Application Note

Cell Analysis and Metabolomics

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Extracellular Flux Analysis and ^{13}C Stable-Isotope Tracing Reveals Metabolic Changes in LPS-Stimulated Macrophages

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Abstract

Eukaryotic cells are known to generate ATP by mitochondrial (oxidative phosphorylation) and non-mitochondrial (glycolysis) metabolism. Agilent Seahorse technology is an invaluable tool to gain access in real time to the bioenergetics activity of the cell by measuring cellular oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) as measures of mitochondrial respiration and glycolysis, respectively. Even though Seahorse provides phenotypic information about the bioenergetic state of the cell, an in-depth understanding of the changes in the metabolic pathways requires measuring the abundances and rates of metabolite interconversion of the molecules involved in the key metabolic pathways (e.g. glycolysis, tricarboxylic acid cycle (TCA) cycle, pentose phosphates pathway). This is achieved by performing stable-isotope tracing analysis on an LC/Q-TOF, providing a detailed view of cellular metabolism. Advancements in LC/Q-TOF technology coupled with integrated qualitative flux analysis software reveal more details by tracking the *in vivo* turnover of metabolites through pathways. In this application note, we present an example of using both Seahorse XF technology and ^{13}C stable-isotope-tracing analysis with Agilent MassHunter VistaFlux software to study the response to lipopolysaccharide (LPS) treatment of RAW 264.7 macrophages. The aim of this application note is to demonstrate the complementarity of phenotypic analysis using Seahorse technology and targeted stable-isotope-tracing analysis using an Agilent 6546 LC/Q-TOF.

Introduction

Immunometabolism research has significantly expanded our understanding of immune cell function in recent years. Modulation of metabolism appears to be crucial for controlling cell fate.[1,2] In this context, Seahorse technology enables insight into the bioenergetic state of the cell in response to stimuli. This is achieved by simultaneously measuring, in real time, the changes in oxygen consumption rate (OCR, a qualitative indicator of mitochondrial oxidative phosphorylation) and the rate of extracellular acidification (ECAR, a qualitative indicator of glycolysis), as shown in Figure 1.

To gain further insight into the mechanism leading to changes in OCR and ECAR in response to stimuli, metabolomics allows a direct snapshot of pathway activities and metabolite regulation. For yet more detailed information on metabolic pathway changes, stable-isotope-tracing analysis (e.g. ^{13}C, ^{15}N, ^{2}H) can be applied. Stable-isotope labeling provides a different picture of intracellular metabolism than metabolomics. Although untargeted metabolomics provides the abundance of different metabolites within metabolic pathways, several metabolic changes do not *a fortiori* result in an increase or a decrease in the metabolite level. Stable-isotope-tracing provides information not revealed by conventional untargeted metabolomics by measuring the rates of metabolite interconversion as a readout of metabolic enzyme regulation. This makes stable-isotope-tracer studies a powerful option to probe metabolic changes in complex biological systems.

Insights into the full picture of cell metabolism from the combination of Seahorse and LC/Q-TOF data can inform biological research as answers from one platform can drive experiments on the other, allowing a feedback loop for follow-up experiments (Figure 2).

Such workflows can provide accurate answers to biological questions much faster as both experiments are targeted (specific assays on the Seahorse and pathway-driven experiments on the LC/Q-TOF).

Within immunometabolism research, macrophages are known to play a central role in pathogen recognition, exhibiting

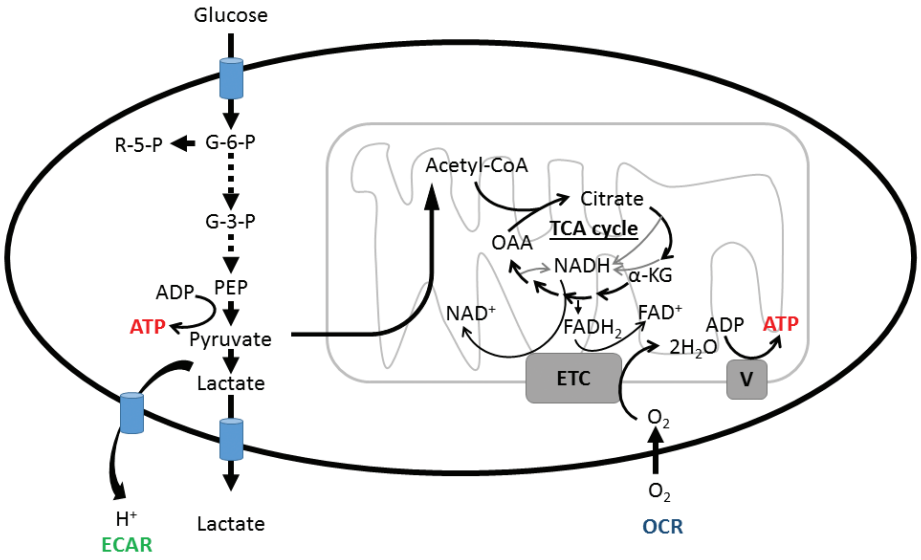


Figure 1. Schematic illustration of cellular metabolism pathways and the link between phenotypic measurements using Agilent Seahorse (ECAR and OCR) and the metabolites from relevant metabolic pathways (e.g., glycolysis, TCA cycle, and mitochondrial respiration) using stable-isotope-tracing analysis by LC/Q-TOF. G-6-P: glucose-6-phosphate; R-5-P: ribose-5-phosphate; G-3-P: glyceraldehyde-3-phosphate; PEP: phosphoenolpyruvate; ECAR: extracellular acidification rate; OCR: oxygen consumption rate; ETC: electron transport chain.

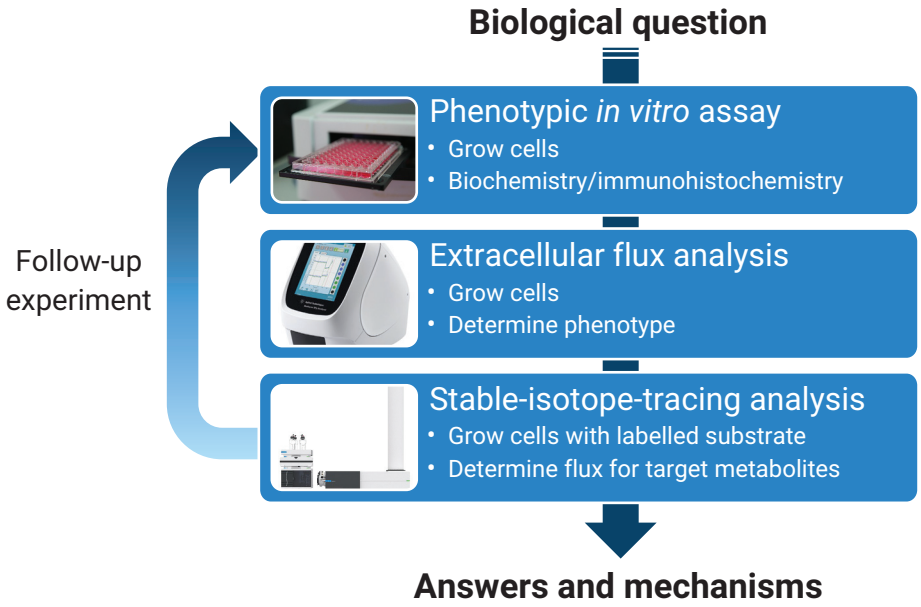


Figure 2. Workflow used to address biological questions by combining conventional phenotypic assays, Agilent Seahorse measurements, and stable-isotope-tracing analysis on an LC/Q-TOF.

an array of immediate physiological remodeling in response to pathogen stimuli. Elucidation of the mechanisms regulating metabolic pathways and the balance of metabolites is crucial for understanding macrophage function and their remodeling upon external stimuli. One such example is macrophage activation. Classical activation of macrophages is performed by pro-inflammatory stimuli such as LPS and/or interferon gamma (IFNγ). Several studies have demonstrated that activated macrophages undergo a metabolic switch that includes immediate up-regulation of glycolysis, remodeling of the TCA cycle, and the inhibition of mitochondrial respiration.[3] These processes follow an orchestrated sequence of metabolic adaptions that can be followed in real time via extracellular flux analysis.[10]

For example, itaconic acid, a non-amino organic acid derived from *cis*-aconitate by the action of *cis*-aconitate decarboxylase (cadA), is known to be one of the most highly induced metabolites following LPS stimulation in bone marrow derived macrophages (BMDMs).[4,5] This process is known to be energy costly for the macrophages as they lose the ability to perform mitochondrial substrate-level phosphorylation.[6]

In this application note, we present an example of the combination of Seahorse XF technology with ^{13}C stable-isotope-tracing analysis using MassHunter VistaFlux software to understand the response of RAW 264.7 macrophages to 4 hours of LPS treatment. The presented workflow can be adapted to different cell types to decipher the molecular mechanisms underpinning immunometabolism.

Experimental

Cell culture and reagents

RAW 264.7 cells were prepared from the frozen stock (–80 °C) and cultured in a tissue culture flask (~0.5 × 10^5 cells/mL) in DMEM medium (Dulbecco's Modified Eagle's Medium, Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS). After 48 hours of incubation at 37 °C in CO_2, confluent cells were split and cultured in fresh media. The process of splitting was repeated five to six times until the cells were fully recovered from being frozen.

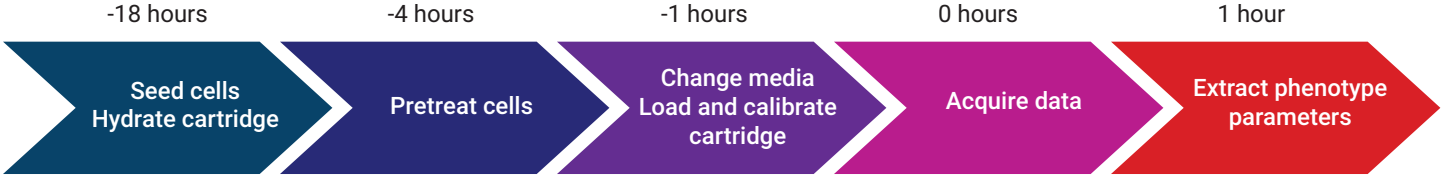
Phenotypic *in vitro* assay-cytokine measurement by ELISA

The RAW 264.7 cells were cultured in a six-well plate and incubated at 37 °C in CO_2 for three to five hours to adhere, then treated with LPS at 100 ng/mL final or plain media for the next four hours. The supernatant was collected for cytokine TNFα analysis. The TNFα production by RAW 264.7 macrophages was determined using the mouse TNFα ELISA kit (Ready-SET-Go; Affymetrix eBioscience; Invitrogen 88732486) according to the manufacturer's instructions. Absorbance readings were measured at 450 nm.

Extracellular flux analysis on the Seahorse XFp Analyzer

RAW 264.7 cells were seeded on an eight-well miniplate at cell densities of 4 × 10^5 cells/mL and left to adhere overnight. Cells were or were not treated with LPS at 100 ng/mL final or plain media, and after four hours, the OCR and ECAR were measured using an Agilent Seahorse XFp Analyzer. The day before the assay, the sensor cartridge was hydrated by filling the utility plate's moats and wells with 400 and 200 µL of double-distilled sterile water, respectively, and incubating overnight at 37 °C in a non-CO_2 incubator. On the day of the assay, the water was replaced with 200 µL of prewarmed Agilent XF calibrant and incubated for another one hour. Also, the culture medium was replaced with 180 µL of Agilent Seahorse XF RPMI medium, pH 7.4, that was supplemented with 10 mM glucose, 2 mM sodium pyruvate, and 2 mM glutamine, and the cells were incubated at 37 °C in a non-CO_2 incubator for one hour. The Seahorse miniplate with cells was placed into the XFp Analyzer for subsequent analysis at 37 °C. Measurement cycles consisted of three minutes mixing and three minutes measuring. Baseline rates were measured at 37 °C three times before sequentially injecting the following mitochondrial inhibitors: oligomycin (2 µM), carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP, 2 µM), and a rotenone/antimycin A mixture (0.5 µM) all from the Agilent Seahorse XFp Cell Mito Stress Test kit. After the addition of each inhibitor, three readings were taken. OCR and ECAR

Seahorse XFp Analyzer workflow



were automatically calculated by the Seahorse XFp Wave software. Each data point represents an average of three different well measurements that were normalized to the number of cells per well. For a more complete understanding of the general metabolic changes, a phenotype analysis was conducted using the Cell Mito Stress Test data result file.

Qualitative flux analysis

Metabolite extraction

RAW 264.7 cells were seeded on a six-well plate at a cell density of 5×10^5 cells/mL and incubated for three to five hours to adhere. After this time, the media was changed for the media supplemented with [U-$^{13}C_6$]-glucose at 4.5 mg/mL. Cells were or were not treated with LPS at 100 ng/mL media concentration and in the presence of [U-$^{13}C_6$]-glucose at a concentration equivalent to a 1:1 ratio of ^{12}C and ^{13}C glucose present in the culture medium, then incubated for four hours. The microplate was placed on ice, and the media was removed carefully. The adhered cells were washed three times with cold PBS before addition of a metabolite extraction buffer (acetonitrile, methanol, and water at a ratio of 40:40:20 v/v/v at –20 °C) at a density of 10^7 cells/mL. Cells were then scraped and transferred to an Eppendorf vial. A 100 µL aliquot of the metabolite solution was then mixed with 100 µL of acetonitrile with 0.2% acetic acid at –20 °C and centrifuged for 10 minutes at 17,000 g at 4 °C. The final concentration of 70% acetonitrile was compatible with the starting conditions of the HILIC chromatography. The supernatant was then transferred to an Agilent LC/MS V-shape vial (p/n 5188-2788) and 4 µL was injected into the LC/MS.

LC/Q-TOF data acquisition

Data were acquired on an Agilent 1290 Infinity II LC coupled to an 6546 LC/Q-TOF system. Chromatographic separation was performed on an Agilent InfinityLab Poroshell 120 HILIC-Z, 2.1 × 100 mm, 2.7 µm column (part number 675775-924). The HILIC methodology was optimized for polar acidic metabolites (details in Table 1). For easy and consistent mobile phase preparation, a concentrated 10x solution consisting of 100 mM ammonium acetate, pH 9.0, in water was prepared to produce mobile phases A and B.

Target metabolite list creation

As Seahorse provides phenotypic information about glycolysis (ECAR) and mitochondrial oxidative phosphorylation (OCR), a target list of metabolites related to those pathways was created using Agilent MassHunter Pathways to PCDL software. From a public pathways database, the software generates a target personal compound database (PCDL) that contains names, formulas, and various identifiers. Addition of metabolite retention times in the target PCDL were provided from a pre-existing in-house database.

Table 1. Liquid chromatography conditions.

LC Conditions	
Column	Agilent InfinityLab Poroshell 120 HILIC-Z, 2.1 × 100 mm, 2.7 µm (p/n 675775-924)
Mobile Phase	A) 10 mM ammonium acetate in water, pH 9 with 5 µm Agilent InfinityLab Deactivator Additive (p/n 5191-4506) B) 10 mM ammonium acetate, pH 9 in 10:90 (v:v) water/acetonitrile
Flow Rate	0.5 mL/min
Gradient	Time (min) %A %B 0 0 100 11.5 30 70 12 0 100 15 0 100 20 Post time
Column Temperature	30 °C
Injection Volume	1 µL
Multisampler Temperature	6 °C

Table 2. Mass spectrometry conditions.

MS Conditions	
MS System	Agilent 6546 LC/Q-TOF
SWARM Autotune Selection	*m/z* 50 to 750 fragile ion mode
Ionization Source	Agilent Jet Stream
Polarity	Negative
Gas Temperature	200 °C
Drying Gas Flow	10 L/min
Nebulizer Pressure	40 psig
Sheath Gas Temperature	300 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	3000 V
Nozzle Voltage	0 V
Fragmentor	115
Acquisition Range	*m/z* 40–1000
Reference Mass	*m/z* 68.995758 and 980.016375

Batch isotopologue data analysis

Agilent MassHunter Profinder 10 is the batch-processing module of VistaFlux. Data files were imported and assigned to treatment groups. The chromatographic data were time-aligned against an in-house database of compounds with known retention times. The batch isotopologue extraction wizard was used to extract isotopologues for the target metabolites list as well as to correct for natural abundance of ^{13}C and for the tracer purity. In stable-isotope tracing, results are self-normalized for each metabolite as the isotopologue enrichment percentage is calculated relative to the other isotopologues. The ^{13}C tracer purity in this experiment was 50%, so the result of these corrections is that 100% label incorporation represents full incorporation of ^{13}C into the metabolite. The results from Profinder were transferred to Omix Premium, another module of VistaFlux, for visualization of metabolic fluxes on pathways.

Statistical analysis

Data are presented as the mean ± standard error of the mean from two biological replicates and four technical replicates per condition for all experiments. Unpaired two-tailed Student's t-tests were used to compare values, with $p < 0.05$ considered significant.

Results and discussion

LPS stimulation induces a large increase in TNFα

RAW 264.7 cells are widely used to understand molecular mechanisms of macrophage activation. Here, we assessed macrophage activation by measuring the amount of tumor necrosis factor alpha (TNFα) released in the cell culture medium in response to LPS stimulation. As expected, activation of RAW 264.7 cells by 100 ng/mL of LPS for four hours leads to a 100-fold increase in the abundance of secreted TNFα, confirming the activation state of the cells (Figure 3).

VistaFlux workflow

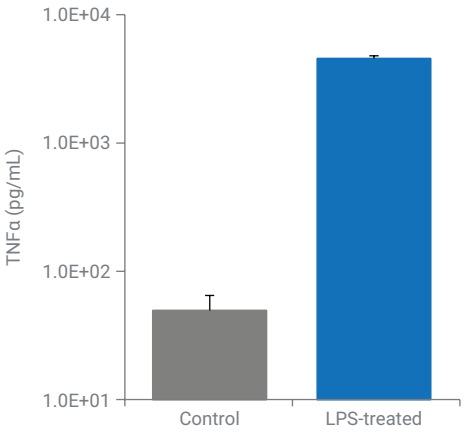


Figure 3. Level of TNFα in response to four hours of stimulation by 100 ng/mL of LPS in RAW 264.7 cells.

LPS stimulation induces a shift towards a glycolytic phenotype

To demonstrate how LPS activation impacts cell bioenergetics in RAW 264.7, we investigated the cell phenotype with the Seahorse XFp Analyzer (Figure 4). The Seahorse phenotype analysis software shows the metabolic activity of cells under basal conditions and under stressed conditions to reveal the metabolic potential. The analysis showed a general shift of the RAW 264.7 towards a more glycolytic phenotype when stimulated with LPS in the basal as well as in the stressed phenotype. This is in line with the literature showing that a glycolytic switch is an essential component of the pro-inflammatory function of macrophages.[7-9]

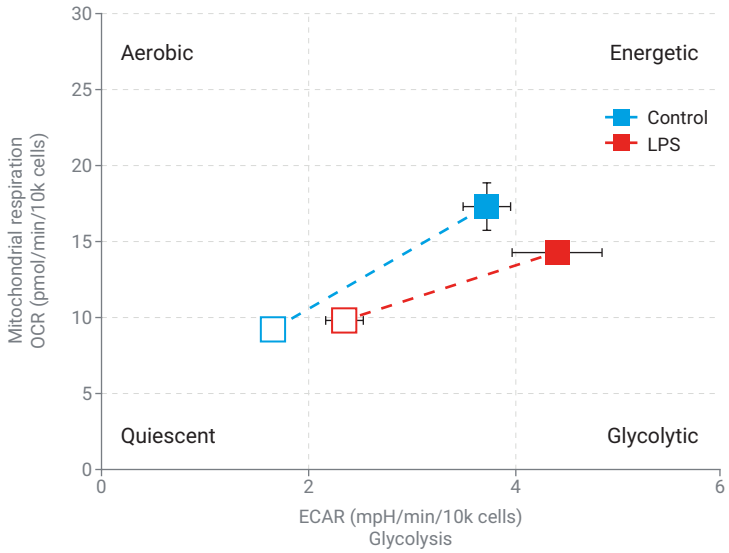


Figure 4. OCR versus ECAR plot for metabolic phenotype analysis measured in triplicates. Open quadrats represent the basal phenotype and closed quadrats the stressed phenotype (metabolic capacity after injection of oligomycin and FCCP). Four hours of stimulation by 100 ng/mL of LPS in RAW 264.7 cells induce a shift towards increased glycolytic activity.

LPS stimulation results in a redirection of carbon flux towards itaconic acid synthesis

As shown previously by Seahorse XFp analysis, LPS stimulation of RAW 264.7 cells increases ECAR. As discussed earlier, ECAR informs on glycolysis. Therefore, to gain insight into the changes in metabolite turnover in response to LPS stimuli leading to changes in ECAR, we undertook [U-$^{13}C_6$]-glucose stable-isotope tracing focusing on metabolites involved in central carbon catabolism and the TCA cycle (Table 3). RAW 264.7 cells were simulated or not with LPS for four hours in a culture medium containing [U-$^{13}C_6$]-glucose, and incorporation of ^{13}C was determined by LC/MS.

As shown in Figure 5, both lactate and pyruvate display very high levels of ^{13}C incorporation, reaching up to 95% labeled independently of the treatment applied. However, the major change occurs at the level of *cis*-aconitate, and specifically itaconic acid, where LPS activation drastically alters the isotopologue distribution. Effectively, upon LPS stimulation, a large increase in ^{13}C incorporation is observed for M+1, M+2, M+4, and M+5 concomitant with a decrease in M+0. This is consistent with the literature where LPS activation is known to promote the production of itaconic acid.[4,5] Based on the XF data presented here, LPS stimulation leads to an increase in glycolysis. We therefore investigated the change in the isotopologue distribution of metabolites involved in this pathway. As shown in Figure 5, we observed an increase in ^{13}C incorporation in metabolites involved in glycolysis as well as the oxidative branch of the TCA cycle leading towards the production of itaconic acid. An associated decrease in metabolites found in the reductive branch of the TCA cycle was observed, confirming an inhibition of the turnover of metabolites from the reductive branch of the TCA cycle upon LPS stimulation. Taken together, these data suggest that upon LPS stimulation, RAW 264.7 cells redirect their carbon flux towards itaconic acid production, leading to an alteration in ^{13}C turnover in the TCA cycle.

Table 3. Target metabolite list for isotopologue extraction showing mean measured retention time of eight control samples.

Information Pathway	Metabolite	Formula	RT (min)	CAS
ECAR	β-D-Glucose 6-phosphate	$C_6H_{13}O_9P$	9.60	15209-12-8
	D-Fructose 6-phosphate	$C_6H_{13}O_9P$	8.22	643-13-0
	β-D-Fructose 1,6-bisphosphate	$C_6H_{14}O_{12}P_2$	10.2	488-69-7
	D-Glyceraldehyde 3-phosphate	$C_3H_7O_6P$	7.03	591-57-1
	Dihydroxyacetone phosphate	$C_3H_7O_6P$	7.95	57-04-5
	Pyruvic acid	$C_3H_4O_4$	1.40	127-17-4
	Lactic acid	$C_3H_6O_3$	2.21	50-21-5
OCR	Citric acid	$C_6H_8O_7$	8.88	77-92-9
	cis-Aconitic acid	$C_6H_6O_6$	7.86	585-84-2
	D-*threo*-Isocitric acid	$C_6H_8O_7$	6.94	6061-97-8
	Oxoglutaric acid	$C_5H_6O_5$	6.04	328-50-7
	L-Glutamate	$C_5H_9NO_4$	6.25	56-86-0
	Succinic acid	$C_4H_6O_4$	6.54	110-15-6
	Fumaric acid	$C_4H_4O_4$	6.88	110-17-8
	L-Malic acid	$C_4H_6O_5$	6.79	97-67-6
	L-Aspartic acid	$C_4H_7NO_4$	1.22	56-84-8
	Itaconic acid	$C_5H_6O_4$	6.68	97-65-4

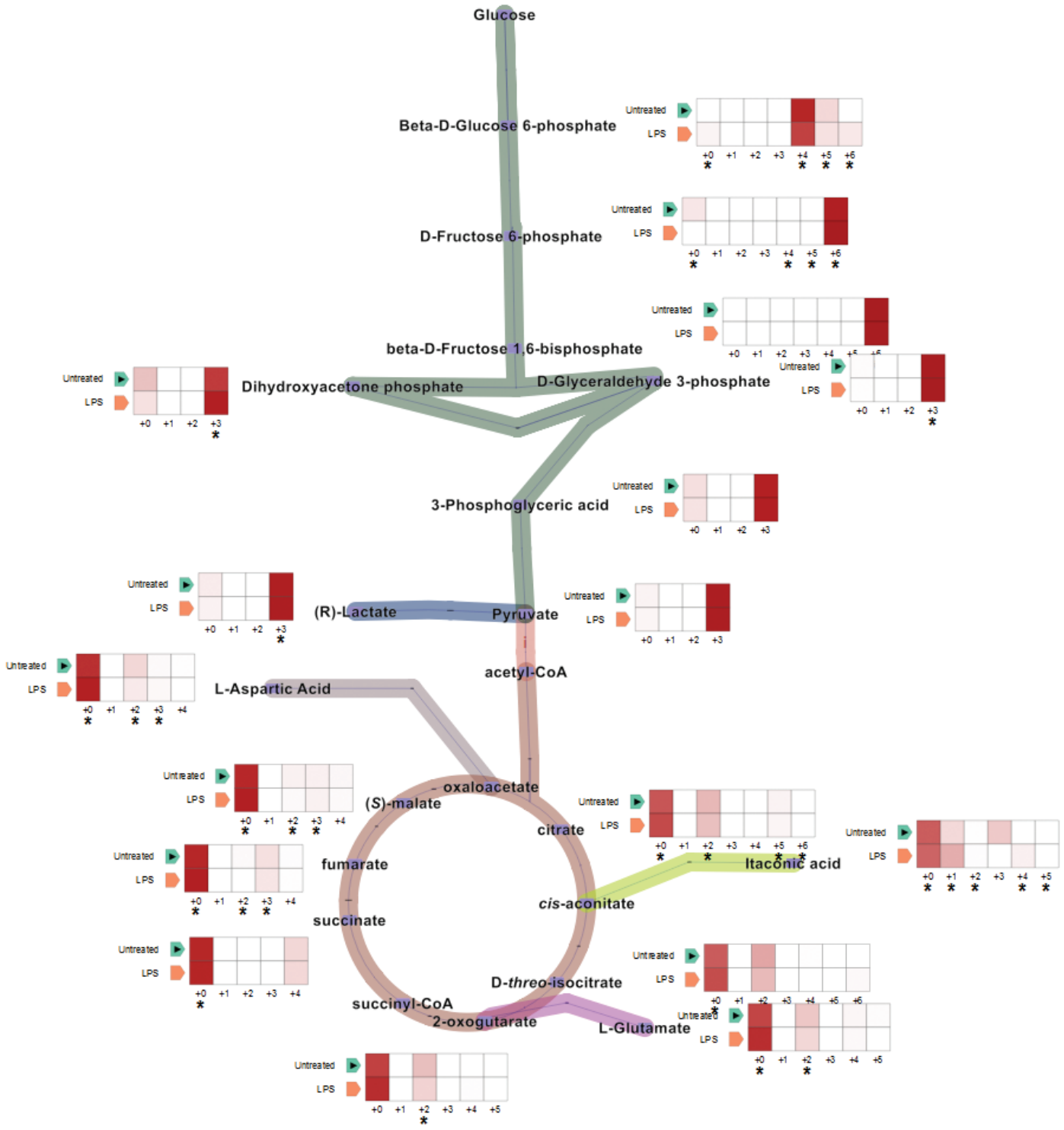


Figure 5. Summary of [U-$^{13}C_6$]-glucose stable-isotope-tracing analysis of RAW 264.7 cells stimulated for four hours by 100 ng/mL of LPS compared to untreated RAW 264.7 cells (control). Results are displayed as quilt plots on the pathways where each quilt plot shows corrected abundance for each isotopologue for control and treated cells. Statistical significance is denoted by an *.

Conclusion

Combining real-time extracellular flux information with ^{13}C stable-isotope-tracing allows greater insight into the mechanism of macrophage activation. Where Seahorse technology provides information about the metabolic phenotypes and the activities of the two major energy pathways from a macroscopic view, ^{13}C stable-isotope-tracing analysis allows a microscopic insight into the pathways leading to these changes.

Seahorse XF assays provide real-time, live-cell metabolic analysis. With this technology, a glycolytic shift in the phenotype of the living macrophages was shown, which is a prerequisite for pro-inflammatory macrophage activation. This was confirmed when looking into the metabolic details by qualitative flux analysis. The increased glycolytic rate was confirmed by the increased incorporation of ^{13}C into glycolysis metabolites relative to the basal case. Flux analysis also showed an increased production of itaconic acid, a potent antimicrobial compound produced by pro-inflammatory macrophages, and thereby provided a more detailed picture of the changes in metabolism.

This workflow and the technologies used can be applied to other cell types to study cell metabolism at the phenotype and metabolite levels to uncover molecular mechanisms of cell metabolism in response to external triggers or genetic manipulations.

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DE.5524421296

Printed in the USA, April 14, 2020
5994-1846EN

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