Monitoring energy metabolism changes in metabolic disorders, cancer and immune cells

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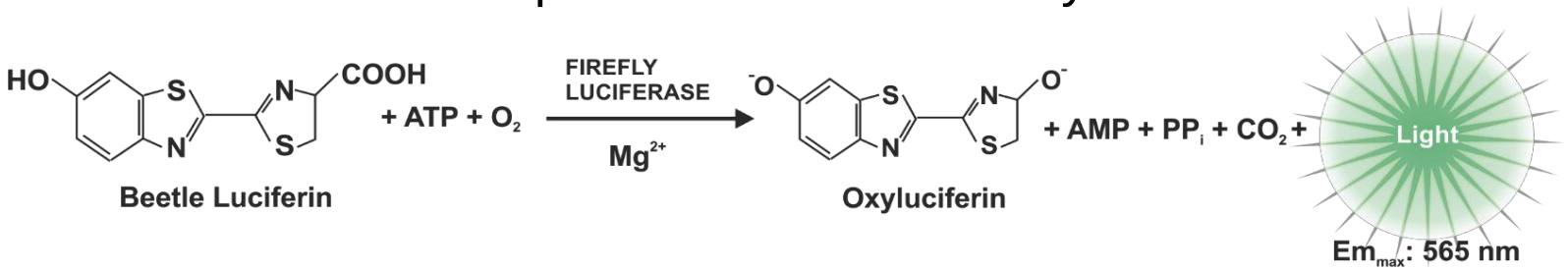
Today‘s Agenda

1. Luciferases and their basic features
2. Basics of cellular metabolism and how we can measure it
3. Studying insulin biology with metabolic and Lumit assays
4. Metabolic assays in cancer and immunology
5. News flash from cell-biology portfolio

- The presentations will be provided in pdf after the seminar
- Please fill in the satisfaction survey, any feedback is welcome

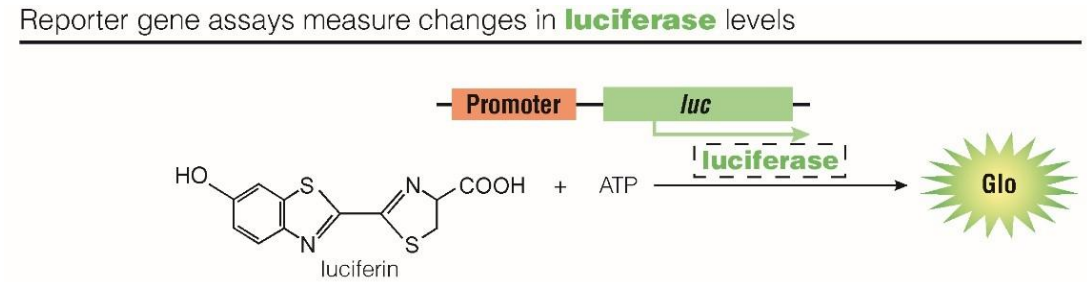
Firefly Luciferase Basics

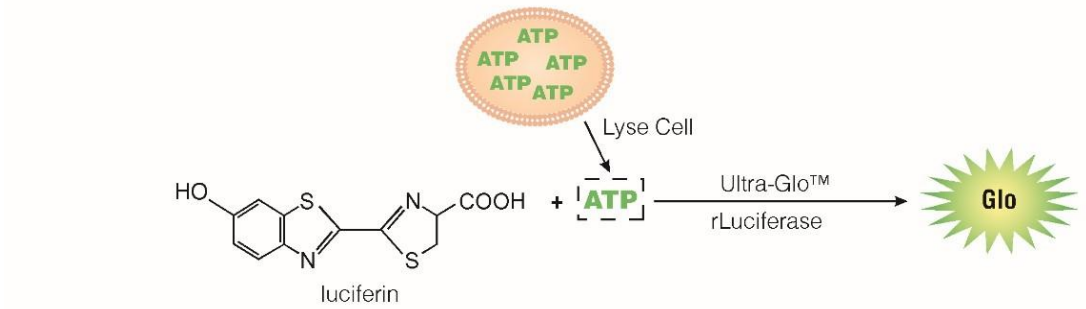
- Bioluminescence = production of light by living organisms
- Enzymatic oxidation and excitation of luciferin substrate by luciferase enzyme
- Firefly luciferase – most widely used enzyme
  - ATP-dependent luciferase
  - Universal reaction adaptable for various assays

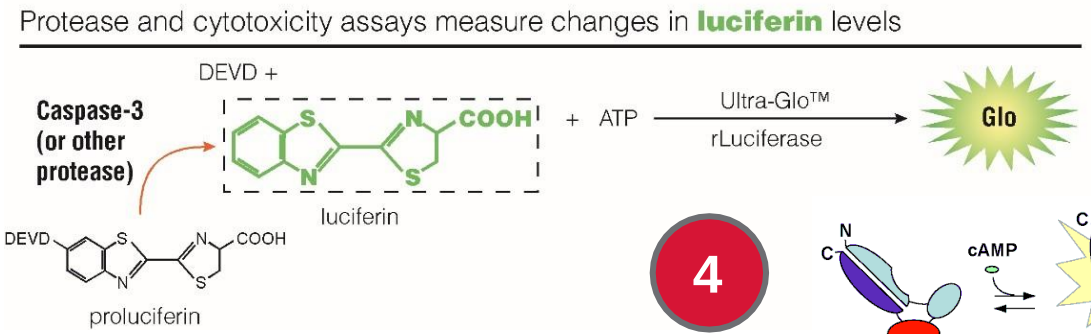


  - Ultra-Glo™ rLuciferase – mutated version of *P. pennsylvanica* luciferase
  - Higher stability in the presence of detergent and reducing agents
  - More robust signal – less temperature signal variance

Firefly Luciferase Applications







1. Measure luciferase expression

- Reporter gene assays
- GPCRs
- Nuclear receptors
- Pathway activation

2. Measure ATP levels

- Cell viability
- Kinase assays
- cAMP assays
- Phosphodiesterase assays

3. Measure luciferin release

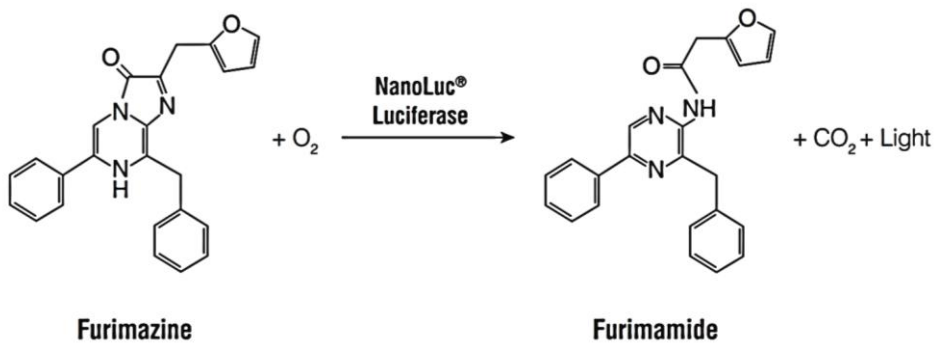
- Protease assays
- CYP450 assays
- Oxidative stress assays
- HDAC/SIRT assays

4. Measure modified luciferase activity

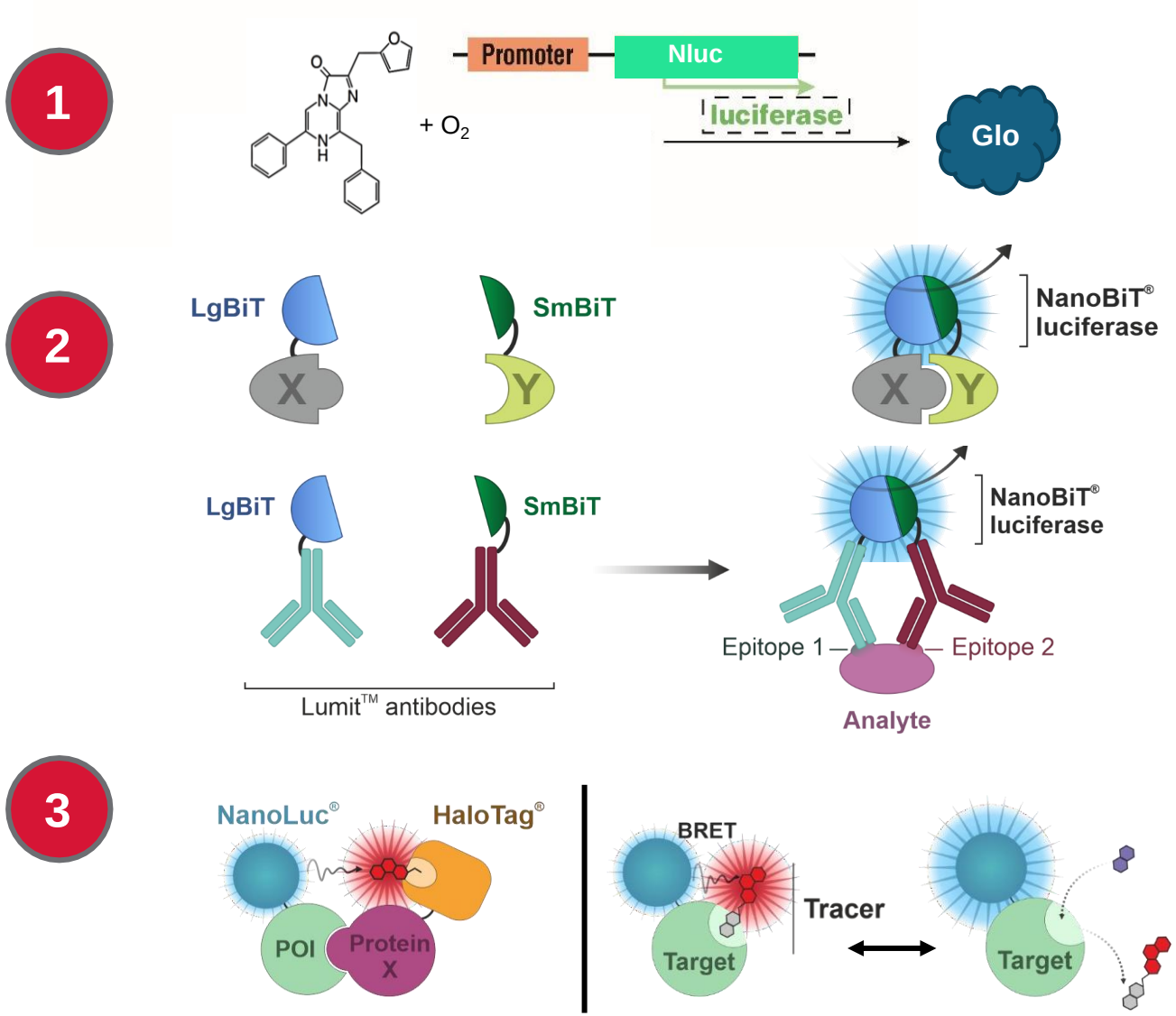
- GloSensors (cAMP, cGMP, proteases)

NanoLuc® Luciferase – Novel Experimental Reporter

- ATP-independent luciferase from a deep sea shrimp
- 100x brigther than Rluc and Fluc, glow-type luminescence
- Smallest luciferase – 19,1 kDa
- Enables the setup of highly sensitive assays
- No overexpression of reporter required
  - ~100-fold fewer molecules of NanoLuc® than Firefly to get measurable signal
  - Work at physiologically relevant expression levels



NanoLuc Luciferase Applications



1. Measure luciferase expression

- Reporter gene assays
- GPCRs
- Nuclear receptors
- Pathway activation

2. Measure split luciferase reconstitution

- Protein:protein interaction assays
- Real-time apoptosis measurements
- Lumit immunoassays

3. Combine NanoLuc with fluorophores in NanoBRET assays

- Protein:protein interactions
- Small molecule permeability and binding

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2. **Basics of cellular metabolism and how we can measure it**
3. Studying insulin biology with metabolic and Lumit assays
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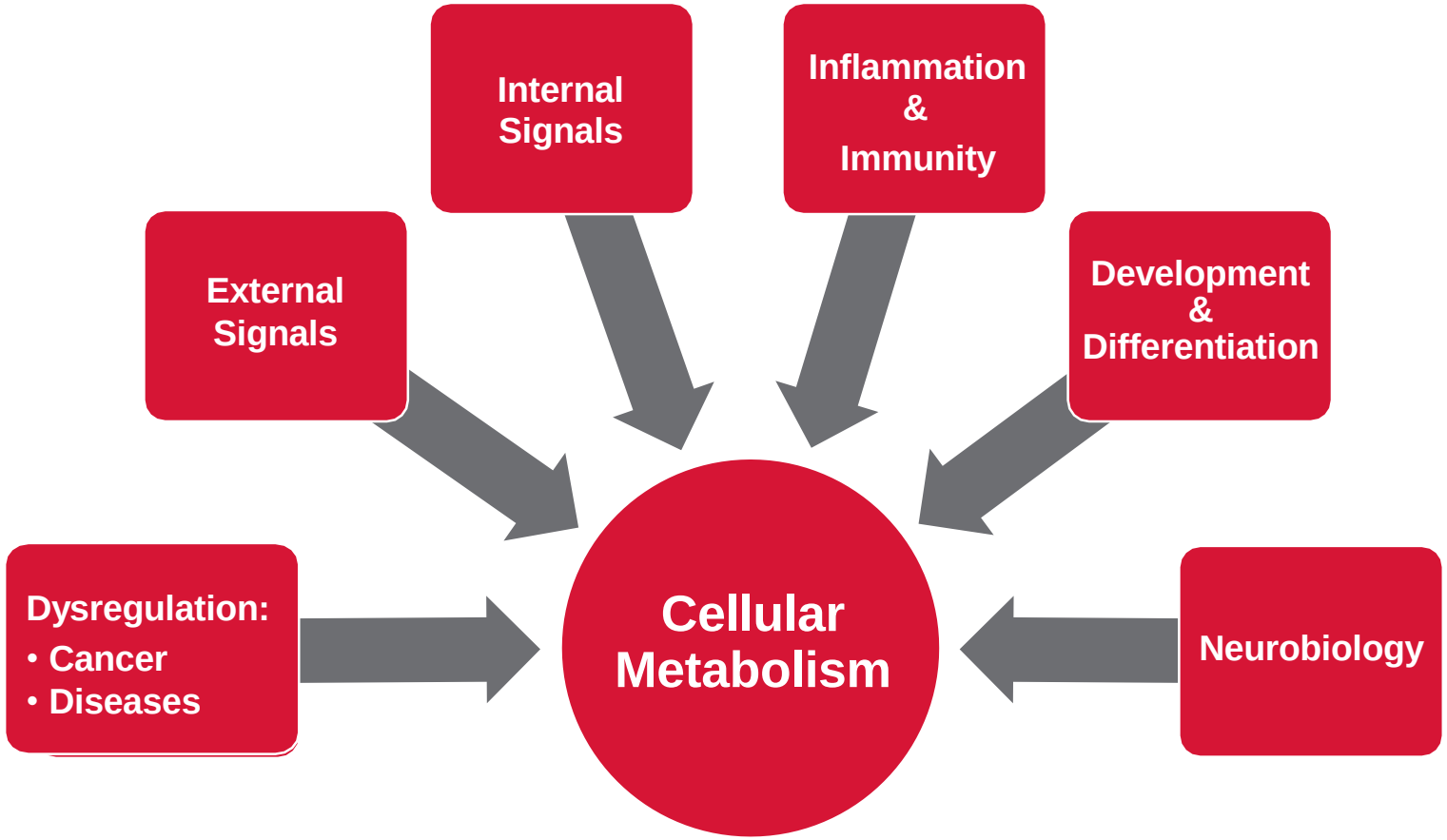
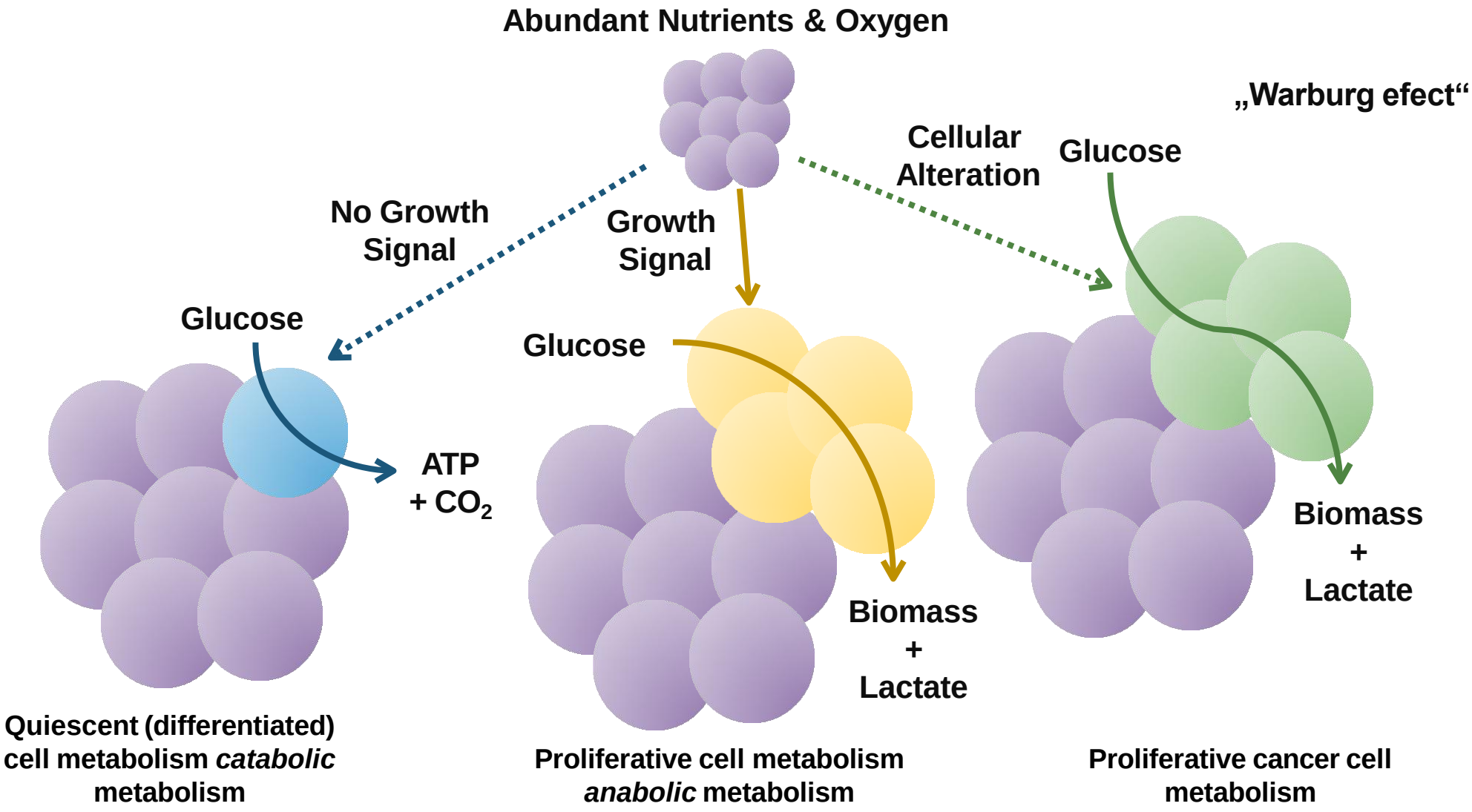
5. News flash from cell-biology portfolio

Why Study Cellular Metabolism?

Metabolism: *Chemical processes that occur within an organism to maintain life*

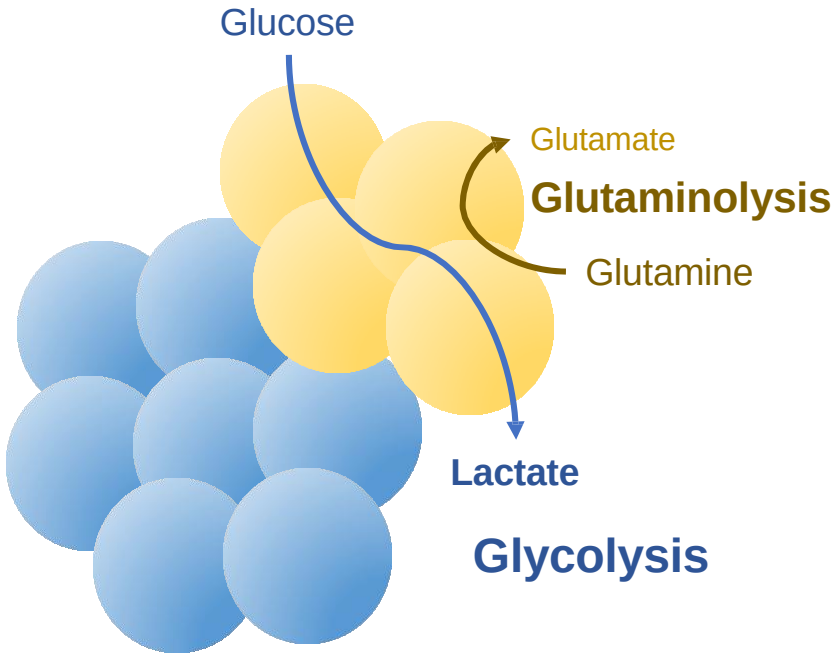


Cellular Metabolism Shifts



Studying Cellular Metabolism Shifts

- Metabolic status can help define the differentiation state
- Evaluate immune system response
- Profile a cancer cell, indication of aggressiveness and invasiveness
- Profile response to treatment

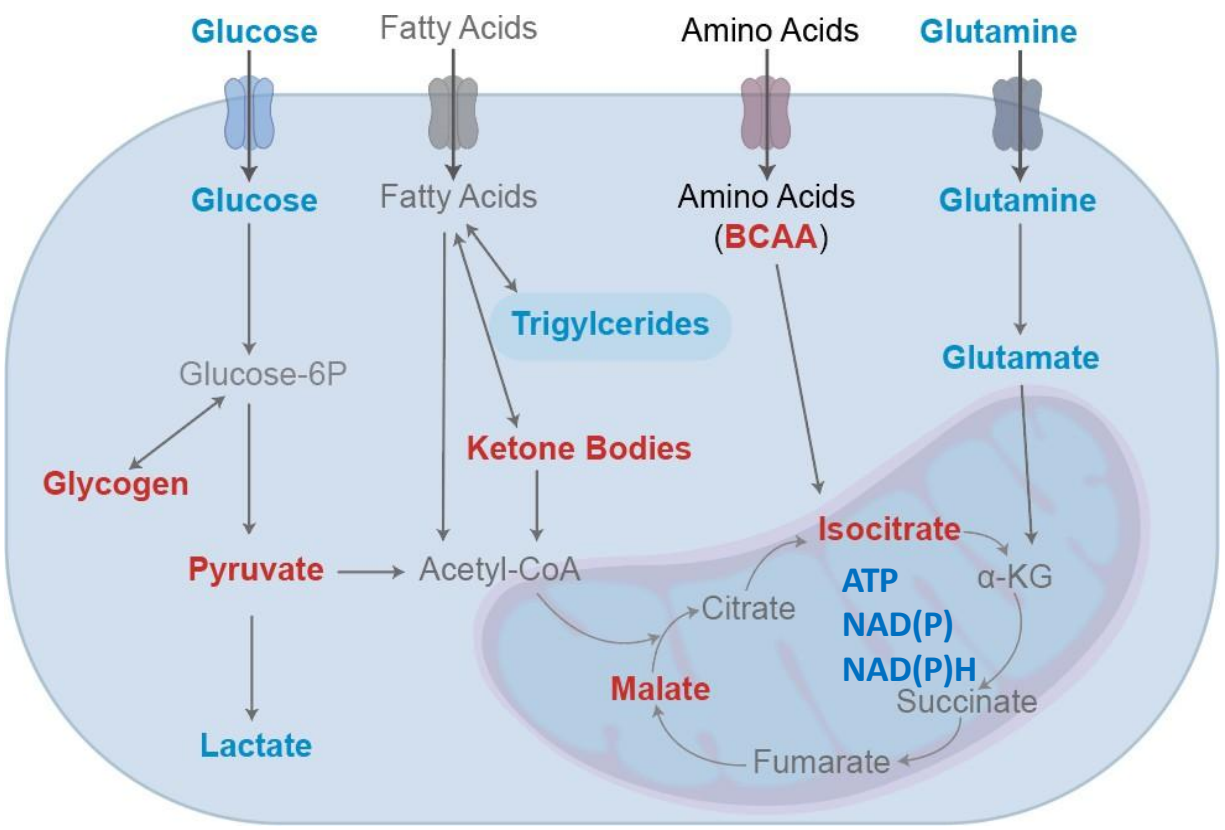


Proliferative Cell Metabolism

Studying Cellular Metabolism Shifts

Metabolomics	• Untargeted approach • Measures 1000s of metabolites per sample • Requires organic extraction • Requires specialized instrument or outsourcing • Limited sample number
Clinical Analyzer	• Focus on key metabolites • Designed to monitor blood or serum samples • Lower sensitivity • Requires specialized instrument
Seahorse XF	• Measures oxygen consumption rate, extracellular acidification rate • Extrapolates data on glycolysis and mitochondrial respiration • Requires specialized instrument
Plate-Based Assays	• Many sample types • Larger number of samples (96-well plates) • Minimal sample preparation • Plate reading luminometer

Bioluminescent Assays for Metabolism Research



Metabolic co-factors
- ATP
- NAD(P)/NAD(P)H-Glo

Glucose Metabolism
- Glucose Uptake-Glo
- Glucose-Glo
- Lactate-Glo
- Glycogen-Glo

Amino acid Metabolism
- Glutamine/Glutamate-Glo
- BCAA-Glo

Lipid Metabolism
- Triglyceride/Glycerol-Glo
- Cholesterol/Cholesterol Ester-Glo
- BHB-Glo (β-hydroxybutyrate)
- Fatty Acid Oxidation

ROS
- H_2O_2
- Super Oxide
- Nitric Oxide

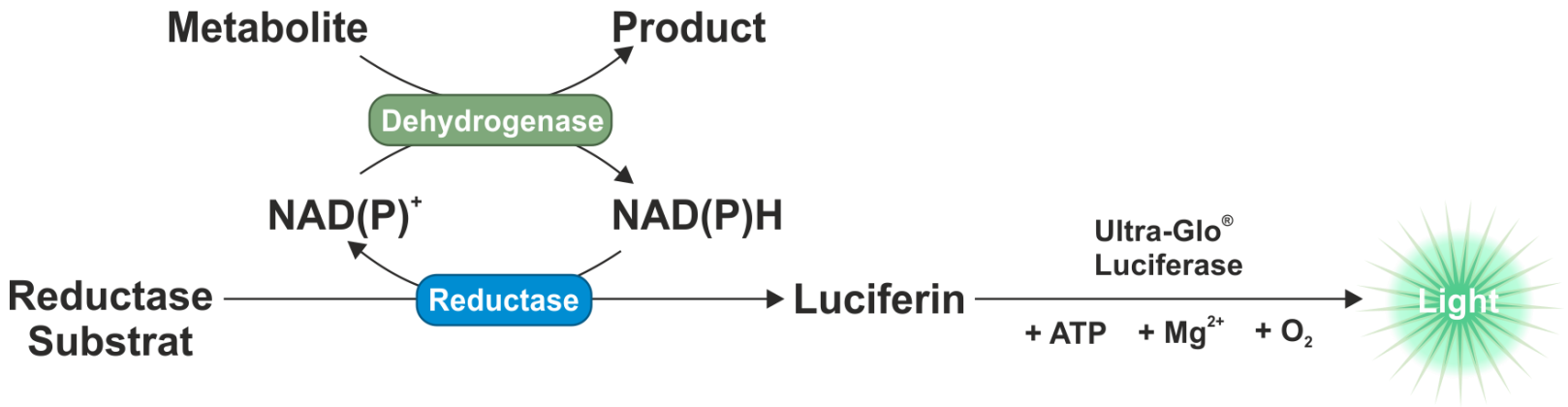
Glutathione
- GSH-Glo
- GSH/GSSG-Glo

TCA Cycle
- Malate
- Isocitrate
- Pyruvate

Existing Products
In Development

Metabolite Assays – One Reaction to Rule Them All

Metabolite-selective dehydrogenases coupled to bioluminescent NAD(P)H detection



"With the aid of auxiliary enzymes nearly every substance of biological interest could be measured with a pyridine nucleotide system"

Oliver Lowry JBC (1961) 236, 2746

- ✓ Broad linear range of up to 3 logs (0.1 – 100 µM)
- ✓ Wide dynamic range S/B < 100
- ✓ High sensitivity, requiring only small amounts of sample
- ✓ Simplified protocol applicable to many sample types

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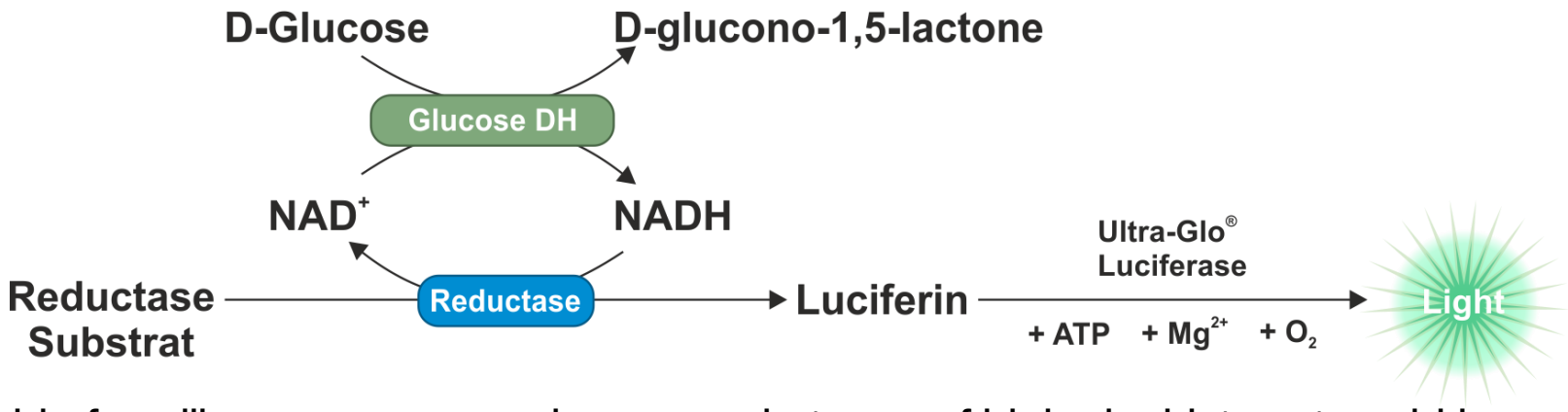

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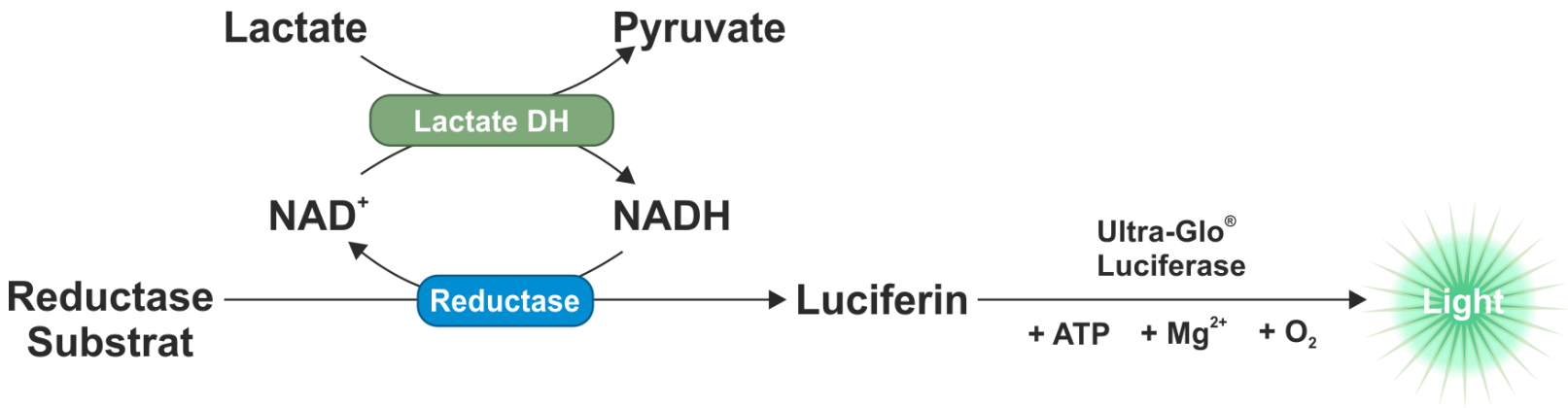

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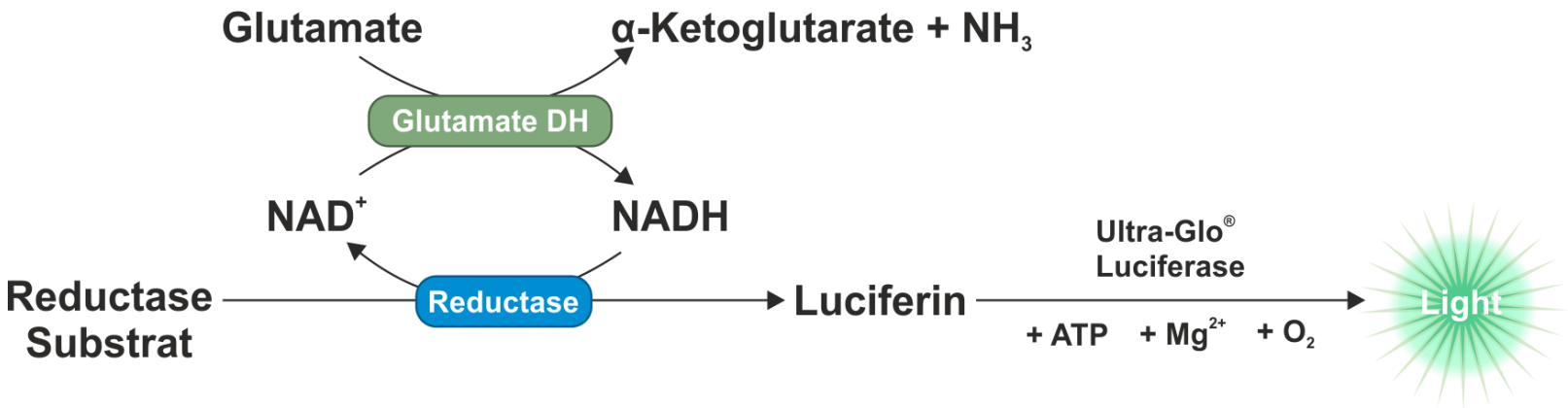

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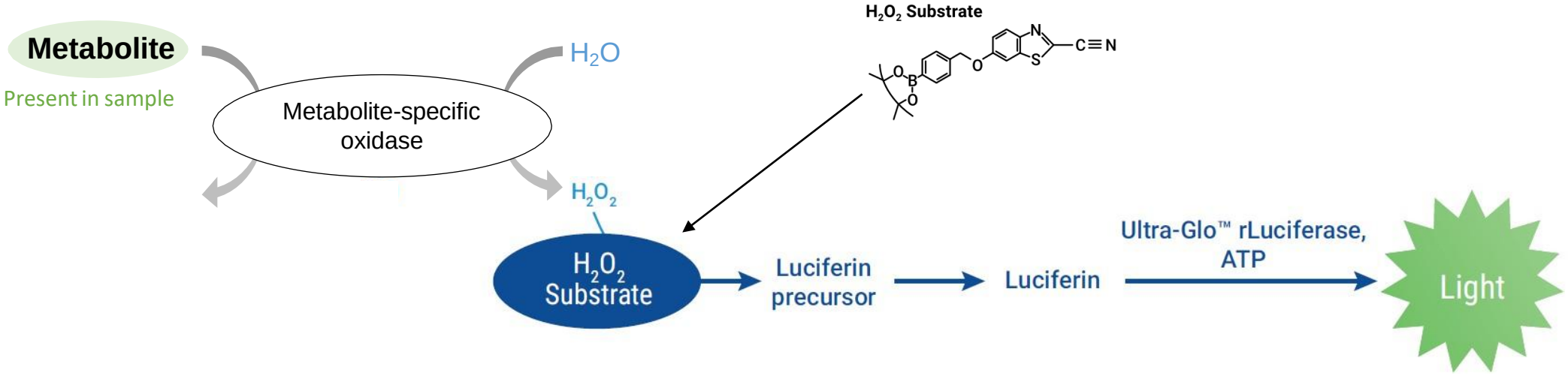
Metabolite Assays – ~~One~~ Two Reactions to Rule Them All

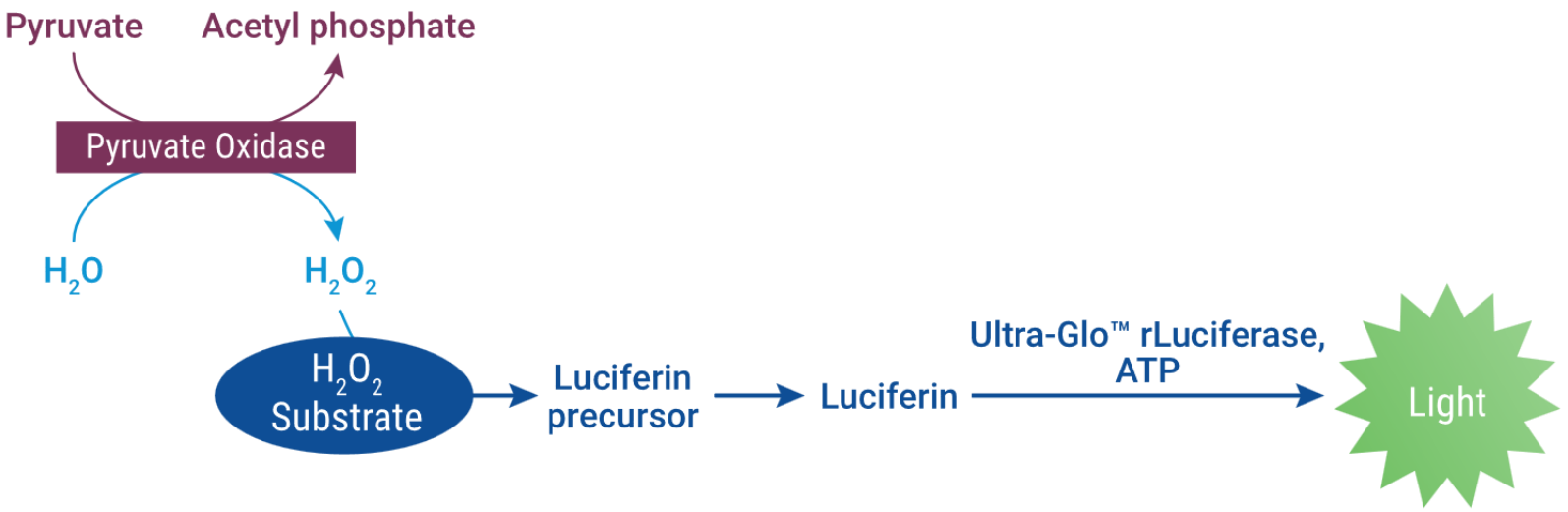
Metabolite-selective oxidase coupled to bioluminescent H_2O_2 detection



- Coupled metabolite reduction by metabolite-specific oxidase with a concomitant oxidation of H_2O to H_2O_2
- H_2O_2 substrate then reacts with H_2O_2 to generate luciferin precursor that is converted to luciferin after reaction with D-cysteine
- Luciferin is detected in Ultra-Glo™ rLuciferase ŕeaction that generates light

Pyruvate-Glo Assay – Utilizing H_2O_2 Detection

Metabolite-selective dehydrogenases coupled to bioluminescent H_2O_2



- ✓ Broad linear range of up to 3 logs (0,4 to 50µM)
- ✓ Wide dynamic range S/B > 150
- ✓ High sensitivity, requiring only small amounts of sample
- ✓ Measure from lysate or supernatant

Sample Compatibility & Protocol

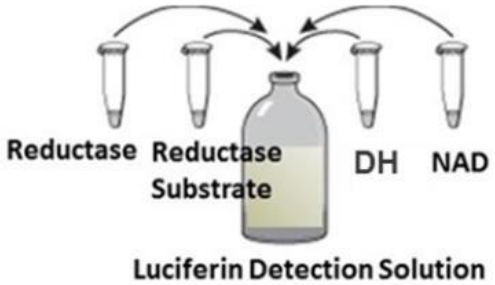
Compatible sample types

- Cells in culture: 2D & 3D
- Intracellular metabolite content
- Total metabolite content
- Cell culture media samples (2–5µl)
- Plasma and serum samples
- Tissue homogenates
- Perifusate samples
- Other biological fluids

Prepare Samples

According to sample type, dilution and/or acid treatment & neutralization

Prepare Detection Reagent



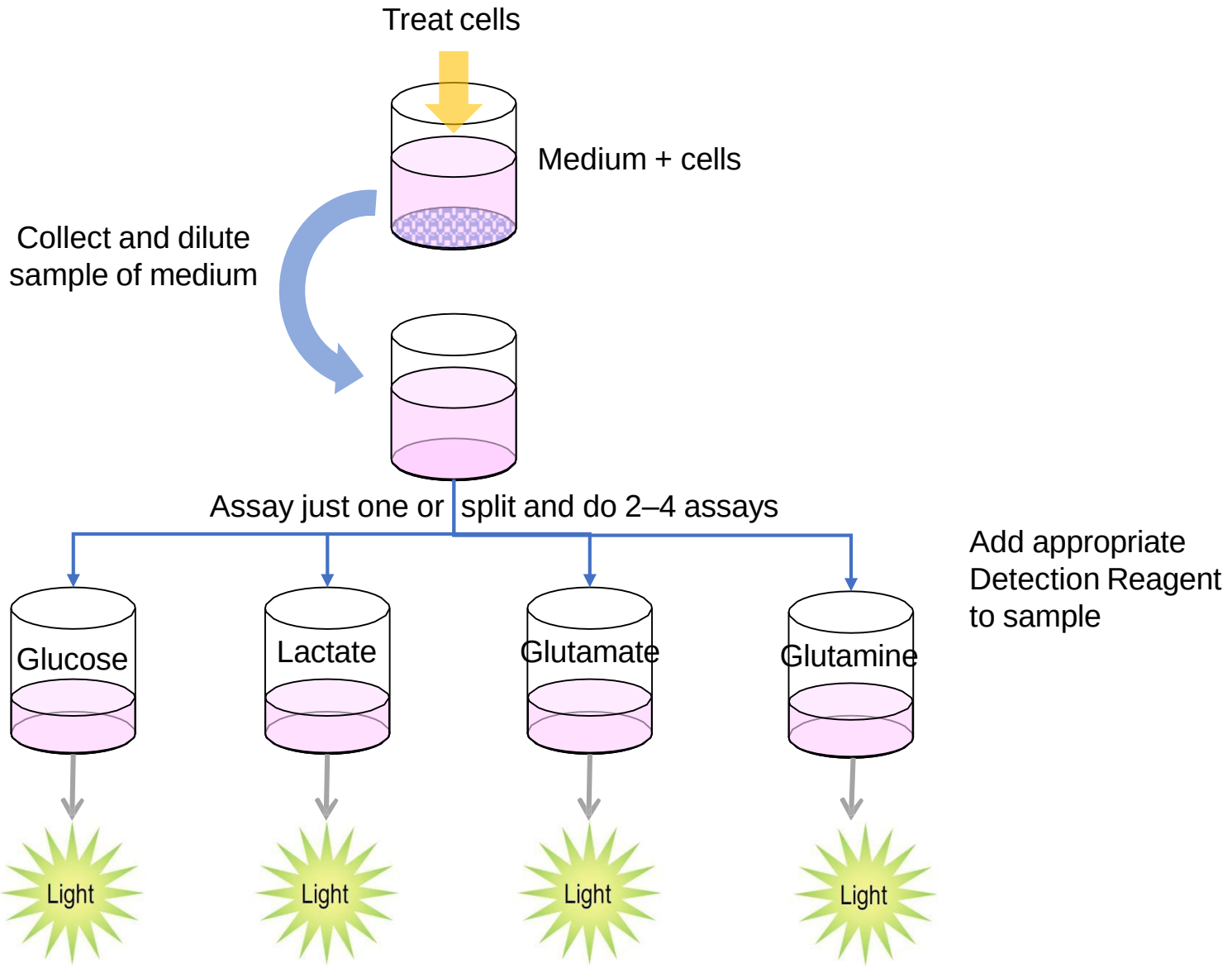
Combine 1:1

Incubate

1-2 hours

Read Luminescence

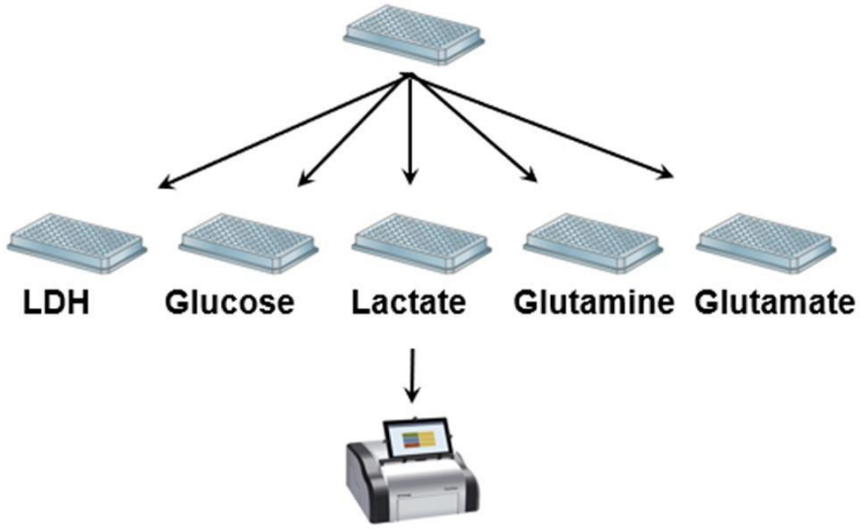
Detecting Metabolites in Supernatant



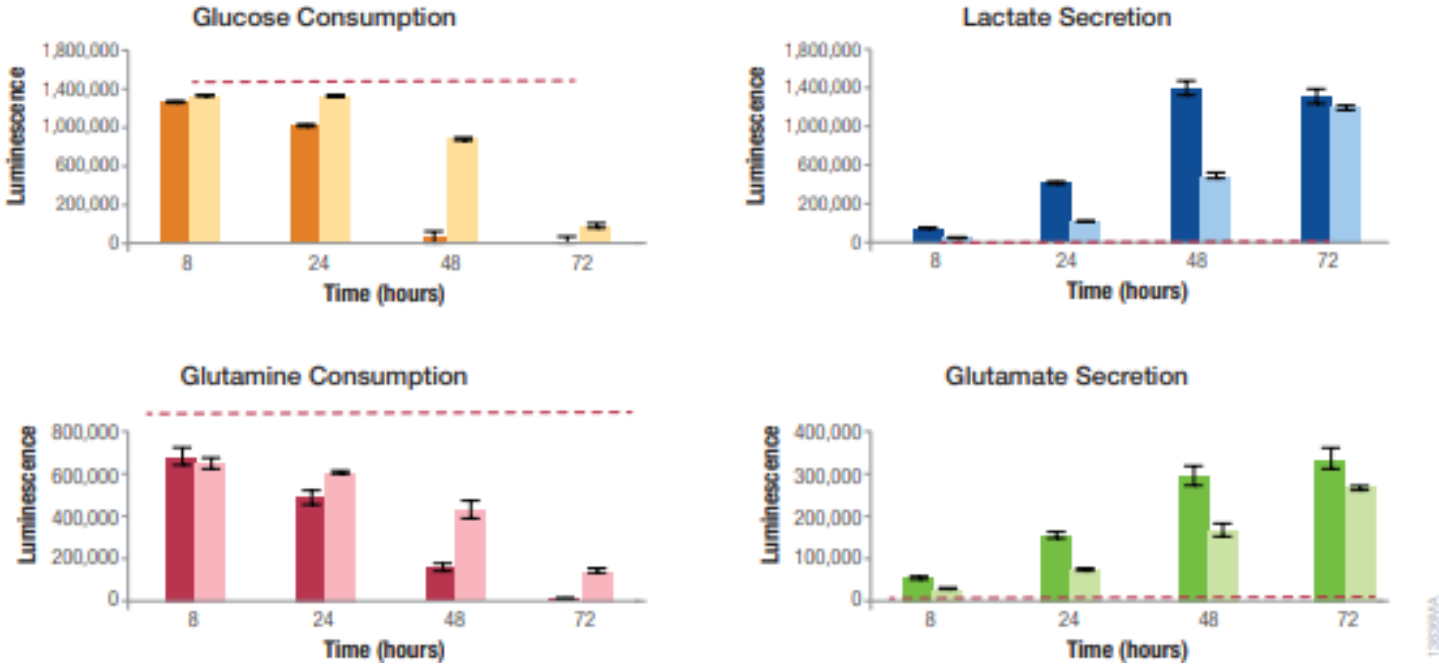
Measuring Multiple Metabolites from a Single Sample

Sample

Diluted and/or acid treated & neutralized



Measure light output



A549 cells were plated at 15,000 (dark bars) or 5,000 (light bars) cells/well in 100µl DMEM with 5mM glucose, 2mM glutamine and 10% dialyzed serum. At the indicated time points, 2.5µl of medium was removed, diluted in 97.5µl PBS and stored frozen at –20°C. At the end of the experiment, samples were thawed and aliquots were transferred to a 384-well plate. Each sample was transferred into four wells, one for each metabolite. The following volumes were used from the thawed sample to detect each of the four metabolites: 25µl for lactate, 12.5µl plus an additional 12.5µl PBS for glucose, 12.5µl for glutamine and 12.5µl for glutamate. The metabolites were then detected using the Lactate-Glo™, Glucose-Glo™, and Glutamine/Glutamate-Glo™ Assays, respectively. Luminescence was recorded using a Tecan instrument. The red lines depict the signals from control wells containing medium but no cells.

Leippe, D., *et al.* (2017) *SLAS Discovery* 22, 366-77

Detecting Intracellular Metabolites in Lysates

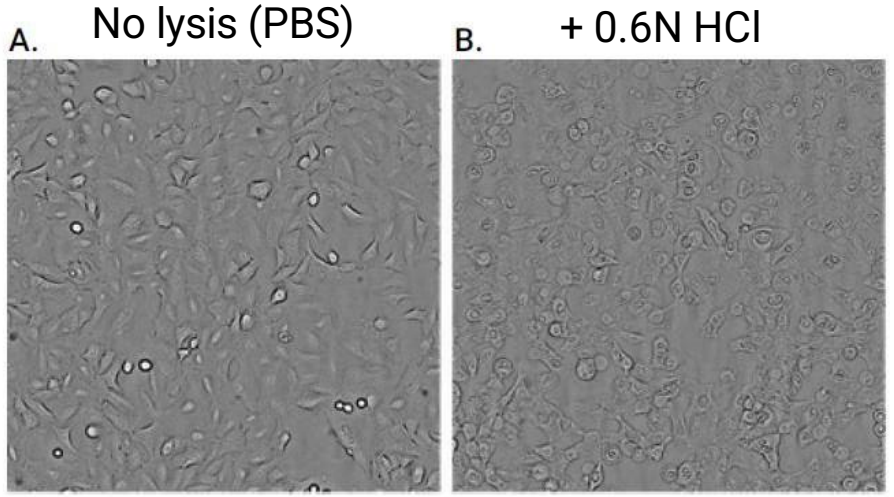
- Acid treatment followed by neutralization
  - Use 0.6N HCl and Neutralization Buffer provided in assay kits
- Complete in ~5 mins
- When dealing with difficult-to-lyse samples such as 3D cultures, Triton®X-100 can be added during lysis

<u>Purpose of this treatment:</u>

- Cell lysis (access to intracellular metabolites)
- Inactivation of endogeneous enzymes (stop metabolism)
- Adjust the pH for subsequent luminescent reaction

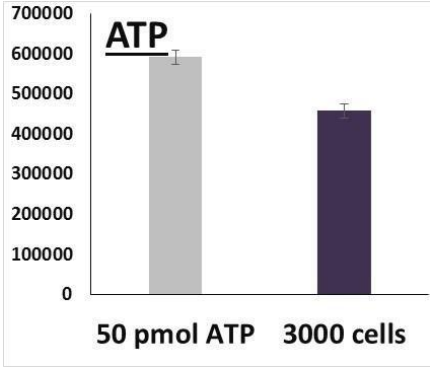
<u>Benefits:</u>

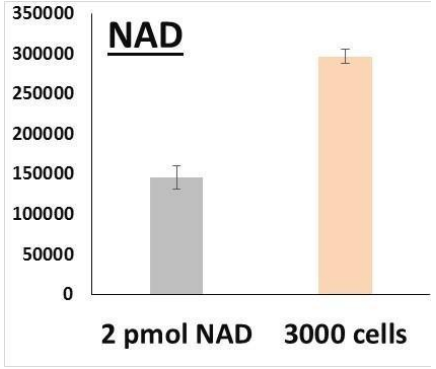
- Can perform lysis directly in-well with cells, no transfer steps
- No sonication or centrifugation required

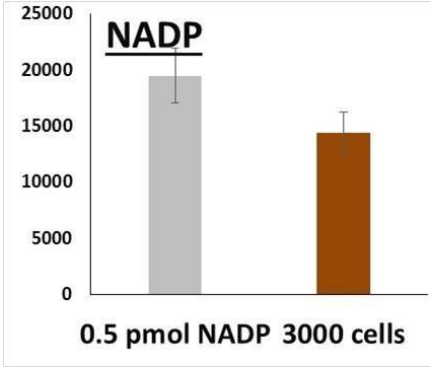


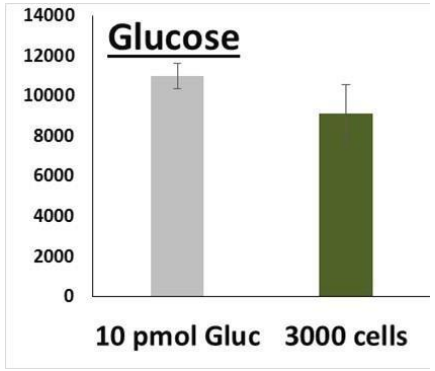
Although cells might appear intact, acid treatment destroys membranes and intracellular metabolites have been released.

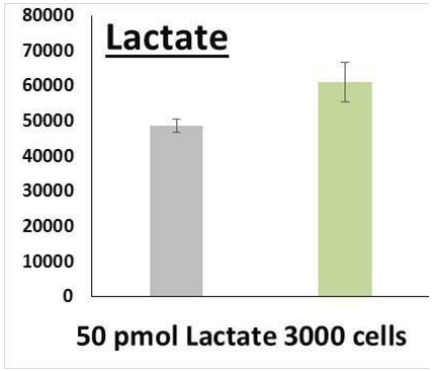
Multiple Metabolites Can Be Measured from a Single Well of a 96-well Plate

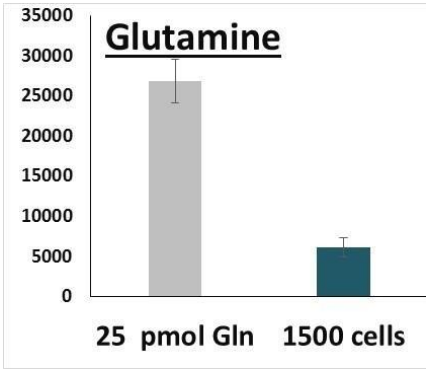


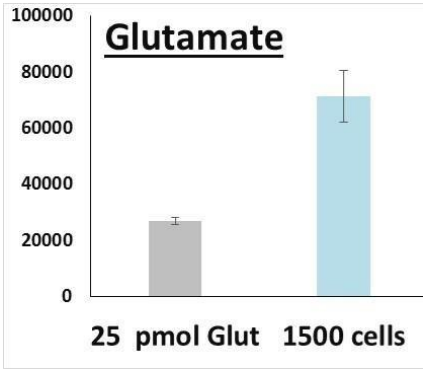










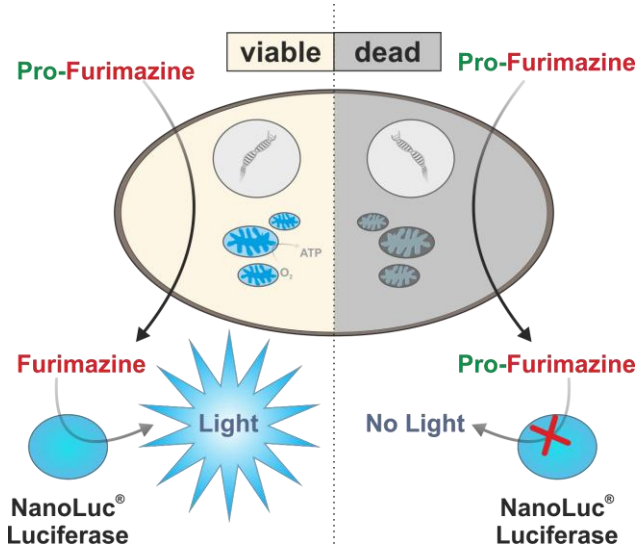


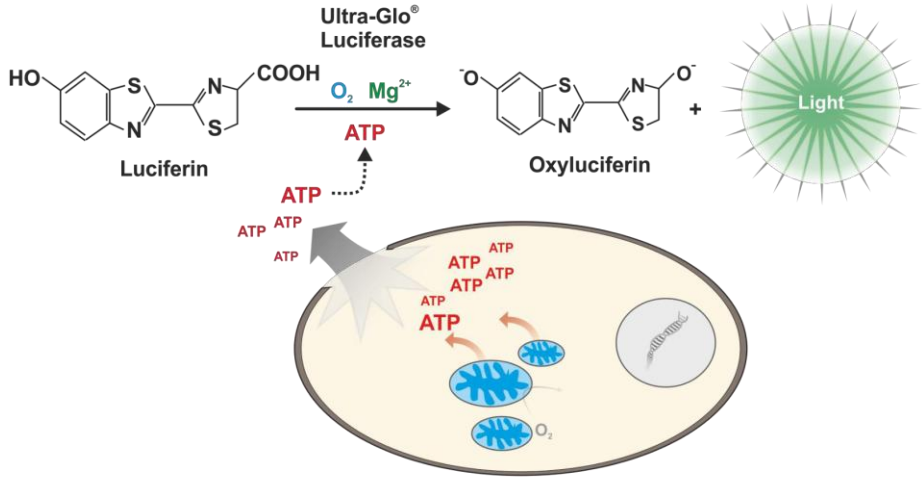
Metabolites	ATP	NAD	NADP	Glucose	Lactate	Glutamine	Glutamate
fmol/cell	6	0.8	0.05	0.8	13.6	3.8	44

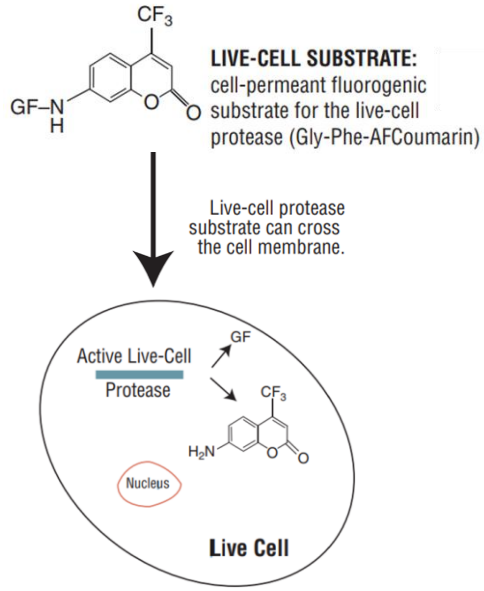
Medium Recommendation

- Be wary of your medium composition and selection
- Start with a defined medium containing known amounts of nutrients, i.e. glucose and glutamine
  - Start with DMEM without glucose and glutamine and add glucose and glutamine at the desired concentrations
- To measure consumption: for glucose, start with 5mM glucose rather than 25mM; for glutamine start with 2 or 4mM concentration
- Use dialyzed FBS: 10% FBS can contribute up to 500µM glucose, but more importantly it can add 1mM lactate and 100µM glutamate
  - Dialyzed FBS has 100X less of these metabolites, making it possible to detect lactate and glutamate at lower levels and at earlier time points
- GlutaMAX™ L-alanyl-L-glutamine dipeptide is not recognized by the glutamine and glutamate detection assays

Viability Assays for Data Normalization







RealTime-Glo MT

- Non-lytic kinetic assay for realtime viability measurements
- Detects reduction of NanoLuc substrate pro-furimazine in viable cells via NAD(P)H
- Suitable for kinetic measurements up to 72 hours

CellTiter-Glo 2.0

- Lytic endpoint assay detecting intracellular ATP released from cells
- ATP is consumed in a firefly luciferase reaction

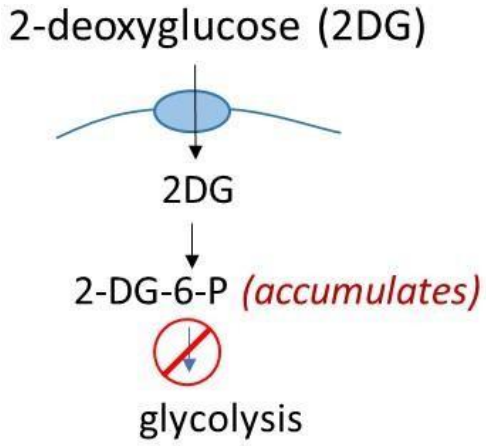
CellTiter-Fluor

- Nonlytic endpoint assay detecting live-cell protease activity
- After the substrate is cleaved, the free AF-coumarin fluorescence is enhanced

Glucose Uptake Assay for Measuring Glucose Transporters

- Glucose is transported by two major glucose transporters **GLUT1** and **GLUT4**
  - **GLUT1** is ubiquitous and is overexpressed in cancer cells and activated T-lymphocytes to support high proliferation
  - **GLUT4** is expressed internally and is translocated to the plasma membrane upon insulin stimulation. The lack of translocation is associated with a wide range of pathologies.

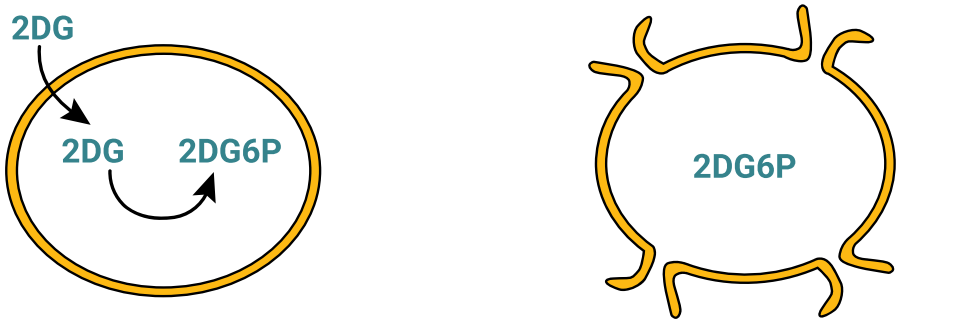
- Standard method for glucose uptake relies on radioactivity
  - Measures accumulation of nonhydrolyzable glucose analog 2-[3H]-deoxy-D-glucose-6P
  - Unincorporated 2-[3H]-DG has to be removed by washing
  - Detection of β-radiation from the accumulated phosphate product



Enzymatic Assay for Measuring 2DG6P Accumulation

Add 2DG to cells.

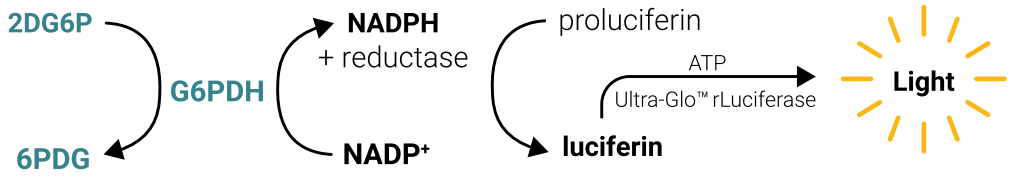
Add Stop and Neutralization Buffers to end reactions, lyse cells and eliminate NADPH.



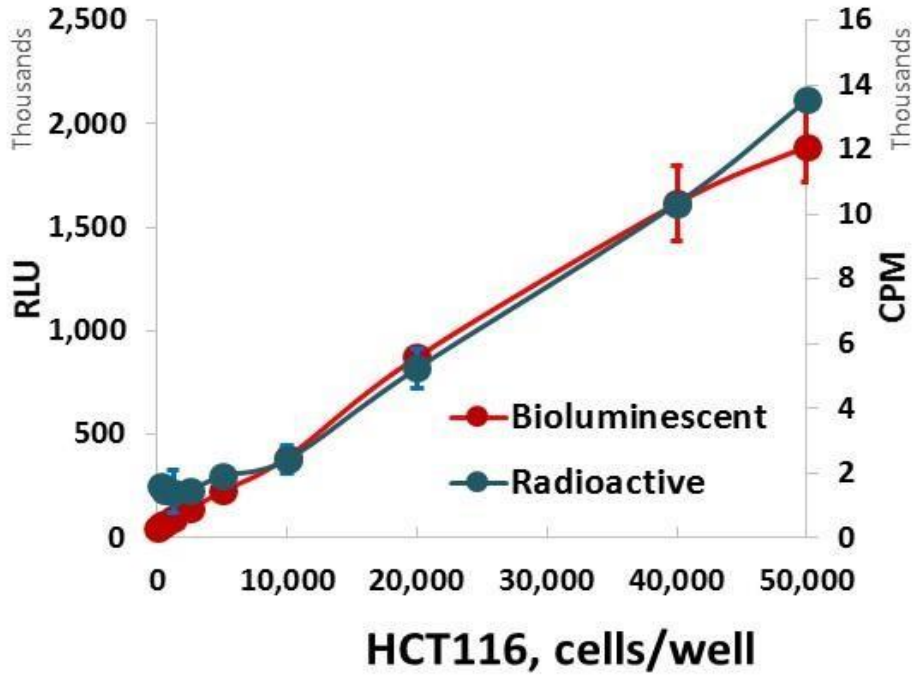
2DG Deoxyglucose

2DG6P 2-Deoxyglucose-6-Phosphate

Add 2DG6P Detection Reagent.



G6PDH Glucose-6-Phosphate Dehydrogenase

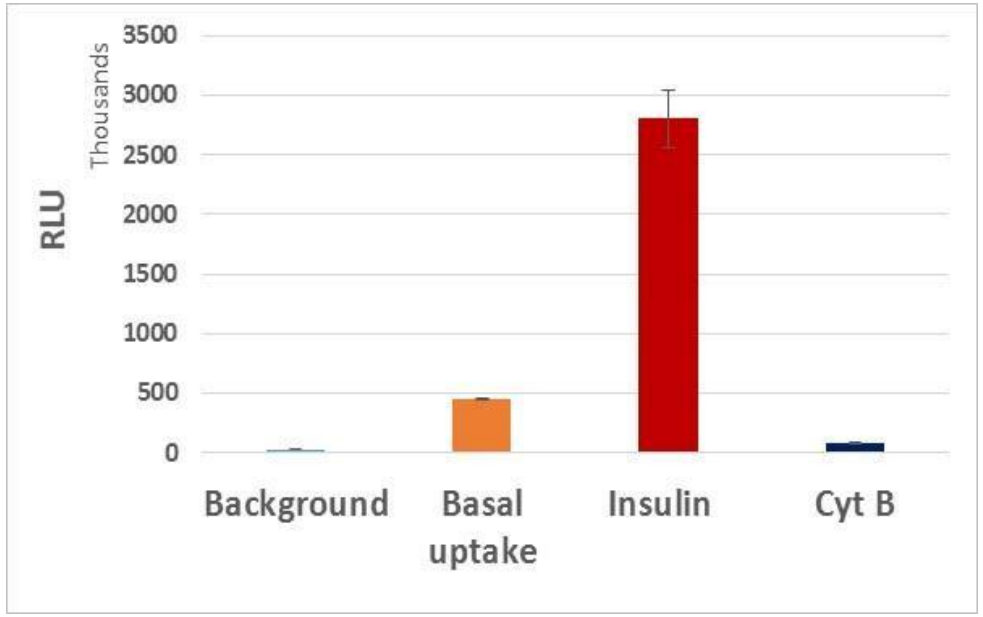


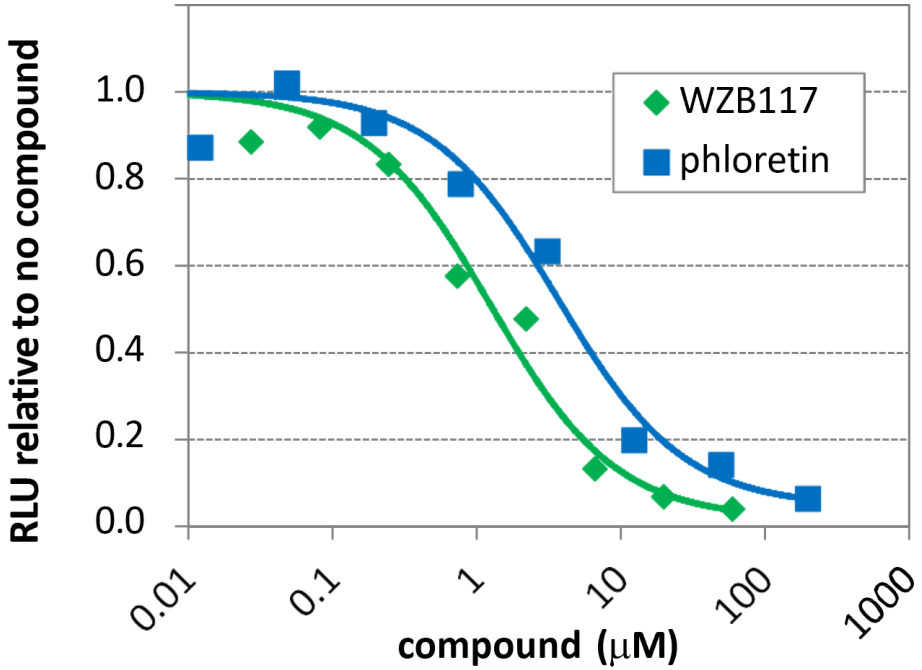
- **Linear from 1,000 to 50,000 cells/well**
- **S/B> 14 fold with 5,000 cells per well**

Valley M. et al. Analytic Biochemistry. 2016, 505, 43-50

Example of Glucose Uptake Assay

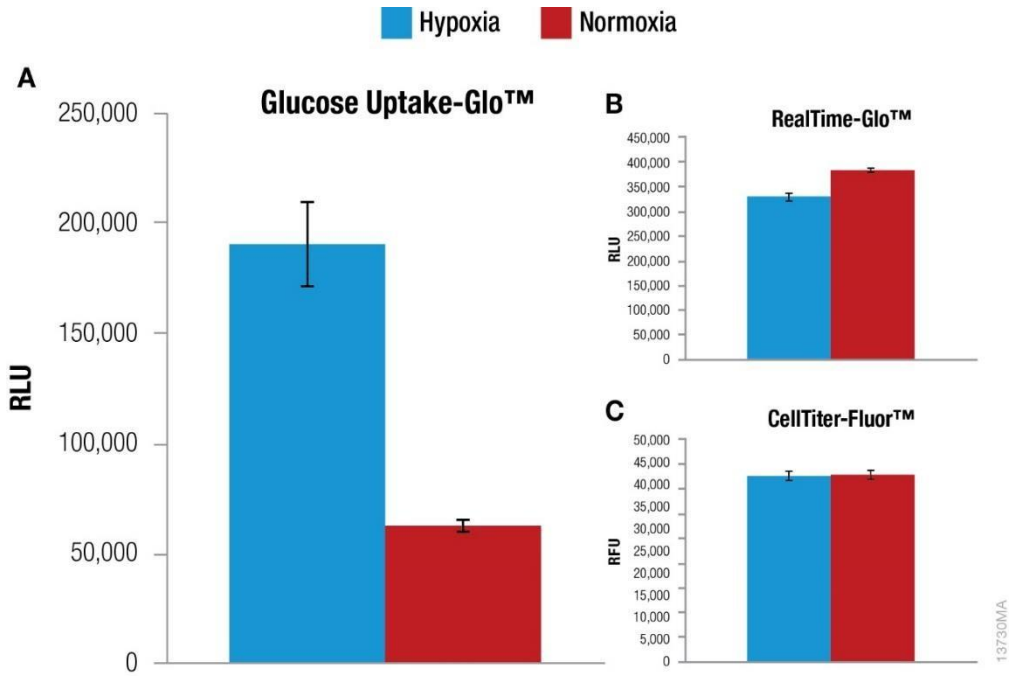
Inhibition of glucose uptake by known glucose transport inhibitors





- Cytochalasin B reversibly binds and inhibits glucose transporters
- WZB117 inhibitor of basal glucose transport
- Phloretin is a type of natural phenol that was shown to inhibit active glucose transport

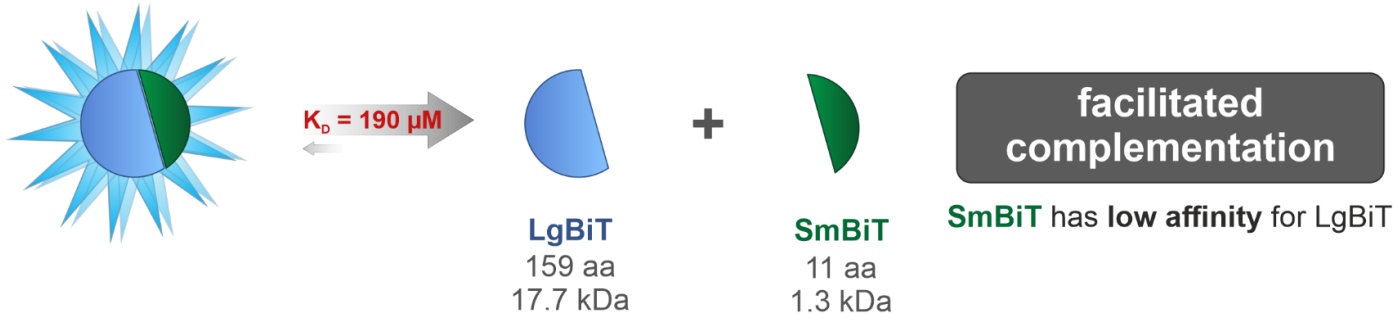
Example of Glucose Uptake Assay



- MCF-7 cells grown under hypoxia show an increase in glucose uptake
- The same cells demonstrate no significant change in viability using the RealTime-Glo™ and CellTiter-Fluor™ Assays

NanoLuc® Binary Technology (NanoBiT®)

A Structural Complementation Reporter Designed for Biomolecular Interaction Studies



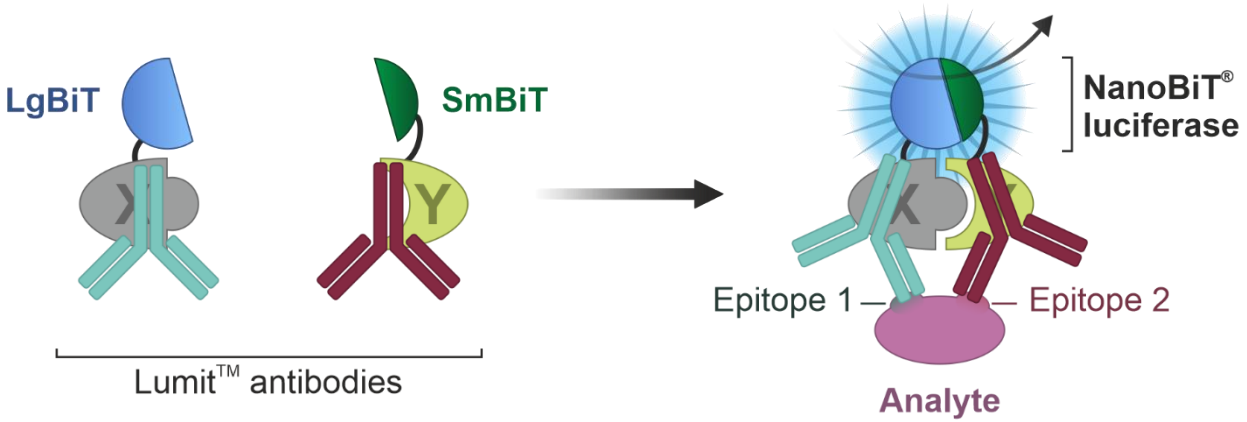
Complementation facilitated through …

(direct protein-protein interaction)

(indirect protein-protein interaction)

→ NanoBiT® PPI System

Dixon et al. 2016, *ACS Chem. Biol.*



Analyte Quantification

Lumit Immunoassays

The Powerful Alternative to Conventional Immunoassay Approaches

Traditional ELISA Workflow

- Traditional ELISA is a heterogenous multistep process involving several wash / incubation steps

Lumit Immunoassays

The Powerful Alternative to Conventional Immunoassay Approaches

Lumit™ Immunoassay Workflow

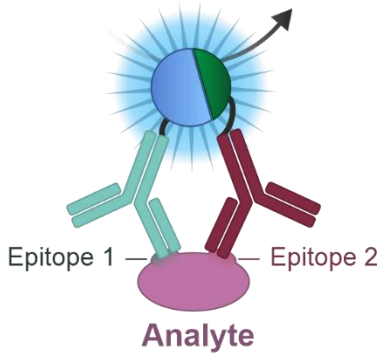
- Traditional ELISA is a heterogenous multistep process involving several wash / incubation steps
- Lumit™ Immunoassays
  - ✓ Easy workflow with short assay time (30 – 120 min)
  - ✓ High sensitivity (low number of cells)
  - ✓ Broad dynamic range (3 – 4 logs)
  - ✓ Flexible formats (96- or 384-well)
  - ✓ Homogenous and HTS compatible

Hwang, B. *et al.* (2020) Commun Biol. 3:8

Lumit Immunoassays

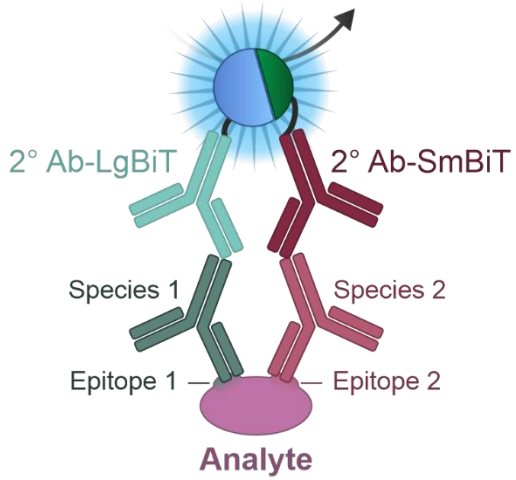
Different Formats for Maximum Flexibility

Direct



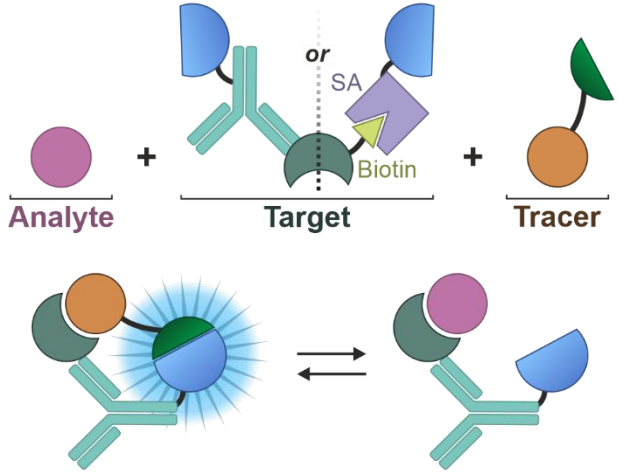
- Requires labeling of 1°Abs
- Validated for cytokines, peptide hormones, …
- *Ready-to-use* assays for
  - ✓ IL1-β, IFN-γ, IL-2, IL-6, IL-10, IL-4, IL-18, TNF-α, VEGF, insulin, glucagon, HMGB1, p24, Ki-67

Indirect



- Avoids labeling of 1°Abs
- Generic pre-labeled 2°Abs (different species available)
- Validated for intracellular PTMs, e.g. phosphorylation

Competitive

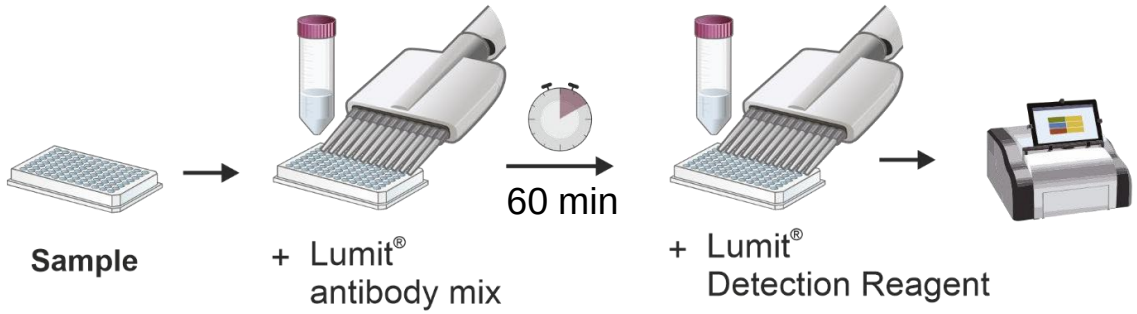


- Requires target and tracer labeling
- Establish competitive (antibody) binding assays
- *Ready-to-use* assays for
  - ✓ Lumit™ FcRn Binding Immunoassay
  - ✓ Lumit™ hFcγR Binding Immunoassays

    I , IIa (H131), IIa (R131), IIIa (V158), IIIa (F158)

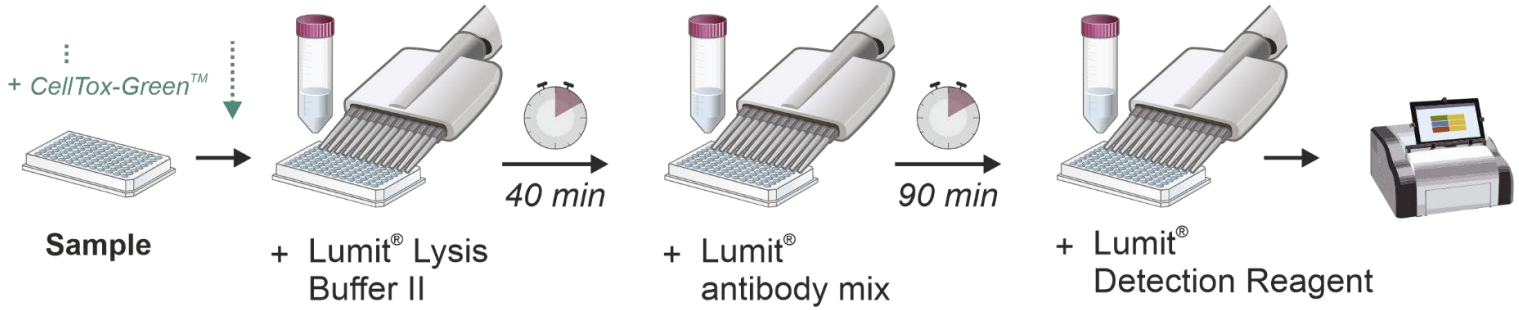
Lumit™ Immunoassays Workflow

Flexible Protocol Options



Secreted proteins and peptides

- Cytokines
- Peptide hormones



Intracellular proteins

- Phosphorylated proteins in cell signaling
- hKi-67
- P24 capsid protein

Today‘s Agenda

1. Luciferases and their basic features
2. Basics of cellular metabolism and how we can measure it
3. **Studying insulin biology with metabolic and Lumit assays**
4. Metabolic assays in cancer and immunology
5. News flash from cell-biology portfolio

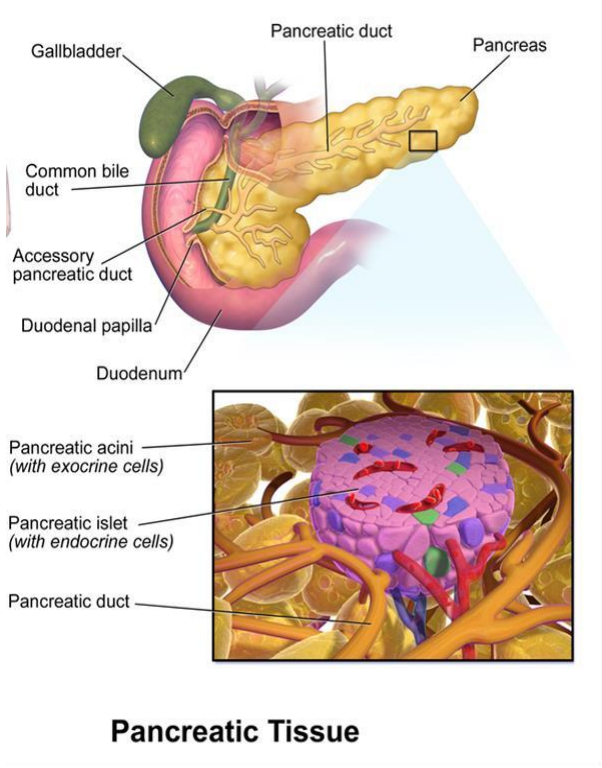
Insulin and Glucagon - Regulation of Glucose

Glucose homeostasis

- Insulin – signals uptake of glucose from blood by tissues
- Glucagon – signals release of glucose stored in tissues

Pancreas Islets of Largerhans

- The islets secrete both hormones
- Insulin – secreted by beta cells
- Glucagon – secreted by alpha cells
- Interplay between the two hormones
- Intra-Islet communication between cells



Pancreatic Tissue

From Wikipedia 102318

Islets of Langerhans (1869)
~3 million in a human pancreas
~100 um in diameter, spherical

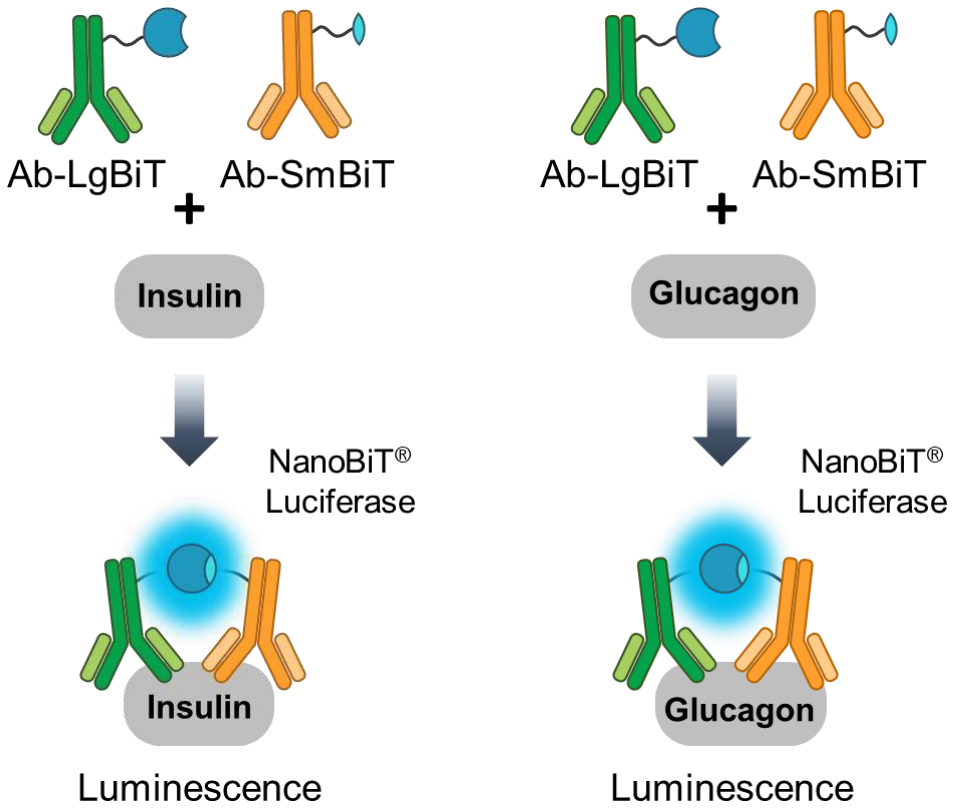
~60% of the cells are beta-cells, which secrete insulin
~20% are alpha-cells, which secrete glucagon

Can be seen under microscope and collected for studies

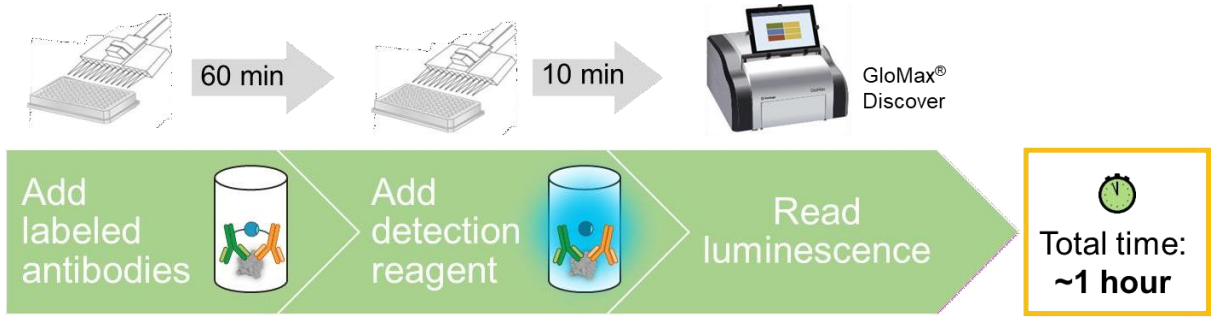
Goal: Develop Immunoassays for monitoring insulin and glucagon secretion

Lumit Immunoassays for Insulin and Glucagon

Lumit™ Insulin and Glucagon Immunoassays



Protocol



Features:

- In-solution immunoassay; no washing steps required
- Time-saving
- Flexible format can be measured in 96 and 384-well plates
- No specialized instrumentation needed

Assay Performance

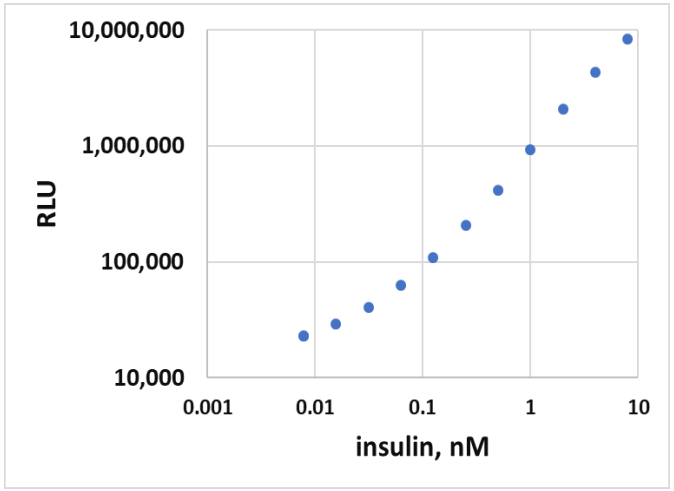
- 96-well plate format
  - 50 µl positive control
  - + 50 µl antibody mix
  - + 25 µl Detection

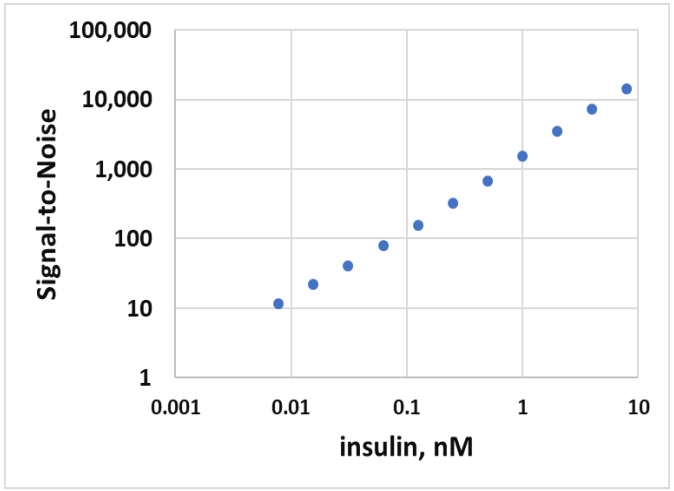
Reagent

- Linear range: pM to nM
- Sensitivity: pM

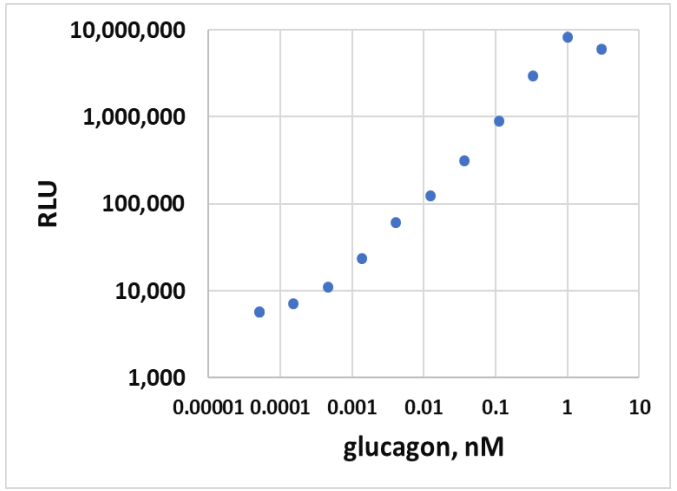
	Insulin	Glucagon
Linear Range	~10 pM to 8 nM	~1 pM to 1 - 2 nM
Signal-to Background, maximum	~500	~500
L.O.D (at S/N = 3)	~ 10 pM (58 pg/ml)	~1 pM (3.4 pg/ml)

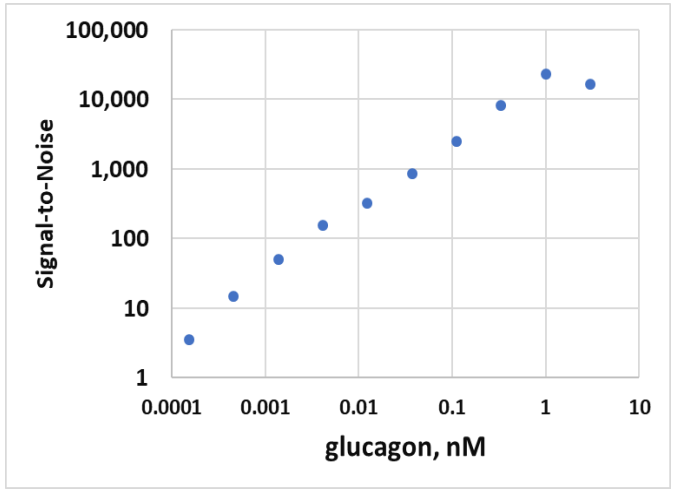
Insulin





Glucagon





Hormone Secretion in Response to Glucose

Typical Workflow

Islets or Cells
In wells of 24- or 96-well plate

Add buffer with no/low glucose
to achieve basal levels

Incubate at 37°C
~60 min

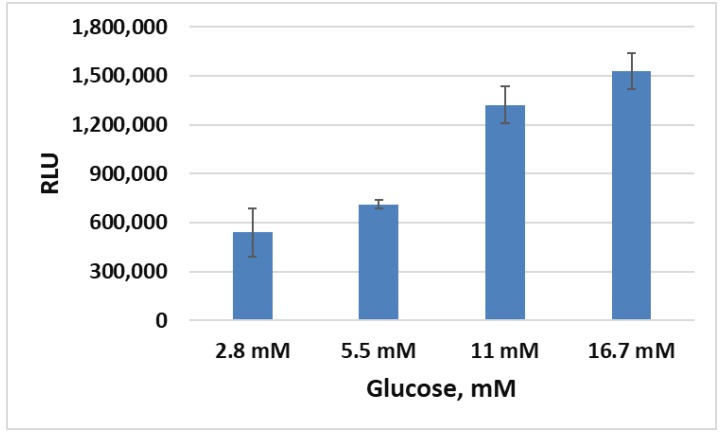
Add buffer with glucose (e.g., 2-25 mM)
to induce insulin secretion

Incubate at 37°C
~60 min

Collect buffer and Assay

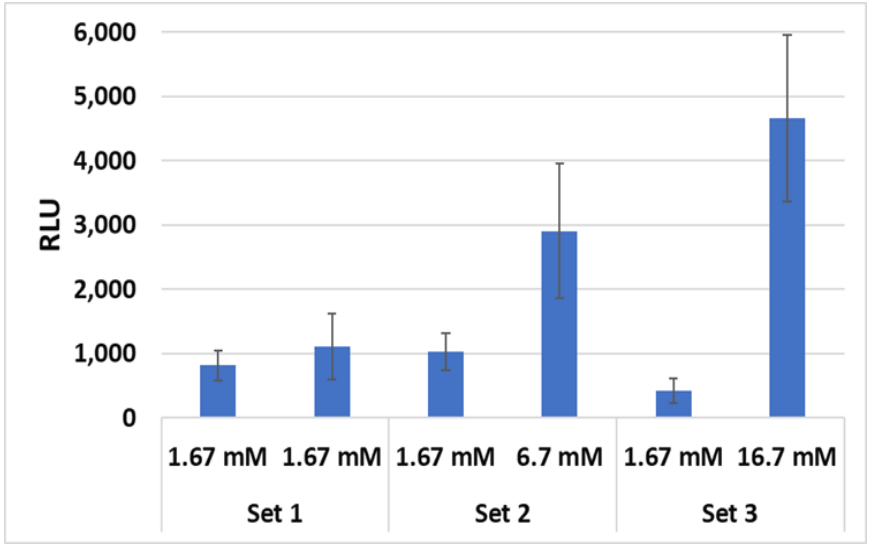
Static/ Endpoint Mode
One glucose concentration per well
Each well generates one or two samples for testing

Insulin Release Increases with Glucose Concentration



- MIN-6 cells were plated in 96-well plates
- Glucose treatment was in 100 µl
- 50 µl was used for the assay in 96-well plates

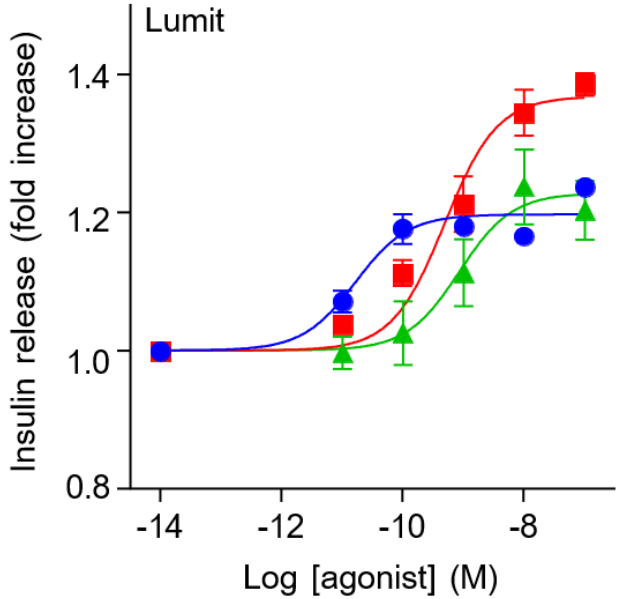
Glucose-Stimulated Insulin Secretion in Rat Insulinoma Cells



- **INS-1 cells in 3D InSphero microtissues (InSphero AG)**
  - Individual islets were cultured per well in a 96-well plate
  - Microtissues were firstlyassayed for basal insulin secretion by incubating in a 50 µl of 1,67 mM glucose
  - Each set was then incubated with increasing concentrations for glucose and 10 µl of supernatant was used for the immunoassay

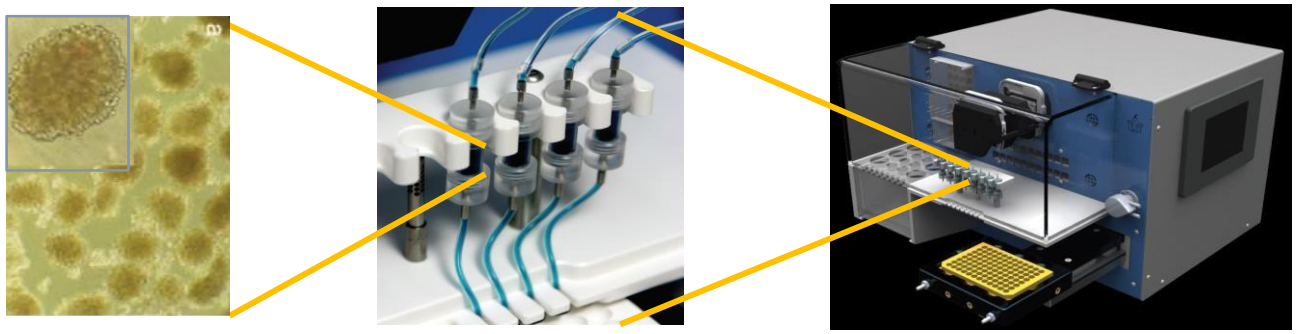
Insulin release after GLP-1 Receptor Agonist Treatment

- GLP-1R is found on beta cells in pankreas and upon activation stimulates insulin release
- INS-1 Cells treated with 3 different GLP-1 receptor agonist drugs (each represented by a different color)



Measuring Insulin in Perfusion Systems

- Perfusion systems allow periodic collection of samples over time
- Helps study the dynamics and kinetics of insulin release in time
- Provides ability to sequentially treat one set of islets (a valuable resource) with different nutrients or compounds



Isolated islets
e.g., from control and experimental mice

50-100 islets per chamber
Up to 8 or 12 parallel chambers

Flow solutions through chambers
Collect fractions over time
e.g., 100 µl every min for 60 min

Samples numbers add up fast:
Each chamber or "well" generates
60 min x 1 per min = 60 samples

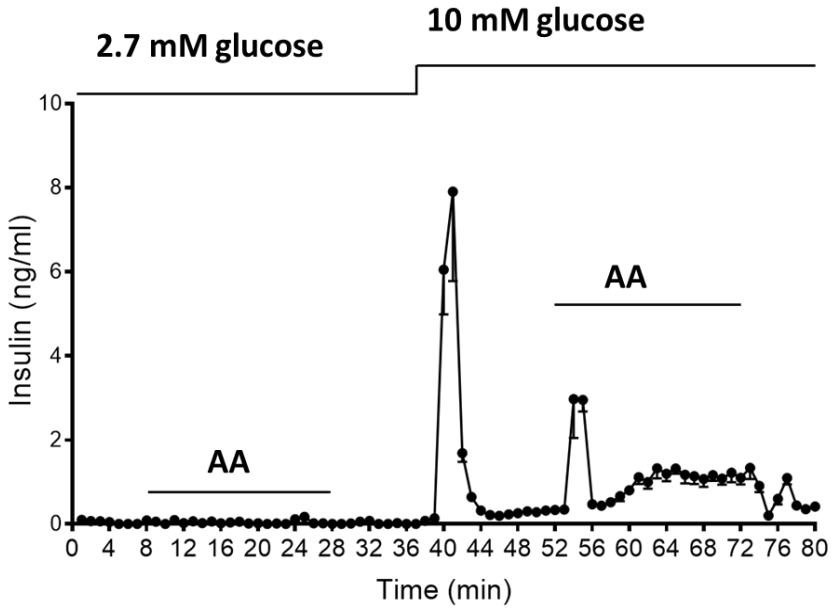
60 x 8 chambers = 480 samples

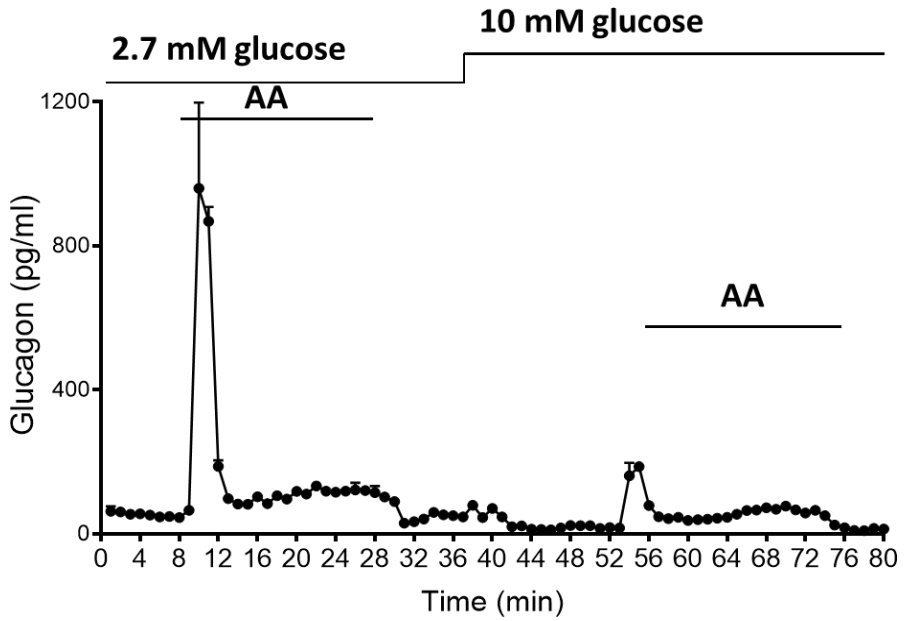
Assay for insulin = 480 assay points

= 5 x 96-well assay plates

Measuring Insulin & Glucagon Secretion from Mouse Islets

- Mouse islets (~80 per chamber), in triplicate perifusion chambers
- Islets treated with glucose and amino acids (AA)
- Samples collected every minute for 80 min, 10µl for assay in 384-well plates
- **AA level increase stimulates glucagon secretion**, particularly at low or normal glucose levels
- Glucagon promotes **gluconeogenesis** and **glycogenolysis**, which helps **prevent hypoglycemia** after a high-protein meal
- Aas can stimulate both insulin and glucagon, insulin with AA uptake and protein synthesis while glucagon prevents hypoglycemia by maintaining glucose levels.

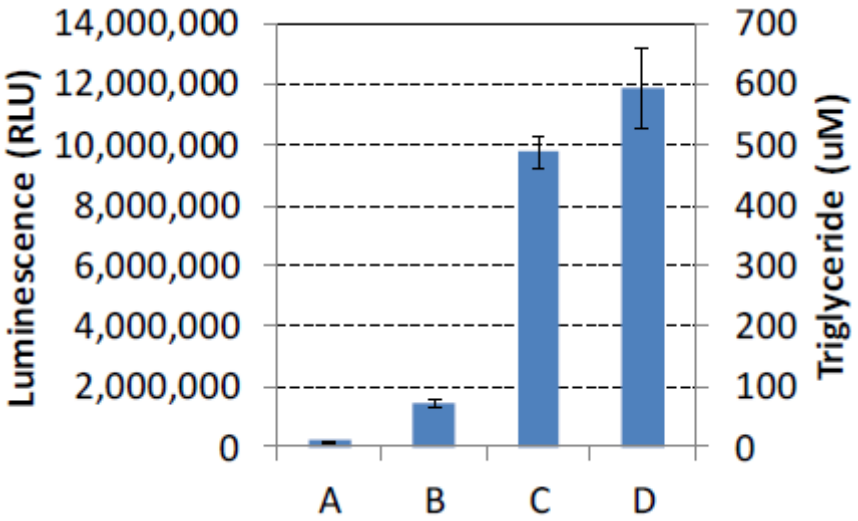


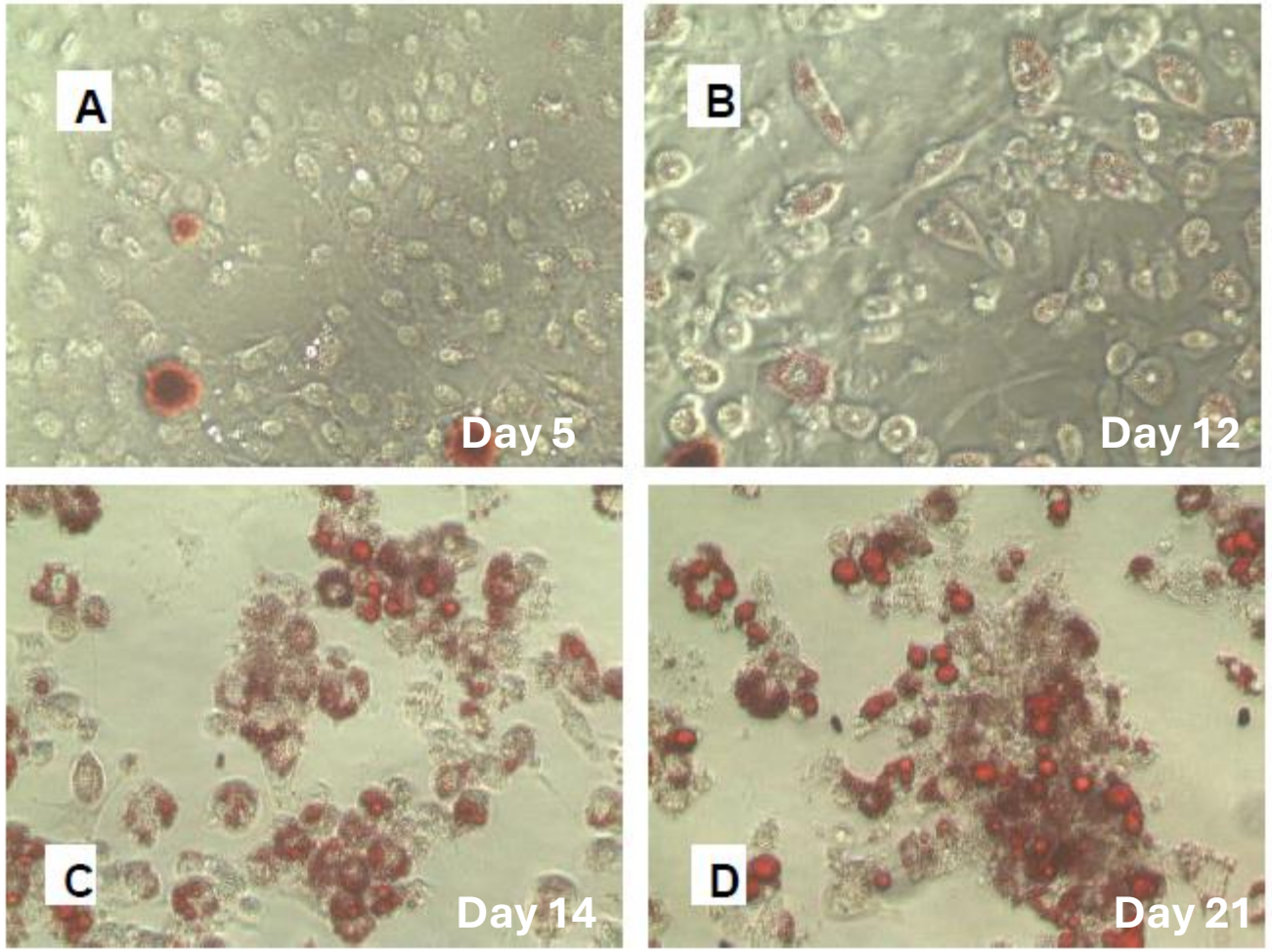


Data provided by dr. Matt Merrins laboratory, Faculty of Medicine, University of Wisconsin

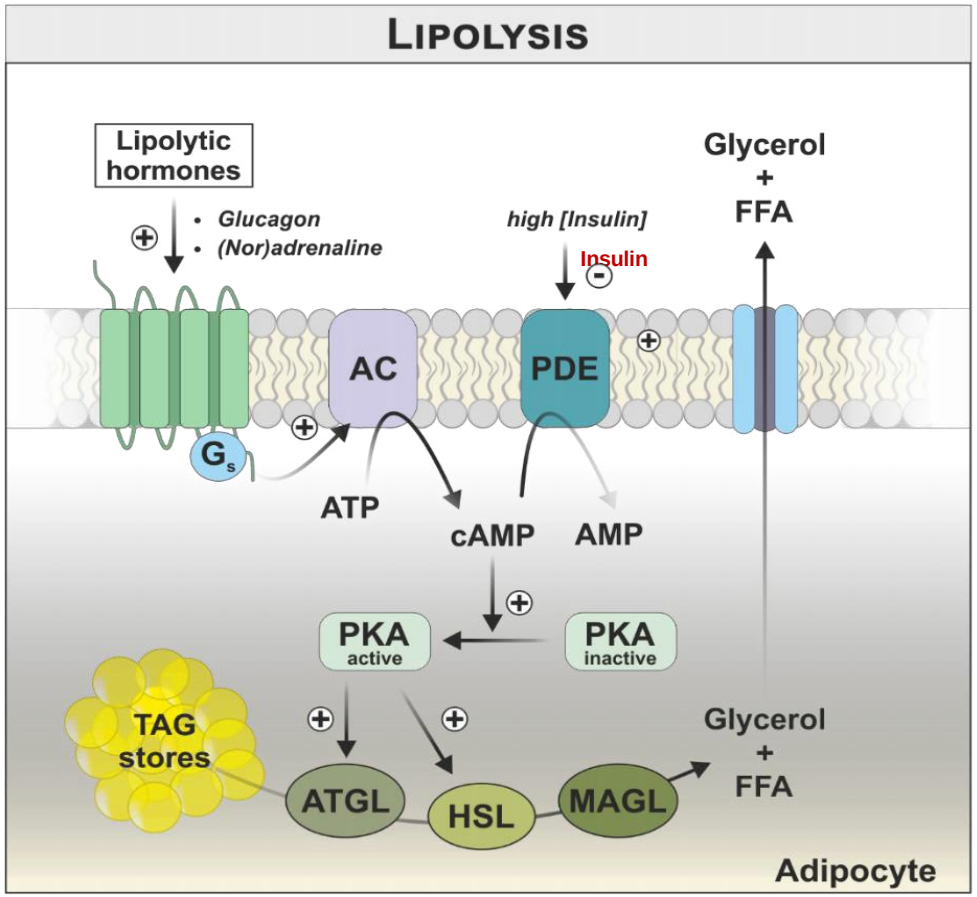
Investigating Metabolic Changes in Adipocytes and Hepatocytes

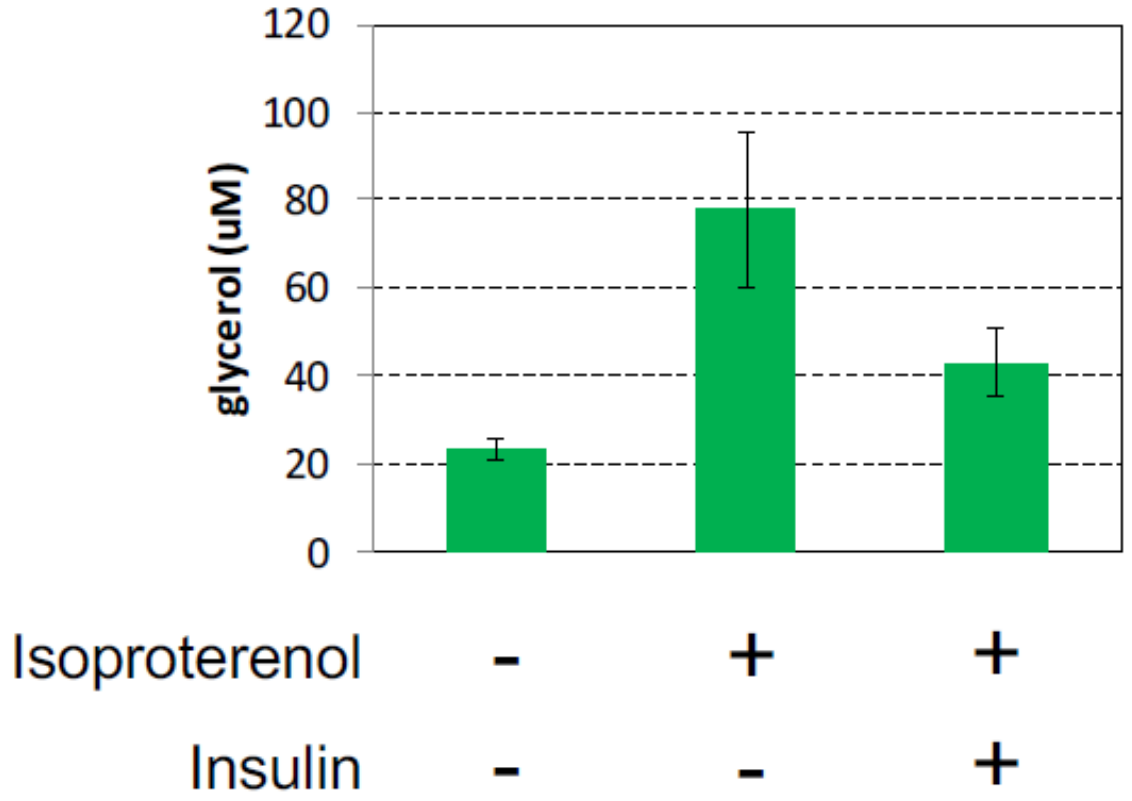
- Triglyceride assay and Cholesterol/Cholesterol Ester assays allow monitoring metabolic changes in adipocytes and hepatocytes
- 3T3L1-MBX fibroblasts were differentiated to adipocytes for the course of 21 days
- Cellular lipids were stained with oil red and triglyceride levels measured by the Triglyceride-Glo assay





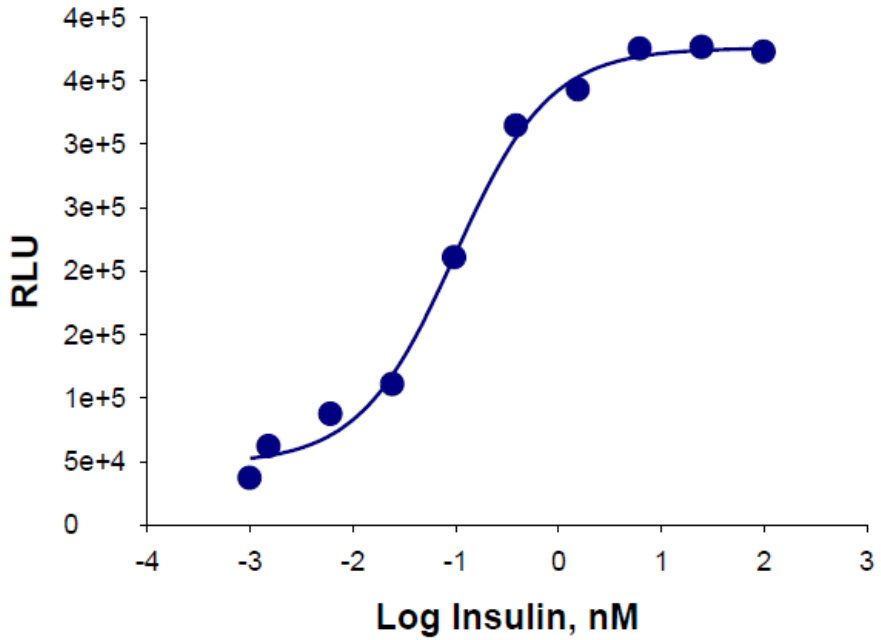
Stimulation & Inhibition of Lipolysis



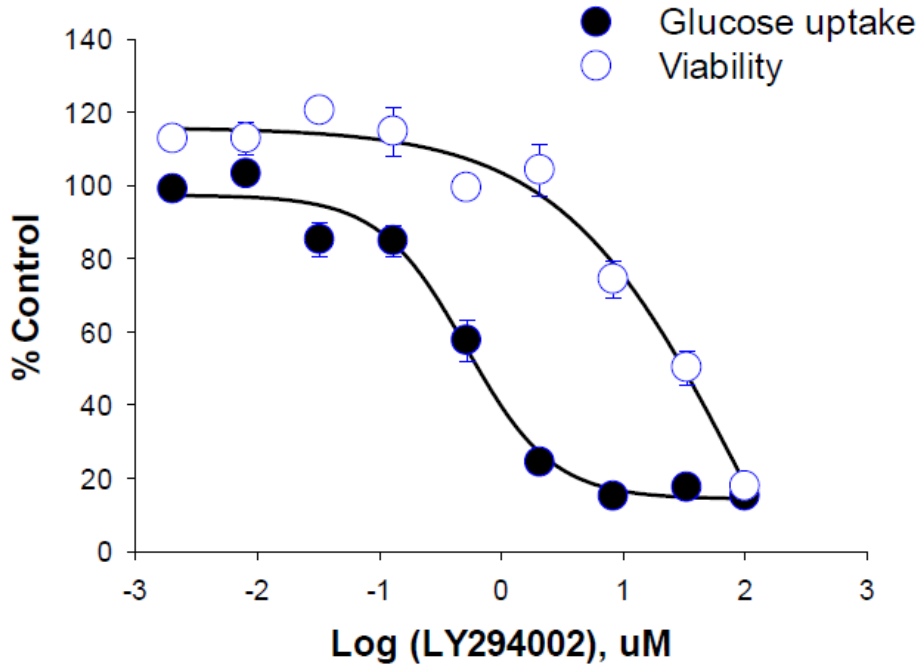


- 3T3L1-MBX fibroblasts plated at 20,000 cells/well and differentiated for 2 weeks to form adipocytes
- Treatment with 25nM isoproterenol and 150nM insulin for 90 min

Stimulation of Glucose Uptake in Adipocytes by Insulin

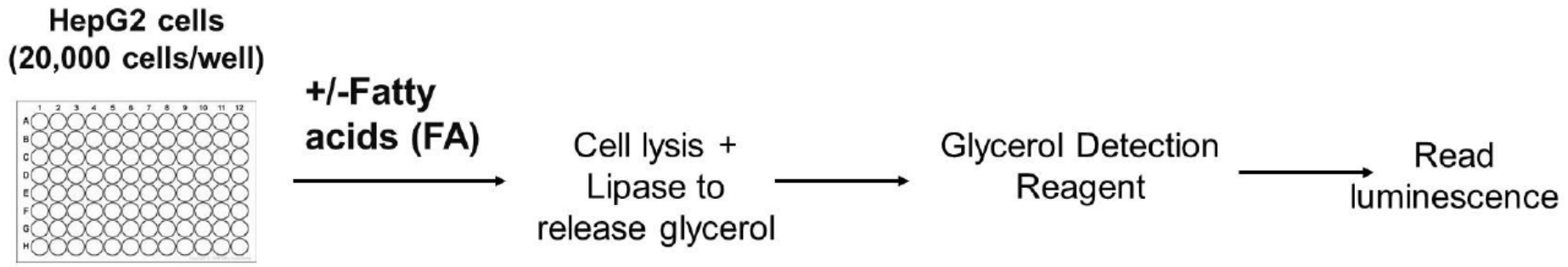


- Insulin stimulates translocation of GLUT4 in 3T3-L1 MBX adipocytes
- 10-fold increase in glucose uptake as measured by the Glucose Uptake-Glo Assay with an EC_{50} value of 0,1 nM

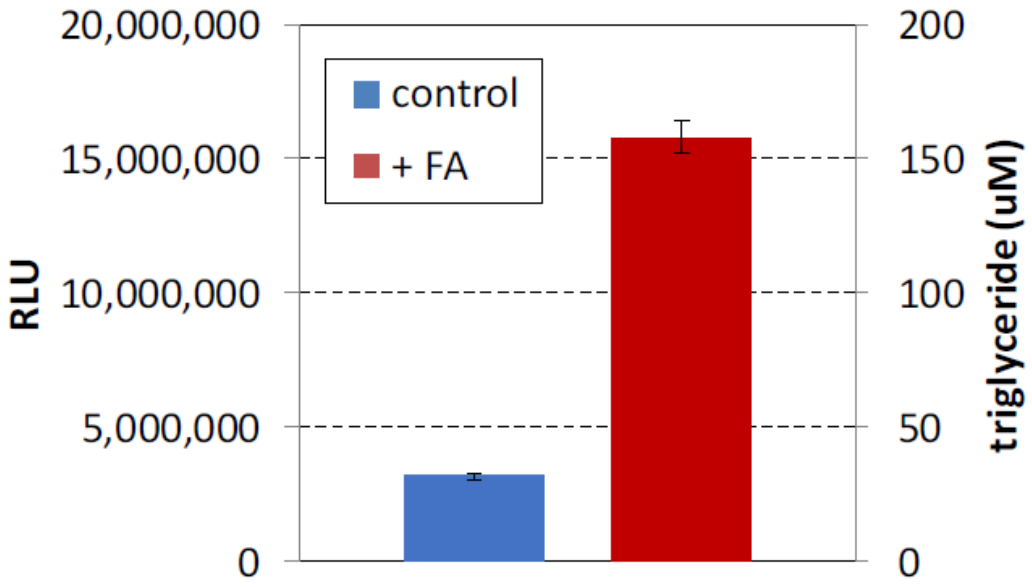


- Insulin stimulated glucose uptake could be inhibited by the PI3K inhibitor LY294002.
- Cells were treated with inhibitor for 30 min and then 100 nM insulin for 1 hr.
- Viability and glucose uptake were then measured, yielding IC50's of 88 µM and 0.5 µM, respectively.

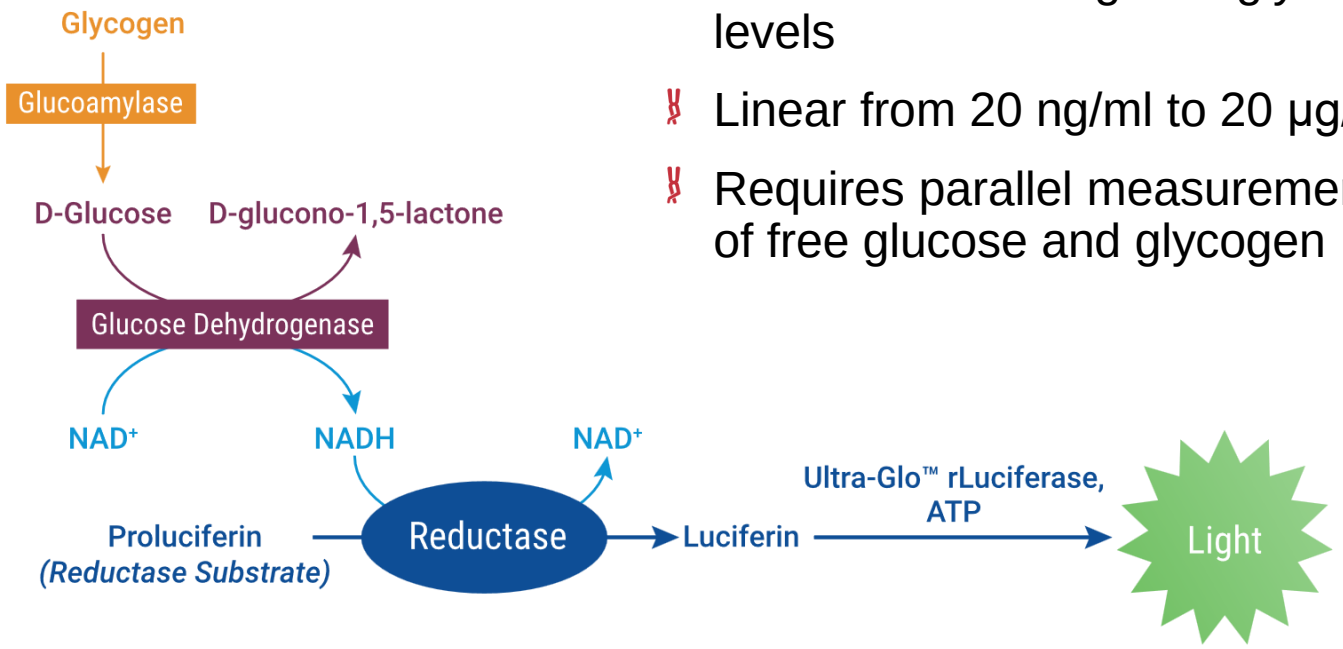
HepG2 Cells as a Model for NAFLD



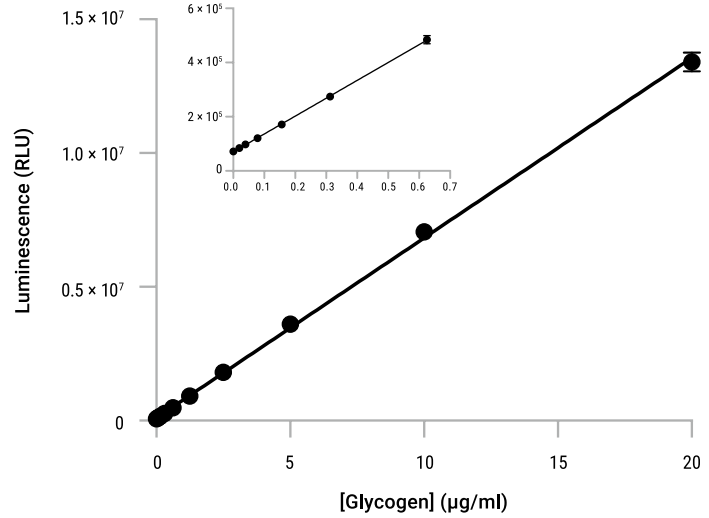
- 20 000 HepG2 cells seeded per well
- Overnight incubation in the absence or presence of 0,3mM BSA-bound linoleic and oleic acids to induce normal and steatoic conditions
- Cells were washed with PBS and assayed with triglyceride detection assay



Measure Glycogen Levels with Glycogen-Glo Assay



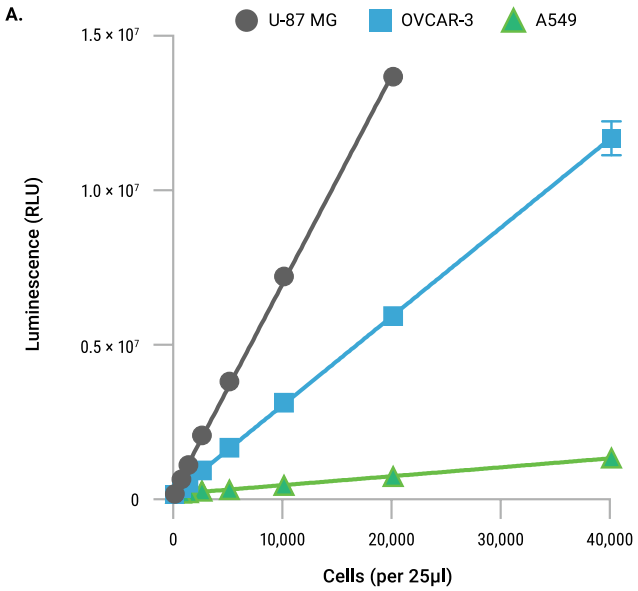
- Detect small changes in glycogen levels
- Linear from 20 ng/ml to 20 µg/ml
- Requires parallel measurements of free glucose and glycogen



$$[\text{Glycogen}] = [\text{Glucose}]_{\text{total}} - [\text{Glucose}]_{\text{free}}$$

$[\text{Glucose}]_{\text{total}}$
Plus glucoamylase
= *glucose from glycogen*
+ free glucose

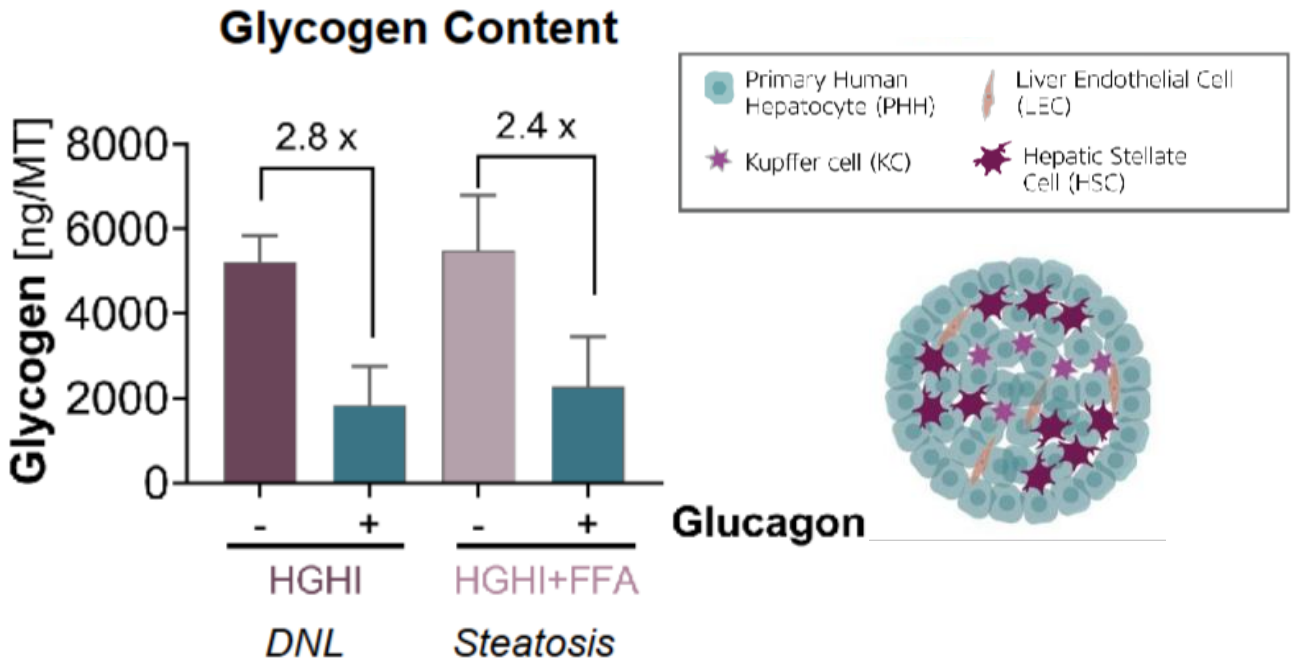
$[\text{Glucose}]_{\text{free}}$
Without glucoamylase
= *free glucose*



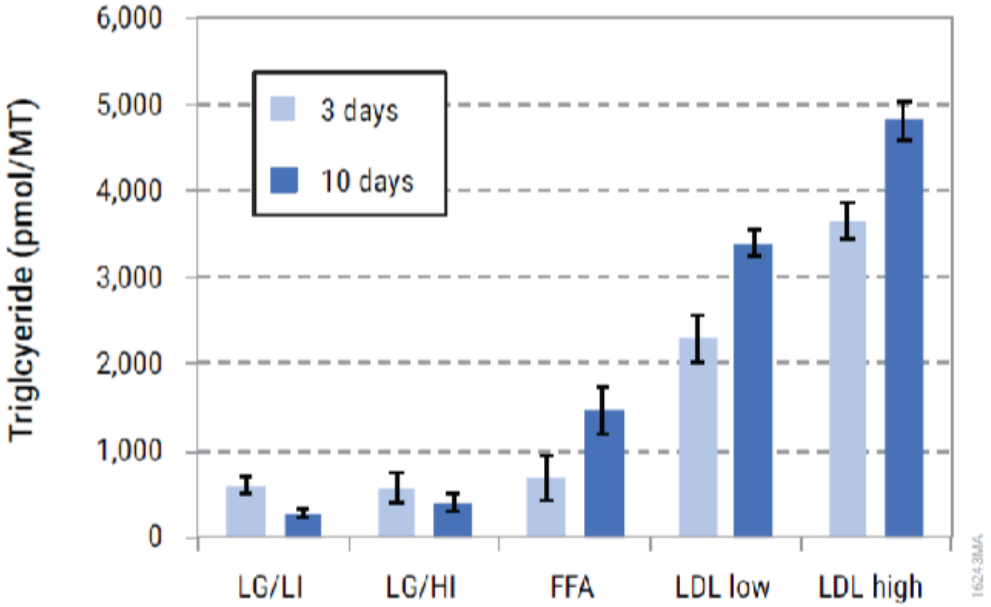
Monitoring Glycogen and TGAs in Liver Microtissues

Microtissues were treated with the following conditions to mimic disease states:

- Healthy - Low glucose, low insulin (LGLI)
- De novo lipogenesis - High glucose, high insulin (HGHI)
- Steatosis - High glucose, high insulin and free fatty acids (HGHI + FFA)



Glycogen metabolism – 3D liver microtissues were treated for 7 days with high glucose, high insulin (HGHI; DNL model) or high glucose, high insulin, and free fatty acids bound to BSA (HGHI + FFA; Steatosis model). The tissues were starved for 24 hours and treated glucagon or a control for 24 hours. Glucagon stimulated glycogenolysis and decreased glycogen levels in both the DNL and steatosis models.



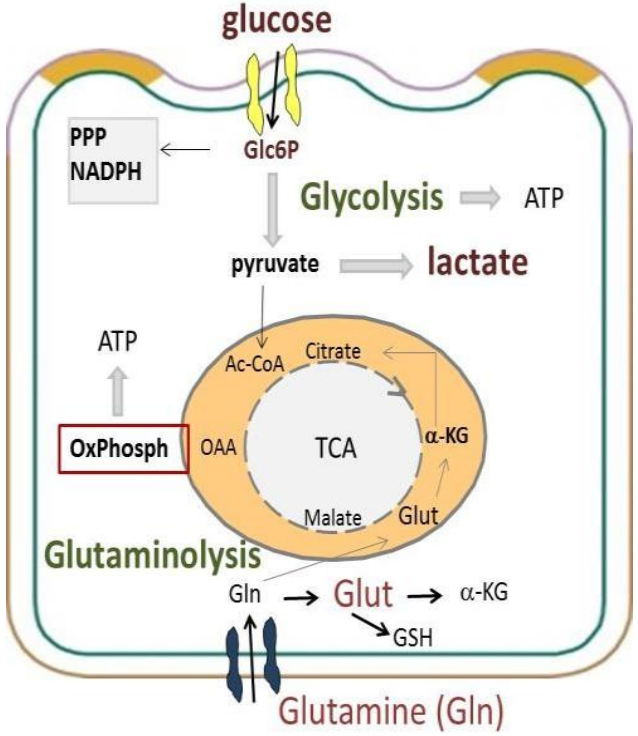
Lipid metabolism in 3D Liver Microtissues: 3D InSight Human Liver Microtissues were incubated for 3 and 10 days in medium with physiological (LG/LI) or supraphysiological (LG/HI) levels of glucose and insulin and supplementation with either FFA bound to BSA (FFA) or LDL plasma fraction (LDL). The microtissues where washed twice in PBS and assayed for total glycerol content according to the Triglyceride-Glo protocol

Today‘s Agenda

1. Luciferases and their basic features
2. Basics of cellular metabolism and how we can measure it
3. Studying insulin biology with metabolic and Lumit assays
4. **Metabolic assays in cancer and immunology**
5. News flash from cell-biology portfolio

Cancer Cells Fuel Their Proliferation Through Cathabolic Pathways

- Highly proliferative cancer cells shift to **glycolysis** and **glutaminolysis** to fuel the protein synthesis and pentose cycle to produce nucleotides
- Various cancer cell lines exhibit differences in the rate of glycolysis and glutaminolysis



Ovarian cancer cell lines are a good example:

- OVCAR-3 - low invasiveness
- SKOV-3 – highly invasive, glutamine “addicted”

→ **Glucose consumption**

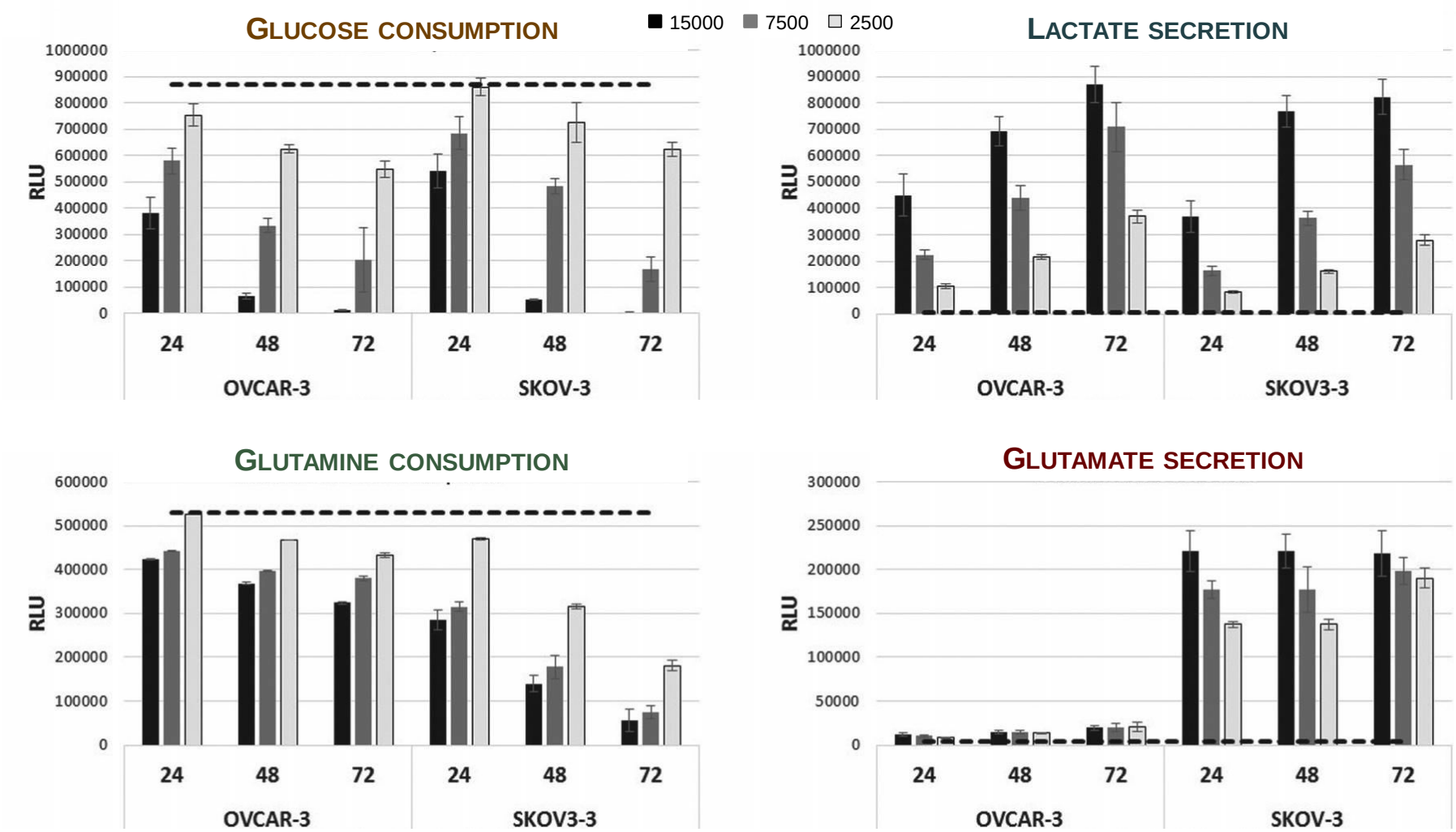
→ **Lactate secretion**

→ **Glutamine consumption**

→ **Glutamate secretion**

Adapted from Leippe, D *et al.* (2017), SLAS Discov. 22(4):366-377

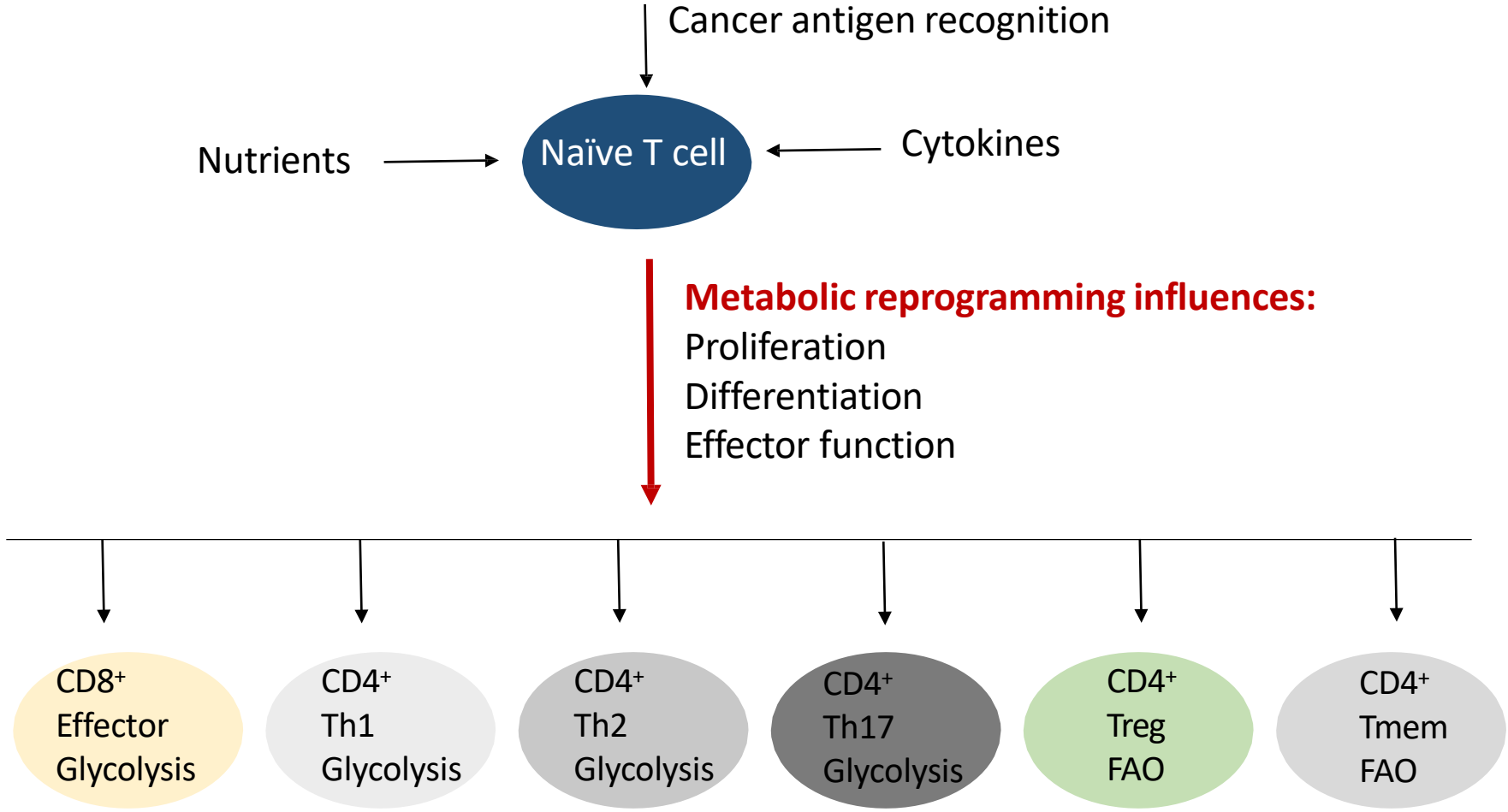
Glutamine Metabolism – Different Cell, Different Profile



- Both cell lines showed comparable glucose consumption and lactate secretion rates
- Glutamine consumption rate was higher in more invasive SKOV-3 with even greater difference in glutamate secretion

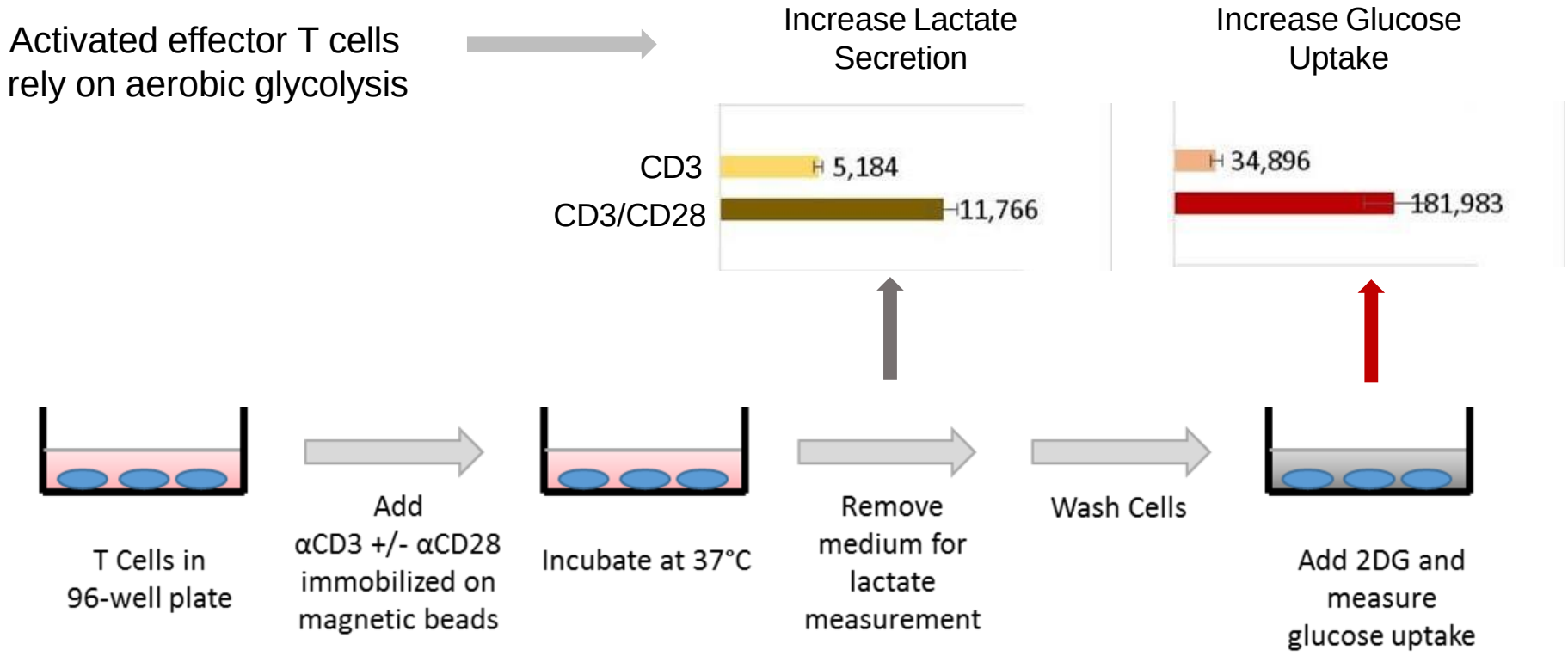
Adapted from Leippe, D *et al.* (2017), SLAS Discov. 22(4):366-377

Metabolic Reprograming of Activated T-Cells



Measuring T-Cell Activation – Endpoint approach using Glucose Uptake

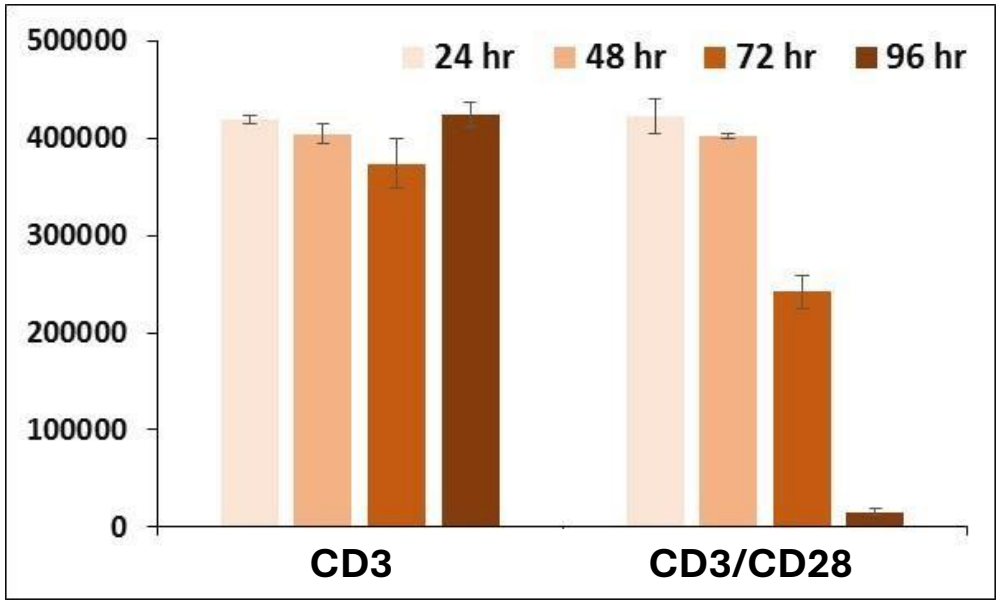
- Human peripheral blood T cells (250,000 in 100µl medium per well) were activated with anti-CD3 anti-CD3/anti-CD38 antibodies immobilized on magnetic beads
- After 24hr activation media was used for determining lactate levels, while the cells were used for glucose uptake measurements.



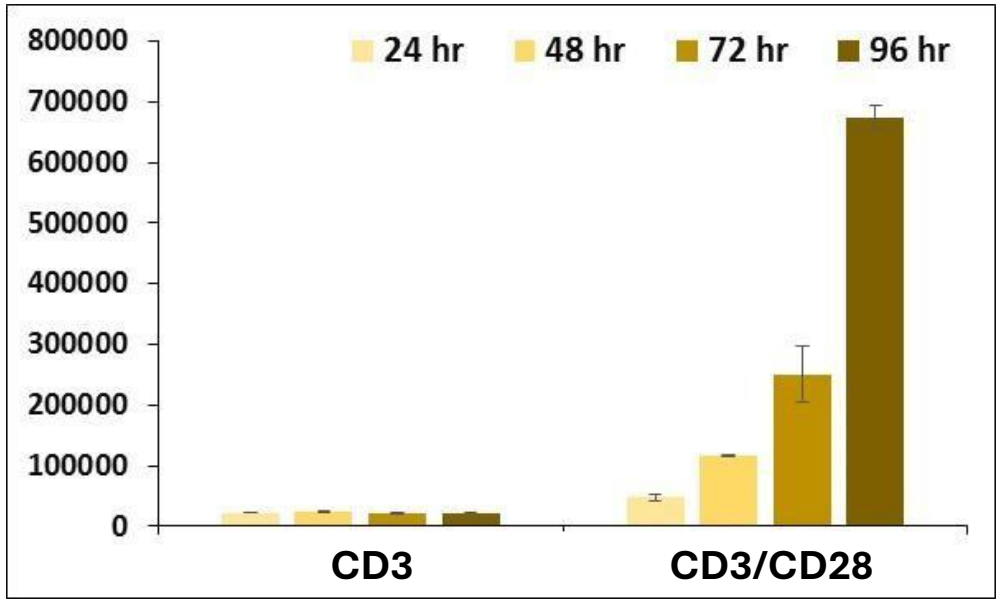
Measuring T-Cell Activation – Kinetic Approach Using Glucose and Lactate Assays

- Human peripheral blood T cells were activated with anti-CD3 alone or anti-CD3 plus anti-CD28 antibodies
- Changes in glucose and lactate concentrations in medium were measured at indicated time points
- The data indicate increase in aerobic glycolysis upon activation of T cells with anti-CD3/anti-CD28 antibodies

Glucose consumption

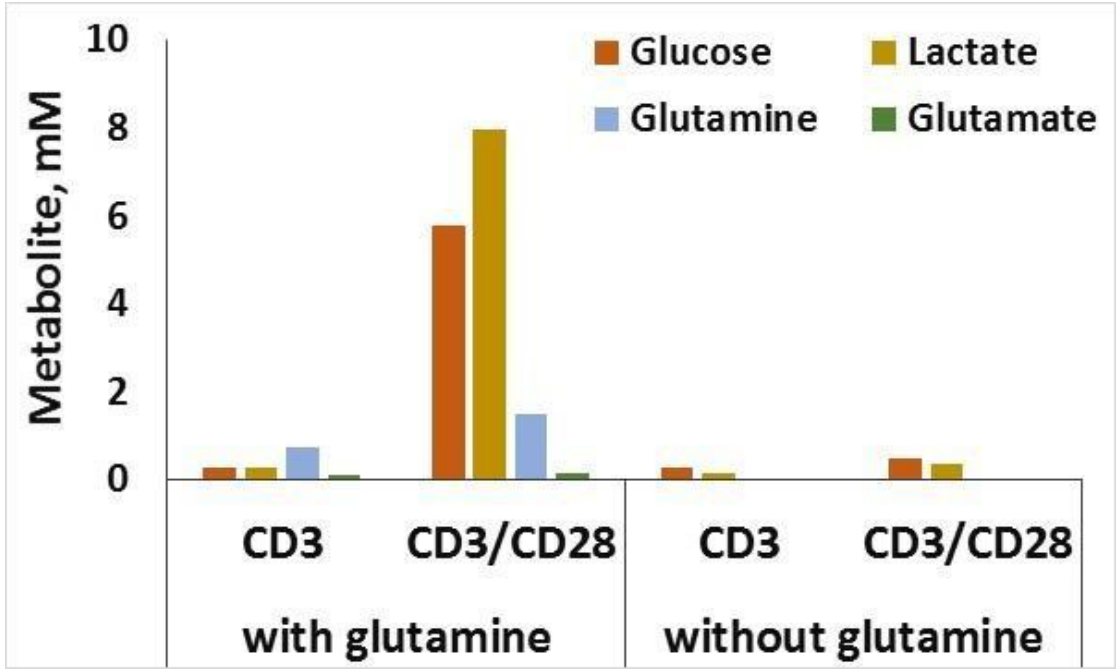


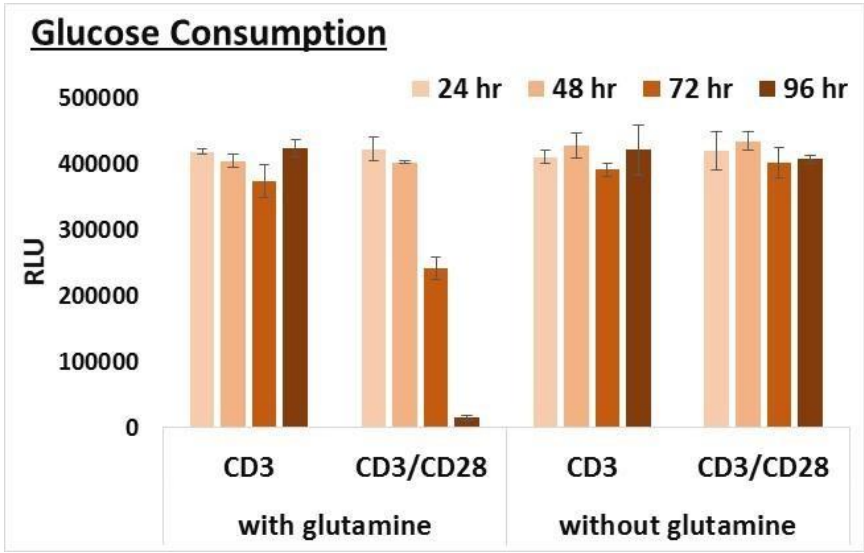
Lactate secretion

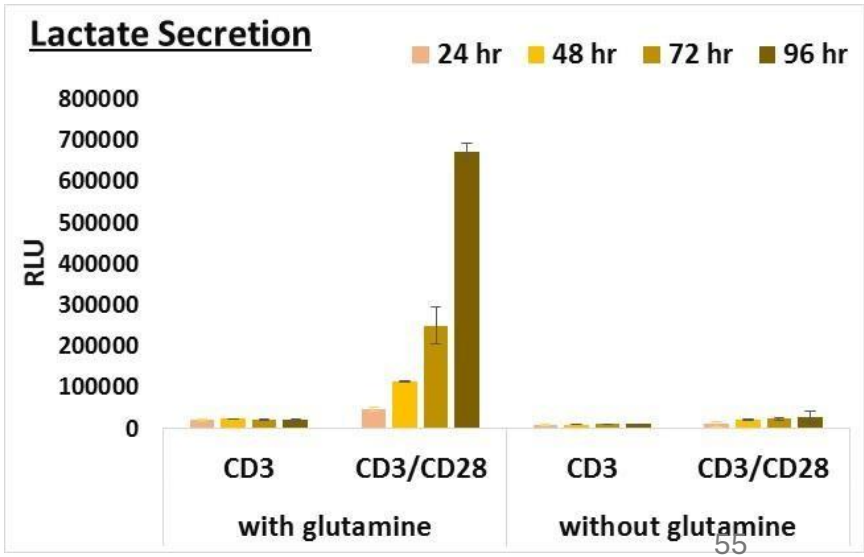


T Cell Activation Depends on More than One Fuel Source

- Glutamine is required to support anabolic metabolism of effector T cells
- There is no increase in aerobic glycolysis without glutamine
- Activated T cells exhibit increased glutamine consumption

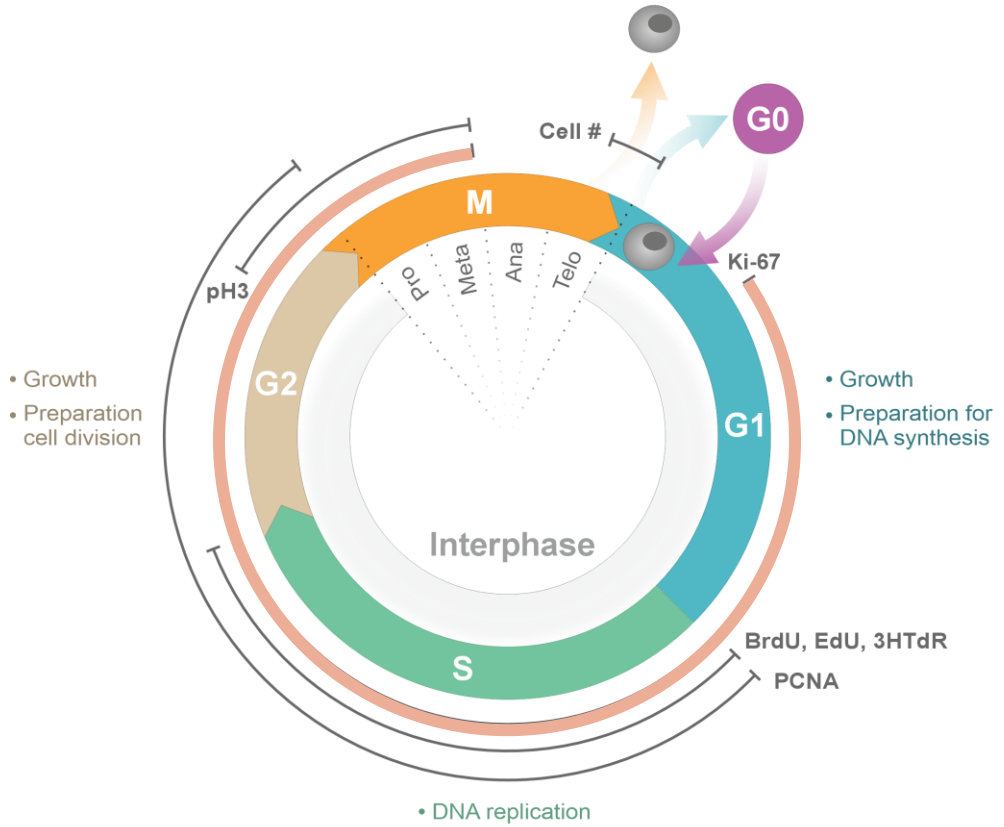






Lumit™ hKi-67 Immunoassay for Cell Proliferation

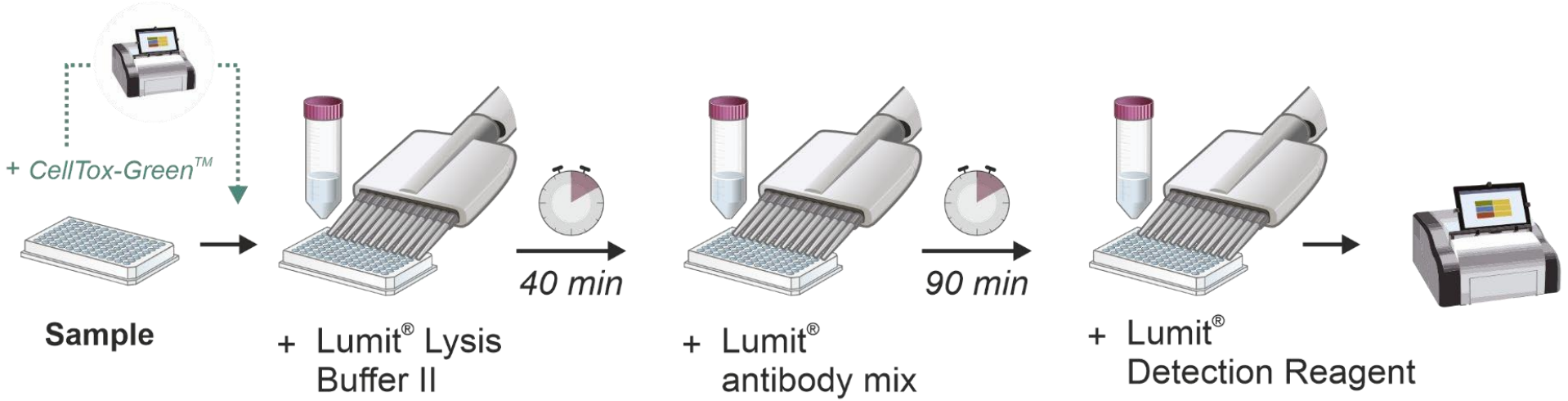
The proliferation marker Ki-67



- Expressed in proliferating cells
  - Expressed in G1, S, G2 and M cell cycle phases
  - Ramps up from G1 until peaks early in M phase
- Absent in resting, non-dividing cells (G0) (quiescent, senescent, or terminally differentiated)

Lumit™ hKi-7 Immunoassay for Cell Proliferation

Workflow

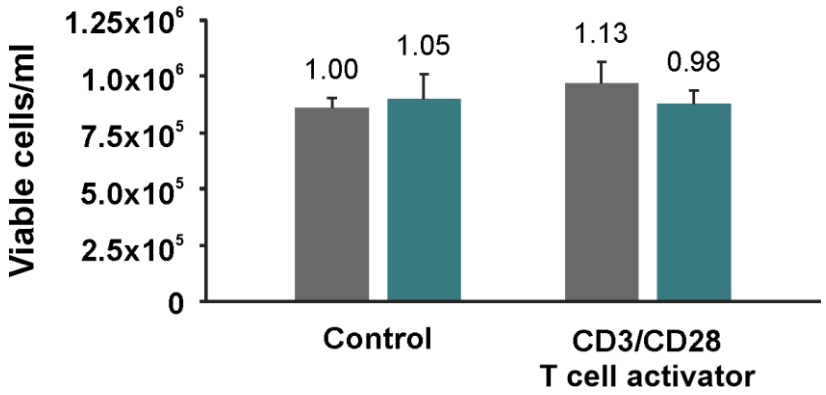


- Completely homogeneous assay with no transfer or wash steps
- CellTox-Green™ Cytotoxicity Assay fluorescence readings for loss of membrane integrity (cell death) must be taken before initiating the Ki-67 assay protocol
- Antiproliferative activity is indicated by decreased Ki-67 levels without cell death

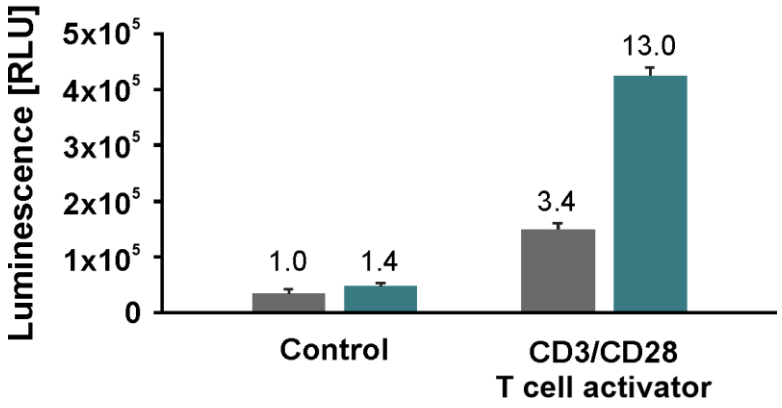
Lumit™ hKi-67 Immunoassay for Cell Proliferation

Ki-67 is an early indicator of cell proliferation

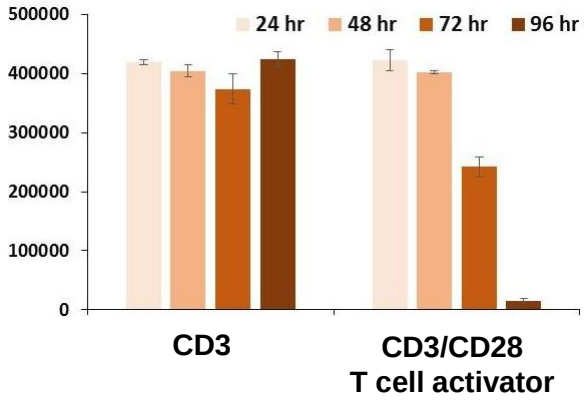
Cell Count



Ki-67 Level



Glucose Consumption

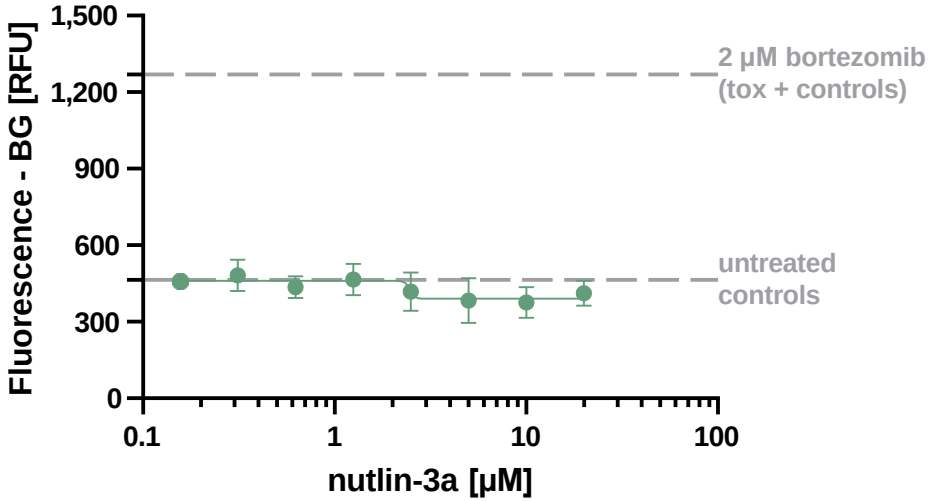


- Human CD8+ T cells (80,000/well) were treated with T cell activator (+/- IL-2) for 48 h
- Upregulation of Ki-67 is observed before T cell proliferation (which begins > 72 h after activation; data not shown)

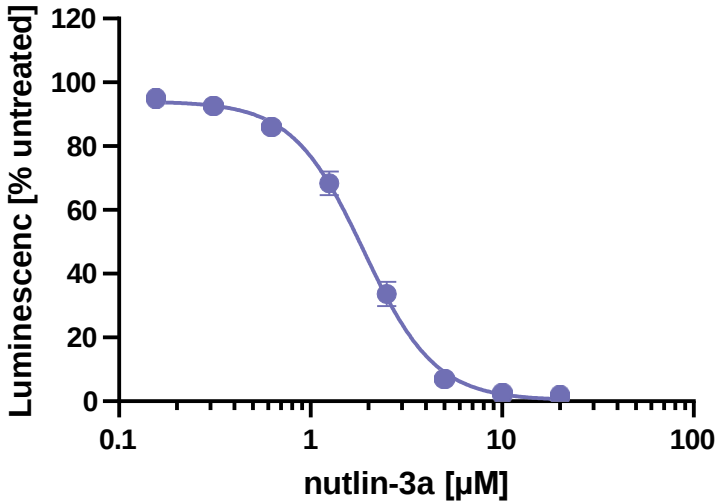
Lumit™ hKi-67 Immunoassay for Cell Proliferation

Ki-67 is an early indicator of cell proliferation

CellTox-Green™



Lumit® hKi-67 Immunoassays

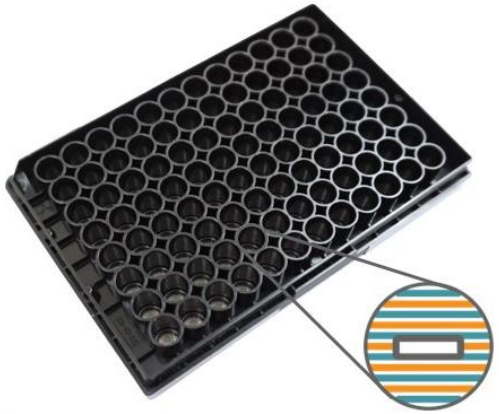


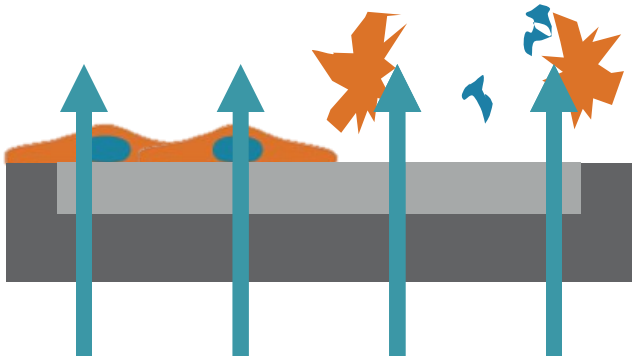
- HCT 116 cells (10,000/well) were treated with antiproliferative agent nutlin-3a for 48 hours
- Ki-67 expression was reduced in a dose-dependent manner without inducing cytotoxicity

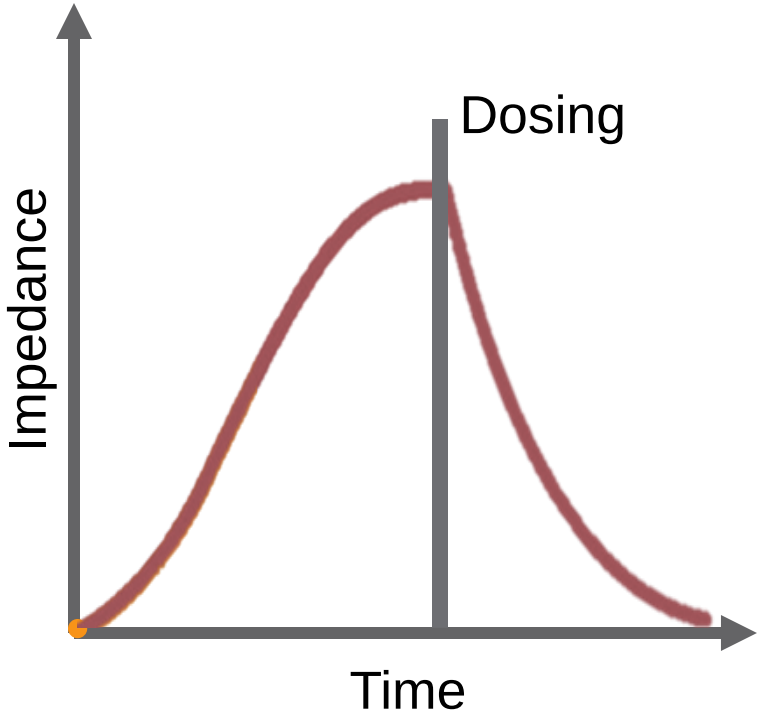
Immunoassay	Limit of Detection (LOD; 3 SD above background)	Dynamic Range	Minimal Detectable Dose (MDD; 2 SD above background)
Lumit® IL-2 (Human)	11.2pg/ml	28.2pg/ml–25ng/ml	7.2pg/ml
Lumit® IL-4 (Human)	6.7pg/ml	18.2pg/ml–25ng/ml	4.5pg/ml
Lumit® IL-6 (Human)	7.5pg/ml	18.2pg/ml–25ng/ml	5.0pg/ml
Lumit® IL-10 (Human)	7.4pg/ml	18.2pg/ml–25ng/ml	4.9pg/ml
Lumit® IFN-γ (Human)	1.7pg/ml	7.3pg/ml–10ng/ml	1.1pg/ml
Lumit® TNF-α (Human)	2.9pg/ml	18.2pg/ml–25ng/ml	2.0pg/ml
Lumit® IL-12 (Human)	4.5pg/ml	18.2pg/ml–25ng/ml	3.0pg/ml
Lumit® IL-1β (Human)	10pg/ml	22pg/ml–40ng/ml	7pg/ml
Lumit® HMGB1 (Human)	1ng/ml	4–1,000ng/ml	—
Lumit® Active IL-18 (Human)	≤10pg/ml	11pg/ml–20ng/ml	—
Lumit® IL-8 (Human)	1pg/ml	7.29pg/ml–10ng/ml	1pg/ml
Lumit® IL-17A (Human)	3pg/ml	18.2pg/ml–25ng/ml	2pg/ml
Lumit® IFN-β (Human)	5.3pg/ml	18.2pg/ml–25ng/ml	3.6pg/ml
Lumit® VEGF-A (Human)	3pg/ml	18.2pg/ml–25ng/ml	2pg/ml
Lumit® MCP-1 (Human)— Coming soon	—	—	—
Lumit® TGF-β_1 (Human)— Coming soon	—	—	—
Lumit Insulin	58 pg/ml	—	—
Lumit Glucagon	3,4 pg/ml	1pM-2nM	
Lumit hKi-67			

Impedance Assays Principle

- Measures how easily signal passes the electrode-cell interface
- Resistance increases as coverage and attachment increases
- Can detect:
  - Proliferation
  - Viability
  - Cell-cell coupling strength (barrier function)
  - Migration
  - Cell signaling

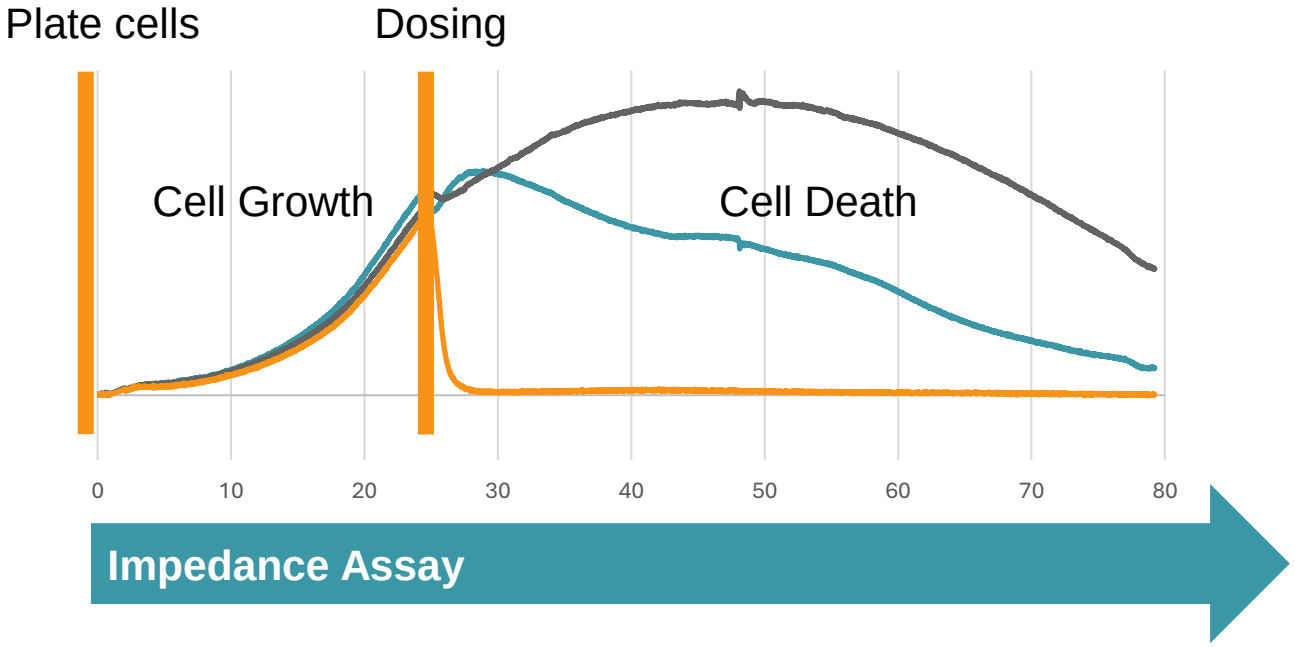




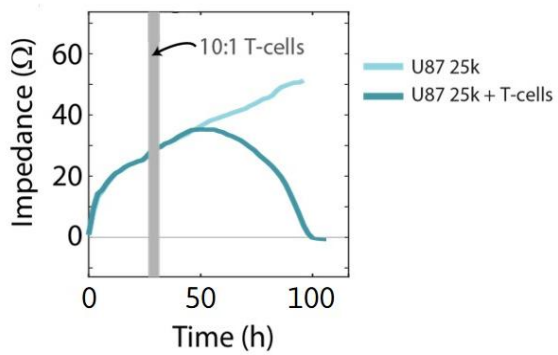


Impedance Assays vs. Traditional Assays

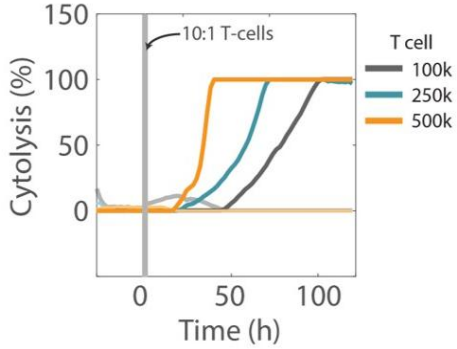
- Captures all stages of an experiment
  - Cell growth and death
  - Acute or chronic treatments
  - TEER measurements
- Hands-free data collection
  - Plate cells, add treatments, done
- Label-free
  - Measurement doesn't impact biology
  - No optimization of labels, dyes, or incubation times required
  - Multiplex with bioluminescent and fluorescent cell-based assays



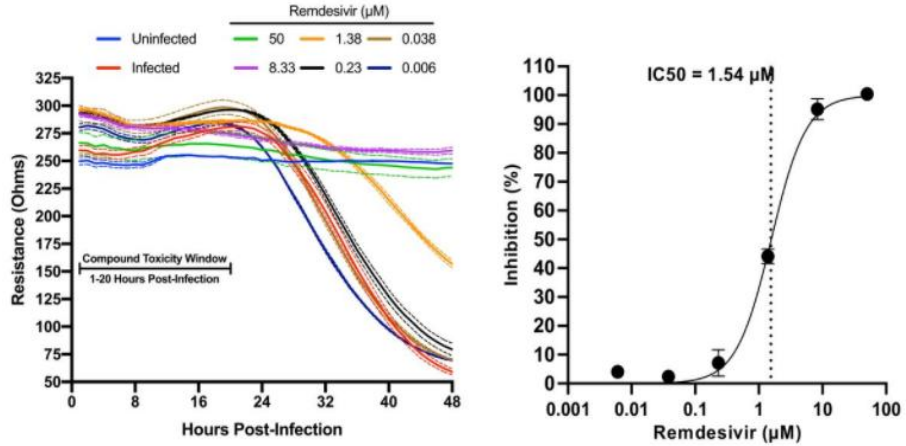
Impedance Measurement Applications



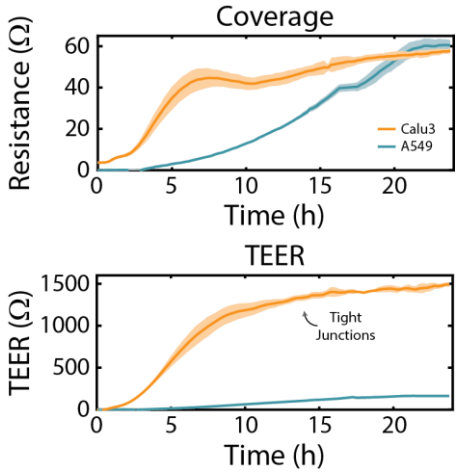
Immune cell kill assay

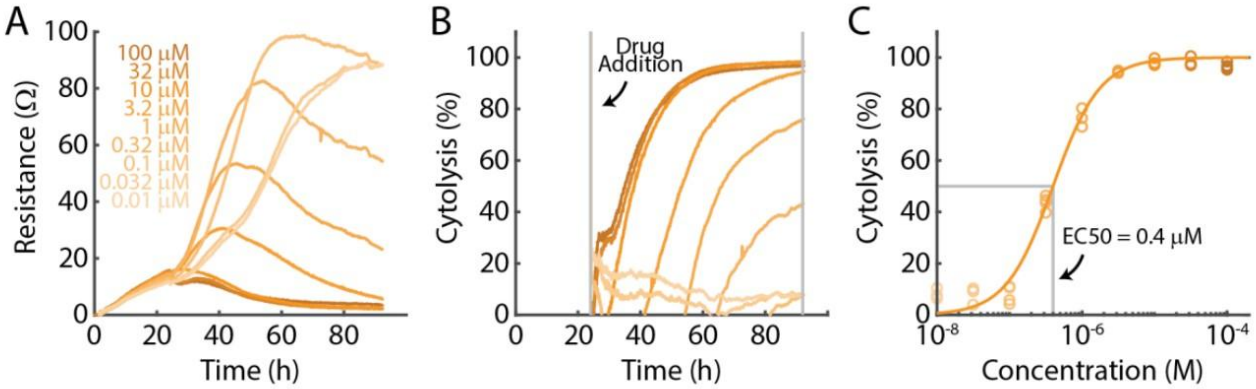


Kill curve



- **CAR-T therapy development**
- **Viral cytopathic effects**



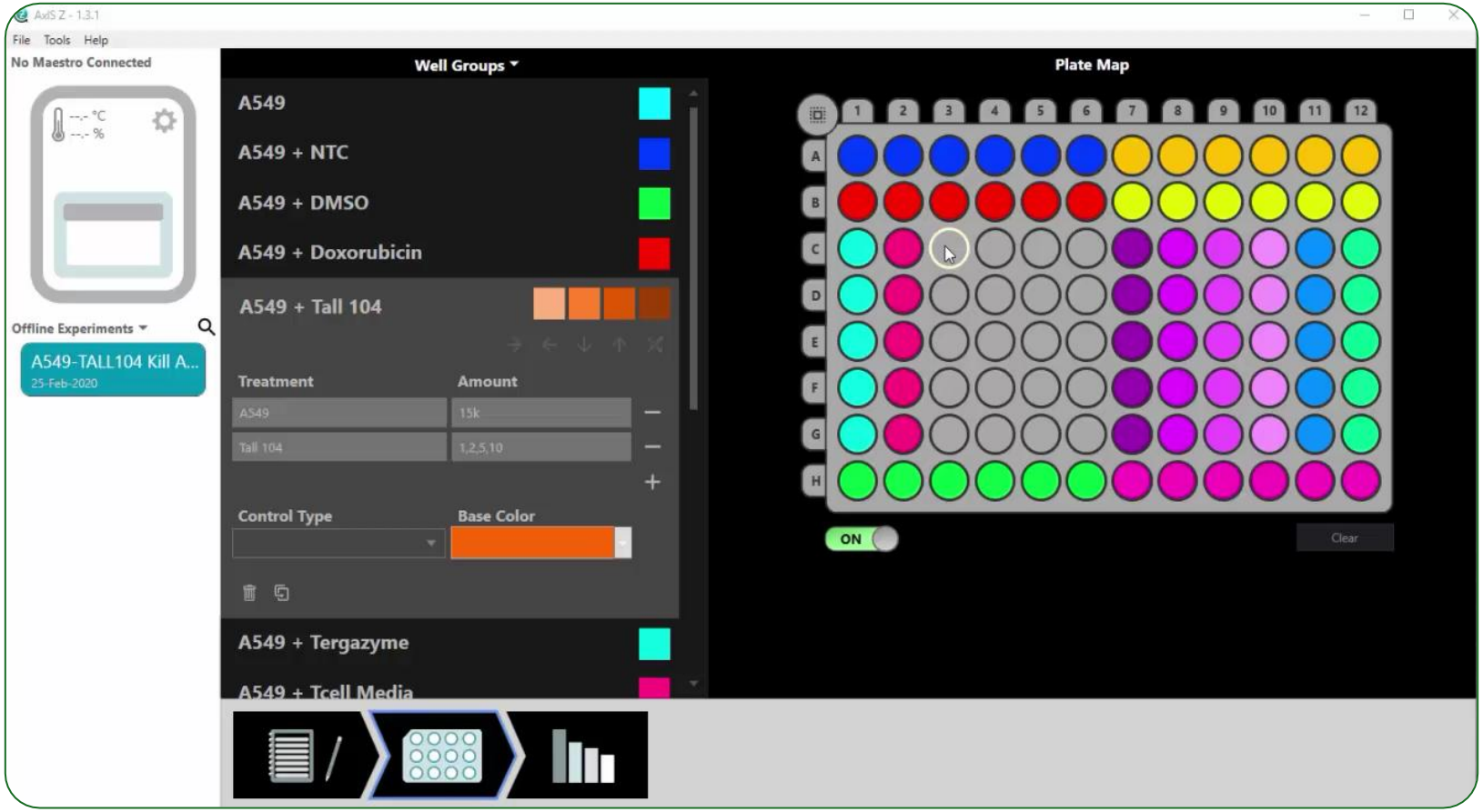


SKOV3 cells treated with 9 different concentrations of doxorubicin

- **Transepithelial electrical resistance (TEER) changes**
- **Dose-response analysis**

Label-free, continuous monitoring of cell behavior

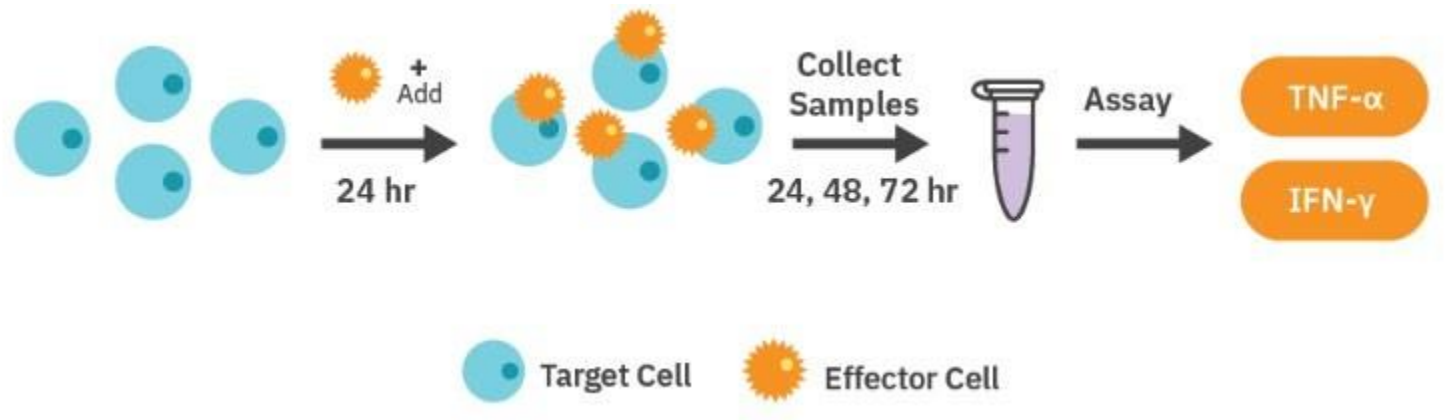
Quantitatively track cell proliferation, viability, and cytotoxicity



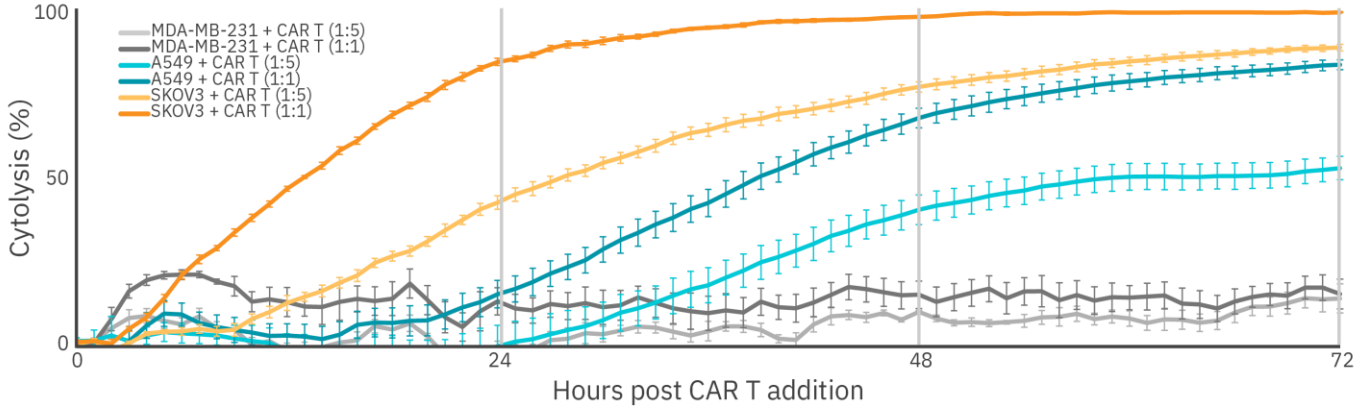
The only impedance assay that can be performed with a single-click

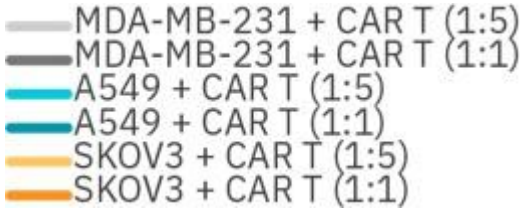
Combination of Impedance with Lumit Immunoassays for CAR-T development

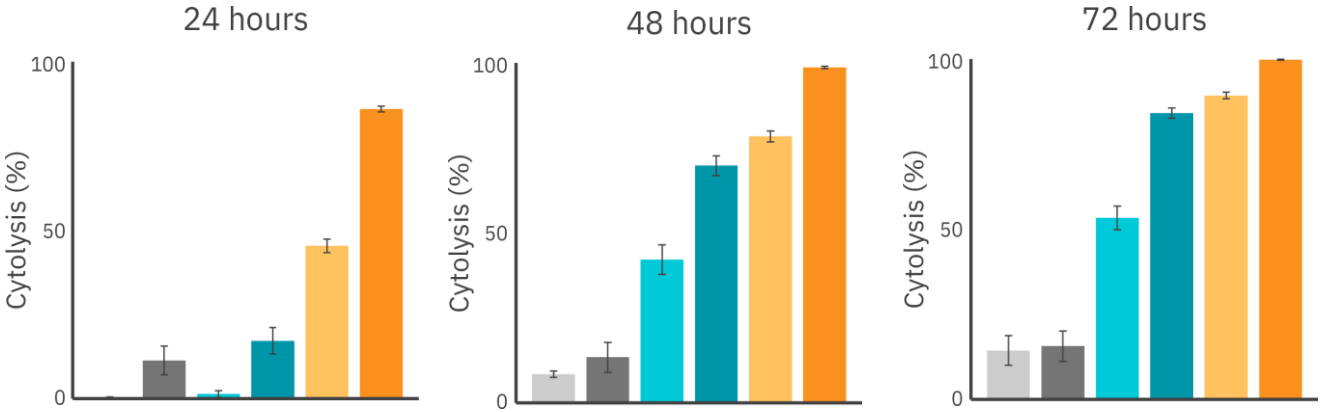
- Does tumor antigen density impact CAR-T Cell performance?
- HER2 CAR-T cells were cocultured with:
  - SKOV3 (high HER2 expression)
  - A549 (low HER2 expression)
  - MDA-MB-231 (no HER2 expression)
- CAR-T cell killing monitored by impedance, TNF-α & IFN-γß detected by Lumit Immunoassays



Combining Impedance with Bioluminescent Assays for CAR-T development

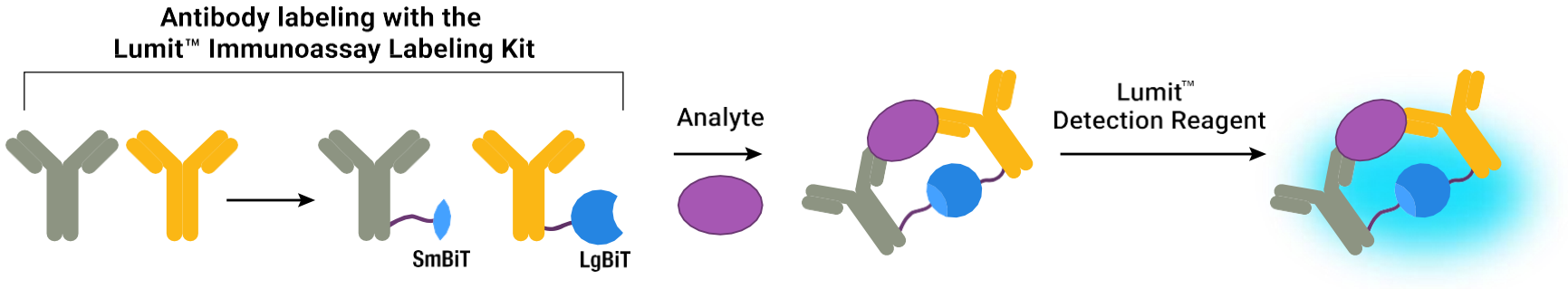




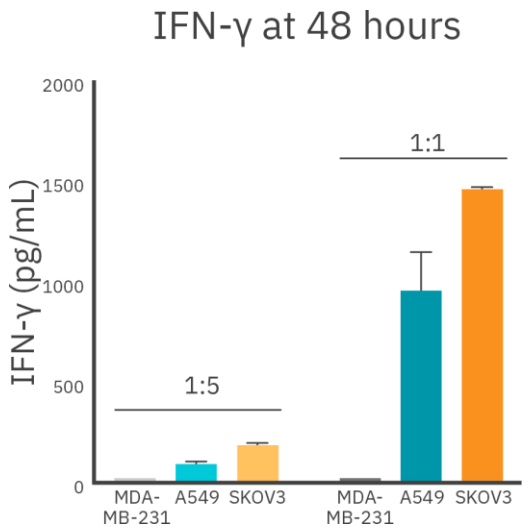


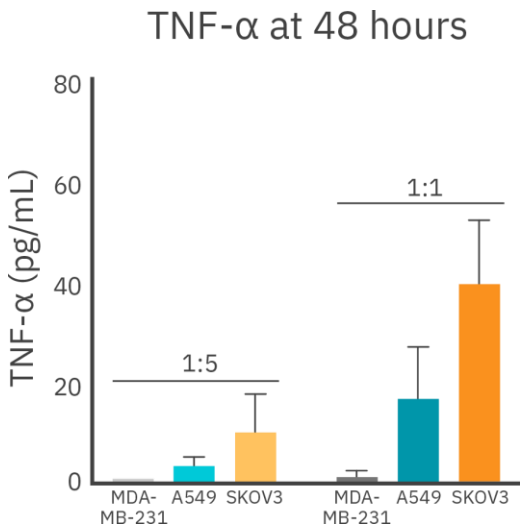
- CAR-T cell killing correlated with antigen expression levels
- Observed differences in cell killing change over time
- MDA-MB-231 cells showed 20% cytolysis due to nonspecific killing

Combining Impedance with Bioluminescent Assays for CAR-T development



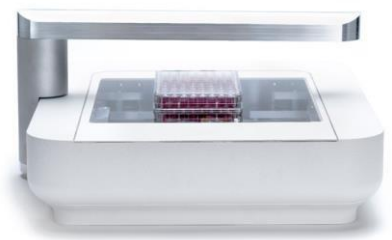
- CAR T cells co-cultured with SKOV3 (high HER2) released 41.6% more IFN-γ compared to A549 (low HER2)
- CAR T cells co-cultured with SKOV3 released 80.5% more TNF-α compared to A549
- CAR T cells co-cultured with MDA-MB-231 (no HER2) did not release detectable TNF-α or IFN-γ

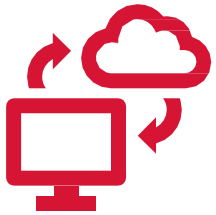


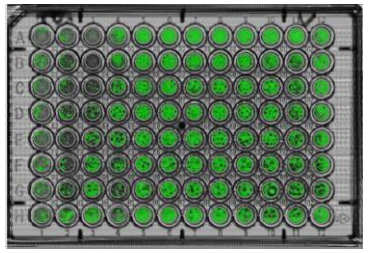


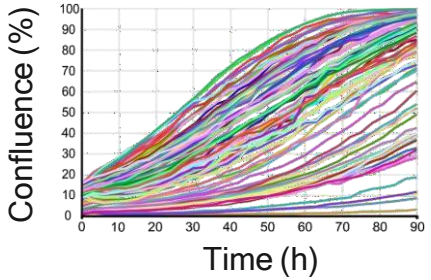
Omni Full Plate Scanner

- On demand, in incubator imaging

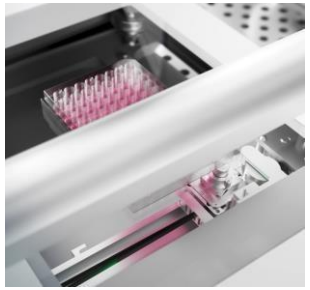






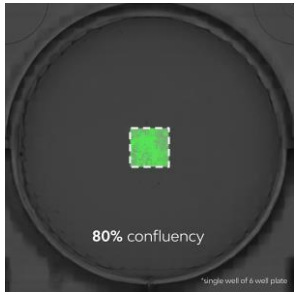


Scan → **Cloud Upload** → **Auto Stitching** → **Image Analysis** → **Plot Data**



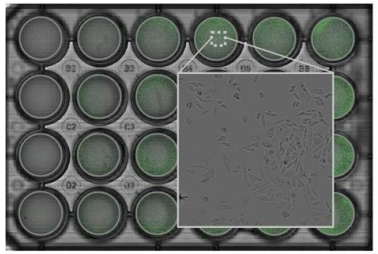
Continuous motion imaging

- Automated whole-plate imaging
- Continuous motion recording
- 9,000 images per scan (e.g., hour)
- Undisturbed image acquisition (weeks)



Full well readout

- Scans entire well and instantly stitches images to give a full well view



CytoSMART Omni AI

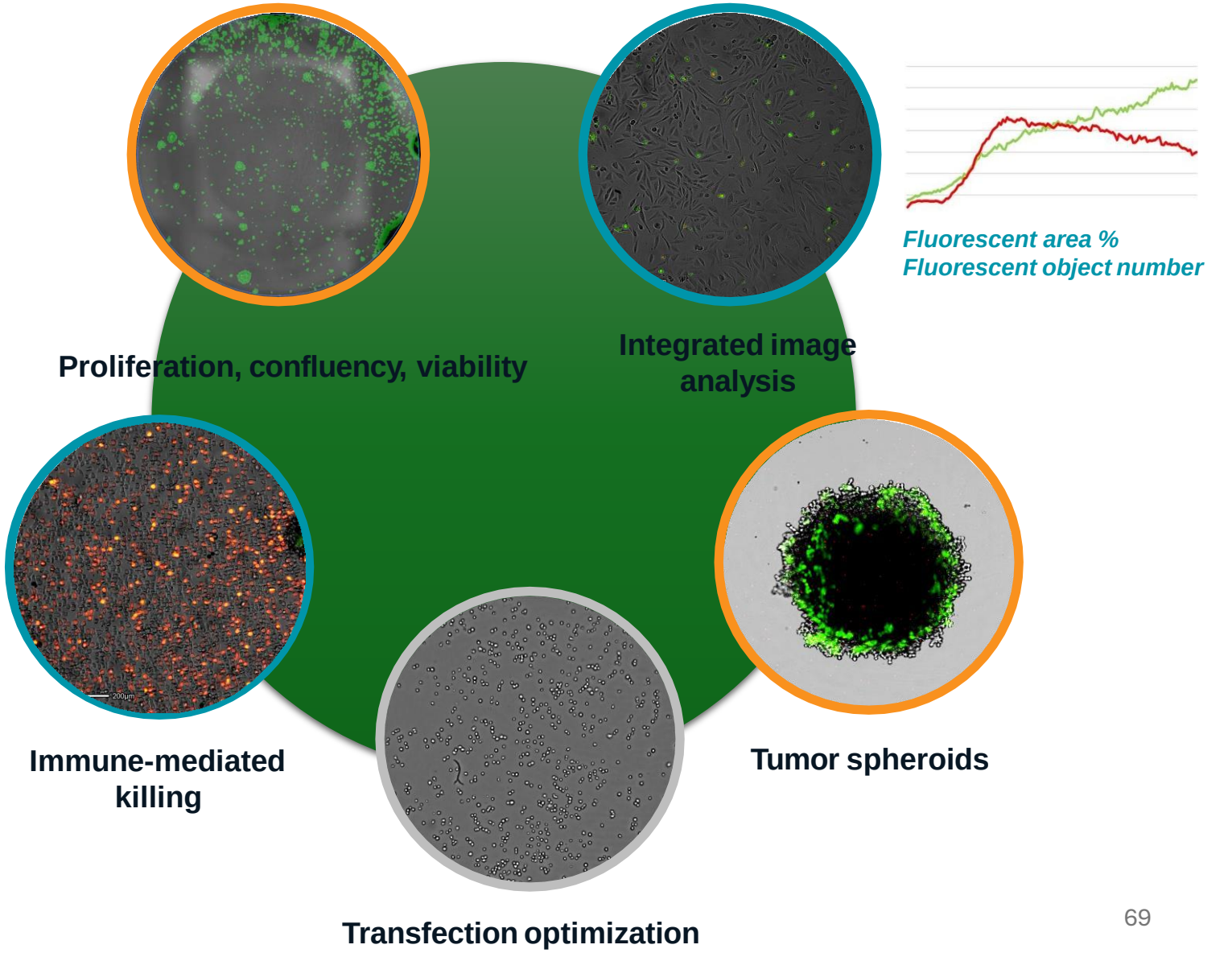
The AI image analysis enables:

- Automated stitching of 9,000 images/scan
- Automated ROI detection
- Automated detection & quantification within ROIs

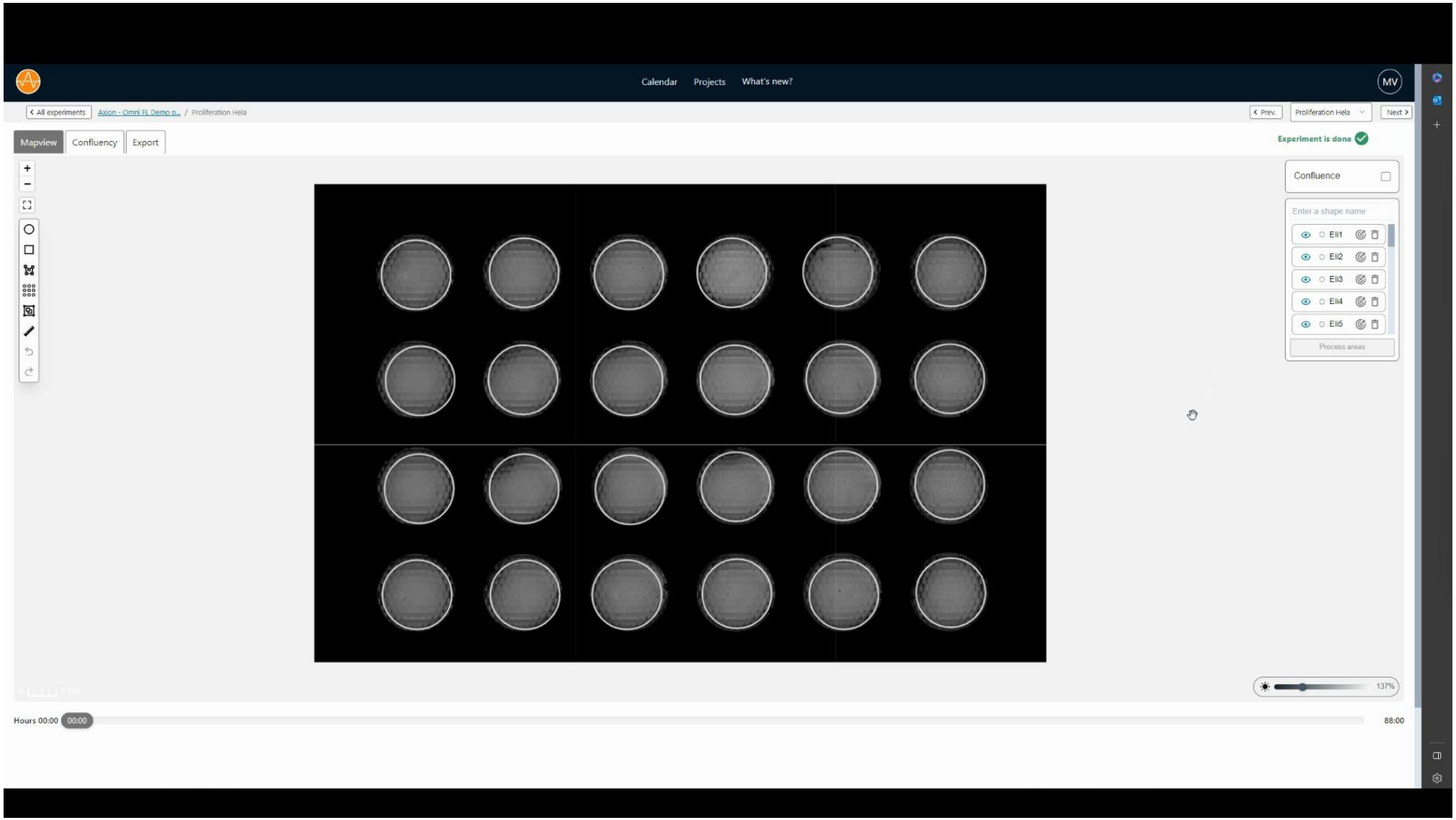
Application Examples

Applications

- Proliferation, viability
- Cytotoxicity assays
- Cell-cell interaction
- Stem cell differentiation
- Transfection efficiency
- Organoid growth
- Cell Migration

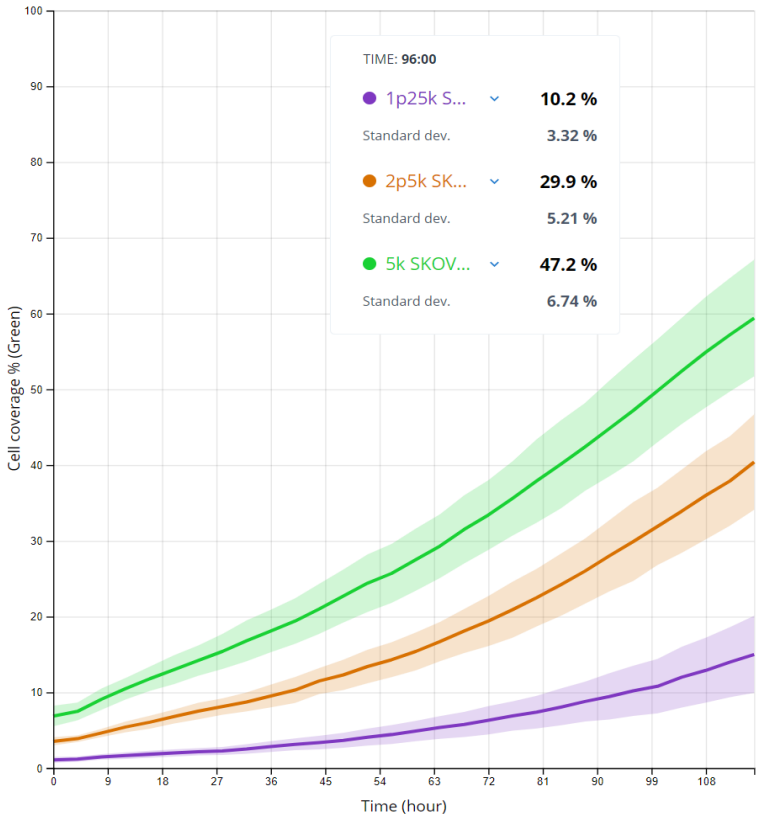


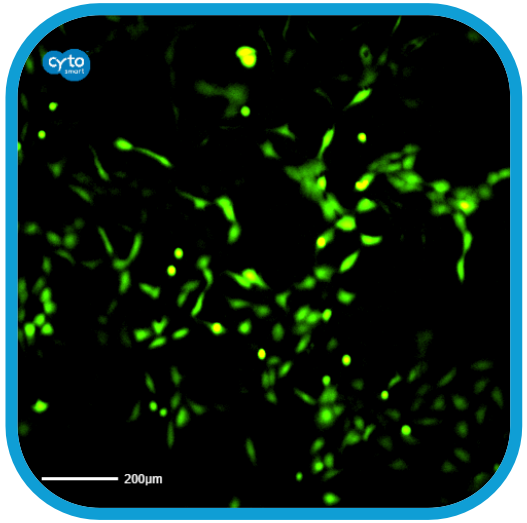
Confluency Module

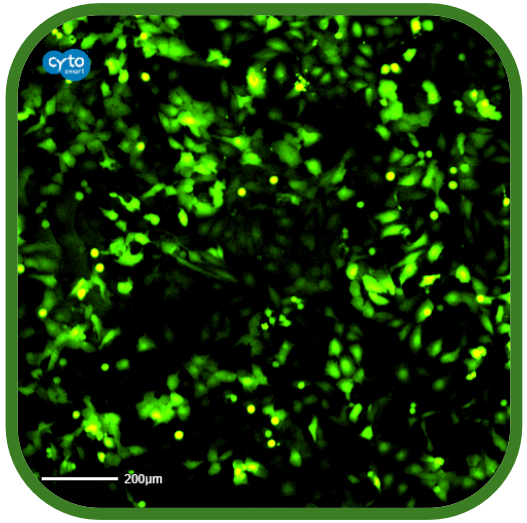


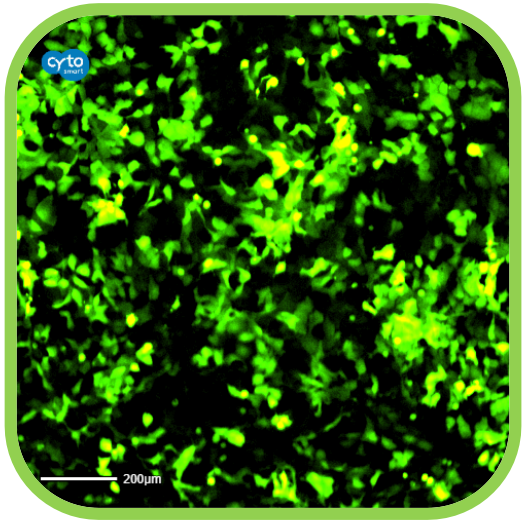
Cell Culture Quality Control

Cell density sweep of GFP+ ovarian cancer cells (Skov3)







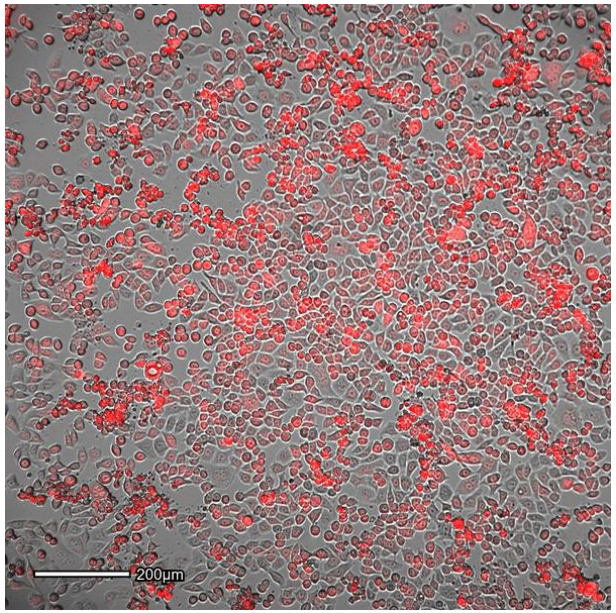


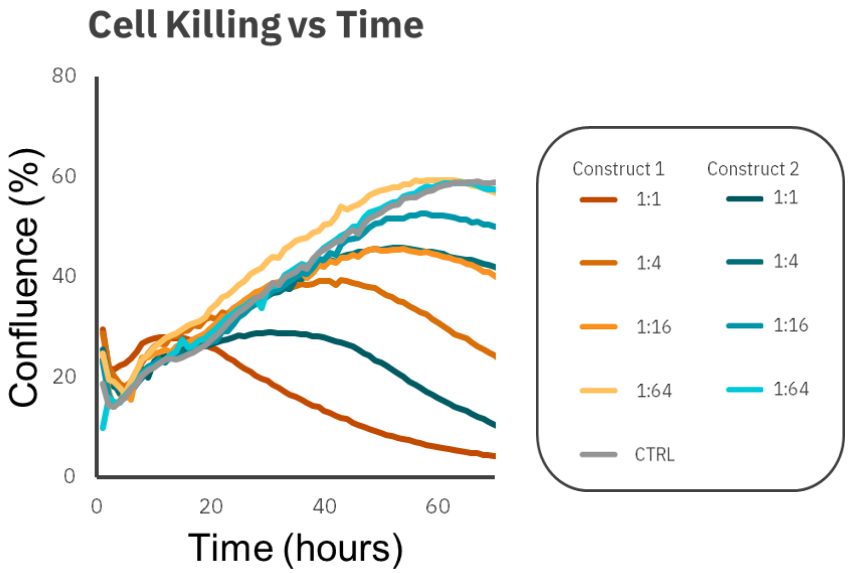
- Images of the green fluorescent channel of the 3 different cell densities.
- Cell confluency over time for cells plated at different densities.

Cell Killing Assay for CAR Optimization for Effective Immunotherapy Development Using Live-Cell Imaging

The Omni live-cell imaging system was used to compare the killing of different CAR constructs and showed that cell death was observed in a dose-dependent manner.

Leucid





Confluency of RFP-labeled cancer cells after the addition of CAR T cells is measured with the Omni platform. Two different CAR constructs were compared across E:T ratios and Construct 1 showed a greater potency across all ratios.

"The Omni live-cell imaging platform was a straightforward way to *measure the immune cell killing of our CAR constructs* in vitro. Its simple interface and analysis gave fast, clear results and enabled us to quickly assess *multiple conditions* without disturbing the underlying biology."

Marc Davies, VP of R&D
Leucid Bio

Today‘s Agenda

1. Luciferases and their basic features
2. Basics of cellular metabolism and how we can measure it
3. Studying insulin biology with metabolic and Lumit assays
4. Metabolic assays in cancer and immunology
5. News flash from cell-biology portfolio

GloMax Galaxy Bioluminescent Imager

- Use NanoLuc® technologies to study rare events and analysis of mixed cell populations
- Study protein dynamics and cellular physiology
- Living & fixed cells & tissues
- Ideal for assay development

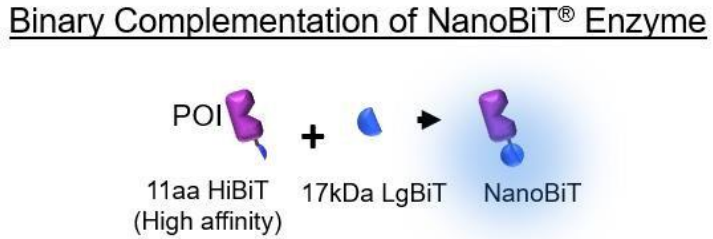
LUMINESCENCE	FLUORESCENCE	BRIGHTFIELD
Protein dynamics and localization	Cellular reference markers	Morphology

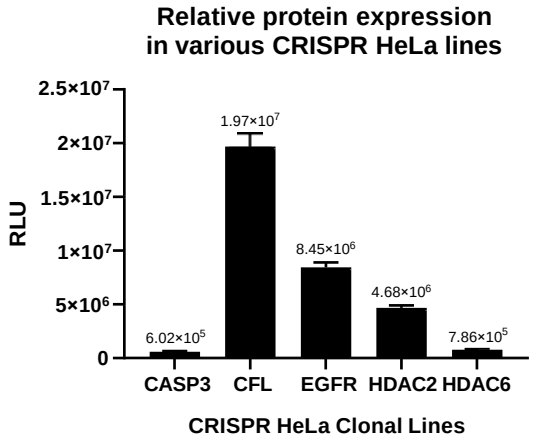
Affordable, Easy to use, Low-throughput

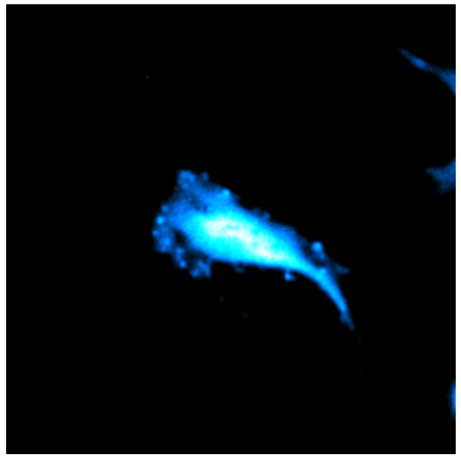
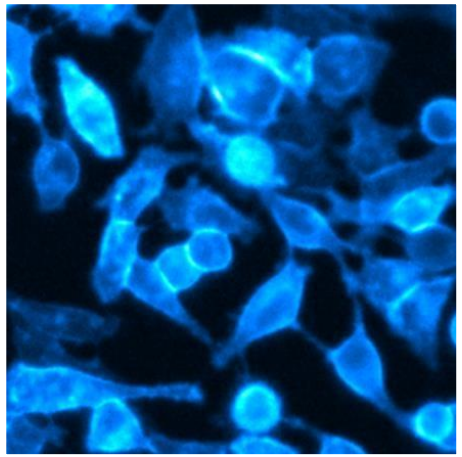
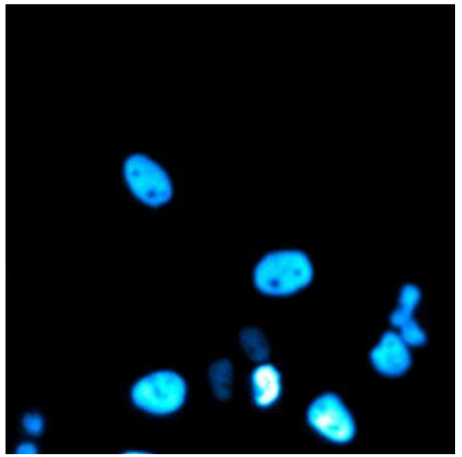
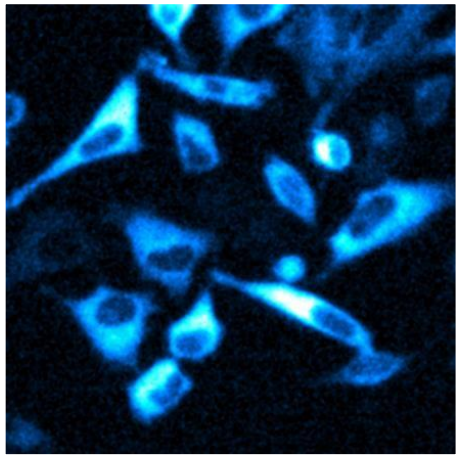
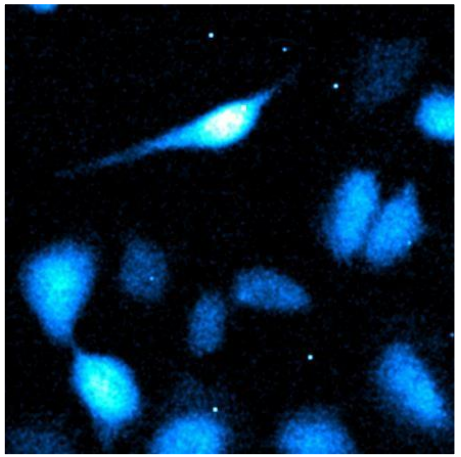
- Includes PC and monitor
- Compatible with slides, microchambers, dishes, and plates
- Motor-driven focusing and alignment
- 20X objective lens (10X overall magnification)
- Accessory: Environment Chamber (temperature, humidity, gas)

Imaging Low Abundance Endogeneous Proteins

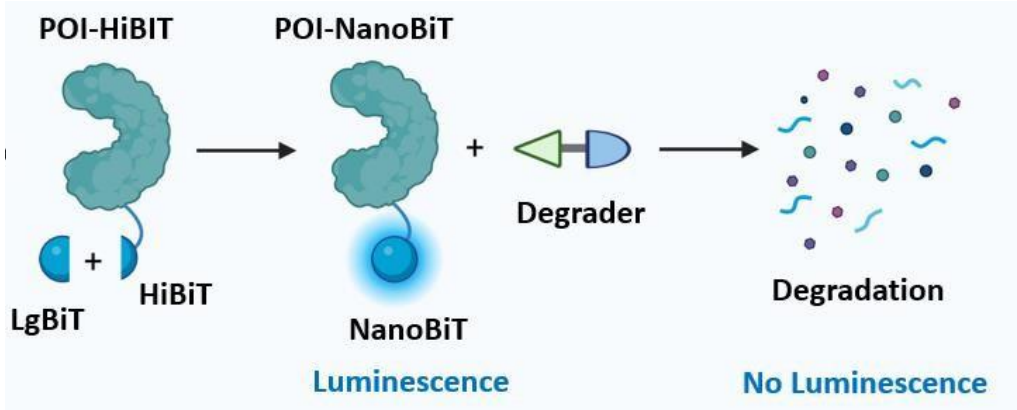
- HiBiT inserted to genomic locus via CRISPR/Cas9 in HeLa cells
- LgBiT expressed ectopically



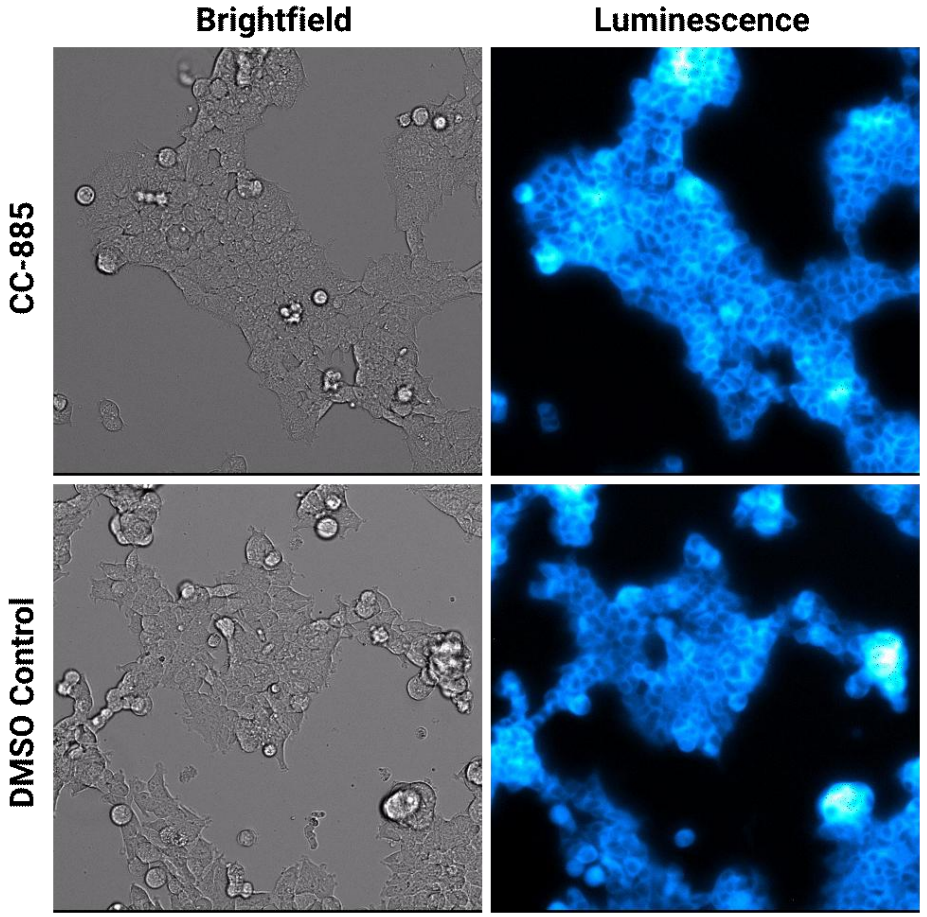


Cofilin	EGFR	HDAC2	HDAC6	CASP3
				
1-minute exposure	1-minute exposure	1-minute exposure	Low Expression ~3-minute exposure	Very Low expression ~5-minute exposure

Targeted Protein Degradation of Endogeneous GSPT1



- HEK293 cells expressing endogenous HiBiT-tagged GSPT1 and stably expressing LgBiT were treated with CC-885 degrader or DMSO control treatment.
- Assayed with Nano-Glo® Vivazine Live Cell Substrate and imaged over 5 hours in stagetop incubator.
- Molecular-glue degrader, CC-885, facilitates targeted protein degradation of GSPT1, and acts as an anti- tumor agent.



GloMax Plate Readers – Configurations

GloMax® Navigator

96-well

✓ Luminescence

GloMax® Explorer

6-, 12-, 24-, 48-, 96- and 384-well

✓ Heating
✓ Shaking
✓ Luminescence
✓ Fluorescence

Available Upgrades
✓ Vis Absorbance
✓ UV/Vis Absorbance
✓ BRET / FRET

GloMax® Discover

6-, 12-, 24-, 48-, 96- and 384-well

✓ Heating
✓ Shaking
✓ Luminescence
✓ Fluorescence
✓ UV/Vis Absorbance
✓ BRET / FRET

Monochromator-Based Microplate Readers

MOLECULAR DEVICES

SpectraMax® ABS

- ABSORBANCE
- WAVELENGTH: 340–850 NM
- MICROPLATE: 96 WELLS
- TEMPERATURE CONTROL
- PRECONFIGURED METHODS
- GXP COMPLIANCE AND VALIDATION
- AUTOMATION READY
- SPECTRADROP MICRO-VOLUME MICROPLATE
- ELISA
- BRADFORD/BCA
- NAD/NADP CONVERSION
- CELL VIABILITY

SpectraMax® ABS Plus

- ABSORBANCE
- WAVELENGTH: 190–1000 NM
- MICROPLATE: 96- AND 384-WELLS
- TEMPERATURE CONTROL
- PRECONFIGURED METHODS
- GXP COMPLIANCE AND VALIDATION
- AUTOMATION READY
- SPECTRADROP MICRO-VOLUME MICROPLATE
- CUVETTE PORT
- PATHCHECK SENSOR
- ELISA
- BRADFORD/BCA
- DNA/RNA
- NAD/NADP CONVERSION
- CELL VIABILITY
- PROTEIN A280

SpectraMax® i3x

- ABS
- FL
- LUMI
- TRF/TR-FRET
- FP
- ALPHA-SCREEN
- INJECTOR
- WESTERN BLOT
- CELL IMAGING

SpectraMax® iD5

- ABS
- FL
- LUMI
- TRF/TR-FRET
- FP
- INJECTOR
- WESTERN BLOT

SpectraMax® iD3

- ABS
- FL
- LUMI
- INJECTOR

Primary Cells, Stem Cells and Media

Lonza

- Primary cells - over 150 human and animal cell types available
- Clonetics media a growth factors for wide spectrum of primary cells
- Stem cells together with media
- Blood and immune cells from vast collection of donors and sources
  - Specialized X-Vivo™ media
  - Specialized GMP T-Vivo media

TheraPeak® T-Vivo Medium

Lonza

TheraPEAK® T-VIVO® Medium is a **chemically defined** media that contains **no animal origin** components

Achieves **high performance without serum**

Exceeds performance compared to various commercial media that require human AB serum

Delivers greater consistency and process control, and simplifies regulatory approval for faster time-to-market

Demonstrated **performance across multiple platforms**

- T-flask, Spinner flask, G-Rex®, Xuri™, Cocoon® Platform

High efficiency using **virus transduction and Nucleofector® Technology**

High Quality Cell Culture Media and Sera

- German company established in 2013
- Specialises on the production of high quality sera and cell culture media and reagents
- Possibility of custom manufacturing from 20 liters

Sera
- FBS
- FBS Xtra
- FBS Advanced
- Human sera

Cell Culture Media
- Classic liquid media
- Classic powdered media
- Special culture media
- Cryopreservation

Cell Culture reagents
- Supplements and additives
- Antibiotics
- BSA
- Trypsin
- Cell separation

Balanced Salt Solutions
- Liquid buffers
- Powdered buffers

Diagnostics
- Virology media
- Cytogenetics

High Quality Sera for Cell Culture

FBS Standard	FBS Advanced	FBS Xtra
Natural FBS	Slightly reduced raw FBS content	Reduced raw serum content
Low endotoxin	Cost-efficient with defined additives	Chemically defined additives
Consistent quality	Minimal lot-to-lot variations	Reduced lot-to-lot variations
Pricing highly dependant on raw FBS price fluctuations	No further batch testing necessary	More sustainable ingredients
Suitable for all cell types	Best for tumor cells & less demanding cell types	Best for tumor cells & fast-growing cell types

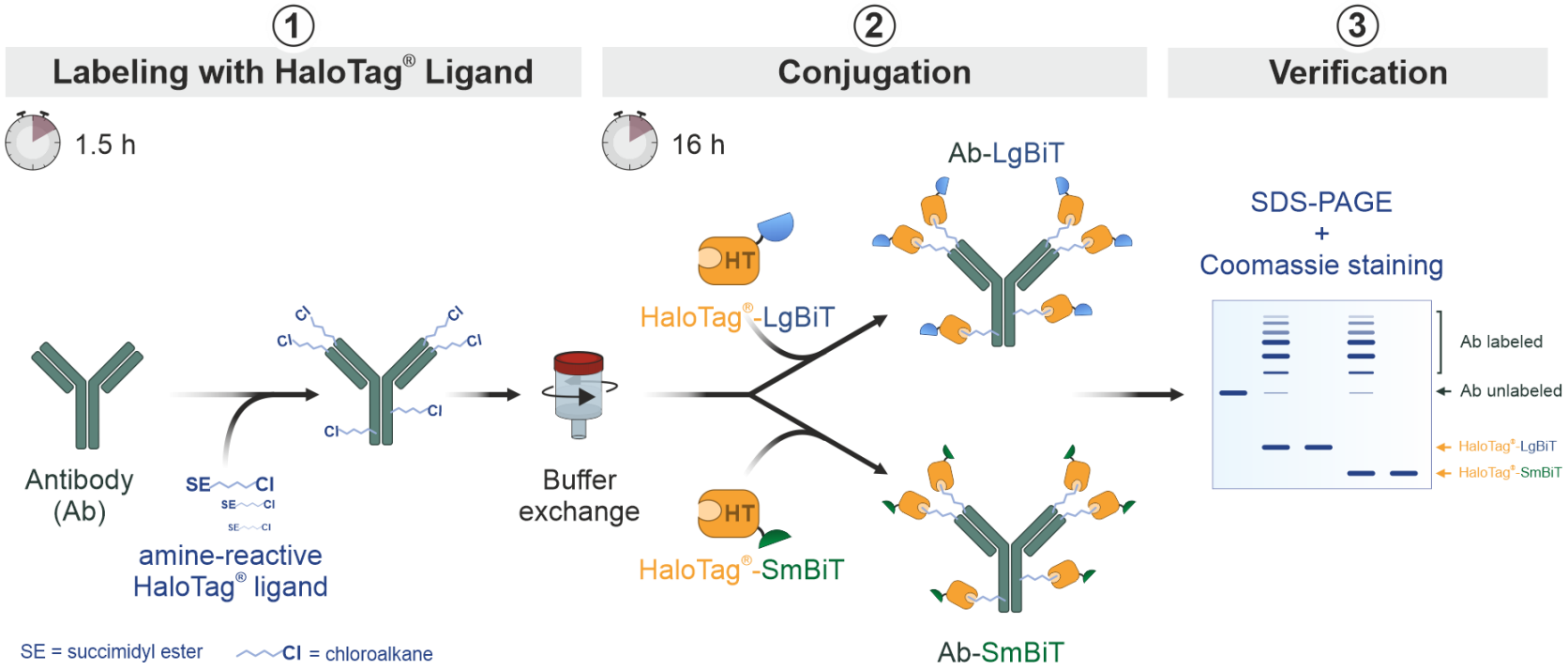
MSC StemPrime – Novel Medium for MSCs

- New chemically defined medium for culture and propagation of mesenchymal stem cells
- The medium comes in both research use and GMP quality (for clinical applications), only difference is presence/absence of phenol red and additional testing procedures for GMP
- Serum-free, xeno-free, animal-free, ultra-low endotoxin levels
- Suitable for isolation, expansion and compatible with all MSC types
- Two component kit – medium and recombinant growth factor supplement
- Easy scaling and transfer from lab to large-scale culture
- **Free-samples for testing available!**


Thank you for your attention!

Build-Your-Own Direct Lumit™ Immunoassay

Step 1: Labeling of Antibodies with the Lumit® Immunoassay Labeling Kit



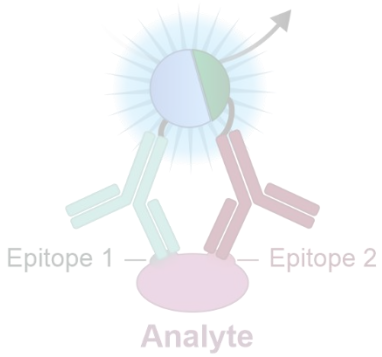
FACTS

- Easy and robust 2-day protocol
- Attachment is highly efficient (> 90%)
- Oriented BiT subunits for maximum activity
- Removal of unbound HT-BiTs is usually not required
- Clean-up can be easily performed using Magne® HaloTag® Beads

Lumit Immunoassays

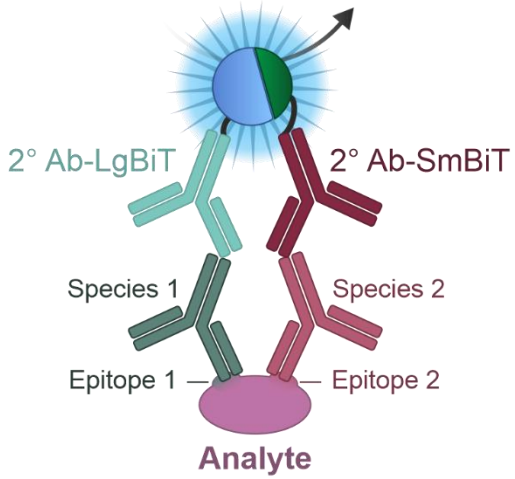
Different Formats for Maximum Flexibility

Direct



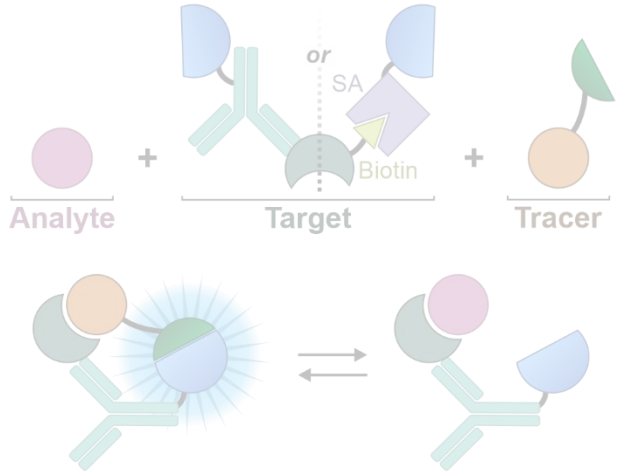
- Requires labeling of 1°Abs
- Validated for cytokines, peptide hormones, …
- *Ready-to-use* assays for
  - ✓ IL1-β, IFN-γ, IL-2, IL-6, IL-10, IL-4, IL-18, TNF-α, VEGF, insulin, glucagon, HMGB1, p24, Ki-67

Indirect



- Avoids labeling of 1°Abs
- Generic pre-labeled 2°Abs (different species available)
- Validated for intracellular PTMs, e.g. phosphorylation

Competitive



- Requires target and tracer labeling
- Establish competitive (antibody) binding assays
- *Ready-to-use* assays for
  - ✓ Lumit™ FcRn Binding Immunoassay
  - ✓ Lumit™ hFcγR Binding Immunoassays
    - I , IIa (H131), IIa (R131), IIIa (V158), IIIa (F158)

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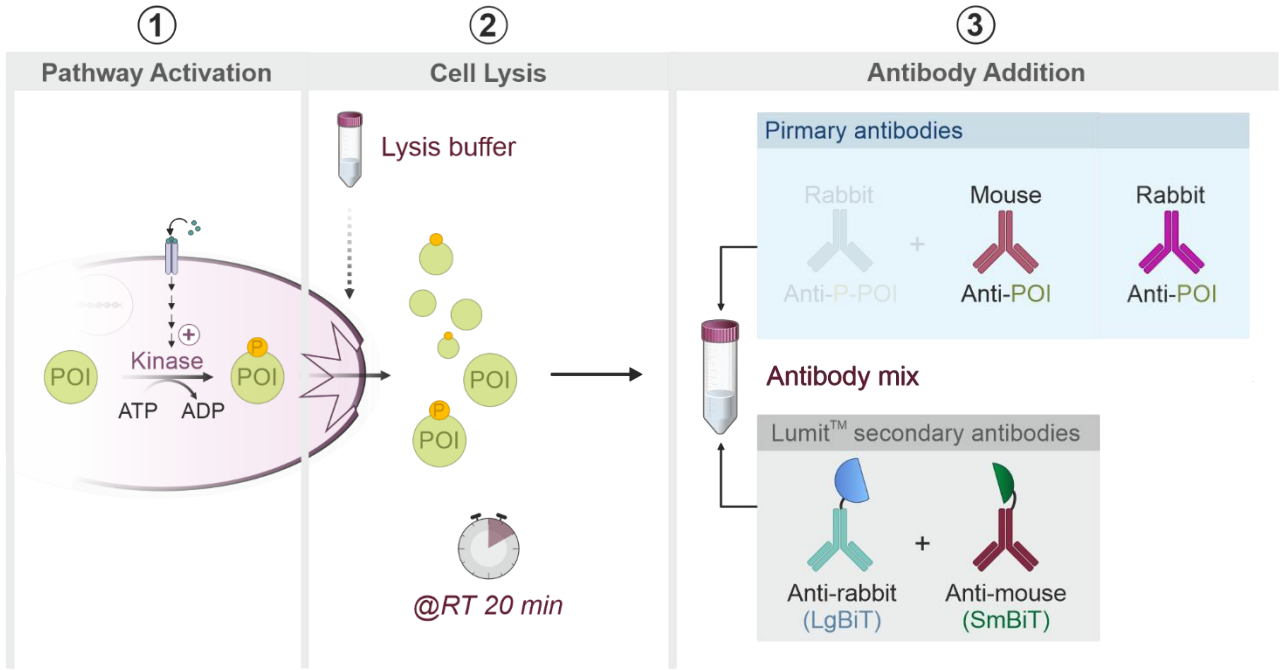
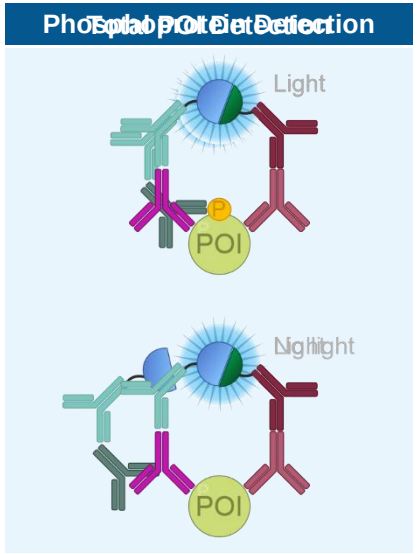
Lumit™ Immunoassay Cellular Systems

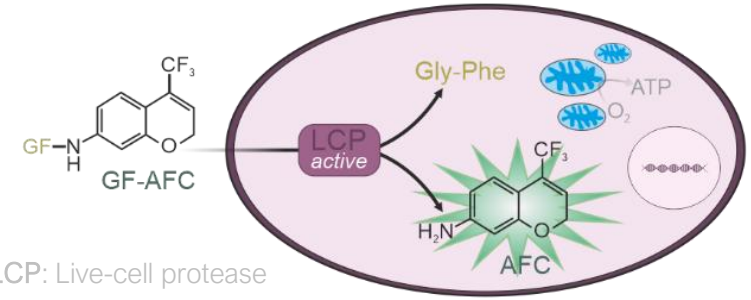
Study Cellular Signaling Events



Normalization to …



Normalization to number of viable cells



LCP: Live-cell protease

Available pre-labeled Lumit™ secondary antibodies:

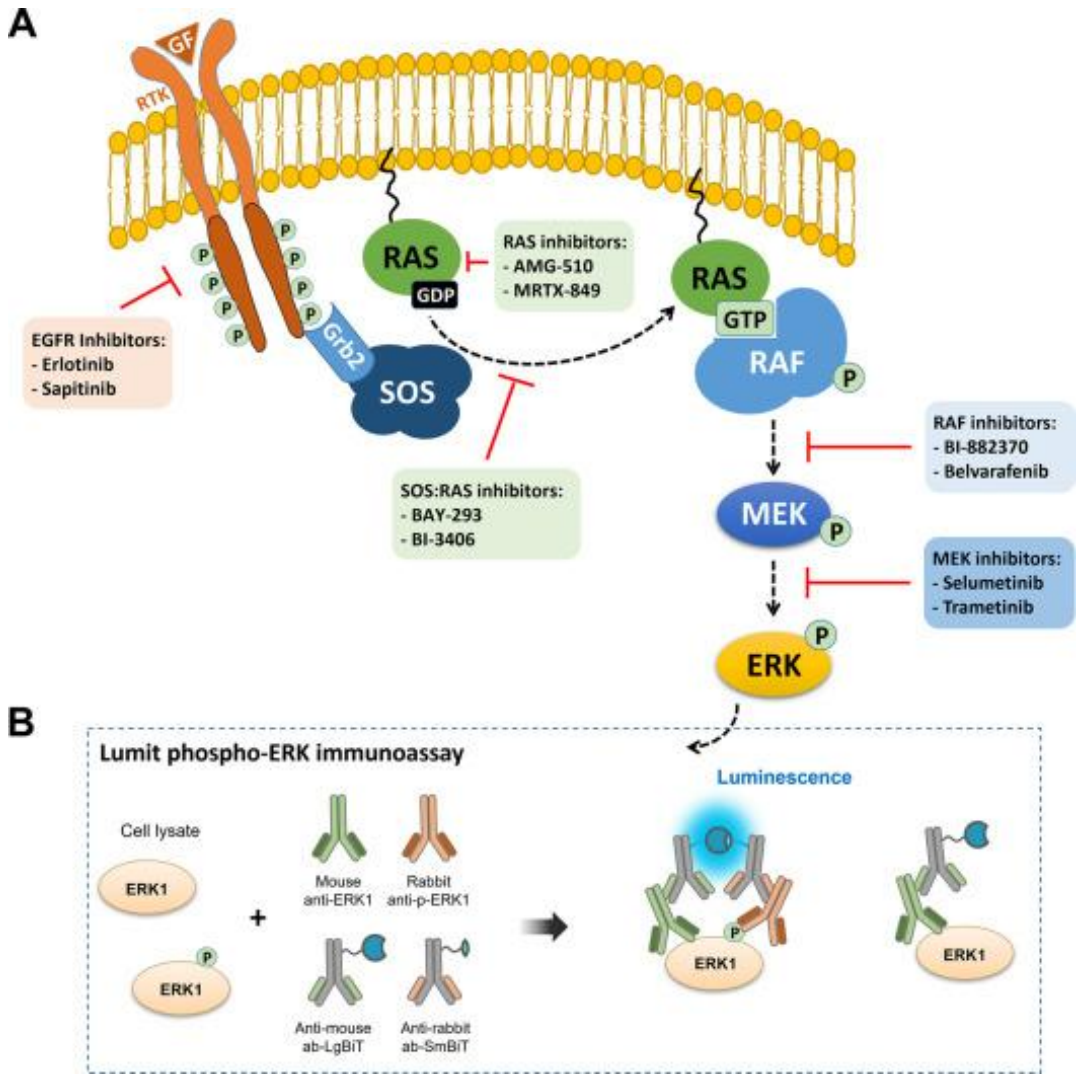
- Anti-rabbit (LgBiT) + anti-mouse (SmBiT)
- Anti-rabbit (LgBiT) + anti-mouse (SmBiT)
- Anti-rabbit (LgBiT) + anti-mouse (SmBiT)

- Anti-rabbit (LgBiT) + anti-mouse (SmBiT)
- Anti-rabbit (LgBiT) + anti-mouse (SmBiT)
- Anti-rabbit (LgBiT) + anti-mouse (SmBiT)

- In live cells, GF-AFC is processed into AFC by LCP
- Fluorescent AFC accumulates over time
- AFC signal correlates with viable cell number

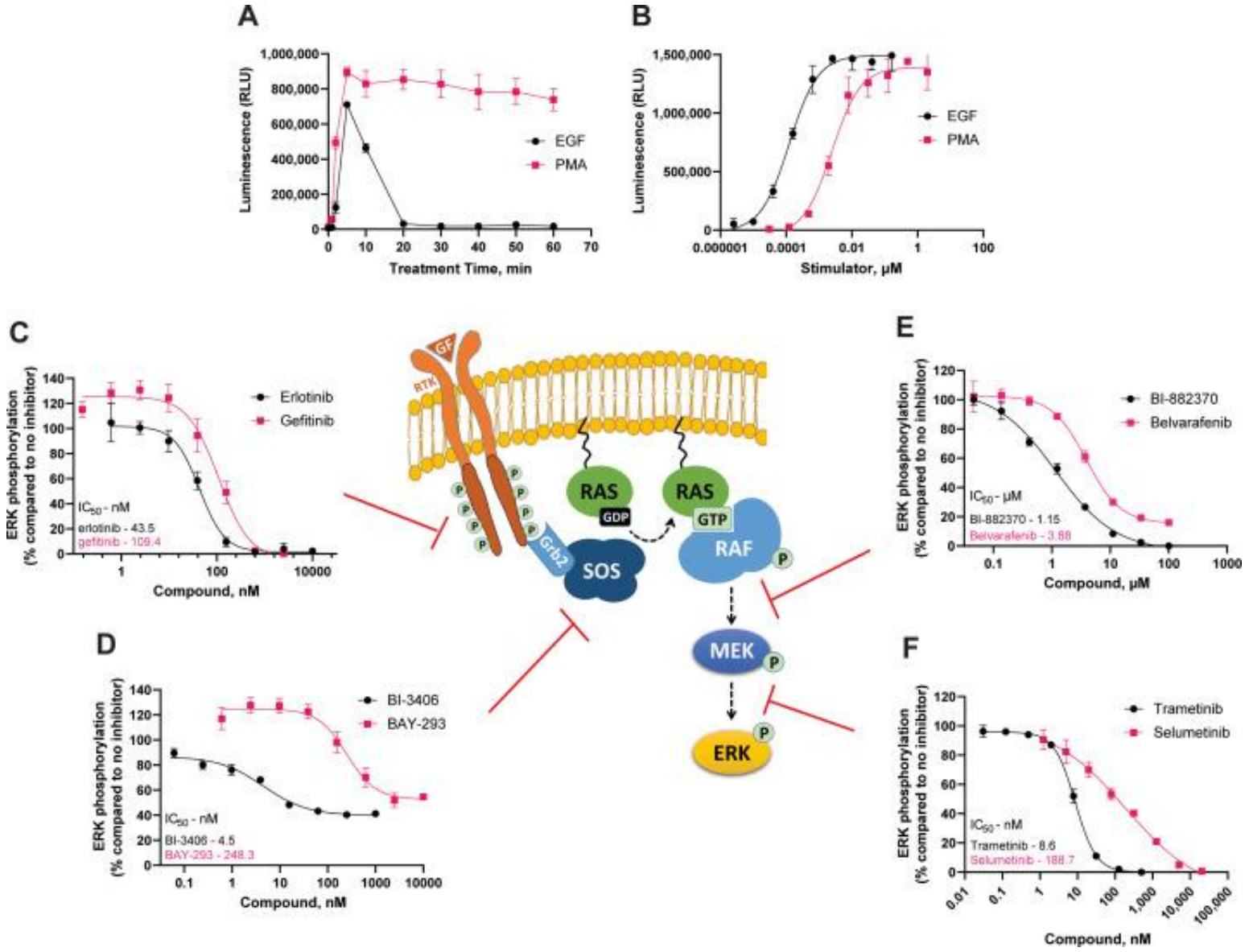
Analyzing RAS Signalling with Lumit Immunoassays

- KRAS is one of the most mutated oncogenes and targeting of its mutant forms has been difficult
- RAS/RAF/MEK/ERK pathway downstream of epidermal growth factor receptor (EGFR) activation
- EGFR activates son of sevenless 1 (SOS1) through the adapter protein GRB2
- SOS1 in turn, mediates the exchange of GDP for GTP within RAS which results in a phosphorylation cascade through the ERK-MAPK pathway, leading to phosphorylation of ERK
- The effect of different pathway inhibitors was monitored by detecting the ERK1 phosphorylation via Lumit indirect immunoassay



Swiatnicki et al. SLAS Discovery, 2022, Issue 4, Pages 249-257.

Inhibiting the RAS Pathway at Different Levels



Swiatnicki et al. SLAS Discovery, 2022, Issue 4, Pages 249-257

Lumit™ Immunoassay Cellular Systems

A Universal Immunoassay to Study Cellular Signaling

Validated with >20 phospho- and total proteins using 8 cell types, suggesting this universal immunoassay can be adapted for any pathway **with the appropriate antibodies**

- AKT (phospho-Ser473 and total protein)
- BTK (phospho-Tyr223 and total protein)
- BCL6 (total protein)
- BRD4 (total protein)
- β-catenin (phospho-Thr41/Ser45 and total protein)
- CHK1 (phospho-Ser317)
- c-Jun (phospho-Ser63)
- cMET (phospho-Tyr1234/1235 and phospho-Tyr1349)
- CREB (phospho-Ser133 and total protein)
- EGFR (phospho-Tyr1068, phospho-Tyr1173 and total protein)
- Estrogen receptor (total protein)
- ERK1 (phospho-Thr202)
- GSK1-3 β(phospho-Ser9)
- H2AX (phospho-Ser139)
- HER2 (phospho-Tyr1196 and phospho-Tyr1221/1222)
- IκBα (phosph-Ser32 and total protein)
- JNK (phosph-Thr183/Tyr185)
- NFκB (p65) (phospho-Ser536 and total protein)
- Retinoblastoma tumor suppressor proteiin (phospho- Ser807/811 and phospho-Ser780)
- Ribosomall proteiin S6 (phospho-Ser235/236, phospho- Ser240/244)
- Smad1 (phospho-Ser463/465 and total protein)
- Smad2 (phospho-Ser465/467 and total protein)
- SMARCA2 (total protein)
- SMARCA4 (total protein)
- STAT1 (phospho-Tyr701, phospho-Ser727 and total protein)
- STAT2 (phospho-Tyr690)
- STAT3 (phospho-Tyr705 and total protein)