Bioluminescent Metabolite Detection Assays for Investigating Metabolic Pathways

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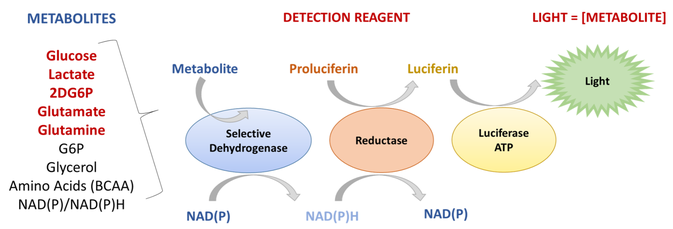
1. Introduction

Nutrient consumption, metabolism and energy production are connected, regulated cell processes. The rewiring of these processes in cancer cells allows abnormal growth and adaptation to the tumor microenvironment. Increasing interest in tumor metabolism has generated a need for assays that have higher throughput to facilitate rapid testing of several samples. We developed bioluminescent plate-based detection assays for measuring metabolites central to glycolysis and glutaminolysis. In these studies we used assays for measuring glucose, lactate, glutamine and glutamate to study metabolic differences between cells and metabolic changes in response to small molecule compounds and altered environmental conditions.

Bioluminescent Metabolite Detection

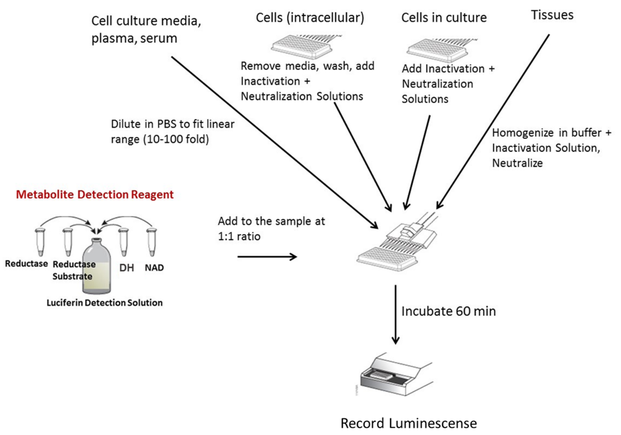


- Metabolite Assays use selective dehydrogenases to produce NAD(P)H in proportion to the amount of metabolite present
- NAD(P)H is detected using a Reductase enzyme and its Proluciferin substrate in combination with a luciferase enzyme to produce light

2. Protocol and Sample Preparation

Assays Detect Metabolites in a Variety of Samples with Minimal Sample Preparation

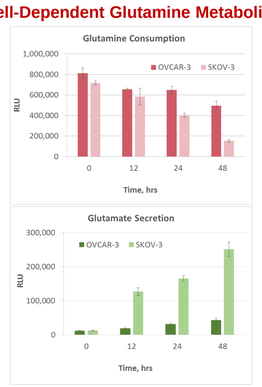
- Sample types include cell lysates, cell culture medium, tissues and plasma
- Sample preparation depends on sample type and may include dilution in buffer or lysis/homogenization in acidic Inactivation Solution (0.6N HCl)
- The use of Inactivation Solution simplifies sample preparation by lysing cells, inactivating proteins and degrading endogenous NAD(P)H in one step



3. Monitoring Nutrient Consumption and Metabolite Secretion

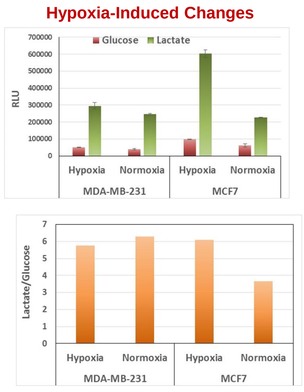
Monitoring metabolites in cell culture medium can provide information about cellular metabolic pathways. For example, glucose consumption and lactate secretion can serve as indicators of glycolysis, while glutamine consumption and glutamate secretion can provide information about glutaminolysis. By sampling small volumes of medium, changes can be monitored over time or after treatments, such as exposure to hypoxic conditions.

Cell-Dependent Glutamine Metabolism



Two ovarian cancer cell lines known to have different glutamine requirements (1) exhibited different patterns of glutamine consumption and glutamate secretion. SKOV-3 or OVCAR-3 cells were plated (10,000 cells/well) in 96-well plates in 100µl defined medium. At the indicated time points 2µl of medium was removed, diluted in 98µl PBS and frozen at -20°C. The ratio of glutamate secreted/glutamine consumed was less for OVCAR-3 cells (~0.17 compared to ~0.42).

Hypoxia-Induced Changes

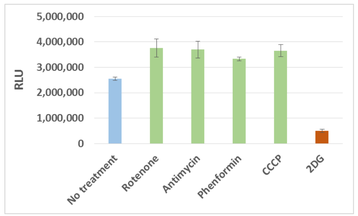


Two different breast cancer cell lines responded differently to hypoxic conditions (1% oxygen). The MCF7 cells shifted to a more glycolytic phenotype with increased lactate secretion, whereas no change was observed for the already highly glycolytic MDA-MB-231 cell line.

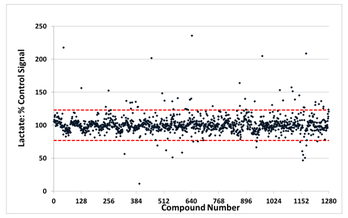
4. Homogeneous Assay for Effectors of Glucose Metabolism

Effectors of glucose metabolism can be identified by using lactate as a glycolytic indicator. To rapidly detect changes in cellular lactate production, we developed an in-well homogeneous assay format. Using this format, plated cells were incubated with compounds for 1hr before adding acidic lysis solution and lactate detection reagent to the wells. This format does not require the removal of medium or the washing of cells, and can facilitate the screening of both inhibitors and activators of glycolysis.

Lactate Production: Compounds that Affect Glycolysis



A549 cells were plated (15,000 cells/well) in defined medium in 96-well plates and then incubated with compounds for 1hr at 37°C. Compounds tested included the glycolysis inhibitor 2DG (10mM) and four mitochondrial inhibitors (5µM rotenone, 5µM antimycin, 2.5mM phenformin and 50µM CCCP).

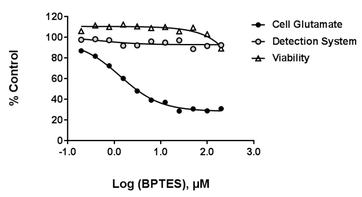


Compounds of the LOPAC®1280 library (Sigma) were incubated with SKOV-3 cells (1000 cells/well) in 384-well plates for 1hr at 37°C before lactate production was measured. Signals were normalized to same-well real-time viability reagent readings before calculating percentage of lactate production as compared to control wells that received no compound. Z' values for all plates were > 0.6 (2).

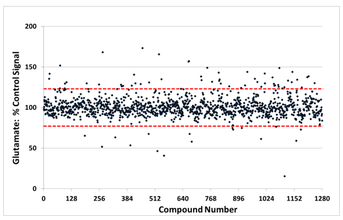
5. Homogeneous Assay for Effectors of Glutamine Metabolism

Analogous to using lactate production as a marker for changes in glycolysis, glutamate production can provide insights regarding glutaminolysis. The homogeneous glutamate assay was validated using a well-known glutaminase inhibitor and then used to screen the LOPAC®1280 library (Sigma) for compounds that both decreased and increased glutamate production. Counter-screens using glutamate controls and viability reagents were included to detect effects on the detection system or viability.

Glutamate Production: Compounds that Affect Glutaminolysis



SKOV-3 cells (4000 cells/well) were added to 384-well plates and incubated with dilutions of BPTES in a final volume of 30µl of defined medium. After 1hr at 37°C, the cells were lysed with 5µl acid solution followed by the addition of neutralization solution and glutamate detection reagent (Ref. 2).

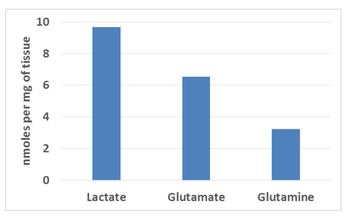


Compounds of the LOPAC®1280 library (Sigma) were also screened for glutamate production following the same protocol described above in Panel 4. Z' values for all plates were > 0.6 (Ref. 2).

6. Metabolites in Tissues and Plasma

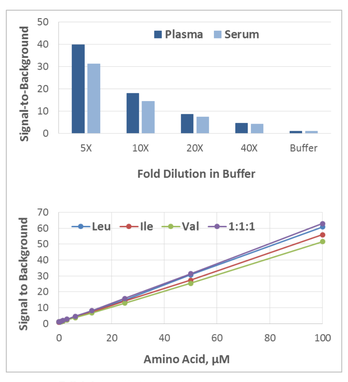
Metabolites can be measured in other sample types such as homogenized tissues or diluted plasma and sera. Only small amounts of sample are needed (e.g.,10mg tissue or 5µl plasma) and one sample can be used to measure multiple metabolites. With the proper selective dehydrogenase, the technology can be extended to other metabolites such as branched chain amino acids (BCAA).

Tissue



Frozen mouse brain tissue was homogenized directly in acidic Inactivation Solution using a Tissue Tearor (BioSpec). After homogenization the sample was neutralized and diluted for the assay. No additional centrifugation or deproteinization steps were needed.

Plasma and Serum

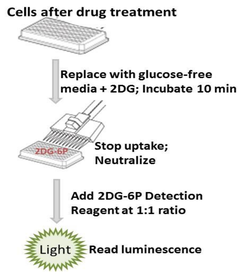


BCAA in dilutions of human plasma and serum were measured using leucine dehydrogenase, a dehydrogenase selective for leucine, isoleucine and valine.

7. Glucose Uptake Assay

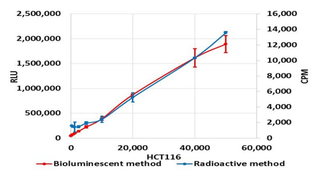
The bioluminescent glucose uptake assay measures the uptake of the commonly used glucose analog, 2DG. Once in the cell, 2DG is phosphorylated forming 2DG6P, a stable, impermeable metabolite that is not further metabolized. Accumulated 2DG6P is detected using the selective dehydrogenase G6PDH.

Protocol

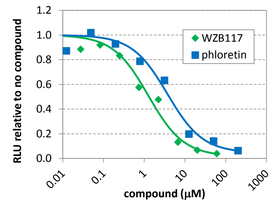


Comparable Performance to Radioactive Methods

Glucose uptake by HCT116 cells



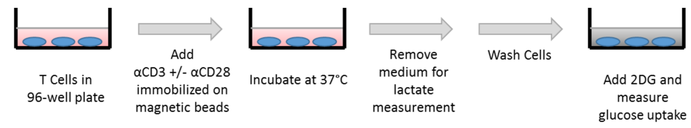
Inhibition of Glucose Uptake

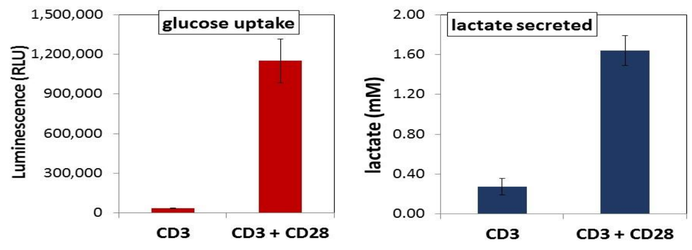


HCT116 cells were plated (10,000 cells/well) and treated for 10min with glucose transporter inhibitors before measuring glucose uptake (3).

8. Immunometabolism: Activation of T Cells

Activation of T Cells Triggers Increase in Glycolysis as Indicated by Increased Glucose Uptake and Lactate Secretion





Human peripheral blood T cells were activated using antibodies to CD3 and CD28. Anti-CD3 alone or anti-CD3 plus anti-CD38 were immobilized on magnetic beads and incubated with T cells (250,000 in 100µl medium per well) for 24hr at 37°C. Samples of the media were used for determining lactate levels, while the cells were used for glucose uptake measurements.

9. Conclusions

Tumor metabolism is an important area of research and a target for the development of new therapeutics. In this poster we have described bioluminescent plate-based assays that can be used to facilitate the analysis of metabolic pathways and the screening of compound libraries.

Bioluminescent metabolite assays can facilitate the study of cellular energy metabolism

- Measurements of key metabolites can provide useful information during studies of glycolysis and glutaminolysis
- Assays are amenable to low volume multi-well plates, automation, and high-throughput formats
- Multiplexing capabilities with cell viability assays provides more information per well and facilitates data normalization

Bioluminescent metabolite assay benefits

- Sensitivity: small amount of sample and low numbers of cells per well (e.g. 1000 cells/well)
- Broad linearity: 2 to 3 logs provides convenience for testing samples at different metabolite concentrations
- Wide assay windows: S/B_{max} > 100 allows for better discrimination of small changes

References:

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2. Leippe, D., *et al.* SLAS Discovery 2017 Advance online publication. doi:10.1177/1087057116675612
3. Valley, M.P., *et al.* Anal. Biochem. 2016 505:43-50

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