New Bioluminescent Assays Enable Easy Measurement of Glucose- Dependent Metabolic Pathways

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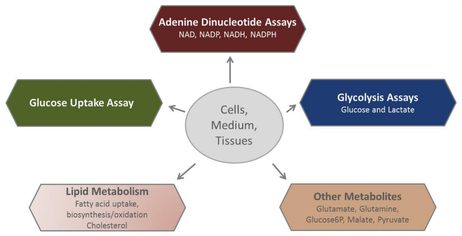
1. Introduction

Energy uptake and utilization in eukaryotic cells is a dynamic process regulated by a series of interacting metabolic networks. Interrogation of this complex network would benefit from rapid, sensitive techniques that do not require extensive sample handling, are easily adoptable to 384-well format and therefore can facilitate novel compound screening and evaluation. Here we describe the development of robust, sensitive and easy to use bioluminescence assays for cellular energy metabolism studies.

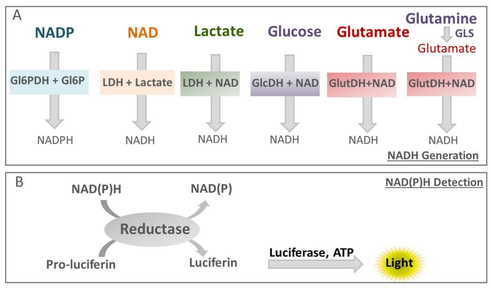


Our aim is to provide tools for studying major metabolic pathways

- Glycolysis
- Glutaminolysis
- Fatty acid/lipid biosynthesis
- Krebs cycle
- Pentose Phosphate Shunt

2. Technology Principle

Each metabolite is used as a substrate by metabolite selective dehydrogenases. Simultaneously NAD(P) is converted to NAD(P)H and is detected with reductase/luciferase coupled reaction. The light output is proportional to the amount of metabolite present in the sample



NAD(P)/NAD(P)H – co-factors of major metabolic pathways such as glycolysis, Krebs cycle (mitochondria function), pentose phosphate pathway (nucleotide, amino acid metabolism)

Glucose, lactate, glutamine and glutamate - markers for two major energy production pathways in cancer cells glycolysis and glutaminolysis

3. Sensitivity with Wide Linear Range and Assay Window

Assay	LOD (nM)	Linearity (µM)	S/B max	CVs	Z'
NAD(P)/NAD(P)H	< 0.5	0.25	> 250	3-9%	> 0.7
NAD(P)H	< 100	25	> 250	2-7%	> 0.7
Lactate	< 400	200	> 400	2-6%	> 0.6
Glucose	< 50	50	> 500	4-9%	> 0.8
Glutamate	< 10	50	> 400	1-6%	> 0.7
Glutamine	< 25	50	> 400	1-6%	N/A

Z' for detecting changes in NAD+NADH or NADP+NADPH using 1K A549 cells (see J. Vidugiriene *et al.*, Assay and Drug Development Technologies, Vol.12, p.514-526, 2014)

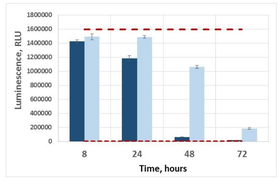
Z' for glucose, lactate and glutamate assays were calculated for glucose consumption, lactate secretion, and glutamate secretion, respectively, by 5K A549 cells in 48 hours.

4. Convenient Analysis of Multiple Metabolites from the Same Sample

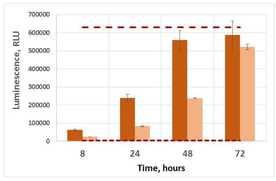
Wider linear range provides flexibility and convenience for experimental set up and data analysis: Samples at different cell densities or at different time points can be analyzed at the same dilution factor without getting out of linear range and data can be directly compared from RLUs

Broad assay window with low CVs makes assay more robust: Changes can be measured with higher signal differences between the samples

Time-dependent glucose consumption



Lactate secretion by A549 cells

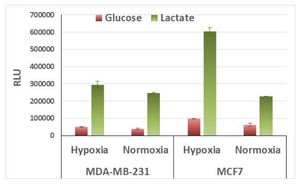


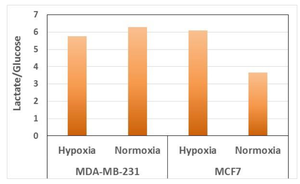
A549 cells at 15K (dark bars) and 5K (light bars) in DMEM + 10% dialyzed serum + 5mM glucose media. At indicated time points 2.5µl of media were removed and diluted to fit into the linear ranges of the assays. Upper and lower lines indicate the linear range of the assays.

5. Convenient Detection of Glycolytic Shift

Cancer cell energy metabolism is a balance between oxidative phosphorylation and glycolysis. Different treatments, including hypoxia (1% oxygen), can perturb this balance, and the adaptive response in energy metabolism varies among cell types

MDA-MB-231 (malignant; highly glycolytic) vs MCF7 cells (less glycolytic; shifts to more glycolytic under hypoxia)





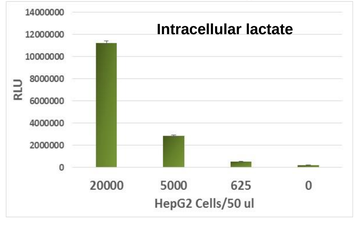
- MCF7 cells become more glycolytic under hypoxic conditions measured by increase in lactate secretion
- Increase in Lactate/Glucose ratio indicates that more of the consumed glucose is converted to lactate under hypoxic conditions

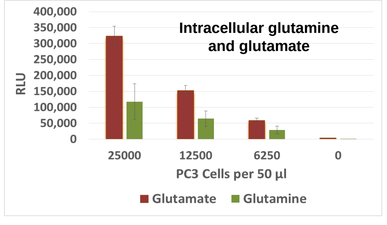
6. Enabling Detection of Intracellular Metabolites

We developed a rapid in-well cell lysis/enzyme denaturation protocol for measuring metabolites in cells and tissues. Sensitivity of the assays allows direct in well detection of multiple metabolites from the same sample:

- Inactivation solution is added to the washed cell monolayers in 96- well plates for rapid cell lysis and inactivation of endogenous enzymes
- The samples are neutralized and Metabolite Selective Reagent is added at 1:1 ratio (lactate panel) or, for analysis of multiple metabolites (glutamate and glutamine panel), aliquots are removed after neutralization and assayed

Measuring intracellular metabolite levels in cells



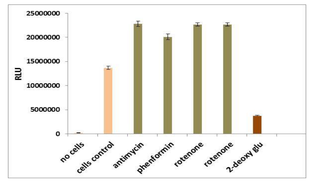


7. Enabling Tools for HTS Screening

Luminescent assays offer homogenous “add and read” format that is well suited for miniaturization and high throughput applications under experimental conditions where total metabolite concentrations are within the linear range of the assays (<50µM).

Measuring inhibitor effect on lactate levels in HepG2 cells

- To compensate for a decrease in ATP, cells increase glycolysis in response to mitotoxins that impact mitochondria function and ATP production
- 2-deoxy glucose inhibits glycolysis by inhibiting glucose consumption

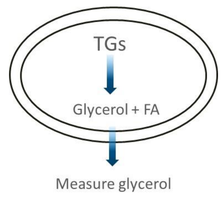


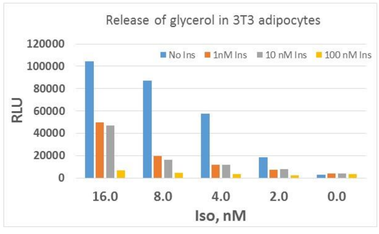
HepG2 cells (10K/40µl) in DMEM no serum + 1mM glucose media were added to the plate with inhibitors. After 1 hour incubation, the cells were lysed with Inactivation Solution (5µl). Lactate Detection Reagent (50µl) pre-mixed with Neutralization Solution (5µl) were added to the samples and luminescence was read after 1 hour incubation.

8. Extending the Technology to Lipid Metabolism

Increase in lipolysis rate with Isoproterenol that is inhibited by Insulin

Lipolysis Assay: measuring release of glycerol in the media





The results are shown at 3h time point using differentiated 3T3 adipocytes

9. Conclusions

The assay represented here demonstrates sensitivity, wide assay linearity and high signals above background. These features translate into multiple benefits:

- Greater flexibility in setting up assays at different cell densities
- Detecting changes earlier with smaller number of cells
- Conveniently following the changes over the time within the linear range of the assays
- Robust discrimination of small changes between samples

Universal detection technology with rapid in well sample preparation for maximal metabolite recovery means:

- Multiple metabolites from the same sample
- Extracellular and intracellular metabolites can be measured from the same sample in 96-well or 384-well plates

For interest in these assay reagents, please contact the corresponding author.

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