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Flux an important, but neglected, component of functional genomics

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Genomics approaches aimed at understanding metabolism currently tend to involve mainly expression profiling, although proteomics and steady-state metabolite profiling are increasingly being carried out as alternative strategies. These approaches provide rich information on the inventory of the cell. It is, however, of growing importance that such approaches are augmented by sophisticated integrative analyses and a higher-level understanding of cellular dynamics to provide insights into mechanisms that underlie biological processes. We argue the need for, and discuss theoretical and practical aspects of, the determination of metabolic flux as a component of functional genomics.

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Introduction

Recent technical advances have transformed biology by accelerating data acquisition to the point where it is often no longer limiting and provides systems-wide information. The most frequently adopted approaches, transcriptomics and proteomics, provide rich information on levels of mRNA and proteins within tissues, respectively. Similarly, developments in metabolite-profiling strategies are currently providing important information about how steady-state metabolite levels respond to genetic, physiological or environmental perturbation [1,2]. Several recent reviews have documented technical aspects of steady-state metabolite profiling [1,3•,4,5•] and have detailed its utility in diagnostics, network analysis and the analysis of gene function [3•,6], Data-driven informatics methods that allow visualization, reduction and comparisons of genomic information are proving invaluable in diagnostics and in the identification of gene function [3•,4,5•]. Such approaches provide global datasets that facilitate the generation of hypotheses about system properties as diverse as gene annotation, physiological regulation and molecular interactions. The resultant hypotheses, as well as those arising from the theoretical modelling of metabolism, require direct testing in a second experimental phase. To gain deeper insights into metabolic regulation, however, these inventories of steady-state mRNA, protein and metabolite contents must be supplemented by sophisticated integrative analyses and a higher-level understanding of cellular dynamics [7,8•]. This review focuses on the measurement of metabolic flux. This is rarely attempted, but is essential to place the static data from transcript and metabolite profiling in a dynamic context. We argue that, in many situations, such information is far more valuable than steady-state determinations.

The need for dynamism

The measurement of steady-state metabolite levels provides clues about the metabolic activity of a tissue and about the metabolic response of an organism to environmental or genetic perturbation [1,2,5•,6]. As the majority of metabolites determined by current metabolite-profiling techniques are not end-products but intermediates of metabolic pathways, these approaches are generally incapable of resolving issues of metabolic dynamics. It has previously been suggested that changes in enzyme activity will have a greater impact on steady-state metabolite levels than on fluxes, and that the measurement of metabolite levels is therefore preferable [9,10]. This property of metabolic regulation was utilized in a proof-of-concept study by Raamsdoonk *et al*. [10]. These researchers performed metabolite-profiling studies on a range of yeast mutants and looked for patterns of co-response between the metabolites; they then used this information to classify 'similar' mutants [9]. There are several examples in plants (and other organisms), however, in which fluxes through a pathway change without corresponding changes in the levels of the metabolic intermediates or even the products of the pathway. One striking example is that the rate of photosynthesis can change several-fold without detectable changes in the levels of metabolites in the Calvin cycle [11]. This occurs because sophisticated regulatory mechanisms allow dramatic changes in the momentary activity of enzymes of the Calvin cycle in response to small changes in the levels of substrates and in the availability of ATP and reducing equivalents. Two recent examples are the increased flux through the sucrose-to-starch transition in the potato tuber following incubation in the sucrose analogue palatinose [12], and the increase in flux to starch without an

increase in starch accumulation following the overexpression of a bacterial ADP-glucose pyrophosphorylase (AGPase) in potato tubers [13]. Although both examples demonstrate that measuring the steady-state metabolite levels can be misleading, the underlying reasons are distinct. The first implies a coordinated increase in flux throughout the pathway without elevation in the pool sizes themselves, whereas the latter was caused by an equal and opposite increase in the rate of starch turnover.

Getting to the crux: general strategies to measure fluxes, and their limitations

Several different approaches have been used to monitor flux through metabolic pathways, including the measurement of the rate of depletion of the initial substrate, the rate of accumulation of the end product, and isotope labelling of metabolites with radioactive or stable isotopes.

Measurements of steady-state metabolite levels can only be used to determine fluxes when the metabolite in question is a genuine starting substrate or end product, and when it is not produced or removed by any other process. It is extremely dangerous to try to estimate fluxes from changes in the levels of metabolic intermediates. These levels often change reciprocally to flux, because enzymes upstream or downstream of them have been activated or deactivated. Measurement of the levels of metabolic intermediates is actually one of the classic strategies to identify steps at which flux is regulated (see [14]). A recent example of this is provided by Tiessen *et al.* [15]. In this study, radioactive-labelling methods were used to show that the detachment of growing tubers from the mother potato plant leads to a rapid decrease in the rate of starch synthesis. Systematic measurements of metabolites levels were used to show that flux was decreased because of the inhibition of AGPase. Levels of all the metabolites upstream of this enzyme in the pathway of starch synthesis, including hexose phosphates, increased upon the detachment of growing tubers. This example illustrates that a combination of measurements of metabolite levels and flux provides far more information than the measurement of fluxes or metabolites on their own.

Methods that use isotopes are essential for the analysis of complex (which means almost all) metabolic networks. Pathways frequently branch so that several products are formed from one substrate. It is also typically possible to generate a given product from several alternative substrates. Indeed, one of the key questions in the analysis of metabolism is to define if and how the patterns of flux change in these highly interactive networks. However, the use of isotope-labelling is complicated by problems of experimental design, including the not-so-trivial question of how to introduce the tracer without altering the system, and by complications in data evaluation related to the dilution of the added isotope by internal pools of unlabeled substrates. We return to these problems later.

A more general obstacle when attempting to infer dynamics from metabolite measurements or labelling experiments is the fact that such inferences are based on the assumption that pathway structure has been established. In reality, this is true of only a few central pathways of plant primary metabolism, and even fewer in secondary metabolism. Definition of the pathways in a network is therefore a prerequisite to measuring the fluxes in the network. One of the principal methods for flux analysis, the use of isotope tracers, has historically proven very effective in the elucidation of pathway structure [16–18]. The development of more-sensitive nuclear magnetic resonance (NMR) and mass-spectrometry (MS) instrumentation is driving a renaissance in this type of analysis [19,20••,21,22••].

An essential prerequisite: determining the structure of metabolic pathways

The use of isotope labelling for the structural elucidation of metabolic pathways is a classical biochemical technique that was employed to a great extent throughout the last century to identify major pathways in plant primary metabolism. Important examples include, but are by no means limited to, the elucidation of the Calvin cycle [16] and the plant pathway for ascorbate biosynthesis [18]. The latter of these illustrates the importance of the biochemical genetic approach in defining metabolic pathways, an approach that has been utilized in several recent studies (see also [21]). The general principal of such approaches is to label a precursor metabolite and to trace the isotope signature through the intermediate metabolite pools that connect precursor(s) and end product(s) of metabolic pathways. At an even more fundamental level, the tracer technique has also been employed to great effect to confirm that metabolites detected in plant extracts were truly of plant origin (e.g. [17]). An alternative approach is that developed by Schuster and coworkers [23], whereby metabolic models are constructed *in silico* in which reactions are described purely in terms of their stoichiometry. It seems likely that, in the future, a combination of experimental and bioinformatics approaches will be necessary to elucidate, or even merely to clarify, metabolic pathway or network structures. Indeed, the first example of the use of such a combined approach in plants has recently been published [22••], and is described in detail later in this review.

What is flux analysis and what does it tell us?

Before detailing how flux analysis can reveal the properties of metabolic regulation, we need to consider practical approaches to flux determination and the challenges that these present in more detail. One possibility for flux determination is to monitor the rate of substrate consumption, but this is a relatively insensitive and inaccu-

rate way to measure fluxes. As mentioned above, the other way to determine a flux is to measure the accumulation of end product over time. This approach is obviously limited to compounds that are not metabolised (or transported) themselves, and to pathways that are not too highly branched (e.g. measuring the CO_2-production rate of a tissue does not always reflect glycolytic flux). In addition, end-product accumulation is not always linear with time, which makes it necessary to restrict analysis to the time-intervals in which a linear relationship between product level and time has been demonstrated. Additional problems arise if fluxes are measured in a growing system. Given that these are huge restrictions, other methods are currently required for the evaluation of fluxes through most metabolic pathways or processes. These range in complexity from the physical measurement of gas exchange or assays of radiolabel transport, through radiolabel tracer experiments, to highly complex network analyses that involve the use of stable isotopes and gas chromatography (GC)-MS or NMR technologies. The level of cellular resolution involved varies from the whole plant down to the organelle [12,15,24,25,26•]. In this review, we concentrate mainly on experiments carried out on isolated organs or tissue slices, and return to whole-plant and subcellular fluxes later.

Practical approaches for flux determination

The simplest measurements of fluxes are most probably the characterisation of nutrient or gas exchange [27–31], in which the rate of the uptake of a radiolabel or the use of carbon dioxide can easily be monitored over time and under a range of environmental conditions. Similar approaches have been used to examine water use efficiency (e.g. [32]). The advantage in these techniques lies in their simplicity, which facilitates high levels of experimental replication and even, in the case of gas exchange studies, their use in the field. The use of flux to characterize the activities of transport proteins is essentially merely a specialized type of enzyme analysis, and we therefore do not describe it in detail here.

Analyses of other fluxes, with the exception of the more complex network-style analyses that rely on NMR or mass spectral quantification, and that are discussed in detail below, largely fit into one of two categories: analysis of end-product accumulation or analysis of flux towards metabolic end-products. As the flux to an end-product is defined as the rate of production of a metabolite over a given time interval, these approaches should give the same answer in the absence of anabolism of the end-product. In the case of potato starch accumulation, this often appears to hold true [33] (despite the fact that there is determinable turnover of starch in isolated tuber discs) but exceptions have already been found [13] and, furthermore, it is unlikely to be the case for every metabolite. For these reasons, a more reliable way of measuring flux is to follow the flow of atoms through metabolic systems using radioactive isotopes or stable isotopes of non-natural mass.

An obvious prerequisite of the tracing of isotope distribution is the ability to detect isotope enrichment in metabolic end-products and, ideally, also within metabolic intermediates. As the complexity of the metabolic system increases, the ability to measure isotope enrichment in intermediates becomes increasingly important. It is also the most effective method for the detection and correction of errors that arise due to isotope dilution when the isotopically labelled substrate is mixed with internal and unlabelled pools of metabolites.

Historically, this approach utilized radioactive isotopes. The metabolic fate of the supplied radiolabel was determined following enzymic and chromatographic fractionation of the metabolic components of plant tissues on the basis of chemical class. Although this method was of high value in determining the structural identity [16,18] and control points [33] of several important pathways of primary metabolism, the resolution it provides is insufficient to allow the evaluation of many other important pathways. The use of stable isotopes in tandem with NMR or mass spectral detection systems circumvents this problem because both methods can yield precise information on the compounds in question [4,34].

NMR and MS methods have distinct advantages and disadvantages with respect to each other for flux quantification. GC- and liquid chromatography (LC)-MS have far greater accuracy than NMR, allowing the measurement of a broader number of metabolites [4,34], and are not restricted in the choice of isotope. On the other hand, NMR has the advantage that it directly measures the properties of the atoms within molecules, and so it is able to provide information concerning positional labelling [22••,35•,36]. Positional-labelling information is exceedingly valuable because it can be used to discriminate and subsequently quantify cyclic, competing and reversible fluxes, which are prominent in plant metabolism [8•]. The methods used to determine these fluxes are extensively detailed elsewhere [8•,35•,36]. Although the use of stable isotopes and NMR probably represent the easiest experimental strategy that can be used to determine the rate of substrate cycling, it is important to note that feeding experiments in which single or multiple radiolabelled substrates were fed independently to plant tissues have yielded similar quantitative results [37–40]. Returning to stable-isotopes, it is also possible both to obtain positional information from GC-MS spectra (by analysing metabolite fragments) and, similarly, to get a broad coverage of primary metabolism by NMR approaches (see below). Moreover, as demonstrated by several comprehensive studies [22••,41], these techniques need not be regarded as being mutually exclusive.

Steady-state or kinetic modelling?

Irrespective of the experimental method used to follow the accumulation of end-product and/or labelled molecules, the experimentalist is faced with a fundamental question: should s/he assume that steady-state is obtained within the experimental time-frame or not? The assumption of steady-state is by no means a pre-requisite for modelling fluxes, but it is imperative that the assumption is empirically tested for every experimental system. For an experiment that monitors only end-product accumulation, it is relatively easy to perform serial sampling over time and to decide which type of modelling to apply after analysis of the data. In an experiment that is based on isotope labelling, however, further complexity is introduced by the addition of an extra variable, the equilibration of labelled and unlabelled molecules. This additional variable means that two steady states must be considered: metabolic steady state and isotopic steady state (see [35•,36,42] for examples). Given that long-term feeding experiments, which assume steady-state, were thoroughly reviewed last year [8•], we restrict our discussion here to two recent manuscripts that have employed this approach [22••,26•].

Sriram and co-workers [26•] took a bold approach to model the metabolism of the developing soybean embryo in a manner analogous to that used by microbial scientists. Their approach is based on the development and application of computer-aided metabolic flux analysis to probe the [^{13}C, ^{1}H]NMR spectra of seed storage protein and starch hydrolysates. Although this type of 'retrobiosynthetic' analysis has been performed previously in plants (e.g. [35•,42–44]), this was the first time that such a large-scale approach had been taken. In their model, Sriram *et al*. [26•] compute 80 metabolic fluxes on the basis of the summed dataset of 155 ^{13}C isotopomers (isotope isomers). This approach clearly has great potential and, given that its reliance on NMR data facilitated the acquisition of information concerning positional labelling, also reveals important information on the compartmentation of metabolism. The authors claim that this approach can be scaled up to high-throughput, and hence it will surely be utilized to address important biological questions in the near future.

The elegant study of Schwender *et al*. [22••] has already demonstrated the involvement of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) in a previously undescribed metabolic context that increases the efficiency of carbon use during the formation of oil in developing green seeds of *Brassica napus* [22••]. This work is an extremely potent example of the value of dynamic analyses because it provided evidence of a powerful mechanism of metabolic regulation in a central metabolic pathway that could not be inferred from measurement of metabolite composition alone. Carbon utilization efficiency in seeds has been a puzzle for many years because the predominant flux of carbon is the conversion of sugars to fatty acids, and the subsequent storage of in excess of 60% of the carbon in the form of oil. The efficiency of this process has been questioned because one carbon is lost in the form of CO_2 for each triacylglycerol incorporated into fatty acids. Schwender *et al*. [22••] analysed the carbon utilization efficiency of *B. napus* by feeding the seeds with uniformly labelled carbon sources, and determining the conversion of these sources into oil, protein, carbohydrates and CO_2. The data obtained were then compared with that theoretically anticipated following elementary flux mode analysis of text-book pathways. Surprisingly, a 3:1 ratio of carbon in oil to carbon liberated as CO_2 was experimentally determined, which was much higher than the 2:1 ratio expected given the action of pyruvate dehydrogenase. Further flux experimentation utilizing [1-^{13}C] or [U-^{13}C]alanine revealed that Rubisco was responsible for the refixation of CO_2, and the absence of label randomisation demonstrated that this enzyme was acting in isolation of the Calvin cycle. In establishing this route, the authors were able to demonstrate that this route of acetyl coA production for fatty-acid biosynthesis was in fact more efficient than the glycolytic route, and that this pathway is responsible for 62% of 3-phosphoglycerate (PGA) production in *B. napus* [22••]. Such a conclusion could only be obtained by quantitative analysis of the relative fluxes. Even though it was known that the translation and transcription of the components of Rubisco were relatively high at this developmental stage, these facts alone could not demonstrate its activity, let alone its participation in the newly defined pathway.

The alternative to the approach used in the experiments described above is not to assume steady-state but rather to model the kinetics of changing pool sizes and labelling patterns. This method is particularly effective in situations in which only a short experimental period is desirable. For example, in potato tuber tissue, there is a very rapid wounding response such that fluxes in isolated tuber discs are dramatically altered within 5h [45,46•], and labelling to steady-state would therefore not give an accurate reflection of physiological fluxes. The kinetic modelling approach works on the principal that the 'average' labelling of an intermediary metabolite pool can be calculated by evaluating the kinetics of labelling of that pool. The flux can then be estimated by dividing the label accumulation in the end product by the proportional labelling of the precursor pool. This approach has been used frequently for interpretation of ^{14}C-feeding experiments in potato tubers [40,45], as well as in combined *in silico* and experimental approaches to model carbon-1 metabolism [47].

Recently, the use of GC–MS has allowed the expansion this approach to include the use of stable isotopes, dramatically increasing the complexity of metabolic net-

works that can be analysed. Roessner-Tunali *et al.* [46•] used stable isotopes to analyse 30 fluxes of primary metabolism in tuber discs that were isolated from wild-type potato and two well-characterised transgenic potato lines. This study, like that of Sriram *et al.* [26•], was essentially technical rather than biological, but some important conclusions could be made on the basis of the data collected. Perhaps most striking of these was the fact that Roessner-Tunali and colleagues were able to confirm the turnover of starch in the tuber. Although this had previously been inferred, on the basis of an increased flux to starch without a corresponding increase in its accumulation in transgenic lines displaying elevated AGPase activity [13], it could clearly be seen from the kinetic analysis of metabolite pool labelling. Furthermore, close inspection of the data reveals that the breakdown of starch dilutes the labelling of the glucose and glucose phosphate pools but not that of the maltose pool. This result is striking with respect to the recent clarification that starch degradation in the leaf yields maltose that is transported to the cytosol via a recently identified maltose transporter [48]. Another application of kinetic modelling allowed theoretical evaluation of the constraints of the pathway of glycine betaine biosynthesis in plants [47], and thereby predicted rational targets for the metabolic engineering of this compound in the hope of conferring stress tolerance. Despite the fact that the kinetic modelling approach is beginning to be used to interpret metabolic regulation, it is clear that the full utility of this approach to address biological questions has not yet been realized.

The examples detailed above concentrate on the simultaneous determination of multiple fluxes, but network analyses concerning a single flux have proven very effective in plants in categorizing reaction steps that are limiting to pathway flux [22••,33,47]. Recent examples suggest that this approach will be equally successful in the analysis of pathways of secondary metabolism [49,50,51•]. The targeted approach also remains highly useful in the determination of pathway structures or the quantitative importance of parallel routes to the same end. Two excellent examples of this approach have recently been published. First, the demonstration, by feeding experiments using deuterium-labelled water, that most carbon leaves chloroplasts at night as glucose, maltose or higher maltodextrins under normal conditions [52]. Second, the use of dideuteriated deoxyxylulose to distinguish the relative contributions of the two alternate pathways of isoprene biosynthesis facilitated the identification of the metabolic regulation of this pathway [51•]. We have recently covered the concepts involved in the targeted investigation of single pathways using starch accumulation in the potato tuber as a case study [33] and so we will not detail them further here, suffice to say that targeted analysis is often more revealing than the unbiased approach.

Spatial resolution of carbon fluxes

Most of the experiments on flux analysis mentioned above were performed in simplified systems, such as cell cultures, tissue slices or isolated organs, which allow the various isotopes to be supplied in an easy and reproducible way. However, these systems, in which the respective tissue has been removed from its *in-vivo* metabolic context, are to some extent artificial: typically, the connections to transport tissues including phloem and xylem are interrupted and internal gas concentrations are changed. One way to overcome this problem is to use a fine hypodermic needle to inject a ^{14}C-labelled tracer directly into the tissue that remains attached to the plant and to follow the metabolism of this tracer. This minimally invasive feeding approach has been used recently to determine the effect of oxygen on *in-planta* fluxes from sucrose to major storage products in growing potato tubers [53], wheat grains [25] and developing seeds of oil-seed rape [54•], and to investigate bottlenecks in the pathway of triacylglycerol synthesis *in planta* [55].

A further possibility for measuring carbon fluxes *in planta* is to feed radiolabelled CO_2 to leaves and to analyse the subsequent movement of labelled assimilates at the whole-plant and tissue levels. If $^{14}CO_2$ is used, this technique allows analysis of carbon flux through a whole plant, from CO_2 fixation and conversion to sucrose in photosynthesising leaves, through the subsequent translocation of sucrose via the phloem to various sink tissues, to the final use of sucrose for storage and growth. Sweetlove *et al.* [24] used this technique for top-down metabolic control analysis by investigating the regulation of carbon flow through potato plants during tuberization. ^{14}C was used in most of the strategies described above because it is a readily available and widely applicable tracer. The low-energy decay products of ^{14}C are difficult to detect, however, and the ^{14}C-tracer usually had to be measured by destructive harvesting of the plant, giving a practical limit to the data resolution in both time and space. A major advantage of short-lived, positron-emitting tracers, such as ^{11}C, is the intriguing possibility of measuring tracer movement *in planta*, providing a detailed on-line time series of tracer data in many locations and opening up powerful new techniques for data analysis [56]. Van Dongen *et al.* [25] used the ^{11}C-tracer technique to measure on-line carbon fluxes to developing wheat grains, and demonstrated that the prevailing low oxygen concentrations within these grains restrict sucrose unloading and affect assimilate transport throughout the entire plant.

Most pathways of carbon metabolism in plants involve more than one subcellular compartment. Therefore, the ability to measure the enrichment of the tracer in metabolic intermediates at a subcellular level is a prerequisite for calculations of absolute rates of carbon fluxes. Non-aqueous density centrifugation of plant material offers the

possibility of measuring subcellular metabolite concentrations. The method was first developed for leaves [57,58] and was recently adapted for use with potato tubers [59]. Tiessen *et al.* [15] used this technique in a systemic approach to measure changes in the subcellular levels of all intermediates in the sucrose-to-starch pathway in response to a change in the flux through this pathway. They were able to identify AGPase as the only site that regulates starch synthesis in response to sucrose. These analyses currently yield estimates for subcellular concentrations of metabolites for the vacuole, plastids and cytosol. The estimated values for the cytosol still include mitochondrial metabolites because it is not possible to separate these compartments using current methodology. In combination with stable isotope labelling and GC-MS or NMR technologies, the use of non-aqueous fractionation techniques will allow the measurement of carbon fluxes on a subcellular scale.

Several other recent technical developments will also aid the analysis of metabolism. These include the development of protocols that allow easier and more-sensitive ways of measuring metabolites [2,60], the recent development of more-sensitive techniques for tissue dissection [61], the development of highly sensitive nanosensor methods that function by fluorescing upon binding to metabolites [62], and the development of techniques that allow the measurement of selected metabolites on tissue slices [63]. By combining such approaches, it is becoming possible to achieve a better spatial analysis of metabolism in complicated organs, including seeds.

Conclusions

A further level of resolution that has recently received renewed attention is that of micro-compartmentation, that is, the existence of physical metabolic channels that allow the operation of metabolic pathways at high efficiency. The demonstration of protein–protein interactions or the co-localisation of pathway enzymes within specific regions of the cell give strong hints for micro-compartmentation but, on their own, such experiments lack proof of functionality. Tracing the metabolic fate of labelled substrates and appropriate co-factors to purified protein complexes allows assessment of their metabolic function and also their efficiency. The power of combining flux analysis with cell biology and subcellular biochemistry was recently demonstrated in studies that confirmed the functionality and efficiency of the enzymes of glycolysis that are associated with the outer mitochondrial membrane [64•]. This example is only one of those demonstrating that, despite the dramatic advances in metabolic flux analysis in plants, it is clear that metabolic regulation itself is best understood by looking at several system components simultaneously. Such analyses have been carried out in microbial and medical research for several years [65•,66,67,68•]. Several general features emerge from these integrated approaches namely that changes in enzyme activities often do not correlate with changes in transcript or protein levels, that a large proportion of metabolic control resides at post-translational levels, and that flux determinations often give results that could not be predicted from observed changes in transcript or protein levels. These factors all argue strongly for the widespread adoption of flux analysis, but the use of the integrated approach has also allowed the identification of several important mechanisms of metabolic regulation in these systems. Integrated genomic approaches that are beginning to be applied to plant systems [3•,69,70,71•] suggest that the features defined above are also characteristic of plant systems. Once available, the incorporation of data from flux [22••,26•,35•] and enzyme [72•] platforms should facilitate function elucidation in genomics strategies.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Fiehn O: **Metabolomics — the link between genotypes and phenotypes**. *Plant Mol Biol* 2002, **48**:155-171.

2. Roessner U, Luedemann A, Brust D, Fiehn O, Linke T, Willmitzer L, Fernie AR: **Metabolic profiling allows comprehensive phenotyping of genetically or environmentally modified plant systems**. *Plant Cell* 2001, **13**:11-29.

3. • Thimm O, Blasing O, Gibon Y, Nagel A, Meyer S, Kruger P, Selbig J, Muller LA, Rhee SY, Stitt M: **MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes**. *Plant J* 2004, **37**:914-939.

The authors describe the first build of software (http://gabi.rzpd.de/ImageAnnotator.html) that, through carefully curated assignment, allows visualisation of complex genomic datasets in a manner that allows facile identification of responses that are conserved or contrasting within and between pathways. This tool has primarily been used for transcriptomics to date but can also be used to place metabolite data within a wider context. The software can combine transcriptomics data with metabolite-profiling datasets and potentially with information about proteins and enzymes such as that obtained in [72•].

4. Sumner LW, Mendes P, Dixon RA: **Plant metabolomics: large-scale phytochemistry in the functional genomics era**. *Phytochemistry* 2003, **62**:817-836.

5. • Fernie AR, Trethewey RN, Krotzky AJ, Willmitzer L: **Metabolite profiling: from diagnostics to systems biology**. *Nat Rev Mol Cell Biol* 2004, **5**:763-769.

A recent review that provides an overview of the utility of steady-state metabolite profiling in medical, microbial and plant sciences. The authors pay particular attention to the state of the art with regard to the use of this technique in diagnostic applications, gene function analysis and systems biology.

6. Stitt M, Fernie AR: **From measurements of metabolites to metabolomics: an ''on the fly'' perspective illustrated by recent studies of carbon–nitrogen interactions**. *Curr Opin Biotechnol* 2003, **14**:136-144.

7. Sweetlove LJ, Last RL, Fernie AR: **Predictive metabolic engineering: a goal for systems biology**. *Plant Physiol* 2003, **132**:420-425.

8. • Schwender J, Ohlrogge J, Schacher-Hill Y: **Understanding flux in plant metabolic networks**. *Curr Opin Plant Biol* 2004, **7**:309-317.

A thorough review of metabolic flux analysis approaches that depend on steady-state labelling. The authors give a brief overview of the use of this technique in the microbial field before discussing detailed case studies in plants including, but not limited to, those described in [35•,42,43].

9. Cornish-Bowden A, Cardenas ML: **Functional genomics: silent genes given voice**. *Nature* 2001, **409**:571-572.

10. Raamsdoonk LM, Teusink B, Broadhurst D, Zhang NS, Hayes A, Walsh MC, Berden JA, Brindle KM, Kell DB, Rowland JJ *et al.*: **A functional genomics strategy that uses metabolome data to reveal the phenotype of silent mutations**. *Nat Biotechnol* 2001, **19**:45-50.

11. Stitt M: **Metabolic regulation of photosynthesis**. In *Advances in Photosynthesis, Vol.3, Environmental Stress and Photosynthesis.* Edited by Baker N. Academic Press; 1996.

12. Fernie AR, Roessner U, Geigenberger P: **The sucrose analog palatinose leads to a stimulation of sucrose degradation and starch synthesis when supplied to discs of growing potato tubers**. *Plant Physiol* 2001, **125**:1967-1977.

13. Sweetlove LJ, Burrell MM, ap Rees T: **Characterisation of transgenic potato (*Solanum tuberosum*) tubers with increased ADPglucose pyrophosphorylase**. *Biochem J* 1996, **320**:487-492.

14. Rolleston FS: **A theoretical background to the use of measured intermediates in the study of the control of intermediary metabolism**. *Curr Top Cell Regul* 1978, **5**:47-54.

15. Tiessen A, Hendriks JHM, Stitt M, Branscheid A, Gibon Y, Farre EM, Geigenberger P: **Starch synthesis in potato tubers is regulated by post-translational redox modification of ADPglucose pyrophosphorylase: a novel regulatory mechanism linking starch synthesis to the sucrose supply**. *Plant Cell* 2002, **14**:2191-2213.

16. Calvin M: **Path of carbon in photosynthesis**. *Science* 1962, **135**:879-882.

17. Graham D, ap Rees T: **Occurrence of fructose 1-phosphate in tissues of higher plants**. *Nature* 1965, **208**:88-89.

18. Wheeler GL, Jones MA, Smirnoff N: **The biosynthetic pathway of vitamin C in higher plants**. *Nature* 1998, **393**:365-369.

19. Gage DA, Rhodes D, Nolte KD, Hicks WA, Leustek T, Cooper AJL, Hanson AD: **A new route for synthesis of dimethylsulphoniopropionate in marine algae**. *Nature* 1997, **387**:891-894.

20. •• Masse G, Belt ST, Rowland SJ, Rohmer M: **Isoprenoid biosynthesis in the diatoms *Rhizosolenia setigera* (Brightwell) and *Haulea ostrearia* (Simonsen)**. *Proc Natl Acad Sci USA* 2004, **101**:4413-4418.

An excellent recent employment of labelling analysis to determine pathway structure. The isoprenoid biosynthetic pathways in two species of diatom were determined. Similar studies have recently also been carried out on the same pathway in higher plants (see [51•]). The work described in these two papers [20••,51•] and in [22••] are fine examples of the use of the traditional approach for the elucidation of pathway structure which is also exemplified in [16–19].

21. Bao XM, Katz S, Pollard M, Ohlrogge J: **Carbocyclic fatty acids in plants: biochemical and molecular genetic characterisation of cyclopropane fatty acid synthesis of *Sterculia foetida***. *Proc Natl Acad Sci USA* 2002, **99**:7172-7177.

22. •• Schwender J, Goffmann F, Ohlrogge JB, Shachar-Hill Y: **Rubisco without the Calvin cycle improves the carbon efficiency of developing green seeds**. *Nature* 2004, **432**:779-782.

An exemplary paper in which the use of elementary flux mode analysis (see [23]) is combined with substrate feeding experiments. Using this approach, the authors were able to elegantly demonstrate the involvement of Rubisco in a previously undescribed metabolic context that increases the efficiency of carbon use during the formation of oil in developing green seeds of *Brassica napus*.

23. Schuster S, Dandekar T, Fell DA: **Detection of elementary flux modes in biochemical networks: a promising tool for pathway analysis and metabolic engineering**. *Trends Biotechnol* 1999, **17**:53-60.

24. Sweetlove LJ, Kossmann J, Riesmeier JW, Trethewey RN, Hill SA: **The control of source to sink carbon flux during tuber development in potato**. *Plant J* 1998, **15**:697-706.

25. Van Dongen JT, Roeb GW, Dautzenberg M, Froehlich A, Vigeolas H, Minchin PEH, Geigenberger P: **Phloem import and storage metabolism are highly coordinated by the low oxygen concentrations within developing wheat seeds**. *Plant Physiol* 2004, **135**:1809-1821.

26. • Sriram G, Fulton DB, Iyer VV, Peterson JM, Zhou R, Westgate ME, Spalding MH, Shanks JV: **Quantification of compartmented metabolic fluxes in developing soybean embryos by employing biosynthetically directed fractional ^{13}C labelling, two-dimensional [^{13}C, ^{1}H]nuclear magnetic resonance, and comprehensive isotopomer balancing**. *Plant Physiol* 2004, **136**:3043-3057.

This paper describes a bold approach to modelling the metabolism of developing soybean embryos in a manner analogous to that used by microbial scientists. The authors' approach is based on the development and application of computer-aided metabolic flux analysis to probe two-dimensional [^{13}C, ^{1}H]NMR spectra of seed storage protein and starch hydrolysates. This approach clearly has great potential and, given that it provides positional labelling information, can also yield information on transfer fluxes and subcellular information. Similar examples of this approach are provided in [35•,42].

27. Long SP, Ainsworth EA, Rogers A, Ort DR: **Rising atmospheric carbon dioxide; plants FACE the future**. *Annu Rev Plant Biol* 2004, **55**:591-628.

28. Carrari F, Nunes-Nesi A, Gibon Y, Lytovchenko A, Loureiro ME, Fernie AR: **Reduced expression of aconitase results in an enhanced rate of photosynthesis and marked shifts in carbon partitioning in illuminated leaves of wild species tomato**. *Plant Physiol* 2003, **133**:1322-1335.

29. Kronzucker HJ, Siddigi MY, Glass ADM, Kirk GJD: **Nitrate-ammonium synergism in rice. A subcellular flux analysis**. *Plant Physiol* 1999, **119**:1041-1045.

30. Narang RA, Bruene A, Altmann T: **Analysis of phosphate acquisition efficiency in different *Arabidopsis* accessions**. *Plant Physiol* 2000, **124**:1786-1799.

31. Gibon Y, Bläsing O, Palacios-Rojas N, Pankovic D, Hendriks JHM, Fisahn J, Höhne M, Günther M, Stitt M: **Adjustment of diurnal starch turnover to short days: depletion of sugar during the night leads to a temporary inhibition of carbohydrate utilization, accumulation of sugars, and posttranslational activation of ADP glucose pyrophosphorylase in the following light period**. *Plant J* 2004, **39**:847-862.

32. Condon AG, Richards RA, Rebetzke GJ, Farquhar GD: **Improving intrinsic water-use efficiency and crop yield**. *Crop Sci* 2002, **42**:122-131.

33. Geigenberger P, Stitt M, Fernie AR: **Metabolic control analysis and regulation of the conversion of sucrose to starch in growing potato tubers**. *Plant Cell Environ* 2004, **27**:655-673.

34. Kopka J, Fernie A, Weckerth W, Gibon Y, Stitt M: **Metabolite profiling in plant biology: platforms and destinations**. *Genome Biol* 2004, **5**:109.

35. • Rontein D, Dieuaide-Noubhani M, Dufourc EJ, Raymond P, Rolin D: **The metabolic architecture of plant cells — stability of central metabolism and flexibility of anabolic pathways during the growth cycle of tomato cells**. *J Biol Chem* 2002, **277**:43948-43960.

A fascinating study on tomato cell suspensions in which flux through central metabolic pathways were calculated from ^{13}C enrichments at near-steady state. Fluxes determined at different time points of the growth cycle were compared. Intriguingly, when assessed relative to the rest of the network, the pathways of carbohydrate oxidation were fairly constant or 'rigid' with growth stage. In comparison, numerous anabolic pathways, including starch accumulation, biosynthesis of cell wall precursors and amino- and organic-acid biosynthesis, were relatively variable. These findings led the authors to the important conclusion that anabolic fluxes of plant metabolism are flexible and can fluctuate in relation to cellular demands for growth.

36. Roscher A, Kruger NJ, Ratcliffe RG: **Strategies for metabolic flux analysis in plants using isotope labelling**. *J Biotechnol* 2000, **77**:81-102.

37. Geigenberger P, Stitt M: **Sucrose synthase catalyzes a readily reversible reaction *in vivo* in developing potato tubers and other plant tissues**. *Planta* 1993, **189**:329-339.

38. Trethewey RN, ap Rees T: **The role of the hexose transporter in the chloroplasts of *Arabidopsis thaliana* L**. *Planta* 1994, **195**:168-174.

39. Fernie AR, Roscher A, Ratcliffe RG, Kruger NJ: **Fructose 2,6-bisphosphate activates pyrophosphate: fructose-6-phosphate 1-phosphotransferase and increases triose phosphate to hexose phosphate cycling in heterotrophic cells**. *Planta* 2001, **212**:250-263.

40. Fernie AR, Tiessen A, Stitt M, Willmitzer L, Geigenberger P: **Altered metabolic fluxes result from shifts in metabolite levels in sucrose phosphorylase-expressing potato tubers**. *Plant Cell Environ* 2002, **25**:1219-1232.

41. Edwards S, Nguyen BT, Do B, Roberts JKM: **Contribution of malic enzyme, pyruvate kinase, phosphoenolpyruvate carboxylase, and the Krebs cycle to respiration and biosynthesis and to intracellular pH regulation during hypoxia in maize root tips observed by nuclear magnetic resonance imaging and gas chromatography mass spectrometry**. *Plant Physiol* 1998, **116**:1073-1081.

42. Dieuaide-Noubhani M, Raffard G, Canioni P, Pradet A, Raymond P: **Quantification of compartmented metabolic fluxes in maize root tips using isotope distribution from C-13 or C-14 labelled glucose**. *J Biol Chem* 1995, **270**:13147-13159.

43. Glawischnig E, Gierl A, Tomas A, Bacher A, Eisenreich W: **Retrobiosynthetic nuclear magnetic resonance analysis of amino acid biosynthesis and intermediary metabolism. Metabolic flux in developing maize kernels**. *Plant Physiol* 2001, **125**:1178-1186.

44. Schwender J, Ohlrogge JB: **Probing *in vivo* metabolism by stable isotope labelling of storage lipids and proteins in developing *Brassica napus* embryos**. *Plant Physiol* 2002, **130**:347-361.

45. Geigenberger P, Fernie AR, Gibon Y, Christ M, Stitt M: **Metabolic activity decreases as an adaptive response to low internal oxygen in growing potato tubers**. *Biol Chem* 2000, **381**:723-740.

46. • Roessner-Tunali U, Liu JL, Leisse A, Balbo I, Perez-Melis A, Willmitzer L, Fernie AR: **Kinetics of labelling of organic and amino acids in potato tubers by gas chromatography-mass spectrometry following incubation in ^{13}C labelled isotopes**. *Plant J* 2004, **39**:668-679.
In this study, a pre-existing GC-MS method for metabolite profiling [2] was adapted such that molecules containing atoms of heavy mass could be readily detected and quantified. Using this approach, the authors were able to trace isotope accumulation in more than 30 metabolites of primary metabolism in potato tuber slices. A detailed analysis of the labelling kinetics of the metabolic intermediates was able to demonstrate the onset of the wound response and also the presence of starch turnover in the tuber slices. However, this same analysis also revealed that the metabolic system was grossly perturbed before isotopic steady-state could be achieved, requiring that a kinetic approach was implemented for the analysis of unidirectional fluxes. This approach offers greater sensitivity than that obtainable by NMR and is more broadly applicable than the steady-state approach but, in this particular application, suffers from being unable to resolve positional isotopomers.

47. McNeil SD, Rhodes D, Russell BL, Nuccio ML, Shacher-Hill Y, Hanson AD: **Metabolic modelling identifies key constraints on an engineered glycine betaine synthesis pathway in tobacco**. *Plant Physiol* 2000, **124**:153-162.

48. Niittyla T, Messerli G, Trevisan M, Chen J, Smith AM, Zeeman SC: **A previously unknown maltose transporter essential for starch degradation in leaves**. *Science* 2004, **303**:87-89.

49. Ramli US, Baker DS, Quant PA, Harwood JL: **Control analysis of lipid biosynthesis in tissue cultures from oil crops shows that flux control is shared between fatty acid synthesis and lipid assembly**. *Biochem J* 2002, **364**:393-401.

50. Verpoorte R, Memelink J: **Engineering secondary metabolite production in plants**. *Curr Opin Biotechnol* 2002, **13**:181-187.

51. • Wolferetz M, Sharkey TD, Boland W, Kühnemann F: **Rapid regulation of the methylerythritol 4-phosphate pathway during isoprene synthesis**. *Plant Physiol* 2004, **135**:1939-1945.
The study described in this paper provides a great example of the use of isotopes to discriminate between different routes to the same end, and to assess the relative importance of each route under various conditions. In this study, the two pathways of isoprene synthesis are investigated in *Eucalyptus globus*. The paper is an interesting complement to [20••], and a valuable reminder of the power of feeding of deuterated precursors to photosynthesising tissues as a tool to deepen our understanding of the metabolic regulation of photosynthesis (see also [52]).

52. Schleucher J, Vanderveer PJ, Sharkey TD: **Export of carbon from chloroplasts at night**. *Plant Physiol* 1998, **118**:1439-1445.

53. Bologa KL, Fernie AR, Leisse A, Ehlers Loureiro M, Geigenberger P: **A bypass of sucrose synthase leads to low internal oxygen and impaired metabolic performance in growing potato tubers**. *Plant Physiol* 2003, **132**:2058-2072.

54. • Vigeolas H, van Dongen JT, Waldeck W, Hühn D, Geigenberger P: **Lipid storage metabolism is limited by the prevailing low oxygen concentrations within growing seeds of *Brassica napus* L**. *Plant Physiol* 2003, **133**:2048-2060.
This paper follows on from [60] in describing the investigation of fluxes, metabolites and, hence, metabolic regulation at a level of spatial resolution that was previously not tractable.

55. Vigeolas H, Geigenberger P: **Increased levels of glycerol-3-phosphate lead to a stimulation of flux into triacylglycerol synthesis after supplying glycerol to developing seeds of *Brassica napus* L. *in vivo***. *Planta* 2004, **219**:827-835.

56. Minchin PEH, Thorpe MR: **Using the short-lived isotope ^{11}C in mechanistic studies of photosynthate transport**. *Funct Plant Biol* 2003, **30**:831-841.

57. Gerhardt R, Stitt M, Heldt HW: **Subcellular metabolite determination in spinach leaves through non-aqueous fractionation**. *Physiol Chem* 1983, **364**:1130-1131.

58. Stitt M, Lilley R, Gerhardt R, Heldt HW: **Determination of metabolite levels in specific cells and subcellular compartments of plant leaves**. *Methods Enzymol* 1989, **174**:518-552.

59. Farre EM, Tiessen A, Roessner U, Geigenberger P, Trethewey RN, Willmitzer L: **Analysis of the compartmentation of glycolytic intermediates, nucleotides, sugars, amino acids and sugar alcohols in potato tubers using a non-aqueous fractionation method**. *Plant Physiol* 2001, **127**:685-700.

60. Gibon Y, Vigeolas H, Tiessen A, Geigenberger P, Stitt M: **Sensitive and high throughput metabolite assays for inorganic pyrophosphate, ADPGlc, nucleotide phosphates, and glycolytic intermediates based on a novel enzymic cycling system**. *Plant J* 2002, **30**:221-235.

61. Eltoum IA, Siegal GP, Frost AR: **Microdissection of histological sections: past, present and future**. *Adv Anat Pathol* 2002, **9**:316-322.

62. Fehr M, Frommer WB, Lalonde S: **Visualisation of maltose uptake in living yeast cells by fluorescent nanosensors**. *Proc Natl Acad Sci USA* 2002, **99**:9846-9851.

63. Borusjuk L, Walenta S, Rolletschek H, Mueller-Klieser W, Wobus U: **Spatial analysis of plant metabolism: sucrose imaging within *Vicia faba* cotyledons reveals specific developmental patterns**. *Plant J* 2002, **29**:521-530.

64. • Giege P, Heazelwood JL, Roessner-Tunali U, Millar AH, Fernie AR, Leaver CJ, Sweetlove LJ: **Enzymes of glycolysis are functionally associated with the mitochondrion in *Arabidopsis* cells**. *Plant Cell* 2003, **15**:2140-2151.
A combination of proteomic, traditional enzymatic and cell biological analyses suggest that the entire glycolytic pathway is attached to the cytosolic face of the outer mitochondrial membrane in *Arabidopsis*. The GC-MS flux-profiling method (described in [46•]) was used to demonstrate the that isotopically labelled glycolytic intermediates were converted into constituents of the TCA cycle, providing the necessary functional evidence of an active glycolysis.

65. • Hellerstein MK: ***In vivo* measurement of fluxes through metabolic pathways: the missing link in functional genomics and pharmaceutical research**. *Annu Rev Nutr* 2003, **23**:379-402.
The authors provide a fascinating and current overview of the use of flux analysis in the medical field. This article highlights the importance of dynamic information in integrated approaches and gives several examples wherein metabolic studies would be severely compromised were such approaches not considered.

66. Yang C, Hua Q, Shimizu K: **Integration of the information from gene expression and metabolic fluxes for the analysis of the regulatory mechanisms in *Synechocystis***. *Appl Microbiol Biotechnol* 2002, **58**:813-822.

67. Even S, Lindley ND, Cocaign-Bousquet M: **Transcriptional, translational and metabolic regulation of glycolysis in *Lactococcus lactis* subsp. Cremoris MG 1363 grown in continuous acidic cultures**. *Microbiology* 2003, **149**:1935-1944.

68. Sauer U: **High-throughput phenomics: experimental**
• **methods for mapping fluxomes**. *Curr Opin Biotechnol* 2004, **15**:58-63.
In this highly readable review, Sauer argues that the capacity to observe functional units directly is a missing link in contemporary functional analyses that focus on the analysis of cellular components. He suggests that quantitative monitoring of metabolic flux analysis could bridge this gap by providing a global perspective of the integrated regulation at the transcriptional, translational and metabolic levels.

69. Urbanczyk-Wochniak E, Luedemann A, Kopka J, Selbig J, Roessner-Tunali U, Willmitzer L, Fernie AR: **Parallel analysis of transcript and metabolic profiles: a new approach in systems biology**. *EMBO Rep* 2003, **4**:989-993.

70. Hirai MY, Yano M, Goodenowe DB, Kanaya S, Kimura T, Awazuhara M, Arita M, Fujiwara T, Saito K: **Integration of transcriptomics and metabolomics for understanding of global responses to nutritional stresses in *Arabidopsis thaliana***. *Proc Natl Acad Sci USA* 2004, **101**:10205-10210.

71. Suzuki H, Achnine L, Xu R, Matsuda SPT, Dixon RA:
• **A genomics approach to the early stages of triterpene saponin biosynthesis in *Medicago truncutula***. *Plant J* 2002, **32**:1033-1048.
Although not incorporating flux analysis, this article describes great examples of the type of integrated genomics approaches currently employed in plants (see also [69,70,72•]). In this instance, the authors mined *Medicago* expressed sequence tag (EST) libraries for sequences that putatively encode three early enzymes of triterpene aglycone formation. The functions of these sequences were subsequently clarified by heterologous expression and metabolite profiling of their reaction products, before looking at the expression and associated metabolite composition *in planta* following jasmonate treatment.

72. Gibon Y, Blaesing OE, Hannemann J, Carillo P, Höhne M,
• Cross J, Selbig J, Stitt M: **A robot-based platform to measure multiple enzyme activities in *Arabidopsis* using a set of cycling assays: comparison of changes of enzyme activities and transcript levels during diurnal cycles and prolonged darkness**. *Plant Cell* 2004, **16**:3304-3325.
The authors describe the development of an inexpensive robotized platform that allows the measurement of the activity of 23 enzymes involved in central carbon and nitrogen metabolism in *Arabidopsis thaliana*. They used this platform to analyze the diurnal changes of enzyme activities in wildtype Columbia-0 (Col-0), in the starchless plastid phosphoglucomutase (*pgm*) mutant, and in Col-0 during a prolonged night. This study revealed that changes in enzyme activity were much smaller than those in transcription. The amplitude of the changes in transcription and enzyme activity were unrelated, with the changes in enzyme activity being strongly delayed. The authors propose that enzyme activities provide a quasi-stable integration of regulation at several levels and provide useful data for the characterization and diagnosis of different physiological states.