CHAPTER

Cell culture metabolomics and lipidomics

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Introduction

Cell culture, the process of maintaining and growing cells in vitro under controlled conditions outside of living organisms, is one of the major tools of biology and biochemistry as well as medicinal and environmental chemistry, systems biology, and biotechnology. Both primary cell cultures, that is dispersed cells that are cultured directly from tissues and have limited lifespan, and cell lines, immortalized cells that can be cultured indefinitely, provide excellent models for studying cell physiology and biochemistry in health and disease or test drugs or toxins. Cell cultures are also used for the production of biologics, vaccine particles and gene therapy components, or for bioprocessing and bioremediation. In all of these and many other applications, metabolomics and lipidomics provide crucial molecular data for the optimization or modeling of cell growth, or analysis of effects of treatments or gene mutations. Metabolomics, the high throughput method measuring metabolites, provides a method for understanding biology, assessing cells' health, monitoring toxins or drugs, determining needs for growth or cell passaging. Metabolomics can also provide, on its own or in combination with other omics methods, data for predictive modeling of cell behavior. Analysis of metabolites gives the closest molecular data to phenotype, showing the outcome of

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combined genetic, epigenetic, and environmental effects on the behavior and characteristics of biological systems.

Interest in the application of metabolomics for analysis and utilization of cell cultures, possibly combined with other types of omics investigations has greatly increased due to a number of technological and analytical advances and a wide range of cell culture applications. Metabolomics has been utilized in many cell culture experiments with some examples including the analysis of microbial extracellular metabolite production (Pinu & Villas-Boas, 2017), test antimicrobial drugs in high throughput (Campos & Zampieri, 2019), explore human cell biotransformation of xenobiotics (Flasch et al., 2020) or define cancer cell characteristics (Li et al., 2019).

In addition to the major ethical and humane advantages of cell cultures compared to animal models, they can be utilized in a fully controlled environment there-by limiting sample variability and providing high statistical power even with small numbers of biological replicates. Additionally, cell cultures are generally a cost-effective method for initial or high throughput testing of, for example, drugs or toxins (Flasch et al., 2020; Muschet et al., 2016). In spite of a number of advantages several possible issues need to be carefully considered in metabolomics examination of cell cultures including cell type and growth media and environment selection, mode of harvesting, quenching of metabolism, cell passage age, data preprocessing including metabolites assignment and quantification, data processing including normalization and finally selection of appropriate analysis tools (Čuperlović-Culf et al., 2010) Table 12.1.

Broadly, application of cell culture metabolomics can be divided into:

1. monitoring of cell culture state and cell biology in single cultures or in different coculturing scenarios;
2. testing effect of a treatment including drugs, growth media, toxins or gene editing approaches;
3. analysis of metabolic processes including metabolic flux under different conditions possibly with isotopic labeling;
4. production of biological material (biologics, vaccines, gene therapy) or bioremediation;
5. cell therapy production.

The cell metabolism can be observed through analysis of: extracellular media (metabolic footprint analysis) which could include media or extracellular particles, cell or cell organelle extract either for bulk or single cell analysis (metabolic fingerprinting) which could include analysis or metabolic extracts or in-cell analysis which includes in vivo analysis of metabolism in cells. For each of the application modes, sample preparation procedures have to be optimized for the experimental goals, cell properties and analytical tools. Sample preparation protocol depends on the cell culture properties (adherent or suspension, 2D or 3D, single cell), cell type, chemical properties of metabolites of interest (in targeted approach) or aims to cover as many metabolites as possible (untargeted),

Table 12.1 Cell culture metabolomics experimentation steps.

Process	Major considerations	Solution examples
Study design	Cell type; Study type; Sample type	Cell culture type has to be well defined Treatment analyses or growth optimization Extra- intra-cellular; hydrophilic and lipophilic
Sample collection and storage	Standard operating procedure; Medium use and addition; Sample quantities	Compatibility between centers and during study Fed batch versus profusion Number of cells as well as amount of material Material storage
Sample preparation	Metabolite extraction possibly with Derivatization	Method selection; selection of metabolite groups; Changing of biochemical properties for measurement
Sample analysis	Method identification	NMR, LC-MS/MS, GC-MS/MS or another
Data analysis	Statistical, Unsupervised or Supervised Machine learning	Correlation; fold changes; Clustering or visualization; Feature selection; classification
Modeling	Mechanistic modeling Machine learning Hybrid methods	Correlation with other data; Pathway and network analysis; Predictive modeling

The most important steps in the cell culture metabolomics experimentation with some important considerations and possible general solutions.

detection techniques [e.g., Nuclear Magnetic Resonance—NMR spectroscopy or Mass Spectrometry (MS)] and type of analysis. In this chapter we will provide several examples of applications of cell culture metabolomics and lipidomics with detailed protocols for sample preparation particularly for mass spectrometric analysis of lipids and metabolites and analysis of extracellular vesicles (EVs). This will be followed by an introduction of approaches for metabolomics analysis for cell processes description, modeling and design.

Sample processing and experimentation for cell culture lipidomics and metabolomics

Methods for optimized metabolite and lipid extractions for cell culture analysis

The metabolome has a major chemical and physical diversity, including both highly hydrophobic lipids such as triglycerides and highly hydrophilic compounds such as sugars, with partition coefficient values spanning 40 orders of magnitude (Cajka & Fiehn, 2016). The huge diversity led to "divide and conquer"

approaches like metabolomics and lipidomics in order to be able to increase coverage of the metabolites measured, wherein water-soluble (polar) metabolites and water-insoluble (hydrophobic) lipids are extracted with different methods. It is important to note here that lipids will partition into the organic phase, whereas most metabolites will partition into the aqueous phase. Thus, either a highly optimized procedure for separation from the same sample or enough replicates are necessary for analysis of both fractions requiring large amounts of biological material for combined metabolomic and lipidomic analysis. One of the important benefits of using cell culture for the analysis of metabolomic/lipidomic changes in response to a variety of treatments, is the possibility for multiple replicates of the same condition as well as possibility for increase of sample size as needed. Exploration of functional and dysfunctional metabolic mechanisms and pathways simultaneously, requires unbiased measurement of a maximal number of lipids and metabolites (Dunn et al., 2005). This section will discuss various lipid and metabolite extraction protocols which can be used to maximize the efficiency for parallel lipidomics and metabolomics analysis of cells.

As an example of the power of parallel metabolite and lipid analysis, we use the study by Zhen et al. which utilized a cell culture model to determine the ecotoxicological effects of chemicals in the aquatic environment (Zhen et al., 2018). In this work zebrafish liver cells were exposed to wastewater treatment plant effluent collected at various distances from the discharging point. They then analyzed both hydrophilic metabolites and lipids. While the effects on the hydrophilic metabolome diminished with increasing distance from the discharge point, the effects on the lipidome increased. The study demonstrated the utility of cell-based systems as a tool to determine impact on both the metabolome and lipidome, as well as the importance of studying both hydrophilic and hydrophobic metabolites in order to be able to fully assess the biological effects of various treatments.

The first step which must be performed in order to be able to analyze changes or disturbances in metabolites and lipids is their extraction from cells and cell media. The general principle of lipid extraction is, simply, mixing of an aqueous solvent with an organic solvent and then separating the phases by centrifugations, wherein the lipids partition to the organic phase, while proteins, many hydrophilic metabolites, as well as many water-soluble contaminants partition to the aqueous phase. The solvent system needs to effectively extract the lipids of interest in an unbiased manner, without promoting the degradation of only specific lipids, and should not introduce contamination by other compounds (Xu et al., 2013). The two most commonly used lipid extraction methods are the ones described by Bligh and Dyer (1959) and Folch et al. (1957) more than 50 years ago involving the use of different ratios of chloroform, methanol and water, wherein the lipids partition to the lower chloroform phase. The methanol is added to the solvent system in order to disrupt the electrostatic forces and hydrogen bonding networks between the lipids and proteins. Other lipid extraction protocols which have been developed more recently use solvent mixtures such as butanol

(Hammad et al., 2010; Löfgren et al., 2012), methyl tert-butyl ether (MTBE) (Byeon et al., 2012; Graessler et al., 2009; Kosicek et al., 2010; Wiesner et al., 2009), and hexane (Hara & Radin, 1978). In general these alternative solvent systems do not show significant differences in the extraction efficiencies of the predominant lipid classes (Byeon et al., 2012; Iverson et al., 2001; Löfgren et al., 2012; Matyash et al., 2008). For example, the MTBE method has been reported to have very similar extraction efficiency to the Bligh and Dyer method in human plasma (Matyash et al., 2008). This method has become very popular for extracting sphingolipids in fluids (Hammad et al., 2010; Wiesner et al., 2009). Specific protocols for these methods, along with their associated disadvantages, are summarized in Table 12.2.

Following phase separation either the organic phase can be collected and transferred to a new tube, or the aqueous phase can be removed and discarded. If one is more interested in having an organic phase as clean of other contaminants as possible, but with the caveat of potentially losing some lipids, especially low abundance lipids, then the aqueous phase, typically the upper phase, can be removed and discarded, and the lower phase containing the lipids can be washed numerous times by multiple additions/removal of aqueous phase (Alecu, Tedeschi, et al., 2017). Alternatively, if the goal is to maximize the amount of extracted lipids, they can collect the lower organic phase containing the lipids, transfer to another tube, and repeatedly re-extract the aqueous phase by repeated additions of organic solvent, which are collected and pooled with the organic phase that has already been collected (Xu et al., 2013).

A variety of modifications to the chloroform-methanol extraction procedure have been made in order to maximize the ability to extract lipids of particular interest with high efficiency. Saunders and Horrocks used isopropanol-hexane (2:3 v/v) to extract lipids from bovine brain with a 12%−37% greater recovery of prostaglandins, compared with traditional chloroform-methanol extraction (Saunders & Horrocks, 1984). The Bligh and Dyer extraction has been modified by a number of groups to use acidified methanol (2% acetic acid) in order to increase the recovery of ether-linked glycerophospholipids, including platelet activating factors (PAFs) (Bonin et al., 2004; Liu et al., 2011; Weerheim et al., 2002; Whitehead et al., 2007). The acidified methanol is added directly to cells being extracted at the time of extraction; however, exposure of the sample to these acidic conditions should be minimized, as extended exposure could lead to the hydrolysis of glycerophospholipids, especially in aqueous solution (Ford et al., 1992; Kayganich & Murphy, 1992). For example, to extract lipids from adherent cells, the cells can be scraped off the plate directly into cold acidified methanol, followed by the addition of chloroform and 0.1M sodium acetate for a final ratio of 1:0.95:0.8 (methanol:chloroform:0.1M sodium acetate). Next, samples are vortexed and centrifuged at 800 × g for 2 minutes. The lower phase is then collected and the upper phase is back-extracted 3 more times by the addition of 2 mL of chloroform. The lower chloroform phase is collected each time and pooled with the other lower phases, and this is then evaporated under nitrogen gas. The dried

Table 12.2 Lipid extraction protocols.

Method	Time	Equipment	Advantages	Disadvantages
Ultracentrifugation, differential centrifugation 700, 2400, 10,000, and 100,000 × g	140–300 min	Ultracentrifugation equipment, rotors and tubes	Isolation from reasonable volumes (upto 1.5 L), low cost if access to UC equipment, sEV cargo, that is protein and RNA not affected	Equipment-dependent, laborious, time-consuming, non-EV contamination, low reproducibility, low yield, low purity, high centrifugation forces cause structural damage to sEVs, higher risk of contamination and low-throughput (only six samples fit in one UC spin)
Density gradient ultracentrifugation, sucrose or iodixanol density gradient after UC	280 min–2 days	Ultracentrifugation equipment, rotors and tubes. As well, sucrose and iodixanol density media	Pure sEVs population; No contamination with viral particles, high sEVs population purity and high separation efficiency after iodixanol UC	Equipment-dependence, low yield, laborious, time-consuming and low-scalability
Tangential flow filtration	110–150 min	Sterile hollow fiber polyethersulfone membrane filter with specific molecular weight cut-off	Pure sEVs population, high sEVs structural integrity, fast, higher reproducibility, better sterility, and large-scale stable production	Lack of method validation, risk of the sEVs being stuck in the membrane pores (filter-plugging), loss of sample, various factors affecting the filtration rate (e.g., temperature), and purified sEVs have small quantity of exosomal proteins

Lipid extraction protocols used for cell culture lipidomics.

lipids can be re-dissolved in 100% ethanol and stored at −80°C in amber glass vials under nitrogen to prevent lipid oxidation (Xu et al., 2013).

In the ecotoxicological study by Zhen et al. (2018), the authors used a modified chloroform/methanol extraction method where they kept both the aqueous and organic phases, wherein one fraction contained the hydrophilic metabolites and the other fraction contained lipids. Cells were homogenized in methanol using a tissue lyser, followed by the addition of 0.24 mL chloroform and further homogenization. The same volume of chloroform was added again to the resulting homogenate, followed by 0.22 mL of deionized water and further homogenization. To separate the phases the mixture was centrifuged at 3000 × g for 15 minutes. The two phases were separated by pipetting and then dried down using a vacuum concentrator (Zhen et al., 2018). It should be noted here that it is important to perform optimization experiments with standards for all metabolites and lipids of interest in order to determine whether using such an extraction method for both hydrophilic metabolites and lipids is biasing the analysis towards specific species. Other such "double" biphasic extraction methods have recently been developed where both the aqueous and organic phases from the sample are used for MS analyses (Villaret-Cazadamont et al., 2020). Villaret-Cazadamont et al., compared the extraction efficiency of a classical water-soluble metabolite extraction with acetonitrile, methanol, and water acidified with formic acid and a lipid extraction with dichloromethane and methanol to a double extraction method. In the double extraction method, both hydrophilic metabolites and lipids were extracted using a quenching solution of cold methanol, acetonitrile, and milliQ water with 0.1% formic acid in a volume ratio of 2:2:1. Samples were centrifuged at 400 × g and 2.5 mL of dichloromethane were added. The upper aqueous phase and lower organic phase were separated and dried down for metabolite and lipid analysis, respectively. Lipid internal standards were added to perform relative quantification of lipids and ^{13}C was added for absolute quantification of metabolites. The absolute concentration of the metabolites was found to be similar for the double extraction compared to the two separate extractions for the majority of polar metabolites. However, lower extraction efficiency was obtained for the amino acids methionine and phenylalanine and the following metabolites linked to energy metabolism: 6-phosphogluconate, pyridoxal-5-phosphate, cytidine diphosphate, a-ketoglutarate, guanosine diphosphate, uridine diphosphate acetylglucosamine and uridine 5′-monophosphate. The polarity of metabolites determines their distribution in aqueous and organic phases during liquid-liquid extractions (Houck et al., 2015), with polar metabolites preferentially partitioning into aqueous phases, hydrophobic metabolites migrating to organic phases (Humbert et al., 2014; Poole & Poole, 2010), and metabolites of intermediate polarity distributing between both phases. This can result in an underestimation of polar metabolites like the ones mentioned above when the concentration is assessed in the aqueous phase. For lipids, the classical extraction protocol showed significantly better extraction efficiency for triglycerides, phosphatidylethanolamines, phosphatidylcholines, and phosphatidylinositol, while the double

extraction protocol demonstrated higher extraction efficiency for ceramides and cholesterol. The relative distribution of different lipid molecular species in each lipid family was not affected by the extraction protocol used.

After extraction of lipids from cell samples, the most common analysis method for their separation and subsequent identification and quantification is liquid-chromatography MS (LC-MS) (Cajka & Fiehn, 2016; Fauland et al., 2011; Zhai & Reilly, 2002). Depending on the lipids of interest, either normal phase chromatography or reverse phase chromatography can be used. In normal-phase chromatography the column packing is polar and the mobile phase is nonpolar for example, hexane, ethyl acetate, etc., and lipids are separated based on their polar head groups. In reverse phase chromatography the column packing is hydrophobic (e.g., silica beads bonded to C18 chains) and the mobile phase is water (buffer) + water-miscible organic solvent (e.g., MeOH), and therefore lipids will separate based on the carbon chain length, double bonds, number of OH groups.

For the analysis of hydrophilic metabolites, gas chromatography-MS was widely used in earlier times and is still used today for the detection of organic acids and amino acids (Kvitvang et al., 2011; Milkovska-Stamenova et al., 2015; Tanaka et al., 1980). However, there are a number of drawbacks to using GC-MS analysis. It is not suitable to use for compounds that are unstable or have high boiling points, such as nucleotides and keto acids and often, complex derivatization methods are required, thereby restricting the range of hydrophilic metabolites which can be analyzed (Hu et al., 2020). Therefore, more and more analysis of hydrophilic metabolites is now also being performed by LC-MS.

The traditional reverse phase columns which are widely used for lipid analysis cannot retain hydrophilic metabolites, as the nonpolar stationary phase cannot form strong interactions with these metabolites. However, a variety of new strategies using different stationary phases and additives to mobile phases have been developed, allowing for broader and more in-depth analysis of these compounds. Wang et al., developed a 2D LC method using both a reverse phase C18 column and a T3 column to be able to separate short-chain, medium-chain, and long-chain Coenzyme A esters (Wang et al., 2017). The T3 column is composed of a trifunctional C18 alkyl phase at a low-ligand density, allowing the metabolites to more easily access the pore structure of the material and therefore greatly improving the retention of polar compounds. However, this interaction is still not able to retain small hydrophilic metabolites (Hu et al., 2020). Currently the column which can retain the largest number of hydrophilic metabolites is the hydrophilic interaction liquid chromatography, HILIC, which was initially proposed in 1990 by Andrew Alpert (1990). HILIC columns consist of polar silica gel (Hemström & Irgum, 2006) which can be modified with functional groups such diol, amide, aminopropyl, and zwitterionic compounds (Jandera & Janás, 2017; Periat et al., 2013), while the mobile phase is an organic solvent containing 2%−3% water. The metabolites partition into the aqueous component of the mobile phase which then forms a layer on the surface of the stationary phase, aiding retention (Jandera, 2008; Wikberg et al., 2011). Recent studies suggest that HILIC columns

modified with zwitterionic sulfobetaine allow for analysis of a wider array of metabolites as well as better chromatographic peak shape and resolution compared to underivatized HILIC columns (Sonnenberg et al., 2019). Even with these improvements in metabolite coverage and retention, poor peak shape and low sensitivity is still a problem in the analysis of phosphorylated metabolites and organic acids. This can be improved by reducing the chelation between these metabolites and metal ions by using medronic acid in the mobile phase (Hsiao et al., 2018).

It is clear that in order to analyze a broad range of both hydrophilic metabolites and hydrophobic lipids, even if a double extraction method is optimized, to be able to separate and identify the largest number of molecular species different chromatographic strategies need to be employed. Therefore, maximizing the amount of biological material analyzed in order to elucidate metabolic pathways and networks through the use of cell culture based models that closely reflect more complex in vivo models is necessary at least as a first step in identifying important nodes in pathways.

Analysis of metabolic processes including metabolic flux

Metabolic processes, pathways, and networks can be elucidated by tracking the metabolic flux of lipids and metabolites in cells of interest. This is critical for the understanding of dysregulation of these processes in pathological conditions and consequently the identification of therapeutic targets to correct these disturbances. Pulse-chase experiments can be used to track the fate of metabolites and lipids, as well as to discover novel metabolites. To do this, lipids tagged with a variety of functional groups such as fluorophores, different numbers of deuteriums or other natural isotopes such as ^{13}C, or alkyne lipids which can later be "clicked" with other functional groups can be used. As an example, the application of mammalian cell culture together with metabolic labeling approaches and differential metabolic analysis was used to discover a novel metabolic pathway for neurotoxic 1-deoxysphingolipids, thus elucidating the reason for increased levels of these lipids in pathological conditions like diabetic sensory polyneuropathy (Alecu, Tedeschi, et al., 2017). Pulse-chase experiments with deuterated 1-deoxysphingolipids led to the discovery of a novel metabolic pathway involving eight never-before measured lipid metabolites (Alecu, Othman, et al., 2017).

1-Deoxysphingolipids are cytotoxic atypical sphingolipids which are implicated in the pathology of the inherited neuropathy, hereditary sensory neuropathy type 1 (HSAN1) and diabetic sensory neuropathy. Due to their molecular structure it was always thought that they are "dead-end" metabolites with no metabolic exit point, thereby continuously accumulating to toxic levels. Alecu, Othman, et al. (2017) demonstrated that this was not the case by treating mouse embryonic fibroblasts with a pulse of d3-labeled 1-deoxysphinganine, an

upstream 1-deoxysphingolipid, for 2 hours. The "pulse" media was then replaced with fresh media without d3-deoxysphinganine for a chase period of 0, 1, 4, 8, 24, and 48 hours. Both the media and the cells were collected at these time points. It is important to note here that sample collection has to be performed as fast as possible to immediately block all enzymatic processes and prevent modifications of metabolites. Therefore, cells and media must be stored on ice/frozen as soon as they have been collected. The collected cell media should be lyophilized before lipid extraction. This step is necessary in order to maintain the appropriate aqueous/organic solvent proportion for lipid extraction without having to extract the media from one plate in multiple batches due to the large volume collected. After lyophilization, the media is re-suspended in the appropriate amount of aqueous phase for example, 200 μL. It is very important here to note the volume of the media lyophilized for normalization of lipid/metabolite levels. Once the lyophilized media is re-suspended, one can proceed with a metabolite or typical lipid extraction such as a Bligh and Dyer extraction described earlier.

The cells are harvested by trypsinization, followed by centrifugation, resuspension of the pellet in PBS in order to wash off any media which could affect the levels of metabolites/lipids measured, and then cell counting. As with keeping track of the amount of media, cell counting is necessary for normalizing the amounts of metabolites/lipids. If cells are harvested by trypsinization plus addition of stop media, it is necessary to pellet the cells, remove trypsin + media, then wash/re-suspend in PBS and pellet again such that lipids in the cell media are not contributing to what is measured in the cells. Detachment of all cells from the plate should be visually confirmed before proceeding with the next steps. There are a variety of other options for cell harvesting, such as scraping adherent cells of the plate in PBS. However, one needs to consider the "harshness" of their harvesting method as it could lead to cell lysis, which would result in inaccurate cell counts and thereby less accurate final metabolite/lipid quantification. A "softer" harvesting technique would be the addition of 10 mM EDTA at 37°C for a total of 10 minutes (Ziemanski et al., 2020). A disadvantage of softer techniques is that all cells may not detach from the plate, thereby decreasing the amount of total lipids and metabolites. This could lead to the inability to measure or quantify these low abundance lipids if their quantities are below the lower limit of detection or quantification.

The next step following the collection of cells and media is the extraction of lipids and metabolites for analysis. Alecu, Othman, et al. (2017) chose to perform an acid-base hydrolysis lipid extraction on both the media and the harvested cells in order to remove the *N*-acyl fatty acid of the 1-deoxysphingolipids. This protocol would also remove the head group of endogenous lipids such as sphingolipids. The acid hydrolysis specifically breaks the *N*-acyl chain, whereas the base hydrolysis leads to a release of the *O*-linked phosphoester or carbohydrate head group. Five hundred microliters of methanol containing 200 pmol of internal standards (D7 labeled sphinganine and sphingosine, the 2 kinds of C18 sphingoid bases) were added to each sample (cell pellets or lyophilized medium re-suspended in

200 μL PBS). Internal standards are necessary in order to account for different extraction efficiencies and to monitor method-accuracy drifts of the MS method. Internal standards selected should not be endogenously present in the sample, and should be different from the labeled lipids/metabolites used for the metabolic flux experiments. If measuring levels of endogenous lipids, at least one internal standard should be used for each lipid subclass of interest.

For the acid hydrolysis, the sample was incubated with methanolic hydrochloric acid (1N HCl/10M water in methanol) for 12−15 hours at 65°C. Next, 40 μL KOH (5M) were added to neutralize the acid, followed by the addition of 4 volumes of 0.125M KOH in methanol for base hydrolysis, 1 volume of chloroform, then 0.5 mL of chloroform and 0.5 mL of alkaline water (Penno et al., 2010). The sample should be vortexed after each step. The aqueous and organic phases are then separated by centrifugation (12,000 × g, 5 minutes). The upper aqueous phase is aspirated, and the lower phase is washed 2 more times with alkaline water in order to remove any remaining contaminants from the organic phase (chloroform) containing the lipids. The chloroform phase is then evaporated under N_2, and the dried lipids should be stored at −80°C until analysis by LC-Ms.

In this case the authors chose to perform the acid-base hydrolysis because they were interested in the total sum of all the lipids with the deuterated 1-deoxysphingoid base backbone in order to be able to monitor the total amount of these lipids. The idea was that if these lipids are a metabolic dead-end, the total sum of exogenously added labeled 1-deoxysphingolipids should be constant. Without the acid-base hydrolysis, some of the low abundance lipids formed, such as a 1-deoxyceramide with an 18:1 *N*-acyl chain may be below the lower limit of detection/lower limit of quantification. If many of these low abundance species were missed, this would have made it impossible to monitor the total sum. Another instance where the user may choose to perform an acid-base hydrolysis lipid extraction would be studying host-pathogen interactions and determining which lipids are produced by the host and which are produced by the pathogen, wherein this protocol would allow for an in depth analysis of the sphingoid base backbone which could differ in length or branching in the pathogen vs the host and may elucidate a potential drug target in the lipid pathway which is host-specific (Lochnit et al., 1997).

There is also the option of simultaneous cell harvesting and extraction. Ziemanski et al. (2020) used the Folch extraction method, adding premixed chloroform−methanol (2:1 v/v, 3 mL), prechilled to −20°C, directly to the petri dish surface with adherent human meibomian gland epithelial cells, and the cells were then scraped off with a stainless steel scraper. Although the benefit of this strategy is that it is much higher throughput than first harvesting the cells, followed by extraction, there are a few factors which need to be considered if undertaking such a protocol. Firstly, the leaching of plastic upon addition of organic solvents to cell culture plates needs to be considered, which would interfere with the detection of metabolites/lipids by MS. Therefore, the cells would need to be

grown in glass dishes. Furthermore, cell counting would present an even bigger problem here than with only cell scraping, as chloroform has been shown to lead to rapid cell lysis (Sapcariu et al., 2014; Vellaichamy et al., 2010).

Once lipids are extracted, they can be identified and quantified by LC-MS as described earlier. There is an optional step of derivatizing lipids by tagging them with specific functional groups before LC-MS analysis. This option is useful for lipids that cannot be efficiently ionized, which is necessary for detection by the MS, or if the lipids lack characteristic fragmentation patterns in tandem MS (MS/MS) analysis which would be necessary for identification of the lipid species (Yang & Han, 2016). In the current example Alecu et al., chose to derivatize the lipids with o-Phthalaldehyde (50 mg/mL in EtOH) in a 0.005:1:99 v/v/v with 3% boric acid and 2-mercaptoethanol in order to improve ionization, and consequently detection, of the lipids (Alecu, Othman, et al., 2017).

Alecu et al. wanted to monitor the time-dependent conversion of 1-deoxysphinganine to its downstream product 1-deoxysphingosine, and to determine whether the total amount of labeled 1-deoxysphingolipids remained constant with time (Alecu, Othman, et al., 2017). This would confirm that there was no further metabolic or catabolic processing of these lipids. The authors chose to collect the cell media in order to determine whether the cells were secreting any of the deuterated 1-deoxysphingolipids which elucidated whether the change in total labeled lipid levels was due to this and not to downstream metabolism. The user could also choose to collect cell media and extract metabolites/lipids in order to monitor changes in the secretome upon treatment with different compounds such as drugs or toxins, or upon mutations in genes coding for specific enzymes. If analyzing the levels of unlabeled, endogenously produced lipids secreted into the media, the use of synthetic serum-free media should be considered such that the amount of lipids already present in the media does not interfere with the identification/quantification of secreted lipids.

Alecu et al. found that the total levels of labeled 1-deoxysphingolipids decreased over time, while the amount of labeled lipids in the media was constant, indicating that further metabolic conversion of these lipids was occurring (Alecu, Othman, et al., 2017). In order to identify these unknown downstream 1-deoxysphingolipid metabolites, the authors used differential analysis of mass spectral data as well as visual analysis of the total ion chromatogram to identify new spectra appearing over the time course of the pulse-chase experiments. For this, the software Sieve from ThermoFisher was used, wherein multiple replicates of two conditions were compared in order to determine the appearance of new lipid molecular species. The basic workflow for this has been previously described (Snyder et al., 2013). A variety of different filters were applied to perform this analysis, including the m/z range and retention time expected for potential lipids of interest, odd-numbered m/z ratios which would indicate that the compound potentially carried the 3 deuterium label, as well as the criterium that the potential molecular formulas generated based on the m/z identified should contain one sulfur coming from the o-phthalaldehyde solution used for

derivatization, since all lipids of interest would have been derivatized. The total ion chromatogram generated for each sample was also visually analyzed scan by scan using the same criteria in order to identify any novel peaks. Sieve software has now been updated to Compound Discoverer which has further features such as pathway analysis.

Another labeling approach was used to elucidate a different aspect of 1-deoxysphingolipid metabolism. In order to determine whether the newly identified downstream metabolites were also formed from de novo synthesized 1-deoxysphingolipids, and not just from those added exogenously, labeled substrates needed for 1-deoxysphingolipid biosynthesis were used (Alecu, 2016). Cells were treated with deuterated versions of both substrates necessary for the synthesis of 1-deoxysphingolipids, methyl-d3-palmitic acid and d4-alanine. Methyl-d3-palmitic acid was given as a 1:1 molar complex with fatty acid-free BSA in order to prevent the fatty acid from sticking to the cell culture dish. For this experiment, 1-deoxysphinganine and 1-deoxysphingosine with mass offsets for +6, +5, +4, +3, +2, +1, as well as the unlabeled mass M, were monitored in order to be able to analyze 1-deoxysphingolipids formed from conjugation of d3-palmitic acid (+3), d4-alanine (+3, as one deuterium is lost upon conjugation), +6 (when both labeled substrates were conjugated), as well as the natural isotopologues arising from this labeling. A similar kind of deuterium exchange assay was performed with 11,11,12,12-d4 palmitic acid in order to determine whether the position of the double bond which is inserted upon the conversion of 1-deoxysphinganine to 1-deoxysphingosine is C14, which is the double bond position in canonical sphingolipids (Alecu, 2016). 1-Deoxysphingolipids with mass offsets of +5, +4, +3, +2, +1 and the unlabeled mass M were analyzed in order to monitor for double bond insertions. Upon being conjugated with alanine, the product formed would be 13,13,14,14-d4-labeled 1-deoxysphinganine. If the double bond was inserted at C14, this would entail the loss of a deuterium at this position, resulting in a d3-labeled 1-deoxysphingosine (meaning a mass offset of +3), compared to the d4-labeled 1-deoxysphinganine (mass offset of +4), which was indeed the case Fig. 12.1.

As illustrated, metabolic labeling and flux experiments in cells can be used to analyze many different aspects of metabolism ranging from the structure of compounds, the substrates used to form metabolites and lipids, as well as novel metabolic pathways. In the bigger picture, this could help elucidate the reason for metabolic dysregulations in pathological conditions, or under different treatment conditions.

Methods and protocols for isolation and metabolomics of small extracellular vesicles from cell culture supernatants

EVs, including small extracellular vesicles (sEVs) or exosomes are naturally secreted in culture by almost all eukaryotic cells except mature red blood cells

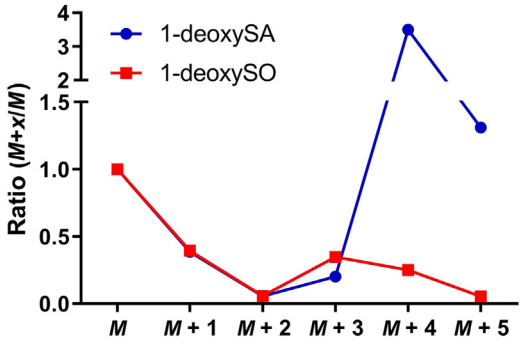

FIGURE 12.1

The majority of de novo formed 1-deoxysphingosine does not have the double bond inserted at C4. The lines on the graph represent labeled upstream 1-deoxysphinganine (*blue*) and downstream 1-deoxysphingosine (*red*) after incorporation of 11,11,12,12-d4 palmitic acid. The highest relative amount of de novo formed 1-deoxysphinganine has a +4 label coming from the d4-palmitic acid. However, the highest relative amount of 1-deoxysphingosine has a +3 label, which indicates that one of the deuteriums was lost from the C12 of the palmitic acid (which would become C14 upon condensation with alanine in the de novo formation of the upstream 1-deoxysphinganine). This would only occur upon insertion of the double bond at C14, and not at C4. *1-deoxySA*, 1-deoxysphinganine; *1-deoxySO*, 1-deoxysphingosine.

under both physiological and pathological conditions (Pegtel & Gould, 2019). sEVs are a subpopulation of membrane-bound, 30−150 nm in diameter vesicles that are formed in the multivesicular bodies, which fuse with the plasma membrane to release sEVs in the extracellular milieu. These nanovesicles harbor a variety of bioactive cargo of cellular components such as nucleic acids (microRNA, mRNA, circular RNA and noncoding RNA), proteins (cytokines, chemokines, receptors and ligands), lipids and metabolites that represent distinct "molecular signatures" of their parental cells (Kalluri & LeBleu, 2020). Therefore, sEVs provide a snapshot of crucial molecular information about the health of its parental cell. Recently, sEVs have emerged as important intercellular communication vehicles exchanging crucial information not only between neighboring cells but also distant organs (Théry et al., 2009). They are stamped with "unique addresses" that dictate their cellular and organ specificity. Increasing evidence demonstrates that sEVs can selectively transfer their cargoes into recipient cells and contribute to the modulation of a wide range of biological processes, including pro-survival, antiinflammatory, antitumorigenic, regenerative and regulation of immune responses. Over the past decade, sEVs have gained clinical utility and are being harnessed for their intrinsic therapeutic properties and also being explored as nanodevices for drug delivery and biomarkers of disease (Andaloussi et al., 2013; Fais et al., 2016).

Under in vitro conditions, cells produce a heterogeneous population of EVs, such as sEVs or exosomes (30−150 nm), microvesicles or ectosomes (100−1000 nm) and apoptotic bodies (1−5 μm) which accumulate in cell culture supernatants (conditioned media) (Raposo & Stoorvogel, 2013). These three types of EVs not only vary in size but also differ in their biogenesis, cargo content and regulation of cellular mechanisms. Recently, it has been demonstrated that cells also release distinct subpopulations of sEVs with different biophysical properties as well as proteomic and RNA repertoires, further emphasizing the heterogeneity of EVs (Willms et al., 2016). Therefore, it is crucial that prior to any metabolomics or lipidomics studies, specific populations of EVs are purified from the biological sample using differential isolation methods. With recent advances in science and technology, many different techniques exploiting the unique physicochemical and biochemical characteristics of EVs, such as size, shape, mass, buoyant density, and molecules on EV surface have been developed for the isolation and purification of sEVs (Sidhom et al., 2020). Here we will describe two protocols that capitalize on the EV properties, such as size and buoyant density for the isolation of sEVs: (1) ultracentrifugation (UC) method, which employs differential centrifugation steps and still remains the gold-standard method of sEV isolation; (2) tangential flow filtration (TFF), an emerging new technique that is coupled to membrane filtration and flow to obtain clinical grade sEVs preparations with high yield, purity and integrity. Both methods are capable of processing large volumes of cell culture medium, for example, for UC several hundreds of liters and for TFF up to several thousand liters. The first part of this section describes the most common protocols used to isolate sEVs, and the second part describes different methods for characterizing and analyzing the purity of the isolated sEVs preparation.

Cell culture for isolation of small extracellular vesicles

Most of the mammalian cells are cultured in media supplemented with 10%−20% fetal bovine serum (FBS), a rich source of nutrients and growth factors, which is important for cell survival. Small EVs are found in almost all biological fluids, including serum (Lässer et al., 2011) and FBS contains many different types of bovine EVs. Since bovine EVs in culture media are bioactive and their presence can influence experimental results (Kornilov et al., 2018; Shelke et al., 2014), they are often removed from FBS prior to addition to the culture media (Théry et al., 2006). Thus far, no standardized protocol for EV-depletion of FBS exists and different laboratories use different depletion protocols. Briefly, cell culture media containing FBS is centrifuged for at least 2 hours at 100,000 × g to remove sEVs. The supernatant is filtered using a 0.22 μm vacuum bottle top filter. Some cell types can be grown in the absence of serum and culture medium without FBS can be used. Several commercial serum alternatives are available, however caution should be exercised in selecting these alternatives for metabolomic

studies as these may contain polyethylene glycol which can interfere with NMR spectra. Cells are usually grown in T-175 flasks at 70%−80% confluency (a total of 20−40 × 10^6 cells/sample or equivalent to 1−2 mg protein/sample) in the presence of serum and then media is changed to EV-depleted media or serum-free media for 24 hours. The user is recommended to include a culture media alone control incubated for 24 hours in the absence of cells. Following incubations, cell culture supernatant (conditioned medium) is collected and subjected to UC or TFF to isolate sEVs as described below.

Isolation of small extracellular vesicles using ultracentrifugation

The traditional EV isolation methods employing UC, namely differential UC and density gradient UC utilize EVs properties, such as size, mass and buoyant density for the separation and purification of sEVs (Romano et al., 2020). Table 12.3 provides the comparison of the inherent advantages and limitations of each method which are important to keep in mind while designing an experiment.

Differential ultracentrifugation

The common method of sEVs isolation is UC, which still remains the gold-standard technique for EV isolation. In brief, conditioned media is sequentially subjected to increasing centrifugal forces and duration to pellet cells at 700 × g, microvesicles at 2400 × g and sEVs at 100,000 × g, as described (Čuperlović-Culf et al., 2020; Kuo & Jia, 2017; Romano et al., 2020; Théry et al., 2006; Witwer et al., 2013). The workflow for the purification of sEVs using differential centrifugation is presented in Fig. 12.2 (top panel). All centrifugations should be performed at 4°C. The low speed spins (<10,000 × g) gradually remove particles with a high buoyant density such as cells, cell debris, apoptotic bodies, and proteins aggregates, while the high speed spin (100,000 × g) sediments small EVs. The sEVs pellet is washed once with 1 mL sterile 1 × PBS and the 100,000 × g step is repeated to obtain an sEVs pellet that can be further purified as below.

Density gradient ultracentrifugation

Although the differential UC EV isolation method provides a reasonably pure sEVs population, it can also coisolate contaminants, such as aggregated proteins and nucleic acids. Therefore, an extra step can be added using density gradient UC to improve the purity of sEVs population (Abramowicz et al., 2016; Zhang et al., 2014). Several gradient medias are available, however sucrose cushions and iodixanol (OptiPrep) gradients coupled with differential UC is most commonly used to isolate different EV populations based on their buoyant densities and

Table 12.3 Standard strategies for isolation of small extracellular vesicles (sEVs).

Method	Time	Equipment	Advantages	Disadvantages
Ultracentrifugation, differential centrifugation 700, 2400, 10,000, and 100,000 × g	140–300 min	Ultracentrifugation equipment, rotors and tubes	Isolation from reasonable volumes (upto 1.5 L), low cost if access to UC equipment, sEV cargo, that is protein and RNA not affected	Equipment-dependent, laborious, time-consuming, non-EV contamination, low reproducibility, low yield, low purity, high centrifugation forces cause structural damage to sEVs, higher risk of contamination and low-throughput (only six samples fit in one UC spin)
Density gradient ultracentrifugation, sucrose or iodixanol density gradient after UC	280 min–2 days	Ultracentrifugation equipment, rotors and tubes. As well, sucrose and iodixanol density media	Pure sEVs population; No contamination with viral particles, high sEVs population purity and high separation efficiency after iodixanol UC	Equipment-dependence, low yield, laborious, time-consuming and low-scalability
Tangential flow filtration	110–150 min	Sterile hollow fiber polyethersulfone membrane filter with specific molecular weight cut-off	Pure sEVs population, high sEVs structural integrity, fast, higher reproducibility, better sterility, and large-scale stable production	Lack of method validation, risk of the sEVs being stuck in the membrane pores (filter-plugging), loss of sample, various factors affecting the filtration rate (e.g., temperature), and purified sEVs have small quantity of exosomal proteins

Key advantages and disadvantages of the standard methods for the purification of sEVs are summarized.

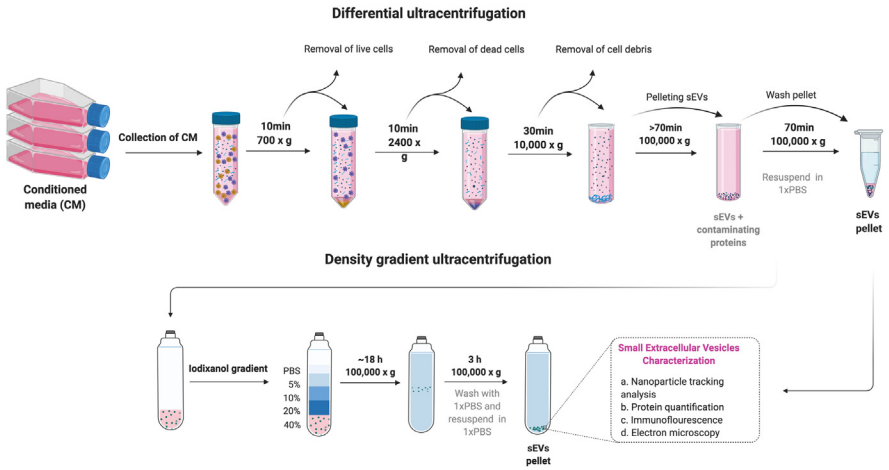

FIGURE 12.2

Schematic representation of common strategies for the isolation and purification of small extracellular vesicles. Flow-chart for the isolation and purification of sEVs based on differential ultracentrifugation (Čuperlović-Culf et al., 2020) and density gradient ultracentrifugation. sEVs indicate small extracellular vesicles.

Illustration created in BioRender (www.BioRender.com).

mass (Araùjo et al., 2008; Graham, 1999). The workflow for the purification of sEVs using density gradient UC is presented in Fig. 12.2 (bottom panel).

Isolation of small extracellular vesicles using tangential flow filtration

Despite the wide use of differential UC for sEVs isolation, this method has major limitations (Table 12.3). Therefore, improved techniques that increase sEVs yield, integrity, scalability and reproducibility have been adapted for sEVs isolation (Furi et al., 2017; Konoshenko et al., 2018). TFF is an emerging ultrafiltration technique that couples membrane filtration and fluid flow for efficient isolation and concentration of sEVs from large volumes of biological fluids (Fig. 12.3). In brief, clarified conditioned media (after 700 and 2400 $\times$ g centrifugation steps to remove cells and cell debris) is concentrated and filtered at the same time using a polyethersulfone hollow fiber filter with varying range of pore sizes or molecular weight cutoff cartridges (10, 50 and 500 kDa) (Lee et al., 2020). The clarified media is pumped using a peristaltic pump system. Multiple rounds of filtration leads to the isolation and concentration of specific sEVs populations. For instance, filtered culture media is concentrated $\sim$10 fold and in the final step culture media is exchanged with 1x PBS and further concentrated $\sim$5$-$10 fold.

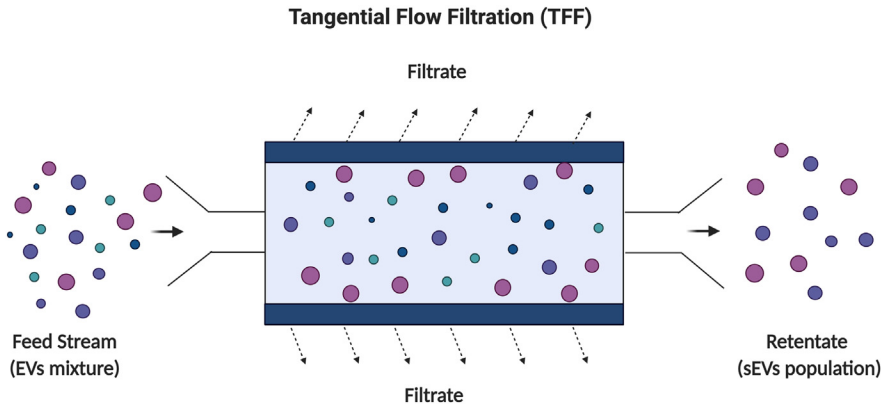

FIGURE 12.3

Tangential flow filtration. Schematic representation of the principle of tangential flow filtration for isolation and purification of small extracellular vesicles.

Illustration created in BioRender (www.BioRender.com).

Characterization of small extracellular vesicles

A large number of methods have been developed to assess the size, concentration, integrity and purify of sEVs. The most common techniques include nanoparticle tracking analysis (NTA), western blotting, scanning electron microscopy, transmission electron microscopy, cryo-electron microscopy, flow cytometry and fluorescence-activated cell sorting (Gurunathan et al., 2019; Noreldin et al., 2021). sEVs originating from a variety of different cell types share common structural and functional characteristics, such as exosomal proteins, tetraspanins (CD9, CD63, CD81), TSG101, Alix and flotillin-1, which can be detected using western blotting. The NTA method (NanoSight and ZetaView) allows real-time visualization and analysis of EVs based on the rate of Brownian motion of individual nanoparticles (EVs) in solution and their ability to scatter light (Bachurski et al., 2019). Hence, NTA allows the measurement of concentration and size distribution of EVs.

Metabolite extractions from cells and small extracellular vesicles

Extraction of polar metabolites using acetonitrile/water method. Metabolomics analysis of the content of sEVs can be performed using MS methods presented above or using approaches that were previously developed for cell and tissue analysis with NMR-based metabolic profiling (Beckonert et al., 2007; Belle et al., 2002; Lin et al., 2007). Since extraction parameters can influence the detection and quantification of metabolites, it is important to consistently adhere to the same extraction protocols to obtain optimum results and ensure experimental reproducibility. The protocols described here can be used as a guide for the

extraction of polar metabolites and the combined extraction of polar and lipophilic metabolites from cells and their sEVs harvested from the conditioned medium of the same culture.

Polar metabolites are extracted using acetonitrile from cells, media and sEVs as previously described (Čuperlović-Culf et al., 2020). Remove cell culture dishes from the 37°C incubator and place them on ice slurry to slow down the metabolism. Collect the conditioned medium which is then subjected to sEV isolation by UC or TFF as described above. An aliquot of medium can be saved at −80°C for the analysis of exometabolomics. All subsequent steps are carried out under ice-cold conditions and making sure that cells and media never approach room temperature. Harvest cells in ice-cold 5 mL of 1× PBS (Ca^{2+} and Mg^{2+} free) by gentle scraping, transfer to 15 mL falcon tubes and centrifuge at 300 × g for 5 minutes at 4°C. Place tubes on ice slurry and aspirate the 1× PBS without disturbing the pellets. Wash the pellets once again with ice-cold 5 mL of 1× PBS to remove any residual medium. Hold the cell pellets on ice slurry for 5 minutes to keep metabolic activity low. Subsequently, resuspend pellets in 1 mL of extraction solvent [50% acetonitrile/50% water (vol/vol) mixture, prechilled at −20°C overnight], which further quenches metabolism and lyses cells. Mix the suspension thoroughly by vortexing and transfer to eppendorf tubes. Centrifuge at 12,000 × g for 10 minutes at 4°C. After centrifugation, the suspension separates into supernatants (contains polar metabolites) and a pellet of cellular proteins, lipids and debris. Transfer the supernatants to fresh eppendorf tubes and evaporate the solvents from the samples under a stream of nitrogen gas or using a SpeedVac concentrator. Alternatively, samples can also be freeze-dried/lyophilized overnight. A similar protocol should be followed for the extraction of intra-exosomal metabolites by adding 200 μL ice-cold acetonitrile/water mixture to the 100,000 × g pellet. The dried samples can be stored at −80°C until NMR analysis.

Extraction of combined polar and lipophilic metabolites using methanol/chloroform/water method: Harvest cells as described above and resuspend pellets in ice-cold mixture containing 2 parts methanol/0.8 parts water to quench metabolic activity (Vuckovic, 2012). Vortex the suspension thoroughly to achieve good mixing. Place samples on ice slurry and sonicate 3−5 times for 1 seconds each time (Folch et al., 1957). Transfer suspension to glass tubes, add 1 part chloroform to a total solution of methanol/chloroform/water (2:1:0.8) and vortex again. Add 1 part chloroform and 1 part water for a final solution of methanol/chloroform/water (2:2:1.8) and vortex again. Hold the samples on an ice slurry for 15 minutes or at 4°C overnight. Centrifuge at 1000 × g for 15 minutes at 4°C. After centrifugation, the suspension separates into three phases: an upper methanol/water phase (contains polar metabolites), an interface of protein/cellular debris (protein disk) and a lower chloroform phase (contains lipophilic metabolites). The protein disk can be saved for proteomics analysis. Transfer the upper and lower phases into fresh glass tubes and evaporate the solvents from the samples under a stream of nitrogen gas. The dried samples can be stored at −80°C. The upper phase is used for metabolomics and the lower phase is used for lipidomics studies. A similar

protocol should be followed for the extraction of intra-exosomal metabolites and lipids, adjusting the volumes accordingly.

Sample preparation and analysis with nuclear magnetic resonance spectroscopy

Samples are prepared in NMR buffer (50 mM sodium phosphate buffer, pH 7.4, in deuterium oxide, 0.1% 4,4-dimethyl-4-silapentane-1-sulfonic acid and 0.5 mM sodium azide) and an internal standard solution (NMR grade). The standard solution is added at 10% of the total sample. For dried samples, reconstitute in 160 μL NMR buffer containing 16 μL standard and for liquid samples, mix 100 μL media with 60 μL deuterium oxide and 16 μL standard. Vortex samples to mix thoroughly. Using gel loading tips, load approximately 10 μL of sample into 3 mm NMR tubes and proceed to NMR analysis.

Although a number of different nuclei can be measured in NMR metabolomics, including ^{13}C, ^{15}N and ^{31}P, ^{1}H NMR spectroscopy measurements are the most significant for general metabolomics profiling and thus far the only approach used for the analysis of sEVs. One dimensional (1D) ^{1}H (proton) NMR spectra with water suppression sequence (NOESY 1D) provides a good combination of speed, excellent water suppression and good lineshape for quantification. NMR experimental techniques and possible pulse sequences that are generally used in metabolomics have been previously reviewed (Čuperlović-Culf et al., 2010; Ranjan & Sinha, 2019) and all the methods for NMR metabolomics described in Chapter 5, Nuclear Magnetic Resonance in Metabolomics, can be applied in this case as well.

Cell culture metabolomics and lipidomics data analysis

Data analysis method selection, application and interpretation depends on the level of background knowledge, metabolomics coverage and sample set size as well as specific goals of the study. Although the majority of analytical methods can be utilized for knowledge discovery, presentation or model development from metabolomics and lipidomics data regardless of the biological source, analysis of cell culture data provides some unique opportunities including a possibility for analysis of cells, organelles and media for the same system in a highly controlled environment, possibly with isotopic labeling and flux analysis. Metabolomics data analysis is described in Chapters 8–11. Here we will only show examples of analysis either specifically applied to cell culture metabolomics or lipidomics or methods that can provide some unique benefits to the cell culture application. This includes:

1. utilization of cell modeling for design and optimization of cell cultures including optimization of growth conditions or productivity in cell bioreactors;

2. utilization of cell culture metabolomics for the development of Artificial Intelligence (AI) methods for optimal design and prediction of behavior of cell and gene therapy modalities including cells and exosomes as therapy carriers.

Both of these groups of applications include analysis of metabolomics data, determination of major metabolic pathways and networks, simulation of cell metabolism and linking these models and data within machine learning models of cell metabolism and cell growth conditions and these steps will be described in some detail below.

Cell culture metabolomics and cell modeling for the design and optimization of cell culture applications

Application of cells for the production of biologics, vaccines or for bioprocessing has transformed therapy fabrication and provided avenues for synthetic biology utilization. A bioprocess is an extremely complex interplay of numerous factors that requires regulation and optimization while still lacking complete understanding. Traditionally around 10 biochemical molecules are monitored including oxygen, CO_2, glucose as well as some toxic by-products, for example, lactate, however these are insufficient to track cell metabolism, growth and productivity. Metabolomics and lipidomics can provide additional quantification of tens to hundreds of metabolites in media or cell extracts providing information about the cells' oxidative state, cell growth or death, metabolic needs or toxins, active pathways, etc. Accordingly, metabolomics can provide a way for finding the perfect media for each application; identify clonal instabilities early in the process and help provide continuous process monitoring for optimization of growth. As an example, the most popular mammalian cells used in bioprocessing are Chinese hamster ovary (CHO) cells and human embryonic kidney 293 cells (HEK293). Although CHO cells remain the most often used, they can result in nonhuman posttranslational protein. Thus, HEK293 is becoming a predominant cell line for expression of recombinant proteins and biologics providing appropriate human cell glycosylation and protein folding appropriate for in vivo use (Dietmair et al., 2012; Petiot et al., 2015) making this cell line of particular relevance in biotechnology. HEK293 cells are explored as a possible way to provide production of difficult-to-express proteins as well as next-generation biologics including bispecific antibodies and weaponized antibodies. HEK293 cells grow easily in suspension serum-free culture, reproduce rapidly, and produce high levels of protein. In this context, metabolomics can be used for testing of gene editing methods, cell productivity and health as well as analysis and optimization of HEK293 growth for biomanufacturing. Metabolomics analysis of HEK293 cells have shown major influence of media on cell metabolism and the measured cell secretome (Daskalaki et al., 2018) necessitating optimization of media for specific application. Metabolomics combined with models of specific cell lines used in the bioprocessing can be directly used in this process however

modeling of metabolism in cell lines requires determination of significant pathways or metabolic interaction network, followed by the development of mechanistic, machine learning of hybrid models.

Determination of major metabolic pathways or network from metabolomics or fluxomics data

Cell function, growth or productivity is largely regulated through the metabolism and metabolites. Metabolic processes are driven through allosteric regulation, posttranslational modifications, inter-compartmental material balance, and signaling control (O'Brien et al., 2020). Mapping metabolomics data on its own or combined with other omics data onto cellular pathways or determination of data-driven interaction networks can be used to establish significant pathways and regulation mechanisms under different conditions. Several highly advanced, freeware pathway analysis tools (Table 12.4) can be used to map metabolomics data and determine statistical significance of the representation of metabolic pathways by a selected metabolite set with or without concentration information.

Mapping of the omics data on the metabolic pathways provides a static representation of relevant processes. Although these methods do not provide any predictive power, some of these tools provide a sophisticated way to determine major metabolic differences between conditions or cell types. As an example Lilikoi analytical method (AlAkwaa et al., 2018) provides metabolite ID matching, feature selection through information gain calculation, ML classification modeling and pathway deregulation score determination. It also transforms metabolite-sample matrix into pathway-sample matrix providing in this way personalized pathway mapping. In another example, MetExplore (Cottret et al., 2018), provides statistical information about the organism-specific metabolic network coverage and gives interactive visualization of metabolomics data on the whole metabolic network, selection of pathways or specific reactions. All pathway mapping methods, by their design only give mapping onto the known pathways included in one of many databases and in this way do not allow determination of novel interactions between biological molecules.

Network analysis in cell culture metabolomics

Cell culture metabolomics provides data that can be utilized for novel mechanistic insight about biological processes under different conditions, stimuli or phenotypes and this move away from the known processes mapping into a hypothesis generation can be a major advancement for omics and systems biology (Rosato et al., 2018). A powerful approach for the data-driven metabolic mechanism analysis can be accomplished through development of interaction, that is correlation, statistical or clustering networks. A biological network in this context is a graphic representation of features (metabolites or lipids)—nodes and their

Table 12.4 Pathway and Network analysis tools in cell cultures metabolomics.

Method	Application	Availability and references
Lilikoi	Group of applications in R for: mapping of metabolites to pathways, dimension transformation to personalized pathway-based profiles using pathway deregulation scores;, feature selection module, and classification and prediction module, which offers various machine learning classification algorithms.	https://github.com/lanagarmire/lilikoi (AlAkwaa et al., 2018)
MetPA	Web-based tool for the analysis and visualization of metabolomic data within the biological context of metabolic pathways combining several advanced pathway enrichment analysis procedures with the analysis of pathway topological characteristics to help identify the most relevant metabolic pathways involved in a given metabolomic study. The results are presented in a network visualization system.	http://metpa.metabolomics.ca
IMPALA	Pathway overrepresentation and enrichment analysis with expression and/or metabolite data. Both gene and metabolite information can be either a list for overrepresentation analysis or values in different conditions for enrichment analysis.	http://impala.molgen.mpg.de/
MBRole	Overrepresentation, enrichment, analysis of categorical annotations for user provided sets of compounds. Provided categorical annotations correspond to biological and chemical information available in a number of public databases and software. Provided is also information about metabolite-protein interaction.	http://csbg.cnb.csic.es/mbrole2/index.php
MetExplore	Web-based collection of interactive tools for metabolic network curation, network exploration and omics data analysis. In particular, it is possible to curate and annotate metabolic networks in a collaborative environment with the contextualization of metabolic elements in the network and the calculation of overrepresentation statistics.	https://metexplore.toulouse.inrae.fr/index.html/

Pathway and Network determination from metabolomics data can be applied to cell cultures metabolomics.

associations—edges. Network can be represented as an adjacency or connectivity matrix of interactions describing the strength of the relationships between any two nodes. In the cell metabolomics context network can represent interactions within cells, extracellularly or across the cell membrane and can be based on topology, stoichiometry, directionality or kinetics of the metabolome. Several reviews have recently described network analysis methods including applications to metabolomics in some detail (Perez De Souza et al., 2020; Rosato et al., 2018; Toubiana et al., 2019) and any of these methods can be applied to cell culture metabolomics data. Several examples that have shown utility in cell culture analysis are outlined in Table 12.5.

Metabolic networks based on correlation analysis can indicate rapid equilibrium between metabolites or presence of conserved chemical groups. They are particularly useful for analysis of changes between different conditions, treatments or phenotypes. Correlation network requires establishment of threshold of relevant correlation with several authors showing that correlation of 0.6 and P value of 0.01 provide good threshold levels indicating lower bound for weak correlations in metabolomics data (Camacho et al., 2005; Ghini et al., 2015; Saccenti et al., 2016). It is important to point out however that lack of strong correlation does not necessarily mean lack of proximity between metabolites in the metabolic pathways and that strong correlation can be observed for metabolites that are metabolically distant. Therefore, even in the context of cell culture analysis, correlation networks may not be sufficient for reverse engineering of metabolic pathways (Rosato et al., 2018). The Debiased Sparse Partial Correlation algorithm (DSPC) was developed as an attempt to regularize correlation methods. DSPC uses a desparsified graphical lasso modeling procedure and assumes that the number of real connections in the network is much smaller than what is determined from correlation analysis. DSPC is implemented in MetScape within Cytoscape (Basu et al., 2017; Perez De Souza et al., 2020).

A number of methods that were originally developed for gene and protein network determination and analysis are finding their place in metabolomics. As an example weighted gene correlation network analysis (WCGNA) can be used to determine modules, clusters of highly correlated features and finding "module eigengene" a representative feature summarizing module profile or an intramodular features that relates modules to one another and to sample trait (Langfelder & Horvath, 2008). WCGNA provides dissimilarity profiles through analysis of topological overlap matrix (TOM) that makes the network less sensitive to distant connections or connections that are missing due to noise. TOM is related to correlation between metabolite pairs. TOM and thus WCGNA assumes scale-free topology which does not apply to all metabolic networks (Broido & Clauset, 2019; Rosato et al., 2018). Use of WCGNA in metabolomics, often with low coverage, requires some modifications to the original method with the detailed tutorial provided for this application by Pei et al. (2017).

A number of other methods have also been developed for gene network determination with few examples of their use in metabolomics, albeit thus-far

Table 12.5 Interaction network methods in cell cultured metabolomics.

Network approach/ availability	Method in brief	Advantages	Disadvantages
Correlation or relevance: Pearson, Spearman or Distance correlation methods Available in Python, R, etc.	Pearson—measure of the linear association between variables; Spearman—nonparametric measure of rank correlation; Distance—dependence of two random vectors of possibly different dimension	Inferred network is a good description of the physiological state of the system	Cannot be used to reverse engineer metabolic pathways
Weighted gene correlation network analysis—WCGNA Available as R package.	Calculates dissimilarity profiles based on topological overlap matrix based on pair correlations between metabolites.	Less sensitive to spurious connection and missing connections due to noise	Assumes power-law probability distribution for correlation and scale-free network design which does not apply to all networks
Context likelihood of relatedness—CLR Available in minet Bioconductor package	Uses Mutual Information (MI) to calculate similarity between pairs of variables and infers direct interactions by accounting for the local context for each interaction.	CLR does not require threshold as it prunes spurious interactions from network by its design.	High variability for smaller sample sets (<100 samples)
Algorithm for the reconstruction of accurate cellular networks—ARACNE Available in minet Bioconductor package	MI is calculated for each pair of nodes and the interactions are pruned by considering each triplet of edges and removing the weakest edge as it is considered as an indirect interaction.	Good at reconstruction of the backbone of association network	Produces very sparse network missing many significant associations
PCLRC available at: http://download.systemsbiology.nl/	Combination of CLR and iteratively sampling of dataset wherein each iteration a subset is chosen and a weighted adjacency matrix is determined using correlation calculations. Final network is calculated from the average of iterations.	Effective at discriminating between direct and indirect correlations	Requires thresholding by user (usually value of 0.9 is imposed) and larger number of samples

Examples of methods for determination of interaction network from data with some of their advantages and disadvantages in the analysis of metabolomics data.

rarely in cell culture metabolomics such as Algorithm for the Reconstruction of Accurate Cellular Networks (ARACNE) and Probabilistic Context Likelihood of Relatedness Algorithm (PCLR) (Suarez-Diez & Saccenti, 2015). Based on detailed analysis of the network reconstruction performance Suarez-Diez and Saccenti (Suarez-Diez & Saccenti, 2015) have shown that as many as 100−400 samples may be necessary to obtain a stable network estimate making utilization of these methods in cell culture applications challenging. With the development of methods that can profile large numbers of cells (e.g., single cell metabolomics) (as described in Chapter 15: MALDI−Mass Spectrometry Imaging: The Metabolomics Visualization) or high throughput metabolomics or lipidomics, application of network design methods will likely become more relevant.

Mechanistic modeling for cell culture optimization, design, and information gathering

Pathway mapping as well as network determinations provide static representation of the metabolic interactions in the system without possibility to predict behavior under changing conditions. Metabolic models can provide a way to explore metabolic complexity and systematically investigate significant cellular properties for a variety of cell culture applications. Importantly, models can be used to infer processes in cells that were not directly measured. Longitudinal cell culture metabolomics and lipidomics can be utilized for the development and optimization of in silico models of cellular metabolism either aiming to explain observed effects, determine possibly significant targets or to provide predictive models for cell or media design. One of the most significant applications of cell metabolomics and metabolism modeling is the optimization of cell and gene therapies particularly through the optimization of bioreactor production (Selvarasu et al., 2012) or for the design of predictable, optimized cells.

Numerous publications have discussed in great detail metabolism modeling approaches based on either kinetic and ordinary differential equation models or genome scale metabolic models and the readers are referred to those (Almquist et al., 2014; Covert, 2017). Cell culture metabolomics provides a unique opportunity for time-course analysis of metabolites in intra or extracellular space as well as cellular or extracellular organelles possibly augmented with the utilization of isotopic labeling. Access to time-course information combined with the investigation of flux for isotopic labels can be used for the development of predictive models and simulations of metabolic pathways as well as genome scale networks. Feeding cells with isotopically labeled nutrients, measuring the isotopic labeling of extra and intracellular metabolites, and computationally inferring flux through the Metabolic Flux Analysis is the most direct approach for determining metabolic flux through metabolic network and pathways on a whole-cell level (Sauer, 2006; Wiechert, 2002). In this way cell culture metabolomics can

help in both bottomup or forward modeling as well as topdown or inverse approach by providing either data for the determination of model reaction constants, for model parameterization or for determination of network structures for the model.

Mathematical modeling of cell metabolism is an essential approach for gaining system-level understanding of cell behavior and development of predictions of cellular behavior, ultimately providing methods for the design of cells of desired properties or cell growth conditions. Mathematical modeling including deterministic kinetic modeling and stochastic and statistical modeling, have been widely used in the application of cell cultures (Richelle et al., 2020). In the mechanistic, mathematical modeling system functions and properties are described as the result of the interaction of the system elements within the cell and with the environment. Thus, mechanistic models can predict behavior of cellular systems or (metabolic) processes when elements of the model, their properties or interactions change (Stalidzans et al., 2020). Different mechanistic modeling approaches have been extensively utilized for the description and interpretation of cell culture metabolomics results with several methods and related freely available tools and some examples of their application listed in Table 12.6.

Changes in the flow through metabolic networks are a reflection of genetic, epigenetic and environmental factors. Measurement and the analysis of the network can be done through the analysis of the flow of a label from isotopically labeled precursors into metabolites (see above). Metabolic flux and concentration do not necessarily correlate as metabolic concentration increase can come from either increased production flux or decreased consumption flux. Thus, metabolite levels and fluxes provide complementary information. Fluxes can not be directly measured, but can be inferred from measurement of isotope tracers (Jang et al., 2018) with some examples of metabolic flux experiments described above. In addition to MS application for flux analysis, NMR spectroscopy can be used for a highly sensitive site-specific label quantification. In cell culture applications isotopic label tracing with NMR can be used for analysis of extracellular, intracellular or organel specific flow. Time-series measurements of metabolome are essential for the development and validation of dynamic models of metabolism (Judge et al., 2019; Sefer et al., 2016). In a typical cell culture metabolomics setting described above, information about the dynamic metabolome change would require significant resources, and sample material. Time-series sampling has to provide sufficient number of replicates, ensure sufficient experiment duration, and the time resolution. Sampling introduces extraction biases and the confounding of biological and analytical variance (Sitnikov et al., 2016; Tabatabaei Anaraki et al., 2018).

Different types of metabolic modeling have been presented for number of significant cell lines including for example for a model of a generic human cell (Brunk et al., 2018; Robinson et al., 2020) as well as number of specific cell types, including HEK293 cells (Quek et al., 2014), CHO cells (Lund et al., 2017;

Table 12.6 Freeware methodologies for mechanistic modeling.

Method	Software application	Examples of some application in cell culture metabolomics
Bayesian modeling	GRASP	Methionine cycle modeling using approximate Bayesian computation
Logical modeling	CellNetOptimizer (http://www.cellnopt.org) GINsim (http://ginsim.org)	Combination of cell line proteomics and metabolomics data logic mechanistic model modeling to explain heterogeneous drug response in cellular cholesterol regulation
Dynamic modeling through Ordinary differential equations	COPASI (Hoops et al., 2006) CellDesigner (Matsuoka et al., 2014) VCell	Many examples of COPASI's use in biotechnology cell modeling are reviewed in; recent example of hybrid cybernetic modeling that combines dynamic modeling between different metabolic states for CHO cells
Stochastic modeling	COPASI (Hoops et al., 2006) StochKit MaBoSS (http://maboss.curie.fr)	Theoretical foundation to study metabolism in conjunction with stochastic enzyme expression has been presented showing metabolic heterogeneity resulting from enzyme level stochasticity
Stoichiometric modeling	COBRA (Heirendt et al., 2019) CobraPy Raven 2.0 (Wang et al., 2018) Merlin	Genome-scale stoichiometric reconstructions and computational models of mammalian metabolism particularly for CHO cells coupled to protein secretion
Agent based modeling	ARCADE	Extensive review of agent based methods for cancer cell modeling

Several freeware methodologies for mechanistic modeling.

Robinson et al., 2020), iPSCs (Chandrasekaran et al., 2017; Shen et al., 2019), cancer cell lines (Ghaffari et al., 2015; Yizhak et al., 2015). Development of large combined models that can take advantage of advanced knowledge of some pathways and ability to simulate large networks continues to be an active area of research (Hameri et al., 2019; Jamshidi & Palsson, 2010; Opdam et al., 2017). Extreme high-throughput metabolomics and lipidomics of cell cultures, with an increasing coverage over time or flux provides information for parameter optimization. At the same time this data can be used for the development of data-driven, machine learning models and hybrid mechanistic-machine learning models with major potential in the design of optimal cells and cell environments.

Machine learning and hybrid models and artificial intelligence for cell design

Current mechanistic models, although increasingly detailed, still can not provide complete simulation and explanation of cellular processes possibly due to the self-regulatory nature of metabolic networks, posttranslational regulation and the topological organization of metabolism (Zampieri et al., 2019; Zelezniak et al., 2018) all making relationship between enzyme function and metabolites highly dynamic and multifactorial and therefore suboptimaly covered with current mechanistic models.

In the development of safe, specific, and affordable gene and cell therapies the ability to design appropriate modalities with predictable behavior in different environments is of particular importance. Machine learning has been extensively used for the analysis of high throughput omics data as well as images of cell cultures with some examples presented above. AI systems that can describe and predict behavior of biological networks of cells will allow more accurate, faster and less expensive innovations in life sciences while at the same time ensuring predictable outcomes. In particular, the full potential for safe and efficient utilization of gene editing and live cell therapies requires an ability for controlled design of these modalities with simulations that allow testing and optimization under different conditions in both production and utilization. However, the current inability to predict the behavior of biological systems including predicting the phenotype from genotype and the inability to extrapolate large-scale or in vivo outcomes from small-scale, ex vivo experiments severely hampers progress of cell therapy development. The lack of sufficient quantity and quality of data hampers the direct use of machine learning for the development of predictive models of cellular systems. Simultaneously, the lack of biological knowledge as well as the extreme complexity of the system makes development of whole cell system mechanistic models impossible at this point.

Machine learning are algorithms that perform pattern formation and classification and establish rules and statistical structures from data without any explicit instructions. Machine learning is widely used in the analysis of cell culture data including analysis of "omics" data as well as metabolomics and lipidomics (Cuperlovic-Culf, 2018; Pomyen et al., 2020). In cell culture applications there is an increasing abundance of data, both metabolomics/lipidomics as well as other types of omics and data for gene knock-out screens of protein inhibition and this resources can be now used to develop data-driven models without any mechanistic assumptions or inclusion of only very well defined theoretical knowledge. Several recent examples show the power of these approaches when linked to cell culture metabolomics (Zelezniak et al., 2018). Zelezniak et al. (2018) have used machine learning modeling of proteomics data to predict metabolite concentrations. The predicted concentrations correlated strongly with measured metabolomics data in yeast cell analysis. Different data transformation techniques and a

large number of different machine learning algorithms were tested and the quality of obtained models was ranked based on the correlation with the measured metabolite concentrations. This analysis has shown that machine learning approaches can provide some information about multifactorial relationships in metabolic networks without information from mechanistic models. In another example, Costello and Martin (2018) used longitudinal proteomics and metabolomics cell culture data to develop machine learning predictive metabolism models. In this approach authors used tree-based pipeline optimization tool to combine, through genetic algorithms, 18 different feature selection algorithms and 11 different machine learning regressors in order to find function f which satisfies: $argmin\Sigma\Sigma\left|\left|f(m^i[t],p^i[t])-m'^i(t)\right|\right|^2$ where $m^i[t]$ and p^i $[t]$ are, respectively, metabolite and protein concentrations at time t. Metabolome and proteomics concentration measurements over time were the input variables into the machine learning model and m'^i (t) is metabolite time derivative (rate of change) is the output of the model.

Machine learning generally performs poorly in prognosis particularly when trained using sparse data. However, these methods can be combined with mechanistic models in order to provide a combination of knowledge-based and data-driven systems for modeling and design. Examples of metabolomics applications that were combining constraint-based metabolism modeling analysis and machine learning have been recently outlined (Zampieri et al., 2019). Method comparison has shown the possibility to link results from mechanistic models with further analysis with machine learning. Machine learning can also be enhanced with the integration of knowledge in the form of driving equations, constraints or boundary conditions in order to reduce the model search space improving handling of sparse, noisy data. Mechanistic models can benefit from machine learning in creating surrogate models, identify networks, system dynamics and parameters from data (Cuperlovic-Culf, 2018; Peng et al., 2020). Metabolomics and lipidomics investigation of cell culture in different applications provides uniquely rich data for creation of better cell models and AI tools for design of cell environment for optimal utilization as well as design of cells with optimal behavior. Cell culture metabolomics also provides a possibility for measurement of metabolite concentrations in whole cells, organelles, extracellular vesicles and media in static or flux mode. All this data can be combined in order to develop predictive cellular models that can be further linked with other information about the cells including other omics measurements or image analysis data and finally implemented in the cell design systems.

Large datasets primarily resulting from single-cell RNASeq analysis are driving development of a number of new AI methods for prediction and modeling of biological data and many of these approaches can be adapted to metabolomics and metabolism modeling in cell culture analysis. Recently published scGen (Lotfollahi et al., 2019) method combines variational autoencoders (consisting of an encoder and a decoder and able to generate new data points) and latent space vector arithmetics for modeling cell behavior from single cell gene expression

data. In another example Graph Convolutional Neural networks for Genes were developed for inferring gene-gene interactions from high throughput spatial gene expression data (Yuan & Bar-Joseph, 2020). This method, through its use of graph structure, can utilize both the gene expression values (in the original use or metabolite concentration in metabolomics) encoded in each node and relationship between cells expressing these genes or metabolites in order to predict extracellular interactions. Modeling methods used in cell culture design have to provide information about the functional outcome as well as mechanisms leading to the outcomes in order to aid in drug or gene editing making "black box" deep learning models in-appropriate for this application. Use of a "visible" neural network was attempted as a method for an interpretable NN for simulation of a basic eukaryotic cell (Ma et al., 2018). The resulting simulation DCell (http://d-cell.ucsd.edu/) provides an excellent simulation of cell growth and allows in silico investigation of the molecular mechanisms underlying genotype to phenotype relationship.

Theory inspired machine learning methods seek casualties by integrating prior knowledge and data. Wide range of information available for cell cultures as well as mechanistic models for a number of pathways as well as genome scale networks can be used to narrow the search space for machine learning models. At the same time, machine learning can be used to reduce the number of dynamic variables and unknown parameters present in mechanistic models while providing uncertainty quantification. Extensive consideration of the power and opportunities of theory inspired machine learning is provided in Alber et al. (2019). Some examples of biological knowledge inspired machine learning models include knowledge-primed neural networks (KPNN) (Fortelny & Bock, 2020) and simulation-based kernel ML (SimKernML) (Deist et al., 2019). In the KPNN method, a biological network is used to define a graph where each node corresponds to a protein or a gene, and each edge corresponds to a regulatory relationship obtained from biological databases or literature. Network that is designed in this way can be trained, that is optimized, with a much smaller dataset then is needed for example for artificial neural networks as fewer free parameters need to be optimized. Additionally, every node and every edge within a KPNN have a corresponding biological interpretation. SimKernML uses mechanistic simulations of biological processes to build machine learning kernel (e.g., support vector machine) and this improves the downstream machine learning performance for small training dataset.

Novel, faster and more accurate theory inspired and "white box" metabolism modeling methods, developed and trained using metabolomics and lipidomics measurements in cell cultures can be utilized for the design of cell growth conditions, explanation of different cell culture test results or design of predictable and safe cell therapies. Addition of novel methods for single cell metabolomics, in-cell analysis, 3D cell cultures and increased metabolite coverage will provide invaluable data for further development of improved applications of cell cultures.

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