InSight Out: Advances in Understanding Metabolism Achieved by High-Throughput Mass Spectrometry

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Understanding metabolism is of great interest in various research areas since metabolism is the functional interconnection between the genome, transcriptome and proteome with the cellular phenotype. Here we frame, based on recent advances in elucidating various nodes of metabolism, a global perspective of our current understanding of metabolism.

Understanding metabolism to an extent that allows prediction and rational design of metabolic behavior is the key goal in various disciplines ranging from bioengineering to targeted drug design. Yet the extracellular uptake and secretion rates as such are too general a readout since various intracellular alterations can lead to the same macroscopic pattern. Thus, inside the cell, the interplay between proteins and metabolites, resulting in flux through distinct metabolic pathways, needs to be quantified in a first step and integrated in a second step to understand regulation and control of metabolism.

Targeting metabolism for the purpose of modulating cellular physiology opened in the last years to decades, profound new ways to address metabolic engineering or disease treatment challenges. Especially, the high-throughput generation of data on the metabolic entities namely enzymes, metabolites and metabolic fluxes defining the operation mode of a cell allowed for the first time a systematic quantification of *metabolic entities*, the integration of such to themselves and to upstream regulatory events to identify *metabolic regulation* and finally the unraveling of *metabolic interactions* between chemical compounds and cellular metabolism or the cross talk of the cellular metabolism from different species being in a symbiotic or pathogenic relation (Figure 1).

Especially mass spectrometry techniques enabled such advances because mass spectrometry is a comparable cheap, highly sensitive, and high-throughput method to quantify enzyme amounts/modifications, metabolite concentrations and flux through the metabolic pathways (Box1). In this review we focus on the insight we gained on metabolism by applying mass spectrometry methods and the advancement such insights have on biotechnology (microbial metabolism) and bio-medical (mammalian metabolism) research.

Metabolic entities

Through which biochemical reaction network is the metabolic operation mode of an organism conducted? This is a key question in various research fields ranging from metabolic engineering to medical biology. While high-throughput genome sequencing enabled the genome scale reconstruction of metabolic networks for various organisms, the activity of the annotated pathways is often unclear or only supported by microarray data, which are commonly none correlated with enzymatic capacities or metabolic pathway flux being the final readout of the metabolic network operation.

Fluxes through metabolic pathways of bio-engineering relevant microbes were and are identified, confirmed and quantified based on mass spectrometry inferred ^{13}C flux analysis. Fuhrer *et al.*¹ compared laboratory strains of *E. coli* and *B. subtilis* to seven metabolically and phylogenetically distinct non-laboratory species. Tang *et al.*², investigated active metabolic pathways in *S. oneidensis*, an organism which is widely used in the microbial fuel cell field, under various growth conditions. Moreover, several groups³⁻⁵ quantified metabolic fluxes in a wide range of yeast species which displayed various degrees of glucose repression. The global conclusion all these groups reached was that although the common metabolic pathways of central carbon metabolism were used by all the tested organisms and in various conditions, the magnitude of flux through them differed greatly. This finding shows the continuing need of determining metabolic fluxes but also the great potential of changing the metabolic operation mode of a cell by redirecting flux.

Especially, in the biomedical research field the concept of rationally designed drugs is the challenge for the future. Using mass spectrometry based ^{13}C flux analysis and metabolite profiling enabled the endeavor of identifying differential pathway usage between healthy and diseased cells, as a first step towards rational drug design. A differential biochemical reaction was found in glioma tumors⁶ or leukemia⁷ tumors harboring a isocitrate dehydrogenase mutation producing the so called onco-metabolite 2-hydroxyglutarate from alpha-ketoglutarate⁶. Inhibiting the production of 2-hydroxyglutarate is thereby a promising strategy to target the oncogenic transformation of isocitrate dehydrogenase mutation associated glioma tumors or leukemia. Moreover, differentially active metabolic

pathways were found in breast (3-Phosphoglycerate dehydrogenase/serine biosynthesis pathway)^{8,9} and lung (glycine decarboxylase/glycine biosynthesis pathway)¹⁰ cancers, and as a general cancer feature (pyruvate kinase isoform 2/glycolysis)¹¹. In the case of pyruvate kinase isoform 2, such findings led to the development of isoform specific inhibitors potentially useful for cancer treatment^{12,13}.

Metabolic regulation

While the large scale identification and quantification of the metabolic entities through mass spectrometry based methods have certainly led to advances in targeted engineering of cellular metabolism and novel drug target discovery, ultimately the regulatory principles underling the metabolic responses need to be revealed to enable a targeted modulation of the metabolic operation mode. In this challenge an important step is to functionally relate the different metabolic entities to each other. While metabolic profiles have been found to be largely organism specific rather than flux magnitude specific¹⁴ a general principle does exist for the relation between enzyme capacity alterations and metabolite concentration changes. Metabolite concentrations in central carbon metabolism are normally close to the K_m values of the catalyzing enzymes allowing reversibility of metabolic reactions¹⁵. Thereby it has been found that metabolic homeostasis can be kept by an inverse relationship between metabolite concentrations and enzyme activity, *i.e.* decreased enzymatic activity is buffered by an increased substrate metabolite pool size to keep metabolic fluxes as constant as possible¹⁶. Thus, this identifies an inverse enzyme capacity – metabolite concentrations modulation as general functional relation between both entities.

In the bio-engineering field the adaption processes of microbes to changing environments are of special interest. Thereby, the adaption processes to variations in glucose concentrations, carbon source switches, and the balance between nitrogen and carbon metabolism for coordinated proliferation, is most important. Mass spectrometry based metabolite profiling revealed that these processes are regulated differentially on the metabolic, transcriptional, and post-transcriptional level. The balancing of nitrogen and carbon metabolism in various environments is controlled in *E. coli* by alpha-ketoglutarate concentrations¹⁷. Alpha-ketoglutarate accumulates during nitrogen limitation since its

conversion to glutamate is limited by nitrogen availability. As a consequence alpha-ketoglutarate directly blocks glucose uptake by inhibiting enzyme I, the first step of the sugar-phosphoenolpyruvate phosphotransferase system, allowing immediate responses to changing nitrogen availability¹⁷. A second line of balancing carbon limitation and nitrogen metabolism in *E.coli* is adenylation¹⁸, a so far less investigated post-transcriptional modification. Adenylation was also found to prevent depletion of alpha-ketoglutarate (substrate for glutamine production via glutamate) in carbon limiting conditions by keeping the alpha-ketoglutarate to glutamate ratio constant¹⁸. Robustness of this systems is achieved by the avidity of a bifunctional enzyme adenylyltransferase to its multimeric substrate glutamine synthetase¹⁸.

Rapidly adaption to changing concentrations of glucose¹⁷ was found to be regulated in *E.coli* by fructose-1,6-bisphosphate levels. Fructose-1,6-bisphosphate is an allosteric activator of phosphoenol pyruvate carboxylase, thus upon depletion of glucose fructose-1,6-bisphosphate, phosphoenol pyruvate carboxylase activity subsequently decreases and the phosphoenol pyruvate pool size increases¹⁹. This buildup of phosphoenol pyruvate is essential for a rapid resumption of glucose metabolism as soon as the substrate is available by so far not understood process¹⁹.

The regulation of a carbon source switch was investigated in *B. subtilis*. It was found that adaption to malate from glucose as a carbon source was rapid and primarily controlled post-transcriptionally compared with the slow, mainly transcriptionally controlled adaptation to glucose from malate as a carbon source²⁰. Interestingly it has been found that transcriptional regulation in *S. cerevisiae* and *E. coli* has only a limited impact on relative metabolic pathway activity^{21,22} if the cell is adapted to the provided carbon source or stress condition. In both organisms transcriptional control is largely limited to the switch between respiration and fermentation^{21,22}. Thus, regulation of metabolic functions is very diverse regarding dynamic manipulations of nutrients but seem to follow general principles when looking at the adapted situation to a certain nutrient source or stress condition.

In mammalian cells so far no systematic study exists that infers the extent to which metabolism is controlled on the metabolic level, by transcription factors, or by post-

transcriptional regulation. However, several interactions between signaling events and metabolism are known^{23,24}, these include e.g. the protein kinases AKT²⁵ and mTOR²⁶, the transcription factors MYC²⁷ and p53²⁸, or the apoptosis regulator Bcl-2²⁹; acetylation to regulate metabolism has also gained great attention recently³⁰. Here, we would like to highlight two examples regarding the regulation of metabolism in mammalian cells that are highly relevant for developing new strategies for cancer treatment.

The main carbon sources of mammalian cells are glucose and glutamine, under standard oxygenated conditions fatty acids are produced mainly from glucose. However, Metallo *et al.*³¹ and Wise *et al.*³² found that HIF1 α stabilization, triggered through genetic loss of VHL or through hypoxia, induces the switch from glucose to glutamine as the main carbon source for fatty acid production. While certainly the HIF1 α target pyruvate dehydrogenase kinase 1 is involved in this regulatory process³¹, the overall principle is not entirely clear yet.

mTOR is a major proliferation regulator in mammalian cells. Ye *et al.*³³ found that the non-essential amino acid serine is a regulator of mTOR activity and that cancer cells achieve high levels of serine biosynthesis to sustain proliferation signals through mTOR due to an allosteric link between serine and pyruvate kinase isoform 2. When serine levels are low³³, pyruvate kinase isoform 2 activity is reduced leading to an accumulation of upstream metabolites which shunts metabolites into the serine biosynthesis pathway³³. Both examples open the idea of drugs targeting intrinsic metabolic network properties rather than focusing on signaling pathways that might lead to extensive off-target effects.

Metabolic interaction

Especially in the field of drug development, the influence of drugs or host-pathogen relationships on metabolic interactions is of special interest. Global questions that can be addressed by mass spectrometry based metabolic analysis are pharmacodynamics and the potential of host cell adaptations during infection for drug treatment. For example, trimethoprim is an antibiotic that inhibits bacterial dihydrofolate reductase, however it was shown that folylpoly-gamma-glutamate synthetase is also inhibited by the drug³⁴. This second inhibition is not a direct interaction but results from accumulation of the dihydrofolate reductase substrate dihydrofolate, which in turn potently inhibits folylpoly-

gamma-glutamate synthetase³⁴. Identification of this second indirect target does explain the complex drug dynamics³⁴ and could lead to an improved patient dosing strategy. Vastag et al. investigated the metabolic responds of mammalian cells to human cytomegalovirus and herpes simplex virus type-1³⁵. Although both viral infections are commonly treated with drugs targeting nucleotide metabolism, their work shows that this seems efficient for herpes simplex virus type-1 but not human cytomegalovirus³⁵. However, due to the metabolic rearrangements induced by human cytomegalovirus targeting fatty acid synthesis in this case seems promising³⁶. Thereby, such studies on host-pathogen interactions open complete new areas to target viral but potentially also bacterial infections.

The task of understanding metabolism by quantifying metabolic entities, identifying metabolic control and revealing metabolic interactions has made dramatic progress in the last decade, obviously not only, but to a substantial amount due to advances in high-throughput mass spectrometry measurements. Yet, while quantification of metabolic entities can be nowadays largely considered as a standard tool for investigating metabolism in the organism of interest, the challenges are to gain further knowledge on the regulation of metabolism and the interactions of metabolism with drugs or pathogenic organisms. Development of comprehensive mechanistic descriptions with generally applicable principles, explaining when metabolic alterations occur and how they can be specifically manipulated, are the tasks of tomorrow.

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Box1: 'omics' data

Proteomics

The proteome describes the entirety of all expressed proteins under a certain condition. Yet the field of proteomics, unlike metabolomics or fluxomics, includes not only the quantification but also the interactions and modifications of proteins³⁷⁻⁴¹. Mass spectrometry based proteomics generally divides into shotgun proteomics versus target proteomics; using shotgun proteomics the whole mass range is scanned, while for the targeted approach only specific masses are monitored. In both methods proteins are first digested into peptides which are separated by liquid chromatography and then quantified based on the mass of protein specific peptides, or in the case of modifications based on the peptide mass with and without modification.

Metabolomics

The metabolome is the entirety of all small molecules that are not encoded by genes but produced in metabolic reactions^{42,43}. The field of metabolomics deals with the quantification of ideally all metabolites that are present in an organism under distinct conditions^{44,45}. One big challenge in metabolomics, that distinguishes it from other omics technologies, is that metabolites belong to various chemical classes such as organic acids, sugar phosphates or amino acids. Thus, multiple methods and variations of methods are needed to measure all metabolites that occur. Using mass spectrometry based detection, the metabolites are normally separated first by liquid or gas chromatography before they are fragmented and detected in the mass spectrometer. Either the whole mass range is scanned (untargeted method) or only the specific masses resulting from the metabolites of interest are scanned (targeted method). Moreover, some metabolite profiling methods rely solely on mass spectrometry identification without any upstream chromatography method which increases the throughput dramatically, but resolution is lost for metabolites with the same mass and fragmentation pattern.

Fluxomics

Using metabolic flux analysis, the *in vivo* metabolic fluxes (net fluxes) in metabolism are estimated^{44,46-48}. In contrast to protein abundances or metabolite concentration, *in vivo* metabolic fluxes cannot be measured directly but must be inferred indirectly via the

stoichiometric network, the extracellular rates, and the intracellular information about the used metabolic pathways^{47,49}. Already with knowledge about the stoichiometric network and the extracellular rates some *in vivo* fluxes can be estimated with mass balancing^{50,51}. The coverage of flux estimations that are based on the stoichiometric network and extracellular rates can be enhanced if several assumptions (e.g. about cofactors) are made since several alternative and converging pathways exist in metabolism. To decrease the number of assumptions necessary to calculate fluxes one can measure experimentally which pathways were used based on stable mass isotopomer tracer (mainly ¹³C) experiments^{44,47,48}. To infer alternative pathways in central carbon metabolism a ¹³C labeled substrate is fed and metabolized by differing enzymes of the alternative metabolic pathways leading to branch point metabolites with ¹³C labeling patterns that reflect the usage of the alternative pathways.

Figure1

The task to understand metabolism can be dissected into three layers, *i*) quantification of the metabolic entities (metabolite concentrations, enzyme amount/activities, flux magnitude), *ii*) elucidation of metabolic control (metabolic, transcriptional, post-transcriptional level), and *iii*) identification of metabolic interactions (drug, pathogen, cell-cell interactions). All the described layers are highly interconnected and their integrated responses lead to the macroscopic metabolic phenotype.

1. Fuhrer, T., Fischer, E. & Sauer, U. Experimental Identification and Quantification of Glucose Metabolism in Seven Bacterial Species. *Journal of Bacteriology* **187**, 1581-90 (2005).
2. Tang, Y.J., Hwang, J.S., Wemmer, D.E. & Keasling, J.D. Shewanella oneidensis MR-1 fluxome under various oxygen conditions. *Applied and Environmental Microbiology* **73**, 718-29 (2007).
3. Fiaux, J. *et al.* Metabolic-Flux Profiling of the Yeasts *Saccharomyces cerevisiae* and *Pichia stipitis* *Eukaryotic Cell* **2**, 170-80 (2003).
4. Cannizzaro, C., Christensen, B., Nielsen, J. & von Stockar, U. Metabolic network analysis on *Phaffia rhodozyma* yeast using ¹³C-labeled glucose and gas chromatography-mass spectrometry. *Metabolic Engineering* **6**, 340-51 (2004).

5. Blank, L.M., Lehmbeck, F. & Sauer, U. Metabolic-flux and network analysis in fourteen hemiascomycetous yeasts. *FEMS Yeast Research* **5**, 545-58 (2005).
6. Dang, L. *et al.* Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. *Nature* **462**, 739-44 (2009).
7. Ward, P.S. *et al.* The common feature of leukemia-associated IDH1 and IDH2 mutations is a neomorphic enzyme activity converting alpha-ketoglutarate to 2-hydroxyglutarate. *Cancer Cell* **17**, 225-34 (2010).
8. Locasale, J.W. *et al.* Phosphoglycerate dehydrogenase diverts glycolytic flux and contributes to oncogenesis. *Nature Genetics* **43**, 869-874 (2011).
9. Possemato, R. *et al.* Functional genomics reveal that the serine synthesis pathway is essential in breast cancer. *Nature* **476**, 346-50 (2011).
10. Zhang, W.C. *et al.* Glycine decarboxylase activity drives non-small cell lung cancer tumor-initiating cells and tumorigenesis. *Cell* **148**, 259-72 (2012).
11. Christofk, H.R. *et al.* The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. *Nature* **452**, 230-3 (2008).
12. Vander Heiden, M.G. *et al.* Identification of small molecule inhibitors of pyruvate kinase M2. *Biochemical Pharmacology* **79**, 1118-24 (2010).
13. Chen, J. *et al.* Shikonin and its analogs inhibit cancer cell glycolysis by targeting tumor pyruvate kinase-M2. *Oncogene* **30**, 4297-306 (2011).
14. Christen, S. & Sauer, U. Intracellular characterization of aerobic glucose metabolism in seven yeast species by ¹³C flux analysis and metabolomics. *FEMS Yeast Research* **11**, 263-72 (2011).
15. Bennett, B.D. *et al.* Absolute metabolite concentrations and implied enzyme active site occupancy in *Escherichia coli*. *Nature Chemical Biology* **5**, 593-9 (2009).
16. Fendt, S.-M. *et al.* Tradeoff between enzyme and metabolite efficiency maintains metabolic homeostasis upon perturbations in enzyme capacity. *Molecular Systems Biology* **6**, 356 (2010).
17. Doucette, C.D., Schwab, D.J., Wingreen, N.S. & Rabinowitz, J.D. α -ketoglutarate coordinates carbon and nitrogen utilization via enzyme I inhibition. *Nature Chemical Biology* **7**, 894-901 (2011).
18. Hart, Y. *et al.* Robust control of nitrogen assimilation by a bifunctional enzyme in *E. coli*. *Molecular Cell* **41**, 117-27 (2011).
19. Xu, Y.-F., Amador-Noguez, D., Reaves, M.L., Feng, X.-J. & Rabinowitz, J.D. Ultrasensitive regulation of anapleurosis via allosteric activation of PEP carboxylase. *Nature Chemical Biology* **8**, 562-8 (2012).
20. Buescher, J.M. *et al.* Global network reorganization during dynamic adaptations of *Bacillus subtilis* metabolism. *Science* **335**, 1099-103 (2012).
21. Fendt, S.-M. *et al.* Unraveling condition-dependent networks of transcription factors that control metabolic pathway activity in yeast. *Molecular systems biology* **6**, 432 (2010).
22. Haverkorn van Rijsewijk, B.R.B., Nanchen, A., Nallet, S., Kleijn, R.J. & Sauer, U. Large-scale ¹³C-flux analysis reveals distinct transcriptional control of respiratory and fermentative metabolism in *Escherichia coli*. *Molecular Systems Biology* **7**, 477 (2011).
23. Cairns, R.a., Harris, I.S. & Mak, T.W. Regulation of cancer cell metabolism. *Nature Reviews. Cancer* **11**, 85-95 (2011).
24. Jones, R.G. & Thompson, C.B. Tumor suppressors and cell metabolism: a recipe for cancer growth. *Genes & Development* **23**, 537-48 (2009).
25. Hers, I., Vincent, E.E. & Tavaré, J.M. Akt signalling in health and disease. *Cellular Signalling* **23**, 1515-27 (2011).
26. Zoncu, R., Efeyan, A. & Sabatini, D.M. mTOR: from growth signal integration to cancer, diabetes and ageing. *Nature Reviews. Molecular Cell Biology* **12**, 21-35 (2011).

27. Dang, C.V. Rethinking the Warburg effect with Myc micromanaging glutamine metabolism. *Cancer Research* **70**, 859-62 (2010).
28. Maddocks, O.D.K. & Vousden, K.H. Metabolic regulation by p53. *Journal of Molecular Medicine* **89**, 237-45 (2011).
29. Krishna, S., Low, I.C.C. & Pervaiz, S. Regulation of mitochondrial metabolism: yet another facet in the biology of the oncoprotein Bcl-2. *The Biochemical Journal* **435**, 545-51 (2011).
30. Wellen, K.E. & Thompson, C.B. A two-way street: reciprocal regulation of metabolism and signalling. *Nature Reviews. Molecular Cell Biology* **13**, 270-276 (2012).
31. Metallo, C.M. *et al.* Reductive glutamine metabolism by IDH1 mediates lipogenesis under hypoxia. *Nature* **481**, 380-4 (2011).
32. Wise, D.R. *et al.* Hypoxia promotes isocitrate dehydrogenase-dependent carboxylation of α -ketoglutarate to citrate to support cell growth and viability. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 19611-6 (2011).
33. Ye, J. *et al.* Pyruvate kinase M2 promotes de novo serine synthesis to sustain mTORC1 activity and cell proliferation. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 6904-9 (2012).
34. Kwon, Y.K. *et al.* A domino effect in antifolate drug action in Escherichia coli. *Nature Chemical Biology* **4**, 602-8 (2008).
35. Vastag, L., Koyuncu, E., Grady, S.L., Shenk, T.E. & Rabinowitz, J.D. Divergent effects of human cytomegalovirus and herpes simplex virus-1 on cellular metabolism. *PLoS Pathogens* **7**, e1002124 (2011).
36. Munger, J. *et al.* Systems-level metabolic flux profiling identifies fatty acid synthesis as a target for antiviral therapy. *Nature Biotechnology* **26**, 1179-86 (2008).
37. Aebersold, R. & Mann, M. Mass spectrometry-based proteomics. *Nature* **422**, 198-207 (2003).
38. Cox, J. & Mann, M. Quantitative, high-resolution proteomics for data-driven systems biology. *Annual Review of Biochemistry* **80**, 273-99 (2011).
39. Gstaiger, M. & Aebersold, R. Applying mass spectrometry-based proteomics to genetics, genomics and network biology. *Nature Reviews. Genetics* **10**, 617-27 (2009).
40. Kirkpatrick, D.S., Denison, C. & Gygi, S.P. Weighing in on ubiquitin: the expanding role of mass-spectrometry-based proteomics. *Nature Cell Biology* **7**, 750-7 (2005).
41. Huang, P.H. & White, F.M. Phosphoproteomics: unraveling the signaling web. *Molecular Cell* **31**, 777-81 (2008).
42. Dunn, W.B. Current trends and future requirements for the mass spectrometric investigation of microbial, mammalian and plant metabolomes. *Physical Biology* **5**, 011001 (2008).
43. Mapelli, V., Olsson, L. & Nielsen, J. Metabolic footprinting in microbiology : methods and applications in functional genomics and biotechnology. *Trends in Biotechnology* **26**, 490-7 (2008).
44. Zamboni, N. & Sauer, U. Novel biological insights through metabolomics and ¹³C-flux analysis. *Current Opinion in Microbiology* **12**, 553-8 (2009).
45. Reaves, M.L. & Rabinowitz, J.D. Metabolomics in systems microbiology. *Current Opinion in Biotechnology* **22**, 17-25 (2011).
46. Nielsen, J. It Is All about Metabolic Fluxes. *Journal of Bacteriology* **185**, 7031-7035 (2003).
47. Sauer, U. Metabolic networks in motion: ¹³C-based flux analysis. *Molecular Systems Biology* **2**, 62 (2006).
48. Wiechert, W. ¹³C metabolic flux analysis. *Metabolic Engineering* **3**, 195-206 (2001).
49. Stephanopoulos, G. Metabolic fluxes and metabolic engineering. *Metabolic Engineering* **1**, 1-11 (1999).
50. Covert, M.W. *et al.* Metabolic modeling of microbial strains in silico. *Trends in Biochemical Sciences* **26**, 179-86 (2001).

51. Varma, A. & Palsson, B.O. Metabolic Flux Balancing: Basic Concepts, Scientific and Practical Use. *Nature Biotechnology* **12**, 994-998 (1994).