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# A novel platform for automated high-throughput fluxome profiling of metabolic variants

Stéphanie Heux [a,b,c,*], Poinot Juliette [a,b,c], Massou Stéphane [a,b,c], Sokol Serguei [a,b,c], Portais Jean-Charles [a,b,c]

[a] Université de Toulouse; INSA, UPS, INP; LISBP, 135 Avenue de Rangueil, F-31077 Toulouse, France
[b] INRA, UMR792, Ingénierie des Systèmes Biologiques et des Procédés, F-31400 Toulouse, France
[c] CNRS, UMR5504, F-31400 Toulouse, France

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ABSTRACT

Advances in metabolic engineering are enabling the creation of a large number of cell factories. However, high-throughput platforms do not yet exist for rapidly analyzing the metabolic network of the engineered cells. To fill the gap, we developed an integrated solution for fluxome profiling of large sets of biological systems and conditions. This platform combines a robotic system for $^{13}$C-labelling experiments and sampling of labelled material with NMR-based isotopic fingerprinting and automated data interpretation. As a proof-of-concept, this workflow was applied to discriminate between *Escherichia coli* mutants with gradual expression of the glucose-6-phosphate dehydrogenase. Metabolic variants were clearly discriminated while pathways that support metabolic flexibility towards modulation of a single enzyme were elucidating. By directly connecting the data flow between cell cultivation and flux quantification, considerable advances in throughput, robustness, release of resources and screening capacity were achieved. This will undoubtedly facilitate the development of efficient cell factories.



## 1. Introduction

In the field of biotechnology, metabolic engineering and synthetic biology, fluxomics has been identified as a key analytical technology (Ellis and Goodacre, 2012; Feng et al., 2010; Sanford et al., 2002; Stephanopoulos, 1999) not only for the rational design of cells but also for comprehensive understanding of the link between genotype and phenotype. Fluxomics, i.e. the cell-wide quantification of metabolic fluxes, reveals the actual operation of metabolic networks in given environmental conditions, resulting from the integrated flow of interactions between all molecular components - genes, mRNAs, proteins and metabolites (Sauer, 2004, 2006). Hence, metabolic flux analysis is the most comprehensive description of the metabolic phenotype at the cellular level (Wittmann and Portais, 2013). In practice, $^{13}$C-fluxomics is still a tedious and rather time consuming process. The method combines both $^{13}$C-labelling experiments and mathematical modelling of biochemical networks. Cells are gown on $^{13}$C-labelled substrates to metabolic and isotopic steady state and the labelling patterns of metabolites are monitored by mass spectrometry (MS), nuclear magnetic resonance (NMR), or both. The labelling information can be collected on metabolic end-products such as protein-bound amino acids, which accumulate to larger extents than true metabolic intermediates (Sauer, 2004; Szyperski, 1995; Wiechert, 2001). Combined with quantitative physiological data and a detailed metabolic model of metabolism, the labelling patterns give access to the *in vivo* reaction rates (i.e. fluxes) associated with the cellular network of an organism. When this information is not available, multivariate statistics can be applied to the isotopic data to provide detailed phenotyping of biological systems without any prior knowledge (Raghevendran et al., 2004; Zamboni and Sauer, 2004).

*Abbreviations:* MS, mass spectrometry; NMR, nuclear magnetic resonance; HT, high throughput; OD600, optical density at 600 nm; CDW, cell dry weight; RSD, relative standard deviation; G6PDH, glucose-6-phosphate dehydrogenase; PCA, principal component analysis; WT, wild type; R5P:C1, C1 of ribose-5-phosphate; AKG:C4, C4 of alpha-ketoglutarate, OAAA/PYR:C3, C3 of the combined pool of oxaloacetate/pyruvate; PYR, C3C3 of pyruvate; R5P:C3, C3 of ribose-5-phosphate; PPP, pentose phosphate pathway; LiHa, Liquid Handling; HEPA, High Efficiency Particular Air Filter; DO, dissolved oxygen; TSP-d4, 3-(trimethylsilyl)-2,2′,3,3′-tetradeuteropropionic acid; HCA, hierarchical clustering analysis; TCA, tricarboxylic acid cycle; ED, Entner–Doudoroff; FTBL, flux table format

* Corresponding author at: Laboratoire d'Ingénierie des Systèmes Biologiques et des Procédés, INSA Toulouse, 135 Avenue de Rangueil, 31077 Toulouse Cedex 4, France. Fax: +33 561 55 96 89.
*E-mail address:* stephanie.heux@insa-toulouse.fr (S. Heux).

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The rapid expansion of fluxomics and its areas of application is driving the need for high-throughput approaches to enable comprehensive metabolic investigations of a growing number of organisms, engineered mutants and physiological conditions (Ellis and Goodacre, 2012). Significant advances have been made in the field in the last decade. Miniaturized cell cultivation systems have been used to perform $^{13}$C-labelling experiments with less effort and cost in labelled substrate (Balcarcel and Clark, 2003; Betts and Baganz, 2006; Ge et al., 2006; Girard et al., 2001; Huber et al., 2009; Isett et al., 2007; Kocincova et al., 2008; Tang et al., 2009). The sensitivity, speed, and robustness of both NMR-based and MS-based isotopic analysis have been improved (Boisseau et al., 2013; Cahoreau et al., 2012; Fan and Lane, 2008; Fischer et al., 2004; Giraudeau et al., 2012; Giraudeau et al., 2011; Massou et al., 2007a; Peng, 2012). Tools have been developed for large scale isotopic data processing (Millard et al., 2012; Poskar et al., 2012) as along with improved algorithms and software for flux calculation and statistical analysis of isotopic data (Antoniewicz et al., 2007; Quek et al., 2009; Raghevendran et al., 2004; Sokol et al., 2012; Weitzel et al., 2012; Zamboni et al., 2005; Zamboni and Sauer, 2004). These developments have allowed large-scale flux analysis to be applied to microorganisms (Amador-Noguez et al., 2010; Blank et al., 2005; Fischer and Sauer, 2005; Fischer et al., 2004; Haverkorn van Rijsewijk et al., 2011; Wittmann et al., 2004) and mammalian cells (Munger et al., 2008).

However, despite all the above improvements, HT fluxomics is still in early development. In particular, there is no existing HT fluxomics platform which combines all experimental and in silico steps of the complete workflow in a single and fully integrated manner. The design and development of such a platform is hindered by several technical challenges that need to be addressed in parallel. This includes (1) the tight control of cultivation parameters to ensure metabolic and isotopic steady state; (2) the automated monitoring of growth parameters to collect labelled material once steady state is achieved (3) parallel cultivation to increase throughput; (4) miniaturization of working volumes to reduce the cost of labelled substrates and to facilitate the parallel processes; (5) automated, rapid, parallel sampling of labeled material to avoid degradation of the metabolites; (6) rapid, sensitive measurement of isotopic profiles to cope with both the small amounts of biological material and the large number of samples; (7) automated extraction of labeling information from raw analytical data, which today is still mainly done manually, thereby saving time and effort and improving data robustness (8), data interpretation tools that provide valuable metabolic information in a high throughput manner, i.e. with reduced user supervision.

For the first time, these challenges were met with the development of a fully integrated solution for fluxome analysis that combines a robotic cultivation and sampling workstation for $^{13}$C-labelling experiments with NMR-based isotopic profiling, and tools for processing and interpreting isotopic data. The automation, parallelization, optimization and integration of all steps in the workflow are described in detail. As a proof of concept, the new platform was applied to a set of *Escherichia coli* mutants with varying levels of a single enzyme and grown on two different $^{13}$C-labelled carbon sources. The power of the overall approach to provide discriminating metabolic information for a large number of mutants and conditions as well as its value in generating valuable metabolic knowledge was demonstrated.

## 2. Materials and methods

Fig. 1 is a schematic diagram of the complete workflow developed in this study.

### 2.1. Bacterial strains

*E. coli* MG1655 strains used in this study are listed in Supplementary Table S1. The over-expression and under-expression mutants (Pzwf) were created by replacing the native promoter of *zwf* by artificial promoters of different strength (Meynial-Salles et al., 2005; Nicolas et al., 2007), resulting in the gradual expression of glucose-6-phosphate dehydrogenase. The deletion mutant (Δzwf) was obtained with a one-step disruption protocol and the *in vitro* activity of the glucose-6-phosphate dehydrogenase was measured in crude cell extracts (Nicolas et al., 2007).

### 2.2. Media and cultivation conditions

All *E. coli* cells were grown on minimal synthetic medium containing (per liter) 2.5 g of either glucose or xylose, 17.4 $Na_2HPO_4 \cdot 12H_20$, 3.03 g of $KH_2PO_4$, 0.51 g of NaCl, 2.04 g of $NH_4Cl$, 0.49 g of $MgSO_4$, 4.38 mg of $CaCl_2$, 15 mg of $Na_2EDTA \cdot 2H_2O$, 4.5 mg of $ZnSO_4 \cdot 7H_2O$, 0.3 mg of $CoCl_2 \cdot 6H_2O$, 1 mg of $MnCl_2 \cdot 4H_2O$, 1 mg of $H_3BO_3$, 0.4 mg of $Na_2MoO_4 \cdot 2H_2O$, 3 mg of $FeSO_4 \cdot 7H_2O$ and 0.3 mg of $CuSO_4 \cdot 5H_2O$, 0.1 g of thiamine. All the cultivations were performed at 37 °C. *E. coli* cells were freshly inoculated from a glycerol stock on a LB (10 g/l of tryptone, 5 g/l of yeast extract and 10 g/l of NaCl) liquid medium for 6 h. Liquid pre-cultures containing minimal synthetic medium were inoculated from the LB cultures. Cultures were inoculated with 0.57 mg CDW $l^{-1}$. The minimal synthetic labelled medium for $^{13}$C-labelling experiments contained 13.9 mM of a mixture containing either 20% (mol/mol) [U-$^{13}$C]-glucose and 80% (mol/mol) [1-$^{13}$C]-glucose or [U-$^{13}$C]-xylose (99% of $^{13}$C atom, Eurisotop, France) and 80% (mol/mol) [1-$^{13}$C]-xylose (99% of $^{13}$C atom, Eurisotop, France). Cells were grown in mini stirred tank reactors.

### 2.3. Automated cultivation and sampling platform

The system presented here incorporates a bioreaction block developed by Kusterer et al. (2008), Puskeiler et al. (2005) in an automatic workstation (Freedom EVO 200, TECAN, Switzerland) (Fig. 1). The bioreaction block has a capacity of 48 stirred tank bioreactors equipped with solid state sensors for oxygen and pH and a working volume ranging from 8 to 15 ml. A lid with magnetic impellers on the central hollow axles enabled stirring, circulation and distribution of sterile inlet air into the head space. A series of 48 holes in the lid allowed the Liquid Handling (LiHa) robotic arm to access each bioreactor. The temperature of the bioreaction block was regulated using a water circulation bath. The bioreactor block was also designed to minimize evaporation and contamination of the medium in each individual bioreactor. For a detailed description of this bioreactor block, see (Kusterer et al., 2008; Puskeiler et al., 2005). The robotic workstation was equipped with three robotic arms to handle liquids, transport tubes, microtiter plates, centrifuge buckets, filters, height modules (i.e. a barcode reader, a cooling module, a heating module, a plate washer, a plate reader, a HCL dispenser, a centrifuge and a filter station) and a high efficiency particulate air filter hood to provide sterile conditions. The robot was activated via control software (EVOware Pipetting Software and Pegasus Event Control Software, TECAN). A database (SQL Manager 2008) made it possible to collect the barcodes of each destination tube, values of pH, dissolved oxygen (DO) and optical density ($OD_{600}$) with a time stamp for each bioreactor, a volume and time stamp for the liquids dispensed into each bioreactor, a volume and a time stamp, and the origin (i.e. name of the bioreactor) of the liquid dispensed into each destination tube and the name of the protocol used. The automated protocol used in this study is described in Supplementary Fig. S1 and Supplementary Note.