Quantification of microbial phenotypes using 13C-Fluxomics

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Systems biology is an emerging tool in microbiology that helps us to understand cellular processes and to optimise microbes for production purposes[1]. It strongly relies on the use of large datasets created using omics tools followed by data mining and modelling in order to gain new insights into biology. The creation of the datasets is usually comprised of *genomics* defining the overall capacity of a microbe, *transcriptomics* and *proteomics* as a measure of the active set of reactions within the overall capacity and more recently *metabolomics* as a measure of the available building blocks and (if performed quantitatively) of the thermodynamic driving forces governing the intracellular reactions. The latter can define feasibility of pathways as well as reaction reversibility, which can be important constraints for the analysis of metabolic networks. However, all these omics techniques fail to quantitatively assess the metabolic phenotype in its ultimate form: The reaction rates, or metabolic fluxes inside the cell that define the material transfer rates from one metabolite pool to another and from pathway to pathway. The 'omics technology that enables the quantification of fluxes is metabolic flux analysis, or *fluxomics*.

This article will introduce this important, but still underrepresented omics technology.

When analysing flux distributions in microbes, only the most peripheral fluxes are usually directly measurable while intracellular fluxes need to be inferred from stoichiometric models[2]. Besides dynamic approaches, that remain localised approaches, and global constraint-based flux balancing approaches[2], one powerful fluxomics approach that was developed over the last two decades is ^{13}C-based metabolic flux analysis (^{13}C-MFA)[3].

^{13}C-MFA is typically focused on central carbon metabolism where rearrangements of carbon atoms can be used to infer metabolic activity. It delivers a flux estimate that describes the metabolic state of a cell at a given time. ^{13}C-MFA has been reviewed in detail by different authors[4-6]. Despite some efforts to extend ^{13}C-MFA to mammalian cells and to complex environments, it is still most successfully applied to bacteria and yeast growing on simple chemically defined media.

The central assumption for ^{13}C-MFA is that the metabolic network under study is at pseudo steady-state. This means that the molar flux through a metabolite pool is orders of magnitude larger than changes in concentration of this metabolite over time. During exponential growth, in stationary phase and in continuous cultures this assumption is generally valid, while invalid during transient states. Accumulation of large amounts of compatible solutes inside cells also poses a problem in this context. These should be treated as products of the network rather than intermediates. A second assumption is that the system is well mixed and thirdly that no significant isotope effect on enzyme activity is present. Finally, the systems should ideally be at isotopic steady state (stable labelling enrichments). These assumptions have been demonstrated in the past to be valid for single cells growing suspended in chemically defined medium.

^{13}C-MFA is usually performed in four different stages (Figure 1): Experimental design (i), flux experiment (ii), quantitative metabolomics (iii) and flux modelling (iv).

Experimental design is an essential step, since the wrong design may render expensive, labelled substrates ineffective. The design will require the definition of stoichiometry and atom transitions of the reaction network. Once this is established, simulation of labelling outputs of the network for metabolites at given labelling inputs from substrates under different flux ranges will define which metabolite labellings are sensitive for the flux ranges tested.

When performing the *flux experiment* it will be necessary to assure metabolic and isotopic steady state. This can be achieved by measuring rates and labellings of metabolites during an initial experiment. Closing of mass balances around carbon, nitrogen and/or degree of reduction will ensure that the datasets are consistent. The best vessels to perform such experiments are fully controlled bioreactors[7], but the successful use of shake flasks[8] and even microtitre plates has been demonstrated before[9].

Quantitative metabolomics will deliver the datasets necessary. This includes the relative labelling distributions of metabolites measured by MS[10] or NMR[11], quantitative analysis of all substrate

uptake and product secretion rates (measured fluxes) as well as the compositional analysis of the biomass itself, if no reliable literature data is available (for example, carbohydrate, DNA/RNA, protein content and so on).

At the end of the process stands the *flux modelling*, which includes the actual estimation followed by a sensitivity analysis. This will provide the flux optimum and a confidence interval for each flux parameter. The estimation is an iterative process: Firstly, random fluxes are chosen and using the stoichiometric and atom mapping networks labelling outputs are calculated while fulfilling the experimentally observed fluxes (uptake, growth and secretion rates). Then the calculated and the measured labelling sets are compared. Subsequently the fluxes are changed until the difference between both labelling sets is minimised.

The flux experiment and the quantitative metabolomics stages require state-of-the art fermentation equipment and analytical tools for quantification of rates and labelling distributions. The experimental design and the flux modelling require sophisticated modelling software. Over the last few years, several modelling tools have been implemented[12,13]. After Antoniewicz *et al.* redefined the way the labellings are mapped throughout the network with the EMU approach[14], we recently implemented an open source software package, OpenFLUX[15] (https://sourceforge.net/projects/openflux/). This software enables the user to perform experimental design as well as flux estimation and sensitivity analysis and is available free of charge.

I hope that this article will spark the interest in ^{13}C fluxomics in the microbiological community in Australia and that I have been able to shed some light on this important omics technology.

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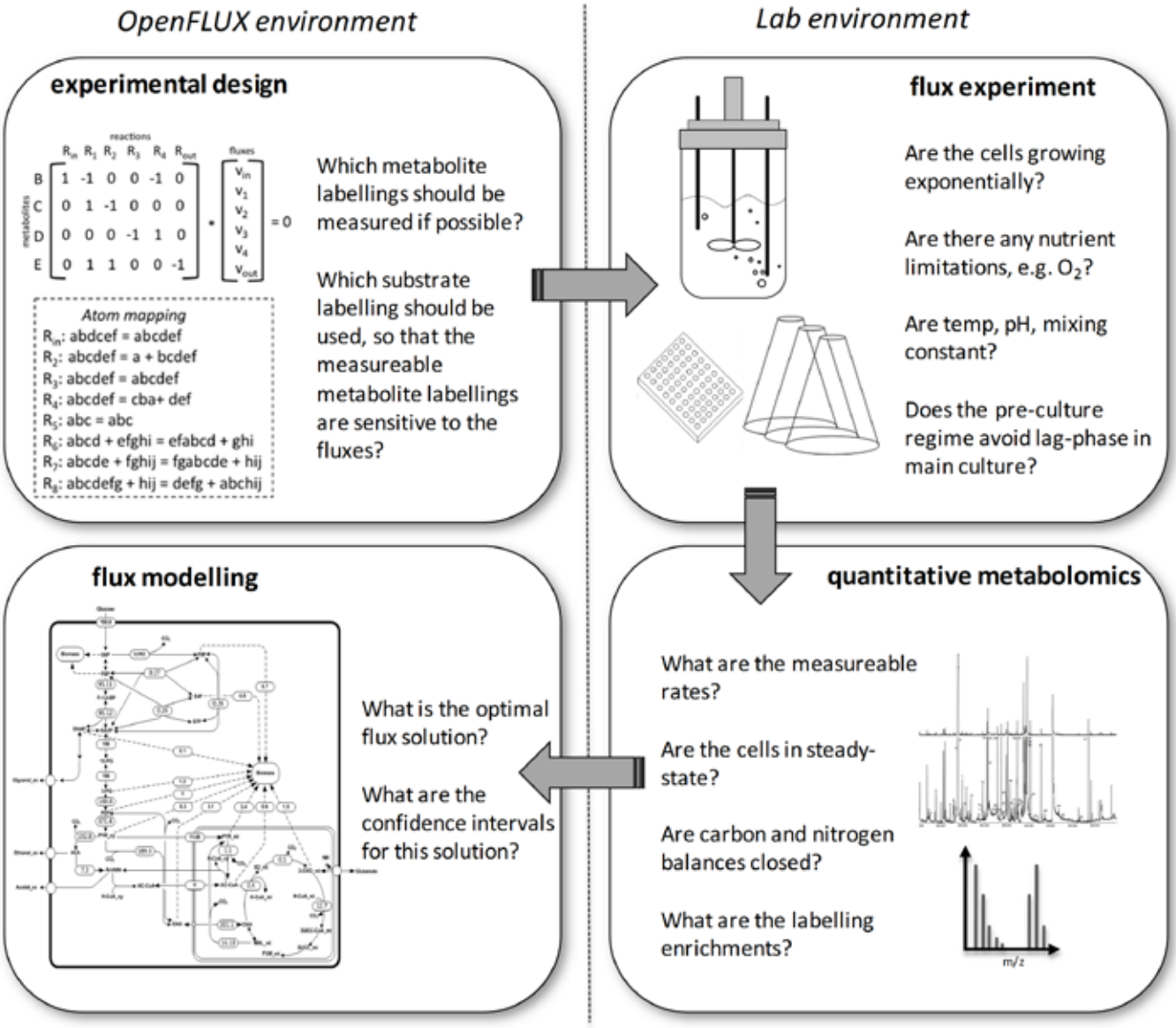

Figure 1. The four phases of ^{13}C metabolic flux analysis and essential questions to be answered in each phase. The simulations can be performed in the OpenFlux environment [15].

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Biography

In 2006, **Jens O Krömer** received a PhD in the area of Systems Biotechnology from the Saarland University, Germany. His thesis on the L-methionine production in *Corynebacterium glutamicum* led to a number of publications but also significantly contributed to three international patents filed by the commercial project partner BASF. His thesis was recognized with the Dr-Eduard-Martin-Preis in 2007, an award for the best PhD thesis in the faculty.

Since his arrival at University of Queensland (UQ) in October 06, Jens focusses on the development of systems biological tools, especially metabolomics and fluxomics, as platform technologies and the adaptation to industrially relevant processes. Together with Prof Lars Nielsen, he established a critical mass in the area of metabolomics, fluxomics and *in-silico* analysis of industrial microbes at the UQ. As part of his role in establishing this research field at UQ he designed and until recently managed the NCRIS 5.1 funded Queensland Node of Metabolomics Australia. This state-of-the-art analytical facility provides non-targeted metabolite profiling, quantitative analysis of metabolites in various biological matrices and supports ^{13}C fluxomics in collaborative projects.

Retirement of Associate Professor David Ellis from the Editorial Board

Associate Professor David Ellis is Head of the Mycology Unit at SA Pathology, Women's and Children's Hospital, Adelaide and an Associate Professor in the School of Molecular and Biomedical Science at the University of Adelaide. He graduated from La Trobe University Botany Department with BScHons, MSc and PhD in mycology and has been in charge of the Medical Mycology Unit for the past 33 years. David is an Honorary Fellow of the Royal College of Pathologists of Australasia, and a Fellow and past President of the Australian Society for Microbiology. He has also served as General Secretary and is the current President of the International Society for Human and Animal Mycology. His current research interests include the epidemiology and ecology of medically important fungi, especially *Cryptococcus*, fungal taxonomy and antifungal susceptibility testing. David is also actively involved in the teaching of medical mycology and is a recipient of the Australian Society for Microbiology David White Teaching Award and the Australasian College of Tropical Medicine Ashdown Medal. David has been a member of the Editorial Board of *Microbiology Australia* since 1998. He will retire on 11 November 2011. We wish him all the best for the future.