Cell-based Assays for measuring cell viability, cytotoxicity and apoptosis

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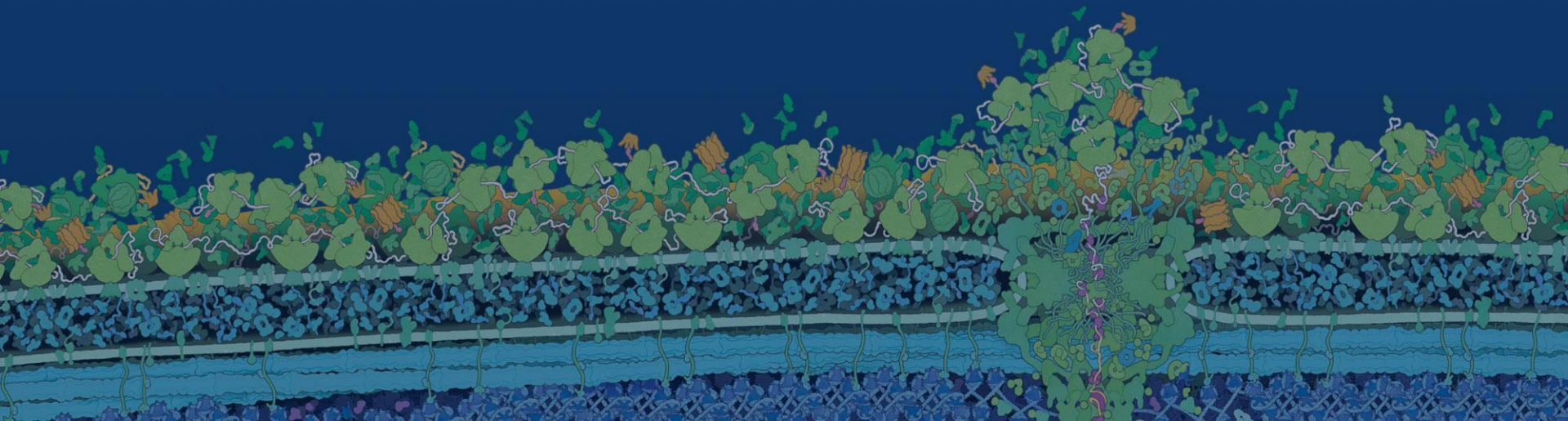
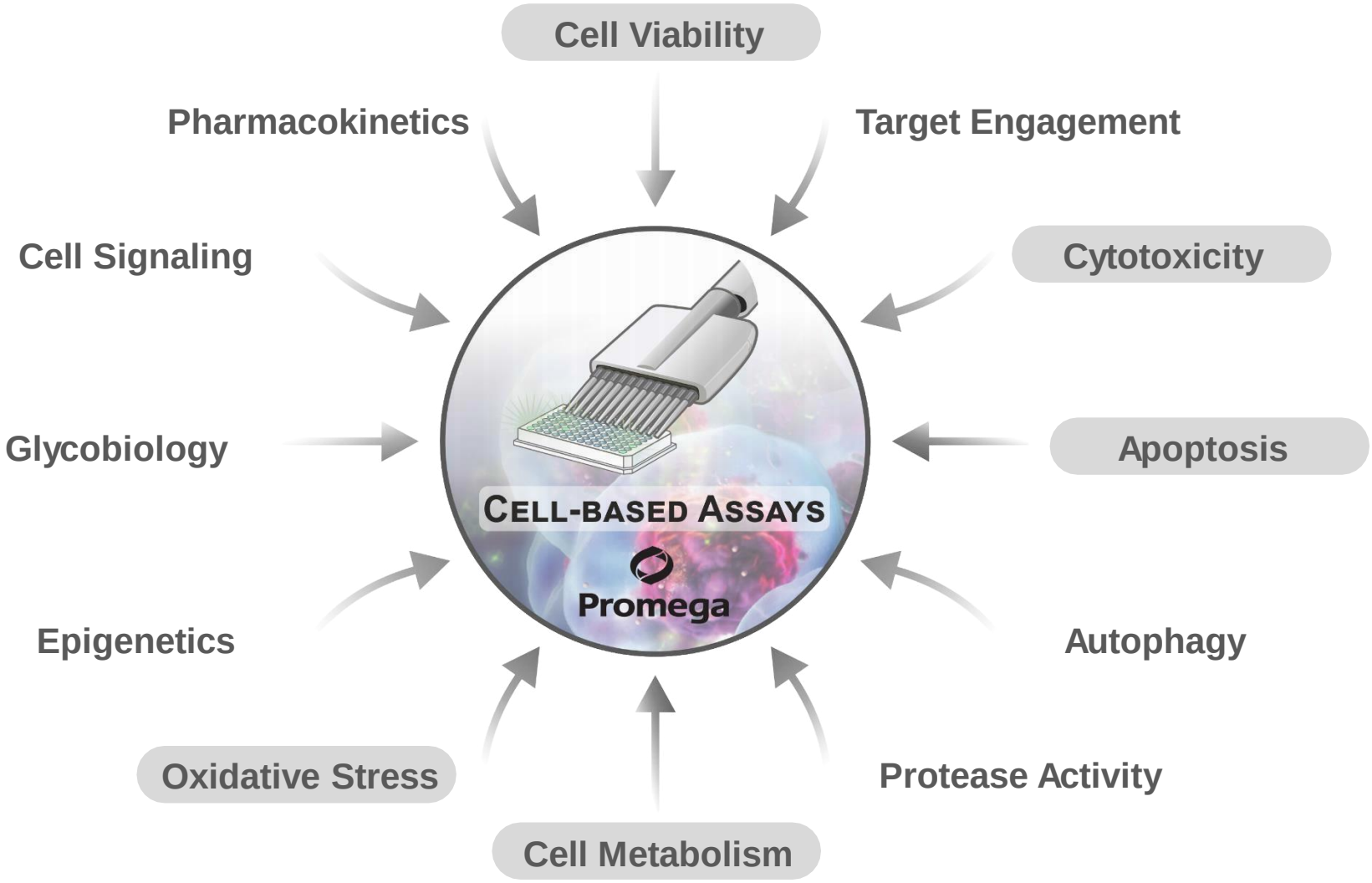
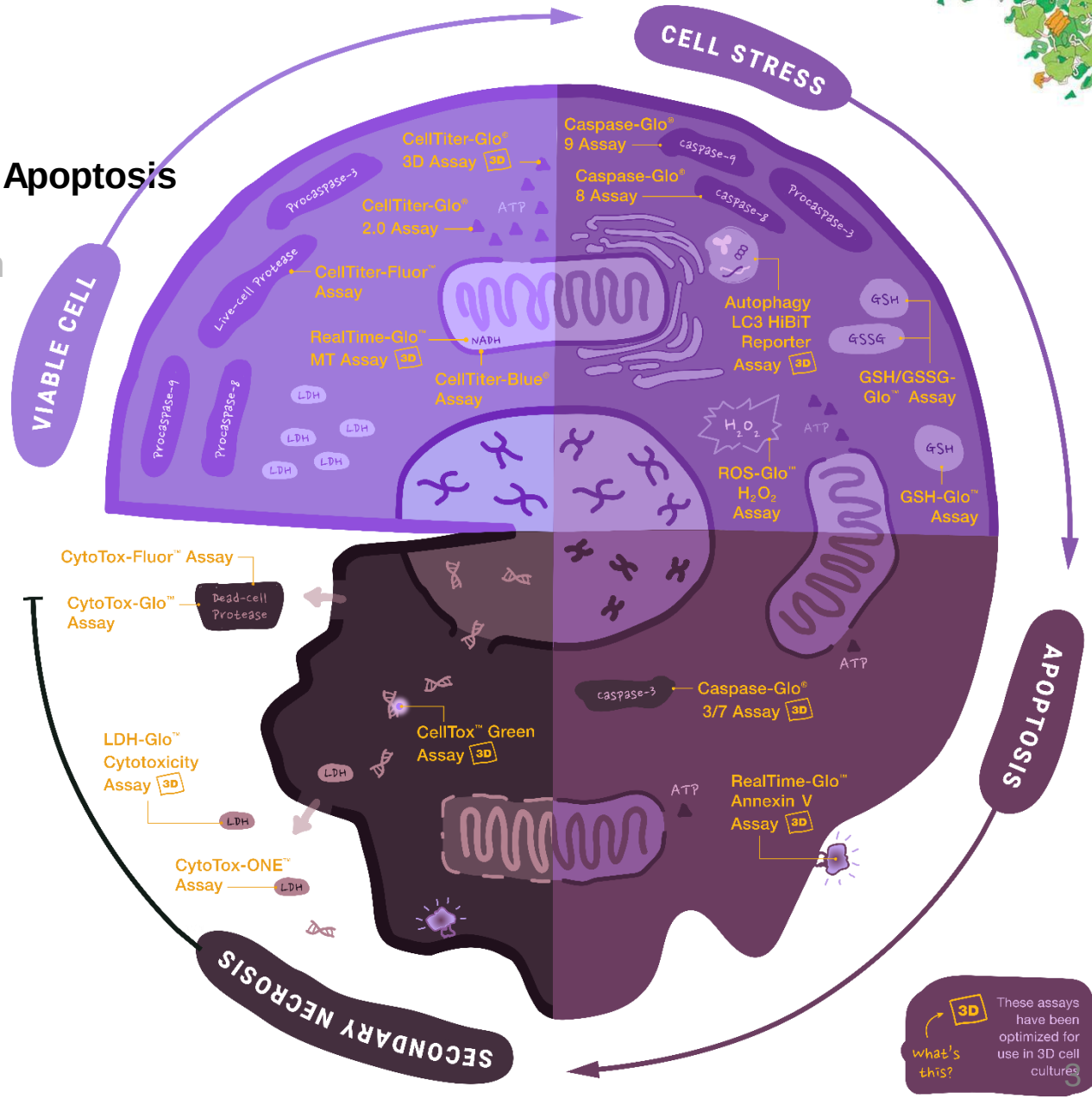


Promega's Cell-based Assay Portfolio



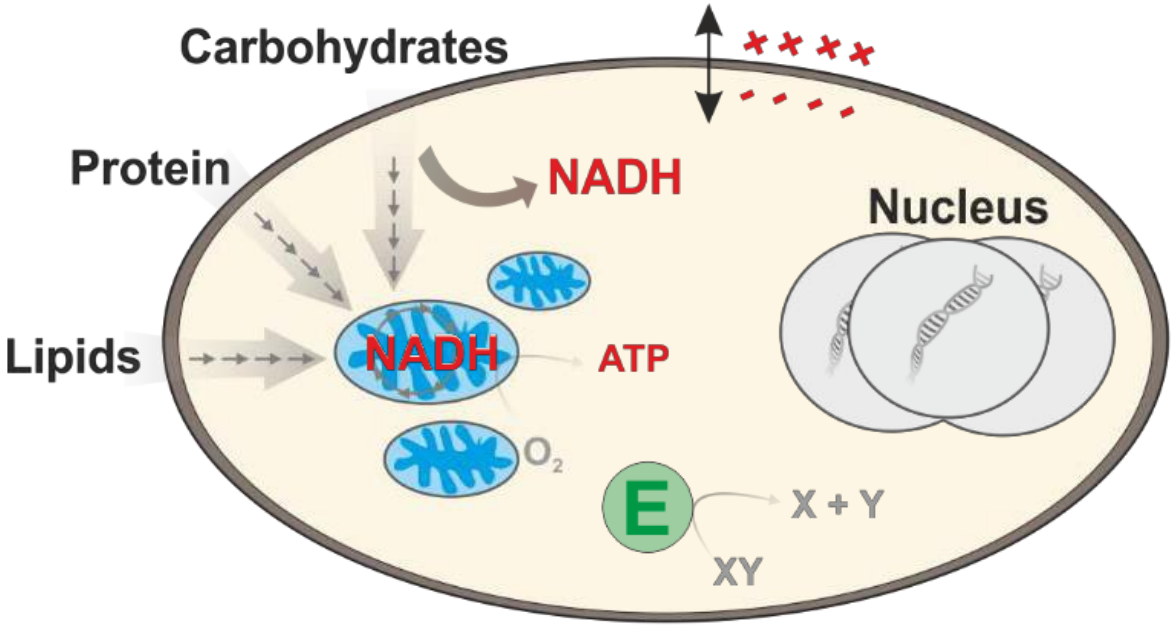
Outline

- ✓ **Cell Viability, Cytotoxicity, Apoptosis**
- ✓ Energy / Lipid Metabolism
- ✓ Oxidative Stress



Biomarkers of Cell Viability

Cell viability assays determine the relative amount of living cells within a population.

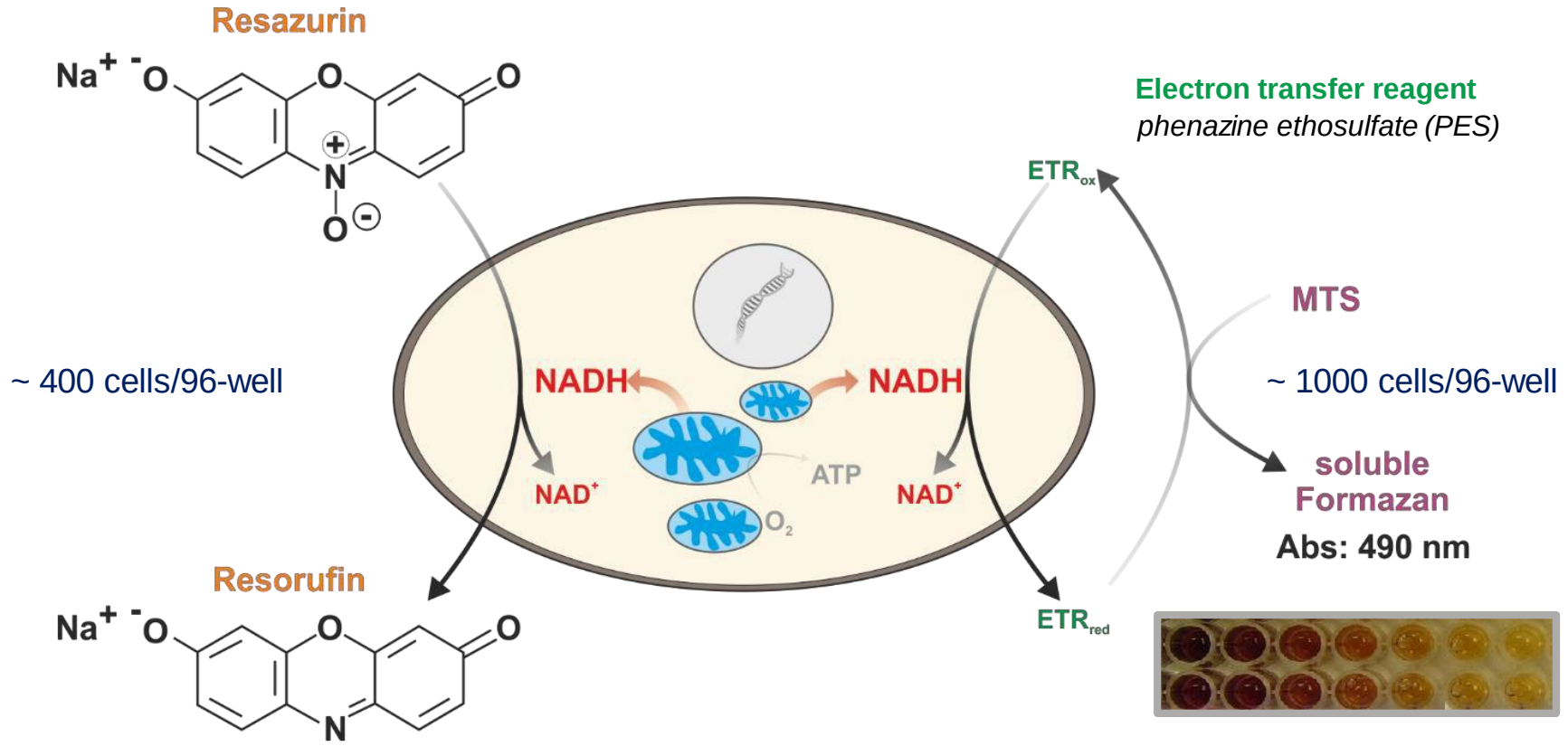


BIOMARKER

1. Reduction equivalents
2. ATP content
3. Division / DNA content
4. Membrane potential
5. Enzymatic activity

Metabolic Markers – Reduction Equivalents

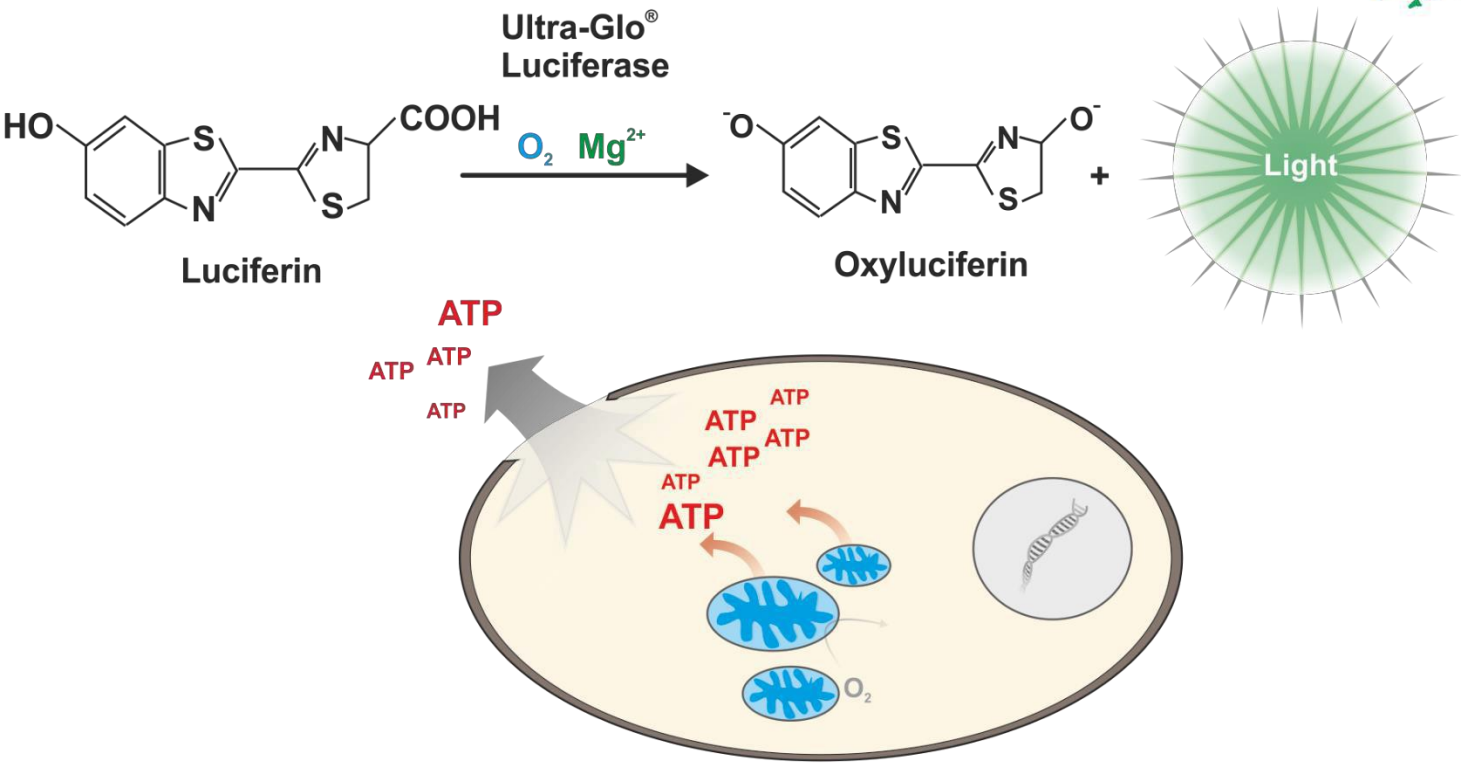
CellTiter-Blue® Cell Viability Assay	CellTiter96® AQueous Proliferation Assay



Ex: 560 nm
Em: 590 nm

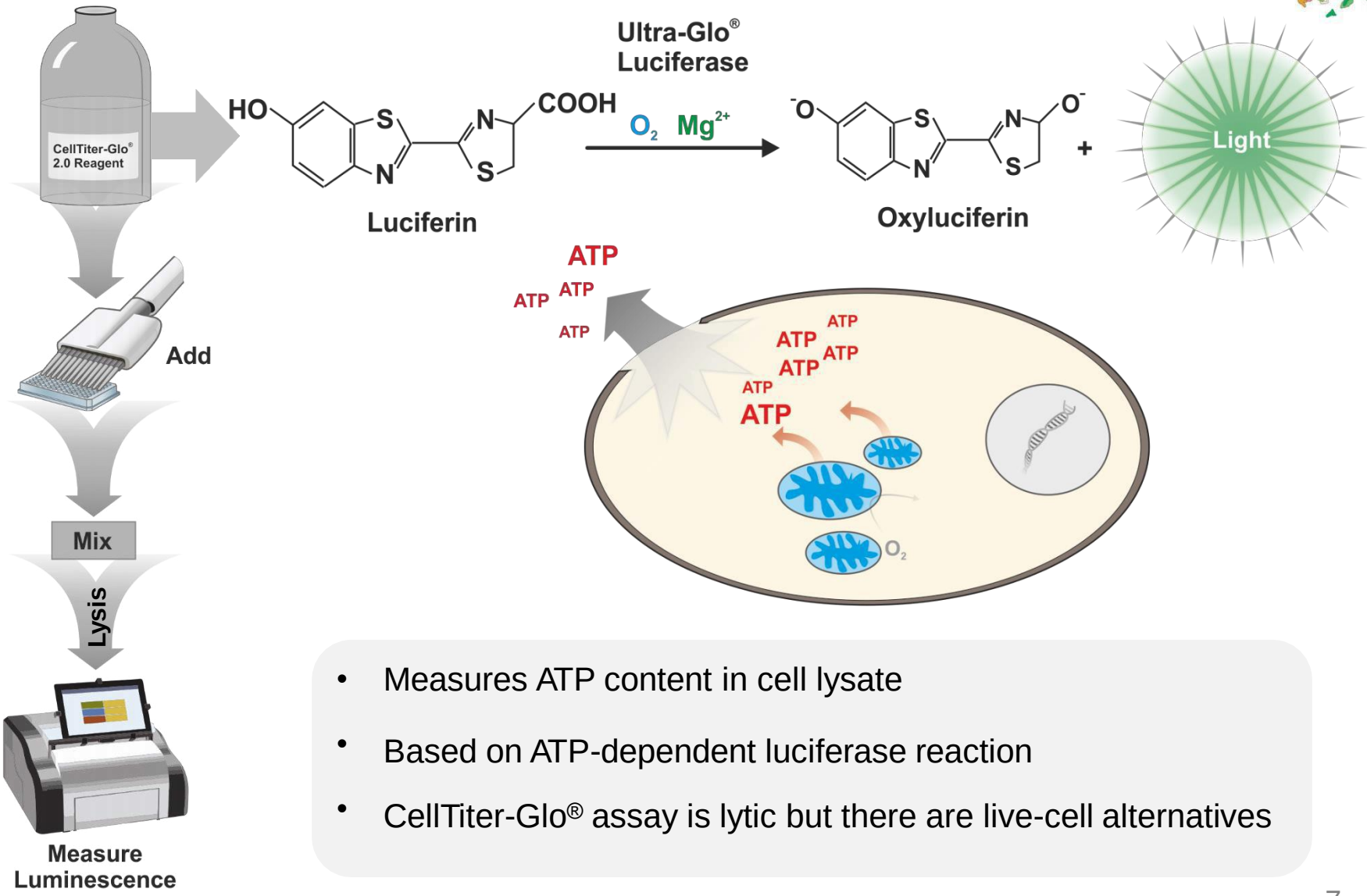
require 1 - 4 hours incubation with assay reagent

CellTiter-Glo® Assay – Metabolic Marker ATP



- Measures ATP content in cell lysate
- Based on ATP-dependent luciferase reaction
- CellTiter-Glo® assay is lytic but there are live-cell alternatives

CellTiter-Glo® Assay – Metabolic Marker ATP



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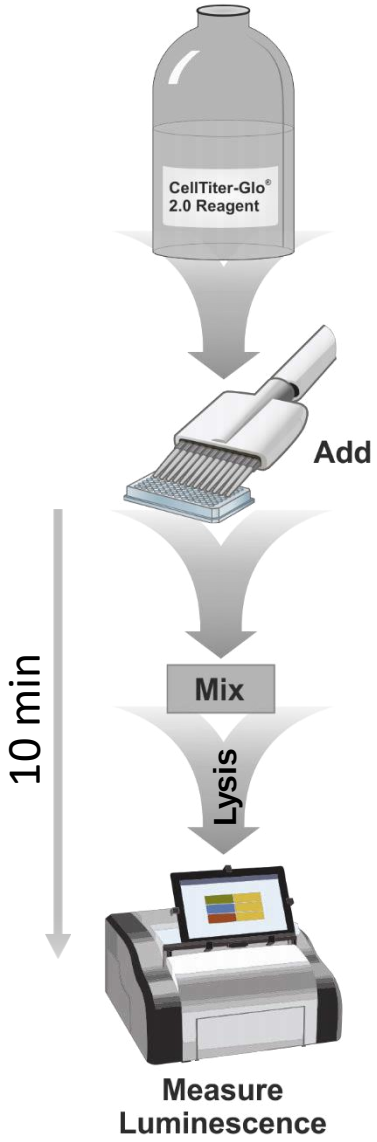
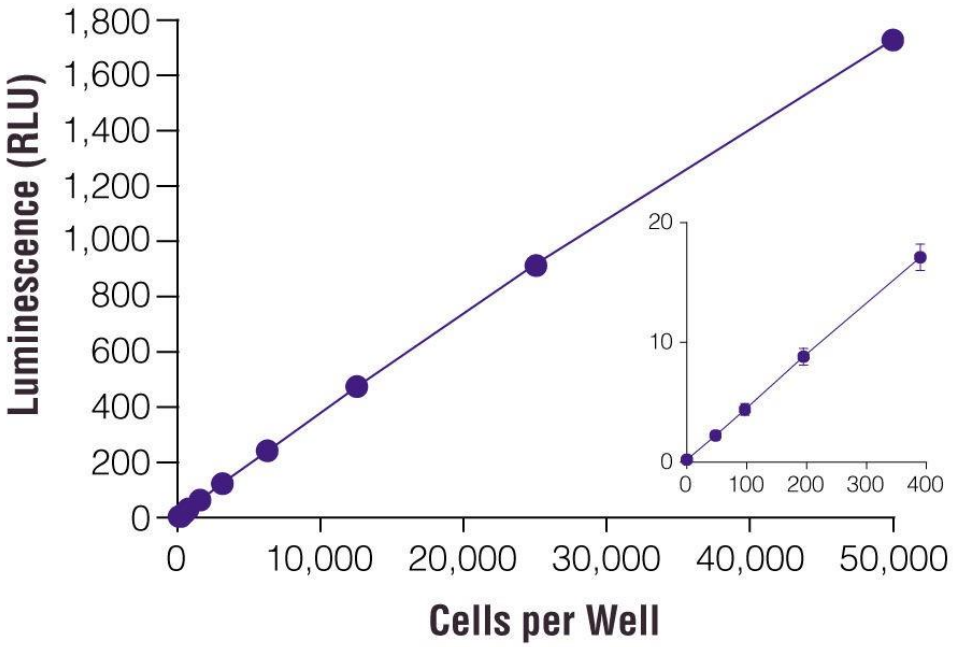
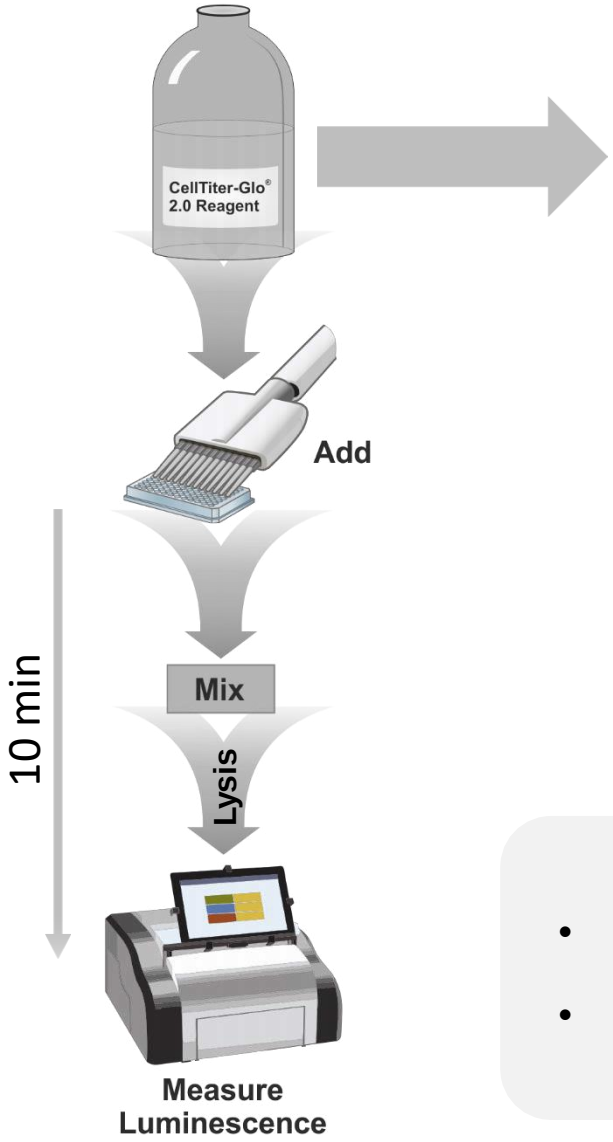
CellTiter-Glo® – Large dynamic range

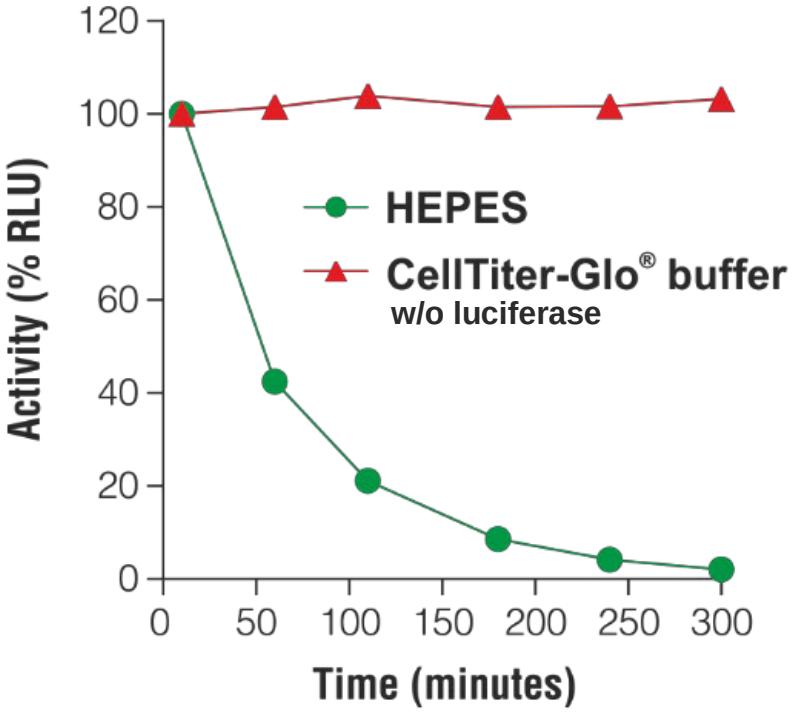




- Linear range: 10 – 50,000 cells
- Signal stability: Half life > 3 hours, robust: High Z‘-factor
- With callibration curve allows precise quantification of ATP concentration in cells

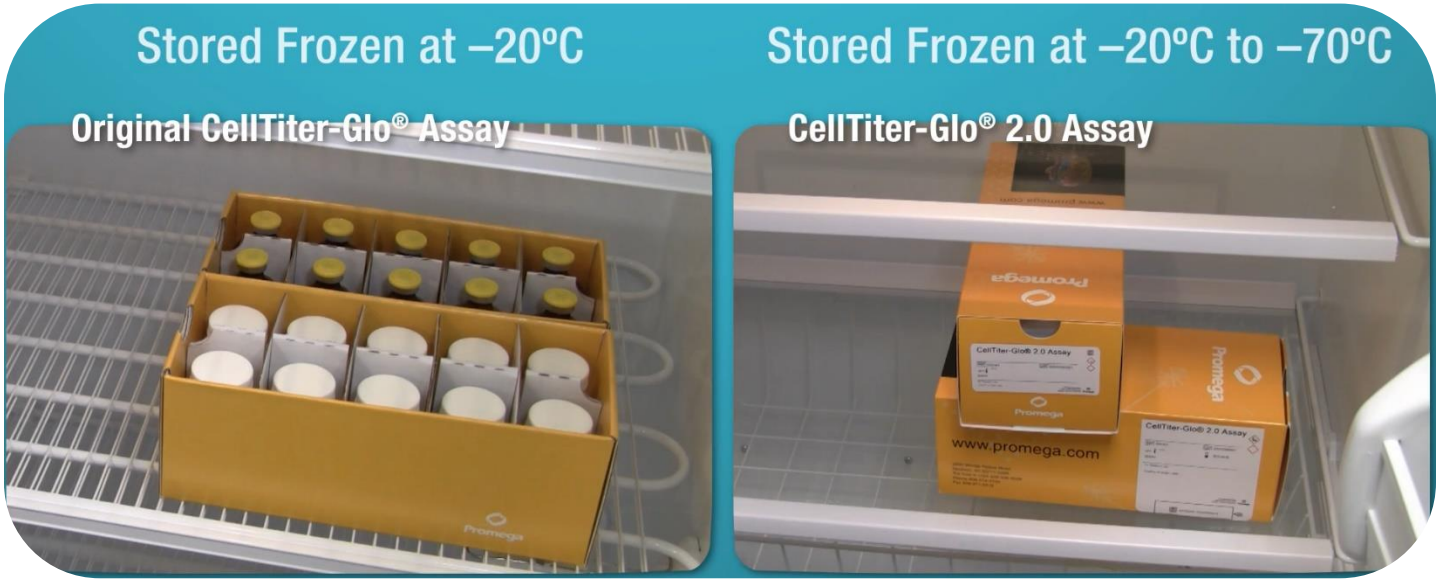
CellTiter-Glo® – ATPases inhibited by assay components





- After cell lysis, ATP is rapidly degraded by cellular ATPases
- Endogenous ATPases are fully inhibited by the reagent

CellTiter-Glo® 2.0– more stability and less pipetting

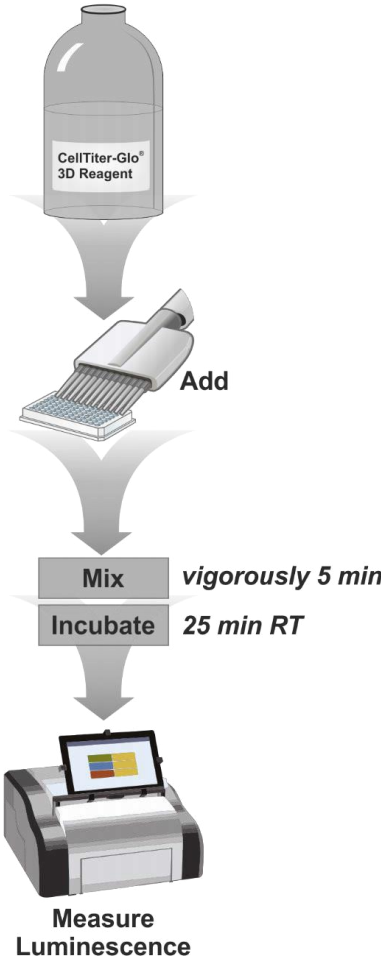


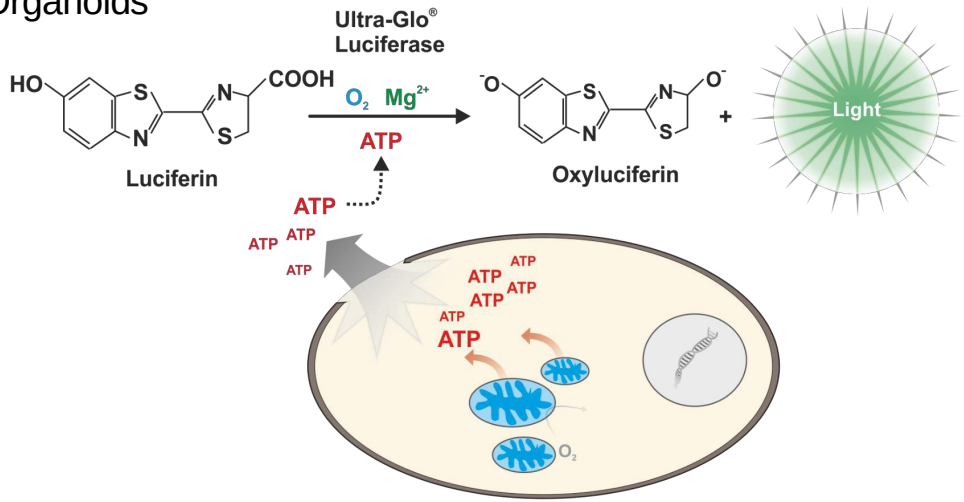
- Form of a single solution, will last 2 months in the fridge without significant loss of activity.
- However, long term storage best in -80°C.
- Can undergo five cycles of freeze-thaw without loss of activity.
- Same great performance as the classic CellTIter-Glo.

3D version of CellTiter-Glo

Optimized and validated for 3D – Spheroids & Organoids

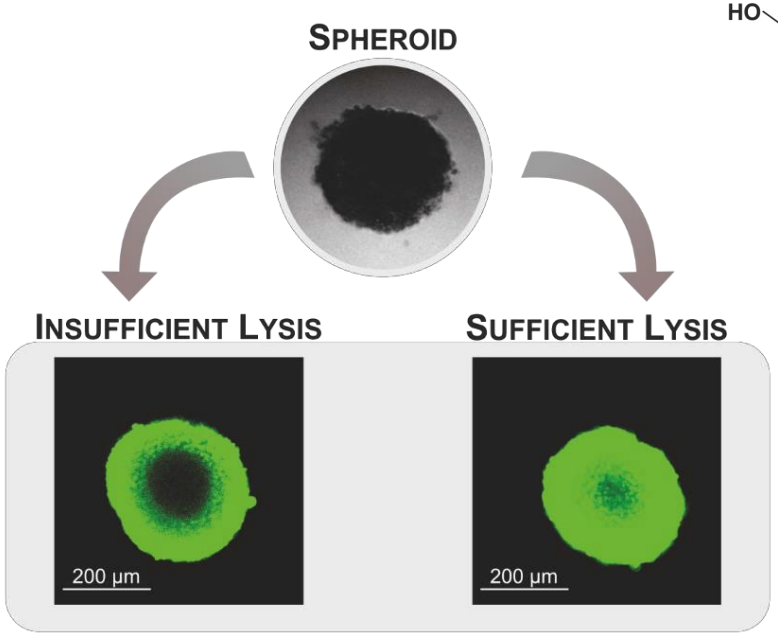
Change in protocol very important



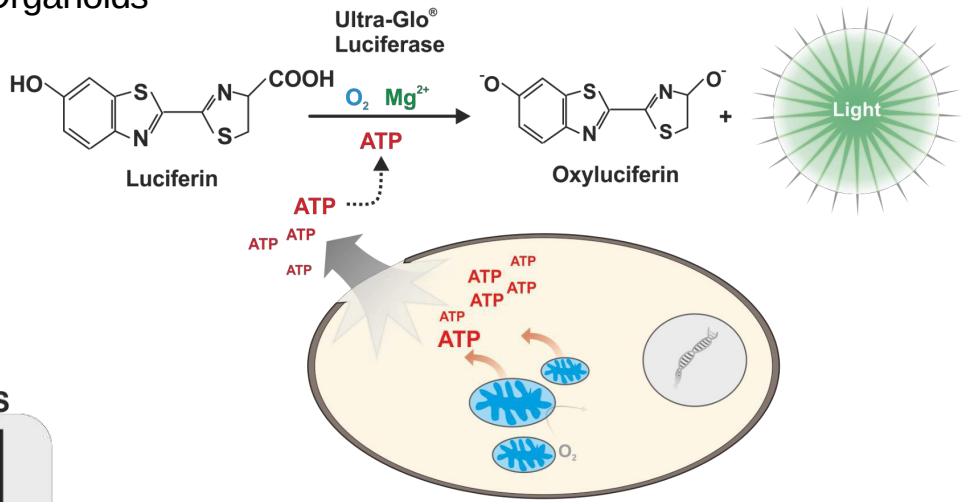


3D version of CellTiter-Glo

Optimized and validated for 3D – Spheroids & Organoids

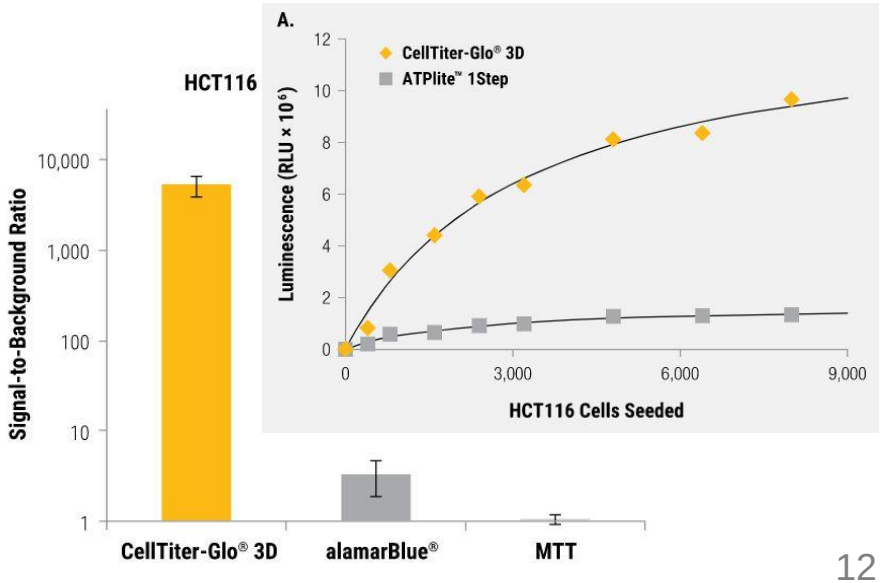


ATPlite CellTiter-Glo 3D



CellTox-Green™

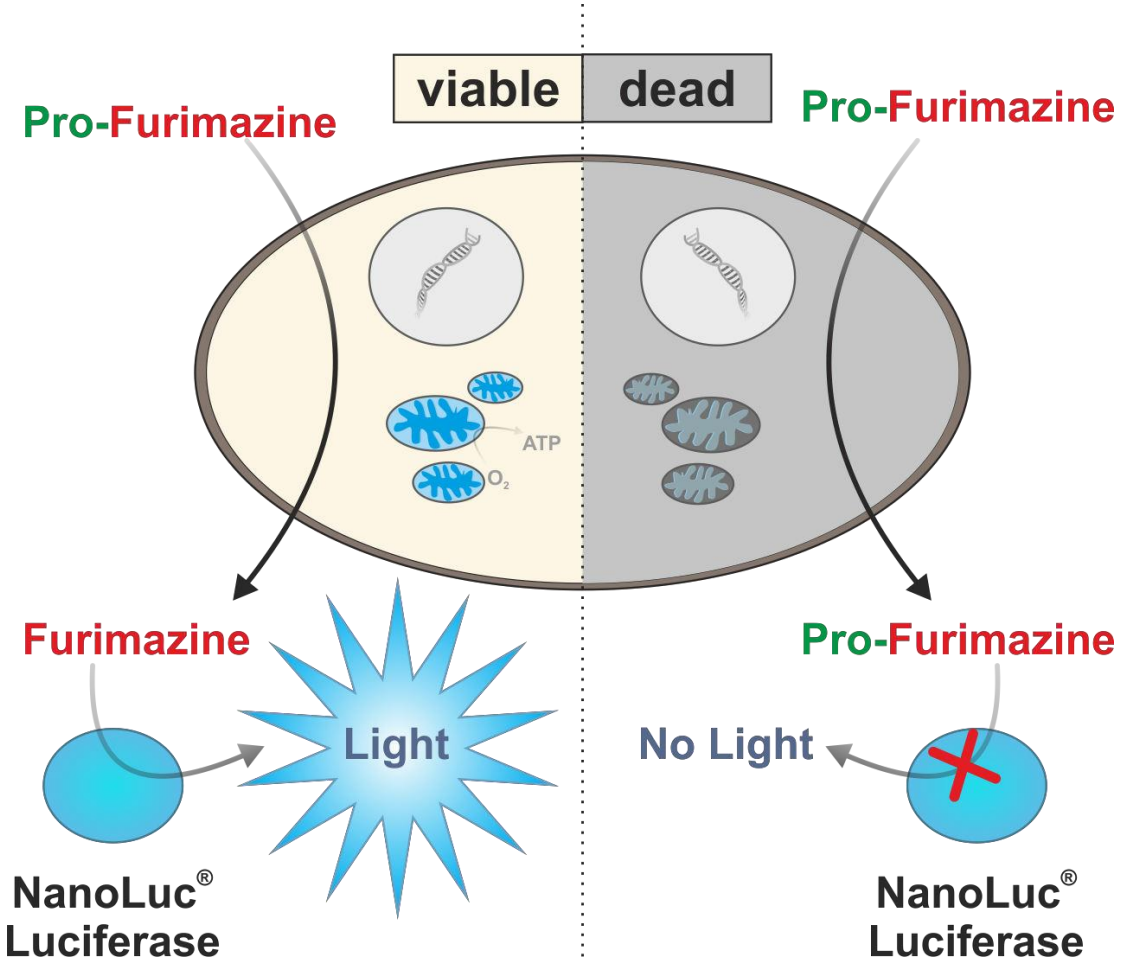
Complete lysis was validated by staining with cell-impermeable fluorescent dye.



CellTiter-Glo features and recommendations

- Ultra-Glo luciferase-proprietary recombinant luciferase (a different enzyme than in the luciferase reporters) is resistant to inhibition by small molecules or detergents-in lysis buffer, also is thermostable and gives stable glow type signal with half life of 3 hours. No competitor has such a good Firefly luciferase enzyme.
- Keep assay reagent in the dark. Never heat above laboratory temperature.
- Neither serum nor phenol red interferes with CellTiter-Glo. However prepare the ATP calibration curve in serum-free medium (residual ATPases).
- Always first equilibrate the plate with cells to lab temperature before adding the reagent. The cooling down of the cells has negligible effect on ATP content.
- Do not ever use overgrown cells or too many cells per well in the assay, their ATP content will be low or the signal half life will be short.
- Generally, some cell types can have much lower or higher ATP content, you need to test.

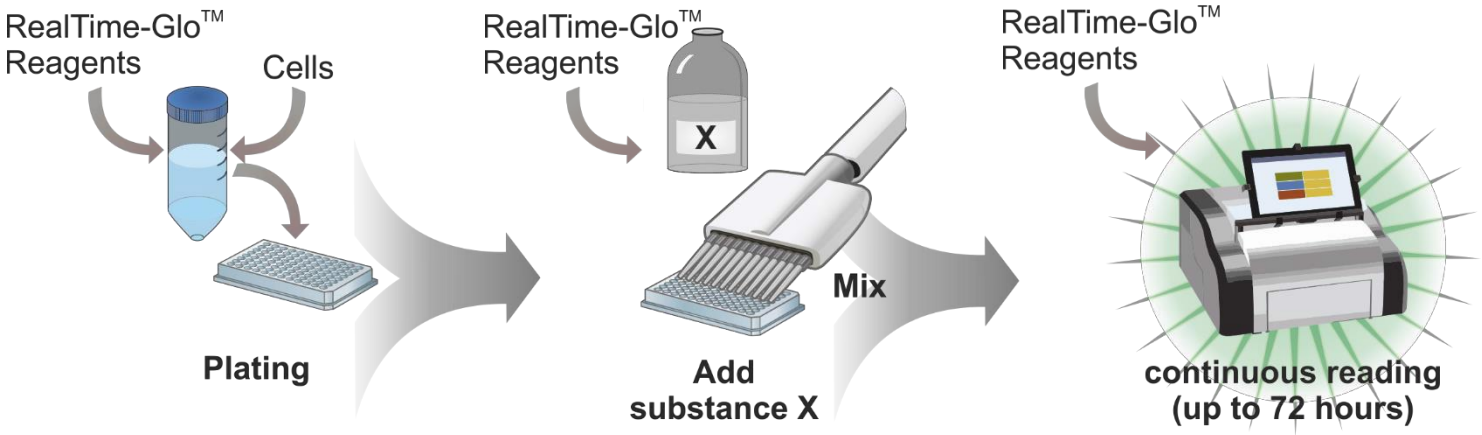
Real Time-Glo MT Cell Viability Assay

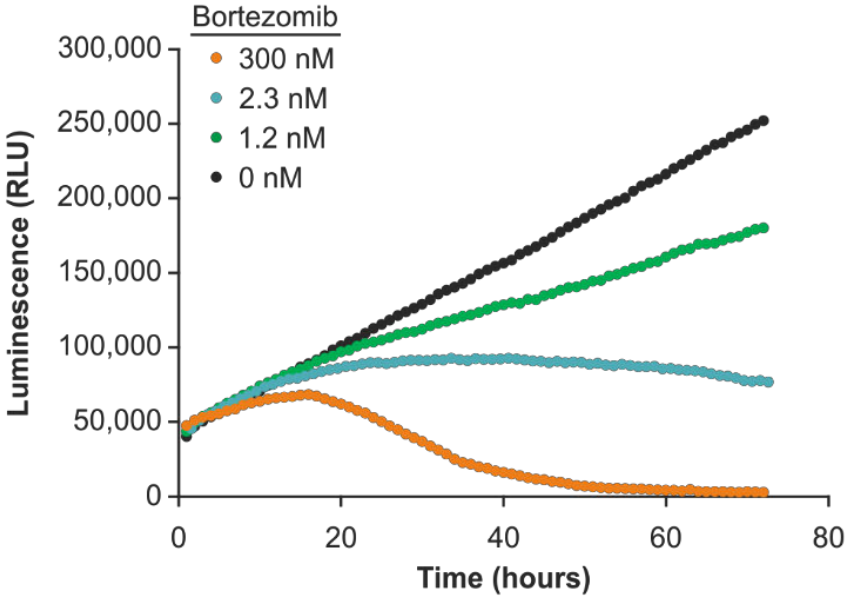


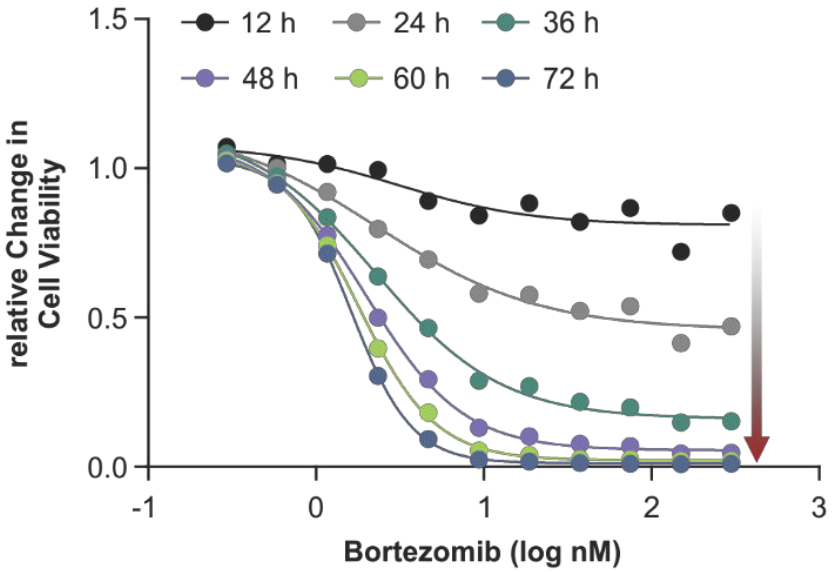
FACTS

- NanoLuc® luciferase is present in culture medium
- Cell-permeable pro-substrate “Pro-Furimazine” is intracellularly reduced to form Furimazine
- Furimazine diffuses from the cell and is rapidly consumed by NanoLuc® to produce light
- Supports kinetic measurements up to 72 h

Real Time-Glo™ Assay – Workflow and Data

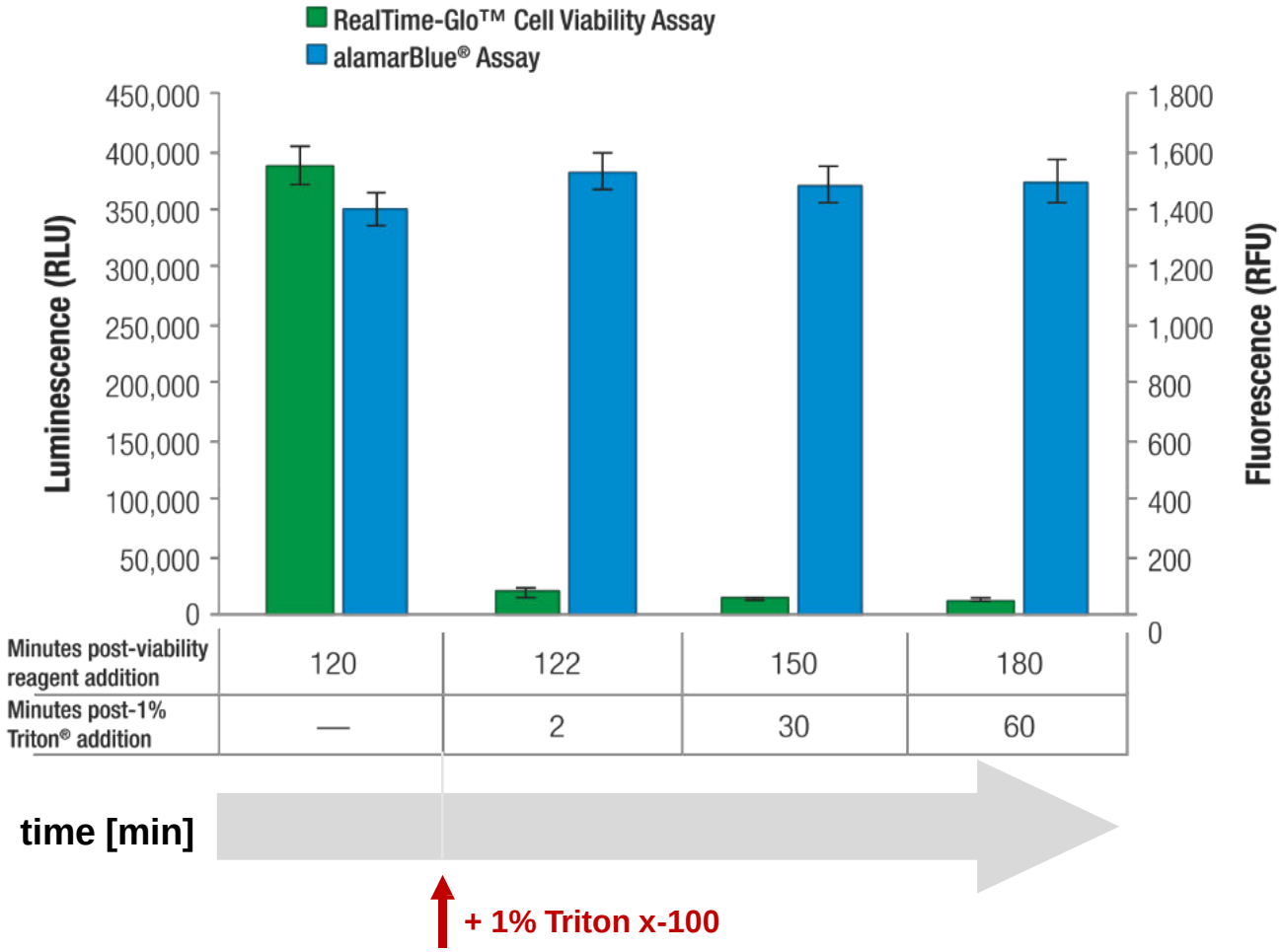






True real time assay – Furimazine Does Not Accumulate

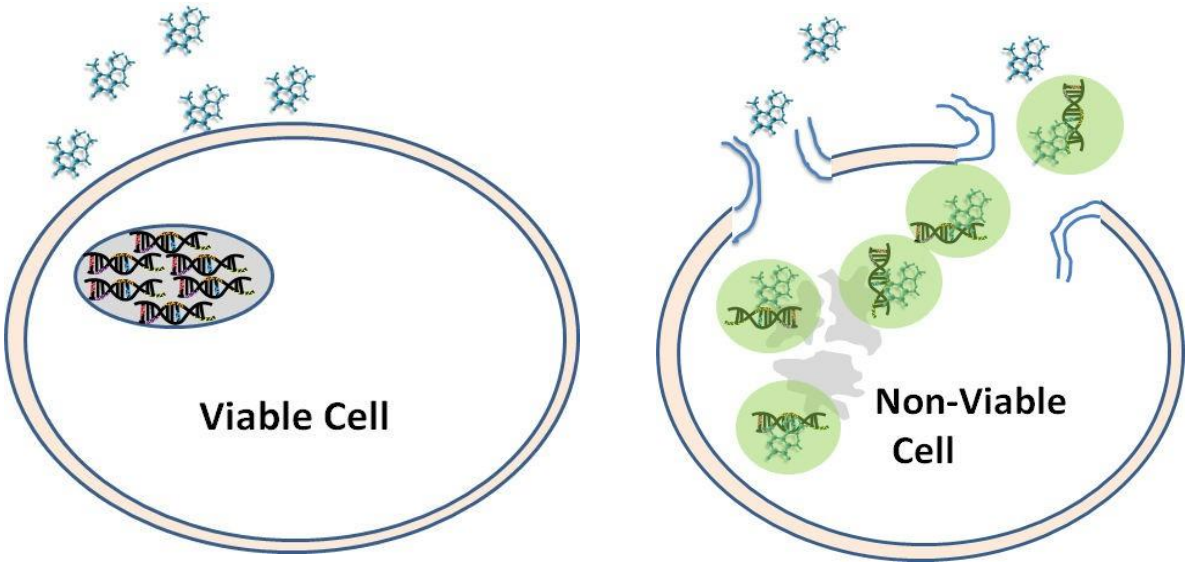
- Limited cell permeability
- Shows true „real time“ situation



CellTox™ Green Cytotoxicity Assay

How to define cytotoxicity?

Membrane integrity "sensed" by environmental dye.



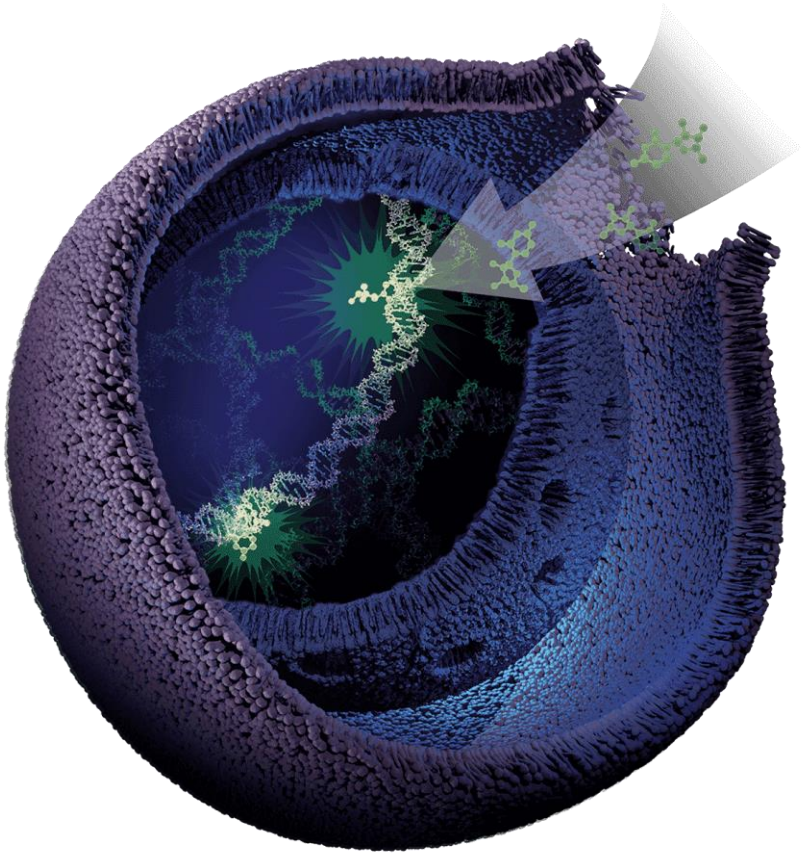
Excluded dye yields no increase in fluorescence.

Non-excluded dye yields increase in fluorescence

Main advantages

CellTox™ Green Cytotoxicity Assay

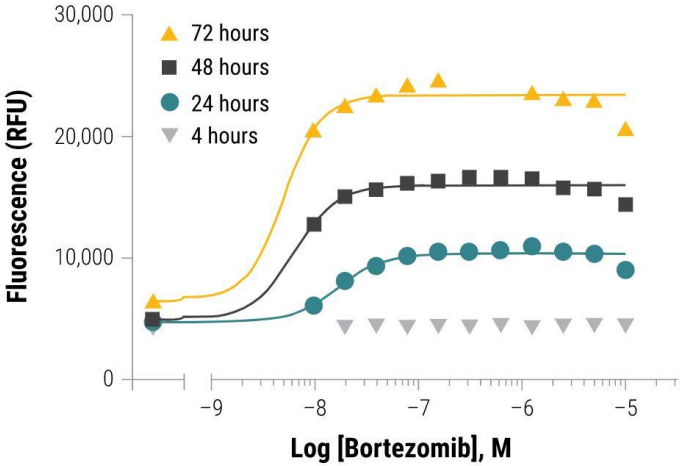
- Kinetic measurement (72 hrs.) Ease of use (no touch assay) Flexible protocol options
- Multiplexing possible (e.g. with RealTime-Glo™ Cell Viability Assay)
- Downstream use of cells possible
- Suitable for 3D cultures
- Detection by plate reader, flow cytometry, microscopy possible
- GFP/FITC filter set comatible
- Cheap (1000x dilution)

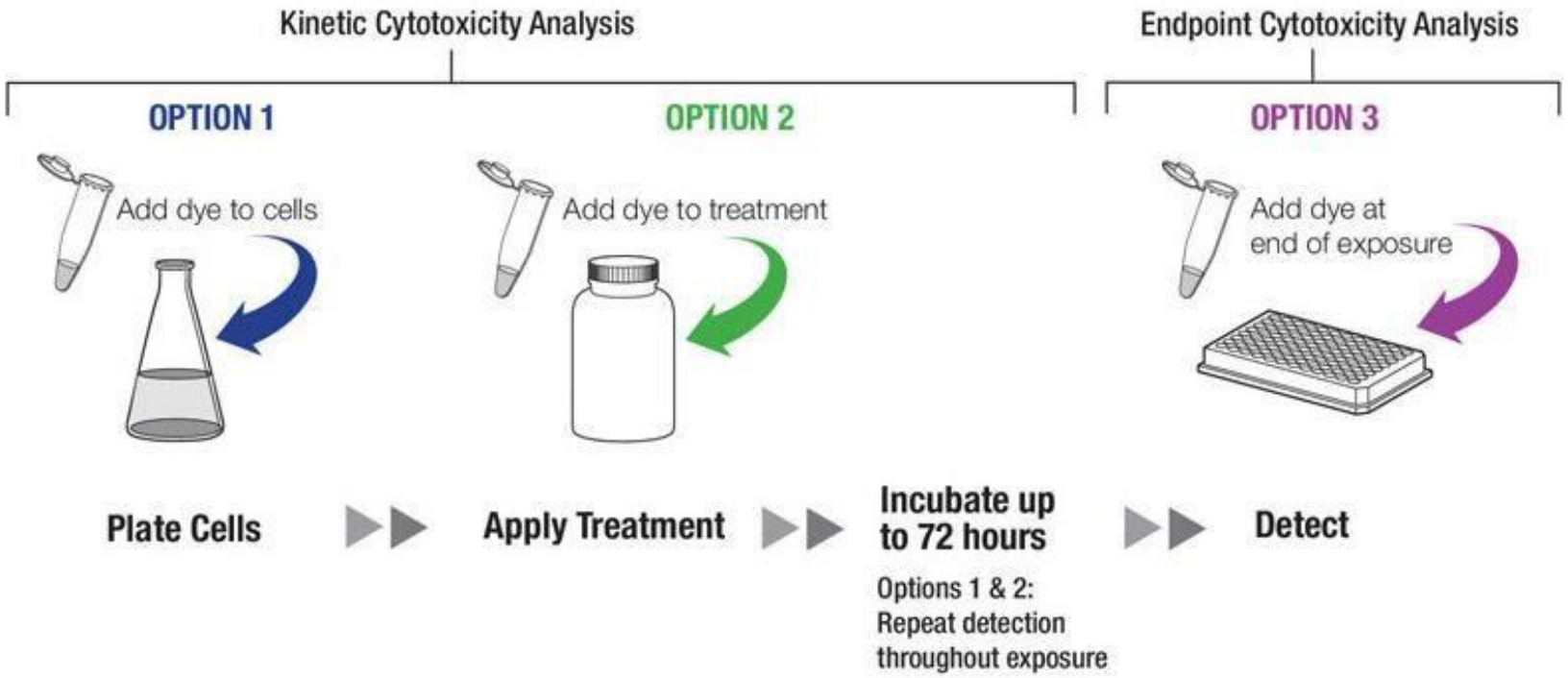


Flexible protocol

The reagent can be added

- When cells are seeded
- When adding the treatment to cells
- At the end of experiment

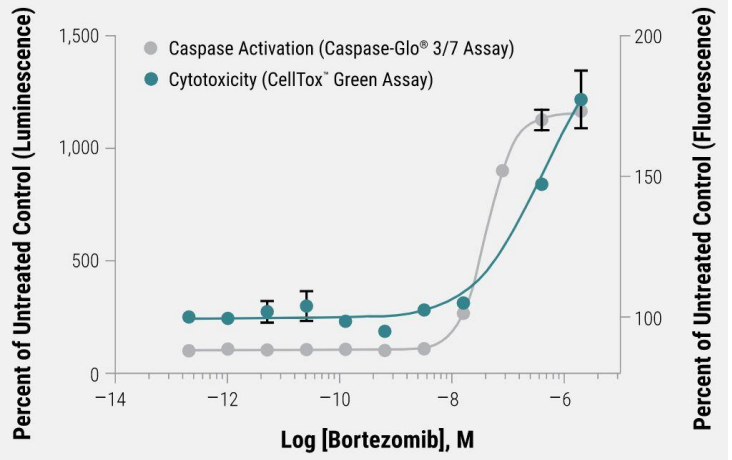


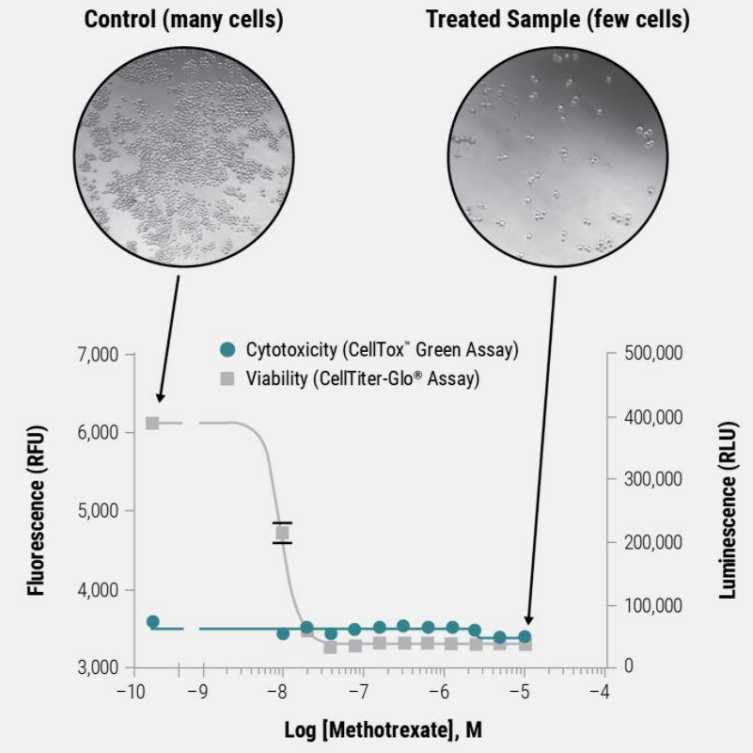


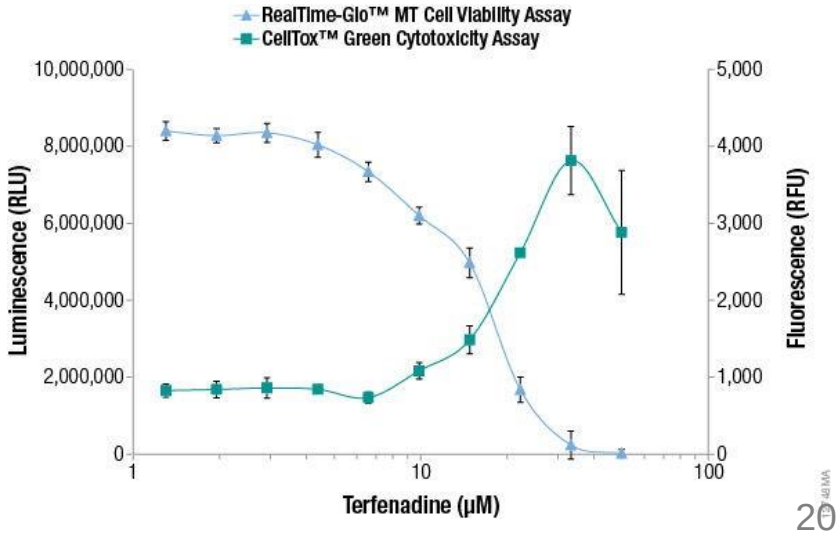
Assay was designed for multiplexing

CellTox™ Green Cytotoxicity Assay

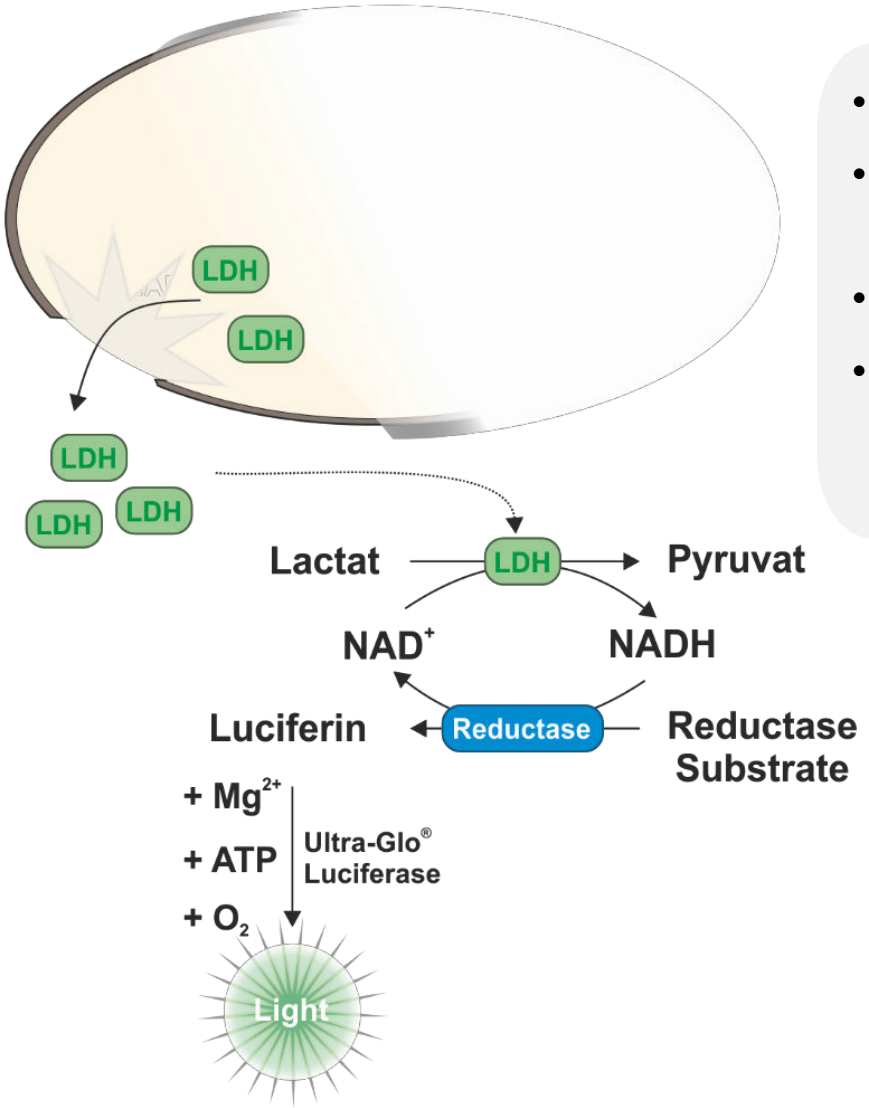
- Natural multiplexing with kinetic assays-CellTox Green can be present in the same well the whole time
- Will differentiate cytotoxic from cytostatic efects
- Can help estimating when to measure apoptosis





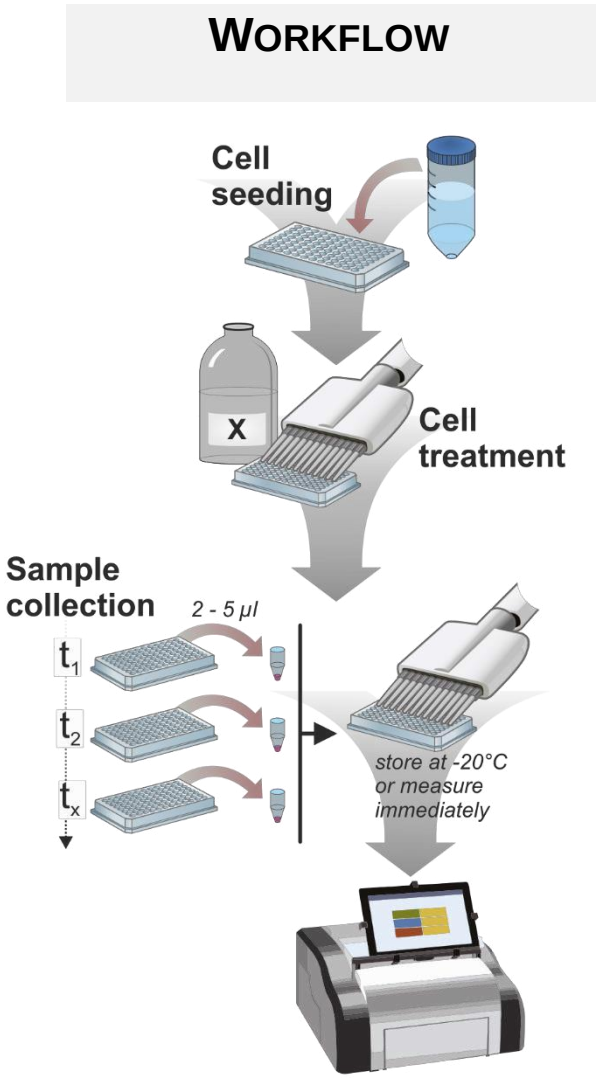


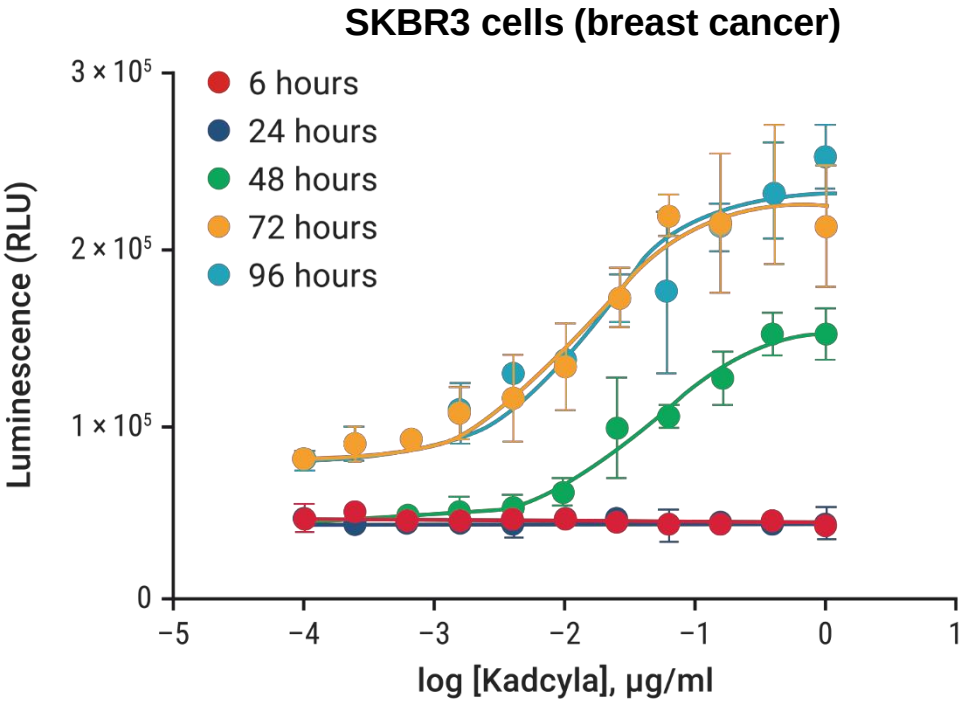
LDH-Glo® Assay – measures LDH leaking from cells



- Coupled enzymatic reaction to detect LDH
- Pro-Luciferin (Reductase Substrate) is being reduced
- Works well also for 3D cell cultures
- can be used to measure antibody-dependent-cell mediated cytotoxicity (ADCC)

LDH-Glo™ can be run as a kinetic assay

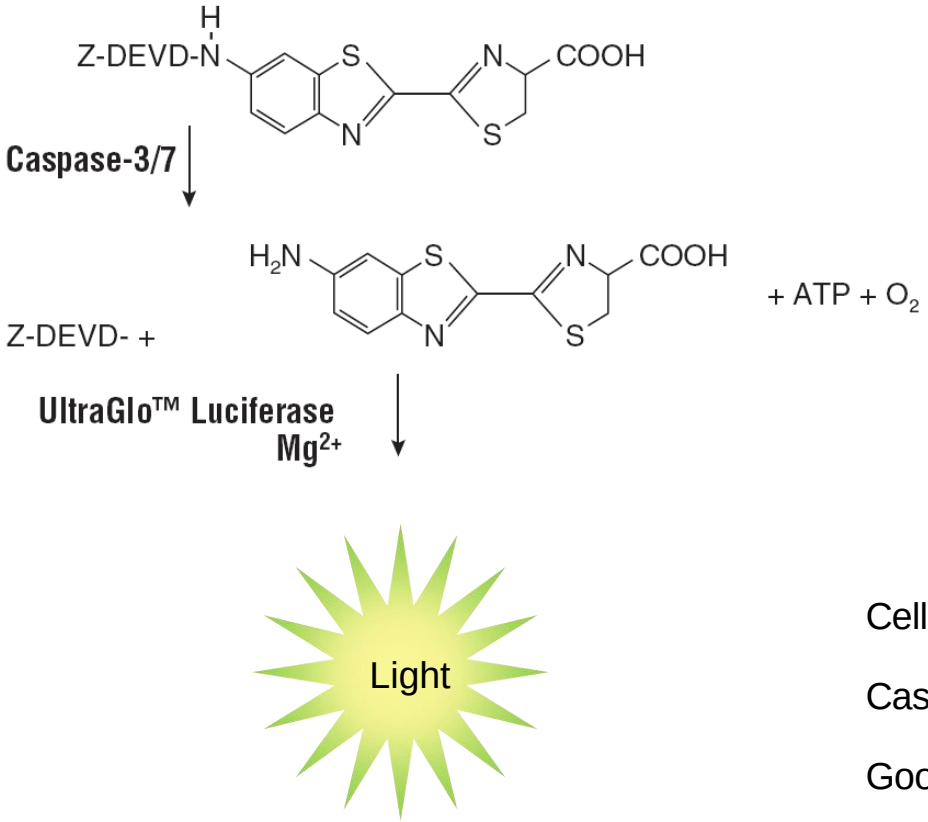


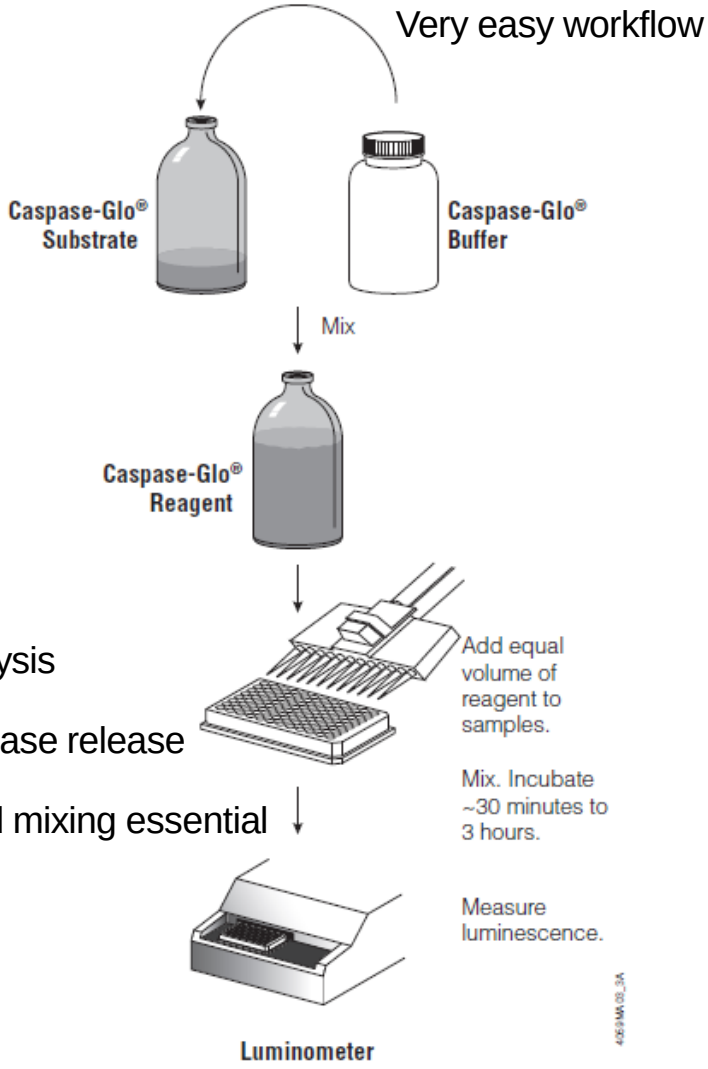


- Activity-based cytotoxicity marker
- Supports kinetic analysis
- Heat-inactivated FCS = sensitivity ↑

Measuring apoptosis

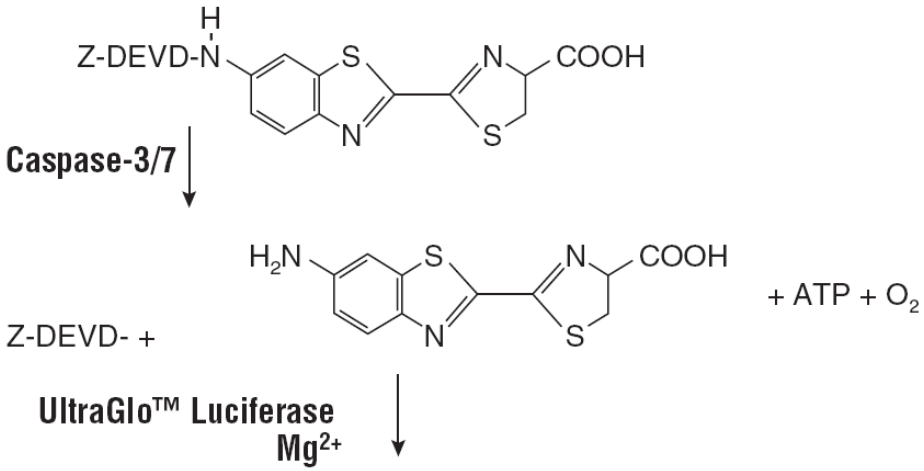
Measuring apoptosis–Caspase-Glo principle



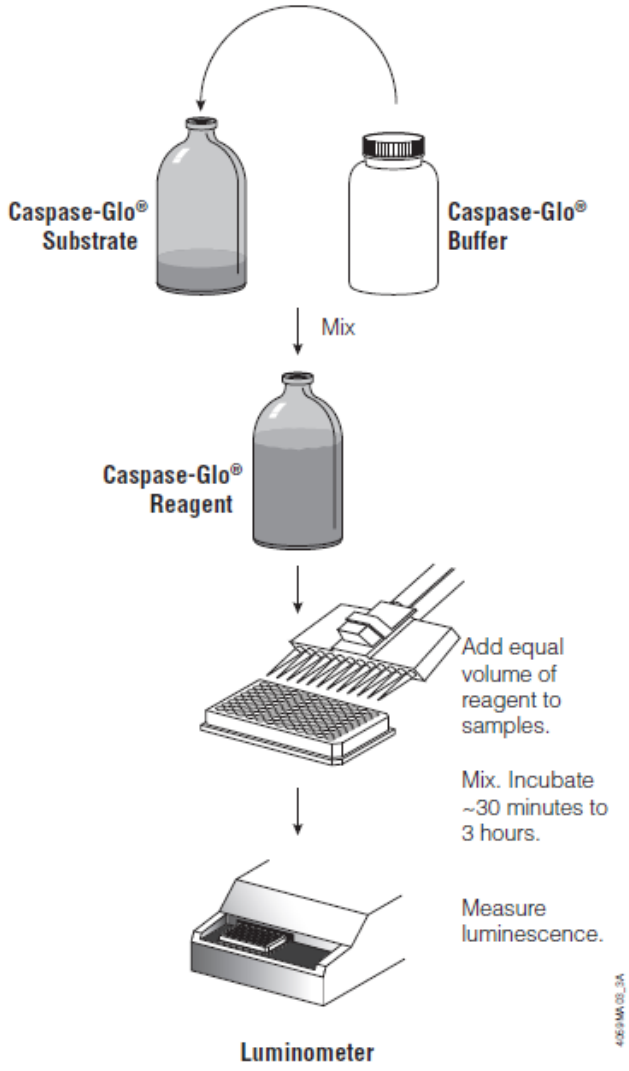


- Measurements in cell lysate but in vitro purified enzyme measurements also possible

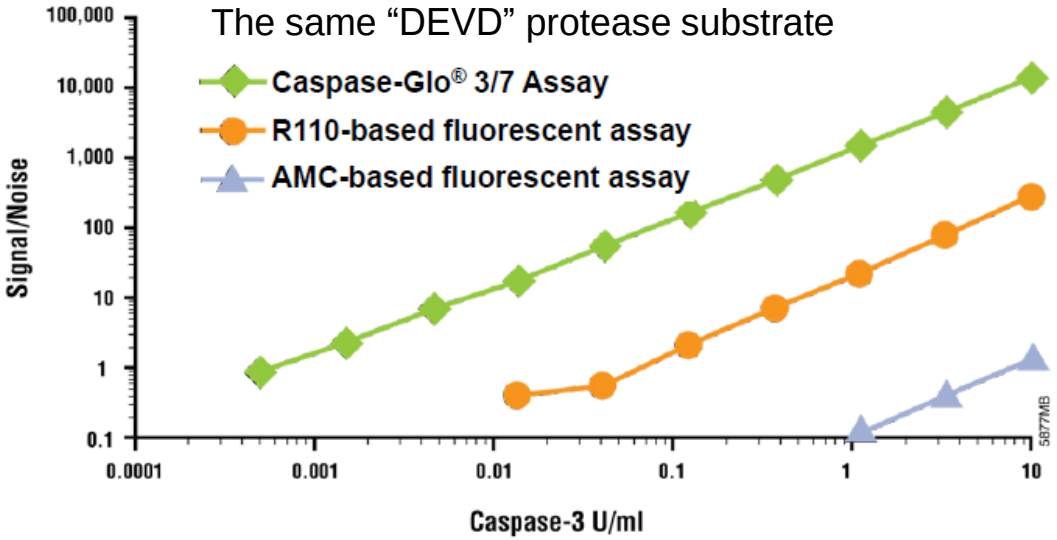
Measuring apoptosis – substrate for each Caspase



Popular caspase assays	
Substrate	**Caspase-Glo™ Assay**
Z-W**EHD**-aminoluciferin	Caspase 1
Z-**DEVD**-aminoluciferin	Caspase 3/7
Z-**LETD**-aminoluciferin	Caspase 8
Z-**LEHD**-aminoluciferin	Caspase 9



Superior sensitivity of luminiscence



The Caspase-Glo® 3/7 Assay can detect caspase 3/7 activation at lower levels than fluorescent methods.

- Luminescence measurements have minimal background (unlike fluorescence)
- The detection is very sensitive
- Broad range of concentrations, where the assay is linear
- Why does it matter?

Caspase-Glo recommendations

- We can also detect measurable caspase activity in the control "untreated" sample.
- Serum also has residual measurable caspase activity.
- You must include **two controls** in the experiment, cells without treatment and wells with medium only.
- It is good to adhere to SOPs for cell culture to reduce this source of variability. Poorly cultured cells also have a different response to your "treatment".
- You need to find out the appropriate time point for measuring caspase activity after adding your "treatment". Caspase activity is present **only transiently** in dying cells. “The treatment should not be long enough to affect the rate of cell proliferation / cause necrosis - there it would be necessary to normalize with CellTiter-Glo, etc.

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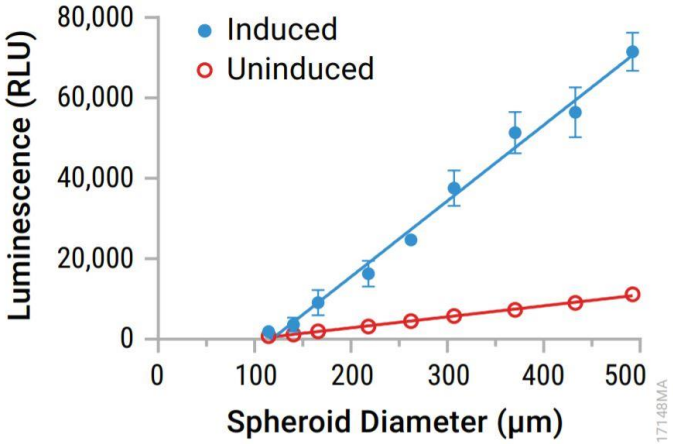
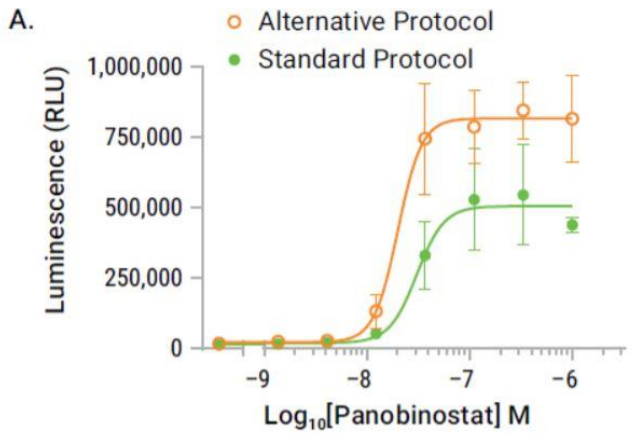
- A suitable measurement interval is **30 min to 1 hour** after the addition of Caspase-Glo, that luciferase and caspase activity reach steady state, the signal is stable for several hours and then slowly decreases. Waiting 1 hour helps to reduce the background from self-cleaved substrate.
- Let the plate / plates equilibrate to room temperature before adding the assay.
- In Caspase-Glo 8 and 9 the proteasomal activity can interfere with the assay, so the kits contain the proteasomal inhibitor MG-132.
- The assay is resistant to DMSO up to a high concentration of 5–10%

Caspase-Glo 3/7 3D

- The familiar essay, but with an enhanced lytic capacity.
- Ultra-Glo luciferase is modified so that its enzymatic activity is maintained even in harsher lytic conditions.
- Modified protocol for optimal results with 3D cultures, 30 to 60 s shaking.
- Thoroughly validated with spheroids created in ultralow attachment plates, by hanging drop method or those growing in matrigel.
- For cells in matrigel optimized "alternative protocol" must use Cell recovery solution (Corning), it provides higher RLUs.





Data normalization

Start the experiment with as similar number of cells in each well as possible, e.g. do transfection in bulk and seed the cells later.

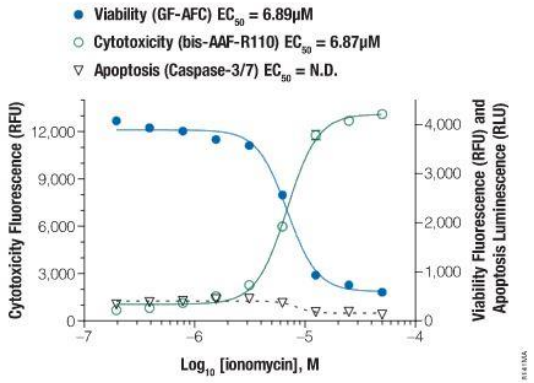
- To control for the toxicity of tested compounds, two similar options are the best.
- **Multiplex** the assay with a cell viability assay (fluorescent) in the same plate.
- **Parallel measurement** in two plates. CellTiter-Glo as an ideal assay for normalization.
- Other methods, like normalizing to total protein-Bradford, BCA introduce unnecessary error.

- **ApoLive-Glo** – combination of Caspase-Glo 3/7 and a fluorescent cell viability assay
- GF-AFC = measures live cell protease activity
- Fluorescent supplementary assays are also sold separately and can be combined in various ways.

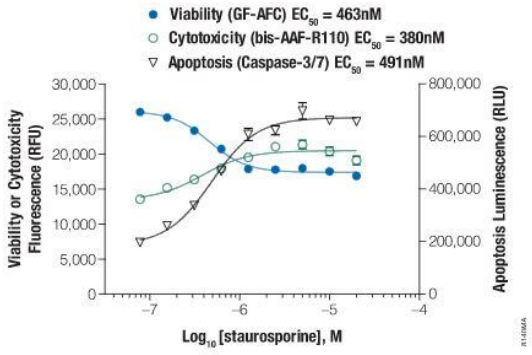
Apotox-Glo – triplex assay

- Combination of Caspase-Glo 3/7 with two more fluorescent assays detecting live cell protease and dead cell protease activity.
- GF-AFC substrate = peptide-modified coumarin that begins to fluoresce (blue fluo) upon cleavage of the peptide inside the cell by live cell protease activity. It passes spontaneously across the cell membrane.
- bisAAF-R110 substrate = peptide-modified rhodamine, which is cleaved by protease activity released from necrotic cells and begins to fluoresce in red. It does not pass spontaneously across the cell membrane.

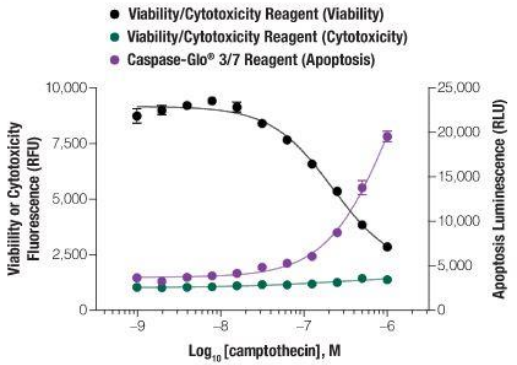
Necrosis Results



Apoptosis Results

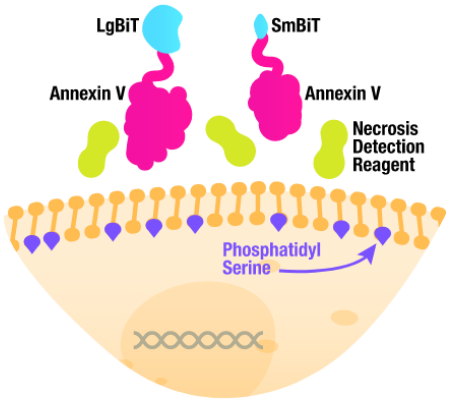


Cytostasis Results

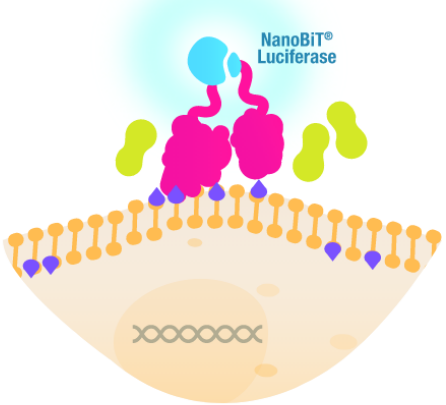


RealTime-Glo Annexin V assay – the kinetic advantage

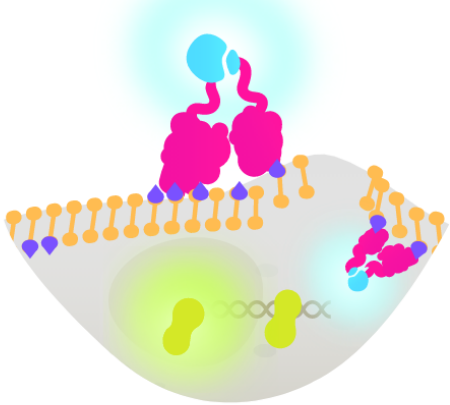
- Based on the NanoBiT split luciferase technology. LgBiT=large subunit, SmBiT=small subunit of NanoLuc (NanoBiT).
- Contains: Annexin V-LgBiT fusion, Annexin V-SmBiT fusion and luminescence substrate. All three are added into the cell culture media.
- Annexin V proteins bind phosphatidylserin in the outer cytoplasmic membrane leaflet and bring LgBiT and SmBiT into proximity. They complement and form a functional NanoLuc luciferase.
- Typically combined with CellTox Green cytotoxicity measurement.



PS confined to inner leaflet
Cell membrane intact
Luminescence (RLU) negative
Fluorescence (RFU) negative



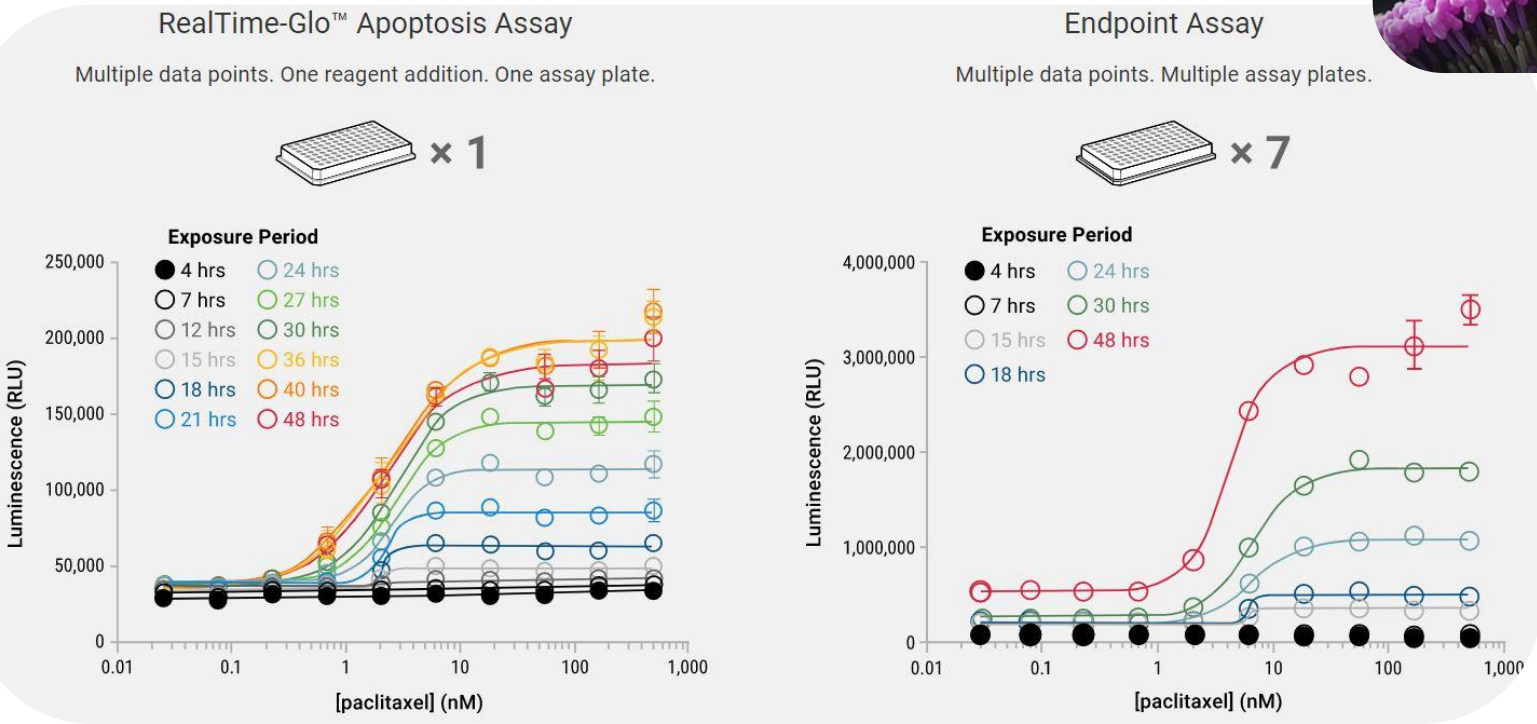
PS translocation to outer leaflet
Cell membrane intact
Luminescence (RLU) POSITIVE
Fluorescence (RFU) negative



PS on inner and outer leaflet
Cell membrane compromised
Luminescence (RLU) POSITIVE
Fluorescence (RFU) POSITIVE

RealTime-Glo Annexin V assay – the kinetic advantage

- Significant savings of reagents if we need to determine apoptosis onset.
- We get a lot more information from each well
- Often the kinetic information is important in itself. Different drugs trigger apoptosis with different kinetics.



We are also an exclusive distributor of Lonza

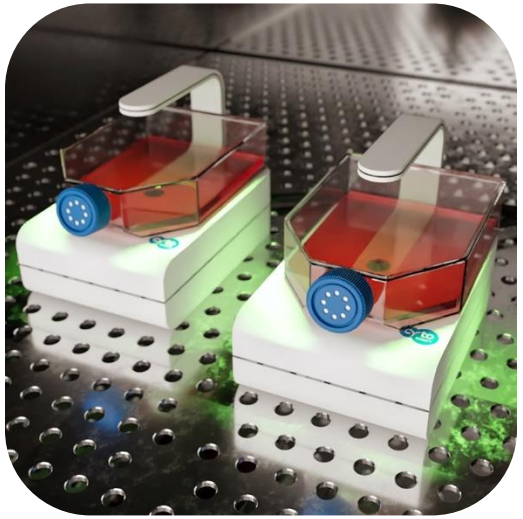
- Standard media like DMEM, serum-free media
- Media supplements
- Primary cells mainly human, mouse, stem cells
- Specialized media to culture these cells
- Cell transfection – Nucleofector
- Mycoplasma testing, endotoxin testing

Possibility of demo: Cytosmart OMNI, Lux2

Live cell imaging in 6 – 96 WP, culture flasks

Microscope that fits into the cell culture incubator

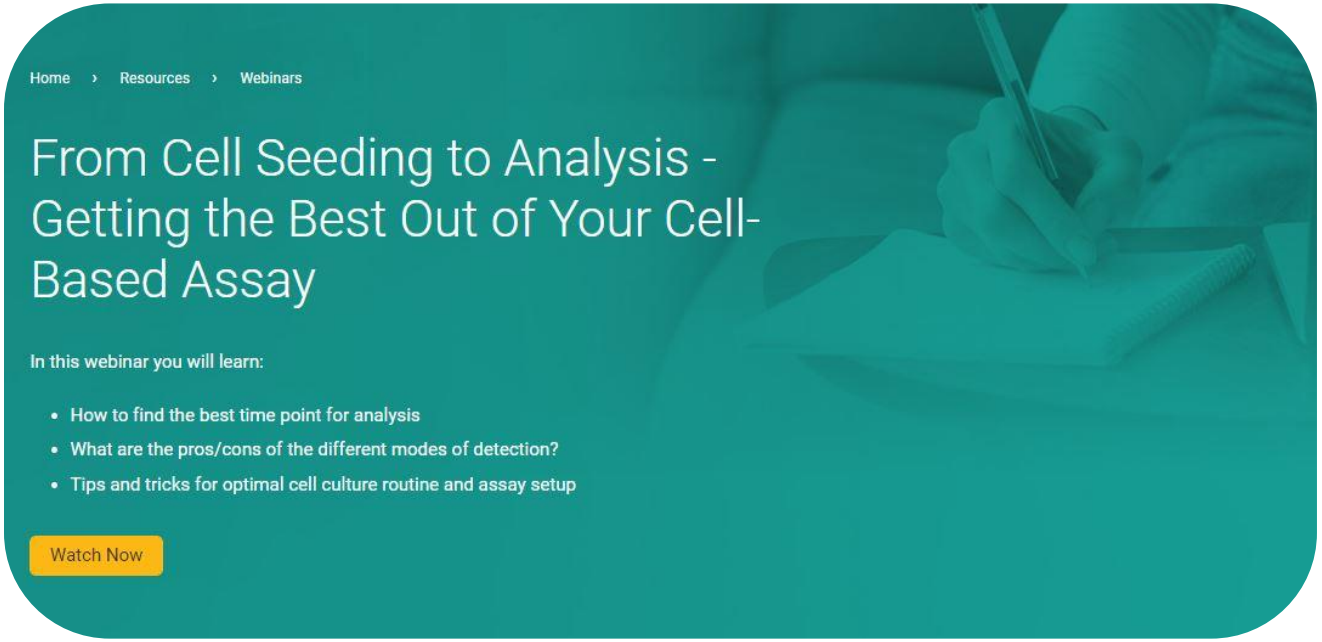
One week free non-binding demo in your lab possible



Promega Webinar Library

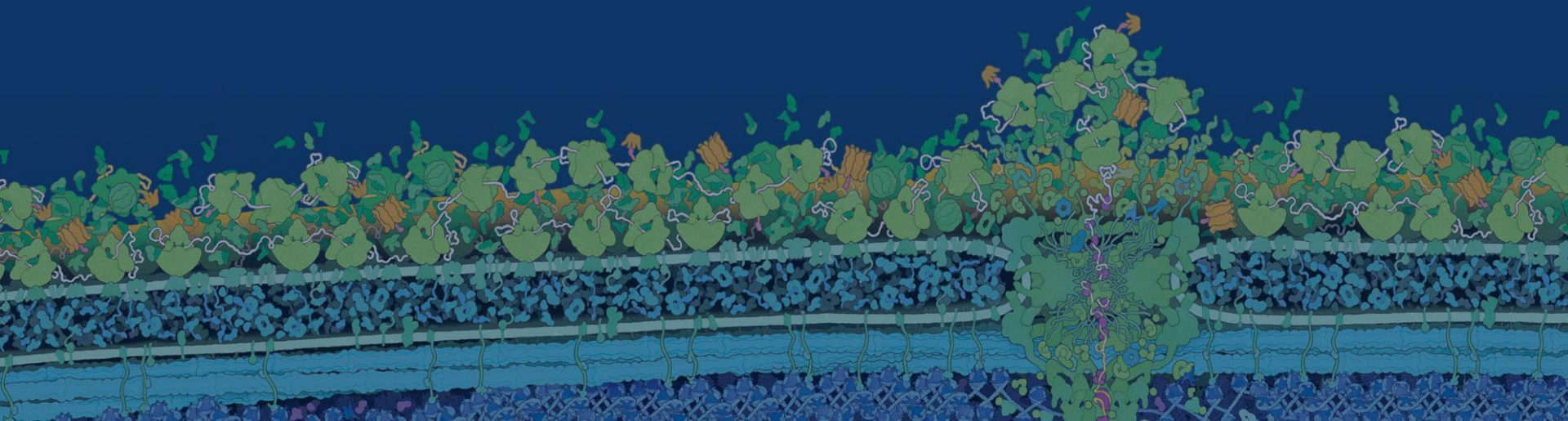
https://www.promega.com/resources/webinars/

- 3D cell culture models and assays
- NanoLuc and HiBiT technologies
- Energy metabolism



Summary

- I showed you assays to measure Cell Viability/Cytotoxicity, apoptosis in standard cell culture and 3D, in end point as well as in kinetic format.
- Based on consuming ATP, chemically modified luciferase substrates requiring reduction or cleavage by protease, split NanoLuc (NanoBiT) luciferase technology.
- Many assays are applicable in cells as well as in vitro. They are usually designed as a complete experiment with positive and negative controls. Promega has strict QC.



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- Many assays are applicable in cells as well as in vitro. They are usually designed as a complete experiment with positive and negative controls. Promega has strict QC.
- There are rich resources in the form of application notes, webinars, just ask for them
- We offer a complete solution for cell biology: cell culture media, transfection reagents and instruments, primary cells, cell culture microscopes and other small lab equipment...
- Demo possibilities for instruments and sample packages for reagents are available.