AUTOMATED HIGH-THROUGHPUT FLUX ANALYSIS OF NON-SMALL CELL LUNG CARCINOMA CELLS GROWN IN VITRO IN TWO AND THREE DIMENSIONS

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INTRODUCTION

Mammalian cell lines are widely used to study human disease and treatment. Monolayer (2D) cell cultures are commonly used for this purpose. However, this geometry of multicellular cell cultures is believed to be inadequate to recreate the biological microenvironment of natural occurring cells[1,2]. Spheroid (3D) cell cultures are considered more viable in vitro alternatives since they better mimic the in vivo cellular growth environment. The potentially different outcomes from 2D and 3D culture systems may have however a significant impact on the relevance of experimental findings. The results of a small case study using dedicated software for the processing and analysis of metabolic flux data of 2D and 3D non-small cell lung carcinoma cells lines treated with ^{13}C-glucose are presented.

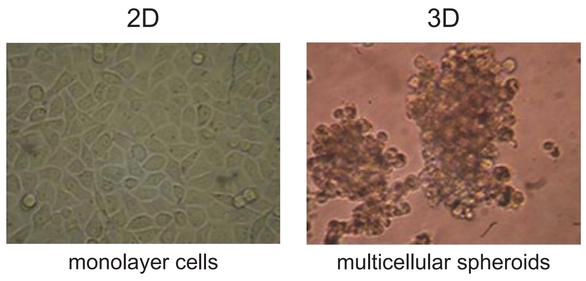

Figure 1. Biological microenvironment at in vivo cell level.

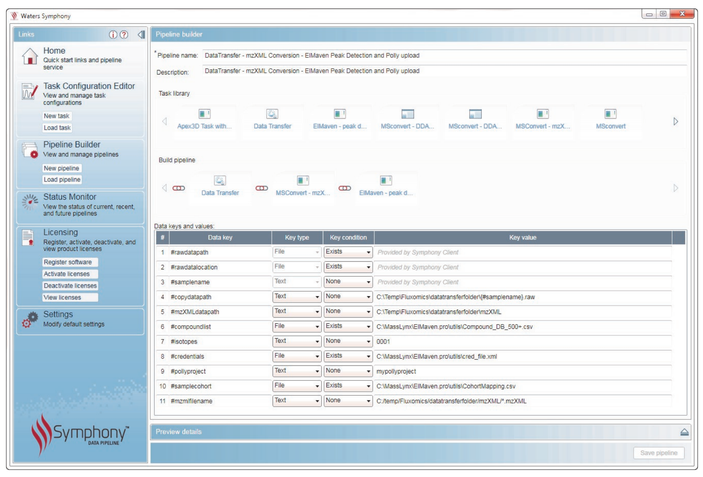

Figure 2. Symphony Pipeline Builder interface showing the task library and the metabolic flux analysis pipeline with key user parameters being passed to the tasks.

METHODS

Growth of monolayers and sphere cultures/cell extracts

H1299 cell lines were grown and extracted as previously described.[3] Cell lines were grown as monolayer in complete Dulbecco's Modified Eagle's Medium. To create 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, an spheroid cultures with 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. 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.

LC-MS conditions

LC conditions

LC system:	ACQUITY UPLC I-Class
Column:	BEH C18, 1.7 µm, 2.1 mm × 50 mm
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 (9:1, 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:	MSE data independent acquisition
Resolution:	30,000 FWHM

Data Management

MS software:	MassLynx
Informatics :	Symphony, MSConvert, El-MAVEN, Polly

Figure 3. Symphony pipeline chains data transfer followed by MSconvert, raw to mzXML data conversion and El-MAVEN peak detection, integration, annotation and upload.

RESULTS

Automation

Figures 2 and 3 show the software user interface and components of a Symphony pipeline, a client/server application that is triggered by the data acquisition system. A server request is executed, which consists of a list of tasks based on data input. Tasks are requests that cover the execution of command line interface modules (exe, bat, script).

The pipeline applied here consists of three basic components: Data transfer from the acquisition to a storage server or processing PC, conversion of the native MassLynx format into mzXML standard by MSconvert, and import of the converted data by El-MAVEN and Polly for peak detection, curation, natural abundance correction and pathway level visualizations.

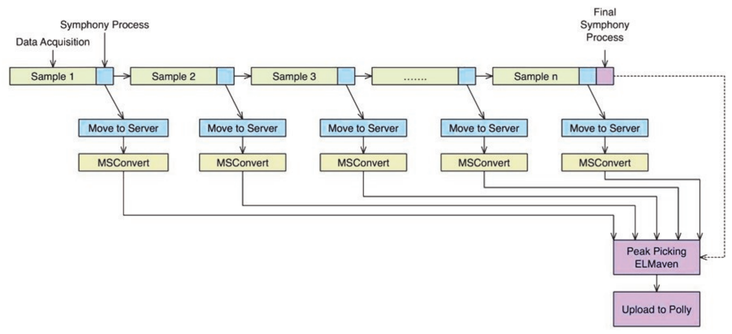

Figure 4. Schematic flow diagram showing the sequence of the Symphony tasks.

In total, 100 LC-MS data files were collected to study the metabolic flux in human lung cell lines. This included all relevant QCs, experimental conditions, and ionization modes. Once moved, converted and processed, a series of cloud based applications can be applied.

Typical steps performed in the Polly environment include a data quality check, isotopic natural abundance correction, and visualization of the data mapped to pathways, as shown in Figure 5. Individual metabolites can be reviewed to inspect their extracted ion chromatograms and relative responses across study groups.

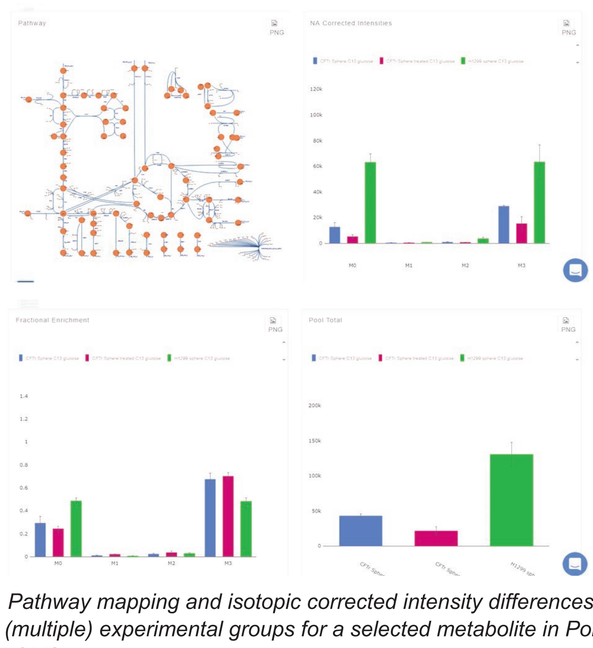

Figure 5. Pathway mapping and isotopic corrected intensity differences between (multiple) experimental groups for a selected metabolite in PollyPhi Relative LCMS.

Application

A subset of the data, with focus on H1299 cell geometry only, were analyzed separately in more detail. Example data of some polar metabolites are shown in Figure 6.

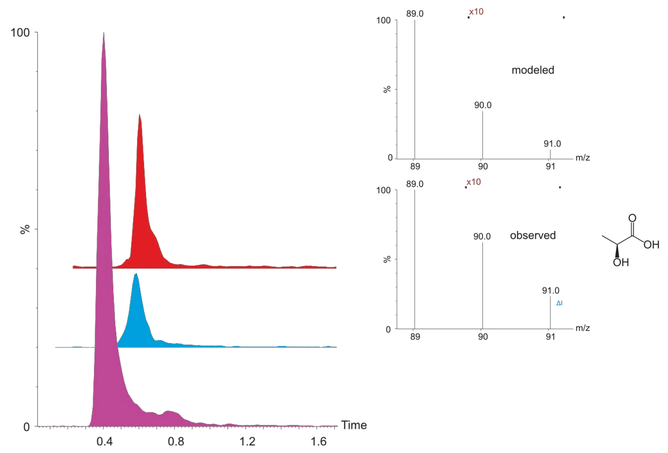

Figure 6. Example metabolites XICs (lactate (blue), malate (red), and glutamate (blue)) and modelled/observed isotopic distribution of lactate (pre isotopic abundance correction, illustrating ^{13}C incorporation).

Following format conversion and local El-MAVEN peak detection, the data are automatically uploaded to Polly for further analysis. Data can be automatically reviewed using features such as, the peak picking shown in Figures 7.

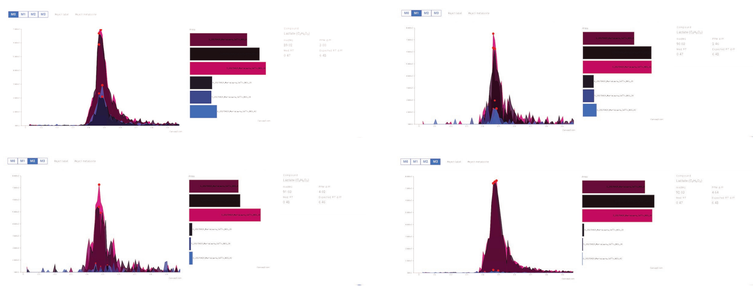

Figure 7. PollyPhi Relative LCMS Peak Picking showing extracted and integrated ion chromatograms for the detected lactate isotopologues and relative peak areas across samples (color coded by experimental group(s)).

The lactate fractional enrichment ($^{13}C_3$ isotopologue) was found to be significantly higher in spheroid cell cultures compared to monolayer cell cultures, as illustrated in 8. 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.

Figure 8. Results Visualization showing relatively high lactate $^{13}C_3$ isotopologue and $^{13}C_2$ isotopologues in TCA and adjoining metabolites in 3D spheroid cell culture as opposed to 2D monolayer cell culture indicates upregulated glycolysis and TCA cycle.

Moreover, key intermediates in the tricarboxylic acid (TCA) cycle, have higher $^{13}C_2$ isotopologues. $^{13}C_2$ isotopologues in TCA 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 U-$^{13}C_6$-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 9.

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.

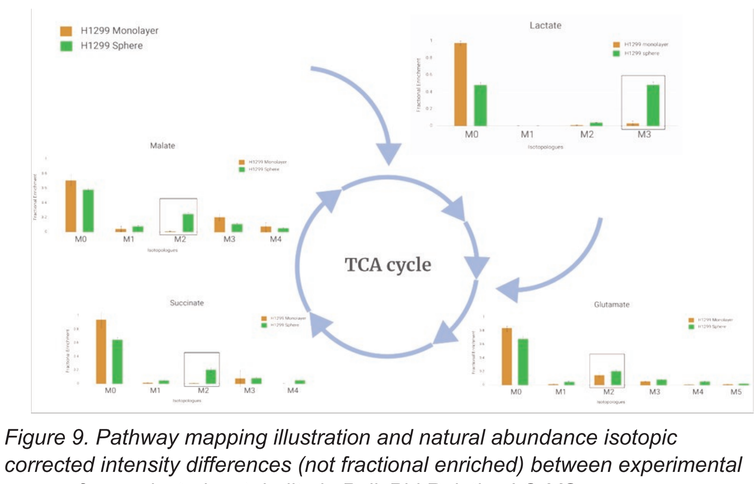

Figure 9. Pathway mapping illustration and natural abundance isotopic corrected intensity differences (not fractional enriched) between experimental groups for a selected metabolite in PollyPhi Relative LC-MS.

A prominent example is highlighted in Figure 9, 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.

CONCLUSION

- **Automated fluxomics analysis has been demonstrated using pipeline software/informatics that can be executed from a sample acquisition list, thereby providing increased and improved throughput**
- **The direction of glucose in the same cell line can change dramatically depending on whether they are grown as 2D monolayers or 3D spheroids**
- **Increased glucose flow through the glycolysis and TCA pathways were observed when they were grown as spheroids in contrast to monolayers**
- **Analysis of the monolayer cell culture in isolation would have made translating the findings from the *in vitro* model to the *in vivo* model 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.
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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.

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