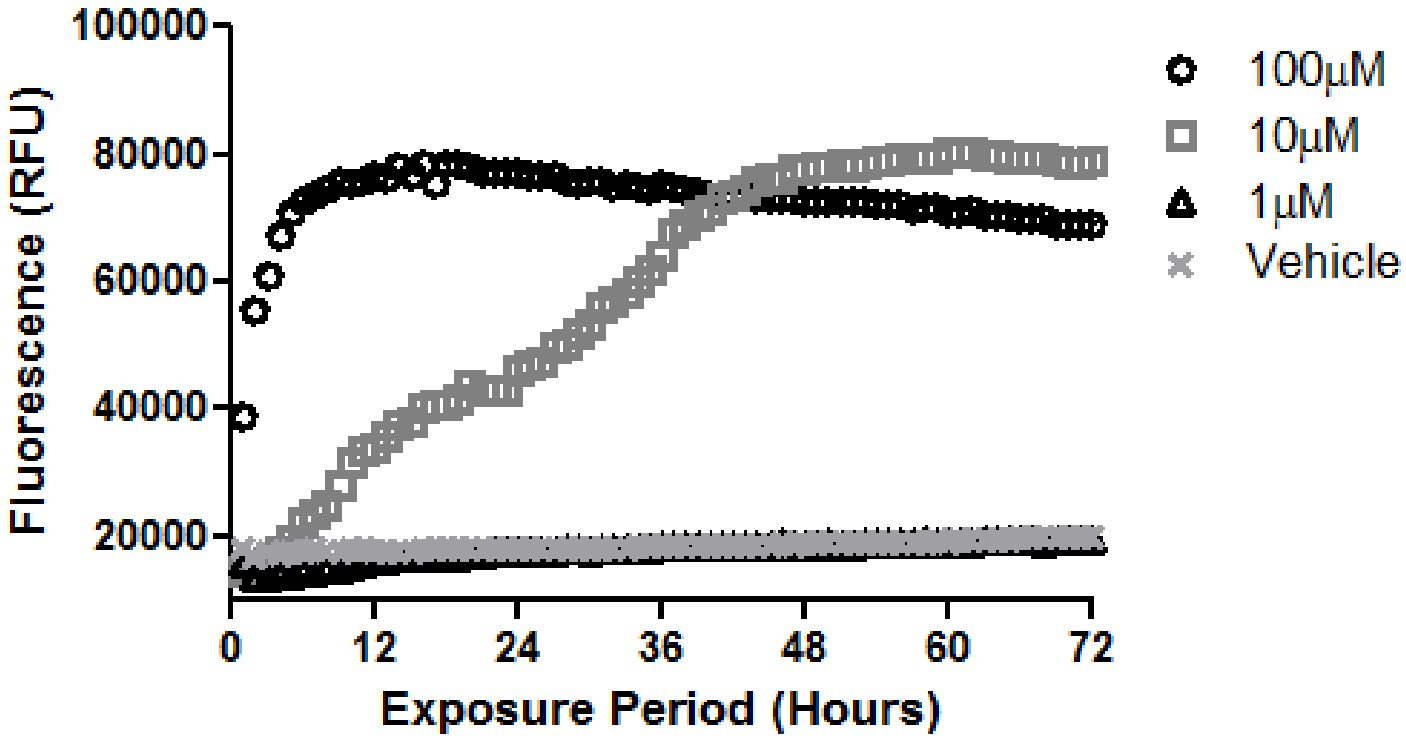
DNA Dye Staining to Detect Dead Cells
(Overcomes some limitations of short half-life markers)

Non-permeable DNA dye

Staining of dead cells results in a fluorescent signal that is stable.



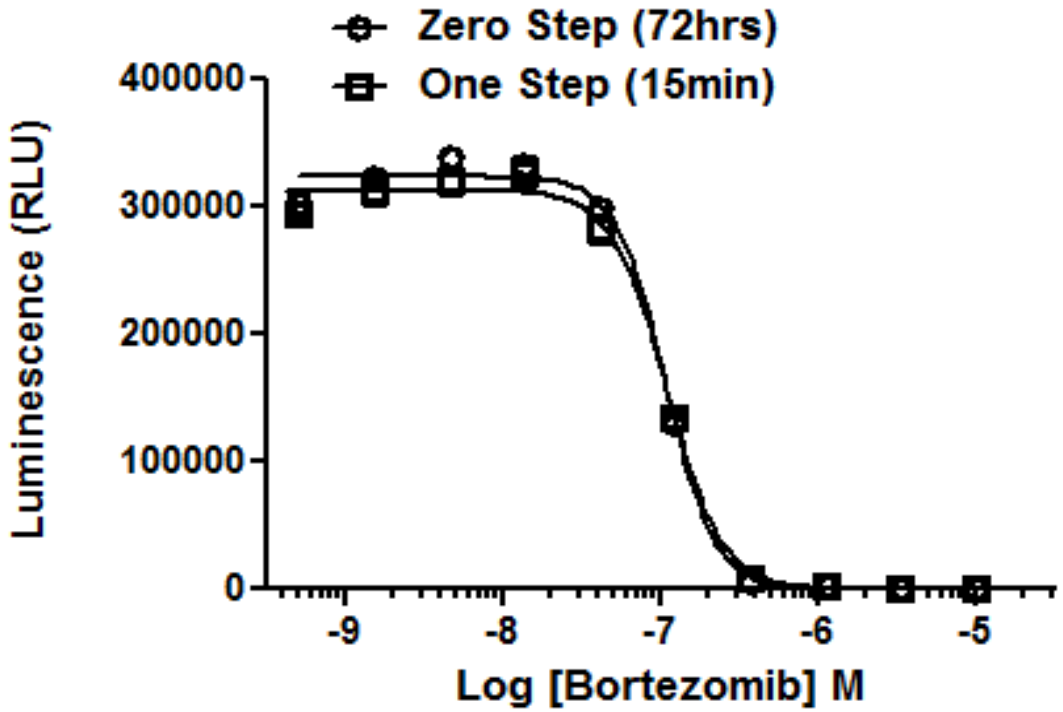
CellTox™ Green Dye Measures Accumulation of Dead Cells in Culture



HepG2 cells were treated with various doses of Terfenadine. CellTox™ Green Dye was added and fluorescence was measured every hour for 3 days. Increasing fluorescence indicates an increase in the number of dead cells.

CellTox™ Green Dye is Not Toxic to Cells

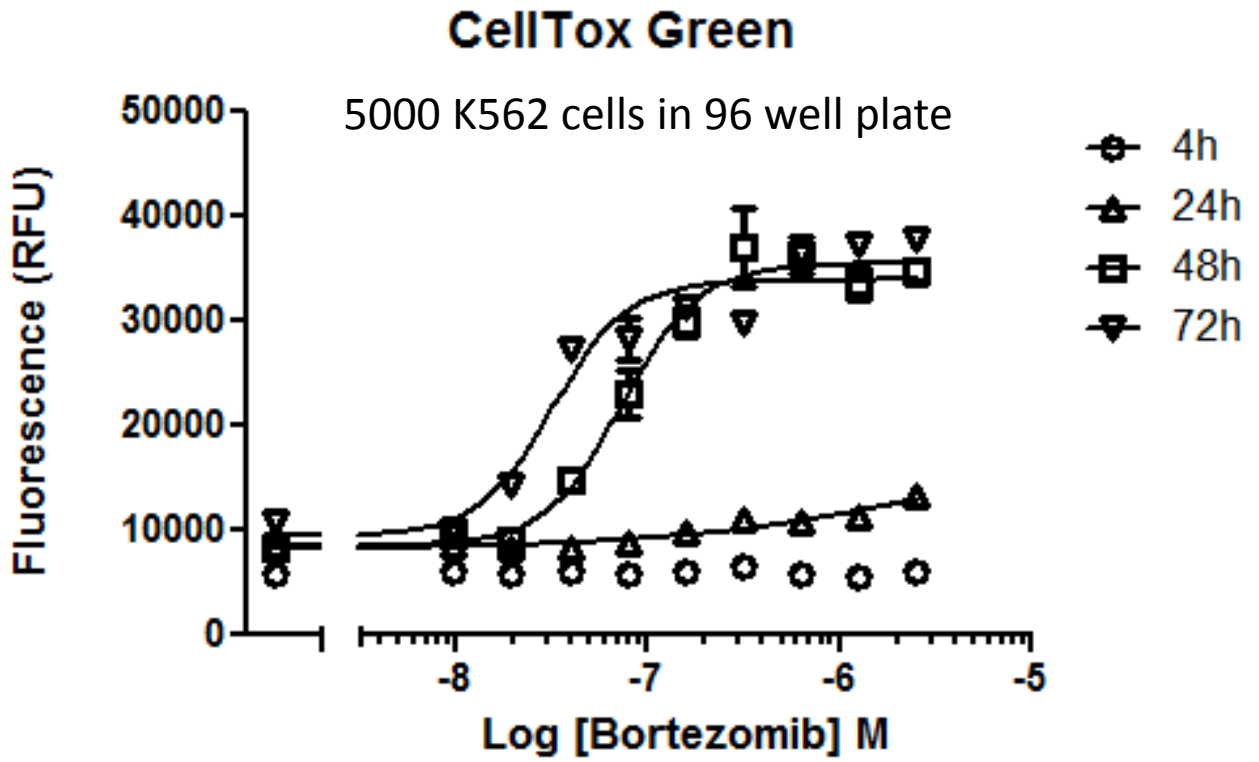
…and does not affect response to other toxins



ATP assay data showing viability of cells exposed to DNA binding dye for 15 minutes or 72 hours.

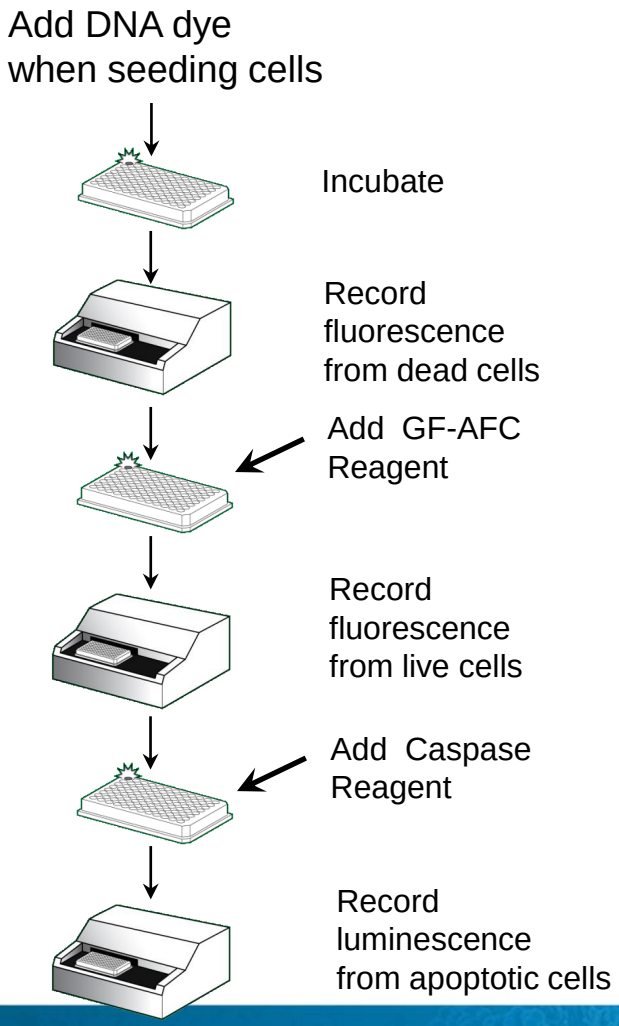
- Dye is non-toxic for at least 72 hours
- No effect on IC_{50} value of test compounds

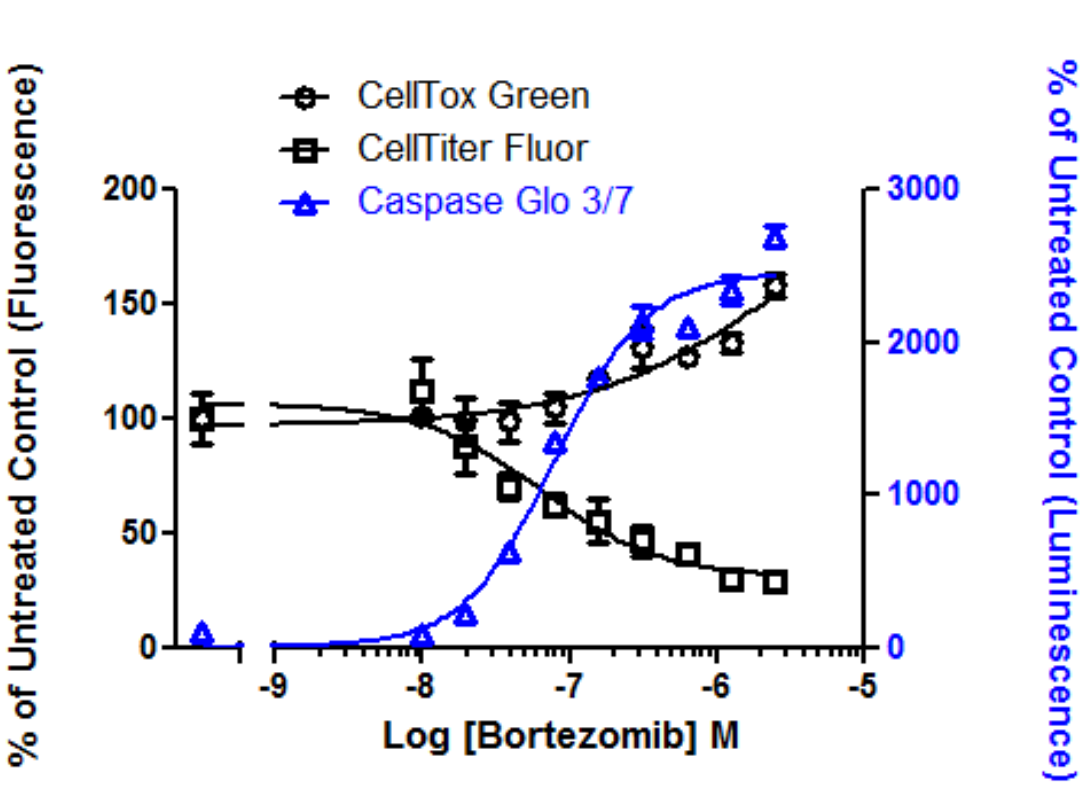
Reading the Same Plate Multiple Times to Detect the Onset of Cell Death



First appearance of cell death may trigger further experimentation with the same sample.

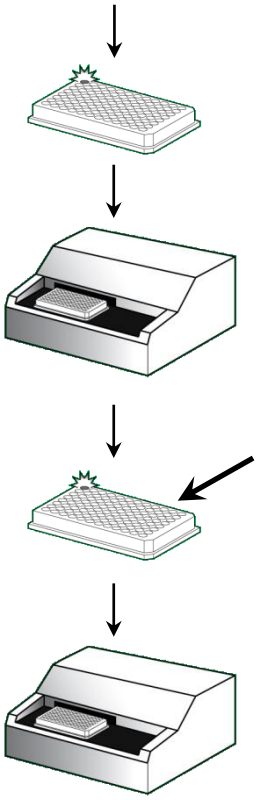
Samples Stained with DNA Dye can be Multiplexed with Cell Viability and Apoptosis Assays





Multiplexing DNA Staining and ATP Assays

Add CellTox™ Green Dye
when seeding cells

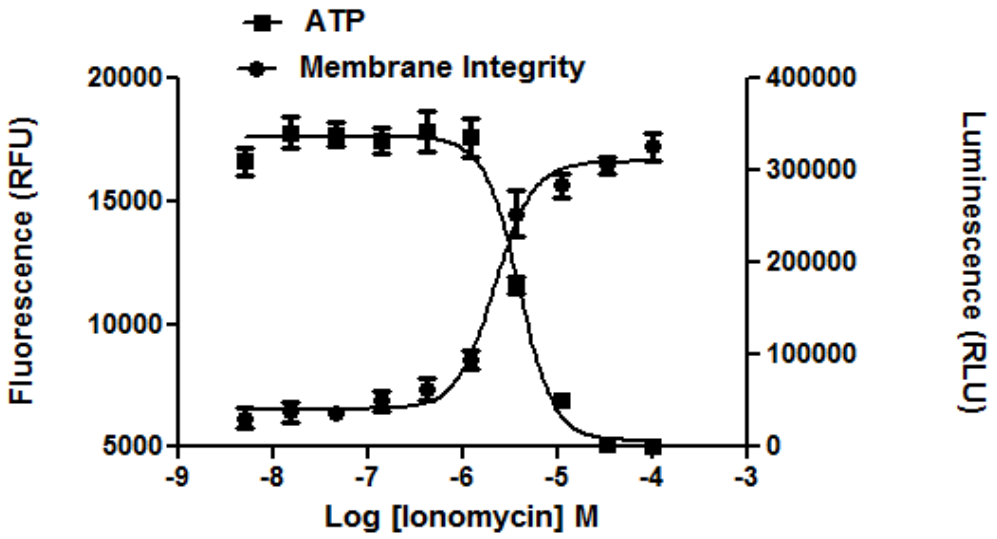


Incubate
72hr

Record
fluorescence
from dead cells

Add ATP Detection
Reagent

Record
Luminescence
from live cells



	Membrane Integrity	ATP
IC50	2.177e-006	3.950e-006

Advantages of DNA Staining

Advantages:

- “Real Time” DNA staining of dead cells produces a fluorescent signal that lasts much longer than the signal from enzyme release.
- DNA staining dye overcomes the major disadvantage of enzyme release assays.
- Numerous multiplex opportunities because cells remain viable

Disadvantage:

- Signal window is not as great as enzyme marker assays which can be amplified by enzymatic generation of product

Summary

A novel assay has been developed to measure viable cell number in "real time":

- Repeated kinetic luminescent measurements indicate cell growth or death over time
- Reagents are not toxic, thus cells remain viable for subsequent multiplexing assays

A non-toxic non-permeable DNA dye can measure dead cell number in real time:

- Repeated fluorescence measurements indicate appearance of dead cells
- DNA dye is non-toxic, thus cells remain viable for subsequent multiplexing assays

Real time detection methods provide flexibility during assay development:

- Kinetic measurements of cell health from the same plate eliminates the need for multiple parallel plates during development and optimization of phenotypic assays
- Multiplexing real time assay methods can provide an internal control to verify viable cell number simultaneously with a variety of other phenotypic assays

Acknowledgments

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Questions Welcome

For additional information visit Promega booth #605

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