Reviews • KEYNOTE REVIEW

Cell culture metabolomics: applications and future directions

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Metabolomics represents a global quantitative assessment of metabolites within a biological system. The metabolic analysis of cell cultures has many potential applications and advantages to currently used methods for cell line testing. Metabolite concentrations represent sensitive markers of both genomic and phenotypic changes. Consequently, the development of robust metabolomic platforms will greatly facilitate various applications of cell cultures – including, for example, the understanding of the *in vitro* and *in vivo* actions of drugs – and aid in their rapid incorporation into novel therapeutic settings. In addition, metabolomic analysis of cell lines provides information, either independently or in conjunction with other omics measurements, essential for system level analysis and modeling of biological systems. This review outlines some of the applications of metabolomics in cell culture analysis and some of the issues that need to be addressed to make this approach more relevant.

Introduction

Metabolomics, initially defined as the global analysis of all metabolites in a sample [1,2], and metabonomics, perceived as the analysis of metabolic responses to drugs or diseases [3], are nowadays often interchangeable terms broadly referring to the multi-component analysis of metabolites in a biological system [4]. Metabolomics can be viewed as a 'reinvention' or extension of the approaches of analytical biochemistry of the 1960s; however, there are some major differences between modern metabolomics and the analytical biochemistry of the past. First is the introduction of highly advanced and reliable instrumentation, such as nuclear magnetic resonance (NMR) and mass spectrometers (MS) for parallel, quantitative analysis of complex biological samples. These advanced analytical tools provide the high degrees of sensitivity, selectivity, matrix independence and universality required for metabolomic-scale experiments. Second is the introduction of a novel data-driven approach aiming to observe all measurable metabolites without any preconception or preselection. This approach requires the development

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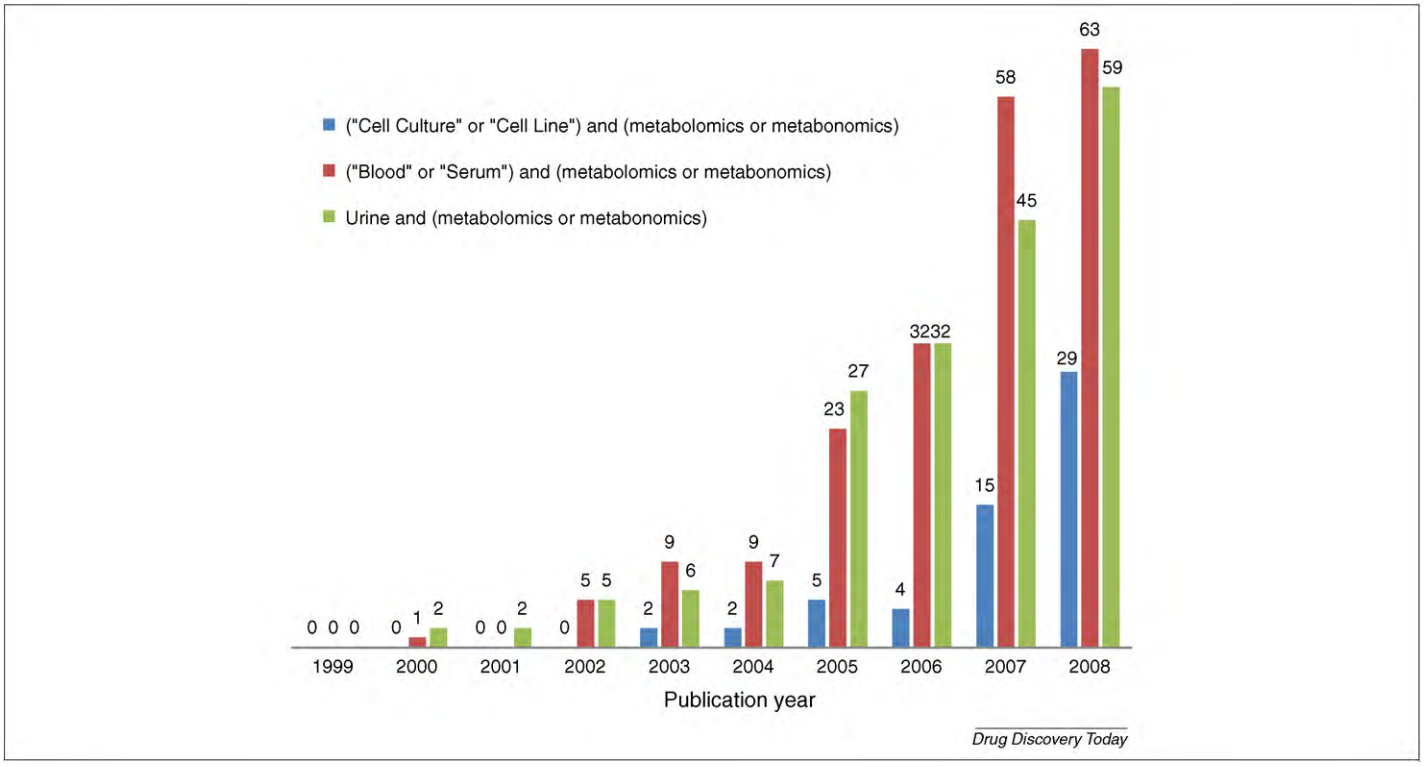

FIGURE 1

PubMed search using keywords outlined in the legend. The numbers presented show only the general trend in publications in the past nine years and should not be considered as absolutely correct because one of the major journals in the field is still not part of PubMed database.

of novel metabolite extraction procedures that assure high recovery of all different classes of compounds in parallel, both reliably and reproducibly. The third difference is the introduction of various data analysis procedures, computational tools and methodologies that are able to quantitatively and accurately analyse large amounts of data. These tools must handle, store, preprocess and analyse complex and often large datasets.

The focus of metabolomics in studies related to human biology and health has, thus far, been largely holistic in terms of biological systems analysed and has focused mostly on the analysis of body fluids for various clinical applications [4]. This approach has since been proven to have a large clinical, as well as biopharmaceutical, potential (reviewed in Refs. [5–7] with several recent examples: [8–10]). Future clinical applications are still awaiting the collection of larger datasets that can account for variations in human metabolic profiles and can lead to the discovery of appropriate metabolic biomarkers. Major efforts are being made in this regard [11] with high expectations in the development of novel diagnostic methods, disease risk factor assessments and toxicity analysis of various stimulants. The focus on the metabolic profile of the whole organism, however, does not provide relevant information about specific cell types under different conditions, which is crucial for a more holistic understanding of cell properties and functions and for the development of drugs and markers for specific cellular phenotypes. An overview of publications for various applications of metabolomics (Fig. 1) clearly shows that cell culture or cell lines efforts are lagging behind metabolic analysis of body fluids. In this review, we focus on the major aspects of the application of metabolomics in the analysis of cultured mammalian cell lines. Data provided by metabolic profiling of individual cells can be complementary to the whole system results. Cell line applications are easier to control, less expensive and easier to interpret than analysis of both animal models and human subjects. Furthermore, some extremely difficult problems facing other metabolomic applications – such as individual variations across different subjects and time points, difficulty in population control, and various confounding factors such as age, gender, overall health, environmental exposures and contributions from different tissues – are not issues in cell culture applications. In addition, analysis of cell cultures does not require the same level of ethics consideration as is required for applications in animal and human subjects. Focusing on a specific cell type can reduce variability and provide a more constant background against which more subtle metabolic changes become apparent. Cell line measurements of metabolites can be directly correlated with genomics or proteomics data. Furthermore, metabolomic data – independently or in conjunction with other data – can be used for the development of models of biological pathways and networks. At the same time, metabolomic analysis can greatly aid other cell culture studies in the interpretation of various results [12].

The development of cell culture metabolomics, however, has been impeded by several challenges specific to this application. Some of the major issues include the following [13–17]: 'quenching' of cell cultures – halting cellular metabolism, harvesting and processing of samples to prevent alterations in metabolic profiles during the extraction procedures; variability of growth medium formulation and additives; differential rates of proliferation for cell lines based on growth conditions and age (passage number) of the

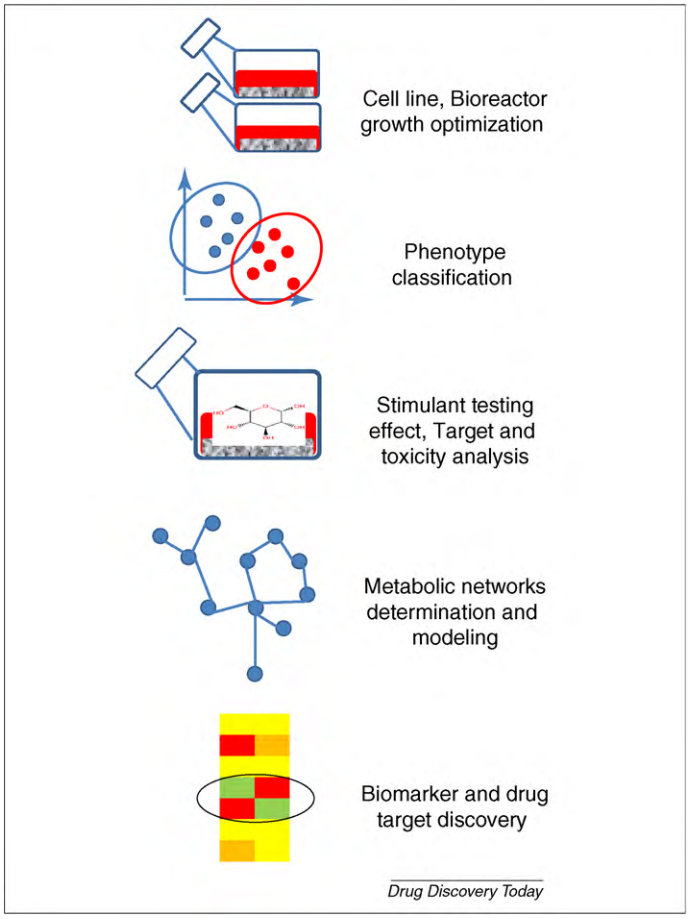

FIGURE 2

Five major application areas of mammalian cell line metabolomics. Many different applications and approaches can be envisioned for each major group.

culture and possible downstream effects on metabolite concentrations; addition of foreign molecules during metabolite extraction procedure and their possible effects on metabolic profiles; time-consuming metabolite extraction procedures that can lead to degradation of labile metabolites, as well as long hands-on time, making high-throughput screening and experimentation problematic; and difficulties in obtaining sufficiently large numbers of cultured cells for analysis. Recent efforts have provided methodologies that alleviate some of these problems, thereby making cell line metabolomics much more accurate, faster and more informative. Some of these new methods will be outlined in this review.

In addition, some of the general problems of metabolomics are also prominent in cell culture applications, including [18,19] an inability to measure, identify and quantify all present metabolites and issues in data analysis (the curse of dimensionality, as well as subjective problems of insufficient attention to statistical test requirements, insufficient replication, and omission of cross-validation and independent validation). Many of the issues facing data analysis can be resolved by appropriate experimental design and the application of appropriate analysis methodologies. Combined use of different types of metabolomic measurements can lead to the determination of approximately 1000–2000 metabolites [8], and although this does not cover all the metabolites present in the system, it does provide a large information pool and good coverage of many metabolic processes in cells.

There are many possible applications for cell line metabolomics, and some prominent examples are outlined in Fig. 2. Currently, the greatest interest focuses on: cell phenotype analysis – cell culture and bioreactor optimization, as well as classification of cellular phenotypes aimed at, for example, molecular classification of disease subtype; system biology (defined as an interdisciplinary field that focuses on complex interactions in biological systems) – metabolic network determination can be achieved through metabolomics alone or in conjunction with other omics data; metabolic network and pathway models that can be based on metabolic profiling in time (i.e. fluxomics); toxicology and drug-testing studies, tracking both pharmacokinetic (metabolism of drug) and pharmacodynamic (drug effect on metabolism) profiles after treatment; and determination of the metabolites with the most significant differences in their concentration levels in different phenotypes aimed at, for example, discovery of markers of sample phenotypes or drug targets.

Some published examples of these different applications will be outlined throughout the text. For any application, a cell culture metabolomic experiment can be divided into five general steps: experimental design, cell culture growth and/or stimulation, quenching and metabolite extraction, metabolomic measurement, and data preprocessing and analysis. In the following text, we review major issues related to each of the five steps leading from the initial research question to a final answer from metabolomic results.

Experimental design

The biological variability is expected to be small in cell lines grown under the same conditions and in the same population context [20], compared with the variability that is observed in animal models and human subjects. In addition, using standard operating procedures, metabolomics analytical methods can result in data that are highly reliable across different instruments and over time [21,22]. It is still essential, however, to include proper experimental design to reduce bias and variance in the parameters of interest, while ensuring that the planned experiment is feasible and that the questions of interest can be answered with the resulting data. In this sense, experimental design must incorporate randomization, replication and local control in a manner similar to other fields. Epidemiological issues that have to be carefully observed in whole-organism or population-based studies of metabolome are of reduced concern in cell culture applications. Randomization protects an experiment against extraneous factors of chance. In cell line metabolomics, factors of chance can include cell line population effects [20], variability caused by different personnel (e.g. differences in yield or efficiency in metabolite extraction and cell culture maintenance) or instrumental variability, such as drift in the MS. It is crucial to understand all possible sources of variability and minimize their effects by randomizing the samples accordingly and by introducing quality control standards (as was shown for the correction of MS drift [21]). Replication is used to increase statistical accuracy of the results. In cell culture applications, it is crucial to include both biological and technical replicates. Cultures grown independently (in separate flasks) and subsequently treated with the same compound would represent biological

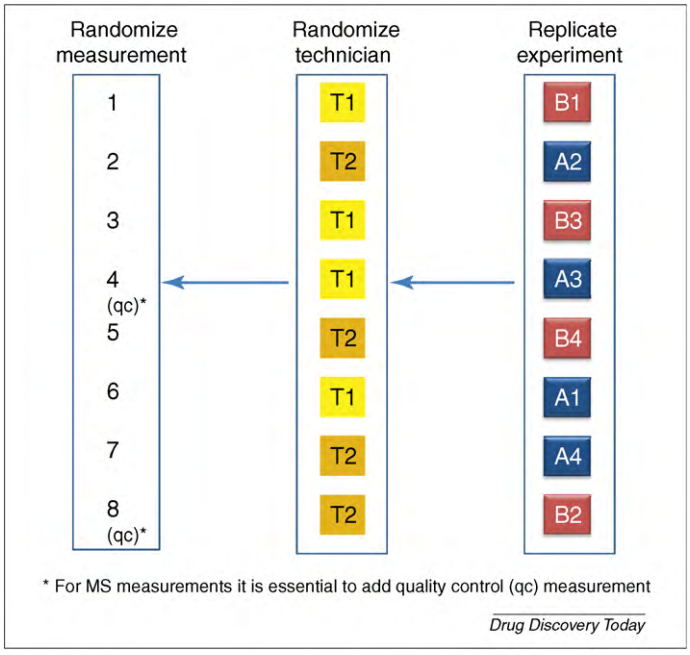

FIGURE 3

Example of experimental design for a simple experiment with two groups of samples performed by two technicians. In this experiment, it is essential to perform replication (experiments A and B are replicated four times) and randomize known causes of unwanted variance (experiments A and B are randomized between two technicians). Finally, measurements are performed randomly with the possibility of inserting quality control samples (essential for MS measurements).

replicates. Technical replicates would be different aliquots of cells harvested from the same culture flask after treatment or even multiple measurements of the same extracts. The experiment should include replication of all the steps applied to independent cell line cultures (biological replicates) with several technical replicates of all subsequent cell processing steps. In cell culture metabolomics, at least three technical replicates are essential to ensure accuracy of measurements. The exact number of replicates needed depends on the variability between samples, the expected range of variability, variance between observed groups (window of effect) and the power of the test being performed. The number of replicates, therefore, will need to be determined for each specific experiment from preliminary measurements. Finally, local control requires that the known sources of variability are either completely removed (if possible) or deliberately made to fluctuate widely (by collecting many samples with large variation) so that their effects can be measured and eliminated (i.e. averaged out) from experimental error. In a cell culture experiment, local control is reflected in strict regulation of the cell source and growth conditions. A simple experimental design comparing two cell cultures performed by two technicians is shown in Fig. 3.

Cell culture growth, stimulation

The differences in optimized cell culture growth conditions present another major concern for cell line metabolomics. This is particularly an issue in studies involving comparative analysis of several different cell types, all of which might require different growth medium formulations. These formulations might contain different levels of glucose, glutamine and lactate, as well as other nutrients and additives, which will probably lead to differences in the metabolome of the cells. If possible, it is recommended to use the same growth medium for all cell lines in the study to reduce variance in metabolic profile that can be caused by the medium. Studies with equalized growth medium were performed on acute myeloid leukemia cell lines [23] and primary fibroblast cell lines [24]. Unfortunately, using suboptimal media formulations for cell lines affects their growth behavior and might ultimately provide a different metabolic profile to the same lines grown under more optimized conditions. In several other studies, it was not possible to achieve the same growth conditions for different cell lines because the same conditions would either lead to cell death in some lines or result in large variations in cell growth rate. Examples of cell metabolomic analyses with different cell line growth conditions are the analysis of metabolic differences in breast cancer and normal cell lines of Meadows and co-workers [25], the analysis of different types of breast cancer cell lines [26] and the investigation of lung carcinoma cell lines showing different levels of drug resistance [27].

The standard enhancement of cell culture medium with serum of animal origin can add another level of complexity in cell growth condition optimization. Variations in serum can lead to contamination with exogenous metabolites and alterations of endogenous cell metabolite. An early application of NMR metabolite analysis outlined large effects that different media, including serum type, can have on cellular metabolic profile [28]. A more recent analysis exploring the changes in metabolic profiles in cells grown under serum-enriched, serum-reduced or serum-free medium or serum substitutes [29] have shown that the supplementation of cell culture medium with serum can influence the metabolism of cells and the observed profiles even when there are no observable effects on cellular morphology. To the best of our knowledge, however, an exploration of the effects of batch differences in standard calf serum on the metabolic profiles of cell lines is still lacking. Thus, addition of serum should be considered as a possible source of variation and effort should be made to use the same serum and, ideally, the same batch of serum for each group of experiments.

Although differences in growth condition do add variance to the data, important conclusions have been obtained even when growth conditions between cell types could not be optimized. Previous efforts have shown that phenotypic differences influence metabolic profile more than cellular growth conditions [25–27]. Proper experimental design is crucial to minimize the effects of these variables on the final results of metabolomic experiments. Nonetheless, more effort is required in the future for the determination of metabolic differences caused by various growth conditions, cell culture age and/or passage number for different cell lines.

Quenching and metabolite extraction

The goal of metabolomics is to analyze all or, at least, as many as possible different metabolites without selectivity for any particular molecular type and/or characteristic. This requirement creates a major problem in the development of metabolite extraction procedures because any extraction method is unavoidably selective towards some molecular types (such as polar *vs.*

non-polar molecules). Thus, sample extraction can led to major loss of particular cellular components. In the analysis of body fluids, the metabolic profiling can often be performed directly from the sample with only minor sample processing. Measurement of cell metabolites can be done from the whole cells with NMR, but a more informative approach requires metabolite extraction from cells and the removal of large biomolecules (i.e. protein, DNA and RNA) and/or the isolation of separate metabolites from different subcellular compartments (i.e. organelles). It should be mentioned that whole cell metabolomics profiling can be performed using NMR spectroscopy. ^{1}H NMR has been employed to study intact human cells; however, these measurements resulted in broad, non-informative spectral profiles [15]. High-resolution magic angle spinning (HRMAS) ^{1}H NMR has lead to large resolution improvement in the analysis of whole cells with several different applications for the analysis of cell type [30], cell differentiation [31], genetic modification [32] and response to drug exposure [33,34]. Recent work has attempted to establish optimal cell manipulation, freezing and storage conditions for HRMAS NMR procedures [15]. The work of Duarte *et al.* [15] has shown that although cell integrity is generally well preserved in both fresh cells and those frozen in cryopreservative, there is a higher degree of membrane degradation observed in fresh cells. Cell lyses by mechanical methods or by freeze–thaw cycles without cryopreservative showed notable changes in lipid profile when compared to intact cells. For studies of cellular lipids, HRMAS was a more accurate method than solution-state NMR [15]. HRMAS of lysed cells gave enhanced information on lipids and comparable resolution for smaller molecules in comparison to solution-state measurement of cell extracts.

For the complete analysis of a cell culture, it is important to measure both extracellular (footprint) and intracellular (fingerprint) metabolic profiles. Metabolic footprinting is technically simple because it requires only centrifugation to separate culture media and cells before the analysis. Metabolic fingerprinting, although much more technically challenging because it requires metabolite extraction from cells, provides more complete information about cellular metabolic processes.

Over the past few years, several optimized protocols have been developed for the extraction of intracellular metabolites [13–17]. The fundamental requirement for metabolic fingerprinting is that all enzymatic activities are stopped (i.e. quenched) as quickly as possible. It is also highly important that the procedure is reproducible and rapid to enable sufficient replication. A procedure proposed by Sellick and co-workers [13] involves quenching the cells with 60% methanol supplemented with 0.85% ammonium bicarbonate at −40 °C. This fast and reproducible procedure was able to generate a profile representative of the physiological status of the cells. Another extremely simple, fast and highly reliable quenching and extraction procedure was proposed by Teng *et al.* [14]. This procedure, developed for adherent cell lines, involves rinsing cells with ice-cold phosphate buffed saline and quenching using only methanol. Use of effective quenching methods is crucial, not only for observing differences between cell samples but also for establishing a representative cellular context or background profile against which any metabolic changes can be assessed. After cell quenching is metabolite solvent extraction, typically by dividing lipophilic (in chloroform) and hydrophilic (in water) metabolites. Extraction is followed by lyophilization and reconstitution in MS- or NMR-friendly solvents.

Independently, it is very difficult to determine the optimal combination of quenching and extraction procedures. Recently, however, an extremely valuable study appeared comparing different metabolite extraction protocols for mammalian cell cultures [35]. In this work, the authors compared 12 different extraction methods; according to their results, extraction in cold 50% aqueous acetonitrile was superior to other methods. Wisely, Dietmair *et al.* [35] have tested these 12 extraction methods on a known mixture of many standard metabolites. This analysis enabled the authors to very precisely determine the ability of different procedures for 100% recovery of metabolites. Furthermore, the authors have tested several quenching procedures on CHO cells using fluorescence staining to determine whether cell quenching might lead to metabolite leaking through the membrane. From this analysis, authors have concluded that the only quenching method acceptable for fragile animal cells was cold 0.9% (w/v) NaCl, and all the other methods outlined above render cell membranes prone to leaking.

Metabolite measurements

The metabolite measurement approaches for cell cultures do not differ from other metabolomic applications. Thus, once metabolites have been extracted, analysis consists of separation (in the case of chromatography-based methods), identification and, if possible, quantification. Many analytical instruments and methods exist for these applications. Currently, the majority of metabolomic measurements are performed using NMR and MS, with a range of different experimental and technological variations depending on the specific questions and focus of the experiment. The general requirements for metabolomic instruments are: excellent sensitivity and resolution for a wide range of molecule types; the ability to handle a large range of concentrations (from pM to mM) for different molecular types; the ability to identify and quantify different molecules; short analysis time, to enable the measurement of many samples without sample degradation during the measurement [12]; and reproducible measurement across different centers and in time.

Several reviews have dealt with the application of NMR and MS in metabolomics [5,36,37]. NMR is a non-invasive, non-destructive, highly discriminatory and fast method that can analyse rather crude samples. NMR spectroscopy can be performed without extensive sample preprocessing and separation and provides several different experimental protocols optimized for mixture analysis and molecular formula or structure determination. The results of NMR measurements have proven highly replicable across centers and instruments [22]. NMR can provide measurements for different types and sizes of both polar and non-polar molecules through analysis of different spectral windows [10,11]. In addition, NMR instruments are highly versatile and with only minor changes in probes, users can obtain spectral information for different nuclei (^{1}H, ^{13}C, ^{15}N, and ^{31}P among others) in solvent or solid samples and even *in vivo* [37,38]. Many different experimental protocols in NMR (i.e. pulse sequences) can show molecular spectra at different levels of resolution. NMR is also the only method used in metabolomics that currently enables direct

measurements of molecular diffusion (i.e. the separation of different components in the mixture base of diffusion coefficients, through – for example – diffusion-ordered spectroscopy experiments [19,39] or line shape analysis [40]), interactions and chemical exchange (from, for example, the exchange spectroscopy experiment). Furthermore, the above-mentioned HRMAS NMR enables measurement of metabolic profiles of whole cells, thus reducing losses owing to extraction protocols. Various pulse sequences can lead to highly quantitative and easily assigned measurements (as in 2D experiments such as total correlation spectroscopy, which provides information about all coupled spins, including ones that are not directly coupled, or heteronuclear single-quantum coherence, which provides correlation between protons and heteronuclear atoms, such as ^{13}C or ^{15}N, that they are attached to) or extremely fast measurements – as with 1D experiments. Several databases and methods are currently being developed that enable metabolite identification and quantification from NMR spectra (Table 2). The major problem with NMR technology as applied to metabolomics is its low sensitivity, which limits the majority of currently available instruments to measurement of fewer than 100 metabolites. Recent technological advances such as CryoProbes (readily available for modern instruments) and newly developed methods such as signal amplification by reversible exchange [41] are leading to considerable improvements in NMR sensitivity.

The role of MS in metabolomic research is constantly expanding, whether the focus is on profiling (targeted analysis) or pattern-based analysis (i.e. fingerprinting or footprinting) [36]. Recent technological advances in separation science, ion sources and mass analyzers have considerably increased the sensitivity, selectivity, specificity and speed of metabolite detection and identification by mass spectrometry. Electrospray ionization, along with the complementary ion sources of atmospheric pressure chemical ionization, atmospheric pressure photoionization and atmospheric pressure matrix-assisted laser desorption and ionization have expanded the range of metabolites well beyond the volatile and thermally stable analytes measured by gas chromatography–MS [42].

There are five important considerations that need to be dealt with in any global metabolite analysis by MS, namely: (i) the efficient and unbiased extraction of metabolites from the sample matrix, (ii) separation or fractionation of the analytes by chromatography, (iii) ionization of the analyte molecules, (iv) detection of mass signals, and (v) analyte identification. Separation of analytes before mass spectrometry detection is an important step leading to detection of more features, effectively increasing the overall 'peak capacity' of the analytical platform. Separation methods include condensed-phase separation methods (high- or ultra-performance liquid chromatography [43] and, less commonly, capillary electrophoresis) and gas-phase analyte separation (gas chromatography or, more recently, ion mobility spectrometry [43] and/or high-field asymmetric waveform ion mobility spectrometry [44] in combination with Atmospheric Pressure Ionization-MS). Direct infusion mass spectrometry [45] relies solely on the mass spectrometer to perform separation and offers a notable advantage in terms of speed and sample throughput. The number of identified features in a MS measurement can also be increased by changing the polarity of the ion source. Positive ion mode electrospray is generally optimal for basic metabolites (e.g. amines). Negative ion mode provides optimal measurement for acidic metabolites. Contemporary mass spectrometers can rapidly switch between negative and positive polarity during the same experiment so that a complete set can be acquired from one injection of the sample. Several types of MS can also be used for ion analysis. Quadrupole-based mass spectrometers (linear quadrupoles and ion traps) are the most common and generally the least expensive; however, they typically offer the lowest resolution. Triple quadrupoles have the distinction of being the quantitative workhorse owing to their wide dynamic range and excellent selectivity. Time of flight, Fourier transform ion cyclotron resonance (FTICR) and Orbitraps offer very high resolution and mass accuracy, leading to the assignment of exact empirical formulae to mass spectral peaks. Knowledge of the empirical formula based on exact mass can often be used to assign one or a few putative identifications that can then be used for searching metabolic or chemical databases (Table 2).

TABLE 1

Comparison of characteristics of major experimental methods for metabolomic analysis.

Analysis	NMR	MS
High throughput – metabolites	No	Medium
High throughput – samples; automation	Yes	No
Quantitative	Yes	Yes
Availability in clinic	No	No
Equipment cost	High	High
Maintenance cost	Medium	High
Per sample cost	Low	High
Required technical skills	Yes	Yes
Sensitivity	Medium	High
Reproducibility	High	Low
Data analysis automation	Yes	Yes
Identification of new metabolites	Difficult	Possible
Chemical exchange analysis	Yes	No
Stereoisomers analysis	Yes	Difficult
Sample preservation	Yes	No
***In vivo* measurement**	Possible	Impossible

A comparative outline of the characteristics of NMR and MS methodologies as applied to metabolomics is provided in Table 1. The two methods are highly compatible and, thus, an ideal approach is to combine the results from NMR and MS measurements. Although both methods are readily available to only a handful of research groups, an increasing number of excellent examples of dual-measurement applications have recently appeared in the literature [45–51] with few examples of the application of this synergistic approach in cell line analysis [48–51]. This synergetic approach has, thus far, led to highly informative models of cellular pathways [48–50] and a novel method for testing drug metabolism in cell lines [51]; these examples will probably stimulate further applications.

Another beneficial experimental method for cell culture metabolite analysis involves stable isotope labeling followed by either MS or NMR measurement. This approach enables pathway tracing, easier metabolite assignment and metabolic flux measurements

TABLE 2

Some major non-commercial databases of metabolomic standard data for quantification and assignment.

Name and availability	Instrument	Additional information
Human Metabolome Project [81] (http://www.hmdb.ca)	NMR, MS	Biological data; chemical and clinical data specific to humans
BMRD (http://www.bmrb.wisc.edu)	NMR	Database search for NMR peaks assignment
Prime (Akiyama [82]**)** (http://prime.psc.riken.jp)	MS, NMR	
Golm metabolome database (http://csbdb.mpimp-golm.mpg.de)	MS	Specific to plants
METLIN metabolite database (http://metlin.scripps.edu)	MS	Drug and drug metabolites; specific to humans
NIST Chemistry WebBook (http://Webbook.nist.gov/chemistry)	NMR, MS, IR	
Madison metabolomics database (http://mmcd.nmrfam.wisc.edu)	MS, NMR	
NMR Lab of biomolecules (http://spinportal.magnet.fsu.edu)	NMR	Database search for NMR peaks assignment

(see Refs. [50,51] and references therein). Isotopic labeling has previously enabled detailed determination of pathways leading to the production of certain metabolites [52] and the development of highly accurate mathematical models of these pathways [53].

In addition to the application of NMR and MS methods previously developed for many other applications, new variations of these instruments and their applications are emerging specifically for metabolomic applications. These innovative applications can lead to more sensitive measurements in small samples [54], more specificity and sensitivity to a subset of metabolites [55], and more sensitive *in vivo* measurement of specific metabolites [56].

Data preprocessing and analysis

Qualitative and quantitative cell metabolomic data analysis – general points

Data analysis approaches in metabolomics can broadly be divided into qualitative and quantitative. The type of analysis approach defines the necessary preprocessing steps. In qualitative metabolic fingerprint analysis, complete metabolomic spectra or spectral regions are used. The simplest analysis can be visual, based mostly on the inspection of the differences between metabolic profiles. Differences in metabolic profiles can sometimes be so pronounced that even a cursory examination of the spectra can lead to the determination of different phenotypes. Furthermore, with current methods (particularly with NMR spectroscopy), only a limited number of highly prevalent metabolites are actually measured, making it possible to see differences directly from spectral data. Although this cursory approach is highly subjective and can lead to erroneous results and oversights and, thus, should not be encouraged, it has resulted in some useful discoveries in the past [29,57–60]. A more accurate but still qualitative approach involves chemometric and statistical analysis of spectral patterns and intensities of the mixtures. Spectra can be compared statistically to classify samples (unsupervised analysis). Alternatively, spectral data can be used to identify the relevant spectral features or regions that distinguish between sample classes – phenotypes (supervised analysis). Once these major features are identified, different approaches can be used to assign them to the corresponding metabolites [61,62] through, for example, comparison with publicly accessible databases of known metabolites (Table 2). By contrast, quantitative metabolomics initially performs compound identification. Once compounds have been identified and quantified, the data can be used for various applications, including the development of system biology models, or for biomarker discovery (reviewed in Ref. [64]). If the quantitative measurement of metabolite concentrations is performed over time, the method is called fluxomics. The major steps and applications of qualitative and quantitative data analysis are outlined in Fig. 4 with some of the terminology outlined in Box 1.

The qualitative approach has a range of clinical applications for sample classification and in this context has some advantages over the quantitative approach [7]. Application of spectral data, however, leads to specific problems such as peak drift caused by different experimental conditions and overlapping and broad peaks. The issue of result normalization is still unresolved, and the application of a suboptimal method can lead to errors and biases in the results. Use of spectra also leads to the inclusion of spectral regions that represent background noise in the analysis. This leads to the use of unnecessarily large datasets and can also result in inaccuracies in classification caused by noise regions. Furthermore, overlapping of metabolic peaks can lead to erroneous conclusions. Quantitative metabolomics can, in principle, alleviate some of these issues by determining concentrations of individual metabolites from spectra. In turn, availability of metabolite concentrations can lead to many more applications of metabolomics. A major concern in quantitative metabolomics is the problem of spectral assignment. Unlike transcriptomics (where gene assignment is trivial thanks to highly specific hybridization of genes to specially designed, unique probes), the high-throughput analysis of metabolic mixtures, peak assignment and the measurement of abundance for each metabolite requires spectral deconvolution and availability of measurements under the same conditions for all metabolites that can possibly be present in the system. Some of the major problems faced by the qualitative approach remain in quantitative metabolomics, including issues with the overlapping spectra and changes in spectra of various compounds under different conditions (i.e. pH-induced changes in ^{1}H NMR spectra). In addition, unidentified metabolites present a great challenge to quantitative metabolomic analysis. Possible experimental solutions are the use of more complex experiments (multi-dimensional NMR), more involved preprocessing procedures (particularly for MS [63]) and/or the combined use of different methods (e.g. the use of both MS and NMR methods or a combination of high-throughput liquid and gas chromatography MS [8], with or without isotopic labeling). Thus far, the assignment and quantification of metabolites requires comparison with

BOX 1

Explanation of some of the major terminology used in the field and in this article

Chemometric analysis: Chemometric analysis involves recording and statistical comparison of spectral patterns without prior compound identification. The focus of this type of analysis can be either sample comparison from complete spectra or the determination of relevant spectral features that distinguish between sample classes. Metabolites corresponding to relevant spectral features can be determined subsequently.

Quantitative analysis: In this approach, compounds (i.e. metabolites) are usually identified and quantified initially in comparison to the spectral reference library obtained from pure, reference standard compounds. The quantitative information about individual compounds is then used for further sample classification, biomarker discovery or determination of information related to cellular pathways.

Exploratory data analysis: Exploratory data analysis is a preliminary data analysis step that leads to the formulation of hypotheses that will be further tested. In this sense, data are used to create hypotheses rather than simply for testing previously defined assumptions. The most popular method in metabolomics exploratory data analysis is principal component analysis (PCA) and variations of PCA. PCA is a mathematical procedure that transforms several possibly correlated variables into a smaller, easier to visualize, number of uncorrelated variables – principal components. PCA provides visual representation of the major variance in the data. PCA is a very useful first step in testing overall data behavior.

Unsupervised analysis: Unsupervised analysis includes methods used for grouping of features (sample, metabolites and spectral features) according to the molecular data measured. These methods are used for the analysis of features when no prior information is available about the system. Depending on the method, the analysis might or might not require the user to define the number of clusters. In terms of cell culture metabolomics, this method is ideal for discovery of novel classes.

Supervised analysis: Supervised analysis defines methods for sample grouping or classification and for selection of major sample defining features. In supervised analysis, a set of features is pre-assigned to a class and it is used as a training set for the method of choice to define a classifier that will be used for classification of an unknown sample. Supervised analysis creates a model from the training set and, thus, can only be accurately used for classification of a different dataset (i.e. supervised analysis requires application of cross-validation for the determination of accuracy of the classifier).

Features: In metabolomics, features can correspond to either individual metabolite measurement (quantitative metabolomics) or spectral point (chemometric approach).

standard measurements on individual molecules or isotopically labeled standards. Several databases that include NMR and MS data for known metabolites are currently under development, and some major non-commercial examples are outlined in Table 2. The spectral assignment is performed using methods for line comparison of the pure compound measurements and mixture spectra using 1D or 2D NMR spectra (e.g. [65,67]) with either manual or semi-automatic spectral assignment (e.g. [68,69]). Several tools for metabolic data processing, including quantification, are presented in reviews of the field [64]. In addition, quantification of the signal on NMR can also be done using the electronic spiking called electronic reference to access *in vivo* concentrations (ERETIC) [65]. An example of the application of digital ERETIC, which is now readily available with most modern NMR spectrometers, is provided by MacIntyre *et al.* [66] in the serum metabolomic analysis of lymphocytic leukemia patients. In the digital ERETIC method, signal is electronically added to the spectrum of an unknown sample and from peak comparison absolute concentration values of molecules in a complex sample can be accurately determined using the known concentration of an unrelated, external reference sample. This method avoids issues of nonspecific interactions between commonly used internal standards. Peak assignment, however, remains an issue that can thus far only be resolved through the comparison with standards.

A qualitative approach can be particularly beneficial in testing the general effect of stimulants, such as drug treatment, or for the optimization of cell culture growth conditions. Insight into the metabolic processes and metabolite functions, however, can be achieved only through the quantitative analysis of metabolites.

Unsupervised and supervised data analysis in cell metabolomics

In both the qualitative and the quantitative approaches, data can be analysed in an unsupervised or a supervised fashion. In the unsupervised approach (Box 1), complete data (either spectra or quantified metabolites) are used for the classification of samples, metabolites or spectral features. This type of analysis for cell culture metabolomics can provide information regarding the sample class – cell culture phenotype classification or overall similarities between cultures after various stimulations. Unsupervised classification of quantitative metabolite information over different cell culture samples can lead to information about possible metabolic pathways and networks, as well as possible involvement of co-clustered metabolites in related processes. The most popular unsupervised method, particularly in qualitative metabolomics, is persistently principal component analysis (PCA). This is not a clustering method but rather a visualization method that is best suited for exploratory data analysis. Only a handful of true clustering methods have been used in metabolomic analysis and particularly in cell culture metabolomics. Those include self-organized maps [67] and fuzzy *K*-means clustering [68]. Indeed, many other classification methods exist [69] and should be tested in metabolomics in the future.

Supervised methods provide more powerful tools for the sample classification and the determination of the major differential features (i.e. biomarkers). Supervised methods can be used for the analysis of unknown samples if there is a set of samples with class (i.e. cell phenotype) assigned that can be used as a training set. In this approach, a search is performed either through metabolite measurements or through the spectral data to determine marker metabolites or spectral regions that are most distinctly different between sample groups. Once this set is obtained, it can be used for the classification of unknown samples in a cross-validation approach [70]. This type of analysis can provide much more accurate classification of samples because only the major features are used for the determination of classes, and the errors caused by features irrelevant for specific sample groups are removed. Regardless of the method used for the feature selection and sample classification, however, it is of major importance to have an accurately classified training set for the feature selection. Furthermore, it is crucial to use cross-validation on an indepen-

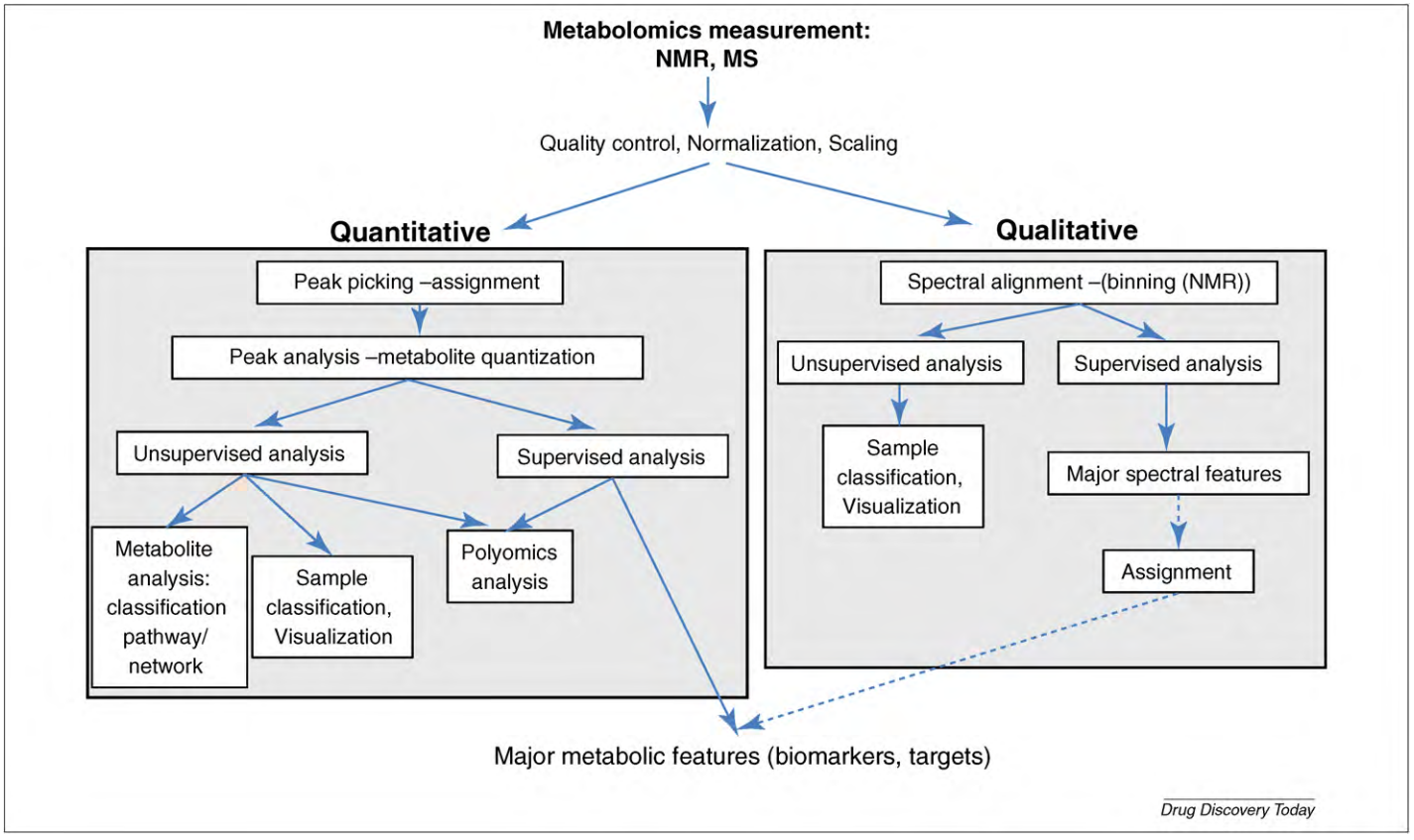

FIGURE 4

Typical major steps performed in quantitative and qualitative metabolomic data analysis. Each of the steps can be done with many different approaches and tools. The choice of an optimal approach for each step depends on the application and data.

dent dataset – test set for the determination of the accuracy of the classifier. Samples in the test set should not be used for feature selection, and their initial assignment to classes, if available, should be used for the determination of the accuracy of assignment of the classifier [70]. In other words, the accuracy of the classifier that was determined using the training set can be determined only through cross-validation on an independent test set. The most popular method for this type of analysis in metabolomics is the partial least squares method [71] and its many variations (such as O-PLS [72]). Many other methods can be used, however, all with their inherent advantages and disadvantages. Because there are no optimal unsupervised and supervised methods, it is essential to explore many different approaches for each individual dataset and application. Only focused, prudent application of different methods can lead to accurate classification of samples or metabolites and more accurate information about targets, markers and cellular changes resulting from stimuli.

Examples of different data analysis approaches in cell line metabolomics

In many applications of cell line metabolomics published thus far, the metabolic changes were so pronounced that even simple visual comparison of spectra led to some important conclusions. This type of qualitative analysis was used for the first metabolomic application in the exploration of the effects of viral infection of cell lines [57], for optimization of growth conditions as serum-free and serum-reduced cell lines [29], comparison of breast cancer phenotypes [58,59] and the analysis of cell differentiation [60]. The majority of these efforts employed NMR spectroscopy for metabolite analysis and only looked at major changes in metabolite concentrations of abundant small molecules such as glucose, lactate, citrate and glutamate. Visual analysis can, however, miss smaller but still highly important changes in less prevalent metabolites. The next level of chemometric analysis includes data transformation, usually through PCA analysis, for clearer separation of subtypes based on overall sample variance. The advantage of this method is that it is unsupervised and, thus, does not make presumptions about the data; therefore, any observed distinction between samples is data driven. This approach was used for optimizing cell cultures for antibody production [73], testing drugs [23,74] and comparing lung cancer cell phenotypes [27]. In all of these examples, the selection of major features leading to the separation of phenotypes observed in PCA plots was performed from PCA loading plots (i.e. the weights for each original variable – spectral position) when calculating the principal components. In these and other examples of chemometric analysis in metabolomics, PCA provided highly intuitive plots that could give general information about the phenotype differences. Therefore, PCA is an excellent method for exploratory data analysis. PCA, however, is highly influenced by major spectral features and thus can lead to suboptimal results in some cases. PCA is likely to lead to errors, for example, when analysing data with very broad spectra or data with many different sources of variance. In addition, PCA is not an optimal method for determining major features for sample classification and whenever possible, supervised methods should be used instead. For unsupervised analysis, it is highly advisable to

use clustering methods following the exploratory phase of analysis with PCA. Two examples of the application of advanced clustering methods in cell culture metabolomics are shown for the analysis of the effect of ionizing radiation of cell lines where self-organized maps were used for sample classification [67] and fuzzy *K*-means clustering for cell phenotype classification [68] where fuzzy clustering, unlike the PCA, enabled separation by cell subtypes. Data analysis methods should be carefully chosen for each particular application and question. In terms of feature selection, supervised methods are much more accurate (for a review, see Ref. [70]) and should be used whenever possible (i.e. whenever there are sufficient data and there is a subset of pre-assigned data).

The quantitative approach is still less popular in cell culture metabolomics, primarily because of the issues outlined above. In addition, line shape analysis methods used for spectral assignment and quantification in NMR might lead to omissions of low-concentration metabolites. Still, assignment and quantization of individual metabolites are essential steps in making metabolomics a truly biologically useful method. Although quantitative metabolomics is still under active development, there have been several interesting applications in the analysis of metabolic pathways and networks. Gas chromatography–MS and high-performance liquid chromatography–MS analysis of metabolites were used for the investigation of the effects of oncogenesis on metabolite profiles [24]. In this three-dimensional screening experiment, the authors analysed four cell lines that were serially transduced with four different oncogenes and five small-molecule inhibitors of metabolic and nutrient-sensing pathways. The resulting quantitative metabolomic data have shown the connection between the effects of oncogenes and the metabolic changes. The conclusion from this study was that metabolic changes are probably the result of the gene changes in cancers. The quantitative metabolic data clearly showed increased glucose consumption and lactate production (indicative of anaerobic glycolysis), increased consumption of oxygen, high levels of nucleotide biosynthesis, changes to the citric acid cycle metabolite concentrations, and changes in mitochondrial biogenesis. These data were in good agreement with the cancer metabolite model emerging from other methods described elsewhere. These experiments, however, led to some unexpected and as yet unexplained observations, including the observation that cells with greater tumorigenic potential consume more oxygen and yet exhibit diminished oxygen-dependent (aerobic) ATP synthesis. This work has shown that quantitative cell culture metabolomics can be used for both data confirmation and hypothesis generation. Several other excellent examples of the application of quantitative metabolomics in cell culture analysis were presented for the analysis of neuroendocrine cancers [75] and in the analysis of cancer cell metabolic phenotype [76]. In both of these applications, it was possible to propose molecular pathways responsible for cellular phenotypes.

Another application of quantitative metabolomics includes the development of computational models of cellular pathways based on the measurement of metabolite concentration changes in time (i.e. fluxomics). These applications required specific isotope labeling to ensure the ability to follow a particular pathway or metabolite. Examples of such analysis include profiling of central metabolism in human cancer cells [48–50], identification of metabolic fluxes in hepatic cells [77] and several other examples that have been reviewed previously [78–80].

It is interesting to note that more recent applications of cell culture metabolomics followed the chemometric approach [27,67,68,73,74]. We are hopeful, however, that the development of more exhaustive databases with metabolite information (Table 2), more reliable and sensitive experimental methods, and more automated software tools for lineshape analysis will lead back to quantitative metabolomics and focus more on detailed biological descriptions of systems through metabolomics.

Concluding remarks

The focus in the majority of cell culture metabolomic publications thus far has been the most abundant metabolites, leading to the observation of changes in only a limited number of metabolic pathways. We expect, however, that as the technology and its applications improve and diversify, metabolomics will become more truly high throughput in nature. In addition, future fundamental research should provide a more complete list of metabolites. Because metabolomics has its solid roots in analytical biochemistry, it is our opinion that it is extremely important to be aware of results from previous non-omics measurements because many of the measurements for major metabolites that are now being performed with modern tools have already been presented. Lack of this consideration poses a great danger that metabolomic research will only repeat earlier work with new instruments but with less attention to detail.

Qualitative and quantitative metabolomics produce voluminous data that can lead to highly useful information about cell biology, but only if appropriate experimentation and analysis methods are used. Therefore, metabolomic experiments in cell culture and other biological media, as well as other omics applications, should always be performed as collaborative efforts between experimental and computational scientists. Only in this way, after detailed experimental design, experimentation and analysis – as was the case with other 'omics' methodologies – will metabolomics provide useful information and become a truly essential analysis component in many different applications of cell lines.

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