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應用手冊

^{13}C Qualitative Flux Analysis with Symphony Software and Polly Software of Non-Small Cell Lung Carcinoma Cells Grown *in vitro* in Two and Three Dimensions

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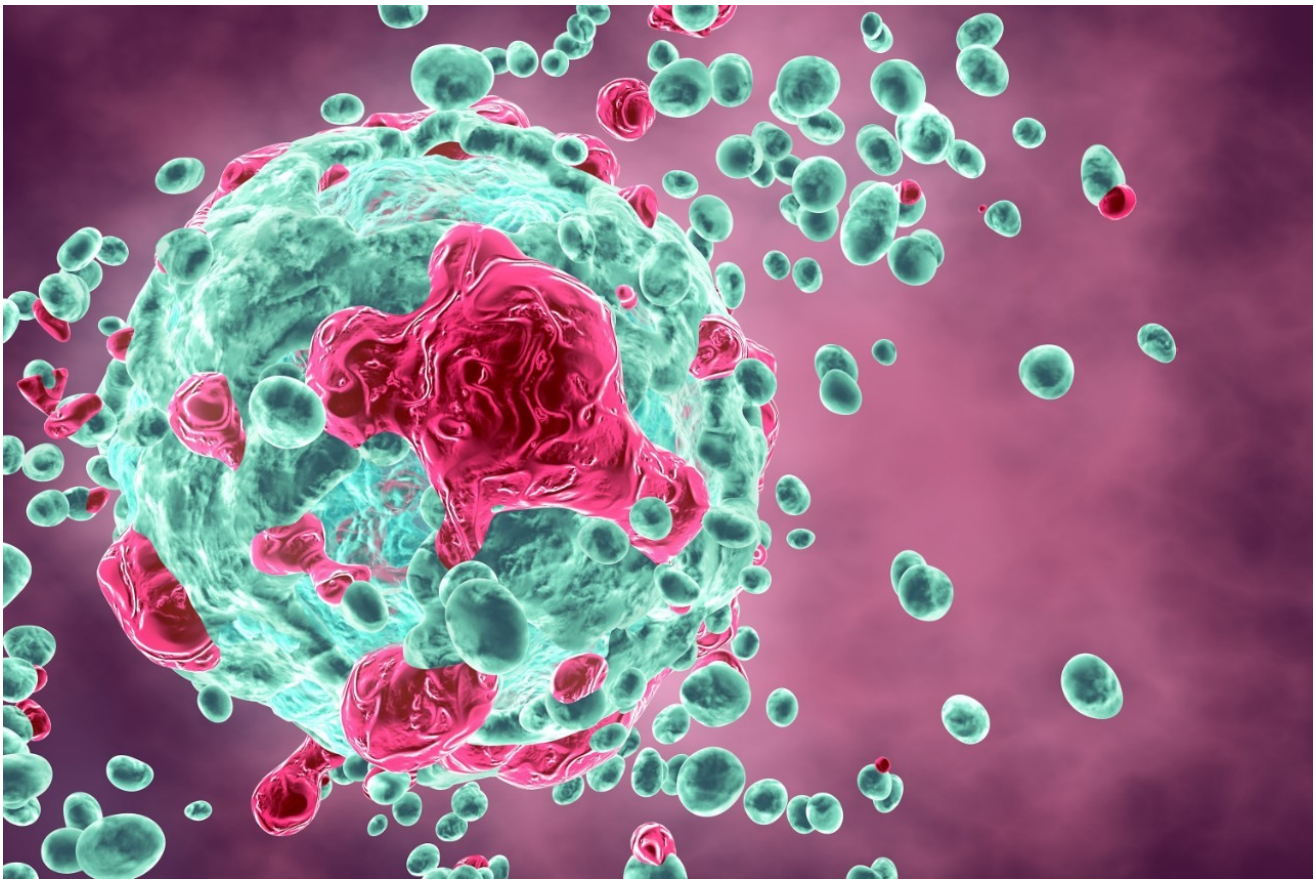

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Abstract

The direction of glucose in the same cell line can change dramatically depending on whether they are grown as 2D monolayers or 3D spheroids. In this application note, we show the results of a small case study where Symphony was interfaced to PollyPhi for the processing and analysis of isotope labeling metabolic relative flux data of 2D and 3D non-small cell lung carcinoma cells lines treated with ^{13}C-glucose.

Benefits

We illustrate the use of Symphony Software and Polly Software for contrasting the flow of glucose within mammalian cells grown *in vitro* in two and three dimensions, monolayers and spheroids, respectively. The more commonly used two-dimensional cell cultures are believed to be inadequate to recreate the in vivo physiologically relevant microenvironment.

Introduction

Mammalian cell lines are commonly used to study human disease and treatment using (2D)/monolayer cell cultures. However, this geometry of multicellular cell cultures is believed to be inadequate to recreate the biological microenvironment of naturally occurring cells, tumor cells in particular.[1,2] Three-dimensional (3D)/spheroid cell cultures are considered to be a more viable *in vitro* alternative since they better mimic the *in vivo* cellular growth environment. The potentially different outcomes from experiments using 2D and 3D culture systems may have a significant impact on the relevance of experimental findings. For example, the effectiveness of a drug treatment studied in (2D)/monolayers does not necessarily predict equivalent effectiveness *in vivo*. In this application note, we show the results of a small case study where Symphony was interfaced to PollyPhi for the processing and analysis of isotope labeling metabolic relative flux data of 2D and 3D non-small cell lung carcinoma cells lines treated with ^{13}C-glucose.

Experimental

Growth of monolayers and sphere cultures/cell extracts

H1299 cell lines were grown and extracted as previously described.[3] Briefly, cell lines were grown as monolayer in complete Dulbecco's Modified Eagle's Medium to generate spheroids, cells were grown in Falcon Bacteriological Petri Dishes coated with 2% pHEMA dissolved in 100% ethanol. Monolayer cells were dissociated using 0.25% Trypsin-EDTA, whereas spheroid cultures were dissociated using StemPro Accutase. Cell extracts from unlabeled cells or from cells labeled with [U-^{13}C] glucose were pelleted and re-suspended in water and lysed by heat shock treatment. Subsequently, chilled MeOH was added followed by the addition of $CHCl_3$. The samples were centrifuged and the resulting phases separated, followed by the addition of chilled acetonitrile and overnight incubation. The samples were centrifuged again and the supernatant collected and dried. Next, the samples were re-suspended and injected onto the LC-MS system. A graphical overview of the experimental design is shown in Figure 1.

Figure 1. Graphical representation of the experimental design for the isotope labeling metabolic relative flux data analysis of 2D and 3D lung cells lines treated with ^{13}C-glucose using Symphony and Polly informatics.

LC conditions

LC system:	ACQUITY UPLC I-Class
Column:	BEH C_{18}, 1.7 µm, 2.1 mm × 50 mm (p/n 186003685)
Column temp.:	40 °C
Sample temp.:	4 °C
Injection volume:	2.0 µL

Flow rate:	0.4 mL/min
Mobile phase A:	0.1% Formic acid in water
Mobile phase B:	0.1% Formic acid in acetonitrile
Mobile phase D:	0.1% Formic acid in IPA/acetonitrile (90/10, v/v)

Gradient:

Time	%B	%D
0.0	5.0	0.0
8.0	98.0	2.0
11.0	98.0	2.0
12.0	5.0	0.0

MS conditions

MS system:	Xevo G2-XS QTof
Ionization mode:	ESI
Acquisition range:	50–1200 *m/z*
Capillary voltage:	3.0 kV
Acquisition mode:	MS^E

Resolution:	30,000 FWHM

Bioinformatics

Data management

MS software:	MassLynx
Informatics:	Symphony, MSConvert, ElMaven, Polly

Figure 2 shows the basic components of a Symphony pipeline, a client/server application that is triggered by the MassLynx data acquisition system. Typically, a server request is executed that consists of a series of tasks that are executed based on input. Here, the pipeline transfers data and converts the native MassLynx format into mzXML using MSconvert (http://proteowizard.sourceforge.net/index.html < http://proteowizard.sourceforge.net/index.html>). Next, it imports the converted data into ElMaven (elucidatainc.github.io/ElMaven <https://elucidatainc.github.io/ElMaven/>) and Polly (elucidata.io < https://elucidata.io/>) for peak detection, curation, natural abundance correction, and results visualization. In other words, mapping of the relative abundances and distribution of the detected metabolites on metabolic pathways as illustrated by the example shown in Figure 3. The details and benefits of the informatics pipeline have been described in detail elsewhere.[4]

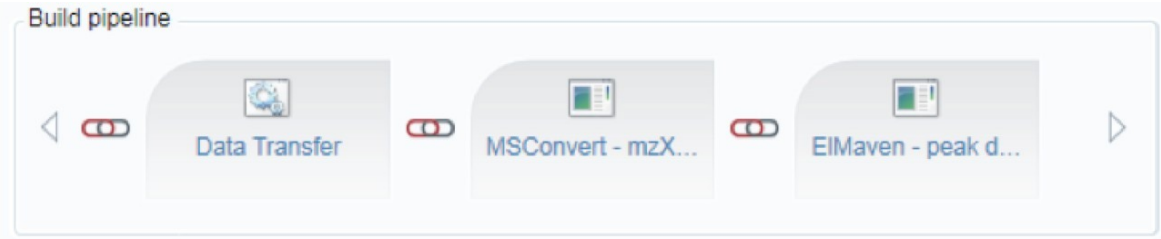

Figure 2. Symphony pipeline chains data transfer followed by MSconvert, raw to mzXML data conversion, and ElMaven peak detection, integration, annotation, as well as upload.

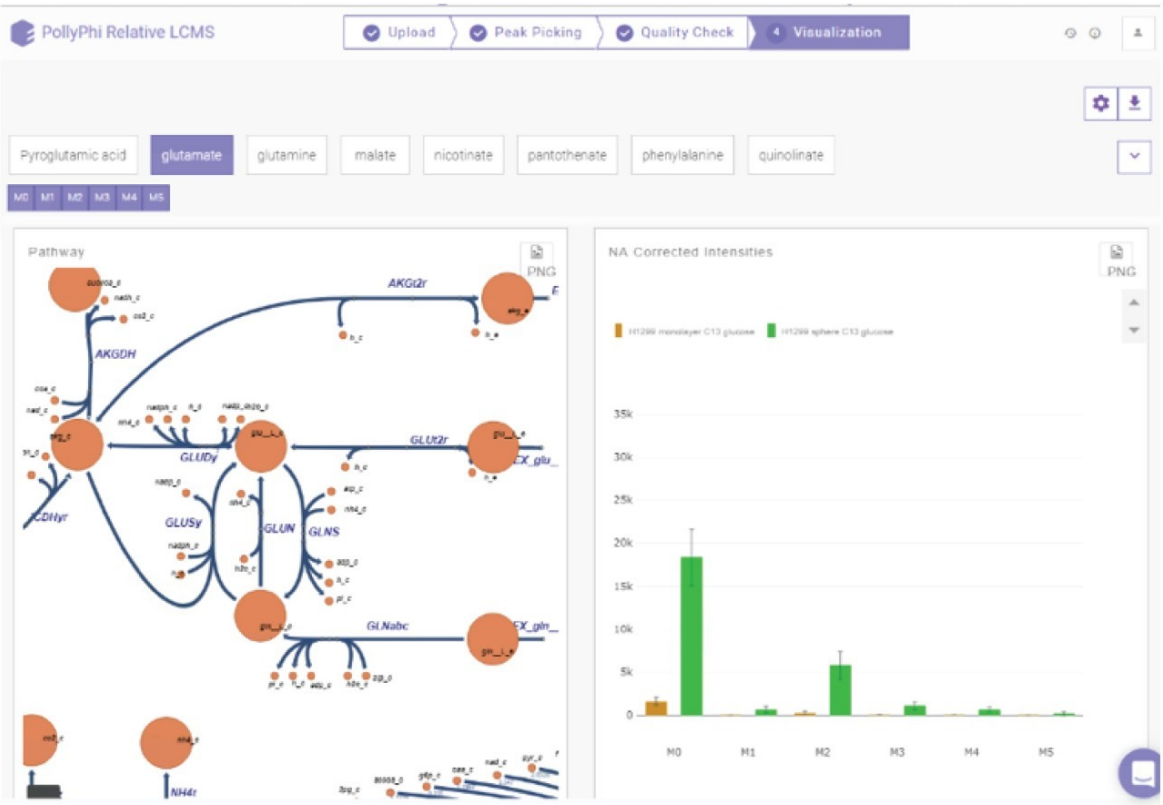

Figure 3. Pathway mapping and natural abundance isotopic corrected intensity differences (not fractional enriched) between experimental groups for a selected metabolite in PollyPhi Relative LC-MS.

Results and Discussion

Upregulation in glycolysis

The lactate fractional enrichment ($^{13}C_3$ isotopologue) is significantly higher in spheroid cell cultures compared to monolayer cell cultures. Since $^{13}C_3$-lactate is the major isotopologue produced from $^{13}C_6$-glucose, the primary energy source, the observations indicate that glycolysis is significantly upregulated in spheroid cell cultures as opposed to monolayers.

Increased glucose contribution to TCA via acetyl-CoA and PDH

In addition, key intermediates in the tricarboxylic acid (TCA) cycle have higher $^{13}C_2$ isotopologues. $^{13}C_2$ isotopologues in the TCA cycle are generally formed from pyruvate $^{13}C_3$ via acetyl-CoA through PDH enzyme. Synthesis of pyruvate $^{13}C_3$ isotopologue can in turn be attributed to $^{13}C_6$-glucose. This observation suggests a

higher contribution of glucose to the TCA cycle via acetyl CoA in spheroid cell culture as opposed to monolayer cell culture. This is consistent with the finding of upregulated glycolysis in spheroid cells as shown and summarized in Figure 4.

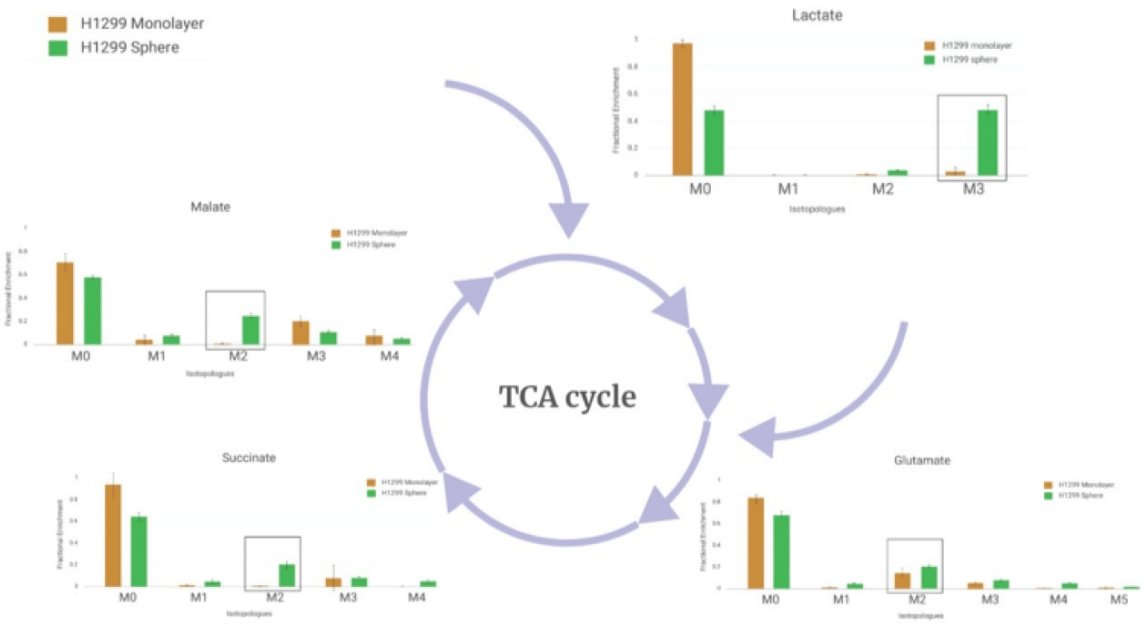

Figure 4. Relatively high lactate $^{13}C_3$ isotopologue and $^{13}C_2$ isotopologues in TCA and adjoining metabolites in 3D spheroid cell culture compared to 2D monolayer cell culture indicates an upregulated glycolysis and TCA cycle.

Conclusion

The direction of glucose in the same cell line can change dramatically depending on whether they are grown as 2D monolayers or 3D spheroids. Specifically, increased glucose flow through the glycolysis and TCA pathways were observed when they were grown as spheroids in contrast to monolayers. Such differences in nutrient utilization has significant implications for the translation of findings based on in vitro models to *in vivo* models. A prominent example has been shown in Figure 4 where the monolayer cell culture of H1299 human non-small cell lung carcinoma cells shows very limited glycolysis. In contrast, the observations and interpretations are very different for lactate. In the spheroid cells, glycolysis rates are increased significantly. Hence, analysis of the monolayer cell culture in isolation would have made translating the findings from the *in vitro* model to the *in vivo* model very challenging.

References

1. Li et al. Survival advantages of multicellular spheroids vs. monolayers of HepG2 cells *in vitro. Oncol Rep*. 2008 Dec;20(6):1465–71.
2. Wallace et al. A Model for Spheroid versus Monolayer Response of SK-N-SH Neuroblastoma Cells to Treatment with 15-Deoxy-PGJ2. *Comput Math Methods Med*. 2016;2016:3628124.
3. Fernandez et al. The mitochondrial citrate carrier, SLC25A1, drives stemness and therapy resistance in non-small cell lung cancer. *Cell Death Differ.* 2018 Jul;25(7):1239–1258.
4. Automating Metabolic Flux Analysis with Symphony and Polly. Waters Corporation. Technical Brief. 720006380EN. 2018.

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