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Boundary flux analysis: an emerging strategy for investigating metabolic pathway activity in large cohorts

Ian A Lewis

Many biological phenotypes are rooted in metabolic pathway activity rather than the concentrations of individual metabolites. Despite this, most metabolomics studies only capture steady-state metabolism — not metabolic flux. Although sophisticated metabolic flux analysis strategies have been developed, these methods are technically challenging and difficult to implement in large-cohort studies. Recently, a new boundary flux analysis (BFA) approach has emerged that captures large-scale metabolic flux phenotypes by quantifying changes in metabolite levels in the media of cultured cells. This approach is advantageous because it is relatively easy to implement yet captures complex metabolic flux phenotypes. We describe the opportunities and challenges of BFA and illustrate how it can be harnessed to investigate a wide transect of biological phenomena.

Address
Alberta Centre for Advanced Diagnostics, Department of Biological Sciences, University of Calgary, 2500 University Drive NW, Calgary, Alberta T2N 1N4, Canada

Corresponding author: Lewis, Ian A (ian.lewis2@ucalgary.ca)

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Introduction

Metabolomics has emerged as a mainstream strategy for understanding the metabolic underpinnings of complex biological systems [1–3]. Over the last decade, the field has advanced dramatically with respect to the analytical tools, software, and standardized methods. These advancements have driven enthusiasm for increasingly in-depth analyses [4,5] that have opened the door to mapping the dark metabolome [6], single-cell metabolomics [7], and efforts to capture genome-scale representations of the metabolic network [8]. Though powerful as tools for cataloguing the full breadth of metabolism, these emerging methods have done little to advance our understanding of metabolic pathway activity.

Complex biological phenomena are made possible by the activities of specific metabolic reactions, the rates with which carbon flows through metabolic pathways, and mass action of entire metabolic networks [9–11]. Perturbations in the activities of these networks are not necessarily manifested through steady-state changes in metabolism. In some cases, the complete inhibition of a metabolic pathway may have minimal observable impact on intracellular metabolite concentrations [12,13]. Consequently, understanding the role that metabolism plays in biological phenomena requires methods for quantifying metabolic fluxes directly. Currently, there are no simple 'fluxomics' methods for measuring metabolic pathway activities on a system-wide scale and the existing techniques are impractical to implement in large-cohort studies. Consequently, there is an unmet need for a technique that can capture complex flux phenotypes in genome-wide knockout studies, pharmaceutical lead screening, and other large-cohort projects.

Brief overview of fluxomic methods

Fluxomic analyses use empirical, computational, and hybrid methods to estimate fluxes through metabolic networks. Empirical methods typically involve the introduction of isotopically labeled metabolite precursors (e.g. ^{13}C, ^{15}N, or ^{2}H) to the medium of cultured cells, which allows downstream metabolic pathways to be traced based on the appearance of the labeled atoms. In some cases, selectively labeled precursors can be used to probe specific pathway fluxes. For example, partitioning of carbon between the oxidative branch of the pentose phosphate pathway and glycolysis can be quantified based on the labeling patterns of lactate produced by cells incubated in 2-^{13}C glucose [14–16]. Alternatively, cells can be incubated for long periods of time in the presence of uniformly labeled precursors (e.g. U-^{13}C glutamine) and steady-state isotopic enrichment in downstream metabolites can be used to quantify the relative contributions of converging pathways to a particular metabolite (flux ratio) [12].

These selective labeling approaches are accurate but are difficult to scale to network-wide analyses. To address this, a kinetic flux profiling (KFP) approach can be used wherein isotope-labeled precursors are added to cultures and metabolites are sampled over time to empirically

determine isotopic enrichment rates in the overall network [9]. KFP is the most direct approach for quantifying intracellular pathway fluxes, but requires costly isotope labels and complicated experimental designs. Moreover, metabolic fluxes differ by orders of magnitude from pathway-to-pathway, which makes capturing the complete diversity of fluxes challenging in a single KFP experiment [9]. Further complicating matters, the selection of commercial isotope-labeled standards is generally restricted to common nutrients (sugars, amino acids, and select carboxylic and fatty acids) [17], which limits the scope of KFP analyses. Although reverse-labeling studies — where cells are fully isotope-labeled, then the catabolism of a select set of unlabeled nutrients is quantified — have captured fluxes from more unusual precursors [18], these studies are difficult to implement for nonspecialist laboratories.

To address these empirical challenges, a variety of computational and hybrid computational/empirical strategies have been developed for fluxomics. Many of these approaches are based on flux balance analysis (FBA), a mathematical modeling approach that uses a system of linear equations to computationally optimize fluxes in a steady-state system (i.e. assuming all intracellular metabolite levels remain constant) [19]. Generally, FBA models are used to understand how metabolic fluxes are balanced to maximize a particular objective function (e.g. biomass production) [19]. In the hands of experts, these models generate surprisingly accurate predictions of fluxes [20] and can provide profound insights into how cells respond to major stressors (e.g. cancer) [21]. However, FBA calculations rely on accurate metabolic network representations for each organism. Given that these networks are built from genomics data, the enzymes present in networks depend on accurate gene annotation and homology searches [22,23]. This is problematic when studying species outside the small selection of model organisms with well-curated genomes as any inaccuracy in the metabolic networks (e.g. orphan reactions, missing enzymes, and incorrectly annotated transporters) can lead to major errors in flux calculations. In addition, FBA calculations rely on a vector of boundary constraints — a range of biologically realistic fluxes describing the rates at which nutrients can enter and exit cells [24,25]. However, these boundary conditions are generally unknown for nonmodel organisms.

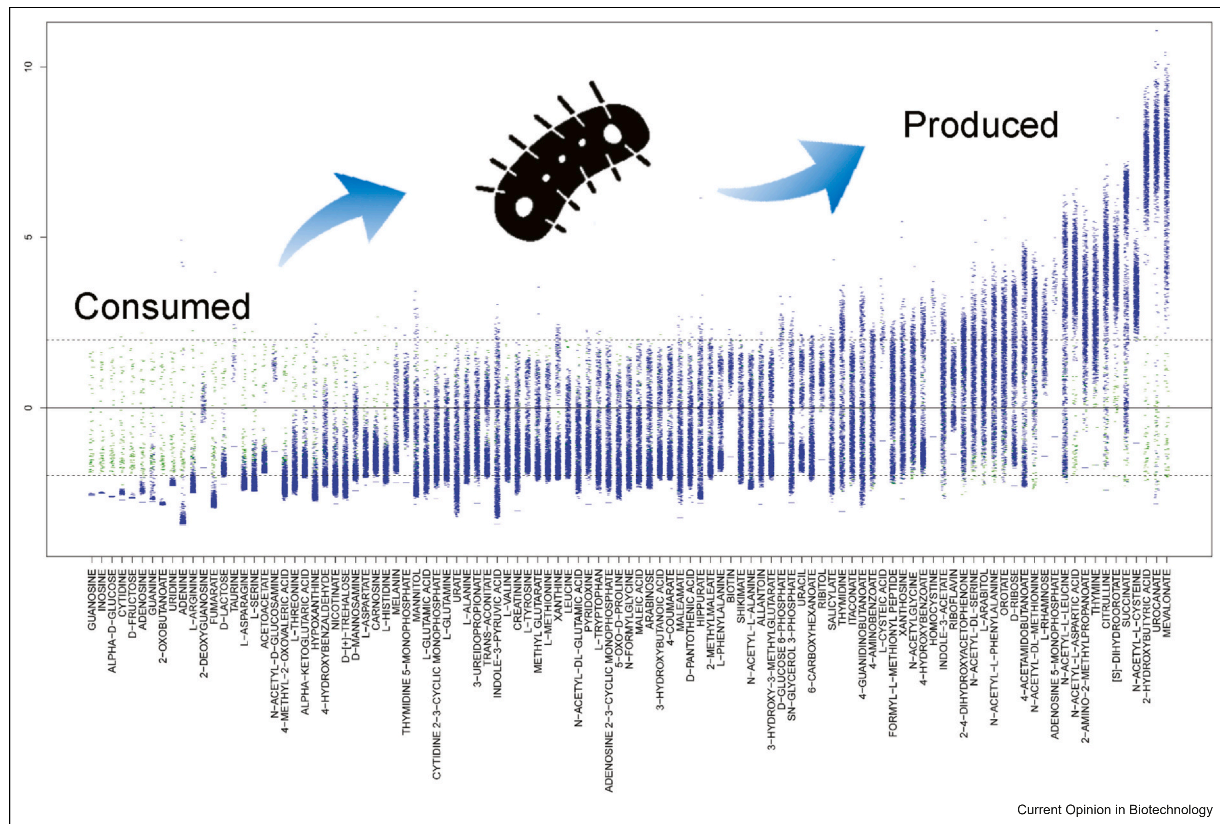
To address these complications, a metabolic flux analysis (MFA) strategy was developed that uses a simplified central carbon metabolism network to capture core fluxes [17,26]. This approach is more computationally tractable and easier to translate between systems. MFA is frequently used in conjunction with isotope labeling (e.g. ^{13}C-MFA) to generate robust metabolic flux maps [12]. Although the use of isotopes raises some of the same challenges faced in KFP studies, ^{13}C-MFA data can be derived from a single steady-state timepoint and are thus more practical to collect [27]. The efficacy of ^{13}C-MFA has made it one of the most widely used modeling strategies, especially in the context of metabolic engineering [26,28,29]. Recently, there has been progress toward the development of other computationally tractable models for simulating fluxes based on an elementary metabolite unit balancing [30,31] and machine learning [32]. In addition, numerous methods have been developed to extend fluxomics to dynamic systems, isotopic nonstationary systems, and other more complex fluxomic applications (e.g. regulatory FBA; dynamic FBA; isotopic nonstationary MFA; dynamic MFA; and COMPLETE-MFA). These emerging methods, which have been reviewed in detail elsewhere [17,33–37], provide a sophisticated vehicle for studying perturbations in well-characterized model systems. Importantly, understanding the output of these models and visualizing their flux projections has become significantly more tractable in recent years due to the emergence of sophisticated new software tools (e.g. Metran, OpenFlux, INCA, 13CFLUX2, and MINNO) [38–42]. However, despite these advances, current methods are largely geared toward detailed analysis of well-characterized model systems and have done little to lowering the barrier to integrating flux analyses into large-scale cohort studies.

Boundary flux analysis

Until recently, empirical methods for characterizing flux-related metabolic phenotypes have centered around quantifying intracellular pathway dynamics. However, boundary fluxes — the flow of nutrients and waste products in and out of cells — play a critical role in constraining the activity of metabolic pathways and are direct reporters of intercellular pathway dynamics [43,44]. Although the targeted analysis of culture media to quantify specific metabolic pathways is commonplace (e.g. glycolysis based on lactate secretion) [45], these targeted studies do not capture the breadth of metabolites needed to constrain metabolic networks. Similarly, metabolomic analysis of growth media is also a common practice [46,47], but these studies are generally not designed to capture uptake/secretion rates of molecules. This distinction is important because it is the rate of change of metabolites — not their presence or absence — that informs network-wide flux phenotypes.

Over the last several years, we have sought to formalize boundary flux analysis (BFA) as a strategy for integrating flux data into large-cohort studies. BFA quantifies changes in the concentrations of all observable metabolites present in the media of cultured cells held at a fixed density over standardized incubation times [44]. These changes in media composition can then be translated into a vector of metabolic uptake and excretion rates, which can serve as stand-alone metabolic phenotypes (Figure 1) or be used as

Figure 1



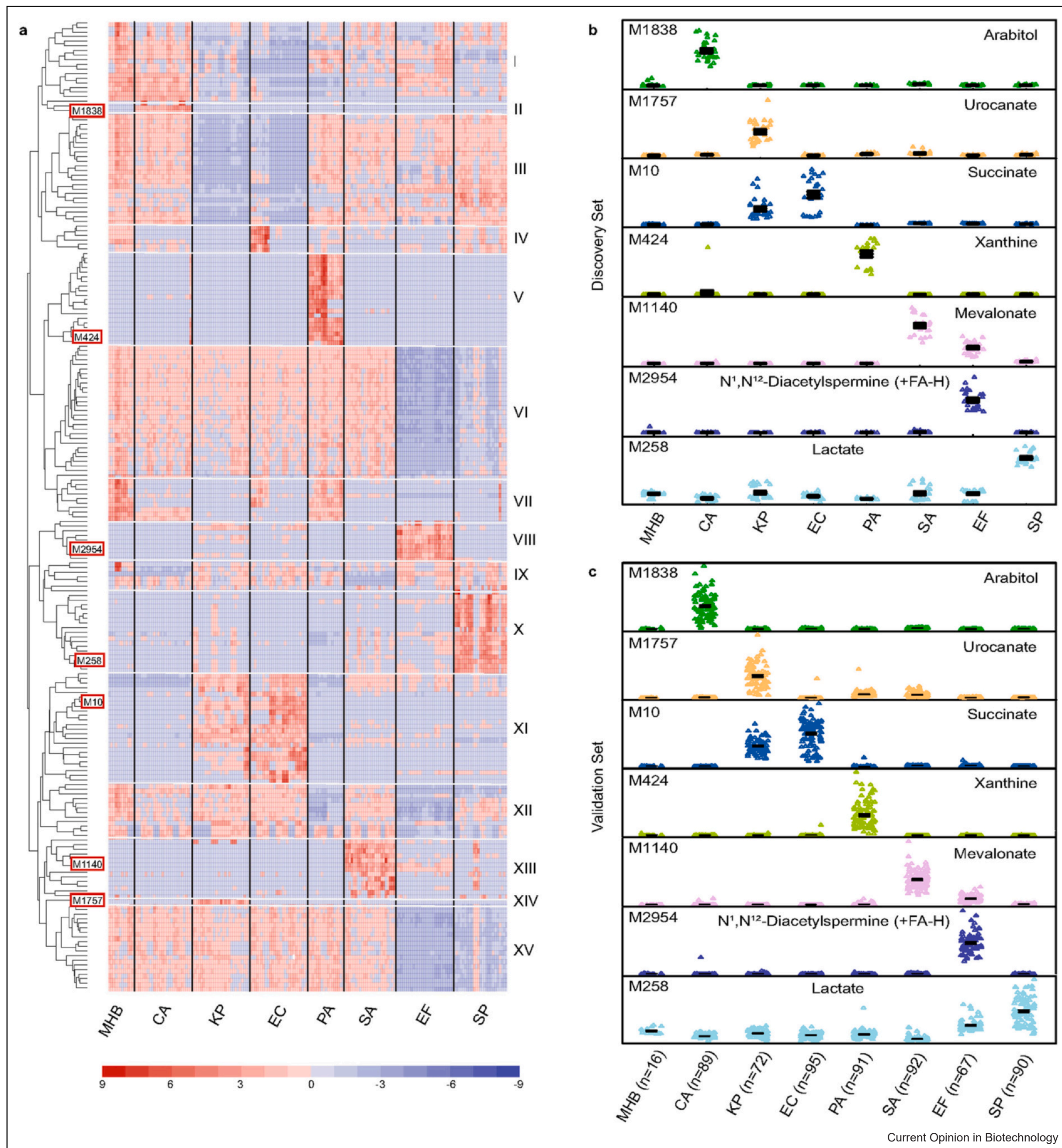
Boundary flux analysis ('Eat-o-gram') of 960 clinical SA isolates. *S. aureus* isolates were cultured in vitro and the fluxes of a transect of 77 central carbon metabolites were measured by BFA, using a high-throughput, two-column HILIC chromatography approach. Enrichment factors (as log-transformed z-scores) were calculated for each metabolite. Dashed lines represent two standard deviations from control. Green points show metabolite levels in sterile media, whereas blue show metabolite levels after a 4-hour incubation with clinical isolates.
(Figure adapted from [43]).

boundary flux constraints for FBA [48]. BFA is more analytically tractable than other empirical fluxomic strategies because 1) the composition of media is generally orders of magnitude less complex than intracellular extracts, 2) even when complex media are analyzed, cells have a limited number of transporters, which restricts the cohort of molecules that contribute to BFA phenotypes, 3) the chemical diversity of metabolites that are soluble in media is much lower than those present in the cytosol and thus can be captured using a narrower selection of analytical methods, and 4) metabolites taken up from or excreted to the medium are at metabolic endpoints and thus do not require extensive time series or complex isotope-based methods to quantify their kinetics. Thus, BFA allows a researcher to capture complex metabolic flux phenotypes in a minimal set of analyses.

Biological applications of boundary flux analysis

Over the last five years, BFA has been applied to a growing transect of applications ranging from clinical microbiology, antimicrobial susceptibility testing, analyses of metabolic interactions between microbes, host-microbiome metabolic relationships, and immunobiological metabolic dynamics. Some of the most exciting recent applications of BFA include the discovery that BFA profiles are reproducible across over thousands of microbial isolates [43] (Figure 1); that BFA profiles can rapidly differentiate species of microbes, including bloodstream pathogens [49] (Figure 2); that BFA profiles are sensitive reports of antimicrobial susceptibility [49], and exposure to environmental toxins [50]; that urinary tract infections can be rapidly diagnosed based on BFA [51]; BFA can help quantify host versus microbial short-chain fatty acid production in mice [52], and elucidate the interplay between microbiota and host factors on the metabolome of mice [53]; viral infection-induced changes in cellular metabolism [54] as well as programmed differentiation of spermatogonia [55] can be mapped using BFA phenotypes; BFA also helped identify optimal strategies for maintaining stem cells in bioreactors [56,57]. Collectively, these

Figure 2



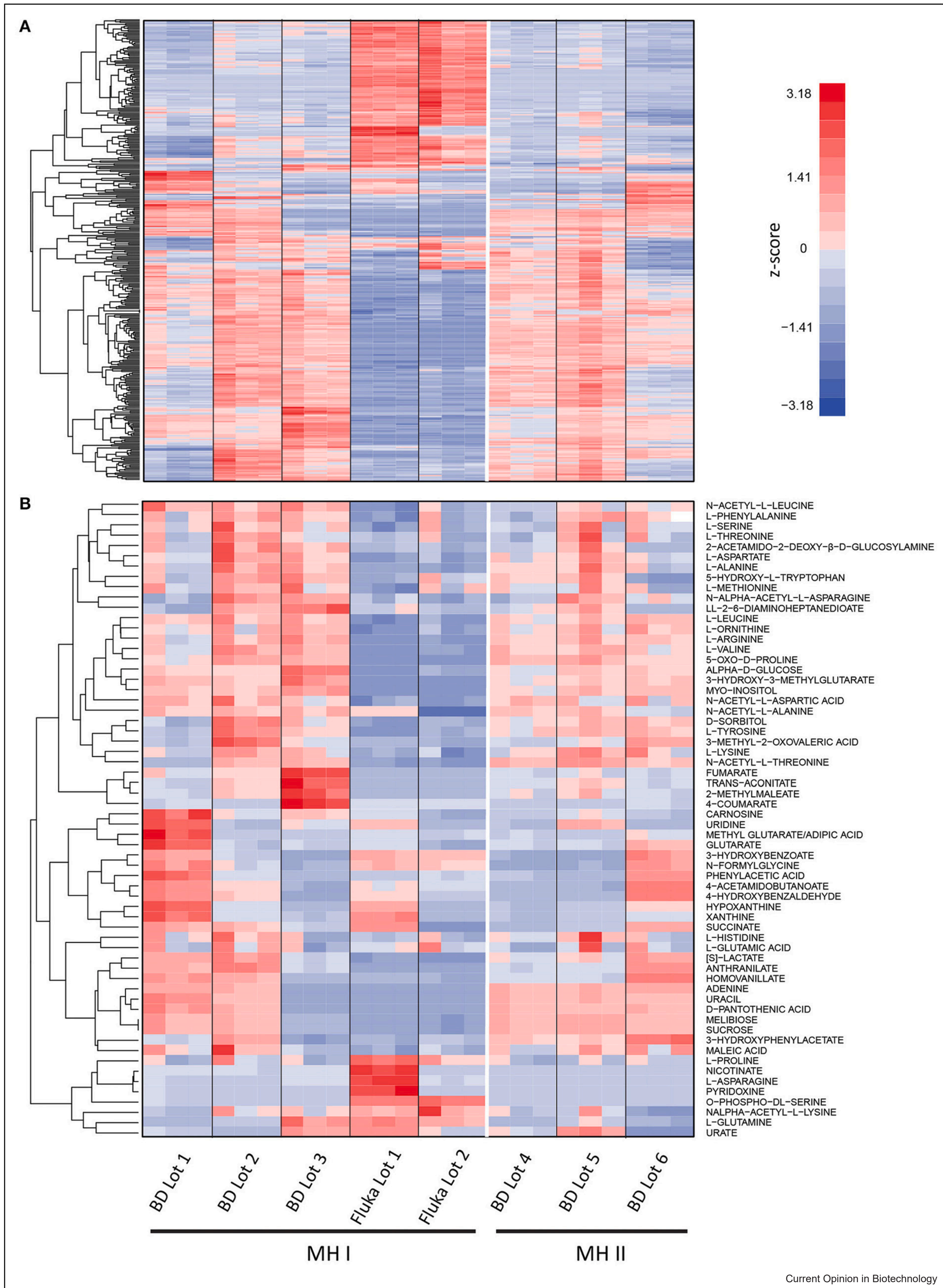
Boundary flux analyses were used to develop a metabolic preference assay that can differentiate bloodstream pathogens after a four-hour incubation. Boundary fluxes of seven metabolite targets were sufficient to identify these pathogens. MHB, Mueller Hinton broth with 10% blood; CA, *Candida albicans*; KP, *Klebsiella pneumonia*; EC, *Escherichia coli*; PA, *Pseudomonas aeruginosa*; SA, *Staphylococcus aureus*; EF, *Enterococcus faecalis*; SP, *Streptococcus pneumonia*.
(Figure from [49], licensed for redistribution under https://creativecommons.org/licenses/by/4.0/).

studies have shown that BFA phenotypes provide a robust mechanism for characterizing network-wide metabolic flux phenotypes that can be implemented in a wide range of biological studies.

Challenges in implementing boundary flux analysis

Although BFA phenotypes are generally easier to capture than KFP or ^{13}C-MFA fluxes, they are sensitive to

Figure 3



Variability in the metabolic composition of microbial growth media. **(a)** Untargeted and **(b)** targeted liquid chromatography–mass spectrometry analyses of Mueller Hinton growth media show variability lot-to-lot and manufacturer-to-manufacturer. These differences contribute to marked differences in metabolic flux phenotypes when microbes are cultured using these undefined media. MFA studies should use a single lot of media or a defined medium to avoid these experimental errors.
(Figure from [58], licensed for redistribution under https://creativecommons.org/licenses/by/4.0/).

their own experimental, biological, and analytical pitfalls that can complicate analyses. One important biological consideration is the composition of the experimental medium. Complex undefined media (e.g. Mueller Hinton) are frequently used in microbiology studies. We have shown that these media — including media sourced for clinical applications — have significant variability in their composition when analyzed using metabolomics approaches [58] (Figure 3). These lot-to-lot and supplier-to-supplier differences have a dramatic impact on BFA phenotypes that can make results difficult to reproduce [59–62]. To address this, we recently developed a biomarker enrichment medium, which is a complex, but chemically defined medium that was formulated specifically to enable reproducible BFA studies in microbial systems [58]. The BFA medium was designed around the nutritional requirements of bloodstream infection pathogens, but we anticipate that it can be used to investigate a wide diversity of aerobic microbes. In addition to being sensitive to the composition of media, BFA phenotypes are also more sensitive to evaporation than traditional isotope labeling methods. Any nonuniformity in evaporation (which are frequently observed in 96-well plate-based experiments) directly translates to errors in BFA phenotypes. We have found that humidified chambers and similar strategies are critical for generating reproducible BFA phenotypes.

One exciting aspect of BFA is that its minimal experimental requirements are amenable to scale-up into large-cohort studies. However, this scale-up has its own analytical complexities. Media metabolites are generally hydrophilic in nature, which are most easily resolved using hydrophilic interaction liquid chromatography (HILIC) [63]. Although HILIC has become a routine chromatographic stationary phase for metabolomics, it is generally less stable sample-to-sample and requires longer equilibration times than other types of chromatographic approaches. This problem is amplified in large-cohort studies. To address this, we developed a dual-column strategy for conditioning columns and an offline column re-equilibration method that enables stable quantification of BFA phenotypes over more than 2000 samples [43]. In addition, we developed a simple consumable product, the microbial containment device, which allows researchers to sample secreted metabolites without contamination from intracellular molecules [64].

Future applications of boundary flux analysis

The systematic quantification of metabolic boundary fluxes is a broadly applicable concept that can be adapted to the analysis of essentially any cell culture model system. The microbial, tissue culture, and bioreactor model systems described to date [43,49,50,52,55–57] represent a small selection of the potential applications of BFA. Most importantly, the scalable nature of the technique makes it amenable to drug screening, knockout library analysis, clinical applications, and similar large-scale projects. One exciting application that we envision is collecting comprehensive BFA phenotypes for a wide transect of microbial isolates that are constituents of our microbiome. Such BFA libraries may provide an important starting point for decoding metabolic phenotypes linked to changes in the microbiome [65–69] and the lung [70–73] as well as metabolic influences on bacterial signaling [74], biofilm formation [75], and microbe–microbe metabolic interactions [76] and may help decode the molecular underpinnings for complex host/microbiome dynamics. Another exciting anticipated application of BFA is using comprehensive boundary flux constraints to refine FBA models, where empirical flux libraries may significantly improve our ability to accurately predict optimal fluxes in complex metabolic networks.

Conclusions

BFA is an emerging strategy that allows network-wide metabolic flux phenotypes to be captured using analytically tractable methods. It is widely applicable to any biological study involving the analysis of cultured cells and can be scaled up to support cohorts of hundreds or thousands of samples. These attributes make BFA an attractive companion to more established metabolomics methods and may allow researchers to evaluate flux-related phenotypes as a routine element of their biological inquiries.

Data Availability

This is a review article, not a primary research article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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