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Frontiers of high-throughput metabolomics

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Large scale metabolomics studies are increasingly used to investigate genetically different individuals and time-dependent responses to environmental stimuli. New mass spectrometric approaches with at least an order of magnitude more rapid analysis of small molecules within the cell's metabolome are now paving the way towards true high-throughput metabolomics, opening new opportunities in systems biology, functional genomics, drug discovery, and personalized medicine. Here we discuss the impact and advantages of the progress made in profiling large cohorts and dynamic systems with high temporal resolution and automated sampling. In both areas, high-throughput metabolomics is gaining traction because it can generate hypotheses on molecular mechanisms and metabolic regulation. We conclude with the current status of the less mature single cell analyses where high-throughput analytics will be indispensable to resolve metabolic heterogeneity in populations and compartmentalization of metabolites.

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Introduction

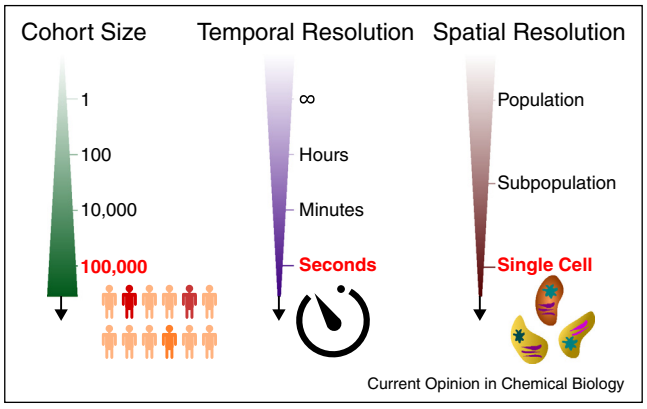
As the most recent member of the omics trilogy, metabolomics rapidly developed during the recent decade, largely driven by improved mass spectrometry (MS) with increased sensitivity and metabolite coverage. Beyond a broad array of targeted methods for accurate analysis of particular metabolite classes, advances include increasingly popular untargeted methods for discovery applications [1], first single cell methods [2], and imaging MS to monitor spatial distributions [3]. Metabolomics has matured to a routinely available and broadly applied technology for standard cell, environmental or biofluid samples. Accuracy, sensitivity, coverage, and speed of spectra acquisition still await major improvements, but the immediate limitation shifts from data acquisition and availability to metabolite annotation and systematic data interpretation. Non-targeted MS methods detect thousands of ion features in a single sample [4], only a small fraction of which can currently be annotated as metabolites, and large community-based efforts are underway to improve annotation [4–6]. The limitation of computational data interpretation concerns both extraction of the most informative features from non-targeted profiling data and the general ability to systematically infer testable molecular hypotheses from any type of metabolite data and its integration with other information [7].

Large scale applications of many samples are feasible with all omics techniques, although costs do not scale equally. In contrast to the MS-based proteomics techniques that still require chromatographic separation and fragmentation of peptides, metabolomics offers the unique advantage of rapid measurements that enable 10–100× higher throughput [8]. This is possible because the metabolome is less complex and tractable by direct profiling of non-fragmented, molecular ions by high-resolving MS instruments. This review focuses on the three frontiers where high-throughput metabolomics measurements are required and expected to create new scientific opportunities in the near future (Figure 1): Firstly, large scale profiling studies; secondly, analysis of dynamic systems at high-temporal resolution; and finally, single cell measurement of cellular populations.

Large scale metabolome profiling

Traditionally, the largest metabolome studies are from human epidemiology and genome-wide association studies, where the complementation of genetic data provided additional markers associated with disease [9••,10], cellular organization [11] or gene–environment interactions [12]. A general outcome is the widespread influence of disease-associated genetic variants on the human metabolome and the potential role of metabolic deregulation in driving several disease states. Such results prompted the use of metabolomics in clinical diagnosis [13•] and personalized medicine [14,15]. For reasons of cost and sample availability, these classical large scale association studies were often performed on static, single time point metabolic snapshots from fluid body samples. Given the complexity of the human system and the scarcity of temporal resolved data, most studies were correlative [16,17] and only sporadically led to testable hypotheses about molecular causality. In single-celled and multicellular model systems, quantitative trait locus mapping with large scale metabolomics is often used for functional genomics [7,18,19], but also unraveled homeostatic mechanisms, such as the role of *ura3* and *ira2* in regulating the

Figure 1



High-throughput metabolomics has presently three thrusts: **(a)** cohort size, **(b)** temporal resolution, and **(c)** spatial resolution. Technological development has allowed these tracks to reach impressive thresholds including large sample sizes (~100 000), fine time resolution (at seconds), and single cell resolution of metabolism.

intracellular abundance of pyrimidine intermediates and S-adenosyl-methionine in *Saccharomyces cerevisiae* [20•]. Similarly, metabolite-based trait mapping with the pathogen *Plasmodium falciparum* identified the role of hemoglobin-derived peptides in reducing parasite fitness [21••], suggesting a functional role for the chloroquine resistance transporter-encoding gene in mediating hemoglobin digestion by the parasite.

Particularly attractive for large scale profiling is the so-called exometabolome of compounds in culture medium because of its simplicity in sampling techniques. Importantly, it provides a direct readout of cellular consumption and production rates that can be exploited for functional interpretation [22]. As an emblematic example, an approximation of the uptake and production rates of 59 cancer cell lines was obtained by single-time point analysis of 219 medium metabolites, revealing the essential role of glycine in promoting rapid proliferation [23]. Furthermore, profiling 71 exo-metabolites in 3901 *Escherichia coli* and 4141 *Shewanella onedeinsis* gene knockouts identified impaired nutrient consumption in 557 and 954 mutants, respectively [24••], suggesting new potential transport and anabolic gene functions.

A key advantage of metabolomics is the capacity to detect responses even in the absence of growth phenotypes, thereby increasing functional readouts by orders of magnitude compared to classical chemical or genetic growth screens [25]. For example, non-targeted mass spectrometry was used to profile intracellular metabolic changes in budding yeast mutants, each lacking one of the known 118 kinases and phosphatases [26•]. Although most mutants were without a detectable growth phenotype, two thirds of them exhibited different steady state profiles. Integrating steady state metabolomics with phosphoproteomic data in a genome-scale stoichiometric model allowed to predict functionality of phosphorylation in regulating the activity of 47 enzymes [26•].

An emerging application is metabolic profiling of cellular responses to drug libraries [27,28]. The combined intracellular and extracellular response of 20 NMR-detected metabolites in mammalian cells challenged with 56 kinase inhibitors, identified inhibitors with similar responses and enabled postulation of new functional and common roles across different kinases [29•]. The results suggested inhibitors of potentially relevant therapeutic targets. With a much higher number of metabolic readouts, non-targeted TOF-based metabolomics was used to screen a library of 234 natural products [30•]. Correlating ion features to high-content cytological profiling assays predicted compound mode of actions. Metabolomics platforms capable of monitoring short-term and long-term metabolic responses to large chemical libraries will largely benefit from a higher throughput and become a valuable tool at early stages of drug discovery, aiding rational drug design and personalized medicine.

The above examples provide a glimpse on mechanistic insights that may be obtained from steady state analysis of metabolites in large cohorts. Typically study sizes exceeded 1000 samples [9••], many reaching 5000 or more, to meet the minimum requirements of power analysis for simple differential or association analyses [31]. This scale can be attained in reasonable time by canonical LC–MS or GC–MS based methods that can analyze up to 100 samples per day and instrument. However, this throughput is insufficient when moving beyond association to unravel the underlying complexity in etiology, with literally thousands of intertwined potential genetic and environmental causes. Hence, there is an urgent demand for experimental methods that are 10–100× times faster. Currently, this is only feasible with a compromise in coverage or by sacrificing separation of isomers in favor of throughput [26•,32•,33]. In absence of chromatographic separation, techniques such as acoustic droplet ejection allow for an exceptional throughput of >1 sample/s, that is, 75 000 samples on a single day [32•].

Increased study power that allows moving beyond mere associations provoked a paradigm shift in the analysis of high-throughput metabolomics data from classical statistics to more sophisticated context and network-based approaches. While several tools and modeling approaches are available for interpretation of genome-based transcript or proteinomics technologies, computational methods capable of exploiting the rich information content of large scale metabolomics studies are still lagging behind [34]. Model-based analysis capable of integrating metabolomics profiles with other omics data [35], rather than exclusive statistical associations [18,36], can facilitate the

process of data-driven hypothesis generation [37••,38]. The major limitation that must be addressed by data interpretation models is the functional and ideally also mechanistic relationship between the measured elements, which is conceptually harder for metabolites and proteins or genes than it is, for example, for genes and proteins. Network-based integration of metabolome and transcriptome data identified functional gene-metabolite modules that characterized phenotypically different macrophage polarization states [35]. More mechanistically, integration of metabolome and proteome data with detailed kinetic models of central *E. coli* and *S. cerevisiae* metabolism unraveled the active contribution of metabolite levels in regulating metabolic fluxes [39,40], but so far such studies remained limited in scope. In parallel, large scale metabolomics may be used to curate and improve current models [41–43]. Driven by increasing analytical throughput, the size of metabolomics studies will rapidly grow, shifting the limitations in number and quality of data driven hypothesis generation even more to the experimental study design and computational data analysis. Since large epigenetic studies and genetic networks in model organisms can guide studies in large human cohorts [44,45], we believe that in the near future systematic metabolome profiling studies charting the relationship between controlled genetic manipulations and metabolic phenotypes will become a powerful tool to understand the functionality of genetic variance in more complex systems.

Time-resolved metabolomics

Metabolite concentrations are influenced by many factors such as enzyme levels, *in vivo* enzyme kinetics, pathway fluxes and regulatory events. Hence, static snapshots of the metabolome represent the integrated consequence of overlapping direct and indirect effects. This situation ultimately hampers static data interpretation. One way out is dynamic metabolomics that can temporally disentangle direct responses from indirect consequences, helping to deconvolute different regulatory layers to identify causal effectors.

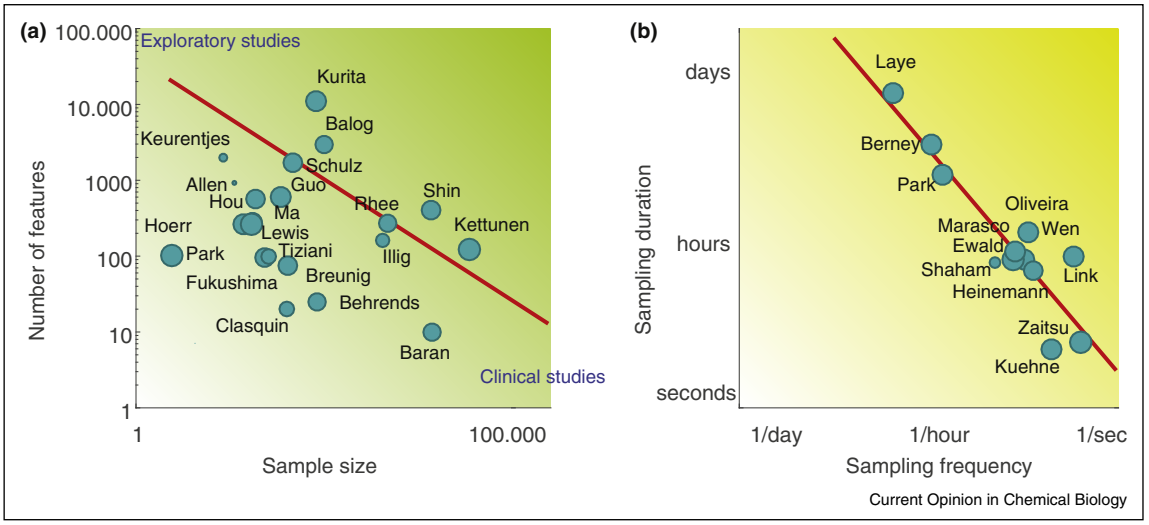
As an emblematic example, it has been a lingering question how NADPH formation can be rapidly activated to alleviate oxidative stress. The dynamic metabolic response of skin cells to hydrogen peroxide revealed an immediate activation of the oxidative pentose phosphate pathway, well before any transcriptional changes [46•]. The previously reported inactivation of the downstream glyceraldehyde 3-phosphate dehydrogenase was too slow to explain the metabolite dynamics and the flux increase [47]. Instead, the data suggested that the release of NADPH inhibition of 6-phosphate dehydrogenase during glucose metabolism opened a valve into the pentose phosphate pathway that was validated independently. Similarly, high temporal resolution of metabolite responses was key to resolve rapid sensing and adaptive mechanisms [48], drug mode of action [49] and homeostasis mechanisms [50].

Beyond short-term responses, dynamic metabolite profiling can also be powerful over extended periods to identify longer-term adaptive mechanisms [51,52]. Tracking 97 metabolites over the course of 2 hours after glucose ingestion in humans helped to reveal an unexpected involvement of bile acid signaling in glucose maintenance by potentially promoting sugar uptake [53]. *In vivo NMR* monitoring of 26 intracellular metabolites at 5 min resolution over 140 min was used in a hepatic cell model to identify off-target effects of the lactate dehydrogenase inhibitor galloflavin [54•]. A highlight of protein candidates, alanine transferase, was deduced from reduced synthesis and recovery of alanine. An almost classical example of periodic long-term changes is the cell cycle with distinct but largely uncharacterized metabolic states in the different cycle phases. Dynamic metabolomics in synchronized *S. cerevisiae* revealed that stored carbohydrates such as glycogen and trehalose are depolymerized to sugars in the G1 and S cell cycle phase to ensure sufficient resources for completion of one replication cycle, regardless of the environmental nutrient [55].

The above studies exemplify the potential of dynamic metabolome measurements but did not yet leverage the high-throughput potential of MS for the temporal dimension. A major hurdle was manual sampling and sample processing such that resolution was either at low-frequency (typically every few minutes) over longer periods [46•,55] or at high-frequency (few seconds) over short periods [47,56] (Figure 2b). More recently, high-throughput methods for online metabolomics were developed based on automated sampling and direct metabolome analysis. Such methods hold promise to sustain high-frequency sampling over longer periods. They also reduce measurement noise because of the simpler, faster, and automated sample processing. In one example, an active probe needle sampled an exposed mouse liver, monitoring α-ketoglutarate and fumarate every 10 s [57]. In two studies, a microfluidic chip was used to extract metabolites from bacteria, blood, and Jurkat T cells [58,59]. In one case the chip separated waste from small molecules by laminar flow diffusion. Accumulated metabolites were injected every 5 min into an ESI-MS system, measuring 77 compounds. In the other case, cells were trapped in a microfluidic chamber, and their desalted exometabolome was automatically injected into an ion mobility-MS with 18 min cycles.

A recently developed real-time metabolomics approach for suspended bacteria, yeast, and mammalian cells allows to profile dynamics at intervals of 10 s for up to the hours range [60••]. Although intracellular and extracellular metabolome were not separated, mechanistic insights could be gained on how *E. coli* resumes growth rapidly

Figure 2



(a) Trade-off between number of measured features and sample size in dynamic metabolomics studies. For each referenced study the number of features (e.g. annotated metabolites or detected ions) considered for the analysis is compared to the corresponding experimental samples size (e.g. number of individuals or mutant strains) (see Table 1). The red line represents the best linear fit. Size of the dots are proportional to the year of publication. While classical clinical applications quantify few metabolites in large cohorts, exploratory studies cover a larger space of metabolic species in selected representative samples. **(b)** Trade-off between the total duration of experiments and sampling frequency (e.g. time resolution) in dynamic metabolomics studies. Typical high resolution time resolved metabolomics studies focus on short term events, while a more coarse grain temporal resolution is used to monitor long term metabolic adaptive changes. **(c)** Tables 1 and 2 reporting the data used to generate figures in panel a and b.

Table 1

List of referenced papers that perform static metabolome profiling. For each study we report the number of collected samples (sample size) and detected ions/metabolites (coverage). Data are displayed in Figure 2a

References	Sample size	Coverage
Park *et al.*, 2016 [77]	3	103
Raamsdonk *et al.*, 2001 [78]	6	7
Fukushima *et al.*, 2014 [25]	50	95
Clasquin *et al.*, 2011 [79]	96	20
Schulz *et al.*, 2014 [26•]	118	1710
Allen *et al.*, 2003 [80]	20	935
Baran *et al.*, 2013 [24••]	8042	10
Behrends *et al.*, 2013 [81]	242	25
Breunig *et al.*, 2014 [20•]	100	74
Keurentjes *et al.*, 2006 [82]	14	2000
Lewis *et al.*, 2014 [21••]	34	279
Shin *et al.*, 2014 [9••]	7824	400
Kettunen *et al.*, 2016 [83]	24 925	123
Illig *et al.*, 2010 [84]	1809	163
Ma *et al.*, 2015 [11]	26	262
Rhee *et al.*, 2013 [12]	2076	271
Hou *et al.*, 2014 [85]	38	562
Balog *et al.*, 2013 [13•]	302	2933
Guo *et al.*, 2015 [14]	80	600
Hoerr *et al.*, 2016 [27]	33	260
Tiziani *et al.*, 2011 [29•]	56	100
Kurita *et al.*, 2015 [30•]	234	10 977

after release from carbon starvation. Kinetic modeling provided the key to interpret the observed antiphasic accumulation of several carbon-rich amino acids and nucleotide metabolites during carbon starvation and their rapid decrease upon growth resumption. Dynamic data were crucial to reveal preservation of costly metabolites during starvation and ultrasensitive allosteric inhibition of

Table 2

List of referenced papers that perform dynamic metabolome profiling. For each study we report the finest time resolution, duration of the time course experiment and detected ions/metabolites (coverage). Data are displayed in Figure 2b

Study	Finest time resolution	Maximum duration	Coverage
Laye *et al.*, 2015 [86]	10 days	40 days	928
Shaham *et al.*, 2008 [53]	30 min	2 hours	191
Park *et al.*, 2015 [87]	12 hours	2 days	1025
Oliveria *et al.*, 2015 [88]	4 min	6 hours	337
Kuehne *et al.*, 2015 [46•]	1 min	5 min	2570
Wen *et al.*, 2015 [54•]	5 min	135 min	26
Ewald *et al.*, 2016 [55]	10 min	135 min	942
Berney *et al.*, 2015 [89]	1 day	6 days	Unknown
Zaitsu *et al.*, 2016 [57]	10 s	6.5 min	26
Heinemann *et al.*, 2014 [58]	3 min	1.5 hours	77
Marasco *et al.*, 2015 [59]	9 min	180 min	Unknown
Link *et al.*, 2015 [60••]	15 s	150 min	419

their biosynthesis upon growth resumption as key mechanism to firstly, enable a jump start of growth; and finally, metabolite-based allosteric coordination of building block biosynthesis.

The current state-of-the-art in sampling allows for a temporal resolution of about 10 s, where over 100 ions increased already within the first sampling period after glucose addition [60••]. This resolution is sufficient for processes that occur in the range of minutes down to about half a minute, which is borderline for investigation of faster processes such as allosteric metabolite regulation, the effect of enzyme phosphorylation, or drug inhibition. The factor limiting temporal resolution is the composition of the buffer that is necessary to maintain cells in a physiologically relevant state. High salt concentrations saturate the electrospray ionization chamber for several seconds before they are cleared by the air stream. Finer-grained resolution at seconds is technically feasible provided that salt interferences are reduced. This could be achieved by firstly, implementing on-line desalting techniques, that is, electrophoretic or membrane-based; secondly, employing MS-friendly buffers such as ammonium acetate; thirdly, improving the design of the electrospray source to enhance ion clearance; or finally, further dilute the samples before injection provided that detection of metabolites is not compromised.

Such virtually instantaneous readout of cellular metabolism opens new avenues and several computational methods could be exploited to elucidate the underlying regulatory architecture. Generally, real-time measurement improves the notorious biological and technical noise problem. Higher frequency measurements enable better statistical differentiation by significance analysis and filtering methods to sift the signal [22,61] and would expectedly decrease the false negative rate of novel allosteric interactions [62,63]. Graph-based activity motif analysis [64,65] or differential equation models [64,65] could be applied to metabolomics to infer ordering of events. Some first applications to dynamic metabolome data have already allowed to find causal effectors or associated pathways from binned functional motifs [60••,66]. Sampling more frequently would allow moving beyond simple clustering of dynamic responses to treat metabolism like electrical signal processing where metabolite abundances are signals and the connecting pathways comprise output (transfer) functions as was shown for expression data [67,68]. Upon pulse or step treatment, the output signals (dynamics of downstream metabolites) could be used to define the function, which encapsulates *in vivo* information about inhibition or cooperation, as demonstrated before [69].

The instant readout of metabolism could also be exploited to actively control metabolism in response to the monitored state, for instance by modulating nutrient availability or by supplementing inhibitors. This opens the fascinating option for automated, comprehensive model generation using robot scientists, an approach successfully applied in gene function elucidation and drug discovery [70,71]. In contemporary examples, metabolic profiles were used to generate an ensemble of potential metabolite–protein interactions that could be whittled after follow up perturbation experiments [40,48,60••,69]. These methods are limited in the coverage of metabolism and the number of candidate associations that can be posed primarily because of the lag time between generation and follow up. A self-learning, automated system could side step such issues by using decision trees to select treatments for maximal information gain. In principle, the robot scientist could converge to a consensus model by selecting the ideal perturbation to apply to a continuous culture or repeatedly setup new experiments while sampling and consequently generate larger, more refined metabolite associations. Independent of the robot scientist, we expect that real-time feedback on the metabolic state will boost elucidation of the underlying regulatory architecture.

Single cell metabolomics

The third frontier of high-throughput metabolomics are single cell analyses. In this context, the use of MS is typically motivated by the need of profiling multiple compounds individually in hundreds of cells to discover and study metabolic subpopulations. This complements the use of fluorescent biosensors and microscopy to analyze single and specific compounds. Regardless of throughput, single cell metabolomics is a challenging task because of the limit of detection imposed by instruments. Under optimized conditions, about 1 amol molecules are necessary for detection using mild ionization techniques such as electrospray or MALDI. For an average yeast cell (50 μm^3), this amount corresponds to an intracellular concentration of 20 μM. Many key primary metabolites pass this threshold and are in fact detectable in single yeast cells [2]. For an average bacterium of 1 μm^3 volume, however, the detection limit is around 1 mM and, thus, prohibitively high to detect most metabolites. Most current work is of technical nature to illustrate the potential of single cell metabolomics by using large cells, which were often cherry-picked because of size.

Focusing on methods with substantial potential for throughput, two basic chromatography-free strategies emerge from recent literature. The first strategy works for very large animal or plant cells that are adherent or embedded in native tissue. It is based on collecting the metabolome content of a single cell in a thin capillary that is subsequently moved to the MS and used as nanoelectrospray-emitter. Organic solvents are added to the tip to support ionization. Fujii *et al.* used the nanospray tip to punch plant cells and suck the cytoplasm [72]. With a similar technique, Zhang and Vertes profiled >70

metabolites in single human hepatocellular carcinoma cells [73]. To avoid the laborious microinjection, rapid and efficient extraction of metabolites from single mammalian cells was demonstrated with the deposition of a small droplet of 2 nL organic solvent on the cell surface but introduced additional drying steps [74]. From sampling to completion of data acquisition, this strategy typically requires 10–20 min for analyzing a single cell due to the numerous and delicate manual steps necessary. The failure rate can attain 30% [74].

The alternative experimental strategy works for suspended cells. The whole culture is pretreated to quench metabolism, diluted, and then deposited on small-sized hydrophilic spots arrayed on a hydrophobic surface [75]. Snap freezing with liquid nitrogen leads to disruption of cells. During drying on the surface, the aqueous phase and hydrophilic content of individual cells is retained on the spots. Metabolites on each spot are profiled by MALDI and high-resolution MS. This approach was used to profile lipids, chlorophylls, and carotenoids in >800 single algal cells of about 100 μm^3 [76•] in about 2 hours (R Zenobi, personal communication). Notably, the measurement was still timely inefficient because the exact position of the cells on the target was unknown. To eventually capture the metabolome signal of a single cell placed somewhere on the hydrophilic spot, this has to be analyzed at 70 distinct and mostly uninformative positions. Hence, this approach offers large room for further improvements in speed.

While single cell metabolomic analysis by MS is readily possible for most eukaryotic cells, its throughput is limited by the poor integration of microscopy and automation of the micromanipulations necessary to obtain concentrated metabolome samples. Further optimization is necessary to achieve routine analysis of large populations. Apart from high throughput, current methods do not allow distinguishing between measurement and biological noise. To eventually achieve a meaningful analysis of metabolomes in single cells, it will be mandatory to implement strategies for normalization and quality control that can monitor or account for differences in cellular volume and sampling.

Conclusion/discussion

Most current large scale studies are based on canonical LC–MS or GC–MS based methods at a throughput of about 100 samples per day and instrument. Exploiting the unique advantage of high resolution MS-based metabolomics for direct profiling of non-fragmented, molecular ions enables rapid measurements at 10–100 times higher throughput. At present, this is only feasible by compromising metabolite coverage or sacrificing separation of isomers in favor of throughput. The achievable throughput, however, opens entirely new scientific and medical opportunities that require firstly, the conception of novel experimental designs and finally, development of novel computational approaches for data integration that move beyond canonical statistical association. Both developments are urgently needed to improve testable hypotheses generation on mechanism and causal relationships in the face of complex and multifaceted networks in even the simplest cells.

High-throughput metabolomics is presently being developed along the three dimensions of firstly, large sample numbers; secondly, temporal resolution; and finally, individual cells. Several large scale applications were already published or are ongoing (Figure 2), and we expected in the near future many more studies with an order of magnitude larger numbers of steady state samples. Likewise we anticipate a major increase of large scale studies that explore the high-throughput potential by sampling dynamic metabolomics across large sample cohorts. High temporal resolution, in part enabled by effective sample processing workflows, is emerging and expected to significantly improve the data basis for computational modeling of cellular metabolism and regulation. Hampered by present MS limitations, poor integration with microscopy, and missing automation of micromanipulations, single cell metabolomics is presently lagging behind, but will eventually be of extraordinarily importance to understand cellular behavior.

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- •• of outstanding interest

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