Cell Health and Mechanistic Toxicity Assays

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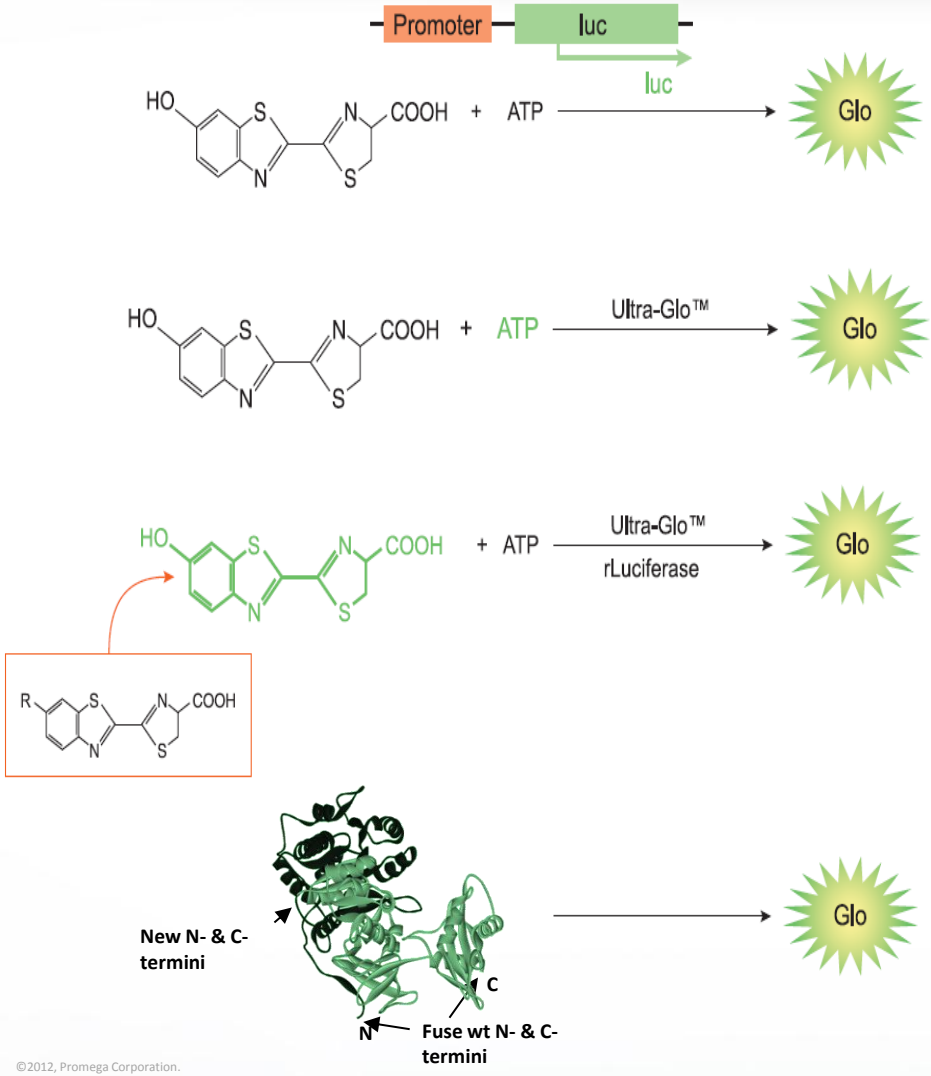
Outline

- Bioluminescent assays
- Live and Dead Cell Assays
- “Real Time” Assays
- Apoptosis
- Stress Events Leading to Cytotoxicity
- Validation of assays for 3D culture models

Bioluminescent Assays Developed From Firefly Luciferase Chemistry



- Reporter gene assays
  GPCR & Nuclear Receptor

- Cell Viability
- Kinase assays
- cAMP & PDE assays
- P-glycoprotein assay

- Caspases/proteases
- CYP450 assays
- MAO assays
- GSH assays
- HDAC assays

- GloSensor cAMP
- GloSensor caspase

Cell Health Assays Overview

Viable cells detected using markers of active metabolism

- **Cellular conversion of indicator dyes (MTT / MTS / Resazurin)**
- **Protease marker**
- **ATP content**
- **Real time viability assay using NanoLuc**

Dead cells detected using marker of membrane integrity

- LDH release
- Protease release
- Dye uptake / staining

Apoptosis detected using caspase activities

Biochemical markers of cell stress leading to cytotoxicity

- Mitochondrial toxicity
- Oxidative stress (ROS and GSH:GSSG ratio)
- NADH

Luciferase reporters of cell stress pathways leading to cytotoxicity

Metabolic & Enzymatic Indicators of Cell Viability

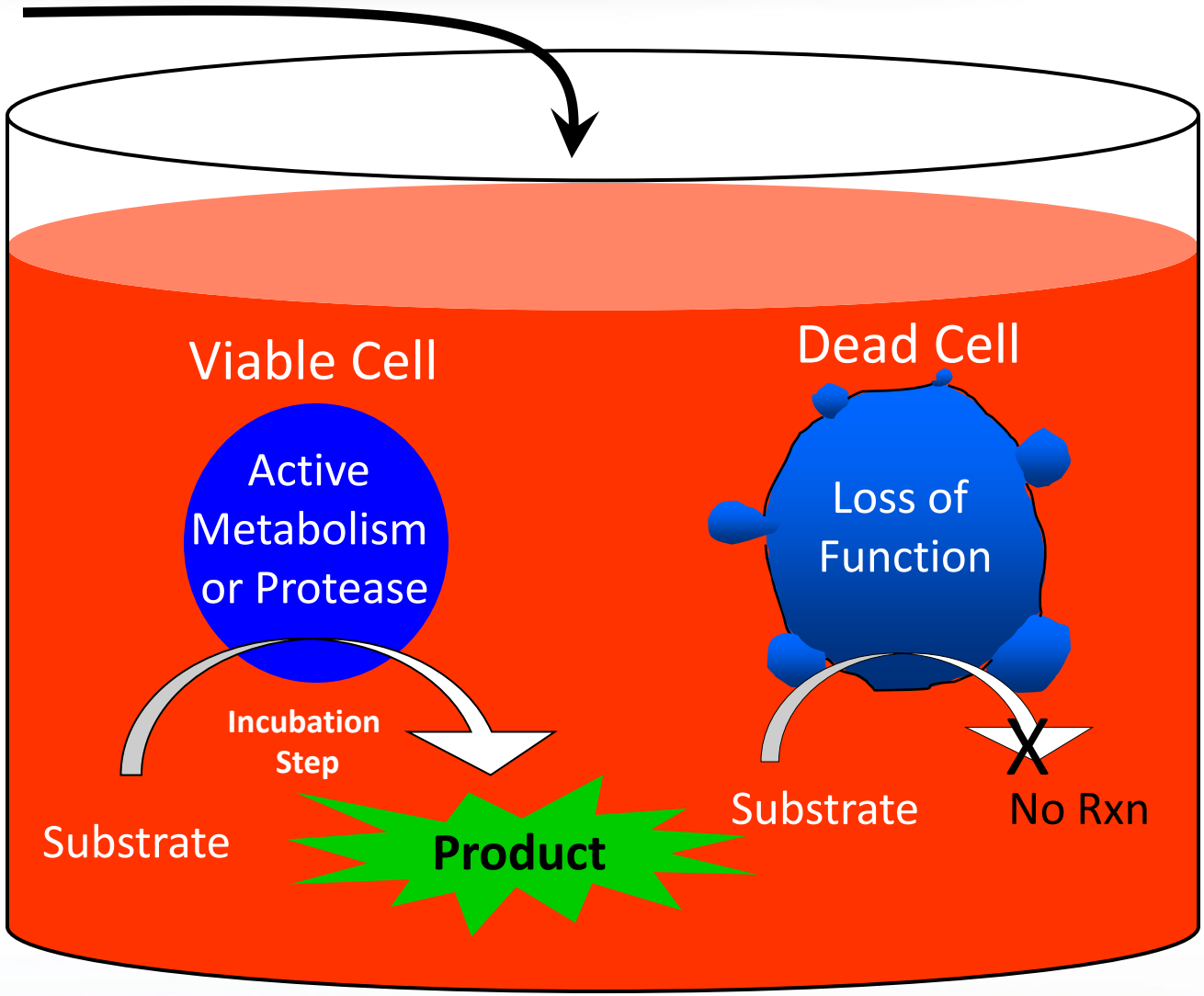
Promega

Reagent

Tetrazolium Reagents
- MTT, MTS, XTT, WST

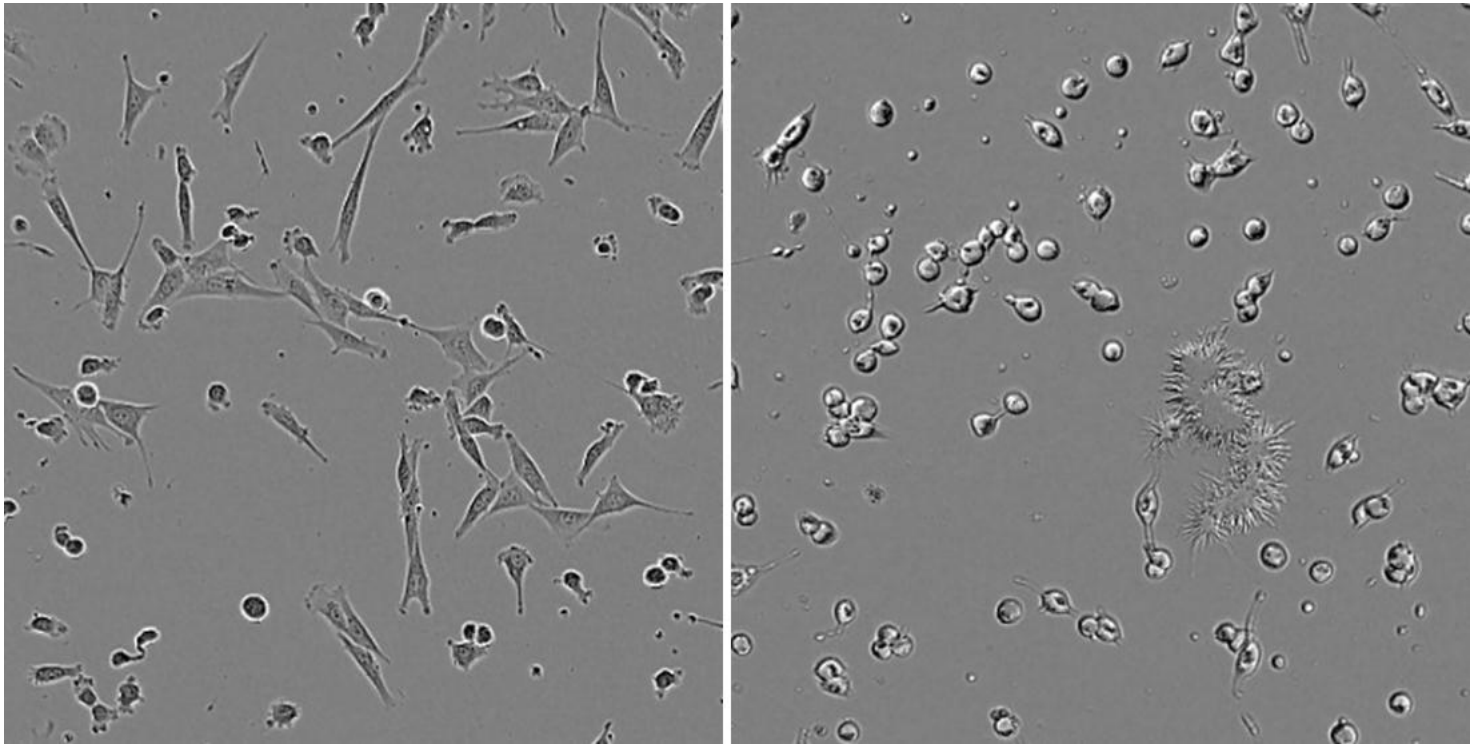
Redox Indicators
- Resazurin

Enzyme Substrates
- Protease Substrates GF-AFC



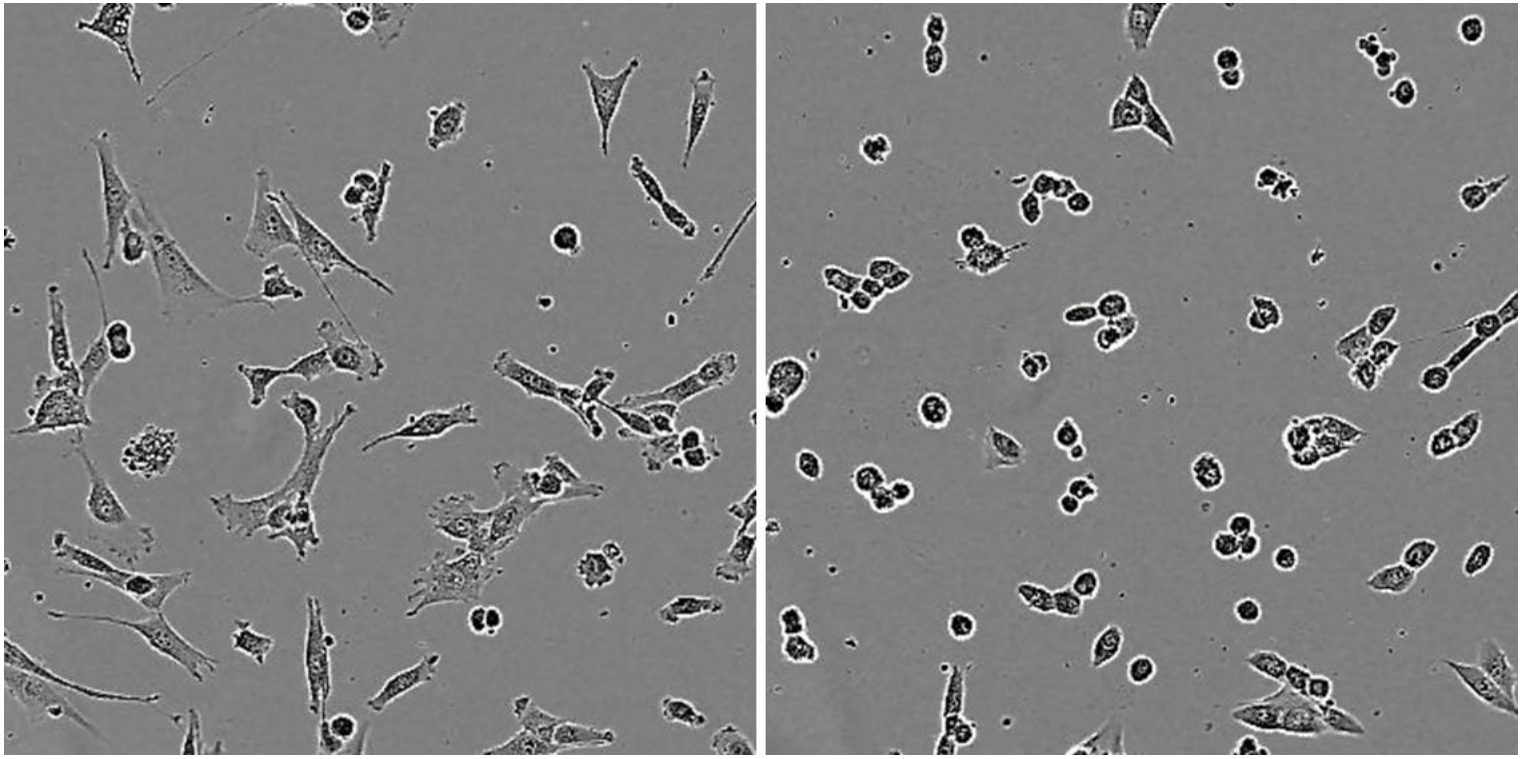
Balb 3T3 Cells Treated with MTT for 4 Hours

Same field of cells imaged immediately after addition of MTT and after 4 hours incubation.



Images captured by Tracy Worzella using Incucyte instrument from Essen Biosciences.

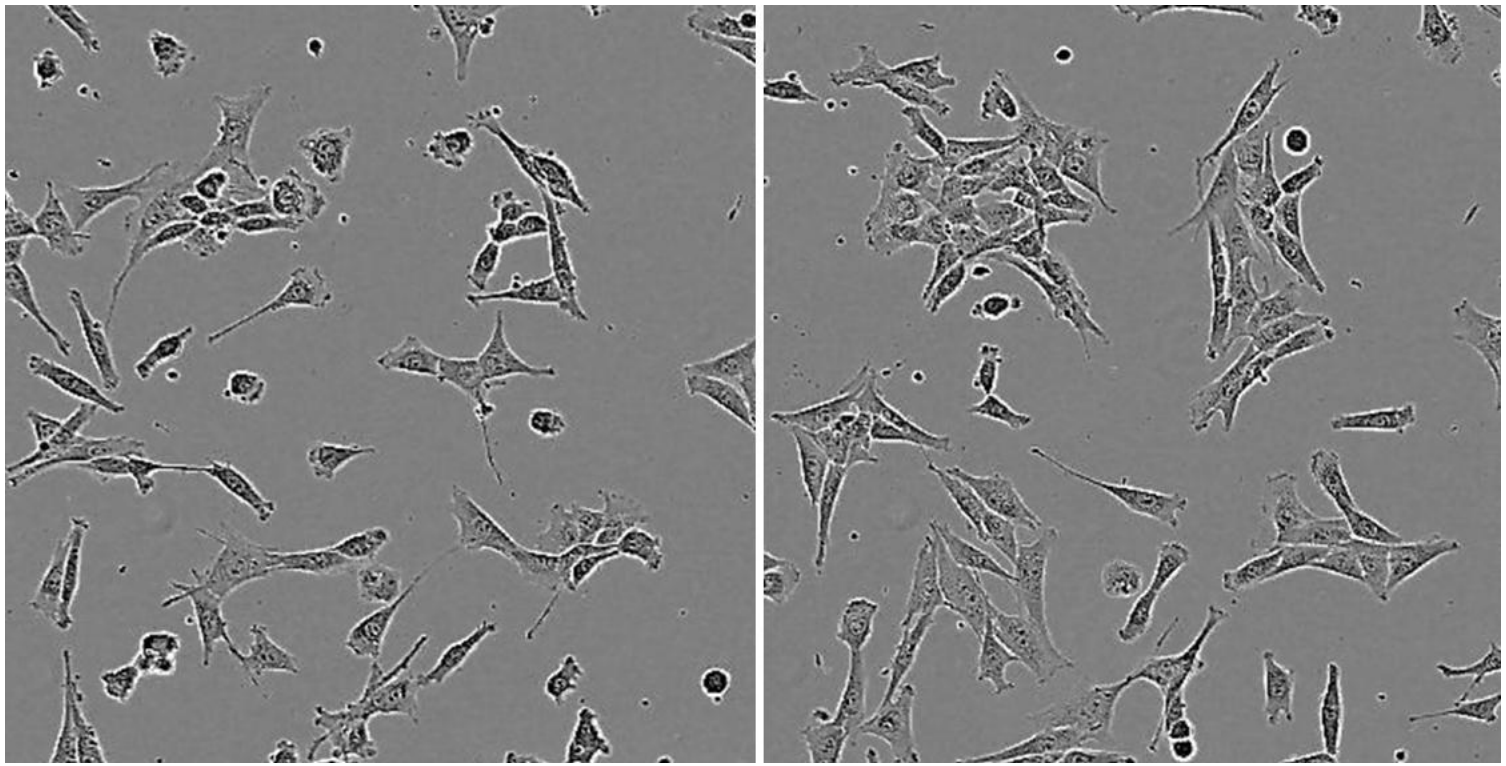
Balb 3T3 Cells Treated with Resazurin for 4 Hours



Images captured by Tracy Worzella using Incucyte instrument from Essen Biosciences.

Balb 3T3 Cells Treated with GF-AFC for 4 Hours

4 h exposure used for comparison; but 30 min is usually adequate

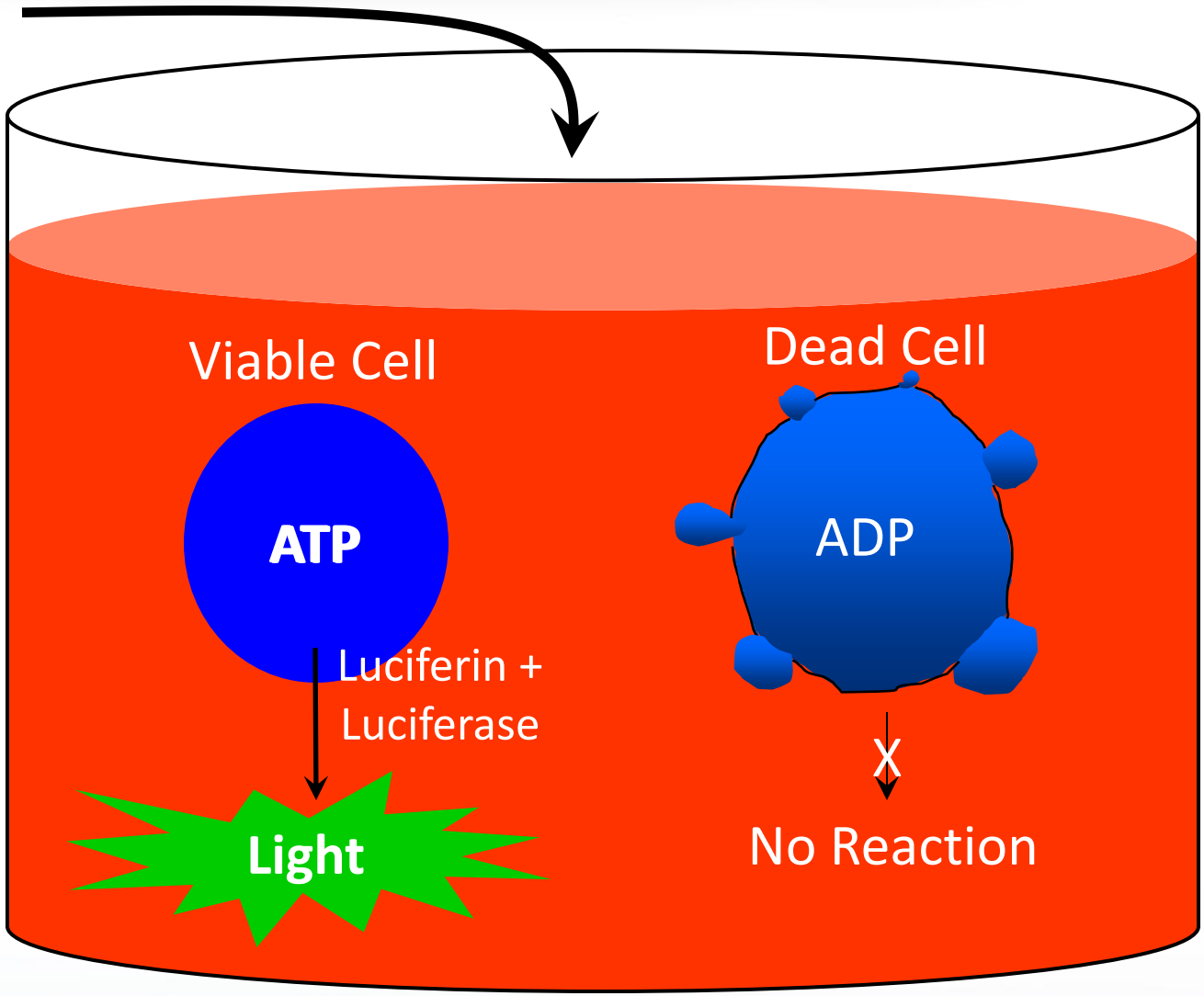


Images captured by Tracy Worzella using Incucyte instrument from Essen Biosciences.

ATP Assay for Cell Viability (immediate lysis)

CellTiter-Glo® Reagent

- Lysis Solution
- ATPase Inhibitors
- **Luciferin**
- **UltraGlo Luciferase**

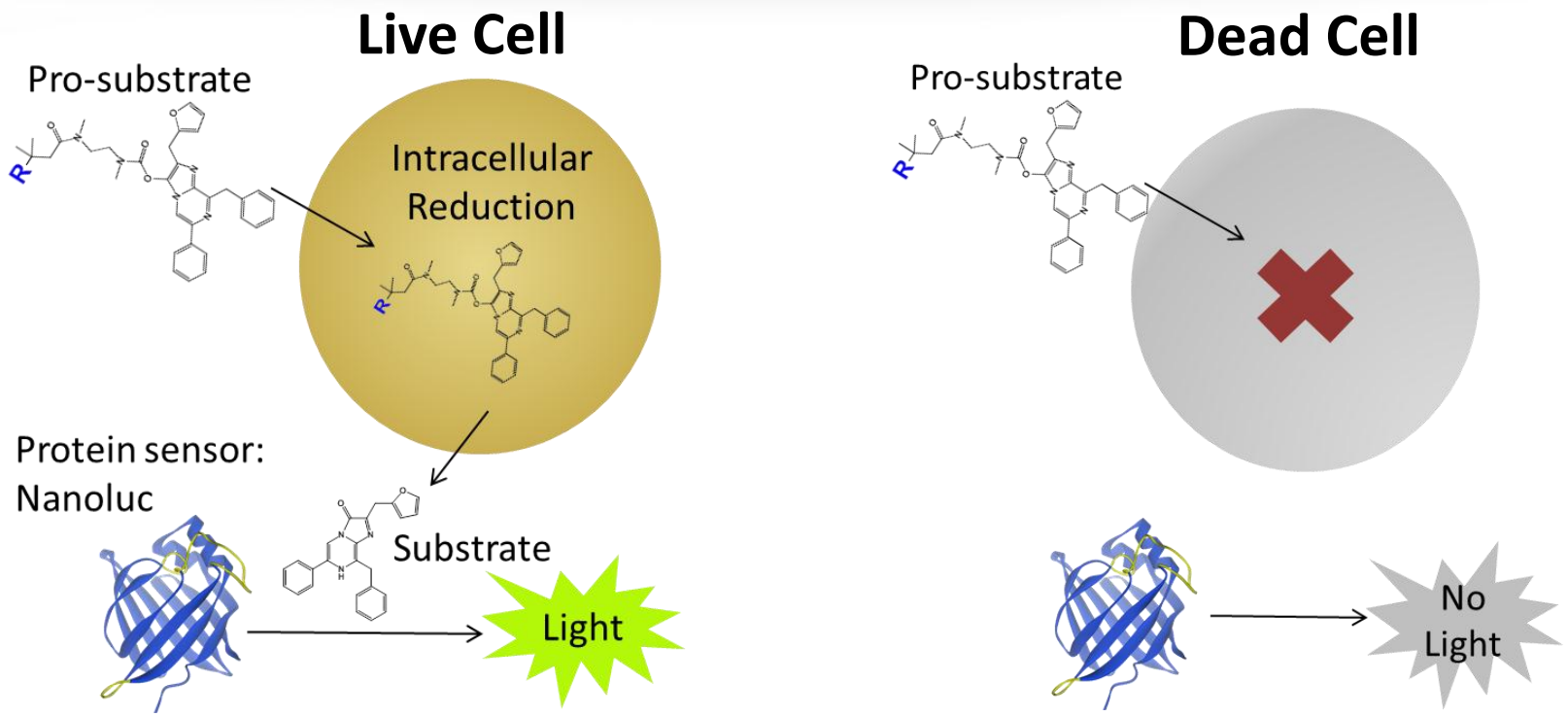


Advantages & Disadvantages of Viability Assays

Assay	Advantages	Disadvantages
MTT / MTS	Widely used Inexpensive	MTT has 2 step protocol 1-4 hour incubation Interference by reducing compounds Toxic to cells Limited sensitivity
Resazurin	Inexpensive Fluorescent readout Good sensitivity	1-4 hour incubation Interference by reducing compounds Toxic to cells Fluorescence interference
Protease	30 min protocol Cells remain viable Better sensitivity than resazurin Good choice for multiplexing	Fluorescence interference
ATP	10 min protocol Best sensitivity No fluorescence interference Lysis step stops reaction immediately (no incubation with viable cells)	Lytic protocol dictates sequence for multiplexing

"Real Time" Cell Viability Assay Based on NanoLuc Luciferase …in Development

Real Time Cell Viability Assay Measures Reducing Potential of the Cell



- NanoLuc protein sensor is present outside of the cells
- Pro-furimazine substrate enters the cell and is reduced by the cell to form furimazine
- Furimazine (substrate) diffuses from the cell and is rapidly used by NanoLuc to produce light

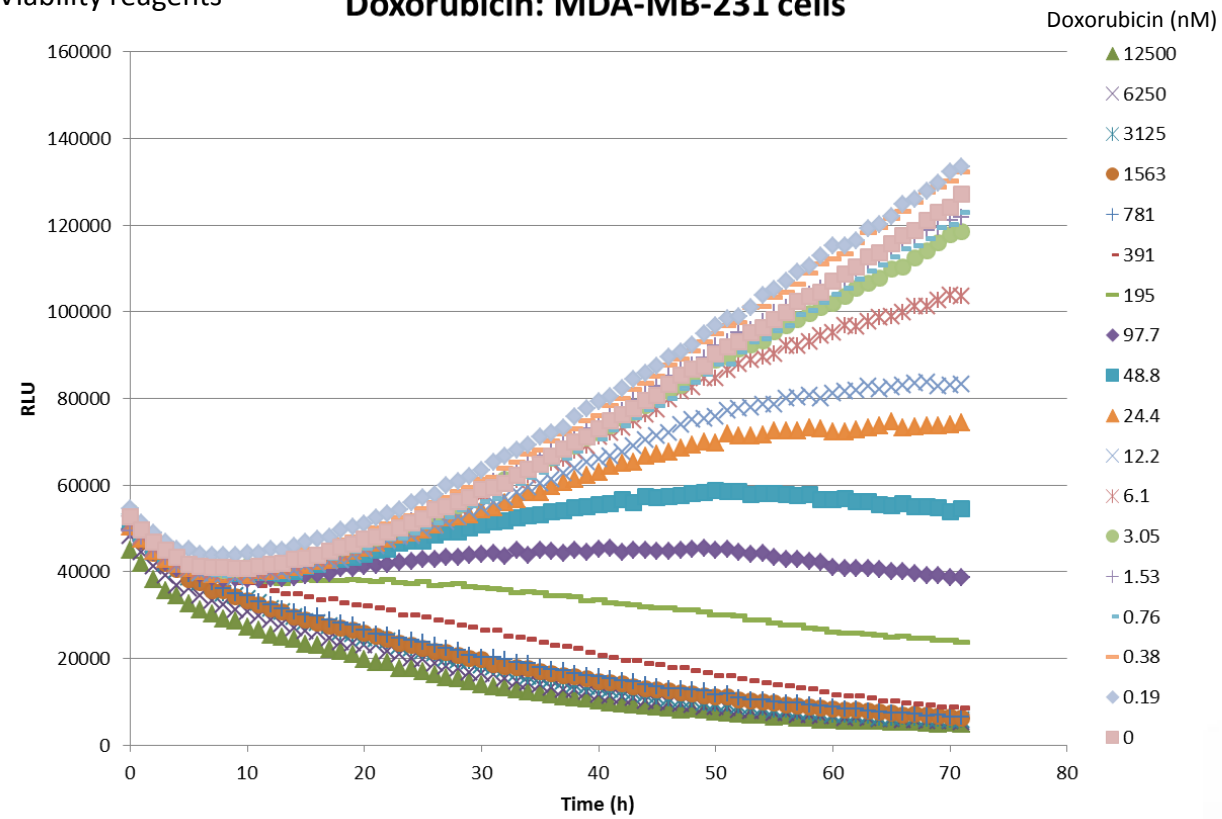
Measure changes in viability over an extended period of time

Cells plated in media containing Real Time Cell Viability reagents

Add drug

Continuous luminescence readings

Analyze viability data

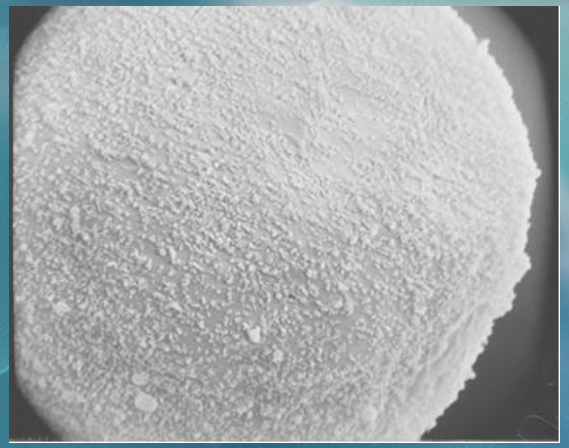


Doxorubicin	
Time (h)	**IC50 (nM)**
8	ND
16	ND
24	556
32	208
40	116
48	81
56	56
64	38
72	27

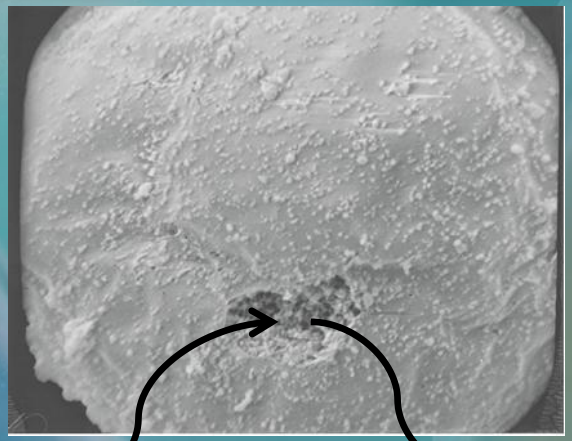
Detecting Dead Cells: Two Basic Approaches

The definition of cell viability is based on membrane integrity.

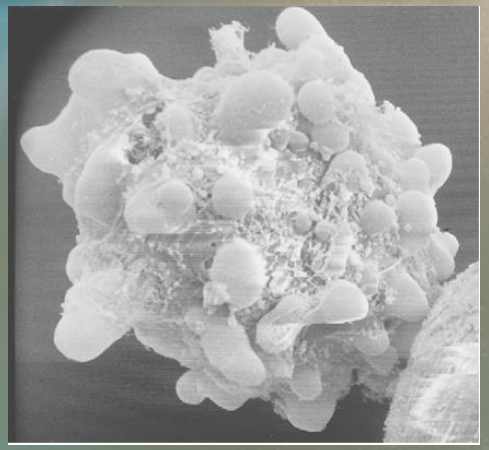
Live



Dead



Apoptotic



Cell Health Assays Overview

Viable cells detected using markers of active metabolism

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Dead cells detected using marker of membrane integrity

- **LDH release**
- **Protease release**
- **Dye uptake / staining**

Apoptosis detected using caspase activities

Biochemical markers of cell stress leading to cytotoxicity

- Mitochondrial toxicity
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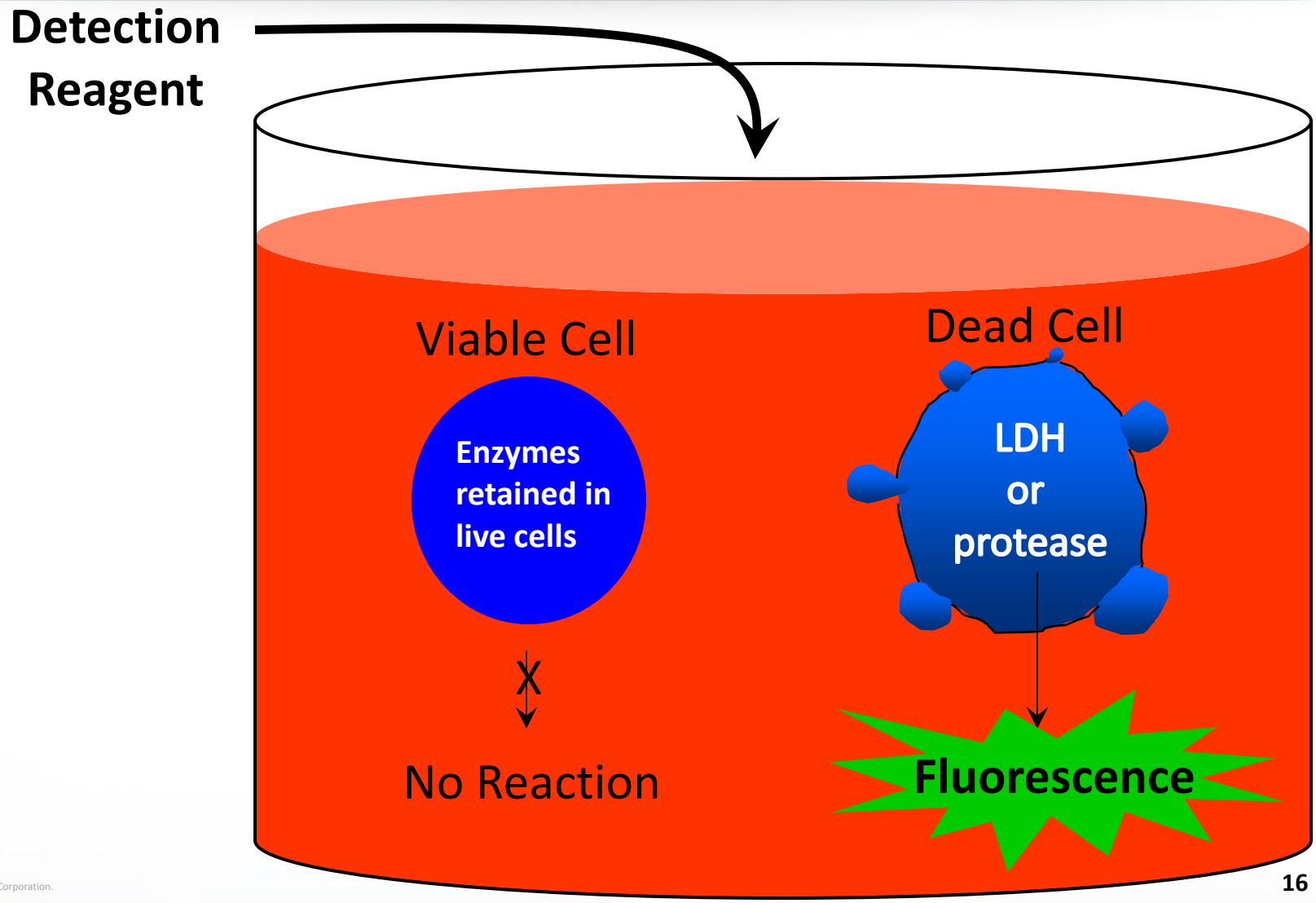
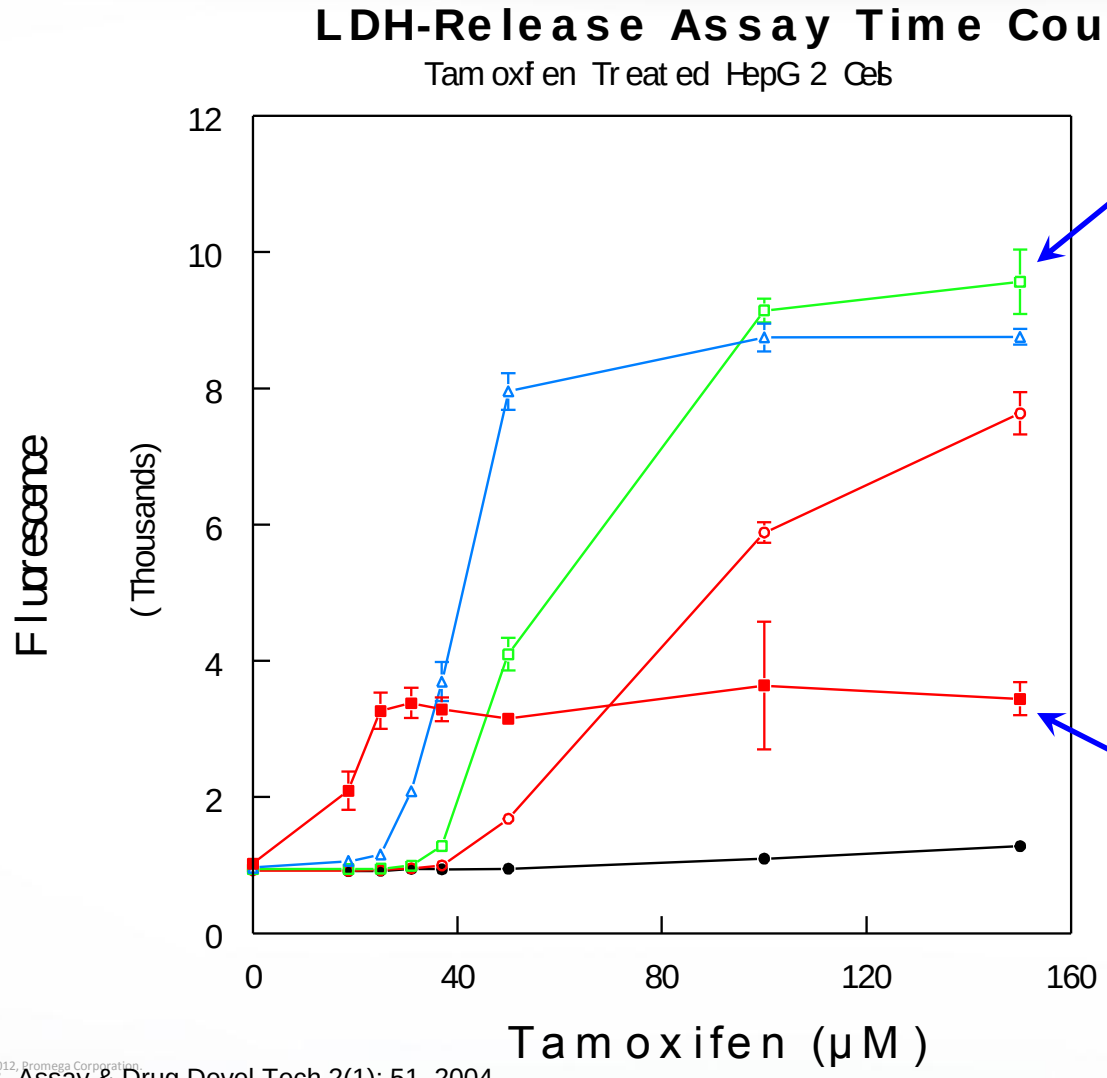
- NADH

Luciferase reporters of cell stress pathways leading to cytotoxicity

Enzyme Marker Release Assay to Detect Dead Cells



Stability of released enzyme activity in culture medium becomes a limitation



Cells are all dead at 2 hr treatment with 150µM Tamox

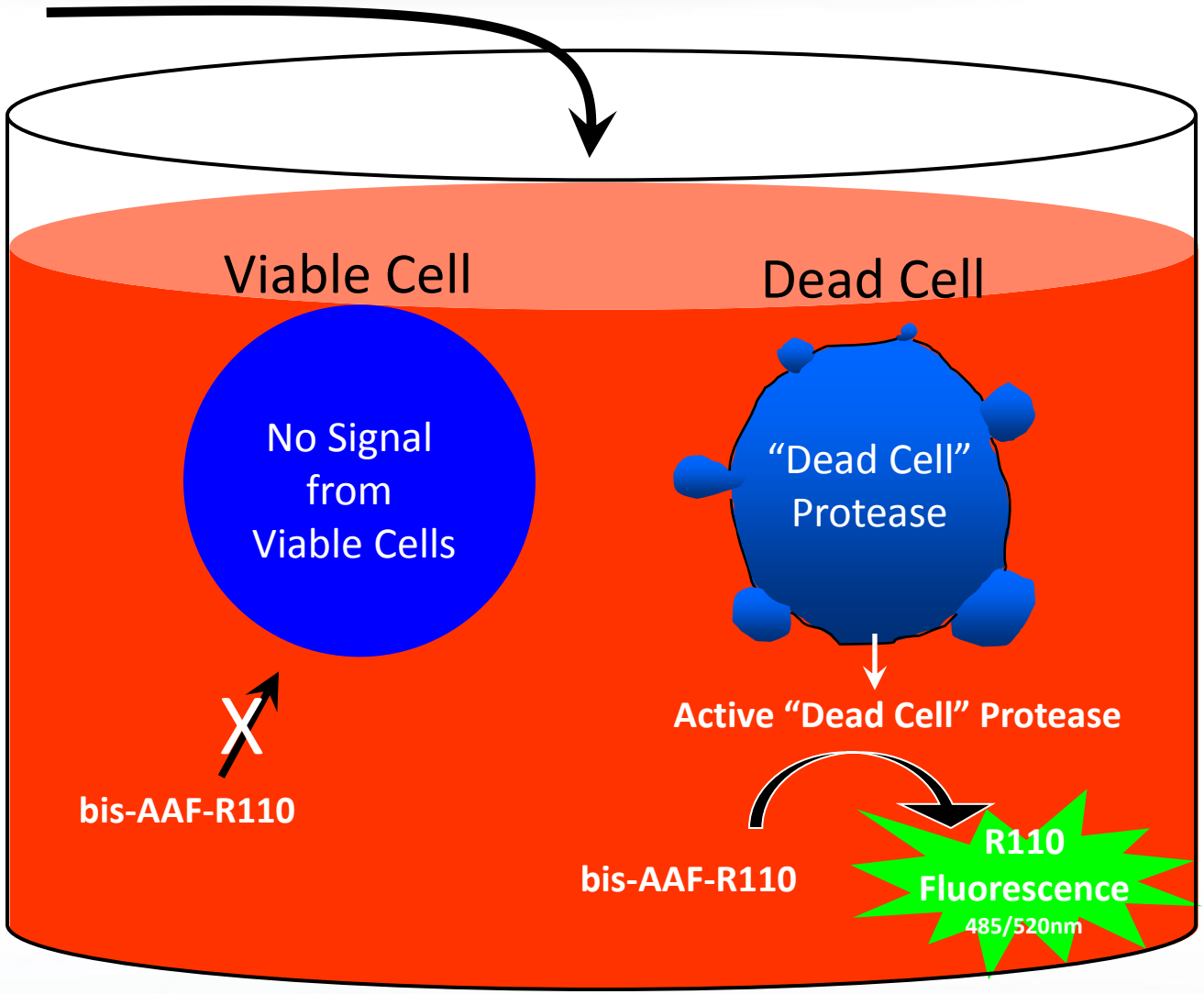
LDH activity in medium decreases after 24 hours

See: Assay & Drug Devel Tech 2(1): 51, 2004

Dead Cell Protease Assay (Fluorescent)

Impermeable Protease Substrate (bis-AAF-R110)

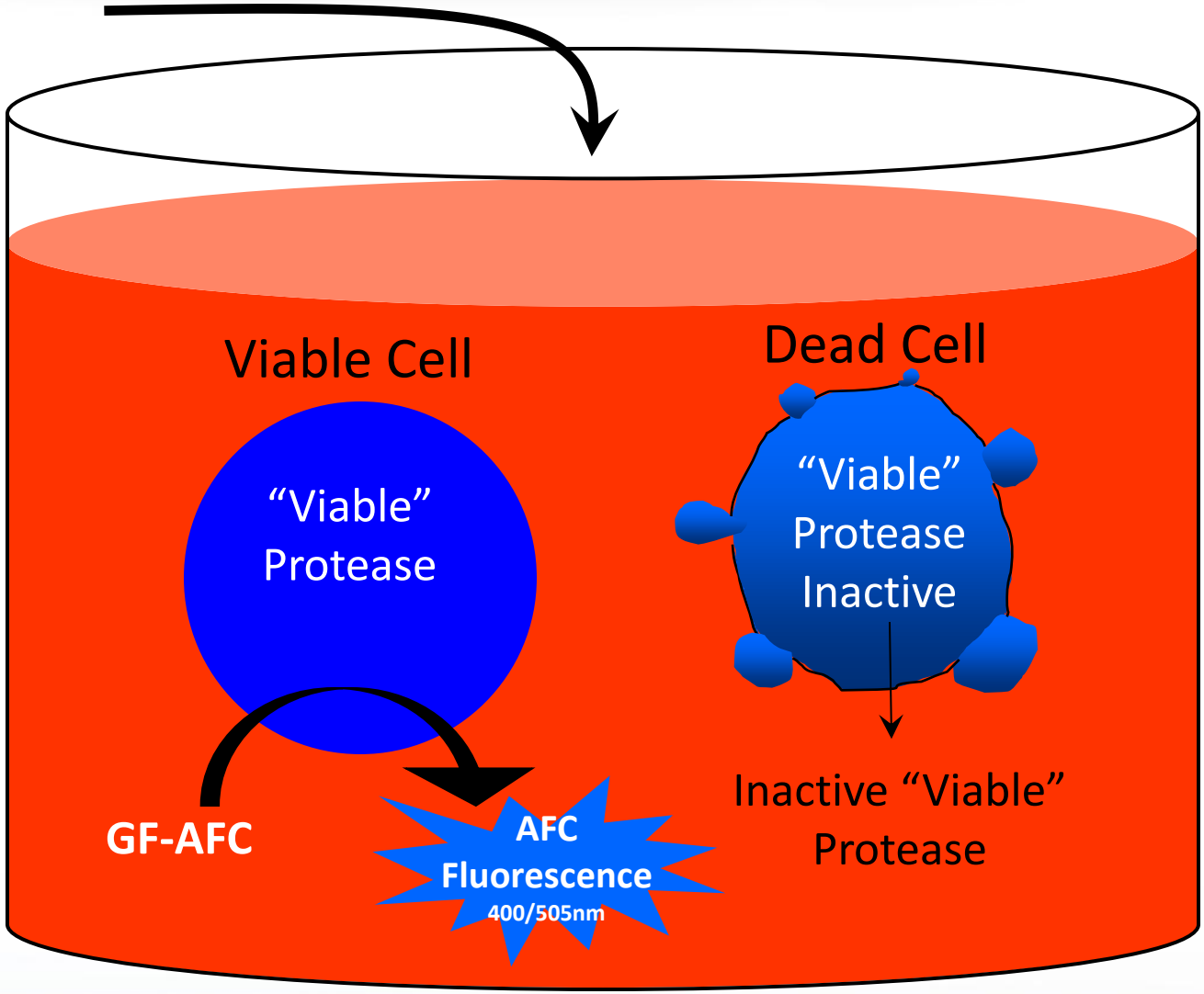
- "Dead" Cell protease remains active long after cell death
- Only signal is from "Dead" Cells
- Impermeable substrate can not enter viable cells



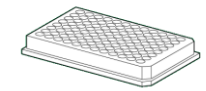
Viable Cell Protease Assay

Cell Permeable Protease Substrate (GF-AFC)

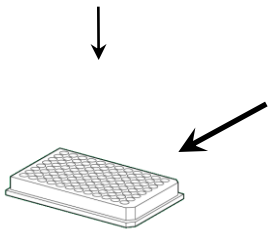
- Viable cells retain protease activity and generate signal
- "Viable" Cell protease becomes inactive upon cell death



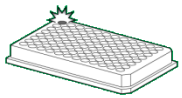
Multiplexing Measurement of Viable Cells & Dead Cells Simultaneously



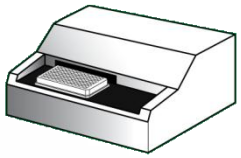
Subjected cells to various treatments



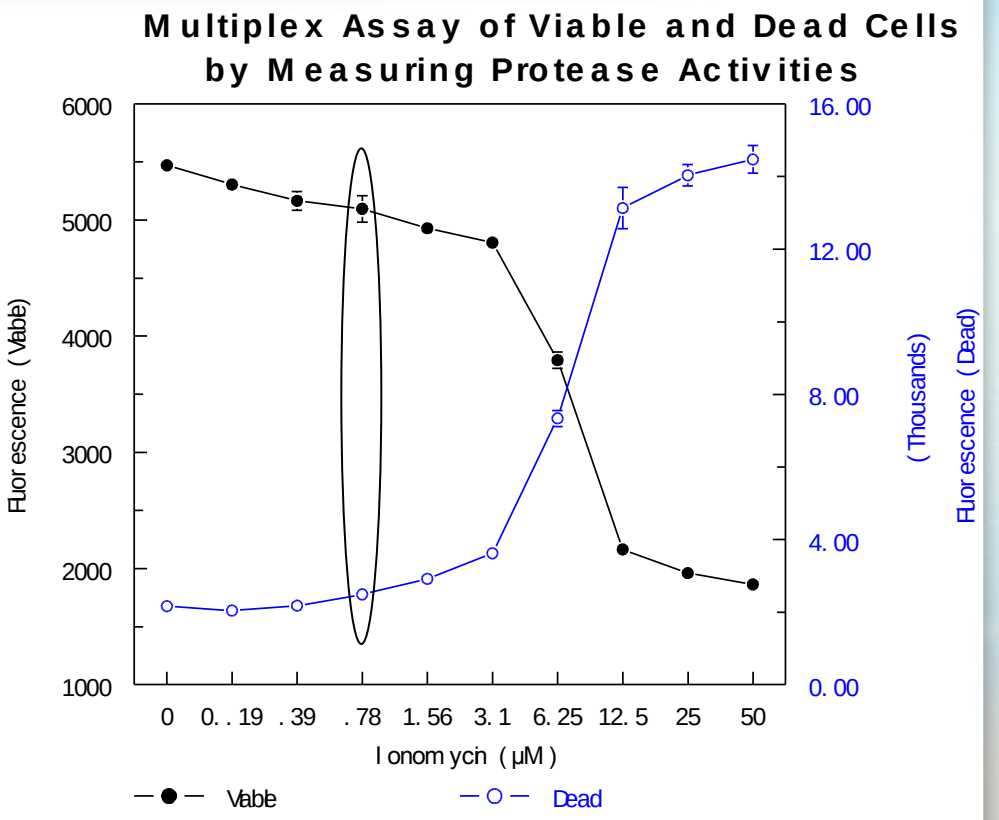
Add Reagent with both Substrates



Incubate 15-30 min



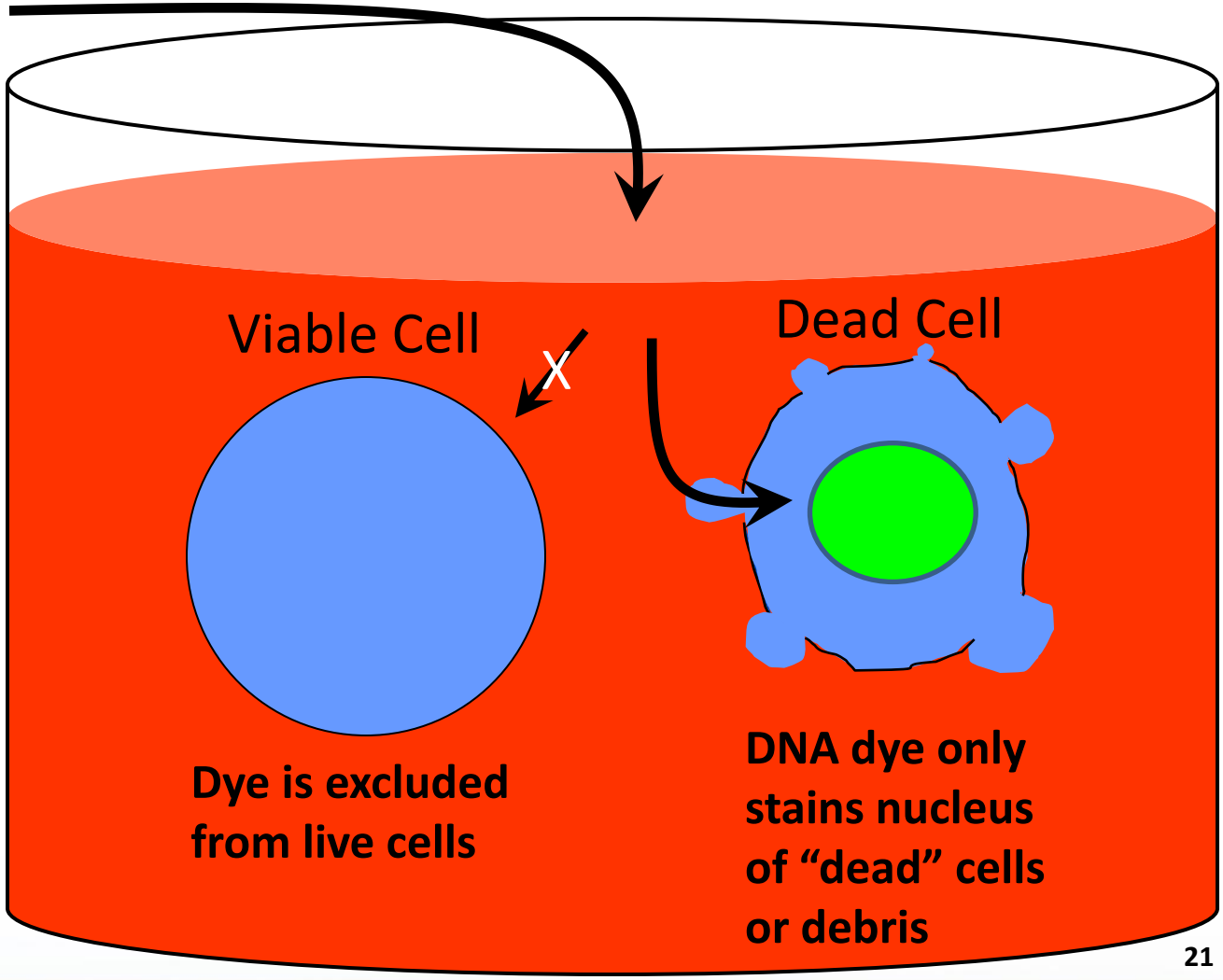
Record Fluorescence at 2 wavelengths



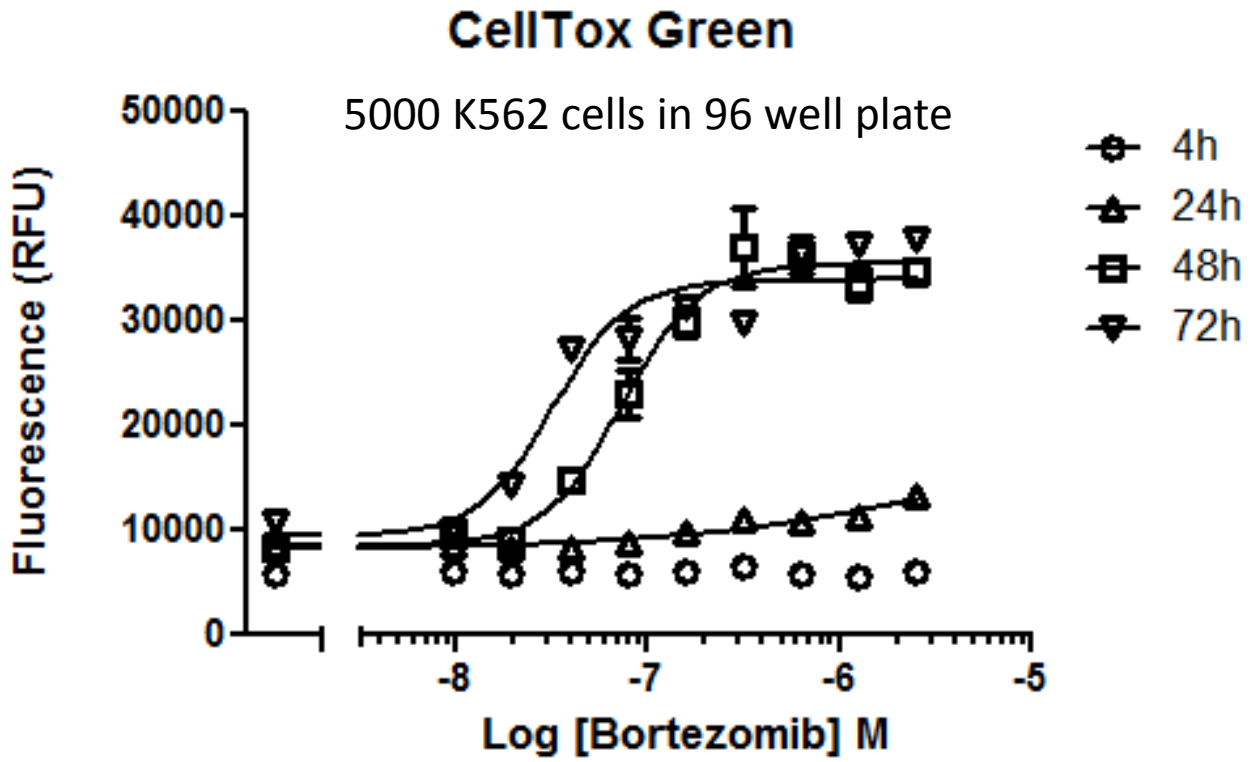
DNA Dye Staining to Detect Dead Cells
(Overcomes some limitations of short half-life markers)

Non-permeable DNA dye

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

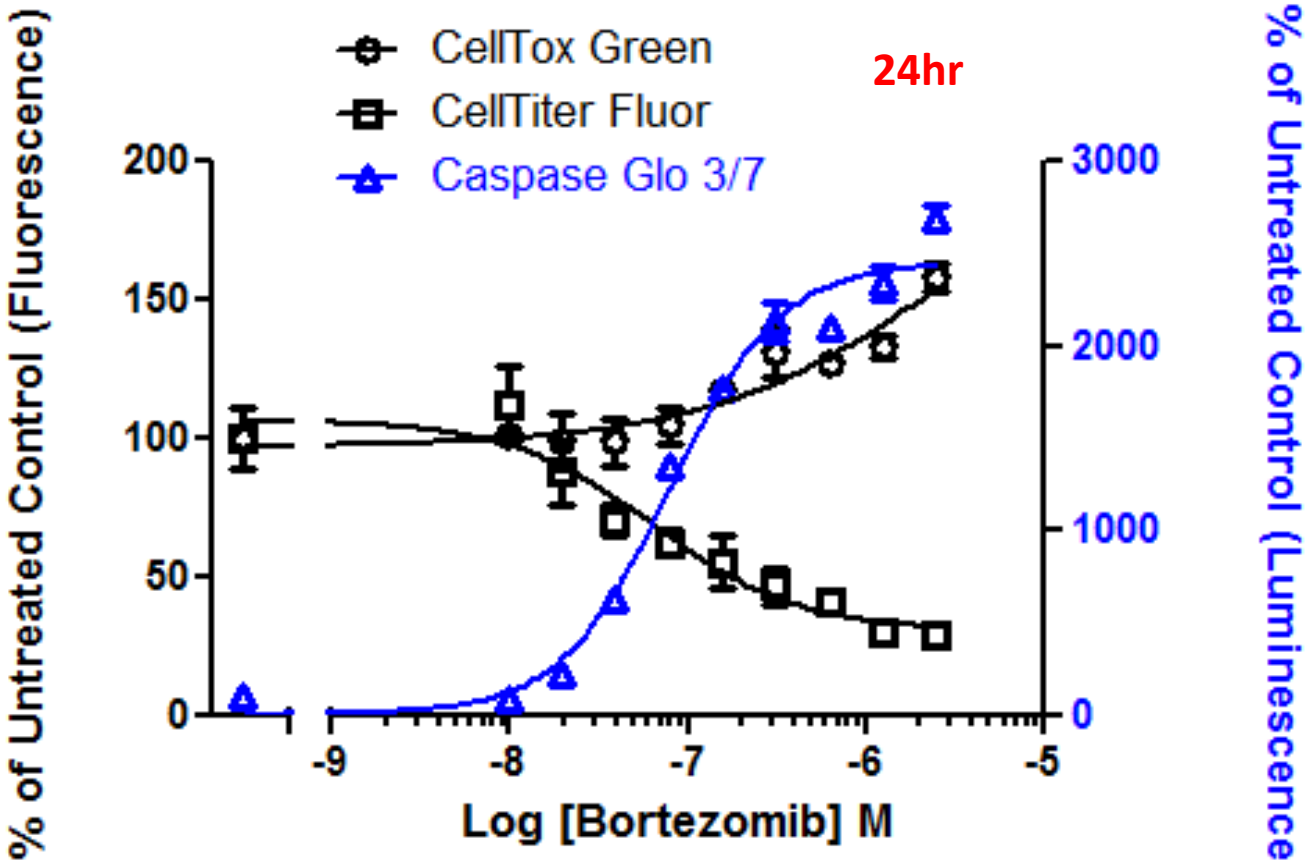


Reading the Same Plate Multiple Times to Detect the Onset of Cell Death (on the fly decision)



First appearance of cell death may trigger further experimentation with the same sample (e.g. How did the cells die? …apoptosis?)

Samples with CellTox Green can be Multiplexed with Cell Viability and Apoptosis Assays



Advantages & disadvantages of assays to detect dead cells

Assay	Advantages	Disadvantages
LDH release	Widely used and accepted Absorbance or fluorescent options	Limited sensitivity Limited half-life of LDH in medium
Protease release	Designed for multiplexing More sensitive than LDH Fluorescent reagent is simpler than formulation for LDH assay Fluorescent or luminescent options	Limited half-life of protease marker Fluorescence interference (fluorescent format only)
DNA Staining	Non-toxic / real time assay Staining persists for 72 hours Good choice for multiplexing	Fluorescence interference Less sensitive than amplified protease release assay

Assays to Determine Cell Stress Events Leading to Toxicity

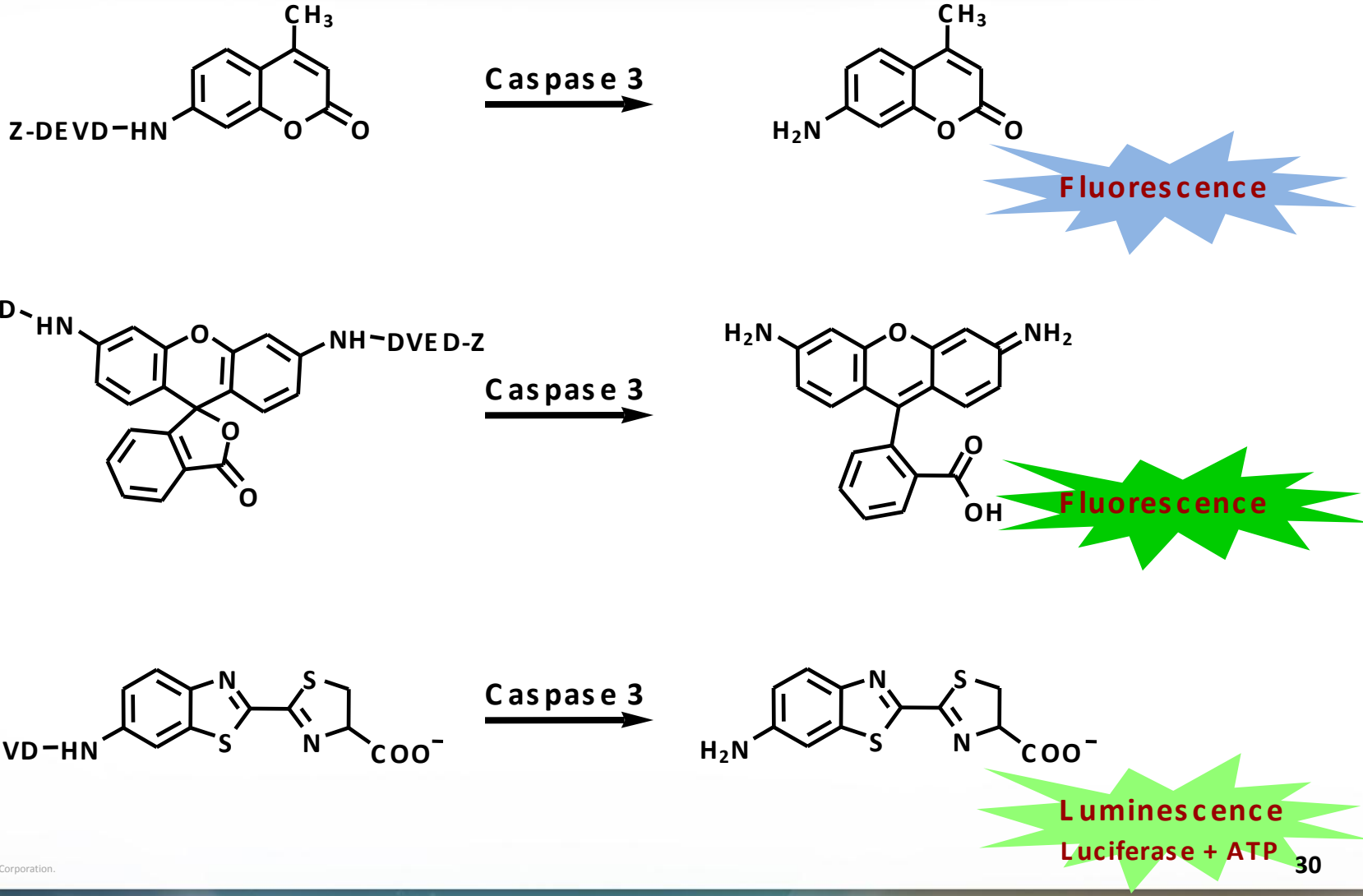
Determining Mechanisms Leading to Cytotoxicity

Going beyond the standard assays available to detect live or dead cells.

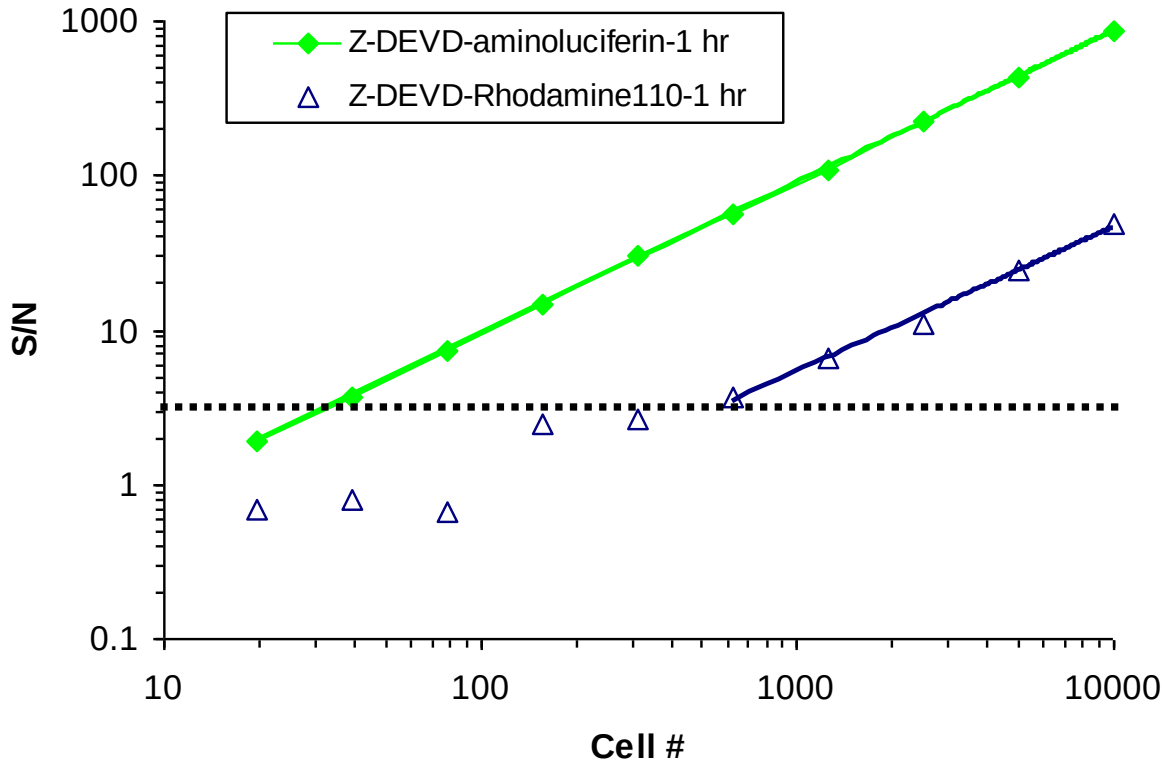
Assay chemistries and approaches to detect…

- Apoptosis
- Oxidative stress (ROS and GSH:GSSG ratio)
- Mitochondrial toxicity
- Genetic reporters to detect stress response pathways

AMC, R110 and aminoluciferin substrates for measuring caspase activity

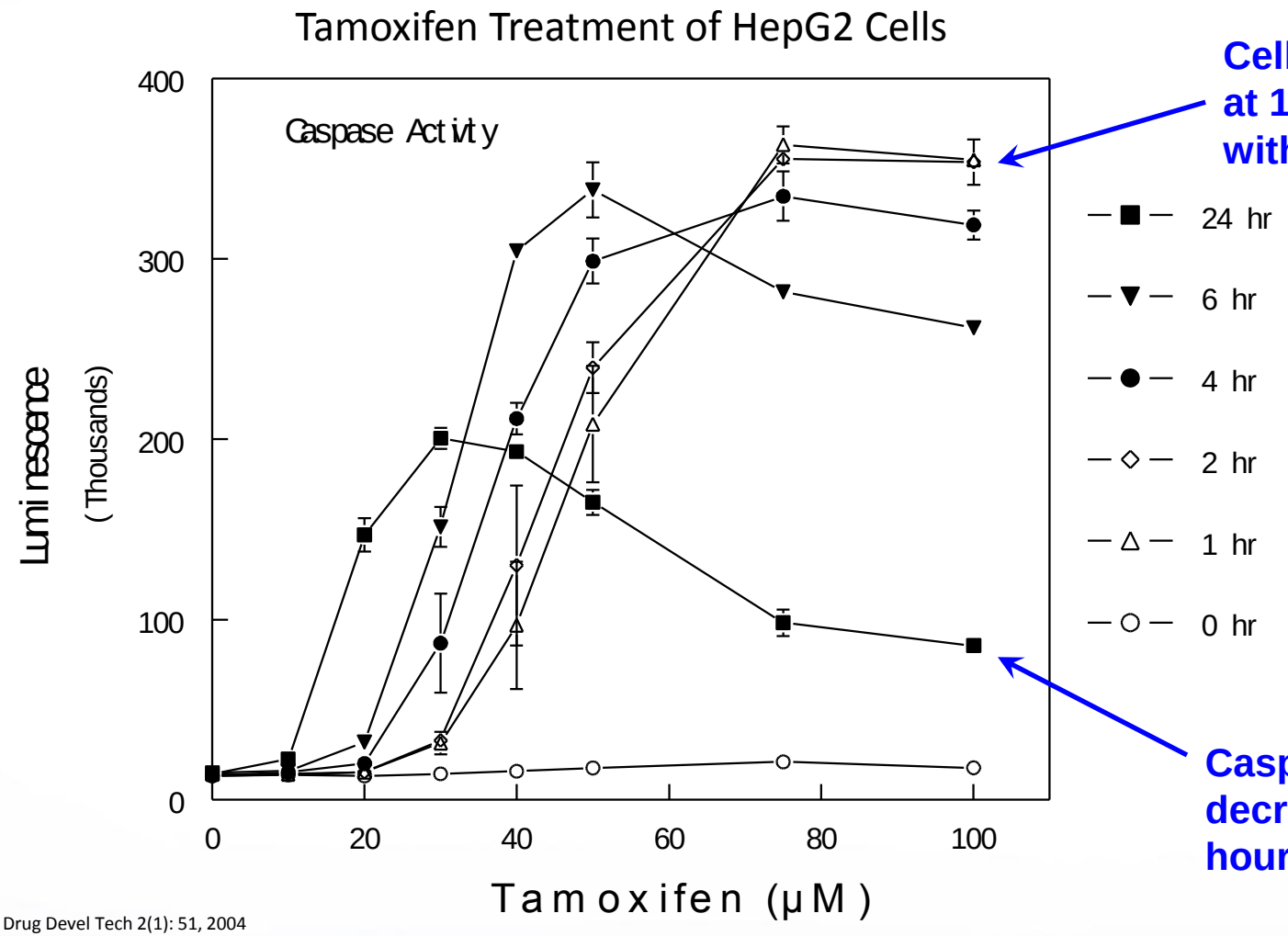


Luminescent assay has better sensitivity and dynamic range



O'Brien, M et al. (2005) Homogeneous bioluminescent protease assays: Caspase-3 as a Model. *J Biomol Screen* **10: 137.**

Caspase-Glo® 3/7 Time Course Indicates Caspase Activity is Transient



Cells are apoptotic at 1 hr treatment with 150µM Tamox

Caspase activity decreases after 24 hours incubation

Assay & Drug Devel Tech 2(1): 51, 2004

Oxidative Stress Assays

Oxidative Stress Assays

Oxidative stress: an imbalance between the production of reactive oxygen species (ROS) and the cell's capacity to detoxify the ROS or to repair the oxidative damage.

Markers of oxidative stress:

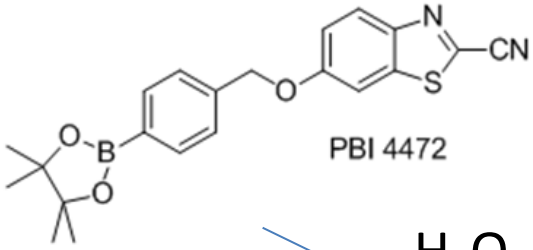
- Altered GSH:GSSG ratio (lowered GSH, increased GSSG)
- ROS (super oxide, hydroxyl radical, nitric oxide, hypochlorite convert to more stable **H_2O_2)**

ROS-Glo H_2O_2 Assay

- Direct H_2O_2 detection without using Horseradish Peroxidase (HRP)
  - Mitigates HRP mediated false hits
- Homogeneous Bioluminescent Assay
  - Add-mix-read
  - No fluorescence interference
- Cell based assay
  - Detects H_2O_2 content of culture wells

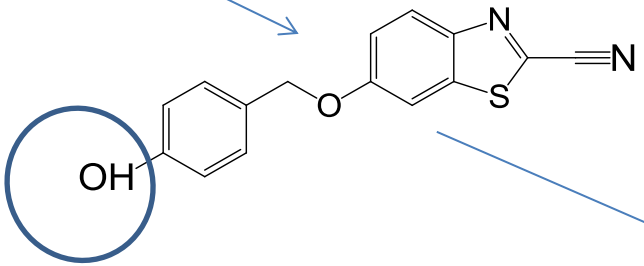
ROS-Glo Assay Chemistry Based on Pro-Luciferin

Modified Pro-luciferin (Peroxide Sensor)



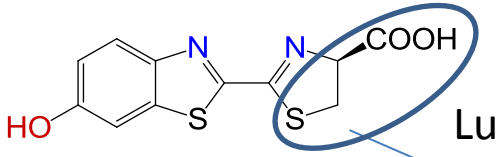
PBI 4472

H_2O_2



LDR (luciferase & ATP)

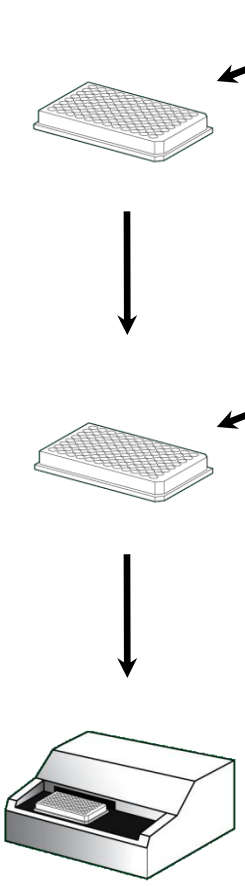
- Self-cleaving linker leaves
- D-Cys cyclization occurs



Luciferase

Light

ROS-Glo™ H_2O_2 Assay Protocol



Add test compound
and modified pro-luciferin
peroxide detector

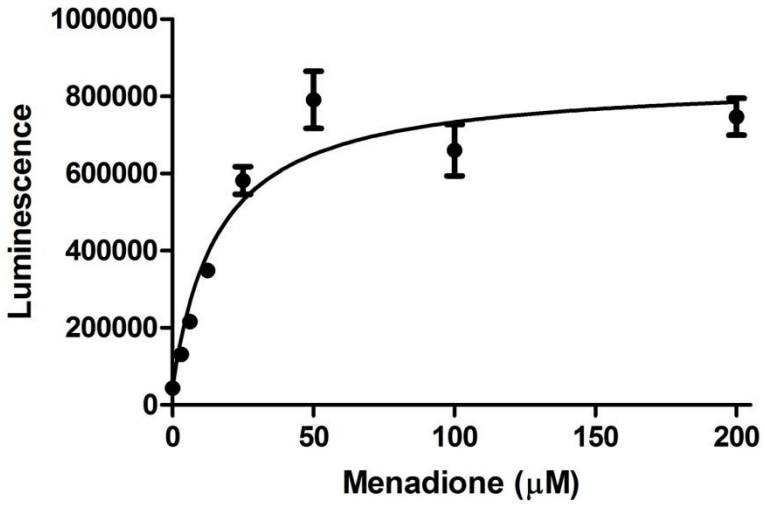
Incubate up to 2 hours

Add luciferin
detection reagent

Incubate 15 min

Record luminescence

ROS-Glo™ H_2O_2 Assay of Hep G2 Cells Treated with Menadione

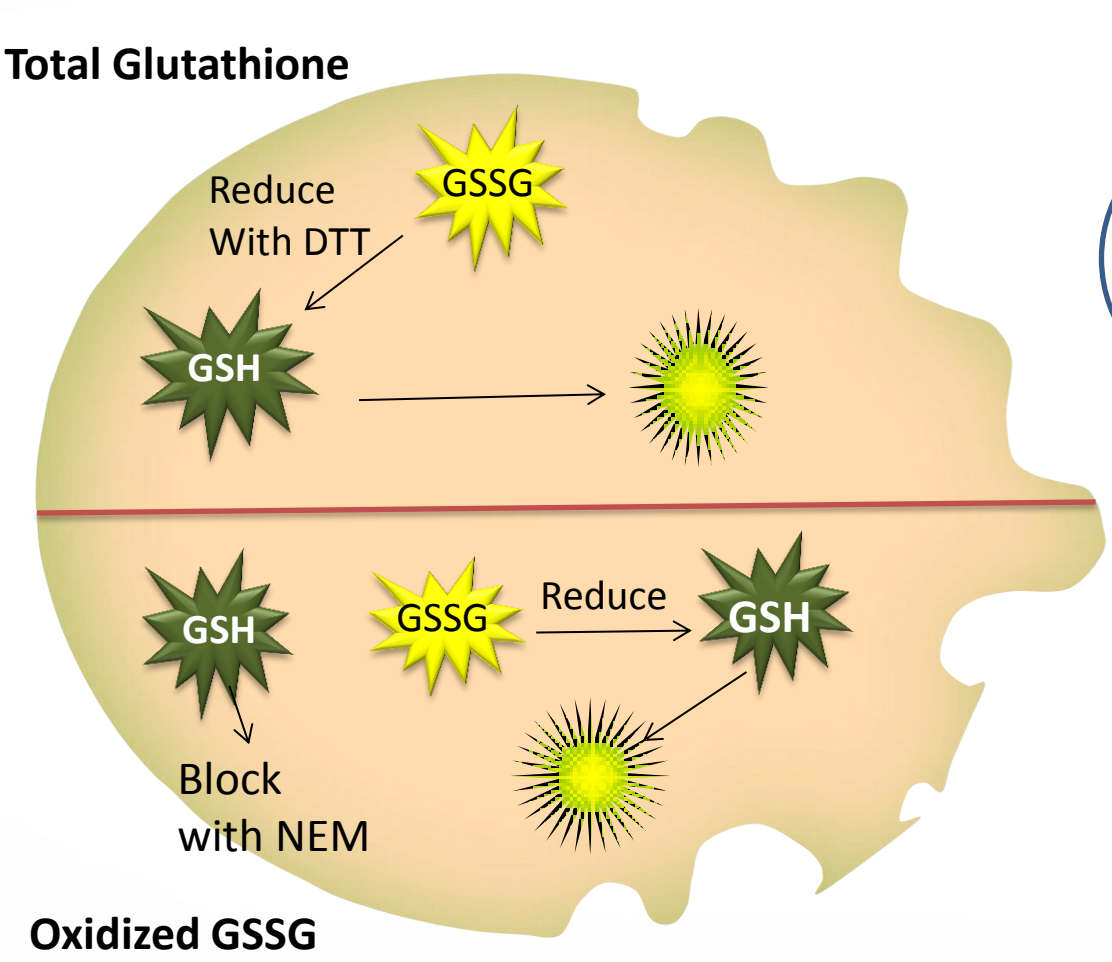


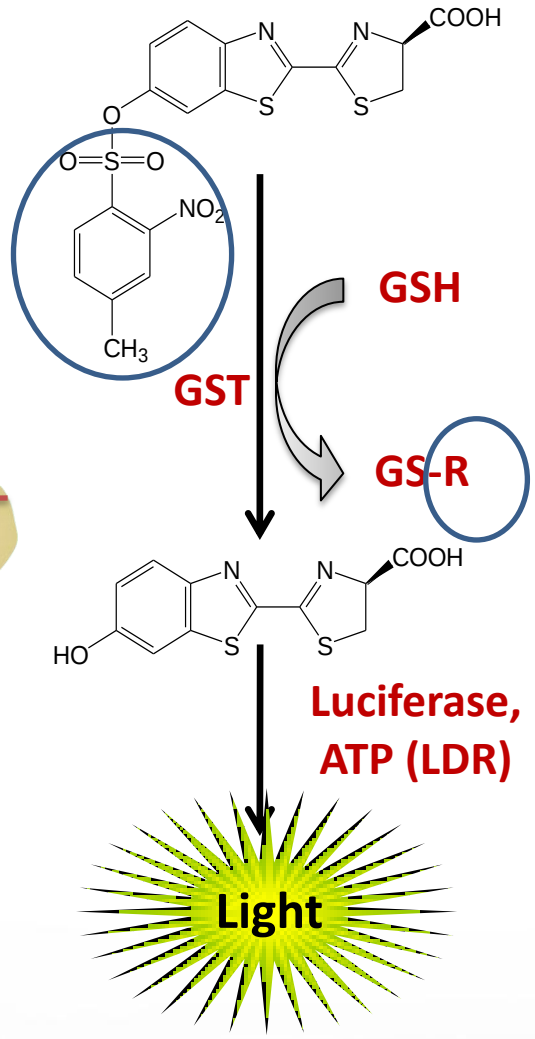
GSH Assay as Marker for Oxidative Stress

- Reduced form of glutathione (GSH) serves as an antioxidant in cells
- Decreased levels of GSH are associated with oxidative stress
- GSH and GSSG can be measured separately with a luminescent assay using Glutathione S Transferase (GST) and luciferase
- A fluorescent cell viability assay can be sequentially multiplexed with the luminescent GSH assay

Principal of GSH:GSSG Ratio Assay

(Assays must be run in parallel in separate wells.)

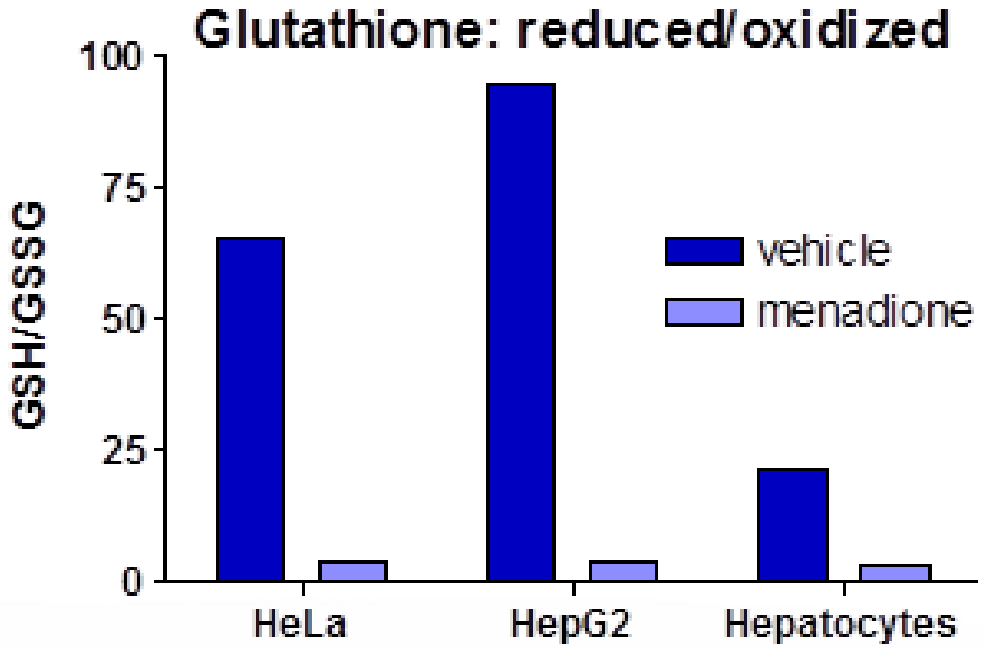




Menadione Treatment Drops GSH:GSSG Ratio

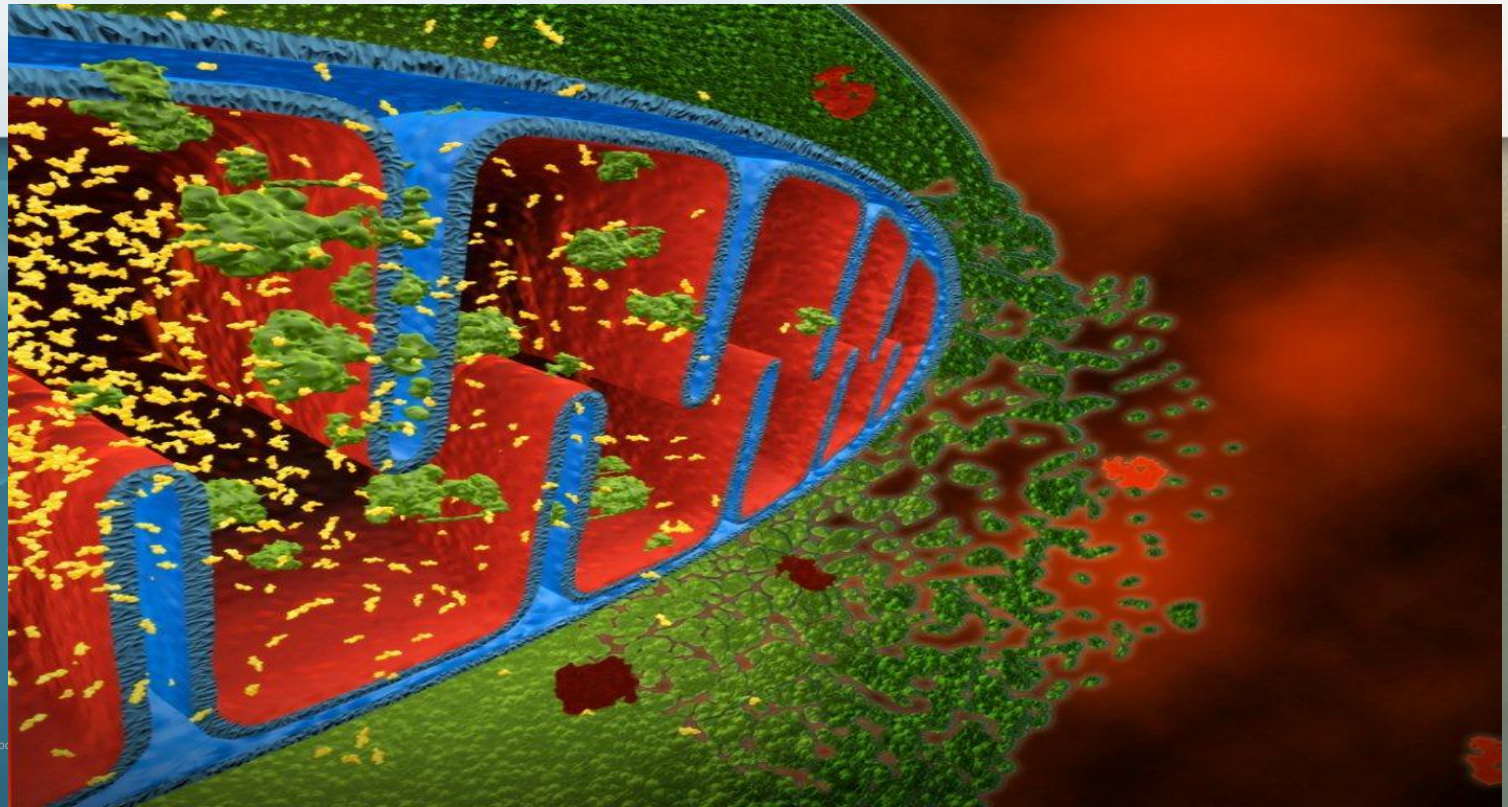
GSH:GSSG changes indicate:

- Oxidative Stress
- Compound toxicity
- Reactive metabolite formation



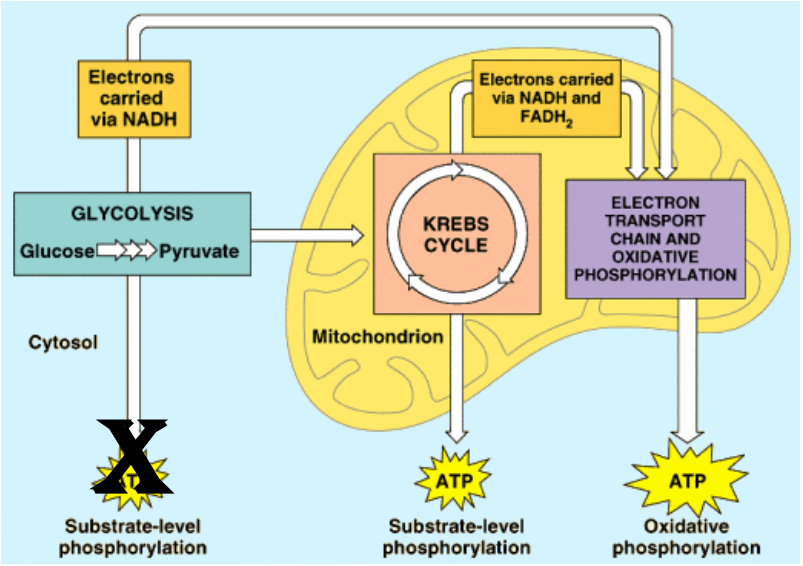
Detecting Mitochondrial Toxicity

Rapid multiplexed cell-based assays using plate reader



Detecting Mitochondrial Toxicity

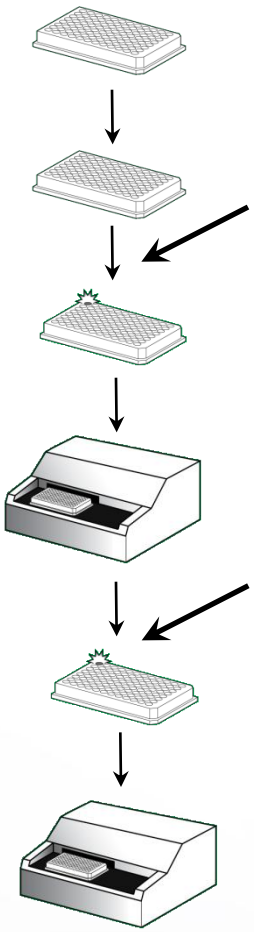
- ATP can be used as a marker of functioning mitochondria
- Net ATP production from glycolysis can be blocked by using glucose-free medium* (without serum reduces background)
- Decrease in ATP marker (without general necrosis) during 1-4hr incubation suggests mitochondrial toxicity
- ATP and membrane integrity assays can be multiplexed



* Lisa D. Marroquin, James Hynes, James A. Dykens, Joseph D. Jamieson, and Yvonne Will. Circumventing the crabtree effect: Replacing media glucose with galactose increases susceptibility of HepG2 cells to mitochondrial toxicants. Toxicol. Sci. 97:539 – 547, 2007.

Mitochondrial ToxGlo™ Assay
Multiplex membrane integrity and ATP content

Change to glucose-free serum-free medium + galactose



Treat cells
30 min - 4hr

bis-AAF-R110
Substrate

Incubate
30 min

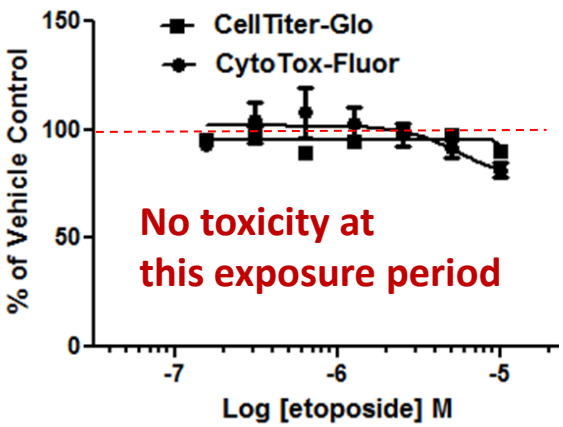
Record
Fluorescence

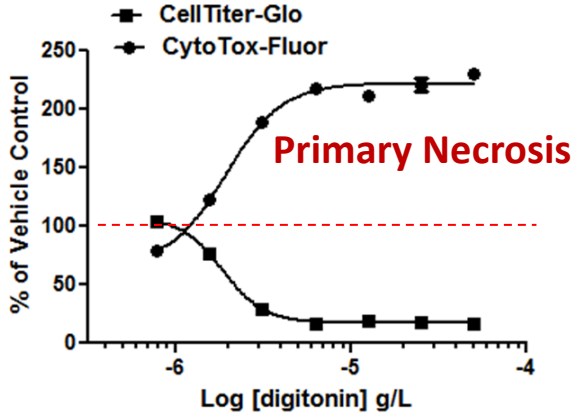
ATP Assay
Reagent

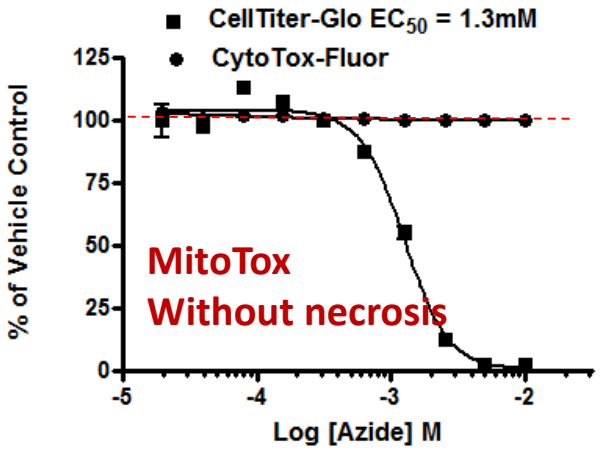
Incubate
10 min

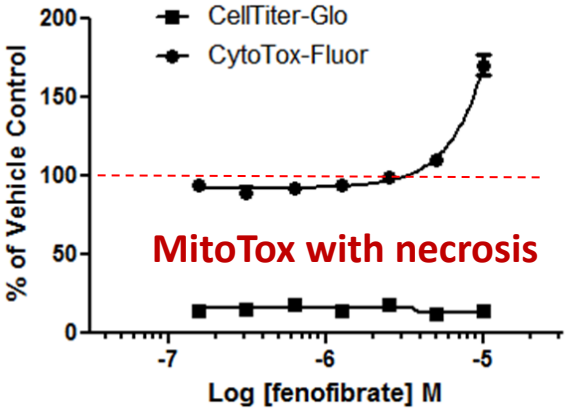
Record
Luminescence

Expected Assay Profiles









Cell Stress Response Pathway Reporters

Stress Response Pathways Leading to Cytotoxicity

- Stress response pathways are toxin activated signal transduction events that modulate transcription factors to trigger expression of cytoprotective genes to enable the cell to attempt to restore homeostasis.*
- Triggering cell response pathways occurs at lower toxin doses or exposure times than what is needed to trigger necrosis or apoptosis.
- If stress cannot be overcome to re-establish homeostasis, the result is induction of apoptosis and removal of the cell.

*Simmons, S.O. et al., Cellular stress response pathway system as a sentinel ensemble in toxicological screening. Tox. Sci. 111(2): 202-225, 2009.

Stress and Toxicity Pathway Vectors

http://www.promega.com/resources/articles/pubhub/novel-pgl4-reporter-vector-panel-for-profiling-cellular-stress-and-chemical-toxicity/

MAPK/JNK	Activation Protein 1 (AP1)	AP1 Response Element (AP1 RE)	pGL4.44[*luc2P*/AP1 RE/Hygro]	E4111
Inflammation	Nuclear Factor κB (NF-κB)	NF-κB Response Element	pGL4.32[*luc2P*/NF-κB-RE/Hygro]	E8491
Oxidative Stress	NF-E2-related factor 2 (Nrf2)	Antioxidant Response Element (ARE)	pGL4.37[*luc2P*/ARE/Hygro]	E3641
DNA Damage	p53	p53 Response Element (p53 RE)	pGL4.38[*luc2P*/p53 RE/Hygro]	E3651
Endoplasmic Reticulum Stress	Activating Transcription Factor 6 (ATF6)	ATF6 Response Element (ATF6 ERSE)	pGL4.39[*luc2P*/ATF6 RE/Hygro]	E3661
Heavy Metal Stress	Metal-Responsive Transcription Factor-1 (MTF1)	Metal Regulatory Element (MRE)	pGL4.40[*luc2P*/MRE/Hygro]	E4131
Heat Shock	Heat Shock Factor 1 (HSF1)	Heat Shock Element (HSE)	pGL4.41[*luc2P*/HSE/Hygro]	E3751
Hypoxia	Hypoxia-Inducible Factor 1α (Hif1α)	Hypoxia Response Element (HRE)	pGL4.42[*luc2P*/HRE/Hygro]	E4001
Xenobiotic Stress	Aryl hydrocarbon receptor (AhR)	Xenobiotic Responsive Element (XRE)	pGL4.43[*luc2P*/XRE/Hygro]	E4121

Cell lines available as custom products

http://www.promega.com/a/forms/custom-assays/custom-assay-services.html

Cell Lines for Toxicity Pathway Analysis

		Pathway	Response Element	Cell line background
☐	1	Antioxidant	ARE	HEK293
☐	2	Hypoxia	HIF1	HEK293
☐	3	Hypoxia	HIF1	HEPG2
☐	4	MAPK	AP1	HEK293
☐	5	Ras/MEK-1	SRE	HEK293
☐	6	RhoA (Gα12/13)	SRF	HEK293
☐	I am interested in a different toxicity pathway cell line			

Validating Performance of Cytotoxicity Assays Applied to 3D Culture Models

One of many Examples to Determine Predictive Value of 3D Culture Models

Activity of Anticancer Agents in a Three-Dimensional Cell Culture Model

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ABSTRACT

Cell-monolayer-based assays for chemotherapeutic drug discovery have proven to be highly artificial compared with physiological systems. The objective of this study was to culture cancer cells in a simple 3-dimensional (3D) collagen gel model to study the antiproliferative activity of known lung cancer drugs. The validity of our 3D model was tested by measuring the activity of 10 lung cancer drugs (Paclitaxel, Alimta, Zactima, Doxorubicin, Vinorelbine, Gemcitabine, 17AAg, Cisplatin, and 2 experimental drugs from the University of Kansas [KU174 and KU363]) in 2 lung cancer cell lines (A549 and H358) and comparing the activity in a traditional 2-dimensional (2D) in vitro cellular assay. Both potency and efficacy of these drugs were calculated to evaluate the activity of the drugs. Our results demonstrate that the activity of these drugs showed significant differences when tested in 3D cultures, which varied with individual drugs and the cell line used for testing. For example, the cytotoxicity of Paclitaxel, KU174, Alimta, Zactima, Doxorubicin, Vinorelbine, KU363, and 17AAg was significantly changed when tested in the 3D model, whereas the potency of Cisplatin and Gemcitabine in H358 cell line remained unaffected. A similar pattern, with some differences, was observed in A549 cells and is discussed in detail in this article. The observed differences in potency and efficacy of the cancer drugs in 3D models suggest that the biological implications of screening configurations should be taken into account to select superior cancer drug candidates in preclinical studies.

INTRODUCTION

Cancer is the second leading cause of death in the United States, with lung cancer having the highest mortality rate.[1] With this high rate of death, it is becoming more imperative to find new molecular entities (NME) that can be rapidly advanced to the clinic by using improved high-throughput screening (HTS) and lead optimization strategies. Currently, NME for biological and pharmacological activities are screened by growing cells as a 2-dimensional (2D) monolayer culture. Cell monolayers are physiologically different compared with 3-dimensional (3D) human tissues or tumors.[2,3] Monolayer cell cultures are not a physiologically relevant environment for NME screening, and therefore may lead to the low predictability of these 2D models in preclinical trials.[4] Although the conventional focus of chemotherapeutic drug development has been on tumor cells, recent studies show that interactions with the stroma (*Fig. 1*) might influence drug sensitivity and development of drug resistance.[5] The extracellular matrix (ECM), which is considered part of the stroma and has been shown to activate signaling pathways, might not have been present when the drugs were tested in 2D cell-based assays, rendering this approach less representative of a human tissue or tumor[6] and therefore could contribute to less success in animal studies and clinical trials.

To closely mimic the *in vivo* behavior of normal or cancerous human tissue, a 3D model needs to be used.[4,6–10] Although 3D cultures of lung cancer cells have been used to study various aspects of tumorigenesis and tumor progression,[6,11] to the best of our knowledge, these models have yet to be used to screen new lung cancer drugs. Using a 3D tumor model as a strategy to test NMEs is more physiologically relevant than the traditional 2D cell culture

ABBREVIATIONS: ANOVA, analysis of variance; AUC, area under the curve; DMSO, dimethyl sulfoxide; ECM, extracellular matrix; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; HTS, high-throughput screening; NME, new molecular entities; PBS, phosphate-buffered saline; 3D, 3-dimensional; 2D, 2-dimensional; VEGFR2, vascular endothelial growth factor receptor 2.

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"The observed differences in potency and efficacy of the cancer drugs in 3D models suggest that the biological implications of screening configurations should be taken into account to select superior cancer drug candidates in preclinical studies."

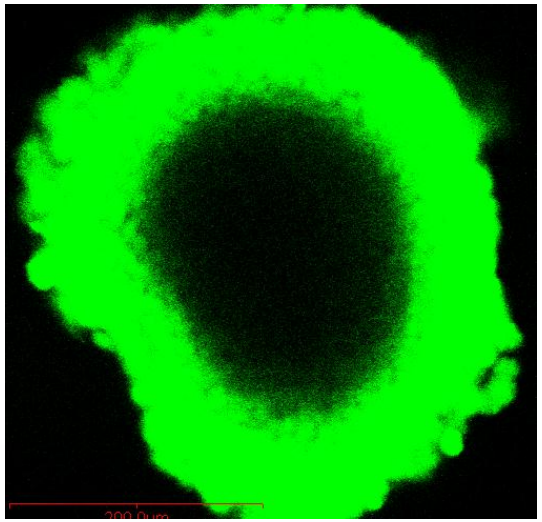
Critical Experiment that Prompted Much of our Effort to Begin Validating Promega Assays

Observation of Lytic Efficiency of ATP Assay Reagents

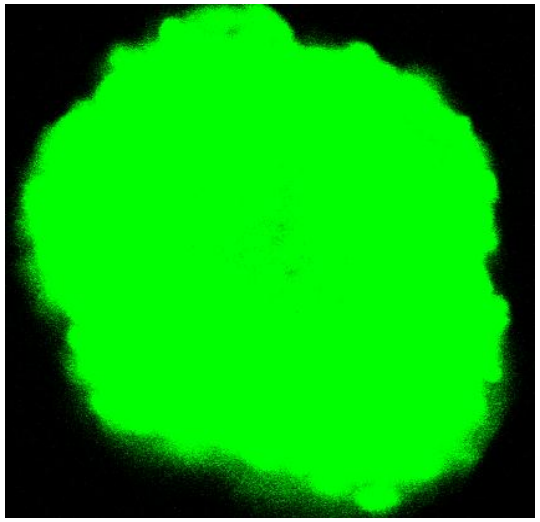
- Microspheres grown to ~350µm using hanging drop method
- Add ATP assay reagents + DNA dye to indicate lytic effectiveness
- Photograph using laser confocal microscopy

~350 µm spheroids

ATPLite 1-Step Reagent



CellTiter-Glo® Reagent



Chad Zimprich, Mike Valley

Improved ATP Assay Reagent Protocol

CellTiter-Glo® Assay

- Equilibrate plate to ambient temperature
- Add equal volume of reagent
- Wait 10 min for signal to equilibrate
- Record luminescence

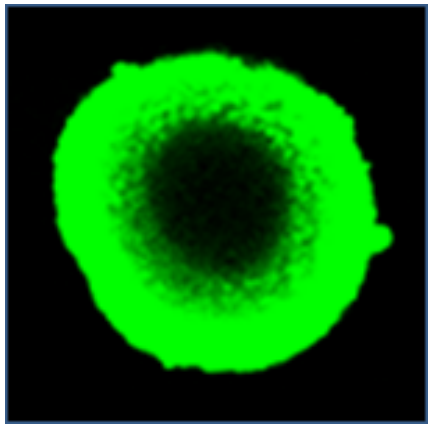
CellTiter-Glo® 3D Assay

- Equilibrate plate to ambient temperature
- Add equal volume of reagent
- **Shake 5 min 450 rpm**
- Wait **25 min** for signal to equilibrate
- Record luminescence

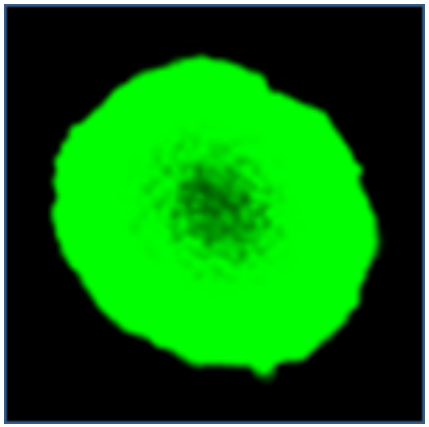
Caspase-Glo® 3/7 Assay: Protocol Optimization to Facilitate Enhanced Microtissue Cell Lysis

- HCT116 cell spheroids grown to ~330µm using hanging drop method
- Add Caspase-Glo® 3/7 assay reagent + DNA dye to indicate lytic effectiveness
- Shake with assay reagent for 5 or 30 min
- Image with confocal after a total of 30 min incubation with reagent

5 min shake



30 min shake



- Increased shake time results in near complete spheroid cell lysis

- ***Note: Human liver microtissues undergo complete lysis and disruption using these conditions (data not shown)***

Take Home Message

- Most cell-based assays were designed for 2D culture models
- Assay effectiveness depends on size of microtissue
- Validation should be done for each assay and 3D culture model
- Promega is in the process of validating many of our cell health and genetic reporter
- Collaborations to validate assays on model systems are welcome

Summary

- Bioluminescent assays
- Live and Dead Cell Assays
- “Real Time” Assays
- Apoptosis
- Stress Events Leading to Cytotoxicity
- Validation of assays for 3D culture models