Online Peptide Fractionation Using a Multiphasic Microfluidic Liquid Chromatography Chip Improves Reproducibility and Detection Limits for Quantitation in Discovery and Targeted Proteomics

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Comprehensive proteomic profiling of biological specimens usually requires multidimensional chromatographic peptide fractionation prior to mass spectrometry. However, this approach can suffer from poor reproducibility because of the lack of standardization and automation of the entire workflow, thus compromising performance of quantitative proteomic investigations. To address these variables we developed an online peptide fractionation system comprising a multiphasic liquid chromatography (LC) chip that integrates reversed phase and strong cation exchange chromatography upstream of the mass spectrometer (MS). We showed superiority of this system for standardizing discovery and targeted proteomic workflows using cancer cell lysates and nondepleted human plasma. Five-step multiphasic chip LC MS/MS acquisition showed clear advantages over analyses of unfractionated samples by identifying more peptides, consuming less sample and often improving the lower limits of quantitation, all in highly reproducible, automated, online configuration. We further showed that multiphasic chip LC fractionation provided a facile means to detect many N- and C-terminal peptides (including acetylated N terminus) that are challenging to identify in complex tryptic peptide matrices because of less favorable ionization characteristics. Given as much as 95% of peptides were detected in only a single salt fraction from cell lysates we exploited this high reproducibility and coupled it with multiple reaction monitoring on a high-resolution MS instrument (MRM-HR). This approach increased target analyte peak area and improved lower limits of quantitation without negatively influencing variance or bias. Further, we showed a strategy to use multiphase LC chip fractionation LC-MS/MS for ion library generation to integrate with SWATH™ data-independent acquisition quantitative workflows. All MS data are available via ProteomeXchange with identifier PXD001464. Molecular & Cellular Proteomics 14: 10.1074/mcp.M114.046425, 1708–1719, 2015.

Mass spectrometry based proteomic quantitation is an essential technique used for contemporary, integrative biological studies. Whether used in discovery experiments or for targeted biomarker applications, quantitative proteomic studies require high reproducibility at many levels. It requires reproducible run-to-run peptide detection, reproducible peptide quantitation, reproducible depth of proteome coverage, and ideally, a high degree of cross-laboratory analytical reproducibility. Mass spectrometry centered proteomics has evolved steadily over the past decade, now mature enough to derive extensive draft maps of the human proteome (1, 2). Nonetheless, a key requirement yet to be realized is to ensure that quantitative proteomics can be carried out in a timely manner while satisfying the aforementioned challenges associated with reproducibility. This is especially important for recent developments using data independent MS quantitation and multiple reaction monitoring on high-resolution MS (MRM-HR)1 as they are both highly dependent on LC peptide retention time reproducibility and precursor detectability, while attempting to maximize proteome coverage (3). Strategies usually employed to increase the depth of proteome coverage

1 The abbreviations used are: MRM, multiple reaction monitoring; BPC, base peak chromatogram; cps, counts per second; FA, formic acid; FDR, false discovery rate; HPLC, high pressure liquid chromatography; IP, immunoprecipitation; LLOQ, lower limit of quantification; MRM/SRM, multiple/selected reaction monitoring; mfg, Mascot generic file; MudPIT, multiple dimension protein identification technology; RP, reverse phase; rpm, rounds per minute; S/N, signal to noise; SCX, strong cation exchange; SDC, sodium deoxycholate; TEAB, triethylammonium bicarbonate; TIC, total ion chromatogram; UPLC, ultrahigh pressure liquid chromatography.
utilize various sample fractionation methods including gel-based separation, affinity enrichment or depletion, protein or peptide chemical modification-based enrichment, and various peptide chromatography methods, particularly ion exchange chromatography (4–10). In comparison to an unfractoned “naive” sample, the trade-off in using these enrichments/fractionation approaches are higher risk of sample losses, introduction of undesired chemical modifications (e.g. oxidation, deamidation, N-terminal lactam formation), and the potential for result skewing and bias, as well as numerous time and human resources required to perform the sample preparation tasks. Online-coupled approaches aim to minimize those risks and address resource constraints. A widely practiced example of the benefits of online sample fractionation has been the decade long use of combining strong cation exchange chromatography (SCX) with C18 reversed-phase (RP) for peptide fractionation (known as MudPIT – multidimensional protein identification technology), where SCX and RP is performed under the same buffer conditions and the SCX elution performed with volatile organic cations compatible with reversed phase separation (11). This approach greatly increases analyte detection while avoiding sample handling losses. The MudPIT approach has been widely used for discovery proteomics (12–14), and we have previously shown that multiphasic separations also have utility for targeted proteomics when configured for selected reaction monitoring MS (SRM-MS). We showed substantial advantages of MudPIT-SRM-MS with reduced ion suppression, increased peak areas and lower limits of detection (LLOD) compared with conventional RP-SRM-MS (15).

To improve the reproducibility of proteomic workflows, increase throughput and minimize sample loss, numerous microfluidic devices have been developed and integrated for proteomic applications (16, 17). These devices can broadly be classified into two groups: (1) microfluidic chips for peptide separation (18–25) and; (2) proteome reactors that combine enzymatic processing with peptide based fractionation (26–30). Because of the small dimension of these devices, they are readily able to integrate into nanoLC workflows. Various applications have been described including increasing proteome coverage (22, 27, 28) and targeting of phosphopeptides (24, 31, 32), glycopeptides and released glycans (29, 33, 34).

In this work, we set out to take advantage of the benefits of multiphasic peptide separations and address the reproducibility needs required for high-throughput comparative proteomics using a variety of workflows. We integrated a multiphasic SCX and RP column in a “plug-and-play” microfluidic chip format for online fractionation, eliminating the need for users to make minimal dead volume connections between traps and columns. We show the flexibility of this format to provide robust peptide separation and reproducibility using conventional and topical mass spectrometry workflows. This was undertaken by coupling the multiphase liquid chromatography (LC) chip to a fast scanning Q-ToF mass spectrometer for data dependent MS/MS, data independent MS (SWATH) and for targeted proteomics using MRM-HR, showing clear advantages for repeatable analyses compared with conventional proteomic workflows.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Thyroid cancer cell lines SW1736 (anaplastic thyroid carcinoma), TPC1 (papillary thyroid carcinoma), and Nthy-ori 3.1 (immortalized normal thyroid epithelial cells) were cultured in 10% (v/v) bovine serum supplemented RPMI 1640 medium (Invitrogen) at 37 °C in a 5% CO2 atmosphere and grown to 80% confluence.

**Human Plasma**—Blood samples were collected into BD Vacutainer® EDTA (Catalog Number: 366643) tubes, centrifuged within 30 min of collection, then aliquoted and stored at −80°C. Ethics approval was obtained from the Sydney Southwest Area Health Service research ethics committee (CH62/6/2006–132).

**Protein Preparation and Digestion**—Cultured cells were lysed and proteins denatured in 100 mM triethyl ammonium bicarbonate (TEAB, Sigma Aldrich) and 1% sodium deoxycholate (SDC, Sigma Aldrich) buffer (pH 7.8) for 5 min at 99 °C. After lysis, nuclease was added and incubated for 30 min at room temperature to degrade DNA (1:10,000 enzyme/DNA). Human plasma proteins (non-immunodepleted) were denatured in 100 mM TEAB and 1% SDC for 5 min at 95 °C. In both, the cell lines and the plasma samples, cysteine residues were reduced in presence of 10 mM dithiothreitol (Bio-Rad, Sydney, Australia) at 60 °C and alkylated with 10 mM iodoacetamide (Bio-Rad) at room temperature in the dark. Trypsin (Promega, sequencing grade, Melbourne, Australia) was added in a 1:50 ration and Proteins were enzymatically degraded overnight at 37 °C. By adding 1 μl formic acid (FA, Thermo Fisher Scientific, Sydney, Australia) the digest was quenched and the SDC precipitated and removed by centrifugation (14,000 rpm). Samples were lyophilized and reconstituted in 2% acetonitrile (ACN, Merck, Melbourne, Australia) and 0.1% FA.

**Multiphase LC Chip**—Silicon wafers with patterned microfluidic chip design were fabricated using conventional multilayer photolithographic techniques (35, 36). A 10 cm wafer was chemically etched with HF to yield three different etch depths (1.5–10–100 μm). Wafer chips were made through bonding of a pair of wafers. The 2 cm2 diced multiphase chip was composed of a 3 cm microchannel (200 μm) that was divided into three equal segments via four built-in resistors (3 μm); each segment was packed separately via a capillary (100/200 μm ID/OD) and the conduits sealed using EMCAST UV glue (Electronics Material, Inc., Breckenridge, CO). The prototype multiphasic chip reported here has a configuration of a 1 cm SCX phase (5 μm PolySULFOETHYL A™, PolyLC Inc., Columbia, MD) sandwiched between two 1 cm C18 reversed phases (5 μm ChromXP C18CL™, Eksigent, part of AB SCIEX, Redwood City, CA), and is readily placed in a chip holder that is compatible with the existing cHiPLC® system (Eksigent, part of AB SCIEX) as shown in Fig. 1B. The multiphase chip was placed in the connector 2 position as a trap in conjunction with a jumper chip (connector 1) and a separate 15 cm x 200 μm analytical column chip (nano cHiPLC Trap column (ChromXP™ C18-CL 3 μm 120 Å; Eksigent, part of AB SCIEX) in the connector 3 position (37). This allows online sample desalting, peptide fractionation and analytical separation online with mass spectrometry.

**ChipLC**—LC-MS/MS analyses were carried out using a NanoLC™ ultra with cHiPLC® system (Eksigent, part of AB SCIEX). For RP LC-MS/MS, 200 μm x 0.5 mm nano cHiPLC trap column (ChromXP™ C18-CL 3 μm 120 Å; Eksigent, part of AB SCIEX) and 15 cm x 200 μm nano cHiPLC columns (ChromXP™ C18-CL 3 μm 120 Å) were used. Multiphase LC-MS/MS using the prototype multiphase trap chip described above. Samples or salt bumps were transferred onto the traps by auto sampler injection using a 10 μl sample loop followed by...
Online Peptide Fractionation Using Multiphasic chipLC

by 5 min loading with 2% ACN and 0.1% FA. Stepwise peptide elution from the SCX phase was achieved by salt bump injection of 2 mM, 50 mM, 500 mM and 1500 mM ammonium acetate (pH = 3). Each sample or salt bump injection was followed by 60 min increasing ACN gradients (5% to 45%; 90% ACN 0.1% FA). In the multiphase LC-MS/MS experiment the ACN gradient after sample injection is used to transfer peptides from the first RP phase onto the SCX phase (desalting) whereas salt injections are used to transfer peptides from the SCX onto the second RP phase. The following ACN gradient allows for analytic separation and MS/MS detection by electro spray ionization tandem mass spectrometry on a TripleTOF 5600 mass spectrometer (AB SCIEX).

For data dependent MS/MS acquisition the 20 most intense m/z values excluding a threshold > 150 counts per second (cps) with charge stages between 2+ and 4+ were selected for analysis from a full MS survey scan and excluded form for 20 s to minimize redundant precursor sampling.

In data independent acquisition m/z windows of 12.5 Da were used over a range of 400–1150 m/z applying collision energies calculated for 2+ precursors with m/z values of lowest m/z in window + 5 m/z. Retention time scheduled high resolution multiple reaction monitoring (MRM-HR) was based on previous data dependent MS/MS data of nondepleted human plasma proteins. Peptides were excluded if present in more than one fraction, had methionine residues or missed cleavages. Multiphase peptide elution information was used to generate multiphase fraction specific mass lists targeting 503 peptides over four fractions within time windows of 2.5 min and precursor tolerances of 50 ppm in unit resolution (0.7 Da isolation window). Nontargeted peptides were excluded from MS/MS analysis by increasing selection threshold for those to 10,000,000 cps.

Multiple reaction monitoring (MRM) monitoring of peptides selected from SWATH-MS acquisitions were carried out on a QTRAP® 5500 (AB SCIEX) with NanoAcquity UPLC system (Waters). Peptides were injected onto a 180 μm × 2 cm Symmetry trap (waters; C18 5 μm 120 Å) and separated on a 100 μm x 10 cm BEH130 column (Waters; 1.7 μm C18 120 Å). After targeted peptide transition optimization and retention time scheduling, 93 transitions were targeted over a 30 min gradient from 1–50% of 99.9% ACN and 1% FA.

**Protein Identification**—Data dependent MS/MS datasets were searched against the human UniProt database (release April 2014, 20266 entries) using the Mascot 2.4 algorithm (Matrix Sciences) after Mascot generic file (mgf) generation with ProteinPilot™ software 4.2. For Mascot database searches, precursor mass accuracy was set to 50 ppm, fragment tolerance to 0.1 Da for charge stages 2–4+ allowing up to three missed cleavages of fully tryptic peptides. Carbamidomethyl was set as a fixed modification. For MS/MS data from cell lysates protein N termini acetylation, N-terminal formylation, Gin to PyroGlu of N-terminal Gin, phosphorylation and methionine oxidation were allowed as variable modifications. For plasma proteins protein N termini acetylation, N-terminal formylation, Gin to PyroGlu of N-terminal Gin, and methionine oxidation were set as variable modifications. Peptides were considered as present if peptide FDR < 1% (decoy database search), individual ion score > minimal score suggested by algorithm, spectrum hit rank 1 and dendrogram cut-off of 20 (supplemental Table S1).

Spectral libraries for SWATH-MS quantitation were generated with ProteinPilot™ software 4.2 using the Paragon™ algorithm (AB SCIEX) in the thorough ID mode (38) excluding biological modifications but allowing chemical modifications. MS/MS data were searched against the human UniProt database (release March 2012, 20246 entries) with carbamidomethyl as a fixed modification for cysteine residues. An Unused Score cut-off was set to 2.0 (99% confidence) and the FDR analysis was enabled.

**Data Analysis**—Generated Paragon group files were imported into PeakView™ software 2.0 (supplemental Table S2A, S2B) using the SWATH MicroApp 2.0 (release 27/11/2013) and matched against SWATH-MS data. After retention time calibration with endogenous peptides, data were processed using following processing settings; 100 maximal peptides per protein, maximal six transitions per peptide, peptide confidence threshold of 60%, transition false discovery rate < 1%, 10 min extraction window and fragment extraction tolerance of 75 ppm. Transition, peptide and protein areas of processed data were exported and assessed with a SWATH replicate reproducibility analysis template (AB SCIEX; template version 0.994). MRM-HR and MRM data were processed using Skyline 2.5.0.6157. After manual validation of transition peak integration, data were exported and CVs and peak area ratios were calculated.

**RESULTS**

We developed a prototype multiphasic LC microfluidic chip consisting of a 1 cm SCX stationary phase interfaced between two 1 cm C18 reversed-phases, to enable sample desalting and ion exchange peptide fractionation in an integrated “plug-and-play” device located upstream and online with a mass spectrometer (Fig. 1A). The multiphasic chip was designed to be used as a trap chip placed into the trap chip holder (connector 2) of an existing chPLC® system (Eksigent, part of AB SCIEX) with 10-port valve connectivity option as shown in the plumbing scheme in Fig. 1B and supplemental Fig. S1. This configuration paired with upper pressure limits of 4000 psi for the multiphase LC chip enabled microflow sample loading and salt-based ion exchange fractionation in one channel (pump 1 flow path in the “load” position) with nanoflow analytical analysis on an independent 15 cm, 200 μm C18 RP microfluidic chip placed in connector 3 using pump 2 flow path in the “inject” position. Peptide fractionation in this standardized system was conducted by autosampler delivered salt plugs of increasing concentration of ammonium acetate.

**Data Dependent MS/MS Using Multiphase Trap Chips**—The performance of the multiphase LC chip was assessed by analyzing tryptic digests of complex biological samples: thyroid cancer cell line lysates and nondepleted human plasma. Several sample loads, salt concentrations for peptide elution, and sample analysis times were tested with final conditions for evaluation chosen as 5 μg loads and 2, 50, 500, and 1500 mM ammonium acetate steps, with an effective acetonitrile gradient of 60 min following each fractionation step. This leads to a five-step fully automated sample fraction method with run time of 8 h. Fig. 1C shows replicate total ion chromatogram (TIC) profiles of five-step multiphase LC chip separated tryptic peptides from thyroid cancer cells compared with an unfractinated sample. Each fraction shows a unique elution profile consistent with effective SCX fraction and the overlay of three replicate injections indicates high reproducibility in TIC and base peak chromatogram (BPC) in all five fractions (Fig. 1C and supplemental Fig. S2). Although TIC intensities are quite low in the desalting and the 1500 mM salt step, both are crucial in this set up. The desalting step is
required for online sample desalting and for peptide transport from the first C18 RP phase to the SCX resin. The 1500 mM salt step functions to equilibrate the SCX by eluting any remaining bound peptides and other molecules thereby minimizing SCX sample carry-over.

Triplicate analysis of five-step multiphasic chip LC separations using 5 µg of SW1736 thyroid cancer cell lysate identified 2850 ± 4 proteins with 14,021 ± 250 peptides (1% peptide FDR) using an 8 hour hands-free analysis (5 × 1 h effective gradient time + 5 × 10 min sample loading/salt fractionation and 25 min column equilibration). One hour C18 RP separation of 5 µg SW1736 lysate detected only 1804 ± 47 proteins and 7168 ± 208 peptides. To allow equivalent MS/MS sequencing time we repeated the 1 h RP experiment five consecutive times, which consumed five-times more total sample (25 µg). This resulted in the detection of 2395 proteins and 11,193 peptides, which was inferior to the data obtained using only 5 µg and multiphasic LC fractionation (Fig. 2A). Similar advantage using multiphasic LC separation was obtained with 5 µg of nondepleted human plasma with 260 ± 15 proteins and 2134 ± 40 peptides compared with 230 proteins and 1743 peptides for 5 × 1 h C18 RP runs (Fig. 2B).

We investigated the reproducibility of these separations by examining triplicate injections of SW1736 cancer cell lysates. Strikingly, 95% of all identified peptides were uniquely identified in a single fraction (supplemental Table S3A–S3C) and less than 0.9% in more than two fractions (Fig. 2C). Further, we investigated whether the identified peptides in three replicates elute in the same salt fraction or are unequally distributed across salt steps. In total 20,944 peptides were identified...
Online Peptide Fractionation Using Multiphasic chipLC

(a) Graph showing the number of peptides over time with different phases:
- **Multiphase (5 μg)**
- **5 x 1 hr RP (5 x 5 μg = 25 μg)**
- **1 hr RP (5 μg; n = 5)**

(b) Graph showing the number of peptides over time with different phases:
- **Multiphase (5 μg)**
- **5 x 1 hr RP (5 x 5 μg = 25 μg)**
- **1 hr RP (5 μg; n = 5)**

(c) Bar chart showing the number of peptides per fraction:
- Fraction 1: 94.9 ± 0.8%
- Fraction 2: 4.3 ± 0.7%
- Fraction 3: 0.1 ± 0.1%
- Fraction 4: 0.09 ± 0.01%
- Fraction 5: 0.03 ± 0.01%

(d) Bar chart showing the number of peptides per fraction:
- Fraction 1: 81.9 ± 0.3%
- Fraction 2: 53.7 ± 0.4%
- Fraction 3: 3.8 ± 0.2%
- Fraction 4: 0.3 ± 0.1%
- Fraction 5: 0.3 ± 0.1%

(e) Bar chart showing the number of peptides per fraction with different desalting conditions:
- Desalt: 2 mM, 20 mM, 500 mM, 1500 mM

(f) Bar chart showing the number of peptides per fraction with different desalting conditions:
- Desalt: 2 mM, 20 mM, 500 mM, 1500 mM

(g) Graph showing intensity over time for different fractions:
- **50 mM fraction 5 μg Cell Lysate**
- **blank multiphasic LC separation after Cell Lysate (50 mM fraction)**

(h) Graph showing intensity over time for different fractions:
- **50 mM fraction 5 μg Cell Lysate**
- **blank after 50 mM fraction (no trap)**
Online Peptide Fractionation Using Multiphasic chipLC

from three multiphasic LC chip separations of SW1736 cell lysates. Almost a third were unique to one of the three replicates (7485; 35.7%), with the remaining 13,459 peptides identified in at least two of three replicates. Of those remaining peptides, 97.9% (13, 182) were identified in the same salt fraction across the replicates (supplemental Fig. S3). This high reproducibility of the multiphasic LC chip fractionation is an important feature that is needed for robust sample-to-sample peptide quantitation. For nondepleted human plasma, which is dominated by several extremely abundant proteins, triplicate analysis showed that 82% of identified peptides were unique to each fraction, with <5% found in more than two fractions (Fig. 2D). Unsurprisingly, the 13.7% of peptides present in two fractions were predominantly derived from highly abundant plasma proteins; serum albumin, immunoglobulins, haptoglobin, and fibrinogens (supplemental Table S4A–S4C).

We assessed whether the LC chip system with multiphasic trap was susceptible to sample carryover. The peptide carryover occurring on the multiphasic LC chip was monitored by analyzing a five-step fractionation of a blank injection following a five-step fractionation of SW1736 cell lysate (experiment 1). Peptide carryover on the RP analytical column was investigated by acetonitrile gradients following each fraction analysis without usage of the multiphase trap (experiment 2). In both experiments peptides were detectible in the blank injection, which correlated to 5% of all identified peptides in the previous (experiment 1) or parallel (experiment 2) analyzed cell lysate (Fig. 2E, 2F). However, comparison of the TIC profiles of the 50 mM salt elution cell lysate fraction (the most peptide rich salt fraction) with the corresponding 50 mM blank fraction revealed an overall TIC intensity of the blank fraction of less than 1% of the TIC intensity of the cell lysate fraction, thus confirming that peptide carryover from the trap and the analytical column is negligible (Fig. 2G, 2H).

Enrichment of Peptides Representing the Acetylated Protein N Terminus—As the physicochemical basis of ion exchange fractionation is peptide charge, it was of interest to ensure that our data conformed to this principle. We observed that peptides with one lysine, arginine or histidine amino acids were predominantly identified in the lower ionic strength 2 mM or 50 mM ammonium acetate fractions, whereas peptides detected in the 500 mM or 1500 mM fractions had two or more positively charged residues (Fig. 3A). Motivated by this observation we investigated the nature of peptides that did not bind to the SCX media (supplemental Table S5A–S5C), but were captured in the initial desalting step. Among three technical replicates of five-step multiphasic fractionation of SW1736 cell lysates, 1245 of 1379 peptides (90%) present in the desalting fraction contained less than two positively charged residues per peptide. This fraction contained functionally interesting subsets of peptides including C-terminal peptides (155, 11%) and phosphopeptides (43, 3%). More strikingly, the vast majority of peptides in the desalting fraction showed N-terminal peptide modifications generated either chemically or enzymatically, leading to a loss of the amino-terminal positive charge (Fig. 3B). Only 105/1379 (7.5%) peptides had no modification except for potential methionine oxidation. Interestingly, the most common N-terminal modification in the desalting fraction comprised an acetylated form of protein N termini (608 peptides - 44%). N-terminal acetylated peptides found in other salt fractions carried additional positive charge through missed tryptic cleavages or presence of charged amino acids. Among all five fractions, 914 N-terminal peptides from 806 proteins were identified, of which 812 peptides (730 proteins) carried an N-terminal acetylation. For 644 out of 730 proteins, N-terminal protein acetylation is a reported modification in the UniProt database (supplemental Table S6A–S6C). Considering at least the presence of an acetylated N terminus in two of three replicates, multiphasic LC chip fractionation led to the identification of 58 novel acetylated N-terminal human proteins. Because three-quarters of the acetylated protein N termini were identified in the desalting step, the multiphasic LC separation workflow functions indirectly as a facile enrichment strategy for these peptides. It is also noteworthy that the cyclization of N-terminal glutamine to form pyroglutamate (PyroGlu) was also commonly found in the desalting fraction, where almost 60% of all PyroGlu modified peptides were detected (456 peptides). Thirty-four percent of all identified formylated peptides were also detected in the desalting fraction. Thus, the desalting fraction consists of many modified peptide species that are commonly lost in off-line SCX fractionation experiments.

Multiphasic Chip Fractionation for Targeted Proteomics of Human Plasma—Based on data derived from the multiphasic LC chip analysis of undepleted human plasma (Fig. 2A) we selected peptides that were exclusively detected in each salt fraction to design methods for targeted detection using scheduled MRM-high resolution (MRM-HR) MS, otherwise known as parallel reaction monitoring (39–41). In total, 503 peptides representing 173 plasma proteins were targeted with
We generated replicate data sets using multiphase LC-MRM-HR MS and made quantitative comparisons with peptides detected using conventional reversed-phase-MRM-HR MS of the same sample load. In both methods the majority of targeted peptides (>80% in both methods) showed CVs less than 6% (Fig. 4A). In the multiphase fractionation approach all 503 targeted peptides were quantifiable with a median CV of 3.4 ± 3%, with only 10 peptides showing CVs > 15%. This was a superior result compared with conventional RP-MRM-HR acquisition where only 418 of 503 peptides (83%) were quantifiable, with 85 peptides below the LLOQ (S/N 10:1). Moreover, the median peak area was 70% higher with the multiphase LC approach, with approximately one-third of peptides showing mean increases exceeding 10-fold and 39% of all peptides having greater than fivefold increases in peak area (Fig. 4B). Interestingly, we noted a gradient in peak area change with the greatest change observed for peptides eluting in the first 10 min with decreasing peak area changes for later eluting peptides. We examined peptide peak shape for potential overloading in the RP MRM-HR MS experiment but found no evidence of this. Because the same RP material was used in the RP trap chips as well as in the multiphase chips, we suggest the improved binding of hydrophilic mole-
Online Peptide Fractionation Using Multiphasic chipLC

**Discussion**

Automated online peptide fractionation using a multiphasic LC chip incorporating SCX with RP chromatography has been shown to be reproducible and robust for discovery and targeted proteomic workflows and provides superior quantitative performance compared with unfractionated analyses. Furthermore, the advantages of online SCX fractionation over off-line SCX fractionation have recently been shown (10, 43). Magdeldin et al. showed greater peptide recovery and therefore higher protein and peptide identification (5.3%) by comparing 10-step online SCX fractionation with 10-step off-line fractionation each followed by 2 h LC-MS/MS analysis (>20 h MS duty cycle per sample) of 100 μg HEK293 cell lysate in label-free and labeled quantitation applications (10). However, in their report, both the online and the off-line fractionations showed relative high variability in protein and peptide identification. In contrast, in its presented configuration, our multiphasic LC chip workflow is considerably short (8 h per sample, including sample/salt injections and column equilibration steps), completely hands-free and requires only low sample amounts (5 μg), but generates relatively deep proteome coverage with few differences in replicate peptide identifications (supplemental Fig. S5). Therefore, we envisage the multiphasic LC chip will be beneficial for various clinical research studies and other important life-science investigations where valuable specimens are only available in low amounts.
Online Peptide Fractionation Using Multiphasic chipLC

Fig. 5. Accuracy and reproducibility analysis of multiphase LC chip spectral library generation coupled with RP-SWATH acquisition. A, Overlay of RP-SWATH-MS acquisitions for three replicates of SW1736, TCP1 and NThy - ori 3–1. Box plots of log transformed protein peak areas matched against spectral libraries generated by either three individual five-step multiphase LC-MS/MS acquisition of the three cell lines in B, or by five-step multiphase LC-MS/MS acquisition using a pool of the three cell lines in C. D, analyses of transition, peptide, and protein CV distribution of used for SWATH quantitation matching each cell line technical triplicate against a spectral library generated from 5 μg five-step multiphase LC chip separated SW1736 lysate. E, Venn diagram of quantified proteins among three technical replicates of RP-SWATH MS of SW1736, TCP1 and NThy - ori 3–1 matched against spectral libraries generated either by three individual five-step multiphase LC-MS/MS acquisition of the three cell lines or by five-step multiphase LC-MS/MS acquisition of a pool of the three cell lines.
Clearly, depending on the sample type, objective, and desired run time the fractionation method we describe can be readily modified to suit user preferences. For example, separation of weak SCX binding peptides with biological importance, such as acetylated protein N termini, from canonical tryptic and/or under-cleaved peptides with multiple positively charged residues could be obtained by analysis of the desalting fraction and a high salt-step (1500 mM) injection only. Another application might be a background reduction by rapid desalting and eventual low ionic strength salt step prior to a 50 mM salt elution, for example, to target a specific biomarker peptide. This approach would, compared with a conventional RP experiment, consume slightly longer analysis time but would benefit from increased sensitivity because of reduced ion suppression.

It is instructive to compare our optimized multiphase LC fractionation workflow with common alternative approaches. Mass based fractionation using GeLC-MS commonly uses 10–16 gel bands per sample, or offline SCX fractionation commonly collects 12–20 fractions, meaning the total analysis time of one sample can easily exceed 24 h MS duty cycle. In this sense, Kulak et al., presented an in-STAGETip fractionation approach combining SCX, SAX or a reverse phase sulfonate with C18 RP (44). The in-STAGETip prefractionation (6 fractions SCX and SAX, two fractions reverse phase sulfonate) was followed by 4 h LC-MS/MS per fraction. The proteome coverage achieved by this method was prodigious but given it took more than 24 h MS time to analyze a single sample this workflow might be considered prohibitive for high-throughput biology that requires quantitative comparisons of many samples. A microfluidic approach related to our multiphase LC chip system has been described by Fortier et al. (23). Their two-dimensional nanoLC approach used SCX only guard columns that were either in-line with an analytical column (sample loading and salt step injection) or taken off the flow path for analytical peptide separation. This configuration however made it necessary to use a dual LC pump system, one for nanoLC gradient delivery and one for sample or salt injection. The proteome reactor microfluidic devices also deploy SCX material with the primary purpose of retaining enzyme and protein prior to digestion, and peptides following digestion, before elution in a single step (26). Zhou et al. (27); however, showed sequential pH based fractionation using a proteome reactor with either SCX or SAX beads to improve the detection of lower abundance proteins. Although the on chip sample processing in the proteome reactor may offer improvements over batch-mode sample preparation, this device is only partly online because protein mixture injection, reduction, alkylation, and digestion were performed using a pressure chamber before placing the reactor in-line with an analytical RP column that required additional liquid junction connections to be made. A performance comparison in terms of protein identification of our multiphase LC chip system with the discussed microfluidic devices is not feasible because the mass spectrometers used in these previous studies were all low resolution MS instruments, and instrument sensitivity has advanced significantly in proceeding years.

The multiphase LC chip MRM-HR MS application showing superior quantitation over conventional 1D C18 RP MRM-HR MS using same peptide loads and similar analysis time is one of the more striking benefits of multiphase LC chip fractionation. The precise peptide retention characteristics of the two stationary phases led to minimal peptide reoccurrence in multiple fractions, increased signal intensity and enabled targeted quantitation of more than 170 plasma proteins with more than 500 peptides that were exclusively present in specified salt elution fractions of undepleted human plasma. By spreading the elution of analytes into specific fractions this decreased ionization competition, increased transition peak areas and improved LLOQ of targeted peptides compared with conventional experiments. The improvement was especially significant for short hydrophilic peptides, where in several cases signal intensity increased by more than 100%, presumably because of improved RP retention in the presence of organic salt (42).

Data independent MS acquisition experiments are highly topical in modern proteomics (3, 45–48). For in-depth sample characterization and quantitation it is currently a necessity to use reference spectral libraries from peptides that have been identified in previous data dependent LC-MS/MS analyses. Numerous sample fractionation approaches can be used to generate comprehensive spectral libraries or enrich for certain targets to enable detection and therefore subsequent quantitation. Lambert et al. showed immunoprecipitation (IP) coupled with SWATH MS as a tool to investigate the interactome of a bait protein, however, excessive data normalization was necessary to account for LC retention time drifts and the presence and absence of IP contaminants as a nature of those experiments. The approach we chose was a five-step multiphase LC chip analysis of thyroid cell lines for spectral library generation, then a 1D RP-SWATH MS for data independent quantitation. A related approach was described by Zi et al., where they used SDS-PAGE fractionation for spectral library generation and mapped it after retention time correction against 1D RP-SWATH MS (47). Our multiphase chip LC approach utilized this workflow as the current SWATH MS identification software does not support fractionated sample analysis. Nonetheless, the approach of matching 1D RP SWATH MS against a multiphase LC trap generated spectral library proved highly feasible, with ~85% of all proteins detected with no loss of reproducibility, nor increase in bias (mean SRM-MS versus RP-SWATH MS correlation of 0.88).

In conclusion, we showed the advantages of automated multiphase LC chip fractionation for both discovery and targeted proteomic workflows to provide high reproducibility, robustness and precision that is essential for comparative biology. The “plug-and-play” LC chip format enables fast
workflow switching for seamless transition from discovery to targeted studies and provides a way to help standardize cross-laboratory proteomic analyses.

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