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

Viability Assays
- MTT → MTS → Glo

Cytotoxicity Assays
- INT → Resazurin

Apoptosis Assays
- TUNEL Ab’s → Cell-Based R110 → Glo

Ease-of-Use & Sensitivity

CellTiter 96® Assay (MTT)
- 1990

CellTiter-Glo® Assay (Luciferin/Luciferase)
- 1998

CytoTox-ONE™ Assay (Resazurin/Resorufin)
- 1994

Apo-ONE® Caspase Assay (bisDEVD-R110)
- 2002

Caspase-Glo® 3/7 Assay (DEVD-NH₂-luciferin)

Caspase-Glo® 8 & 9 Assay

Caspase-Glo® 2, 6, 8 & 9 Assays

Caspase-Glo® 8 & 9 Assay

ApoTox-Glo Triplex Assay

ApoLive-Glo Multiplex Assay

TUNEL Extracts

Cell-Based R110

GF-AFC

AAF-R110

DEVD-NH₂-luciferin

XXXX-Aminoluciferin

GF-AFC/AAF-R110/DEVD-Aminoluciferin
Other enzymatic markers of cytotoxicity

Assay performed on conditioned media only

Adenylate Kinase

2 ADP → 1 ATP + 1 AMP

Luciferin/Luciferase → Light

GAPDH

NADH → NAD^+ + P_i

Glyc-1,3-diPO_4 → Glyc-3-PO_4

PGK

ADP → ATP

Luciferin/Luciferase → Light
Luminescent methods where not ideal...

![Graph showing % Activity vs. Hours After Cytotoxic Event]

- LDH (Fluorescent)
- Glyceraldehyde-3-PO₄ -Dehydrogenase (luminescent)
- Adenylate Kinase (luminescent)
Two Protease activities = live/dead cell assay

Are my cells living?

- Live-Cell Protease

Are my cells dying?

- Dead-Cell Protease

Live-Cell Protease quickly inactivated outside the cell.

Analytical Biochemistry
366, 197-206.
Dead-Cell Protease more like LDH

Adenylate Kinase (luminescent)

Glyceraldehyde-3-PO₄- Dehydrogenase (luminescent)

% Activity

Hours After Cytotoxic Event

LDH (Fluorescent)

Dead-Cell Protease
Measure Live, Dead or Both

Live-Cell Substrate crosses the membrane

Dead-Cell Protease Substrates cannot cross intact membranes

Compromised membranes allow Dead-Cell Protease access to the substrate

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

Live Cells

Dead Cells

Raw or Signal to Noise

Percentage of maximal signal

\[ R^2 = 0.9998 \]

\[ R^2 = 0.9998 \]
Ratiometric measures address variability

MultiTox-Fluor Assay Data

- 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

Variable number of 50% viability cells plated per well.
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**

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.

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**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

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
2. Caspase-Glo® 3/7 Assay
Signature #1. No Cytotoxic Effect

Compound exposure period and cell type are critical parameter for establishing cellular inertness.
Signature #2. Primary Necrosis

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?

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.”
"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
HDAC inhibition shows target specificity

No apparent cytotoxicity or caspase activation.

Cytotoxicity by apoptosis

Histone Deacetylase Inhibitor
SuberoylAnilide Hydroxamic Acid
(Vorinostat™)
Determine Death Mechanism with ApoTox-Glo™ Triplex Assay

ApoTox-Glo Triplex Assay measures:
• Live Cells
• Dead Cells
• Apoptotic Cells

...and gives profile signatures

- GF-AFC (Viability) EC_{50} = 6.89 \mu M
- bis-AAF-R110 (Cytotoxicity) EC_{50} = 6.87 \mu M
- Caspase-Glo 3/7 (Apoptosis) EC_{50} = ND

![Graphs showing different assays and their EC_{50} values]
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