Multiplexing Cell-Based Assays:
Get More Biologically Relevant Data

Fall 2010

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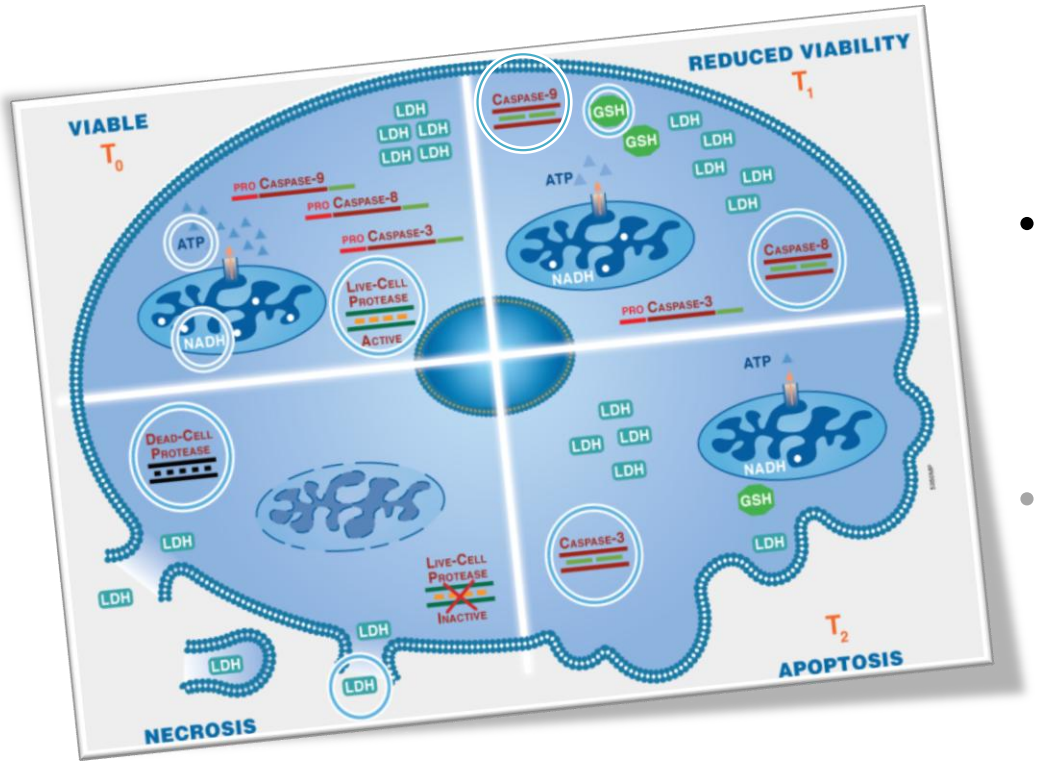
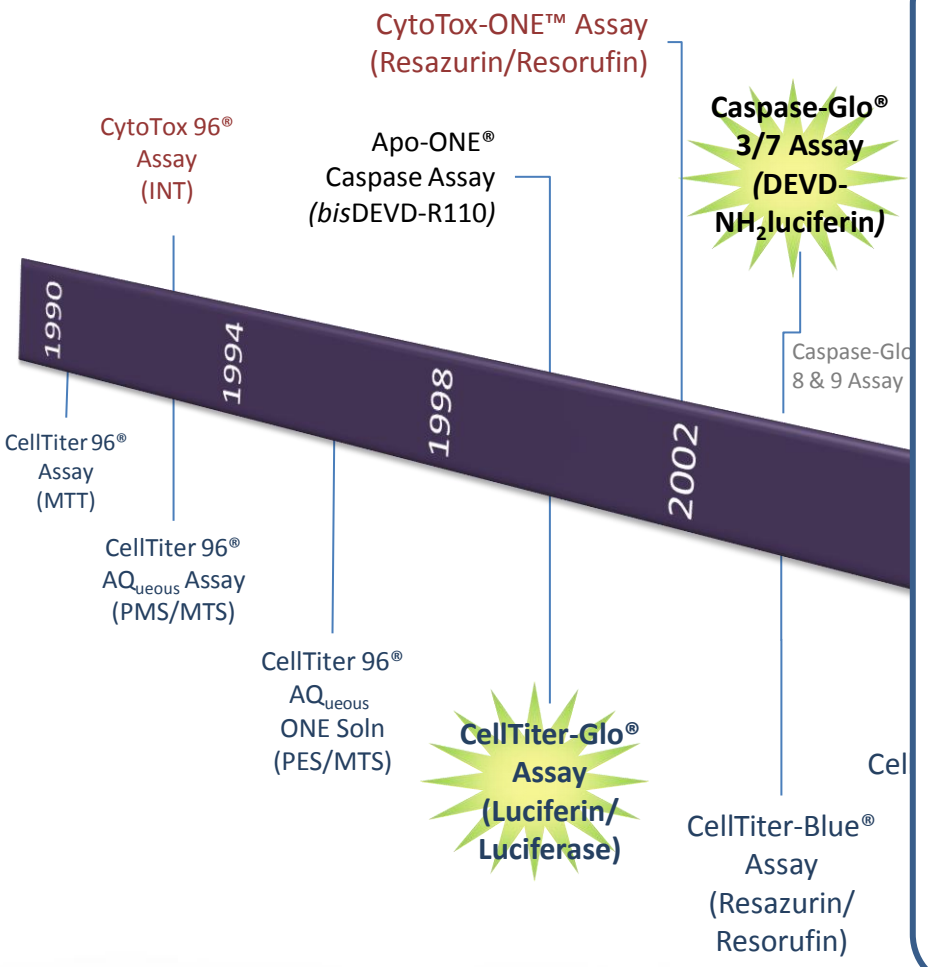
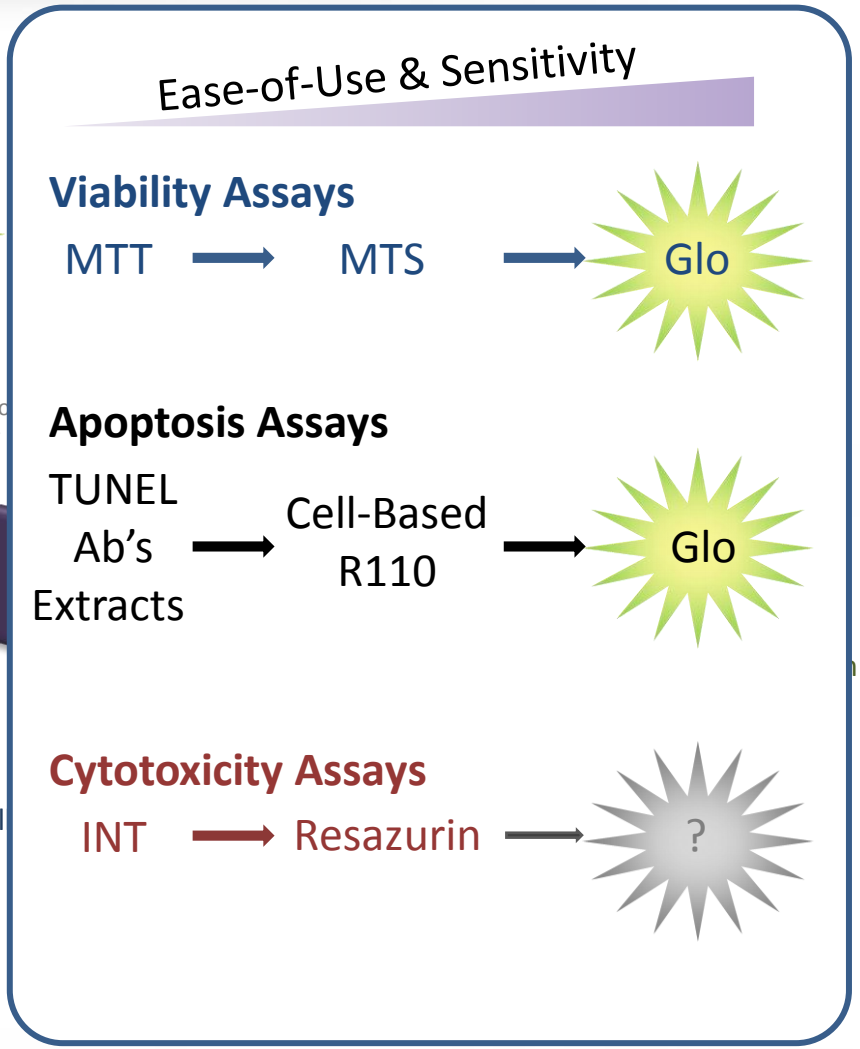
Multiplexing assays for more informative data



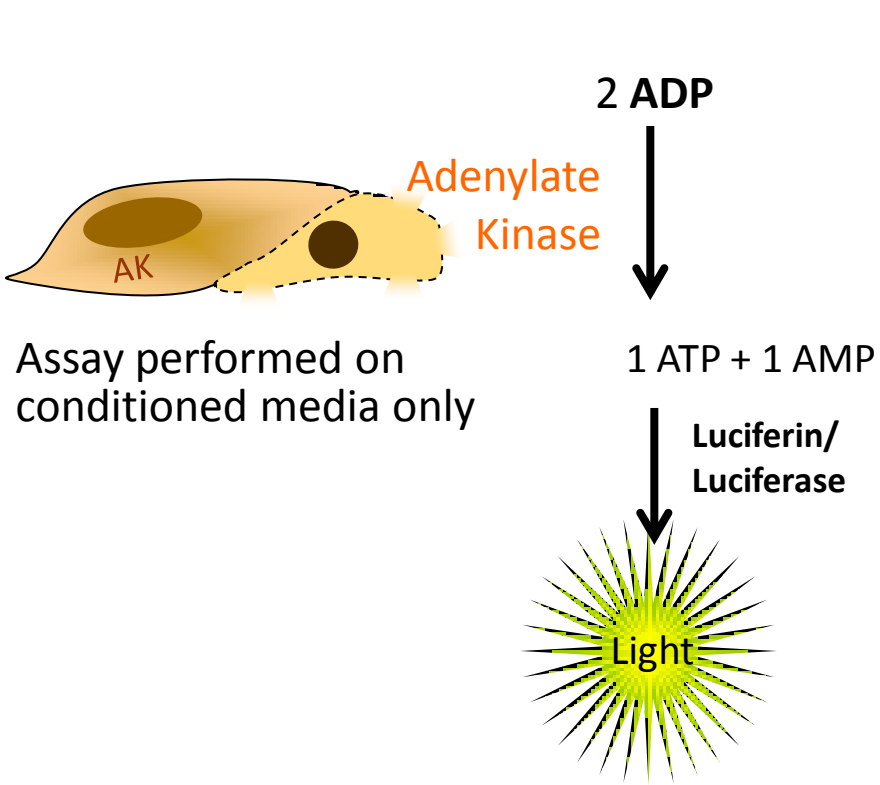
- Plate-based assays for viability, cytotoxicity and apoptosis measurement
- Using multiplex assays to understand cell death mechanism
- Monitoring cell response in multiple applications

Development timeline for cell-based viability, cytotoxicity and apoptosis assays

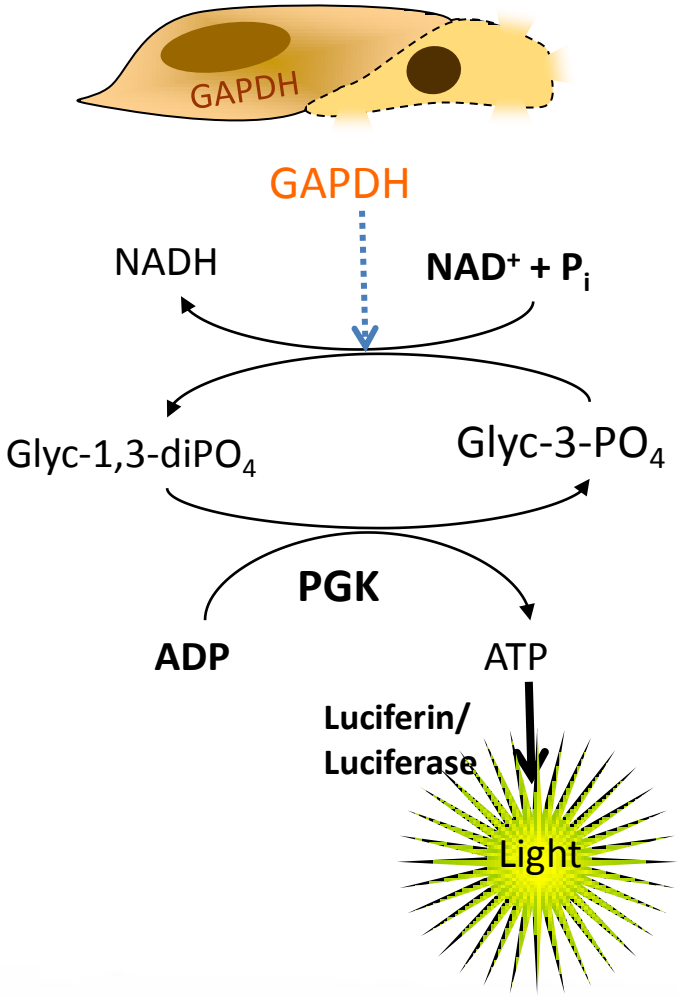




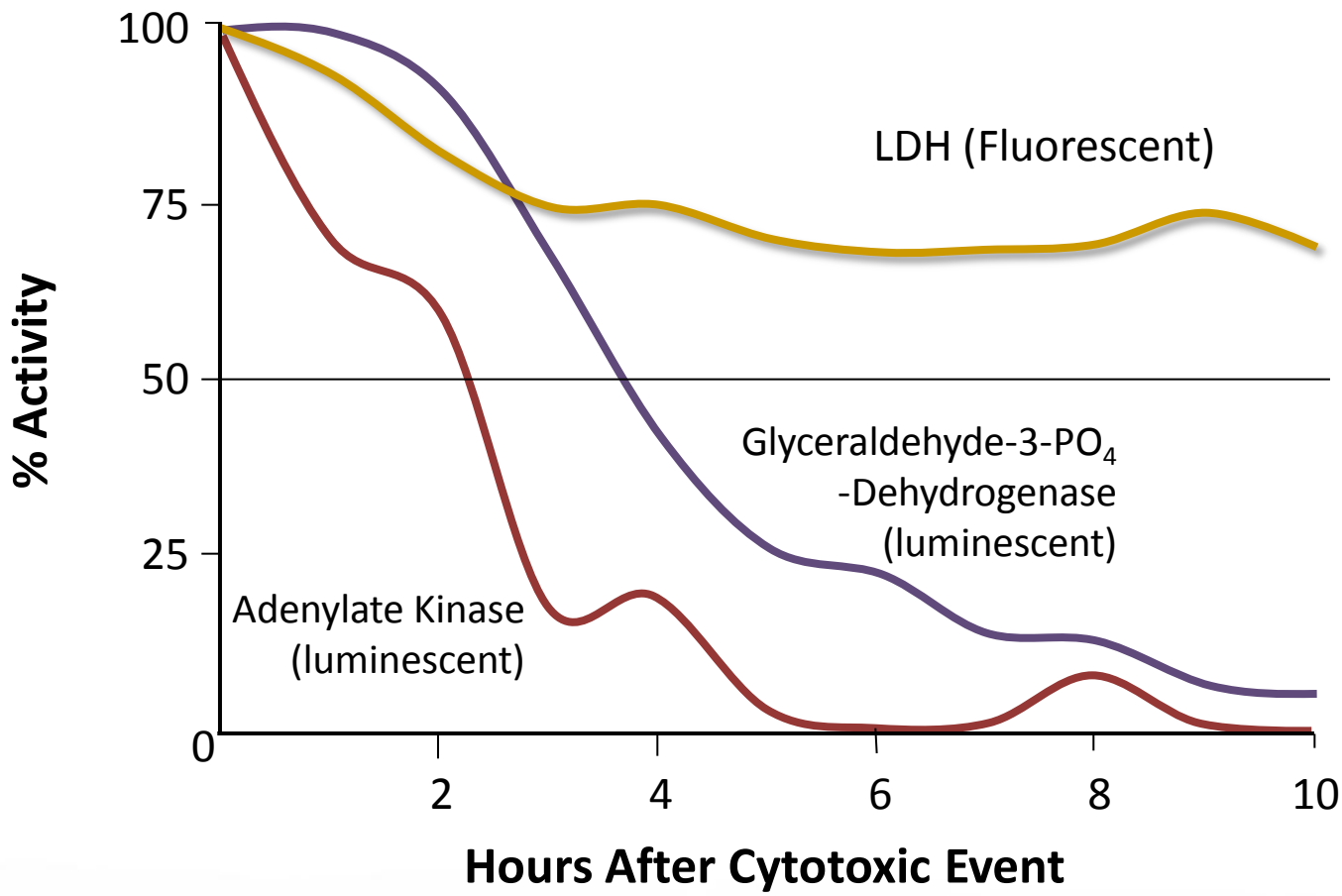
Other enzymatic markers of cytotoxicity



Assay performed on conditioned media only



Luminescent methods where not ideal…



Two Protease activities = live/dead cell assay

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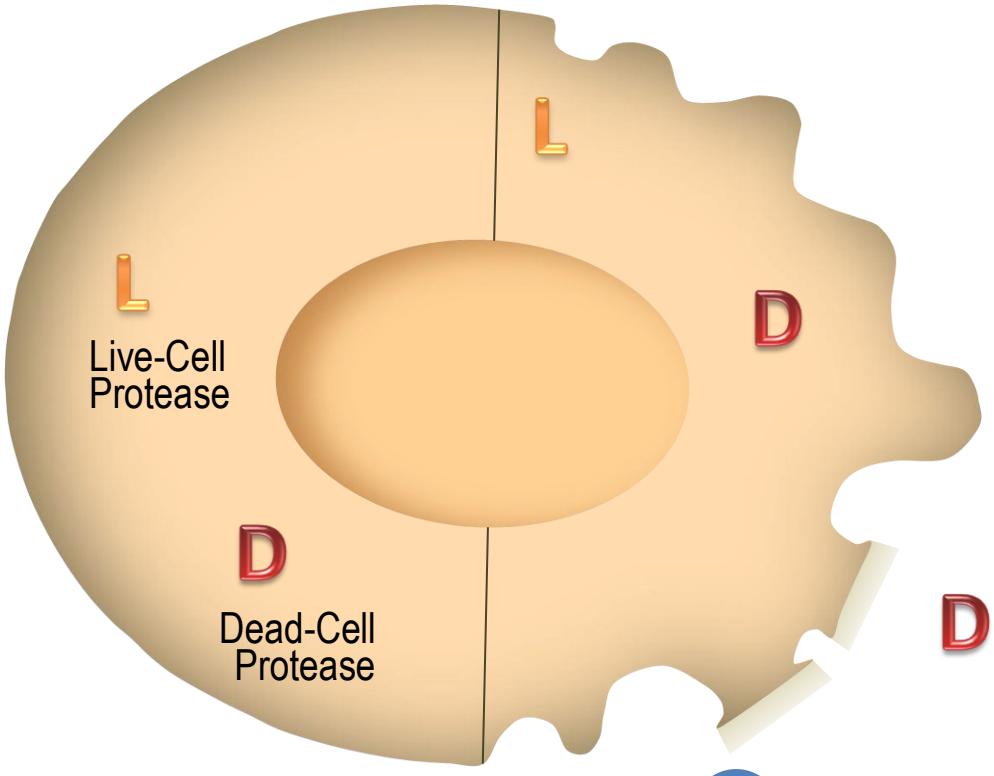
Are my cells living? **Are my cells dying?**



Live-Cell Protease quickly inactivated outside the cell.

Available online at www.sciencedirect.com
ScienceDirect
Analytical Biochemistry 366 (2007) 197–206
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ANALYTICAL BIOCHEMISTRY

A homogeneous assay to measure live and dead cells in the same sample by detecting different protease markers

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Abstract

A method to simultaneously determine the relative numbers of live and dead cells in culture by introducing a combination of two fluorogenic substrates or a fluorogenic and a luminogenic protease substrate into the sample is described. The method is based on detection of differential ubiquitous proteolytic activities associated with intact viable cells and cells that have lost membrane integrity. A cell-permeable peptide aminofluorocoumarin substrate detects protease activity restricted to intact viable cells. Upon cell death, the viable cell protease marker becomes inactive. An impermeable peptide rhodamine 110 (or aminoluciferin) conjugated substrate detects protease activity from nonviable cells that have lost membrane integrity. The multiplex assay can detect 200 dead cells in a population of 10,000 viable cells. The protease substrate reagents do not damage viable cells over the course of the assay, thus the method can be multiplexed further with other assays in a homogeneous format. Ratiometric measurement of viable and dead cells in the same sample provides an internal control that can be used to normalize data from other cell-based assays.
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Keywords: Cell-based; Cytotoxicity; Viability; Multiplex; Fluorescence; Luminescence; Protease; Apoptosis; Homogeneous; High throughput

It is an important and necessary experimental practice to determine the viability of cells in culture after chemical, biological, or physical treatment and manipulation. Maintenance of membrane integrity is a common criterion for cell viability. Measurable changes in membrane permeability include trypan blue exclusion, nucleic acid staining, and ^{51}Cr or lactate dehydrogenase release [1,2]. Conversely, measures of viability by metabolic capacity include tritiated thymidine incorporation, ATP content, tetrazolium dye reduction, and fluorescein diacetate labeling [3].

These existing techniques have a number of technical or practical drawbacks which limit their utility in multiplexed or high-throughput formats. For instance, cellular ^{51}Cr release assays require significant prelabeling preparation, and all assays utilizing radiological tracers or mutagenic/teratogenic dyes impose significant exposure, handling, and disposal issues. In addition, tetrazolium or resazurin chemistries can significantly complicate additional downstream applications by color quenching of fluorescence or luminescence.

Cultured mammalian cells contain a rich milieu of protease, esterase, lipase, and nuclease activities which contribute to homeostatic maintenance. In particular, cytosolic, lysosomal, and transmembrane-bound proteases are involved in intracellular protein degradation, generation of immunogenic peptides, posttranslational modification, and cell division [4–6]. The activity of these enzymes is regulated by various mechanisms including specialized compartmentalization [7]. In response to extreme stress, environmental adversity, or committed progression of the apoptotic program, a loss of subcellular structure or membrane integrity is observed [8,9]. Therefore, we hypothesized that the release of stable protease activity into the

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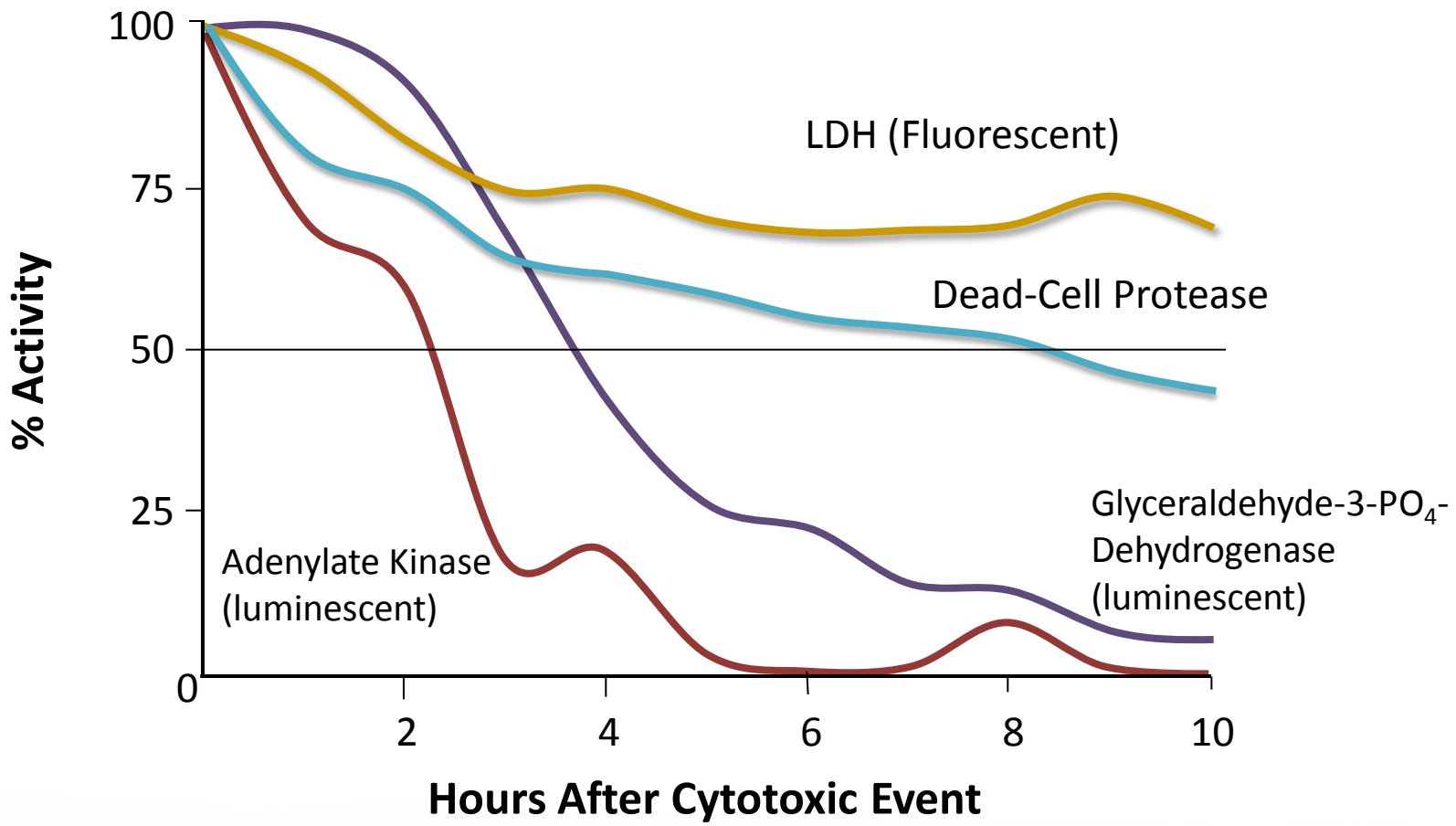
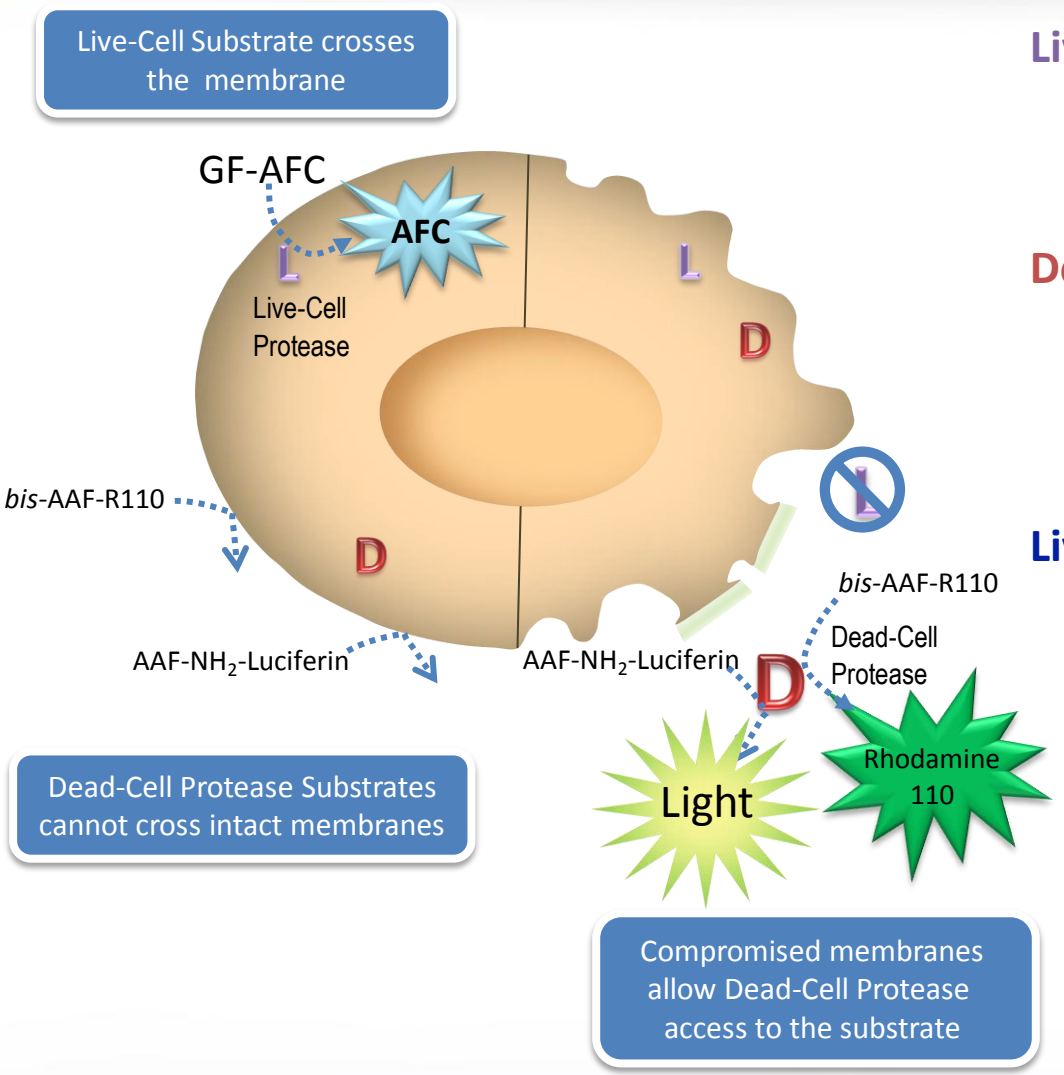
Niles, A.L., *et al.* (2007) *Analytical Biochemistry* **366**, 197-206.

Dead-Cell Protease more like LDH



Measure Live, Dead or Both



Live-Cell Protease Assay

CellTiter-Fluor™ Cell Viability Assay

Dead-Cell Protease Assay

CytoTox-Fluor™ Cytotoxicity Assay

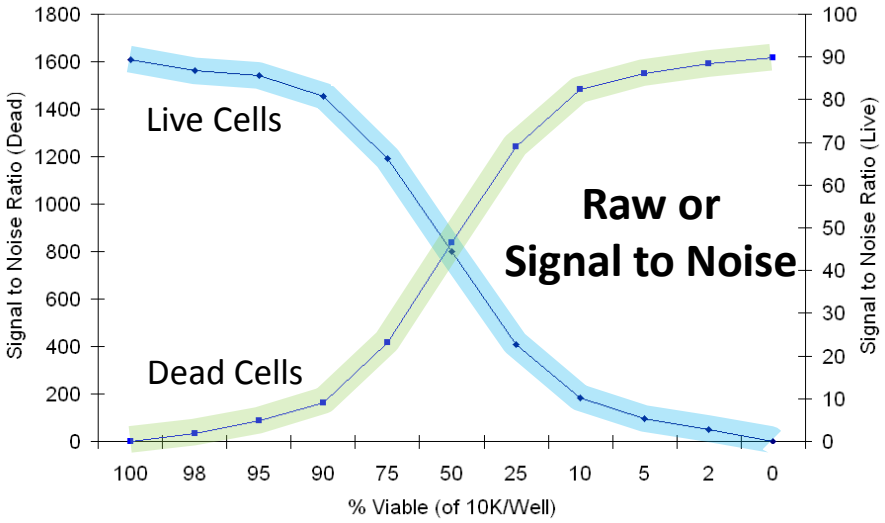
CytoTox-Glo™ Cytotoxicity Assay

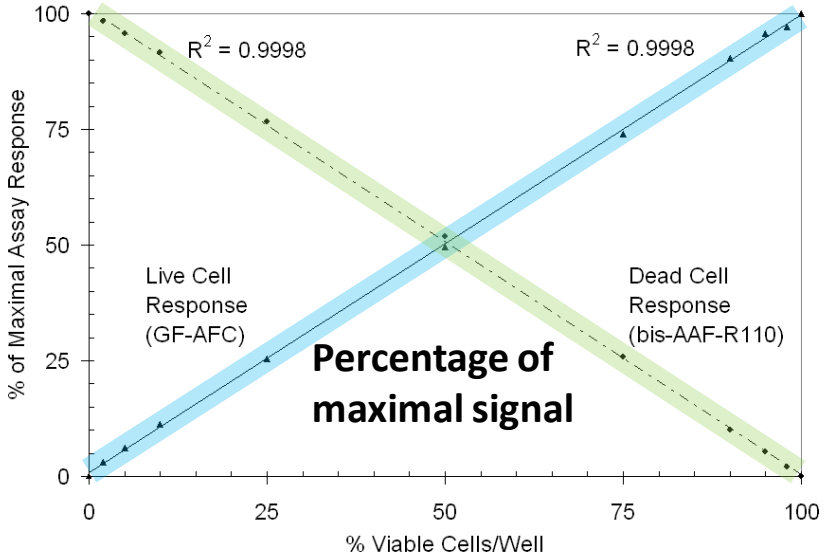
Live- & Dead-Cell Assay

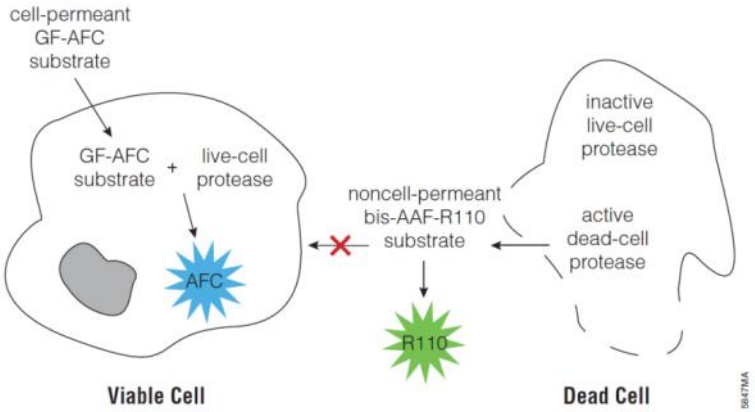
MultiTox-Fluor Multiplex Cytotoxicity Assay

MultiTox-Glo Multiplex Cytotoxicity Assay

Inverse relationship between live & dead cell signals

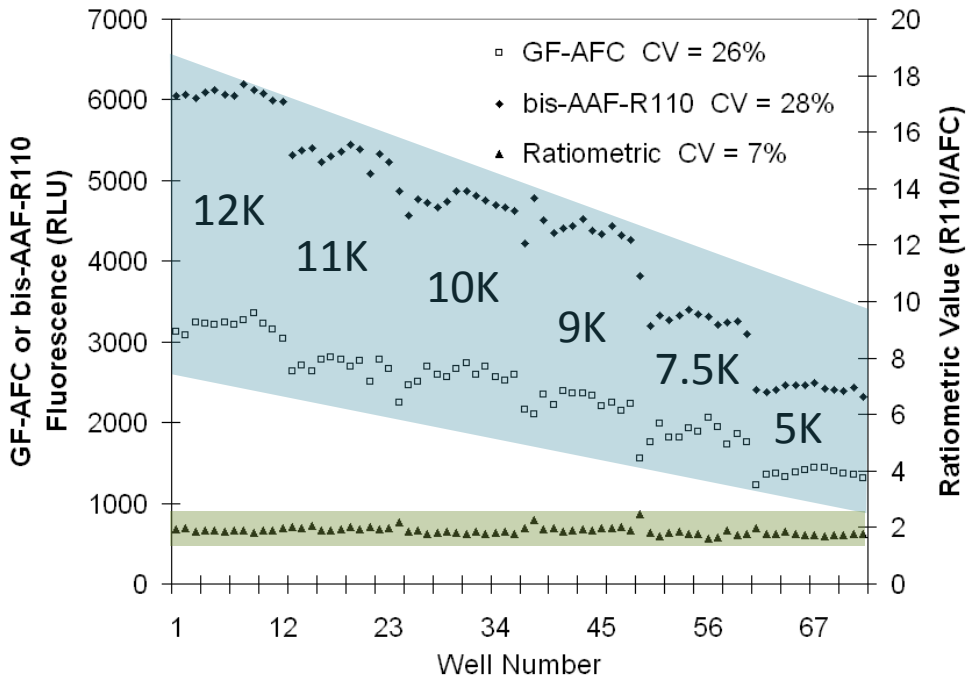






Ratiometric measures address variability

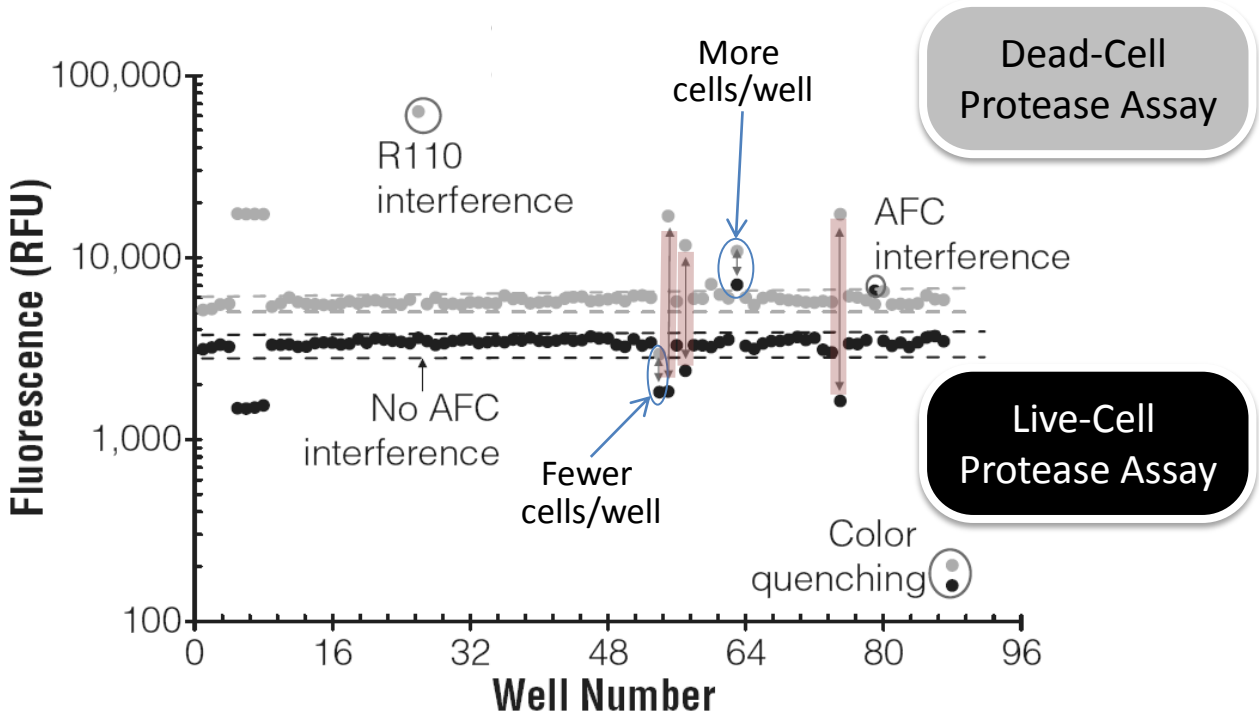
MultiTox-Fluor Assay Data



Variable number of 50% viability cells plated per well.

- Single parameter responses are partially dependent on cell number
- Subtle clumping or pipetting error can make screens difficult to interpret
- Ratiometric measures decrease variation by normalizing the data

MultiTox-Fluor assay improves data confidence for cytotoxicity screens



A cytotoxic event must yield an **increase** in dead-cell protease activity and a **decrease** in live-cell protease activity

MultiTox-Fluor can be the perfect multiplexing partner

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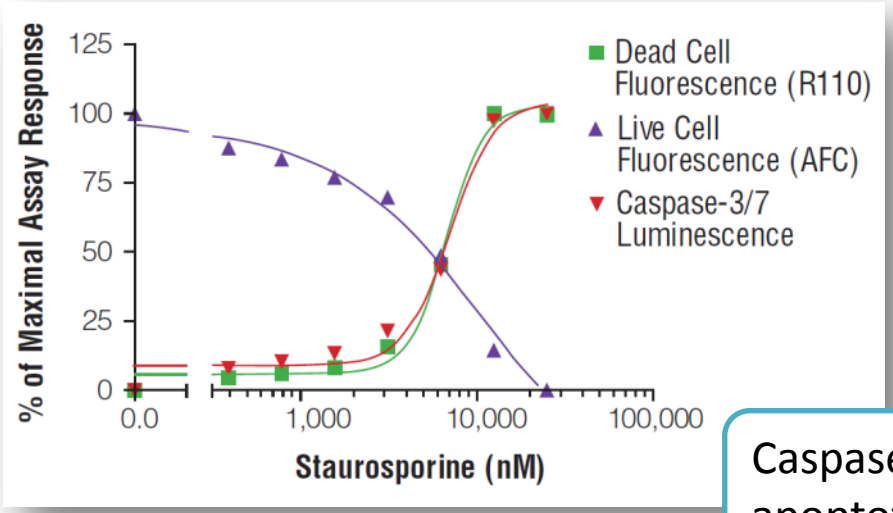
Assays must be chemically & biologically compatible

- Signals must be spectrally distinct (Fluorescence or Luminescence)
- Assay chemistries must be compatible
- The assays must fit in the available volume of the well or be separable.

Multi-Tox Fluor
Non-Lytic
2 data points

Lytic
Luminescent
Assay

Multiplexing with Caspase-Glo® 3/7 Assay



MultiTox-Fluor Multiplex Cytotoxicity Assay matches well with the Caspase-Glo® 3/7 Assay

- Used as the multiplexing example in the MultiTox-Fluor manual

Caspase-dependent apoptotic cell death

This combination can do so much more…

The Cytotoxicity Paradox:
A Simple Concept with Inherent Biological Complexity

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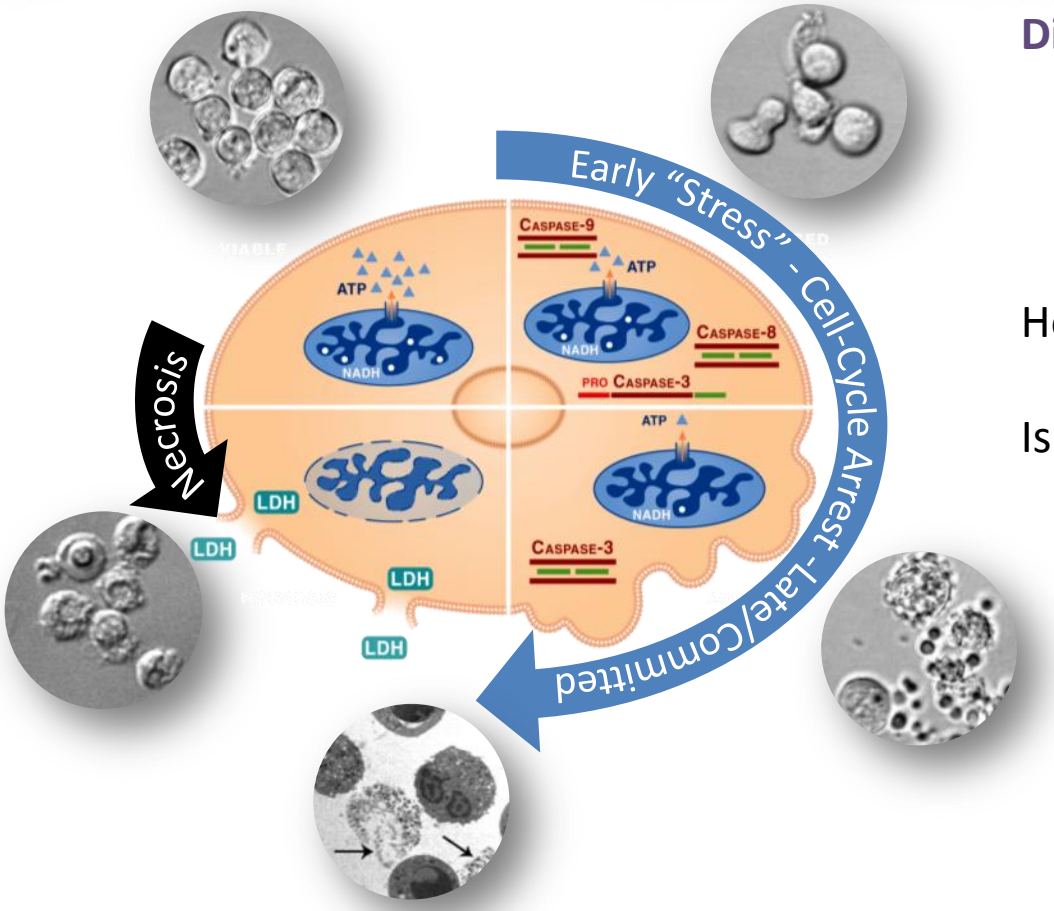


Did the treatment affect cell viability
- -Yes/No?
- -How?
- -When?

How potent was the treatment?

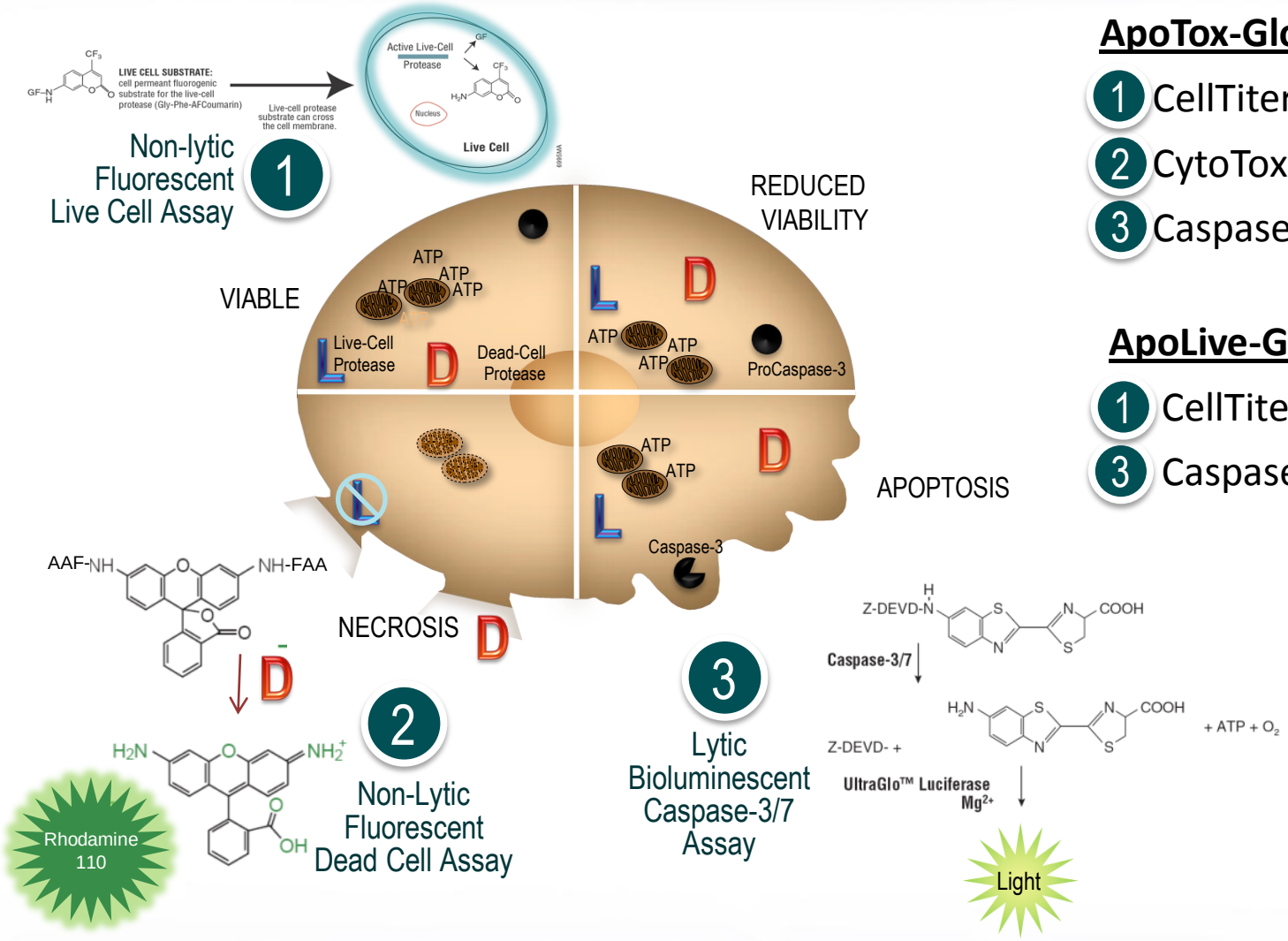
Is the treatment selective?

The cytotoxic phenotype is shaped by multiple factors:

1. **Dosage**
2. **Exposure Time**
3. **Cellular susceptibility**

No single parameter assay can fully characterize cytotoxicity

Deciphering a complicated process: Multiplexed, cytotoxicity signatures



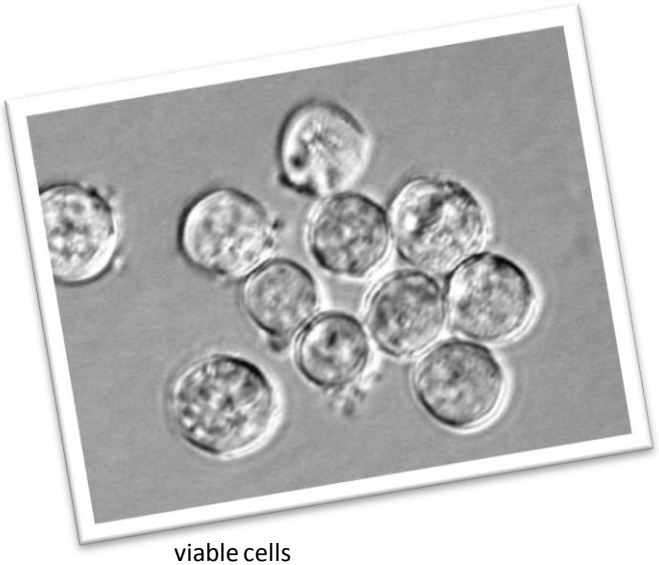
ApoTox-Glo™ Assay:

1. CellTiter-Fluor Assay
2. CytoTox-Fluor™ Assay
3. Caspase-Glo 3/7 Assay

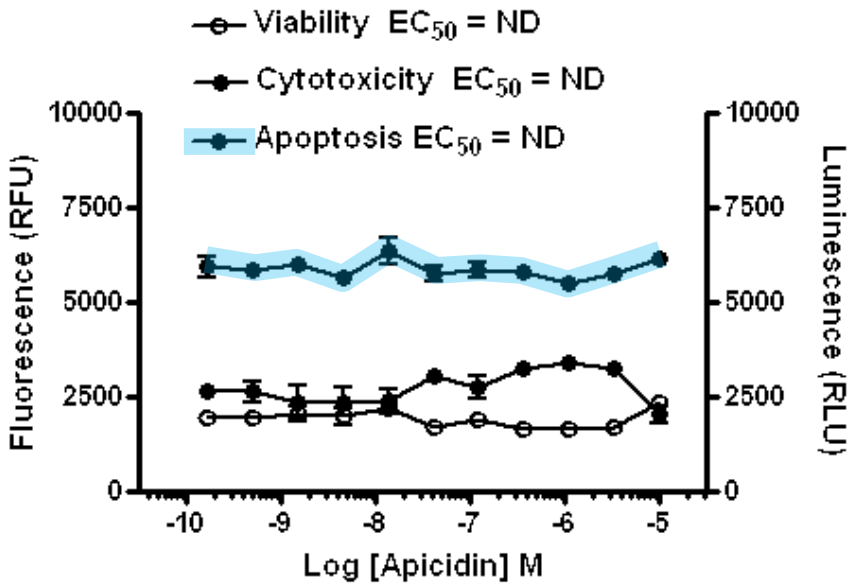
ApoLive-Glo™ Assay:

1. CellTiter-Fluor™ Assay
3. Caspase-Glo® 3/7 Assay

Signature #1. No Cytotoxic Effect

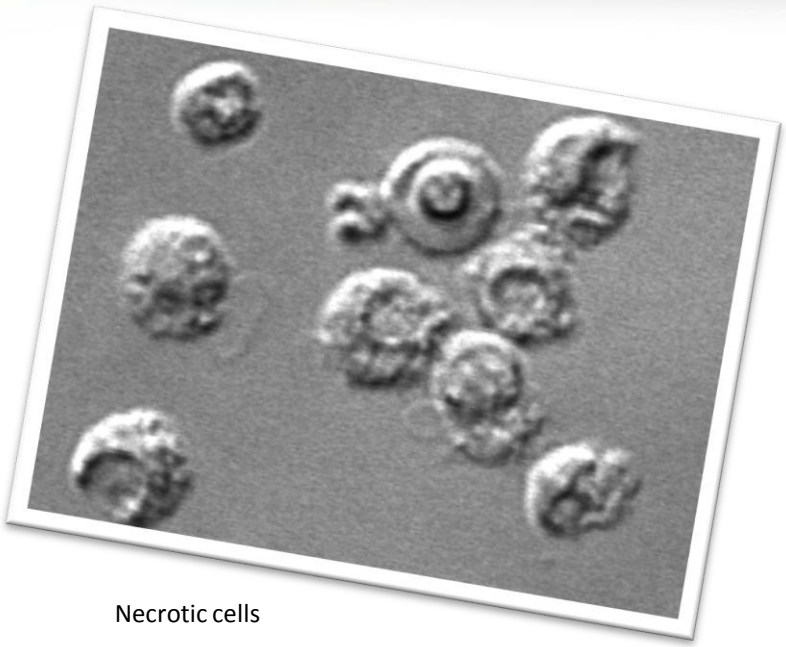


viable cells

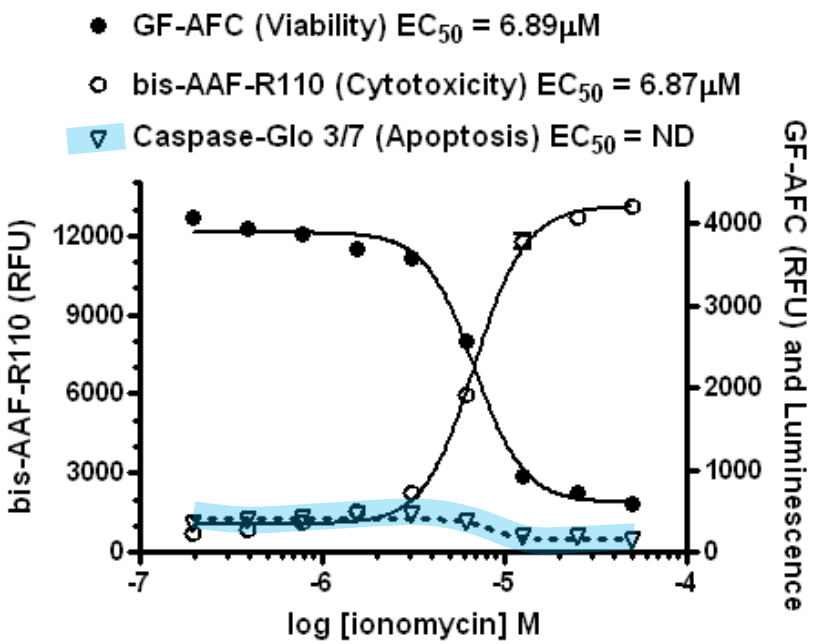


Compound **exposure period** and **cell type** are critical parameter for establishing cellular inertness.

Signature #2. Primary Necrosis

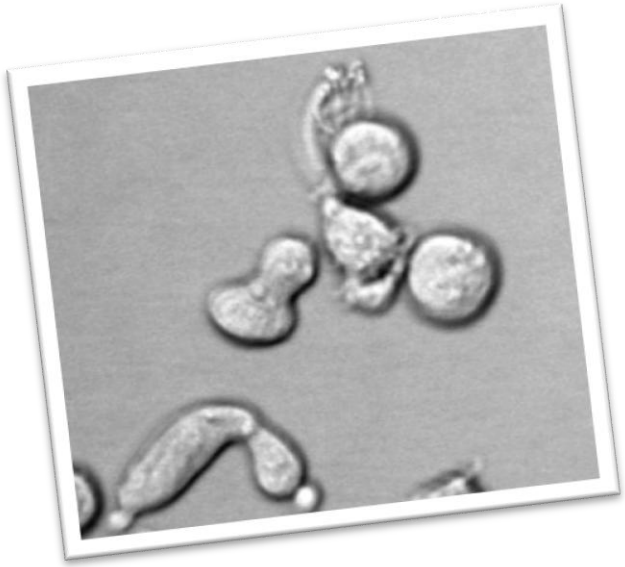


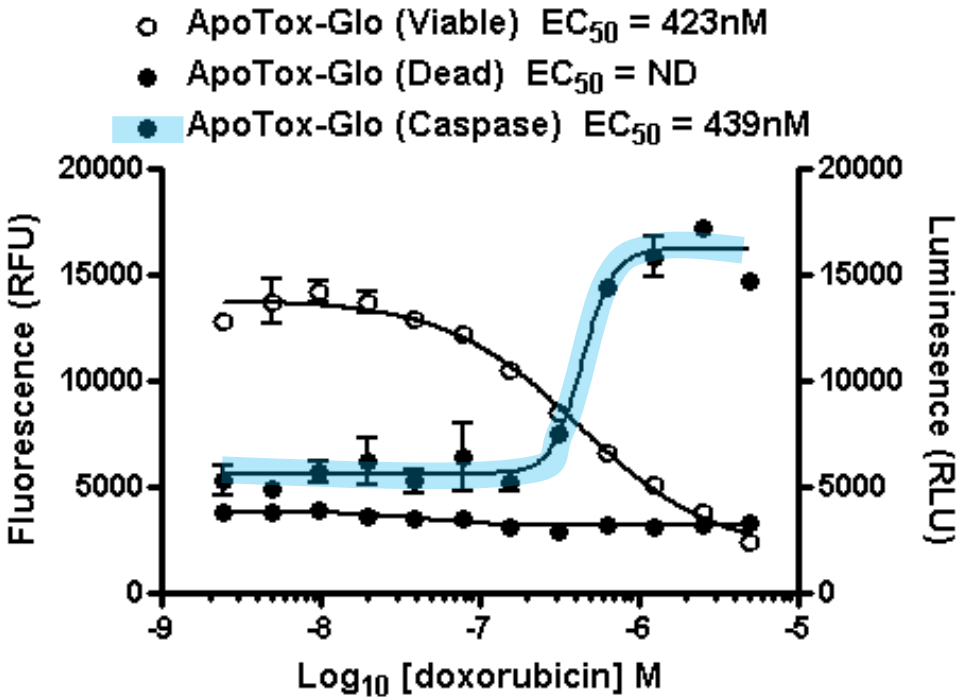
Necrotic cells



Rapid loss of membrane integrity (<4hrs) without caspase activation is strongly indicative of primary necrosis.

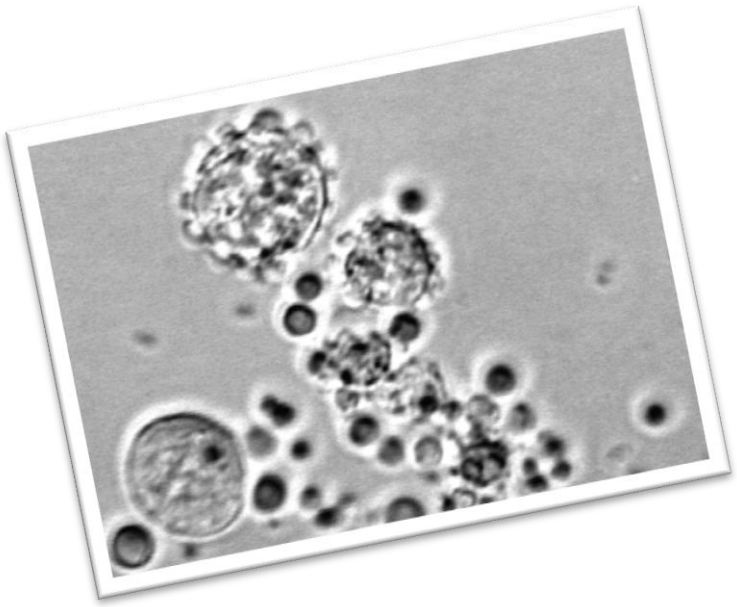
Signature #3
Cell Cycle Arrest…and early apoptosis

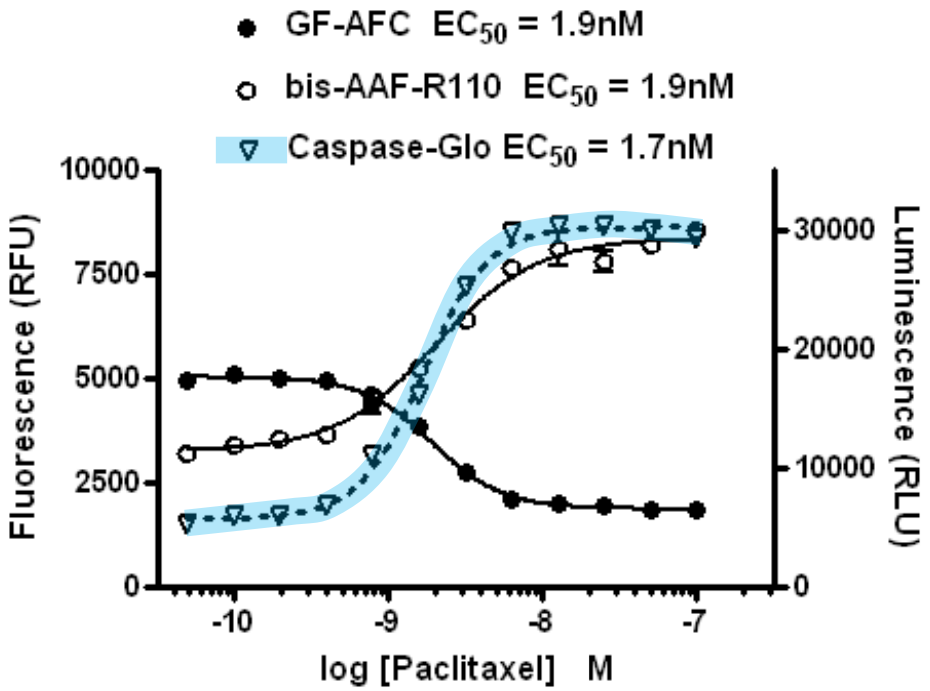




Decreases in apparent viability (viable cell number) with **increases** in caspase activation are consistent with cell-cycle arrest.

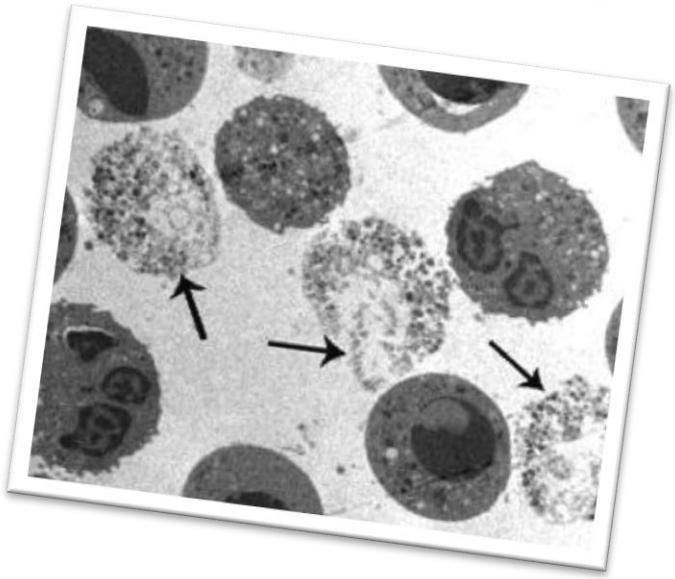
Signature #4: Apoptosis

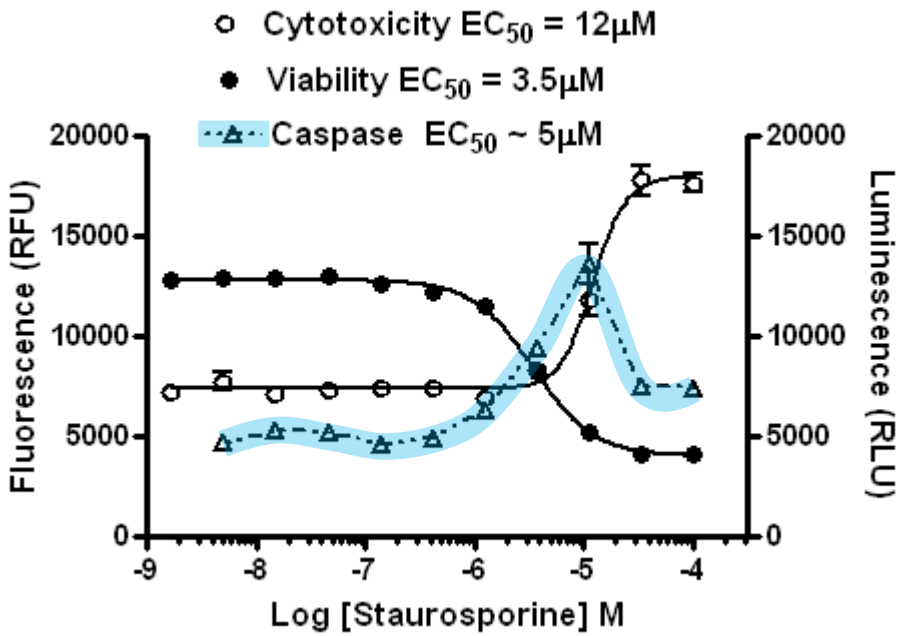




Decreases in viability with a commensurate **increase** in cytotoxicity with caspase activation are consistent with **apoptosis and secondary necrosis**

Signature #5: Late State Apoptosis





Dose-dependent decrease in viability, increase in cytotoxicity with **caspase biomarker degradation** at highest concentrations is consistent with late stage apoptosis.

Does "Biological Relevance" Equate into Translational Relevance?

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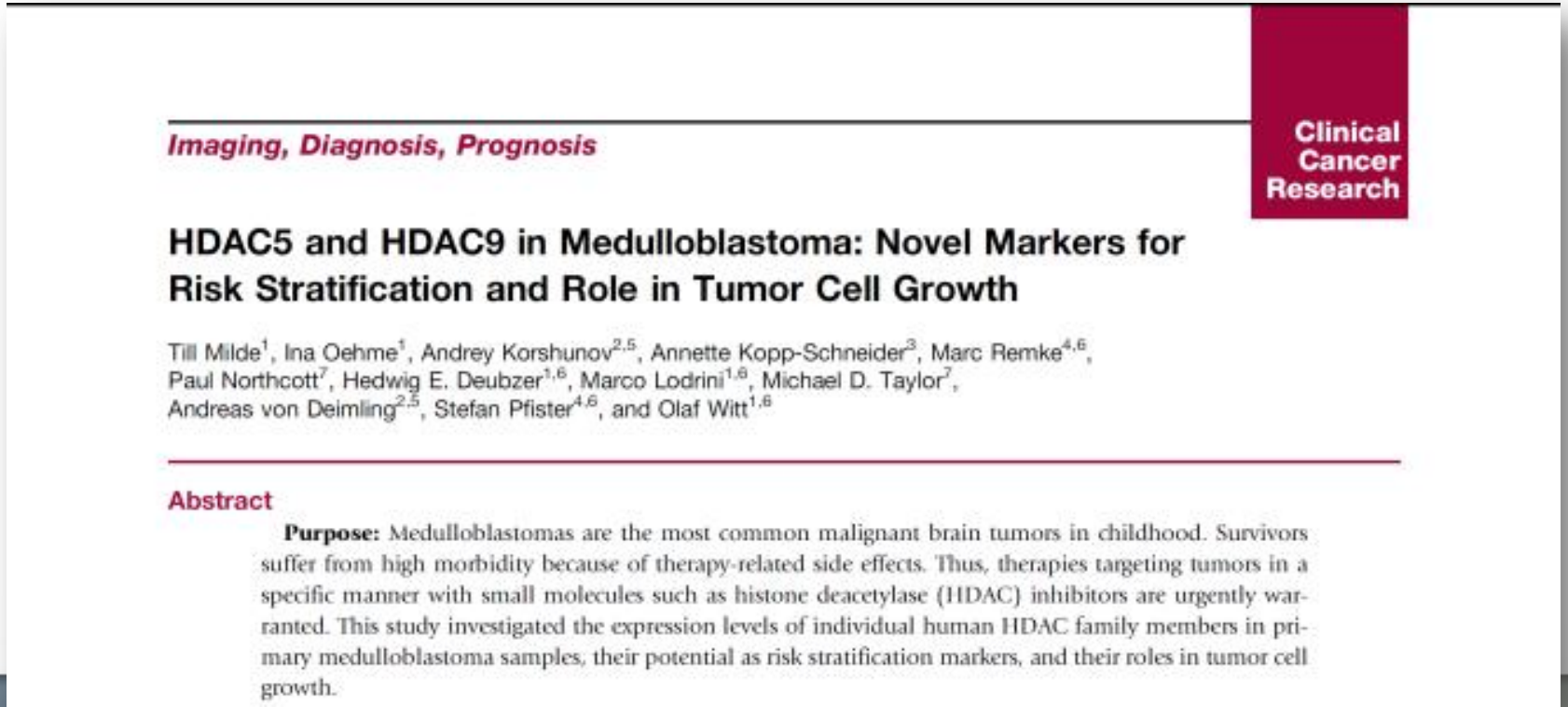
Translational Problem:

"Patients with *[various cancers]* experience poor outcomes, especially in metastasized disease, and treatment of all stages is associated with strong side effects [off-target] resulting in impaired quality of life. Specific therapies for such high-risk patients are therefore urgently needed to resolve this unsatisfactory situation."

– Milde, T., et al. (2010) *Clinical Cancer Research* **16**, 3240-52.



Imaging, Diagnosis, Prognosis

Clinical Cancer Research

HDAC5 and HDAC9 in Medulloblastoma: Novel Markers for Risk Stratification and Role in Tumor Cell Growth

Till Milde[1], Ina Oehme[1], Andrey Korshunov[2,5], Annette Kopp-Schneider[3], Marc Remke[4,6], Paul Northcott[7], Hedwig E. Deubzer[1,6], Marco Lodrini[1,6], Michael D. Taylor[7], Andreas von Deimling[2,5], Stefan Pfister[4,6], and Olaf Witt[1,6]

Abstract

Purpose: Medulloblastomas are the most common malignant brain tumors in childhood. Survivors suffer from high morbidity because of therapy-related side effects. Thus, therapies targeting tumors in a specific manner with small molecules such as histone deacetylase (HDAC) inhibitors are urgently warranted. This study investigated the expression levels of individual human HDAC family members in primary medulloblastoma samples, their potential as risk stratification markers, and their roles in tumor cell growth.

Potency & Safety Evaluation: Validation of ApoTox-Glo™ Assay with Clinical Cancer Therapeutics

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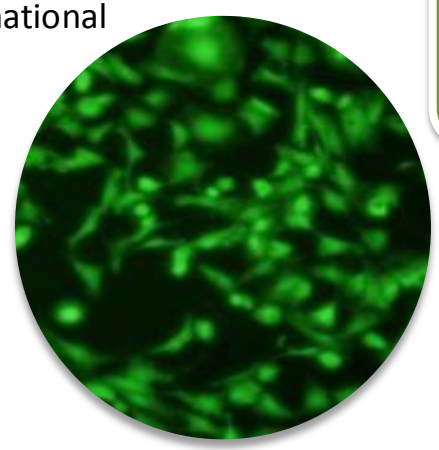
"iCells™ are specifically designed to aid drug discovery and **improve the predictability of drug efficacy and toxicity** screens, weeding out ineffective and potentially toxic compounds early in the pharmaceutical pipeline process before significant time and resources have been invested."
-Cellular Dynamics International



FDA Approved Anti-Leukemia Drugs

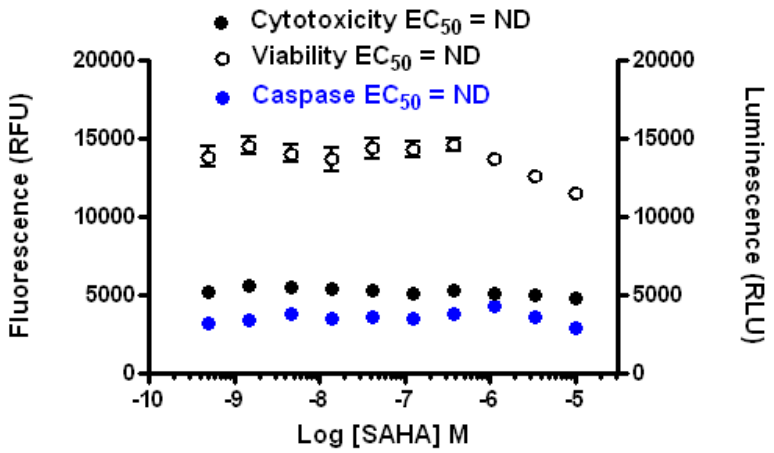
iCells™ Cardiomyocytes
evaluate off-target effects

K562 Human Leukemic Cells
evaluate on-target effects

ApoTox-Glo™
(After 24hr Exposure)

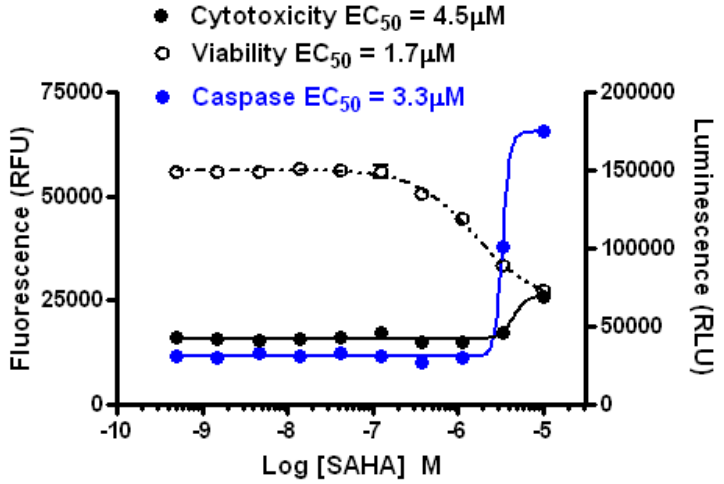
HDAC inhibition shows target specificity

iCell™



No apparent cytotoxicity or caspase activation.

K562



Cytotoxicity by apoptosis

Histone Deacetylase Inhibitor
SuberoylAnilide Hydroxamic Acid
(Vorinostat™)

Determine Death Mechanism with ApoTox-Glo™ Triplex Assay

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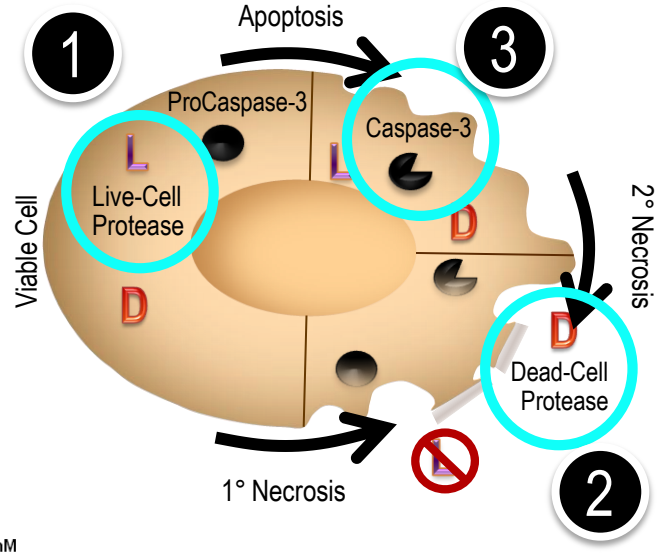
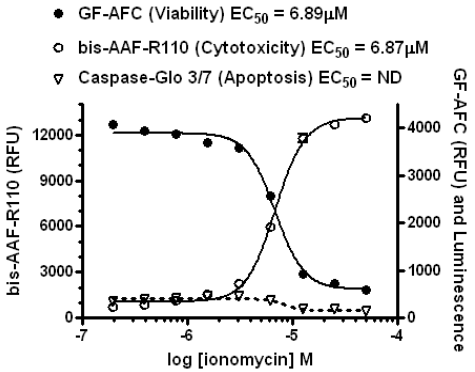
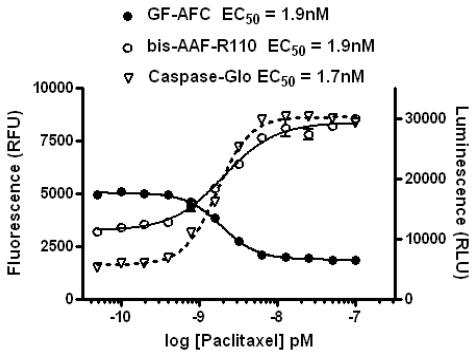
ApoTox-Glo Triplex Assay measures:

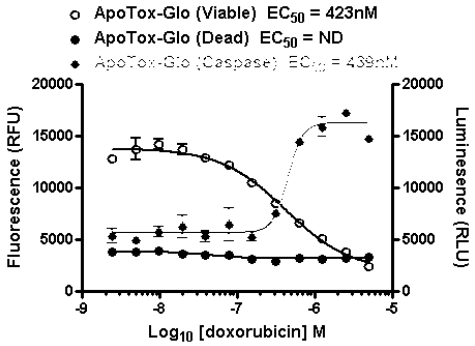
- Live Cells
- Dead Cells
- Apoptotic Cells

…and gives profile signatures









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