Cell Health and Mechanistic Toxicity Assays

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Director of Research

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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

Chemical reactions:
1. Promoter → luc → Glo
2. ATP + luc → Glo
3. ATP + Ultra-Glo™ → Glo
4. rLuciferase + ATP → Glo

New N- & C-termini
Fuse wt N- & C-termini
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

**Reagent**

Tetrazolium Reagents
- MTT, MTS, XTT, WST

Redox Indicators
- Resazurin

Enzyme Substrates
- Protease Substrates
  - GF-AFC

**Diagram:**
- **Viable Cell**
  - Active Metabolism or Protease
  - Substrate
  - Incubation Step
  - Product

- **Dead Cell**
  - Loss of Function
  - Substrate
  - No Rxn
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

Viable Cell

- ATP
- Luciferin + Luciferase
- Light

Dead Cell

- ADP
- No Reaction

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## Advantages & Disadvantages of Viability Assays

<table>
<thead>
<tr>
<th>Assay</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTT / MTS</td>
<td>Widely used&lt;br&gt;Inexpensive</td>
<td>MTT has 2 step protocol&lt;br&gt;1-4 hour incubation&lt;br&gt;Interference by reducing compounds&lt;br&gt;Toxic to cells&lt;br&gt;Limited sensitivity</td>
</tr>
<tr>
<td>Resazurin</td>
<td>Inexpensive&lt;br&gt;Fluorescent readout&lt;br&gt;Good sensitivity</td>
<td>1-4 hour incubation&lt;br&gt;Interference by reducing compounds&lt;br&gt;Toxic to cells&lt;br&gt;Fluorescence interference</td>
</tr>
<tr>
<td>Protease</td>
<td>30 min protocol&lt;br&gt;Cells remain viable&lt;br&gt;Better sensitivity than resazurin&lt;br&gt;Good choice for multiplexing</td>
<td>Fluorescence interference</td>
</tr>
<tr>
<td>ATP</td>
<td>10 min protocol&lt;br&gt;Best sensitivity&lt;br&gt;No fluorescence interference&lt;br&gt;Lysis step stops reaction immediately (no incubation with viable cells)</td>
<td>Lytic protocol dictates sequence for multiplexing</td>
</tr>
</tbody>
</table>
“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: MDA-MB-231 cells**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>IC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>ND</td>
</tr>
<tr>
<td>16</td>
<td>ND</td>
</tr>
<tr>
<td>24</td>
<td>556</td>
</tr>
<tr>
<td>32</td>
<td>208</td>
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<td>40</td>
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<td>64</td>
<td>38</td>
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<tr>
<td>72</td>
<td>27</td>
</tr>
</tbody>
</table>

Doxorubicin is a chemotherapy drug that inhibits DNA synthesis and replication, leading to cell death. It is commonly used in the treatment of various cancers, including breast cancer. The IC50 values indicate the concentration of doxorubicin required to inhibit cell viability by 50%. The time course of IC50 values shows how the effectiveness of the drug changes over time.
Detecting Dead Cells: Two Basic Approaches

The definition of cell viability is based on membrane integrity.

Live                                           Dead                                 Apoptotic

Dye                                           Enzyme Marker
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Apoptosis detected using caspase activities

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Enzyme Marker Release Assay to Detect Dead Cells

Detection Reagent

Viable Cell
- Enzymes retained in live cells
- No Reaction

Dead Cell
- LDH or protease
- Fluorescence

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Stability of released enzyme activity in culture medium becomes a limitation

LDH-Release Assay Time Course
Tamoxifen Treated HepG2 Cells

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

LDH activity in medium decreases after 24 hours

**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 cannot enter viable cells

- **Viable Cell**
  - No Signal from Viable Cells
  - Impervious substrate
  - bis-AAF-R110

- **Dead Cell**
  - Active “Dead Cell” Protease
  - R110 Fluorescence
  - 485/520 nm

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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

Viable Cell

“Viable” Protease

GF-AFC

Dead Cell

“Viable” Protease Inactive

Inactive “Viable” Protease

AFC Fluorescence 400/505nm
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

Multiplex Assay of Viable and Dead Cells by Measuring Protease Activities

Fluorescence (Viable) vs. Ionomycin (µM)

- Viable
- Dead

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.

Viable Cell: Dye is excluded from live cells

Dead Cell: DNA dye only stains nucleus of “dead” cells or debris
Reading the Same Plate Multiple Times to Detect the Onset of Cell Death (on the fly decision)

5000 K562 cells in 96 well plate

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

<table>
<thead>
<tr>
<th>Assay</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDH release</td>
<td>Widely used and accepted</td>
<td>Limited sensitivity</td>
</tr>
<tr>
<td></td>
<td>Absorbance or fluorescent options</td>
<td>Limited half-life of LDH in medium</td>
</tr>
<tr>
<td>Protease release</td>
<td>Designed for multiplexing</td>
<td>Limited half-life of protease marker</td>
</tr>
<tr>
<td></td>
<td>More sensitive than LDH</td>
<td>Fluorescence interference (fluorescent format only)</td>
</tr>
<tr>
<td></td>
<td>Fluorescent reagent is simpler than formulation for LDH assay</td>
<td></td>
</tr>
<tr>
<td>DNA Staining</td>
<td>Non-toxic / real time assay</td>
<td>Fluorescence interference</td>
</tr>
<tr>
<td></td>
<td>Staining persists for 72 hours</td>
<td>Less sensitive than amplified protease release assay</td>
</tr>
<tr>
<td></td>
<td>Good choice for multiplexing</td>
<td></td>
</tr>
</tbody>
</table>
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:GSGG ratio)
• Mitochondrial toxicity
• Genetic reporters to detect stress response pathways
AMC, R110 and aminoluciferin substrates for measuring caspase activity

![Chemical structures](image)

Caspase 3

- Fluorescence
- Luminescence

Luciferase + ATP
Luminescent assay has better sensitivity and dynamic range

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

Cells are apoptotic at 1 hr treatment with 150µM Tamoxifen

Caspase activity decreases after 24 hours incubation
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 $\text{H}_2\text{O}_2$)
**ROS-Glo H$_2$O$_2$ Assay**

- Direct H$_2$O$_2$ 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$_2$O$_2$ content of culture wells
ROS-Glo Assay Chemistry Based on Pro-Luciferin

**Modified Pro-luciferin (Peroxide Sensor)**

![Chemical Structure]

- **H₂O₂**
- **LDR (luciferase & ATP)**
  - Self-cleaving linker leaves
  - D-Cys cyclization occurs

Luciferase

Light
**ROS-Glo™ H₂O₂ Assay Protocol**

1. Add test compound and modified pro-luciferin peroxide detector
2. Incubate up to 2 hours
3. Add luciferin detection reagent
4. Incubate 15 min
5. Record luminescence

**ROS-Glo™ H₂O₂ Assay of Hep G2 Cells Treated with Menadione**

![Graph showing luminescence against Menadione concentration](image-url)
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.)

Total Glutathione

GSH

Reduce With DTT

GSSG

Block with NEM

Oxidized GSSG

GSH

GSSG

Reduce

GST

GSH

GS-R

Luciferase, ATP (LDR)

Light
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

**Mitochondrial ToxGlo™ Assay**

Multiplex membrane integrity and ATP content

**Change to glucose-free serum-free medium + galactose**

1. Treat cells 30 min - 4 hr
2. bis-AAF-R110 Substrate
3. Incubate 30 min
4. Record Fluorescence
5. ATP Assay Reagent
6. Incubate 10 min
7. Record Luminescence

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**Expected Assay Profiles**

- **No toxicity at this exposure period**
  - No change in CellTiter-Glo and CytoTox-Fluor readings.
  - Graphs show no significant drop in luminescence or fluorescence.

- **Primary Necrosis**
  - Graphs show a drop in luminescence and fluorescence, indicating necrosis.

- **MitoTox Without necrosis**
  - Graphs show a decrease in luminescence, indicating reduced mitochondrial activity.

- **MitoTox with necrosis**
  - Graphs show a decrease in luminescence, followed by a further drop, indicating necrosis.

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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.

# Stress and Toxicity Pathway Vectors


<table>
<thead>
<tr>
<th>Pathway</th>
<th>Activation Protein 1 (AP1)</th>
<th>AP1 Response Element (AP1 RE)</th>
<th>Vector</th>
<th>Part No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAPK/JNK</td>
<td>Activation Protein 1 (AP1)</td>
<td>AP1 Response Element (AP1 RE)</td>
<td>pGL4.44[luc2P/AP1 RE/Hygro]</td>
<td>E4111</td>
</tr>
<tr>
<td>Inflammation</td>
<td>Nuclear Factor κB (NF-κB)</td>
<td>NF-κB Response Element</td>
<td>pGL4.32[luc2P/NF-κB-RE/Hygro]</td>
<td>E8491</td>
</tr>
<tr>
<td>Oxidative Stress</td>
<td>NF-E2-related factor 2 (Nrf2)</td>
<td>Antioxidant Response Element (ARE)</td>
<td>pGL4.37[luc2P/ARE/Hygro]</td>
<td>E3641</td>
</tr>
<tr>
<td>Endoplasmic Reticulum Stress</td>
<td>Activating Transcription Factor 6 (ATF6)</td>
<td>ATF6 Response Element (ATF6 ERSE)</td>
<td>pGL4.39[luc2P/ATF6 RE/Hygro]</td>
<td>E3661</td>
</tr>
<tr>
<td>Heavy Metal Stress</td>
<td>Metal-Responsive Transcription Factor-1 (MTF1)</td>
<td>Metal Regulatory Element (MRE)</td>
<td>pGL4.40[luc2P/MRE/Hygro]</td>
<td>E4131</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>Hypoxia-Inducible Factor 1α (Hif1α)</td>
<td>Hypoxia Response Element (HRE)</td>
<td>pGL4.42[luc2P/HRE/Hygro]</td>
<td>E4001</td>
</tr>
<tr>
<td>Xenobiotic Stress</td>
<td>Aryl hydrocarbon receptor (AhR)</td>
<td>Xenobiotic Responsive Element (XRE)</td>
<td>pGL4.43[luc2P/XRE/Hygro]</td>
<td>E4121</td>
</tr>
</tbody>
</table>
# Cell lines available as custom products


## Cell Lines for Toxicity Pathway Analysis

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Response Element</th>
<th>Cell line background</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Antioxidant</td>
<td>ARE</td>
<td>HEK293</td>
</tr>
<tr>
<td>2 Hypoxia</td>
<td>HIF1</td>
<td>HEK293</td>
</tr>
<tr>
<td>3 Hypoxia</td>
<td>HIF1</td>
<td>HEPG2</td>
</tr>
<tr>
<td>4 MAPK</td>
<td>AP1</td>
<td>HEK293</td>
</tr>
<tr>
<td>5 Ras/MEK-1</td>
<td>SRE</td>
<td>HEK293</td>
</tr>
<tr>
<td>6 RhoA (Gq12/13)</td>
<td>SRF</td>
<td>HEK293</td>
</tr>
</tbody>
</table>

I am interested in a different toxicity pathway cell line
Validating Performance of Cytotoxicity Assays Applied to 3D Culture Models
Activity of Anticancer Agents in a Three-Dimensional Cell Culture Model

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.
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
**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
Thank You