Dead or Alive?
New cell-based assays to detect mechanism of toxicity

Dr Réka Nagy, Promega AG
Who we are

Using - the bioluminescence of luciferase - Promega has developed a solid technology platform from which hundreds of unique in vitro cell-based, biochemical and metabolic assays have been configured to provide solutions for understanding of cell biology.
Portfolio of Cell Health Assays Using Plate Readers

- CellTiter 96® Assay (MTT)
- CellTiter 96® AQ<sub>ueous</sub> Assay (MTS)
- CellTiter 96® AQ<sub>ueous</sub> ONE Soln (MTS)
- CytoTox 96® Assay (LDH)
- Apo-ONE<sup>®</sup> Caspase Assay
- Caspase-Glo<sup>®</sup> 3/7
- Caspase-Glo<sup>®</sup> 8 & 9
- CytoTox-ONE™ Assay (LDH)
- CellTiter-Fluor™
- CellTiter-Glo<sup>®</sup>
- RealTime-Glo<sup>®</sup>
- CellTiter-Glo<sup>®</sup> 3D Assay
- CellTiter-Glo<sup>®</sup> 3D Assay
- CellTiter-Blue<sup>®</sup>
- CellTiter-Blue<sup>®</sup>
- CellTox™ Green (DNA)
- CytoTox-ONE™ Assay (LDH)
- MultiTox-Fluor
- MultiTox-Glo
- GSH-Glo™
- ApoTox-Glo™
- GSH/GSSG-Glo™
- NAD/NADH-Glo™
- NADP/NADPH-Glo™
- ROS-Glo™ H₂O₂
- GSH-Glo™
- ApoLive-Glo™
- RealTime-Glo™ MT Assay

Colors:
- Red: Colorimetric
- Green: Fluorescence
- Blue: Bioluminescence
- Black: Fluorescence & Bioluminescence

1990: CellTiter 96® Assay (MTT)
1993: CellTiter 96® AQ<sub>ueous</sub> Assay (MTS)
1996: CellTiter 96® AQ<sub>ueous</sub> ONE Soln (MTS)
1999: CytoTox 96® Assay (LDH)
2002: Apo-ONE<sup>®</sup> Caspase Assay
2005: Caspase-Glo<sup>®</sup> 3/7
2008: Caspase-Glo<sup>®</sup> 8 & 9
2011: CytoTox-ONE™ Assay (LDH)
2014: CellTiter-Fluor™
2015: CellTiter-Glo<sup>®</sup> 3D Assay
Which Assay Should I Use?

Glo!

Fluor

Color

8 orders of magnitude S:N ratio (luminescence)
4 logs (fluorescence)
2 logs (colorimetric)

Signal:Noise

3SD

10pg MDL
100pg MDL
1ng MDL
10ng MDL

Reporter Protein (ng)

10
100
1,000
10,000
100,000
1,000,000
10,000,000
100,000,000
1,000,000,000

2 logs
3 logs
Viability
CellTiter -Glo®, 2.O, 3D

Cell Health in Real Time
CellTox™ Green
RealTime Glo™

Oxidative Stress & Metabolic Assay
GSH/GSSG-Glo™ Assay
ROS-Glo™ H₂O₂ Assay
NAD(P)H –Glo™ assay system
Metabolic & Enzymatic Indicators of Cell Viability

Reagent

Tetrazolium Reagents
• MTT, MTS

Redox Indicators
• Resazurin

Enzyme Substrates
• Protease Substrates

Viable Cell
- Active Metabolism or Protease
- Incubation Step
- Substrate
- Product

Dead Cell
- Loss of Function
- Substrate
- No Product
- X

Promega Corporation
MTS Assay Is Comparable to $[^3\text{H}]-\text{thymidine}$ Assay

Figure 3. Proliferation of B9 cells in response to various concentrations of IL-6 measured using the CellTiter 96® AQgreen Assay and $[^3\text{H}]-\text{thymidine}$ incorporation assays.
U937 Cells Incubated with MTT for 180 Minutes
ATP assay for Cell Viability

CellTiter-Glo® Luminescent Cell Viability Assay

- Lyses cell membranes to release ATP
- Inhibits endogenous ATPases
- Provides luciferin, luciferase and other reagents necessary to measure ATP using a bioluminescent reaction

Glow type signal: half-life is 5 hours

Extremely sensitive - can detect as few as 10 cells
CellTiter-Glo® 2.0 Assay: Complete Liquid Reagent with Enhanced Shelf Life

< 20% change in performance

<table>
<thead>
<tr>
<th></th>
<th>22°C</th>
<th>4°C</th>
<th>-20°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>#CellTiter-Glo®</td>
<td>12 hours</td>
<td>3.5 days</td>
<td>≥ 2 yrs</td>
</tr>
<tr>
<td>CellTiter-Glo® 2.0</td>
<td>7 days</td>
<td>4 months</td>
<td>≥ 2 yrs</td>
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#CellTiter-Glo® stability evaluation performed on reconstituted liquid reagent.
What is the CellTiter-Glo® 3D Assay?

An assay with comparable features to CellTiter-Glo®, but with enhanced performance against 3D cell culture models.

Key new attributes:

- **liquid format** instead of “buffer & cake combo”
- **enhanced lytic effectiveness** (optimized formulation)
- **optimized protocol** for 3D model application
CellTiter-Glo® 3D Provides Effective Lysis of Microtissues

- HCT116 cell spheroids grown to ~350 μm using hanging drop method (InSphero GravityPLUS System)

<table>
<thead>
<tr>
<th>Cells seeded (×10^3)</th>
<th>Diameter (μm)</th>
<th>ATP (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CellTiter-Glo 3D</td>
</tr>
<tr>
<td>6,000</td>
<td>716</td>
<td>1,327</td>
</tr>
<tr>
<td>2,250</td>
<td>533</td>
<td>1,079</td>
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<tr>
<td>1,125</td>
<td>468</td>
<td>799</td>
</tr>
<tr>
<td>563</td>
<td>355</td>
<td>539</td>
</tr>
</tbody>
</table>

**ATP recovery**
- CellTiter-Glo 3D = 93%
- ATPlite 1Step = 27%

- Different lytic effectiveness revealed by membrane-impermeant dye
- *CellTiter-Glo 3D provides near complete lysis of cell spheroid*
The Assay Guidance Manual e-Book

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

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

G. Sitta Sittampalam, PhD, Editor-in-chief, Neely Gal-Edd, MS, Associate Managing Editor, Michelle Arkin, PhD, Douglas Auld, PhD, Chris Austin, MD, Bruce Bejcek, PhD, Marcie Glicksman, PhD, James Inglese, PhD, Vance Lemmon, PhD, Zhuyin Li, PhD, Owen McManus, PhD, Lisa Minor, PhD, Andrew Napper, PhD, Terry Riss, PhD, O. Joseph Trask, Jr., James McGee, PhD and Jeff Weidner, PhD.
Cell Health in RealTime

- Monitor cells continuously over 72 hours, saving time, cell samples, and costs
- Option to add reagent at seeding, dosing of compound, or at the end
- Multiplex with other assays and downstream applications
CellTox™ Green Cytotoxicity Assay
“Real Time” Method to Detect Dead Cells
CellTox-Green Dye is Not Toxic to Cells

ATP assay data showing viability of cells exposed to Bortezomib for 72 hrs
- O cells exposed to DNA binding dye for 72 hr
- □ cells exposed to DNA binding dye for 15 min

Cells exposed to dye for 3 days do not show change in viability or responsiveness to toxin.
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 Assay: Multiplexing a Fluorescent Assay with Luminescent Assays

Glo Reporter Assays

NAD(P) / NAD(P)H Will work

CellTiter-Glo® Cell Viability Assay

CellTiter-Fluor™ Viability Assay

CellTox™ Green™ Cytotoxicity Assay

Caspase-Glo® Assays

CellTiter-Glo® Cell Viability Assay

P450-Glo™ Cell-Based Assays

GSH-Glo™ & GSH/GSSG-Glo™ Assays

ROS-Glo Will work

CytoTox-Glo™ Assay

GSH-Glo™ & GSH/GSSG-Glo™ Assays

HDAC-Glo™ Assay

Nano-Glo™ One-Glo™ Bright-Glo™ Steady-Glo®

Glo Reporter Assays include:

NAD(P) / NAD(P)H Will work

CellTiter-Glo® Cell Viability Assay

CellTiter-Fluor™ Viability Assay

CellTox™ Green™ Cytotoxicity Assay

Caspase-Glo® Assays

CellTiter-Glo® Cell Viability Assay

P450-Glo™ Cell-Based Assays

GSH-Glo™ & GSH/GSSG-Glo™ Assays

ROS-Glo Will work

CytoTox-Glo™ Assay

GSH-Glo™ & GSH/GSSG-Glo™ Assays

HDAC-Glo™ Assay

Nano-Glo™ One-Glo™ Bright-Glo™ Steady-Glo®
Samples with CellTox Green can be Multiplexed with Cell Viability and Apoptosis Assays
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 luciferase to produce light
RealTime-Glo™ Live Cell Viability Assay: Sensitive also as Endpoint Assay

More sensitive, rapid, and robust viability assays that measures the reducing potential of the cell

A549 cells were treated with digitonin for 24 h. The viability reagents were added and signal read at 30 min post-addition (RealTime-Glo) or 4 hours post-addition (AlamarBlue).
Cell Death Can Be Measured in Real Time

Conventional viability assays are accumulation-based and do not allow real time detection of cell death (e.g. AlamarBlue - resazurin is converted to resorufin, which accumulates)

Furimazine is rapidly used by NanoLuc and is unstable, leading to no accumulation.
Multiplexing Viability and Luciferase Reporter Assays

Seed HEK293 cells expressing luciferase in 384 well plate

↓

Incubate overnight

↓

Add RealTime-Glo™ Reagent

↓

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
Multiplexing with Viability/Toxicity Assays

- Multiplexed with fluorescent readouts (e.g. CytoTox-Fluor, CellTox™ Green)

- Due to the rapid decrease in signal upon cell death, this assay can also be multiplexed with lytic assays, including many luminescent assays without the need to inhibit the first reaction or use special filters (e.g. CellTiter-Glo®, ONE-Glo).

MCF7 cells (500 cells/well) dosed with etoposide with RealTime-Glo™ reagents and CellTox™ Green in media. Fluorescence and luminescence measured on Tecan M200 with Gas Control Module (37°C/5% CO2) every 1 h for 72 h.
RealTime-Glo™ MT Cell Viability Assay Applied to 3D Microtissues

Hanging Drop Spheroids of HEK293 Cells

Signal/Background vs. Microtissue Diameter (µm)

- 160 µm
- 212 µm
- 442 µm
- 1075 µm
Viability
Cell Titer-Glo®, 2D, 3D

Cell Health in Real Time
CellTox™ Green
Real Time Glo™

Oxidative Stress & Metabolic Assays
GSH/GSSG-Glo™ Assay
ROS-Glo™ H₂O₂ Assay
NAD(P)H – Glo™ assay system
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™ H₂O₂ Assay

• Direct H₂O₂ detection without using Horseradish Peroxidase (HRP)
  • Mitigates HRP mediated false hits

• Homogeneous Bioluminescent Assay
  • Add-mix-read
  • No fluorescence interference

• Cell based assay
  • Detects H₂O₂ content of culture wells
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 the relationship between Menadione concentration and luminescence.](image)
ROS-Glo™ H$_2$O$_2$ Assay Applications

Cell Based Assays

H$_2$O$_2$ induction in HepG2 cells

Enzyme Assays

NADH Oxidase

NADH + O$_2$ → NAD$^+$ + H$_2$O$_2$

Limit of detection ~ 100 nM H$_2$O$_2$
Low Interference in LOPAC Chemical Library Screen

Many compounds in LOPAC interfere with HRP driven Amplex Red assay.
Multiplex Example
ROS and ATP Assays (Split Sample)

Add test compound and ROS-Glo Substrate

Incubate

Cells

Add CTG Reagent, read

Add ROS-Glo LAR, read

Concentration [µM]
Multiplexing ROS-Glo™ with ATP Assay to Measure Viable Cells
Oncogenes Drive Cancer by Altering Metabolic Pathways

- Cancer cells get reprogrammed-uncontrolled cell growth

- Metabolic pathways get “hi-jacked”: have to balance energy production with increased needs in biomolecule biosynthesis and ROS production

- Increase glucose uptake
- Shift from oxidative phosphorylation to aerobic glycolysis (Warburg effect)
- Increase lactate production
- Activates pentose phosphate pathway
- Increase anabolic reactions
Principal of Bioluminescent NAD(P)H Detection Technology

**Assay principal**

- In the presence of NAD(P)H proluciferin is converted to luciferin by reductase
- Luciferin is used by luciferase to produce light
- The light is proportional to the amount of NAD(P)H in the sample

“With the aid of auxiliary enzymes nearly every substance of biological interest could be measured with a pyridine nucleotide system” by Lowry et al., 1961
We Used the Technology to Develop NAD(P)/NAD(P)H Detection Assays

NAD/NADH-Glo™ and NADP/NADPH-Glo™ Couples NAD(P)H Detection with Enzyme Cycling Reaction

**NAD/NADH-Glo™ Assay** – detects non-phosphorylated forms in cells

**NADP/NADPH-Glo™ Assay** – detects phosphorylated forms in cells
Three Kits Are Available: NAD(P)H-Glo™, NAD/NADH-Glo™ and NADP/NADPH-Glo™

The kits can be used for measuring:

- Total and individual dinucleotides (NAD, NADH, NADP, NADPH)
- Biochemical enzyme characterization
- Dinucleotide detection in cells, tissues and other biological samples
- The NAD/NADH or NADP/NADPH ratio can be determined directly from RLU values
- The actual amount can be calculated from dinucleotide standard curves
## Features of the Assays: Sensitivity, Specificity and Wide Assay Window

<table>
<thead>
<tr>
<th></th>
<th>NAD(P)H-Glo™ Detection system</th>
<th>NAD/NADH-Glo™ Detection system</th>
<th>NADP/NADPH-Glo™ Detection system</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Limit of Detection (LOD)</strong></td>
<td>25nM (625fmol/25μl)</td>
<td>1nM (25fmol/25μl)</td>
<td>1nM (25fmol/25μl)</td>
</tr>
<tr>
<td><strong>Linearity</strong></td>
<td>25nM – 50μM</td>
<td>1 – 500nM</td>
<td>1 – 500nM</td>
</tr>
<tr>
<td><strong>Signal-to-background (S/B max)</strong></td>
<td>~400</td>
<td>~250</td>
<td>~250</td>
</tr>
<tr>
<td><strong>Cells/well for Total dinucleotides</strong></td>
<td>NA</td>
<td>500 - 25,000</td>
<td>500 – 12,000</td>
</tr>
</tbody>
</table>
Expanding NAD(P)H-Glo™ Technology to Different Metabolite Detection

Metabolite detection assays couple selective metabolite oxidation and NAD(P)H production with bioluminescent NAD(P)H detection.

- Metabolite is used as a substrate by metabolite selective dehydrogenases.
- Simultaneously NAD(P) is converted to NAD(P)H.
- NAD(P)H is detected with reductase/luciferase coupled reactions.
- The light output is proportional to the amount of metabolite present in the sample.
Ready-to-use Metabolite Detection Assays Available for Testing: Lactate, Glutamate, Glc-6P, Glucose

The assays provide markers for studying

✓ Metabolic state of the cells (glycolytic versus Oxphosph)
  ❖ Glycolytic: glucose consumption = lactate secretion
  ❖ Oxphosph: decrease in lactate secretion

✓ Mitochondria function
  ❖ Impaired function: increase in glucose consumption and lactate production

✓ Glutamine addiction of cancer cells

✓ Glucose homeostasis in adipocytes, liver, muscles

Note: other metabolite detection assays can be rapidly optimized by using metabolite selective dehydrogenases and coupling it with bioluminescence NAD(P)H detection
Create a custom solution

Our chemists synthesize a variety of specialized enzyme substrates including fluorophores and specialized luciferins for cell-based and reporter assays. Promega can manufacture bulk reagents from 0.5ml to 650 liters, and luciferase-based substrates can be lyophilized in batches up to 21,500 units.

www.promega.com/custom
Thank you for your attention!

Contact details

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