Metabolomics assisted with stable-isotope labeling: exploring neuronal metabolism related to Parkinson's disease

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Chapter 6:

Conclusions and Perspectives

Conclusion

The progressive loss and degeneration of dopaminergic neurons in the mid-brain region is a major pathological hallmark of Parkinson's disease (PD). Due to poorly understood disease pathogenic mechanisms, current therapy is compromised and focused more on treating motor symptoms than slowing down the neurodegenerative process. The onset and progression of PD can be triggered by multiple risk factors, for instance genetic mutation, environmental exposure, and aging. Each of these factors may cause common or unique metabolic disturbances, ultimately converging into a complex metabolic disorder reflected in the diversity of clinical phenotypes. Through metabolome analysis, the full picture of the metabolic landscape depicting a biological system can be revealed. Metabolites function as key elements and direct read-outs of a system's functional status. Alterations in the metabolite concentrations are thus informative for inferring and understanding the underlying metabolic activity. Metabolites are typically characterized by significant physicochemical variability and wide concentration ranges. This diversity creates analytical challenges, and a single analytical platform rarely covers the full range of metabolites of interest [1]. It also makes it important to evaluate the platforms that can be utilized for metabolomics analysis, taking into account the sample type, sample volume, research questions, metabolite concentration, etc. Information derived from broad metabolic profiling can be very instructive in understanding biochemical changes resulting from perturbations. In addition, stable isotope labeling techniques coupled with metabolomics can bring us an extra dynamic vision of the metabolic landscape. Changes in the labeling patterns of metabolites help identify alterations with metabolic fluxes through pathways. For the analytical workflow to capture and process these alterations in metabolite labeling patterns, more analytical and computational developments are still required.

The aim of this thesis was to develop a comprehensive analytical strategy for characterizing the metabolic activity related to PD neurodegeneration, especially focused on the improvement in metabolome coverage and data quality, and facilitating use of stable isotope labeling in in-depth metabolism investigation. In this chapter, we summarize the multifaceted solutions for constructing our comprehensive analytical strategy in PD metabolomics research. The key solutions revolved around tackling the current analytical challenges faced in selecting an appropriate polar stationary phase used in polar metabolome analysis, advancing the analytical method for tracer-based metabolome analysis, and developing a computational workflow for metabolic flux analysis. These methods were applied to investigate metabolic dysregulation of dopaminergic neurons to genetic and environmental factors. The value and contribution of developed methodological

solution in this thesis, as well as the current limitations, are reviewed. Finally, we conclude by proposing opportunities for future research.

Polar metabolome analysis using LC-MS

The ultimate aim of metabolomics is to precisely identify and quantify all metabolites present in a specific biological sample. High-resolution mass spectrometry with an increasing mass resolving power, coupled with enhanced chromatographic resolution, has improved the metabolomics analysis towards broadened feature detection. It has been widely recognized that the combination of multiple analytical platforms in metabolomics is an appropriate strategy to increase global metabolite coverage. Reversed-phase liquid chromatography (RPLC) mainly targets the non-polar and mid-polar metabolome analysis, and hydrophilic interaction chromatography (HILIC) targets the polar metabolome analysis. The chromatographic separation is highly determined by the interaction of the metabolite with the stationary phase, followed by fine-tunings of solvents, pH, additives, column dimensions, and temperature. But due to the limited understanding of HILIC retention mechanisms and lack of guidance on HILIC method evaluation, HILIC is less widespread than RPLC for global metabolomics studies. Polar metabolites account for a large proportion of the metabolome and play important roles in regulating energy and biomass production (e.g. amino acids, carbohydrates, carnitines), interconnecting metabolic modules as a reflection of overall metabolic state (e.g. ATP, NAD(H), S-adenosylmethionine), and producing reporter metabolites in controlling and modulating the activity of signaling pathways (e.g. glycolytic intermediates) [2]. From this, it is reasonable to assume that the comprehensive analysis of polar metabolites involved in major biochemical pathways (e.g., glycolysis, the tricarboxylic acid (TCA) cycle, the pentose phosphate pathway, amino acid metabolism, nucleotide metabolism, neurotransmission, etc.) would allow for an extensive exploration of metabolic dysregulation related to neurodegeneration.

To accelerate the implementation of HILIC-MS analysis as a routine platform for large-scale clinical PD metabolomics studies, the analytical requirements of resolution, reproducibility, robustness, efficiency and result reliability have to be strictly evaluated. In **Chapter 2**, we provided guidance for systematically evaluating hydrophilic interaction chromatography column performance for global plasma metabolomics studies. Plasma was selected as the investigated biological matrix due to its ease of collection and high information content. First, a scoring system was designed to evaluate the chromatographic performance of representative standards involving the aspects of metabolite retention, peak sensitivity (signal-to-noise ratio), peak sharpness (peak height), and peak symmetry (tailing factor). Second, a simple linear regression model could be constructed to analyze the

relationship between metabolite polarity at different pH conditions and elution order, allowing for the investigation of the column retention mechanisms. Third, assessment of the matrix effect and particularly the ion suppression induced by salts helped to identify the vulnerable metabolite classes or the elution range affected by salt ions (clusters). Fourth, a repeatability test monitoring the retention time and peak area of spiked standards in plasma was used to evaluate the column stability for intra- or inter-batch analysis. Finally, a pilot study using an untargeted metabolomics analysis of test plasma from different phenotypes could be carried out to estimate the total metabolic feature coverage and feature retention distribution.

Following this systematic evaluation, we determined the ZIC-c HILIC column (zwitterionic stationary phase) operated at neutral pH was optimal for global polar metabolome analysis due to its superior performance for different classes of compounds, better isomer separation, good repeatability, and high metabolic coverage. We also specifically demonstrated the ion suppression caused by the sodium chloride in the plasma analysis. Thereby, it is recommended to restrict the introduction of salt ions, for instance anticoagulant counter cations (Na+ from citrate in Na-citrate plasma and K+ from K-EDTA in EDTA plasma), as reported by a previous study [3]. Additional difficulties arise because citrate and EDTA show some retention on the HILIC column and will induce ion suppression or enhancement effects on other co-eluting peaks. Considering these obstacles, heparin plasma is preferred for future HILIC-MS global analysis. This evaluation procedure can be selectively applied to any new HILIC column or new biomatrix test in the future. And as current HILIC columns are not yet fully robust, new HILIC columns can be expected to be developed and offered. The optimized HILIC-MS chromatographic method developed in **Chapter 2** served as the basis for the targeted metabolomics study in **Chapter 3**, and the mass spectrometric approach coupled with this separation method was further optimized for tracer-based metabolomics in **Chapter 4**.

Targeted metabolomics provides distinct advantages in the quantification of known metabolites belonging to certain compound classes or common metabolic pathways with a high level of confidence in the accuracy, precision, and repeatability, despite the limited number. To achieve a broad and known metabolite analysis with a wide dynamic range in our PD pathogenesis investigation, we adopted three analytical platforms for targeted profiling of 106 polar metabolites, 50 acyl-carnitines, and over 200 signaling lipids. By utilizing human iPSC-derived midbrain neuronal models, a new way for simulating PD metabolic disease brought on by specific genetic/environmental factors and their interacting contributions was presented. The methodological advancements enabled us to capture a comprehensive picture of metabolomic dysregulation in an in-vitro iPSC-derived midbrain

neuronal model of PD represented by the PINK1 mutation, mitochondrial complex I inhibitor (rotenone), and joint-factor intervention in **Chapter 3**. This gave us a chance to investigate into the hypothesis that genetic deficiency involved in mitochondrial-stress pathways could increase the susceptibility towards neurodegeneration in response to environmental toxins. Through comparative metabolomics analysis, we found similar metabolic dysregulation caused by rotenone and the PINK1 mutation seen in energy failure (reduced fueling by glucose, saturated fatty acids, and branched chain amino acids), impaired redox balance (blocked oxidation of NADH to NAD+), as well as different TCA cycle disturbances, all leading to impaired mitochondrial respiration. Moreover, oxidative damage was found in both the mutated and rotenone-exposed groups. However, the PINK1-mutated model showed a robust anti-oxidative and anti-inflammatory response, while a rather severely disturbed response was seen due to rotenone exposure. Neurons with the inherent PINK1 mutation interacting with exogenous rotenone stress resulted in a more complicated neurodegenerative metabolism. This study clearly unraveled the underlying molecular mechanism of mitochondrial dysfunction induced by a genetic or environmental toxin perturbation and explained the complexity of neurodegeneration from a metabolomics perspective. The supplementation of NAD+ or its precursors has been reported as a promising therapeutic strategy for future clinical PD treatment [4,5]. Targeting the same set of PD neuronal models, this study additionally demonstrated the limited efficacy of NAD+ treatment in ameliorating rotenone-related energy defects, and had no effectiveness for the PINK1 mutated group. Instead, it unexpectedly introduced dysregulation of polyunsaturated fatty acids to the joint-factor group. Overall, our study provides valuable insights into a deep understanding of parkinsonism pathogenesis.

In **Chapter 3**, the combination of multiple metabolomics platforms targeting specific polar and non-polar metabolites opened several windows that allowed us to specifically probe cellular metabolic activity in different metabolic pathway modules. Since just one patient cell line was used as the established neuronal model, a follow-up validation using more patient cell lines with the PINK1 mutation is still required. In the future, improved or novel drug treatments can be evaluated using the established neuronal models and targeted metabolomics platforms. Targeted metabolomics analysis can also be performed on plasma samples from patients in order to highlight the release of unique metabolite markers from neurons into the intravascular fluid. In addition, global plasma analysis will be a crucial and indispensable approach for identifying novel biomarkers with a strong correlation to neuronal alterations as well as a high level of significance, which is achievable by combining the established HILIC-MS method described in **Chapter 2** with the complementary RPLC-MS methods.

Use of Stable isotope labeling for in-depth metabolic flux investigations

The metabolomics study performed in **Chapter 3** to determine the probable changes in the metabolic network in neurons was helpful in identifying relevant biochemical pathways to follow up using stable isotope labeling. Therefore, we focused on central carbon metabolism and its connection to de novo nucleotide synthesis and glutathione metabolism pathways in an effort to capture the reaction changes from energy failure towards oxidative stress related to PD neurodegeneration. Current stable isotope enrichment analysis using mass spectrometry primarily records the total number of labeled atoms and the mass isotopologue distribution (MID) of the intact metabolite structure. Labeling information at the intact level can be used to probe pathway activity. Labeling information at the metabolite substructure (moiety) level also plays an important role in discovering novel pathways and estimating metabolic fluxes. A dissection at the moiety level can bring additional insights into pathway reconstruction, reaction connections, and relevant flux regulation [6–8]. However, there are still technological challenges in detecting intact metabolites and fragmented moiety isotopologues with strong specificity while maintaining high sensitivity and accuracy.

In **Chapter 4**, we developed a HILIC-Zeno MRM^{HR} method for structurally-resolved mass isotopologue distribution analysis, allowing simultaneous acquisition at MS^1 and MS^2 levels in a single analytical run. From an analytical perspective, this method successfully achieved accurate and reproducible MID quantification for intact metabolites as well as their fragmented moiety, with notably high sensitivity in the MS^2 fragmentation mode using Zeno trap pulsing, a system of trapping fragment ions prior to the Time-of-Flight (TOF) injection for duty cycle improvement. Compared to the conventional SWATH method, this method still preserved the relationship between labeled precursor and fragment ions, which was beneficial in accurately identifying the same labeled isotopologue with differential labeling positions. We demonstrated the case by distinguishing two different isotopomers (1,2-$^{13}C_2$-glutamate and 3,4-$^{13}C_2$-glutamate) that belong to the same isotopologue ($^{13}C_2$-glutamate) but are produced by pyruvate anaplerosis (PDH) and pyruvate carboxylase (PC) activity, respectively, from a given D-$^{13}C_6$-glucose tracer.

From an application perspective, the HILIC-Zeno MRM^{HR} method successfully identified flux regulations of glutathione metabolism in relation to rotenone-induced neurodegeneration. By interpreting the intact metabolite and moiety MID data, we first validated the pathway reconstruction of de novo glutathione synthesis in mid-brain neurons from isotopically labeled nutrient sources, separately using D-$^{13}C_6$-glucose and L-$^{15}N_2$-glutamine. Increased oxidation from basal and de novo synthesized glutathione pools under neuronal oxidative stress both contributed to the severely impaired anti-oxidative capability

caused by rotenone exposure. Furthermore, we demonstrated the decreased de novo glutathione synthesis was associated with altered activities of several key enzymes found in the glucose-derived glutamate supply and GSH synthetic reaction. The application of our approach was not limited to elucidating glutathione metabolism but also included studying de novo nucleotide metabolism connected with central carbon metabolism in response to rotenone perturbation. By considering the hypothesis of potentially changed enzyme activity along relevant pathways, the method allows for flexibility in selecting target metabolites for fragmentation. Identification of the key metabolic flux regulations within these metabolic pathways can further augment our understanding of disease mechanisms.

Metabolite labeling patterns in conjunction with targeted metabolomics data are strongly effective in elucidating the cellular flux control of a specific metabolic pathway or reaction under selectively perturbed conditions [9]. This approach works well for hypothesis verification via revealing localized regulation [9,10]. To achieve comprehensive flux phenotyping for studied cell models on a network-wide scale, a quantitative approach to metabolic flux inference has been gradually developed, named fluxomics. This global monitoring approach shows promise for quickly identifying primary flux re-routing for accommodating diseased condition based on the changes of absolute fluxes over multiple metabolic pathways and contributes to new hypothesis generation. However, there is still a long way to go before it finally reaches its true potential for accurately characterizing mammalian cell metabolism at the broader scale. This heavily relies on the constant development and improvement of both analytical and computational methodologies covering reliable reconstruction of a cell type-specific metabolic model, accurate atom mapping in metabolic reactions, measurement of isotopmers encompassing labeled positional information, the development of standardized and high-throughput data processing and analysis pipelines, etc [11,12].

Chapter 5 presented our efforts in constructing a semi-automated pipeline in MATLAB, for computing **flux** with **Tr**acer-metabolomics in **A**tom-resolved **M**odelling (**fluxTrAM**). The first part of the fluxTrAM pipeline could process tracer-based LC-MS raw data and convert them into a standardized MID dataset. This involved multiple steps, including raw MS data format conversion, metabolite peak detection and integration, isotopologue peak correction with naturally occurring isotopic abundance, and basic summarization of metabolite MID results. The peak integration performance of both manual and automated software packages was rigorously assessed using the HILIC-MS method to measure multiple metabolite classes. The optimal package, mzMatch-ISO, was embedded into the pipeline and guaranteed accurate targeted isotopologue integration. Additionally, automated connections between various packages reduced the amount of data format

manipulation and enabled a sequential data processing workflow. The second part of the fluxTrAM pipeline focused on atomically resolving any given genome-scale metabolic model, where the results included a chemoinformatic database of standardized and context-specific metabolite structures and atom mapped reactions. In the final part of the fluxTrAM pipeline, experimental MID data could be exported into external ^{13}C flux analysis programs for flux estimation on a small network scale. Meanwhile, it could also be integrated into the atomically resolved genome-scale model for flux analysis at the genome-scale. The feasible flux distribution obtained from the small and generic central carbon metabolism model served as a good reference solution for determining the flux feasibility obtained from the neuron-specific genome scale model. On the other hand, the genome-flux solution revealed extensive metabolism regulation in the studied cell model that extended far beyond central carbon metabolism. Our work presented the first genome-scale flux solution of human dopaminergic neuronal metabolism based on experimental tracer-based metabolomics data. It helped in revealing many characteristic metabolic phenotypes of neuronal cells while also raising the necessity for studying more PINK1 patient-derived cell lines. In the future, follow-up verification of disease hypotheses generated from genome-flux solutions can be achieved using the combined targeted metabolomics (**Chapter 3**) and tracer-based metabolomics approaches (**Chapter 4**). In addition, new (tracer-based) metabolome data can be used as input for further refining the neuron-specific genome-scale model and predicting new flux distribution, as illustrated in the **Figure 6.1** (a, b, c, e, r1 and r2).

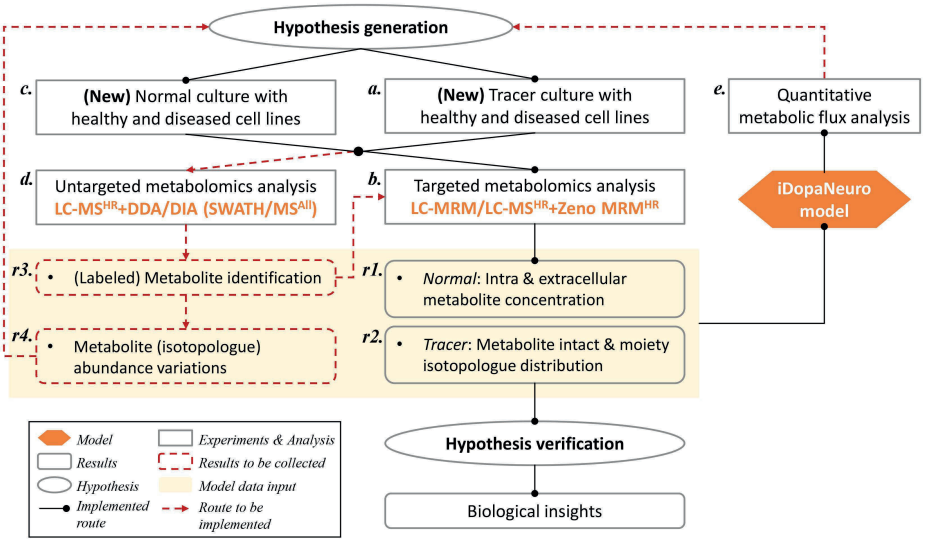

Figure 6.1. A schematic workflow for combining conventional metabolomics and tracer-based metabolomics approaches in the PD neuronal metabolism study. Tracer culture represents cells fed

with stable isotope-labeled substrates, while normal culture is provided with unlabeled substrates. The future implementations are labeled in red dotted lines and the expected results to be collected are labeled in red dotted boxes. a-e: cell culture, analytical experiments, and computational analysis. r1-r4: Metabolome results, as data input for the iDopaNeuro model.

Future perspectives

In this thesis, we demonstrated the capacity of our developed HILIC-MS method for global polar metabolome analysis (**Chapter 2**) and its subsequent application into targeted metabolomics analysis for a Parkinson's disease neuron metabolism study (**Chapter 3**), where relative quantification results were reported. Beyond this thesis with rigorous method validation, targeted absolute quantification so far has been achieved for 106 polar metabolites from nine classes, including amino acids, amines, sugars, sugar phosphates, nucleosides, nucleotides, acyl-carnitines, coenzyme A, and organic acids. By using the increasingly available reference standards and related internal standards, identification and characterization followed by adequate validation will allow to further expand the number of quantifiable targets. Two approaches can be generally followed for extending metabolite coverage. One is the pathway-wise inclusion strategy, which involves adding the missing metabolites from a pathway that is already covered or adding metabolite sets related to a newly given pathway. As an example, as discussed in **Chapter 4**, γ-glutamylcysteine should be added to the HILIC method because it is an important intermediate in the de novo synthesis of glutathione [13]. In addition, the UDP-GlcNAc synthetic pathway is an important pathway to investigate due to its position as a significant hub connecting glucose-, amino acid-, fatty acid-, and nucleotide-metabolism. UDP-GlcNAc is involved in O- and N-linked protein glycosylation, which is important for regulating nutrient sensing and responding to cellular stress [14,15]. The other strategy for prioritizing method extension is metabolite class-wise inclusion, which involves including as many metabolites as possible from a specific class. Growing evidence implies altered polyamine metabolism as a correlate of PD progression [16,17]. Our method has included some polyamine precursors and catabolites, such as L-ornithine and -aminobutyric acid. Many polyamines, including 1,3-diaminopropane, putrescine, cadaverine, spermidine, spermine, agmatine, N-acetylputrescine, N-acetylspermine, and N-acetylspermidine, remain to be tested and should be given priority for inclusion in an extended method.

From a technical perspective, high resolution-based MS and MS/MS methods coupled to a chromatography method can be ideal tools for bridging the gap between untargeted metabolomics profiling and targeted metabolite quantification [18], further extending metabolite coverage in relative or absolute quantitative methods. In terms of large-scale

polar metabolomics quantification, the high-resolution TOF-MS approach can be more susceptible to interferences and have less selectivity than a typical multiple reaction monitoring (MRM) method. However, many QTOF mass spectrometers are able to perform data-dependent or data-independent acquisition (DDA or DIA), such as SWATH or MS^{All}, which allow for recording retention time, high-resolution m/z and MS/MS spectra within the same chromatographic run [19,20]. These three elements are valuable parameters and useful for constructing a comprehensive metabolite MS/MS library database, hence facilitating unknown feature identification in a new sample matrix, and comparing to existing (on-line) databases. Along with further enhanced dimensions in both chromatography and MS instrumentation, other advanced parameters including MS^3 spectra, ion mobility-derived collision cross sections [21], and retention time matching using an orthogonal chromatography can be also recruited for metabolite identification. Newly identified metabolites can be incorporated for later targeted quantification, as illustrated in the left part of **Figure 6.1**. For overcoming the lack of selectivity in quantitative analysis, a high-resolution TOF-MS approach equipped with parallel reaction monitoring (PRM/MRM^{HR}) works as an ideal approach. A recent method coupling HILIC to the general PRM method in a positive/negative ion switching mode achieved simultaneous relative quantification for 237 polar metabolites [22]. Equipping the general PRM/MRM^{HR} method with Zeno trap pulsing could improve MS/MS sensitivity and extend the linear dynamic ranges even further. In Chapter 4, we showed how we used a HILIC-Zeno MRM^{HR} method to quantify 180 precursor isotopologue ions from 25 polar metabolites. This (HILIC-Zeno MRM^{HR}) method can also be easily modified to measure individual metabolites. Based on reduced elution overlap and a scheduled window design, it would have the advantages of providing a lower cycle time and higher method sensitivity for individual metabolite quantification.

The power of stable isotope tracer-based metabolomics, via leveraging additional labeling information to monitor individual reactions or pathway alterations within the interconnected metabolic networks, has been successfully demonstrated not only in our study but in many others as well. However, the potential of stable isotope tracing combined with high-resolution metabolomics technology for characterizing labeled metabolites in an untargeted manner has still not been fully explored. So far, most studies have focused on the targeted analysis of limited known metabolites from anticipated metabolic pathways. This is regarded as the most efficient and accurate way to answer the hypothesis-driven question, whereas a data-driven discovery based on isotope-enriched metabolome (isotopolome) analysis can be of great value to a systematic investigation of metabolism and generate new hypothesis. Untargeted isotopolome analysis is still quite challenging due to the complexity

of isotope labeled metabolomics data. The major bottleneck is the lack of an appropriate workflow to identify unknown labeled features and extract isotopologue information with good accuracy and reproducibility on a large scale.

Through the aforementioned high-resolution LC-MS/MS (DDA or DIA) analysis, metabolite annotation can be first carried out based on collected MS^1 and MS^2 data from unlabeled samples without tracer treatment. Following that, all present isotopologues of annotated metabolites present in labeled samples treated with tracer after a certain period can be extracted. This concept was successfully demonstrated in a recent study named "MetTracer". The study results showed high promise in the unbiased large-scale MID analysis of up to 830 ^{13}C-labeled metabolites and 1725 ^{13}C-labeled isotopologues, which covered 66 metabolic pathways in human embryonic kidney 293T cells [23]. Among the annotated 830 metabolites, each one is assigned three confidence levels. Level 1 means metabolites annotated through matching of retention time, high-resolution m/z and MS/MS spectra with the in-house metabolite database. Level 2 means metabolites annotated through matching high-resolution m/z and MS/MS spectra with public metabolite databases. Level 3 means metabolites annotated based on MS1 and surrogated MS/MS match using in-house identification software. It should be noted that total annotated metabolite at levels 1 and 2 accounted for less than 50%. The use of in-house developed metabolite libraries still has its limits due to standards availability and cost issue. Additionally, it is prone to errors when using external metabolite databases, easily leading to ambiguity and low coverage of identification. To increase confidence in identification, labeled metabolites can provide valuable information on the possible numbers of certain atoms in the molecule. Besides, the metabolite labeling pattern may convey potential connections with other known labeled metabolites. A special effort has been made to analyze MID similarity for pathway contextualization of unidentified metabolites, which provides an alternative solution to further constrain database searches [24]. The proposed approach remains to be tested with LC-MS data and may provide higher metabolome coverage. Available software specializing in unknown labeled metabolite detection and extraction are often bespoke solutions developed to address specific project needs following specific analytical and culture workflows or data structures, for instance, having preconditions for selective ionization techniques, LC-MS or GC-MS, parallel labeling designs, joint analyses for native and highly isotope-enriched biological samples in a single run, etc [25–30]. This requires users to pre-evaluate suitable solutions applicable to their own scenario and tailor the tracer-based metabolomics workflow accordingly. In Chapter 5, we tested and incorporated mzMatch-ISO in fluxTrAM given its excellent capacity for targeted metabolite isotopologue extraction and integration. Future work can add unknown metabolite identification as a pre-

step before targeted processing, also complementing the automated demand of connecting Figure 6.1 d and Figure 6.1 r3. Candidate software can be tested for its performance in the following areas: speed of processing high-resolution LC-MS/MS (DDA or DIA) data, database matching accuracy, cell culturing cost, interoperability with other packages or manual curation, etc. This process can be time-consuming and cumbersome but will be long-term beneficial for future large-scale data analysis in studies involving multiple experimental conditions or serial time-points in dynamic monitoring.

One aspect that still needs to be addressed regarding stable isotope tracer-based metabolomics is the effective utilization of metabolite moiety MID data in metabolic flux analysis. For a localized pathway analysis, moiety MID from a key-node metabolite can be investigated in conjunction with intact metabolite MID to understand the enzymatic regulations of a series of connected reactions [7,8], as demonstrated in **Chapter 4**. For a global network analysis, moiety MIDs act as additional valuable inputs for improving the precision of metabolic flux prediction [31,32]. For instance, they were applied for shrinking the feasible flux solutions for the model of the cyanobacterium Synechococcus sp. PCC 7002 (59 reactions, 53 metabolites) [32]. It is highly promising that in future studies, we can incorporate both intact and moiety MIDs acquired by the HILIC Zeno MRM^{HR} method further into the established genome-scale model, iDopaNeuro, representing human dopaminergic neurons [33]. The computational approaches for performing genome-scale flux analysis assisted with intact and moiety MIDs can highly accelerate the process of biological inference and hypothesis generation for a new stable isotope tracing experiment. Current moiety MID analysis still requires a significant level of manual integration and calculation, once automated software tools are developed in the future, it will be possible to have them evaluated and integrated into the fluxTrAM pipeline.

Aiming at the ideal goal of fully comprehending cellular metabolism regulation in Parkinson's disease, there is still a long way ahead. With the recent analytical and computational developments in revealing metabolite concentrations and flux alterations, it becomes increasingly possible to address ambitious questions. Parallel efforts have been made on constructing the iPSC-derived microglial cell or astrocyte model using healthy and PINK1 mutant cell lines in our group. In the future, we can consider extending our metabolomics investigations into glial response to degenerative neurons. Activated microglial cells and reactive astrocytes are reported to exert both protective and detrimental effects in the neuronal extracellular environment [34,35]. To mimic the intercellular communication between glial cells and neurons and answer how these effects modulate neuronal degeneration, for instance, we can treat the patient-derived neurons with glial cell-conditioned media and perform our proposed tracer-based metabolomics analysis. After

more work is put into developing cellular models of co-cultures in an environment analogous to the human substantia nigra pars compacta, together with their corresponding interactive cellular genome-scale models based on multi-omics data, we expect to design new tracer-based metabolomics experiments to help determine the distinct role of each cell type in the neurodegenerative process. Overall, combining metabolomics profiling based on patient-derived cellular models with metabolic phenotype predictions via in silico genome-scale model analysis would constantly generate new hypotheses and allow us to perform validation or possible enzymatic treatment in a loop fashion. We believe that a better understanding of the metabolic dysregulation leading to PD pathogenesis may bring novel diagnostic and therapeutic approaches into clinical trials. On the other hand, our proposed strategy can also be used to examine any novel medications, providing additional indicators for drug preclinical evaluation. Combined efforts from the analytical, biological, and computational fields will be extremely valuable and will eventually contribute to the development of personalized treatments for Parkinson's disease patients, allowing them to live a high-quality elder life.

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