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Mass Defect-Based *N,N*-Dimethyl Leucine Labels for Quantitative Proteomics and Amine Metabolomics of Pancreatic Cancer Cells

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Abstract

Mass spectrometry-based stable isotope labeling has become a key technology for protein and small-molecule analyses. We developed a multiplexed quantification method for simultaneous proteomics and amine metabolomics analyses via nano reversed-phase liquid chromatography–tandem mass spectrometry (nanoRPLC–MS/MS), called mass defect-based *N,N*-dimethyl leucine (mdDiLeu) labeling. The duplex mdDiLeu reagents were custom-synthesized with a mass difference of 20.5 mDa, arising from the subtle variation in nuclear binding energy between the two DiLeu isotopologues. Optimal MS resolving powers were determined to be 240K for labeled peptides and 120K for labeled metabolites on the Orbitrap Fusion Lumos instrument. The mdDiLeu labeling does not suffer from precursor interference and dynamic range compression, providing excellent accuracy for MS¹-centric quantification. Quantitative information is only revealed at high MS resolution without increasing spectrum complexity and overlapping isotope distribution. Chromatographic performance of polar metabolites was dramatically improved by mdDiLeu labeling with modified hydrophobicity, enhanced ionization efficiency, and picomole levels of detection limits. Paralleled proteomics and amine metabolomics analyses using mdDiLeu were systematically evaluated and then applied to pancreatic cancer cells.

Graphical abstract

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.6b03482. Offline SCX LC-UV chromatogram of mdDiLeu-labeled peptides, evaluation of different NCEs for mdDiLeulabeled proteomics analysis, examples of ID confirmation for metabolite identification, examples of short peptide identification by MS/MS de novo sequencing, protein and metabolite enrichment analyses of PANC1 cells, retention time reproducibilities of nanoRPLC–MS and standard flow RPLC–MS systems, list of identified primary and secondary amine-containing metabolites in PANC1 cells, list of identified short peptides in PANC1 cells, absolute concentrations of representative metabolites in PANC1 cells (PDF)

Notes

The authors declare no competing financial interest.

Quantitative measurements of proteins and small molecules are essential to understanding system biology and disease mechanisms.¹⁻⁵ Mass spectrometry (MS)-based stable isotope labeling is a key technology to quantify proteins and metabolites, where stable heavy isotopes can be differentially incorporated into analytes chemically or metabolically.⁶⁻⁸ Compared to a label-free approach, stable isotope labeling methods allow simultaneous comparison of multiple samples in a single MS run with improved accuracy and reduced systematic variation for quantitative proteomics⁹⁻¹⁴ and metabolomics.¹⁵⁻²⁰

Stable isotope labeling can be categorized into MS¹-centric mass difference labeling and tandem mass spectrometry (MS/MS) reporter ion-based isobaric labeling. Mass difference labeling introduces a fixed mass shift observed in full MS scans of intact precursor ions, with examples such as mass differential tags for relative and absolute quantification (mTRAQ),²¹ metabolic stable isotope labeling by amino acids in cell culture (SILAC),¹⁰ formaldehyde dimethylation,²² and dansylation.²³ Mass difference labeling approaches provide accurate quantification but are mainly criticized for increased mass spectral complexity and overlapping isotopic clusters. In contrast, isobaric labeling reagents incorporate stable heavy isotopes at strategic positions so that each tag produces nearly identical precursor ion mass shifts, but upon MS/MS fragmentation will yield a reporter ion with a unique mass-to-charge ratio (m/z). Isobaric labeling methods, such as iTRAQ,²⁴ TMT,¹¹ and DiLeu,²⁵ provide the advantages of cleaner MS¹ spectra and greater multiplexing capability than mass difference labeling. However, isobaric tags suffer from precursor isolation interferences, compromising the accuracy and dynamic range of quantification.^{26,27} In recent years, researchers have made great efforts to mitigate the negative effects of precursor coisolation and cofragmentation by developing novel strategies, such as the MultiNotch MS³ method,²⁸ computational correction algorithm,²⁹ QuantMode method,³⁰ and using the ion mobility MS platform.³¹

The recent advancements of high-resolution mass spectrometers, such as Fourier transform ion cyclotron resonance (FTICR)-MS and orbitrap MS, enabled the development of hyperplexing isobaric tags³²⁻³⁴ and mass defect-based stable isotope labeling.³⁵⁻³⁸ Mass defect, the difference between an isotope's mass number and its element's mass number, arises from the differences in nuclear binding energy and varies from nucleus to nucleus.³⁹ Employing this mass defect signature, Hebert and co-workers described the novel neutron encoding (NeuCode) protein quantification method which incorporates isotopologues of lysine with subtle millidalton (mDa) mass differences.^{27,40} Ulbrich et al. also expanded the

NeuCode method to the quantification of organic acids with a mass difference of 6.32 mDa between light- and heavy-isotope-labeled analytes.⁴¹

Isobaric *N,N*-dimethyl leucine (DiLeu) reagents have been designed in our lab to have comparable performance to commercial isobaric tags (TMT and iTRAQ), but be obtainable at a greatly reduced cost. The sets of 4-plex, 8-plex, and 12-plex isobaric DiLeu reagents have been successfully synthesized and applied to protein, peptide, and metabolite analyses.^{17,25,32,42,43} Here, we developed a novel MS¹-centric quantification method for both proteins and amine metabolites, called mass defect-based DiLeu (mdDiLeu) labeling. The mdDiLeu reagents are expected to combine the clean MS spectra of isobaric labeling with the accuracy of mass difference labeling with two heavy and light labels whose masses differ by only 20.5 mDa (Scheme 1A).

In light of the significance of linking the metabolome and proteome to understanding systems biology and correlating multiomics to disease phenotypes, increasing numbers of studies have focused upon multiomics integration.^{44,45} However, almost all proteomics and metabolomics analyses have thus far been performed on separate analytical platforms. Most of the multiomics integration was performed to combine genomics and proteomics, while the integration of proteomics with metabolomics was often carried out in the computational realm or via functional pathway analyses.^{46,47} Recognizing the inherent distinct chemical natures of proteins and small molecules, it is challenging to conduct proteomics and metabolomics analyses in parallel simultaneously. Nano liquid chromatography–mass spectrometry (nanoLC–MS) platforms have been extensively used for proteomics analysis, but only few studies have managed to derivatize metabolites for nanoLC–MS.^{16–18} In this study, the mdDiLeu labeling technique enables proteomics and amine metabolomics analyses to be achieved on the same nano reversed-phase liquid chromatography (RPLC)–orbitrap MS platform. By using a single robust analytical platform, instrument cost is reduced and variations between instruments for multiomics analysis are avoided. We further demonstrate the applicability of this method to the quantitative measurements of cellular proteins and metabolites in pancreatic cancer cells.

EXPERIMENTAL SECTION

The overall workflow of the mdDiLeu-labeled cellular proteomics and metabolomics analyses is shown in Figure 1. It integrates cell harvest, protein and metabolite extraction, sample preparation, mdDiLeu labeling, and nanoRPLC–MS/MS analysis.

Pancreatic Cancer Cell Culture

Commercially available PANC1 pancreatic ductal adenocarcinoma cells (ATCC) were routinely maintained in complete media of DMEM/Ham's F-12 (1:1) (ATCC) supplemented with 10% fetal bovine serum (Hyclone), and 1% antibiotic–antimycotic solution (Cellgro). Cell culture flasks were placed in an incubator containing 5% CO₂ and 98% humidity. Cells were used for a maximum of 15 passages and trypsinized using 0.25% trypsin EDTA solution (Gibco) once 80% confluence was achieved. A hemocytometer was used for cell counting, and cell pellets of 2×10^6 cells were rapidly washed twice with phosphate-buffered saline, flash-frozen in dry ice, and stored at -80°C .

Cellular Protein Extraction and Digestion

For proteomics analysis, the cell pellet was resuspended in 8 M urea, 30 mM NaCl, 50 mM Tris (pH = 8), 5 mM CaCl₂, and one protease inhibitor cocktail tablet (Roche Diagnostics). The sample was sonicated in an ice–water bath for 20 min, centrifuged at 14 000 rpm for 5 min, and the supernatant was collected. Protein concentration of the cell lysate was measured by BCA assay (Thermo Scientific). Disulfide bonds from cysteine residues were reduced with 5 mM dithiothreitol (DTT) for 1 h at room temperature. Free thiol groups were alkylated with 15 mM iodoacetamide (IAA) in the dark for 15 min, and the reaction was quenched by 5 mM DTT. Protein sample was diluted with 50 mM Tris solution (pH = 8) to a urea concentration of less than 1 M. Protein digestion was performed with trypsin enzyme at a 50:1 protein/enzyme ratio at 37 °C for 16 h. The digestion was quenched by adding 10% trifluoroacetic acid (TFA) to a pH lower than 3, followed by a desalting step with SepPak C₁₈ solid-phase extraction cartridge (Waters).

Cellular Metabolite Extraction and Metabolite Standards

For cellular metabolite extraction, 800 μ L of ice-cold methanol was added to the cell pellet and the sample was vortexed vigorously. Then, the sample tube was immediately transferred into liquid nitrogen for 10 min, thawed on ice for 10 min, and vortexed for 2 min. The freeze–thaw cycle was repeated twice for cell disruption, and the supernatant was collected after centrifugation at 16 000 rpm for 5 min. Cell pellet was re-extracted with 400 μ L of 50% methanol and water sequentially. The resulting supernatants were combined, freeze-dried, and stored at –80 °C before DiLeu labeling.

Individual metabolite standard solutions were prepared at 1 mM concentration, including representative neurotransmitters and amino acids: serotonin, dopamine, GABA, norepinephrine, valine, leucine, lysine, alanine, histidine, phenylalanine, tyrosine, and tryptophan. Metabolite standards were mixed and dried under SpeedVac for DiLeu labeling.

Synthesis of Mass Defect-Based *N,N*-Dimethyl Leucine (mdDiLeu)

The duplex mdDiLeu reagents were synthesized following a modified procedure as described previously for 4-plex isobaric DiLeu labels²⁵ (Scheme 1B). For the light label, L-leucine-1-¹³C, ¹⁵N was dissolved in 1 N HCl H₂¹⁸O solution (pH 1) and stirred for 4 h at 65 °C for ¹⁸O exchange reaction. Acid was then removed from the solution and isotopic leucine was dimethylated by suspending in H₂O with a 2.5 molar excess of NaBH₃CN. The mixture was stirred in an ice–water bath, and formaldehyde (CH₂O) was added to the solution dropwise. The reaction was monitored every 30 min by ninhydrin staining on a thin-layer chromatography (TLC) plate. For the heavy label, L-leucine was dimethylated in NaBD₃CN D₂O solution with the dropwise addition of heavy formaldehyde (CD₂O) in an ice–water bath. The heavy and light dimethyl leucines were purified by flash column chromatography (MeOH/CH₂Cl₂), dried, and stored in a desiccator at 4 °C.

DiLeu Activation and Labeling Reaction. DiLeu reagents are activated to the triazine ester form to react with amine groups (Scheme 1C). One milligram of each DiLeu reagent was mixed with 1.86 mg of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) and 0.74 μ L of *N*-methylmorpholine (NMM) in 50 μ L of anhydrous

dimethylformamide (DMF). The reaction vial was vortexed at room temperature for 1 h. After activation, metabolite or peptide samples were labeled immediately with DiLeu triazine ester to obtain the optimal labeling efficiency.

Dried metabolite or peptide fraction was redissolved in 0.5 M triethylammonium bicarbonate (TEAB) solution and labeled with a 5× (w/w for peptides) or 20× (mol/mol for metabolites) excess of activated DiLeu.^{17,25} Anhydrous DMF was added to reach 70% of organic/aqueous ratio, and the reaction vials were vortexed for 2 h at room temperature. The labeling reaction was quenched with 0.25% hydroxylamine (v/v), and duplex-labeled samples were dried down separately.

Sample Cleanup with Strong Cation Exchange (SCX)

Offline SCX chromatography fractionation was used to remove unreacted DiLeu reagent and reaction byproducts from the labeled peptides as well as separate each labeled sample into three fractions for subsequent nanoLC–MS/MS analysis. SCX fractionation was performed using an SCX column (PolyLC, 200 mm × 2.1 mm, 5 μ m, 300 Å) on a Waters Alliance e2695 HPLC instrument. Buffer A was 10 mM NH_4HCO_2 , 25% acetonitrile (ACN), pH 3; buffer B was 500 mM NH_4HCO_2 , 25% ACN, pH 6–8. Light- and heavy-labeled peptide samples were redissolved in buffer A and combined for SCX fractionation. The binary gradient at a flow rate of 0.2 mL/min was performed as follows: 0–20 min, 0% B; 20–90 min, 0–50% B; 90–100 min, 50–100% B; 100–110 min, 100% B. Fractions were collected every 2 min, combined into three vials based on UV–vis at 280 nm, and dried down. A typical LC–UV chromatogram is shown in Supporting Information Figure S1. C₁₈ OMIX Ziptip (Agilent Technologies) was used for subsequent peptide desalting, with 0.1% TFA in H₂O as the reconstitution and washing solution, and 0.1% formic acid (FA) in 50% ACN and 0.1% FA in 70% ACN as elution solutions. The eluate was dried down and stored at –20 °C.

For labeled metabolite samples, light- and heavy-labeled samples were resuspended in 0.1% FA water solution and combined for sample cleanup as described previously.¹⁷ OMIX SCX Ziptip (Agilent Technologies) was used to remove unreacted DiLeu and reaction byproducts with 0.1% FA in H₂O as the reconstitution and washing solutions, and 5% NH_3 H₂O in 30% MeOH as elution solution. The eluted sample was dried down and stored at –20 °C.

NanoRPLC–ESI–MS/MS

Labeled PANC1 cellular peptides and metabolites were analyzed on the same analytical platform: a Dionex UltiMate 3000 nanoLC system coupled with a Fusion Lumos Tribid mass spectrometer. Mobile phase A was 0.1% FA in H₂O, and mobile phase B was 0.1% FA in ACN. Flow rate was 0.3 μ L/min. A C18 column was fabricated in-house with an integrated emitter (75.1 μ m × 150 mm, 1.7 μ m, 100 Å). The peptide sample was separated with a 137 min LC gradient starting from 3% B and ramping up to 30% B in 102 min. The top 20 data-dependent acquisitions (DDA) were conducted, and the MS was scanned from m/z 400–1500 at a resolving power (RP) of 240K (at m/z 200) and an S-lens radio frequency (rf) of 30. Parent masses were isolated in the quadrupole with an isolation window of 1.0 m/z and fragmented with higher-energy collisional dissociation (HCD) with a normalized

collision energy (NCE) of 30%. MS/MS scans were detected in the linear ion trap using the rapid scan rate and a dynamic exclusion time of 60 s. Automatic gain control (AGC) targets were 5×10^5 for MS and 2×10^4 for MS/MS acquisitions. Maximum injection times (maxIT) were 50 ms for MS and 20 ms for MS/MS. The optimization of collision energy for proteomics analysis was achieved by conducting a top 1 DDA acquisition multiplexing five different NCE between 20% and 40%. For labeled metabolites, a 40 min LC gradient was conducted from 3% B to 30% B in 10 min, then 30–80% B from 10 to 30 min. Full MS scans were acquired from m/z 100 to 1000 at an RP of 120K and an S-lens rf of 28. MaxIT was 100 ms, and AGC was 5×10^5 . In the top 20 DDA acquisition of labeled metabolites, a survey scan was followed by MS/MS HCD fragmentations with an isolation window of 1 m/z , NCE of 30%, RP of 30K, max IT of 30 ms, and AGC of 2×10^5 .

Data Analysis

Proteomics data analysis was carried out using the MaxQuant software 1.5.3.30⁴⁸ for peptide/protein identification and quantification. Protein identification was achieved by searching against a *Homo sapiens* reference database obtained from UniProt. Methionine oxidation was chosen as variable modification. Cysteine carbamidomethylation, N-terminus DiLeu labeling, and lysine residue DiLeu labeling were chosen as static modifications with tag masses of +145.1200 Da (light) and +145.1405 Da (heavy). The peptide tolerances for the first and main searches in MaxQuant were 10 and 3 ppm, respectively. The NCE optimization data was processed by COMPASS Proteomic Analysis software suite.⁴⁹ The raw data containing five multiplexing NCE channels can be separated into five files to directly compare different collision energies between 20% and 40%. The heavy/light protein ratios of peak pairs were exported to a Microsoft Excel file and corrected for label purities with a previously described method.^{17,42}

Metabolomics data was processed by Compound Discoverer 2.0 software (Thermo Scientific) for aligning chromatograms, detecting unknown compounds, and grouping unknown compounds. For detecting unknown compounds, the mass tolerance was 4 ppm, the minimum peak intensity was 1×10^5 , and the signal-to-noise ratio was 3. The mass tolerance and retention time tolerance for grouping unknown compounds was 2 ppm and 0.05 min, respectively. The compound peak list was exported into an Excel file, and mdDiLeu-labeled peak pairs were identified using an in-house developed software tool. The mass defect for selecting peak pairs is $20.5N$ ($N = 1, 2, 3$) mDa with a mass tolerance of 0.3 mDa and a retention time threshold of 0.05 min. Purity correction was also performed in Excel using the same method as described for protein analysis. Only the detected peak pairs were subjected to metabolite identification by deducting the mass of mdDiLeu tag from the accurate mass and then searching against multiple online databases using MetaboSearch software.⁵⁰ The accurate mass matching threshold was 5 ppm. Chemical structures of identified metabolites were examined, and only primary and secondary amine-containing metabolites were selected. Metabolite identifications were also confirmed via MS/MS fragmentation and spectral matching with light mdDiLeu-labeled standard compounds. The identifications of short peptides were confirmed with MS/MS de novo sequencing using PEAKS Studio 7 software. The parameters for de novo sequencing were as follows: parent mass error tolerance of 5 ppm, fragment mass error tolerance of 0.02 Da, no selected

enzyme, fixed mdDiLeu modification, and variable modifications of oxidation of M and methylation.

RESULTS AND DISCUSSION

mdDiLeu-Labeled Cellular Proteomics Analysis

In order to completely resolve the labeled peak pairs for MS¹-centric protein quantification and at the same time maximize the number of identified proteins, key orbitrap parameters were investigated, including resolving power, automatic gain control, and normalized collision energy. As illustrated in Figure 2, parts A and B, a resolving power of 240K on the Orbitrap Fusion Lumos was sufficient to separate the light- and heavy-labeled peak pairs and provided the highest number of peak pairs and identified protein groups. An RP of 500K enabled further separation of peak pairs but compromised detection sensitivity and duty cycle, consequently resulting in fewer identified proteins than at RP = 240K. Different AGC values were also compared for mdDiLeu-labeled tryptic peptides (Figure 2C). Higher AGC settings allow more ions to be injected into the orbitrap, generating higher signal intensities. However, the trade-off between signal intensity and scan speed needs to be carefully considered. Additionally, if too many ions are injected and trapped in the mass analyzer, the interaction between trapped ion clouds of species with similar *m/z* can cause peak coalescence, which is often observed in FTICR-MS and has also been reported in orbitrap MS in recent years.⁵¹⁻⁵³ Peak coalescence of high-abundance ions was observed with AGC higher than 5×10^5 . Thus, AGC of 5×10^5 was selected, which offered the highest number of identified protein groups (Figure 2C). Different MS/MS NCEs were also compared to provide sufficient backbone fragmentation for protein/peptide identification (Supporting Information Figure S2). An NCE of 30% provided the highest number of identifications and was thus selected as the optimal NCE value.

The mdDiLeu labeling for MS¹-centric quantification is virtually free from precursor interferences and also independent of tandem mass acquisitions where only a portion of all eluting peptides can be selected for MS/MS fragmentation. We evaluated the quantification accuracy, reproducibility, and dynamic range of mdDiLeu labeling strategy for proteomics analysis. Tryptic peptides from PANC1 cells were separately labeled (in triplicate) with the light and heavy mdDiLeu and combined at 1:1, 1:2, and 1:10 ratios. The full MS intensities of light- and heavy-labeled peptide peaks were used for quantification. A total of more than 2000 protein groups were identified and more than 1600 protein groups were quantified using mdDiLeu labeling. The measured median ratios across all quantified proteins were 1:0.92, 1:1.74, and 1:9.56 for theoretical 1:1, 1:2, and 1:10 ratios, respectively. Because the duplex mdDiLeu reagents can generate reporter ions 115 and 118 upon MS/MS fragmentation, mass defect MS¹-based quantification can be directly compared with MS/MS reporter ion-based quantification with the same set of samples (Figure 3). For the reporter ion-based quantification, the measured median ratios of quantified proteins were 1:0.91, 1:1.70, and 1:8.53 for theoretical 1:1, 1:2, and 1:10 ratios, respectively. Both methods achieved accurate quantification of proteins within 15% accuracy. But MS¹-centric quantification was more accurate, particularly for analytes with large fold differences across the full dynamic range compared with reporter ion-based quantification. The accuracy of

MS¹-centric quantification is not influenced by the retention time shift caused by the deuterium effect, though very small retention time shift was observed in the labeled peak pairs. We can possibly alleviate the dynamic range compression in reporter-based quantification by narrowing the precursor mass isolation window or reducing the MS/MS dynamic exclusion time so that multiple MS/MS spectra can be averaged to generate more accurate reporter ion ratios. A multinotch MS³ method or ion mobility MS platform can also be used to mitigate precursor interferences as reported previously.^{14,28,31} Furthermore, due to the subtle mass difference of 20.5 mDa between the light and heavy labels, quantitative information can be easily concealed or revealed by changing MS resolving power and is achieved without increasing spectrum complexity or overlapping isotope distribution. Our results suggested that the mdDiLeu labeling technique yields accurate quantification and uncompressed dynamic range, combining the advantages of isobaric labeling and mass difference labeling.

The optimized proteomics workflow was applied to analyze cellular proteins from PANC1 cells. Go-term enrichment analysis was performed on over 2000 identified protein groups from PANC1 cells using the open-source PANTHER classification system.⁵⁴ Identified proteins, clustered based on their functional annotations, were found to be involved in 13 biological processes and 127 functional pathways (Supporting Information Figure S3). As expected, most proteins belong to cellular and organelle components and are correlated with cellular and metabolic processes. Many identified pathways have been found to correlate with cancer cell metabolism, such as ATP synthesis, p53 pathway, oxidative stress response, hypoxia response, glycolysis, TCA cycle, pentose phosphate pathway, DNA replication, pyruvate metabolism pathway, and glutamine metabolism pathway.¹

Chromatographic Improvements of mdDiLeu-Labeled Amine Metabolite Standards

Despite the wide application of nanoRPLC in proteomics workflow, it is very difficult to detect free metabolites with acceptable signal intensities and peak shapes at low flow rate on typical nanoRPLC columns.¹⁶⁻¹⁸ Thus, metabolomics analysis is routinely performed using standard flow LC systems. Our previous study has demonstrated the improvement of amine metabolites analysis using isobaric DiLeu labeling on a nanoRPLC-QTOF MS platform.¹⁷ As with isobaric DiLeu tags, mdDiLeu labeling increases the hydrophobicity and improves the ionization efficiency of amine metabolites, allowing the reproducible separation of amine metabolites using nanoRPLC-MS. Labeled 12 metabolite standards including representative amino acids and neurotransmitters were analyzed on an Orbitrap Fusion Lumos instrument and compared with our previous results acquired on a Synapt-G2 QTOF MS platform. An average of more than 200-fold improvement of detection limits were achieved on the Fusion Lumos MS, as summarized in Table 1. Two instrument platforms showed similar retention time reproducibility with an average standard deviation of 0.14 min. Retention time reproducibility of the established nanoRPLC-orbitrap MS platform was also found to be comparable to the standard flow RPLC-orbitrap MS, which is routinely used for metabolomics analysis (Supporting Information Table S1). Clearly, combining DiLeu labeling and the advanced orbitrap instrument offered us further improved analytical methodology, which allows more comprehensive profile of amine metabolites in biological samples.

Cellular Amine Metabolomics Analysis Using mdDi-Leu Labeling

When analyzing cellular metabolites on the nanoRPLC–MS platform, label-free metabolites extracted from PANC1 cells showed few peaks with poor signal intensities, while the labeled cellular metabolites (same number of cells) yielded a greatly improved profile, as shown in Figure 4A. Each light- and heavy- mdDiLeu-labeled metabolite presented two peaks with the same LC retention time and a mass difference of 20.5 mDa. Example cellular amino acids are shown in Figure 4B. Isoleucine and leucine can be completely separated after labeling, whereas most of the routine standard flow RPLC platforms are unable to completely resolve them.

Several instrument parameters were evaluated to achieve optimal coverage of the amine metabolome profile in pancreatic cancer cells. First, MS resolving power was optimized in order to completely separate the labeled peak pairs. With the increase of MS RP from 30K to 120K, the light- and heavy-labeled peaks were better resolved and generated more quantifiable peak pairs (Figure 5, parts A and B). The detected total features decline as MS RP rises, and the number of quantifiable peak pairs decreased dramatically at a resolving power of 240K due to the loss of sensitivity and lower duty cycle. A resolving power of 120K was thus chosen as the optimal RP. Next, MS AGC was investigated for the analysis of labeled metabolites. As shown in Figure 5C, increasing the AGC value yielded enhanced sensitivity and more quantifiable peak pairs until reaching a maximum at an AGC value of 5×10^5 . An AGC value of 1×10^6 allows the greatest number of ions to be injected into the mass analyzer at the cost of decreased scan speed and increased susceptibility to peak coalescence. Therefore, an AGC value of 5×10^5 was selected for mdDiLeu-labeled metabolites.

Using the optimized instrument platform, we detected a total of more than 10 000 mass features and more than 1000 labeled small-molecule peak pairs in PANC1 cells. The quantification accuracy was demonstrated by combining light- and heavy-labeled cell metabolite samples at 1:1, 1:2, and 1:10 ratios. As shown in the box plots in Figure 5D, the measured median ratios among all quantified peak pairs were 1:1.07, 1:2.04, and 1:10.72 for theoretical 1:1, 1:2, and 1:10 ratios, respectively. The presence of labeled peak pairs greatly enhanced the confidence of metabolite identification which narrowed the IDs to primary and secondary amine-containing metabolites. The list of identified amine metabolites from PANC1 cells are provided in Supporting Information Table S2. Examples of metabolite ID confirmation are illustrated in Supporting Information Figure S4. We were also able to identify more than 30 short peptides from cells by MS/MS de novo sequencing (Supporting Information Table S3, Figure S5). Additionally, we performed the absolute quantification of selected metabolites in PANC1 cell extract by constructing calibration curves with light mdDiLeu-labeled standard compounds (Supporting Information Table S4).

Metabolite enrichment analysis was carried out using an online tool called MBRole (Metabolites Biological Role) (Supporting Information Figure S3).⁵⁵ The major pitfall of chemical derivatization is that only a subset of metabolites can be labeled. Multiple derivatization methods might be used to increase the coverage of metabolome but at the expenses of greatly increased workload and variations. Despite this shortcoming, a variety of amine-containing metabolites were identified from the PANC1 cells via mdDiLeu labeling,

including amino acids and derivatives, neurotransmitters, biogenic amines, amino fatty acids, and nucleic acids. Identified metabolites belong to different cellular components, including mitochondrial, cytoplasm, extracellular, nucleus, and other cell organelles. Forty-three KEGG metabolism pathways were identified, and seven of them overlapped with proteomics results, including the aminoacyl-tRNA biosynthesis pathway, the glutathione metabolism pathway, the arginine and proline metabolism pathway, the propanoate metabolism pathway, the amino sugar and nucleotide sugar metabolism pathway, the alanine, aspartate, and glutamate metabolism pathway, and the valine, leucine, and isoleucine degradation pathway.

CONCLUSIONS

We demonstrate the first characterization and application of multiplexed mdDiLeu labels for proteomic and amine metabolomic analyses. Paralleled proteomics and metabolomics studies using the mdDiLeu labeling technique enable multiomics analysis to be achieved on the same analytical platform, nanoRPLC–MS/MS. This approach combines the advantages of isobaric labeling and mass difference labeling with excellent quantification accuracy and uncompressed dynamic range. The advanced Orbitrap Fusion Lumos Tribrid MS platform with an ultrahigh-field orbitrap provided superior resolving power and high sensitivity to detect light- and heavy-labeled counterparts with a subtle mass difference of 20.5 mDa. With the established methodology in the present study, future work can be extended to comparative analyses of different treatments and coculture conditions of pancreatic cancer cells. Also possible for future studies is mass defect-based DiLeu tags formulated with as many as 11 heavy isotopes to double the mass difference to ~41 mDa and increase multiplexing capability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Overall workflow of quantitative proteomics and amine metabolomics analyses in pancreatic cancer cells using mdDiLeu labeling. Protein and metabolite fractions were separately extracted from PANC1 cells, prepared, and labeled with duplex mdDiLeu reagents. Instrument analyses were performed on the same platform, nanoRPLC–Orbitrap Fusion Lumos Tribid MS.

Figure 2.

Optimization of MS parameters for cellular proteomics analysis. (A) NanoRPLC-MS chromatograms of duplex mdDiLeu-labeled peptide and MS spectra under different resolving powers. (B) Numbers of sequenced peak pairs and identified protein groups under different MS resolving powers. (C) Numbers of sequenced peak pairs and identified protein groups under different MS AGC values.

Figure 3.

Protein quantification accuracy of mdDiLeu labeling (MS¹-centric quantification) and the comparison with MS/MS reporter ion-based quantification. (A) Box plots represent the measured ratios across all quantified proteins at mixing ratios of 1:1, 1:2, and 1:10. Box denotes 25th and 75th percentiles; line inside the box denotes the median; whiskers denote standard deviation. The dashed lines demarcate the theoretical ratios. (B) The linearity and dynamic range of relative quantification using mdDiLeu (MS¹ quantification) and isobaric DiLeu (MS/MS quantification). Slope = 1 and $R^2 = 1$ represent perfect linearity and accuracy of relative quantification.

Figure 4. Chromatographic performance of mdDiLeu-labeled cellular metabolites in nanoRPLC-MS. (A) Base peak chromatograms of mdDiLeu-labeled (blue) vs unlabeled (green) metabolites from PANC1 cells. (B) Examples of duplex-labeled cellular amino acids, phenylalanine (Phe), isoleucine (Ile), and leucine (Leu). Isoleucine and leucine can be completely separated after mdDiLeu labeling.

Figure 5.

Optimization of MS parameters and quantification accuracy for cellular amine metabolomics analysis. (A) NanoRPLC–MS chromatograms of duplex mdDiLeu-labeled metabolite and MS spectra under different resolving powers. Tryptophan detected from PANC1 cells is used as an example. (B) Numbers of detected total features and quantifiable peak pairs under different MS resolving powers. (C) Numbers of detected total features and quantifiable peak pairs under different MS AGC values. (D) Quantification accuracy of cellular amine metabolites using mdDiLeu labeling. Box plots illustrate the measured ratios across all detected metabolite peak pairs at mixing ratios of 1:1, 1:2, and 1:10, demarcating 25th and 75th percentiles (box), median (line inside the box), standard deviation (whiskers), and the theoretical ratio (dashed line).

Scheme 1. General Structures (A), Synthesis (B), and Activation and Labeling Reactions (C) of Duplex mdDiLeu Reagents^a

^a The light and heavy labels have a mass difference of 20.5 mDa for MS¹-centric quantification.

Table 1
Limits of Detection (LOD) of Metabolite Standards in Different LC-MS Platforms

metabolite	formula	light <i>m/z</i>	heavy <i>m/z</i>	LOD (nM)	
				nanoRP/LC-orbitrap MS	nanoRP/LC-QTOF MS ^a
serotonin	C ₁₀ H ₁₂ N ₂ O	322.2222	322.2428	0.03	89.13
dopamine	C ₈ H ₁₁ NO ₂	299.2064	299.2268	0.46	164.38
GABA	C ₄ H ₉ NO ₂	249.1907	249.2113	0.05	26.90
norepinephrine	C ₈ H ₁₁ NO ₃	315.2011	315.2217	0.35	49.10
valine	C ₃ H ₁₁ NO ₂	263.2062	263.2268	0.14	10.93
leucine	C ₆ H ₁₃ NO ₂	277.2218	277.2426	0.08	30.53
lysine	C ₆ H ₁₄ N ₂ O ₂	219.1804	219.2006	0.27	80.00
alanine	C ₃ H ₇ NO ₂	235.1749	235.1954	1.37	106.87
histidine	C ₆ H ₉ N ₃ O ₂	301.1970	301.2176	0.13	197.58
phenylalanine	C ₉ H ₁₁ NO ₂	311.2064	311.2268	0.14	7.34
tyrosine	C ₉ H ₁₁ NO ₃	327.2013	327.2217	0.04	8.92
tryptophan	C ₁₁ H ₁₂ N ₂ O ₂	350.2171	350.2376	0.02	12.75

^a LOD obtained from previously published method (ref 17).