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Research paper

Analysis of glycogen metabolic pathway utilization by dendritic cells and T cells using custom phenotype metabolic assays

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ABSTRACT

In the field of immunology, there is an increasing interest in cellular energy metabolism and its outcome on immune cell effector function. Activation of immune cells leads to rapid metabolic changes that are central to cellular biology in order to support the effector responses. Therefore, the need for user-friendly and dependable assay technologies to address metabolic regulation and nutrient utilization in immune cells is an important need in this field. Redox-dye reduction-based Phenotype MicroArray (PM) assays, which measure NADH reduction as a readout, developed by Biolog Inc., provide a wide screening of metabolites both in bacteria and mammalian cells. In this study, we delineate a detailed protocol of a customized Biolog assay for investigation of a specific metabolic pathway of interest. The option to be able to easily customize this technology offers researchers with a convenient assay platform to methodically examine specific nutrient substrates or metabolic pathways of interest in a rapid and cost effective manner.

1. Introduction

There is a growing appreciation in the field of immunology that immune cell activation is accompanied by dramatic shifts in cellular metabolism, often highlighted by increased cellular uptake of glucose (Amiel et al., 2012, 2014; Everts et al., 2014, 2012; Krawczyk et al., 2010; Frauwirth et al., 2002; Freemerman et al., 2014; Macintyre et al., 2014, 2008). Cellular utilization of specific metabolic pathways has traditionally been assessed by radioisotope labeling of metabolites and/or indirect measurements of enzymatic activities. While the techniques like carbon isotopic labeling, such as LC-MS approaches, provide reliable snapshots of cellular metabolic fluxes, they demand specialized expertise from the execution of experiments to data analysis. In this work, we outline an experimental method using Biolog Inc. Phenotype MicroArray (PM) technology to perform simple and rapid screenings of cellular energy-producing metabolic pathways that provides fast, cost effective, and clear results.

The Biolog PM assay technology was originally developed for microbial metabolic screening, based on assaying the ability of microorganisms to metabolize distinct nutrient substrates. This assay platform was subsequently developed for a variety of eukaryotic cell applications including assays for evaluating cancer cell drug targets, metabolic disorders, cancer cell metabolism, drug toxicity testing, and general metabolic pathway analyses. For these assays, cells are plated in a 96-well format where each well is pre-loaded with a single distinct metabolic substrate. After allowing cells to equilibrate to their nutrient environment and metabolism to occur, cells are then co-incubated with a colorimetric dye that is reduced in the presence of NADH. If cells are capable of metabolizing a substrate, NADH will be produced resulting in proportional reduction of the dye. This assay is based on the premise that the amount of NADH produced directly correlates the cell's ability to utilize a particular substrate as an energy source (Bochner et al., 2011). Although it is similar to the principle of MTT assays, the Biolog tetrazolium redox dye mix specifically optimized to measure cellular NADH production. It is also water soluble and readily adapted for metabolic rate measurements. This eliminates the solubility issues and the precipitation of formazan, thereby reducing the high background noise that is a typical obstacle for data interpretation in standard MTT methodologies (Bochner et al., 2011). The intensity of color formation that results from dye reduction directly correlates with the amount of cellular NADH production driven by metabolism of a given substrate. In addition, cells can be assayed over time to provide kinetic analysis of substrate usage. Fig. 1 provides a simple illustration of NADH production during glycolytic and mitochondrial metabolism which is read as dye reduction of the Biolog Inc. Dye Mix in these assays.

One of the major advantages of this assay, from the perspective of larger-scale screening assays, is that it can be accomplished with relatively few cells in a total volume of 100 µL (see procedure for details) in each well of a 96-microwell plate. Cells are originally plated in a minimal nutrient media (MC-0) for overnight incubation. During this

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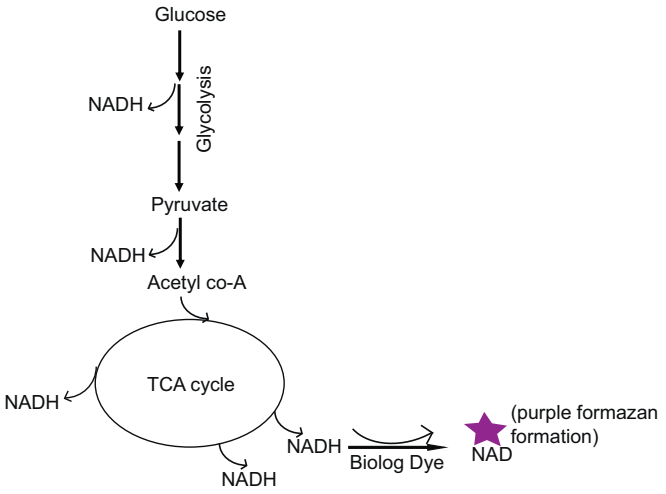

Fig. 1. Illustration of NADH production (with dye reduction) from glucose metabolism.

incubation, the cells will utilize residual glucose provided in MC-0 before they adapt and switch to the saturating single nutrient source in each well of a PM plate. Cellular utilization of metabolic substrates and their kinetics can be simply measured by colorimetric readings of purple formazan formation from Biolog redox dye reduction. The advantage of this technology is that it not only allows an individual to perform a broad screening of multiple metabolites by using pre-designed PM plates, but also provides the flexibility to customize the assay for a thorough investigation of a single metabolite/metabolic pathway of interest, specifically tailored to individual's research needs. Below, we delineate an illustrative example of how researchers can adapt this technology to screen for the efficacy of metabolic-inhibiting drugs for a specific research question.

In a recently published research article, we originally identified that dendritic cells (DCs) of the immune system can use glycogen as an energy substrate from screening a wide number of carbon substrates using Biolog inc., PM assay plates (Thwe et al., 2017). In this screen, we identified that DCs can utilize not only single units of glucose but also various lengths of glucose polymers including the long-chain glucose polymer known as glycogen (Thwe et al., 2017). To systematically examine glycogen usage by DCs, we customized this technology to allow us to specifically screen for the efficacy of drugs that targeted glycogen metabolism in our assay system (Fig. 2). In this case, we built our custom assays using our substrates of interest, glycogen and glucose (Table 1). Because of the extreme flexibility of the assay, we were also

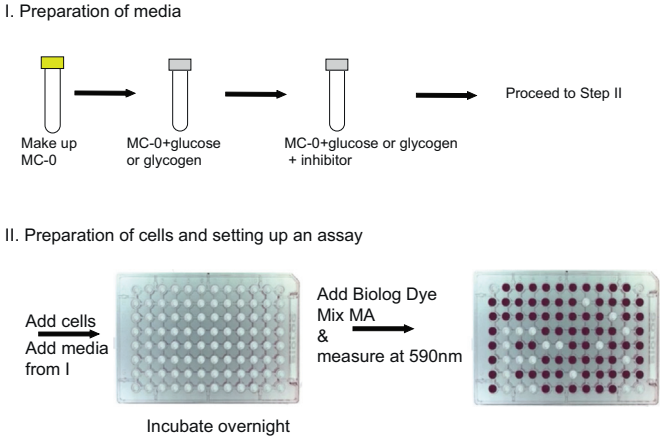

Fig. 2. Workflow for a customized Biolog assay set up (96-well plate picture courtesy of Biolog Inc.)

Table 1
An example of customized plate layout for validating DC utilization of glucose and glycogen metabolic pathways with Biolog plates and the Biolog assay platform with Bone-marrow -derived Dendritic Cells (BMDCs) and Glycogen Phosphorylase (PYG) inhibitor.

MC-0 (no cells)	MC-0 only (with cells)	Glucose	Glycogen	Glucose + PYG inhibitor	Glycogen + PYG inhibitor
	BMDCs	BMDCs	BMDCs	BMDCs	BMDCs
	BMDCs	BMDCs	BMDCs	BMDCs	BMDCs
	BMDCs	BMDCs	BMDCs	BMDCs	BMDCs

able to use this approach to simultaneously examine cellular nutrient utilization in both resting and activated DCs. With this customized approach, we have successfully identified a metabolite of our interest, glycogen, and validated the specificity of multiple inhibitors of glycogen metabolism in DCs as well as other immune cell types at different activation stages. Because of a growing interest in the metabolic pathway utilization of immune cells in response to stimuli, we believe that this assay platform will be attractive to researchers in the field based on the flexibility to customize this technology to query a wide range of metabolic parameters in a quick and cost-effective way.

2. Materials

1. *[a]96-well micro plates (Biolog Inc.); Other 96-well flat bottom plates can be substituted. Please see discussion for more information.
2. *[b]Biolog dye mix MA (6×) (Biolog Inc.) (for some cell types dye mix MB may work better)
3. *[c]IFM-1 (Biolog Inc.); This can be substituted with phenol red free, glucose and glutamine free RPMI.
4. Glutamine 2 mM
5. 5% Fetal Bovine Serum (FBS) (or dialyzed FBS if limiting exogenous glucose is desired)
6. Bovine pancreatic glycogen, 5 mg/mL concentration (Sigma aldrich)
7. CP91149 (CP) glycogen phosphorylase inhibitor I (Selleckham)
8. DAB, 1,4-Dideoxy-1,4-imino-D-arabinitol hydrochloride, glycogen phosphorylase inhibitor II (Santa Cruz)
9. Glucose 10 mM
10. Multi-channel pipette and reservoir (optional)
11. Plate reader at 590 nm wavelength

*These reagents/supplies are obtained from Biolog Inc.,; however, options and limitations to substitute *[a] and *[c] with alternative sources are mentioned in discussion.

3. Procedure

3.1. Murine Bone-marrow -derived Dendritic Cells (BMDCs) are used as an example in this protocol

I. Reagent preparation:

1. Assay Media (MC-0)
   a. MC-0 assay media supplemented with 5%FBS is prepared with the following reagents: for 25 mL of MC-0 medium, add 1.25 mL of FBS, 500μL of Pen Strep, and 50μL of 200 mM stock of glutamine into a 23.5 mL of IF-M1 medium.
   b. Glycogen containing MC-0 medium is prepared from the MC-0 medium from 1a to get a 10 mg/mL (2× concentration). Glucose containing MC-0 medium with a final concentration 10 mM (2× concentration) is prepared similarly by using the MC-0 medium