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Carrier-Assisted Single-Tube Processing Approach for Targeted Proteomics Analysis of Low Numbers of Mammalian Cells

Pengfei Zhang^{†,‡}, Matthew J. Gaffrey[‡], Ying Zhu[§], William B. Chrisler[‡], Thomas L. Fillmore[§], Lian Yi[‡], Carrie D. Nicora[‡], Tong Zhang[‡], Huanming Wu[‡], Jon Jacobs[‡], Keqi Tang[‡], Jacob Kagan^{||}, Sudhir Srivastava^{||}, Karin D. Rodland[‡], Wei-Jun Qian[‡], Richard D. Smith[‡], Tao Liu[‡], H. Steven Wiley[§], and Tujin Shi^{*,‡}

[†]Key Laboratory of Cancer Proteomics of Chinese Ministry of Health, Xiangya Hospital, Central South University, Changsha, Hunan 410008, People's Republic of China

[‡]Biological Sciences Division, Pacific Northwest National Laboratory, Richland, Washington 99354, United States

[§]Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory, Richland, Washington 99354, United States

^{||}Cancer Biomarkers Research Group, Division of Cancer Prevention, National Cancer Institute, Bethesda, Maryland 20892, United States

Abstract

Heterogeneity in composition is inherent in all cell populations, even those containing a single cell type. Single-cell proteomics characterization of cell heterogeneity is currently achieved by antibody-based technologies, which are limited by the availability of high-quality antibodies. Herein we report a simple, easily implemented, mass spectrometry (MS)- based targeted proteomics approach, termed cLC-SRM (carrier-assisted liquid chromatography coupled to selected reaction monitoring), for reliable multiplexed quantification of proteins in low numbers of mammalian cells. We combine a new single-tube digestion protocol to process low numbers of cells with minimal loss together with sensitive LC-SRM for protein quantification. This single-tube protocol builds upon trifluoroethanol digestion and further minimizes sample losses by tube pretreatment and the addition of carrier proteins. We also optimized the denaturing temperature and trypsin concentration to significantly improve digestion efficiency. cLC-SRM was demonstrated to have sufficient sensitivity for reproducible detection of most epidermal growth

*Corresponding Author Phone: 509-371-6579. tujin.shi@pnnl.gov.

Supporting Information

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SRM assays for EGFR pathway proteins, copy numbers of EGFR pathway proteins in a panel of cell lines, quantification of EGFR pathway proteins in small numbers of MCF cell equivalents, evaluation of processing reproducibility of cLC-SRM, and EGFR pathway protein quantification in FACS-isolated 10 and 100 MCF7 cells (XLSX)

Full scan analysis of digestion of different amounts of BSA, LC-MS/MS analysis of MCF7 lysate digests, XICs and calibration curve for PEBP1, H/K/NRAS, K/NRAS, NRAS, GRB2, MAP2K2, PTPN11, ADAM17, CBL, and AKT1, XICs for pathway protein quantification in FACS-isolated 10 and 100 MCF7 cells, selected EGFR pathway proteins for optimization of the TFE digestion protocol and generation of calibration curves, SRM S/N ratio of target peptides in 5–200 MCF7 cell equivalents and intact 10 and 100 MCF7 cells, and supplemental methods (PDF)

The authors declare no competing financial interest.

The Skyline-processed SRM results reported in this study can be accessed at Panorama (<https://panoramaweb.org/gTHtkA.url>).

factor receptor (EGFR) pathway proteins expressed at levels 30 000 and 3000 copies per cell for 10 and 100 mammalian cells, respectively. Thus, cLC-SRM enables reliable quantification of low to moderately abundant proteins in less than 100 cells and could be broadly useful for multiplexed quantification of important proteins in small subpopulations of cells or in size-limited clinical samples. Further improvements of this method could eventually enable targeted single-cell proteomics when combined with either SRM or other emerging ultrasensitive MS detection.

Graphical Abstract

Cell heterogeneity (i.e., single-cell variability) is a characteristic feature of all living organisms and inherent in cellular processes.¹⁻⁴ Multiomics technologies that enable comprehensive characterization of a broad spectrum of subpopulations of tumor cells at single-cell resolution are crucial to achieve a complete picture of tumor heterogeneity at both the genotype and phenotype levels.⁵⁻⁹ Furthermore, clinically important specimens are often available only in low amounts (e.g., 10 tumor cells from needle biopsies), which require using advanced ultrasensitive single-cell platforms for their effective analysis.⁷⁻⁹ Recent advances in nucleic acid sequencing technologies allow for deep characterization of

genomes and transcriptomes of single cells.^{10,11} However, single-cell proteomics technologies are lagging far behind.^{3,6,12-14} Currently, single-cell proteomics measurements exclusively rely on antibody-based targeted immunoassays.^{3,15} This approach has inherent limitations, such as low multiplexing capability and the need to generate high-specificity antibodies, especially for protein mutations and posttranslational modifications.

Mass spectrometry (MS)-based targeted proteomics is highly promising for single-cell proteomics analysis because it is antibody-free and has high multiplexing capability, as well as very high specificity and quantitation precision/accuracy.¹⁶ With recent advances in separations and MS instrumentation, the most sensitive MS platforms can detect peptides at ~10–100 zmol (i.e., 6000–60 000 molecules), and potentially much lower,¹⁷⁻¹⁹ thus enabling the analysis of subnanogram amounts of peptide mixtures.²⁰⁻²⁶ In theory, such sensitivity is sufficient to quantify ~25–55% of the whole proteome of a single mammalian cell (i.e., ~4000–8500 proteins out of ~15 000 proteins in a single HeLa cell²⁷) assuming 100% sample recovery during sample processing and high-efficiency ion generation and transmission to the MS. However, there is an unmet technical challenge in sample preparation for effective processing of single mammalian cells for MS analysis.

Single-cell MS has been implemented for proteomic analysis of very large single cells,²⁸⁻³³ such as early stage embryo cells or oocytes that are ~100–1000 μm in diameter and contain ~0.1–100 μg of proteins per cell.³⁴ It is a much greater challenge to apply these techniques to typical single mammalian cells as most are ~10–100-fold smaller in diameter with ~103–106-fold less protein content (i.e., ~10 μm in diameter and ~100 pg per cell). Recent progress in mass-limited sample processing (e.g., single-tube preparation and online processing)^{35,36} has been reported for effective processing of hundreds and thousands of mammalian cells (i.e., 10–1000 ng of total protein) with identification of ~1000–3000^{20,36} and ~3000–4000 proteins,^{22,32,37-39} respectively. Nevertheless, when sample size becomes smaller (close to single-cell levels), there are increasingly significant losses through contact-surface adsorption, especially with sample processing volumes in the range of 10–100 μL . To significantly minimize surface loss, our group has made significant progress in the development of a nanoPOTS (nanodroplet processing in one pot for trace samples) platform with downscaling of the processing volume to <200 nL.³⁶ When combined with ultrasensitive liquid chromatography–mass spectrometry (LC–MS), nanoPOTS has recently allowed us to identify ~670 and ~1500–3000 proteins from single and ~10–140 mammalian cells, respectively.^{36,40} However, at its current stage of development nanoPOTS is not easily adoptable by other MS-based proteomics laboratories.

To alleviate this situation, we have been exploring alternative more translatable approaches, including the use of exogenous carrier proteins to minimize sample loss, to provide more easily implementable approaches for MS-based proteomics. For example, we have recently developed a facile targeted mass spectrometric approach, termed cPRISM-SRM (carrier-assisted high-pressure, high-resolution separations with intelligent selection and multiplexing coupled to selected reaction monitoring), for enabling proteomic analysis of low numbers of cells.⁴¹ For cPRISM-SRM, we used bovine serum albumin (BSA) (~50 μg) as a carrier to minimize losses in conjunction with highly sensitive PRISM-SRM and found that we could detect low-abundance proteins in as few as 100 mammalian cells as well as

high- to moderate-abundance proteins in single mammalian cell equivalents. This is ~3–4 orders of magnitude lower than the cell number required for current targeted MS methods (typically $\sim 10^5$ – 10^6 cells).^{42,43} However, a key drawback of cPRISM-SRM is its moderate sample throughput because of the need of high-resolution PRISM to reduce the increased dynamic concentration range originating from the addition of the carrier protein.

In this work we have developed an alternative simple and effective targeted proteomics approach, termed cLC-SRM (carrier-assisted liquid chromatography coupled to selected reaction monitoring) that removes the need for extensive sample fractionation, yet is suitable for the multiplexed quantification of proteins from very low numbers of mammalian cells. cLC-SRM utilizes (1) full processing of small numbers of cells in a single tube, (2) low amounts of BSA protein and high amounts of trypsin as an effective carrier combined with tube pretreatment to minimize sample loss, and (3) highly sensitive SRM for protein quantification. cLC-SRM was demonstrated to enable multiplexed detection of target proteins expressed at 30 000 copies per cell in 10 MCF7 cells and at 3000 copies per cell (e.g., SOS1) in 100 MCF7 cells.

EXPERIMENTAL SECTION

Reagents.

Tetrafluoroethylene (TFE), dithiothreitol (DTT), iodoacetamide (IAA), ammonium bicarbonate, formic acid, and BSA were obtained from Sigma-Aldrich (St. Louis, MO). Affymetrix/USB trypsin enzyme was purchased from Thermo Fisher Scientific (Rockford, IL). Synthetic crude heavy peptides labeled with $^{13}\text{C}/^{15}\text{N}$ on the C-terminal arginine or lysine and pure light peptides with >95% purity were purchased from Thermo Fisher Scientific (San Jose, CA).

Cell Culture.

The MCF7 breast cancer cell line was obtained from the American Type Culture Collection (Manassas, VA) and was grown as previously described.⁴⁴ Briefly, MCF7 cells were maintained in 15 cm dishes in ATCC-formulated Eagle's minimum essential medium (Thermo Fisher Scientific) supplemented with 0.01 mg/mL human recombinant insulin and a final concentration of 10% fetal bovine serum (Thermo Fisher Scientific) and 1% penicillin/streptomycin (Thermo Fisher Scientific). Cells were grown at 37 °C with 95% O₂ and 5% CO₂. Cells were seeded into 15 cm culture plates and grown until near confluence.

MCF7 Cell Lysates.

MCF7 cells were rinsed twice with ice-cold PBS and harvested in 1 mL of ice-cold PBS containing 1% phosphatase inhibitor cocktail (Pierce, Rockford, IL) and 10 mM NaF (Sigma-Aldrich, St. Louis, MO). Cells were centrifuged at 1500 rpm for 10 min at 4 °C, and excess PBS was carefully aspirated from cell pellet. Cell pellets were resuspended in ice-cold cell lysis buffer (250 mM HEPES, 8 M urea, 150 mM NaCl, 1% Triton X-100, pH 6.0) at a ratio of ~3:1 lysis buffer to cell pellet. Cell lysates were centrifuged at 14 000 rpm at 4 °C for 10 min, and soluble protein fraction was retained. Protein concentrations were determined by the BCA assay (Pierce, Rockford, IL).

RT-PCR Tube Pretreatment.

Reverse transcription polymerase chain reaction (RT-PCR) tubes (GeneMte) with 0.2 mL of volume were coated with *Shewanella oneidensis* lysate digests. The selection of cell lysate digests rather than cell lysates or standard proteins for coating the tube is because the absorption amount of proteins on the tube surface is hard to determine and the large amount absorption may have severe effects on sample digestion and SRM detection. The tubes were pretreated by the addition of 100 μL of *S. oneidensis* digests at a concentration of 0.2 $\mu\text{g}/\mu\text{L}$ with incubation for overnight at room temperature. The pretreatment solution was removed, and the RT-PCR tube was rinsed three times with HPLC-grade water, and then air-dried in the fume hood. The dried RT-PCR tube was stored until further use. The coated nonmammalian peptides may fall off from the RT-PCR tube wall but should not affect SRM-based targeted quantification of mammalian peptides in terms of sensitivity and accuracy.

MCF7 Cell Equivalents for Optimization of Trypsin Amount and Denaturing Temperature.

A stock of 1 $\mu\text{g}/\mu\text{L}$ of bulk MCF7 cell lysate was diluted to 10 ng/ μL (equivalent to ~100 cells/ μL) with ~10 ng/ μL BSA in 25 mM NH_4HCO_3 . An amount of 1 μL of 10 ng/ μL MCF7 cell lysates was added to the *S. oneidensis*-coated RT-PCR tube with the addition of 100 fmol of crude heavy peptide standards to optimize the single-tube TFE digestion method. To evaluate the impact of trypsin concentration on digestion efficiency, 10 ng of MCF7 cell lysates was digested at different trypsin concentrations (1, 2.5, 5, 10, 15, 20 ng/ μL) with two processing replicates per each concentration. Furthermore, 10 ng MCF7 cell lysates were also used to evaluate digestion efficiency for three different denaturing temperatures (60 $^{\circ}\text{C}$, 75 $^{\circ}\text{C}$, 90 $^{\circ}\text{C}$) with two processing replicates per each temperature.

MCF7 Cell Equivalents for Evaluation of Platform Performance.

A stock of 1 $\mu\text{g}/\mu\text{L}$ of bulk MCF7 cell lysates was diluted to different protein concentrations (0.5, 1, 2, 5, 10, 20 ng/ μL equivalent to 5, 10, 20, 50, 100, 200 cells/ μL) with ~10 ng/ μL BSA in 25 mM NH_4HCO_3 . The sample dilution was performed in the *S. oneidensis*-coated RT-PCR tubes. From each point, 1 μL was transferred to the new *S. oneidensis*-coated RT-PCR tube with the addition of 30 fmol of crude heavy peptide standards. To evaluate the processing reproducibility, three processing replicates were performed with each containing 5 ng of MCF7 cell lysates (equivalent to 50 cells) and 30 fmol of crude peptide standards.

Intact MCF7 Cells Isolated by Fluorescence-Activated Cell Sorting (FACS).

The cultured MCF7 cells were washed with PBS for one time and detached using 0.25% trypsin (Gibco, Grand Island, NY). To avoid cell clumping, after detaching they were dispersed into a single-cell suspension by passing three times through a 25 gauge needle. The cells were suspended in PBS, and then pelleted by centrifuging 5 min at 500g. This process was repeated for five times to remove the remaining PBS and trypsin. After that the cells were resuspended in PBS and passed through a 35 μm mesh cap (BD Biosciences, Canaan, CT) to remove large aggregates. A BD Influx flow cytometer (BD Biosciences, San Jose, CA) was used to deposit cells into the precoated RT-PCR tubes. Alignment into a Hard-Shell 96-well PCR plate (Bio-Rad, Hercules, CA) was done using fluorescent beads (Spherotech, Lake Forest, IL) after which the coated RT-PCR tubes are placed into the plates

for cell collection. For unstained MCF7 cells, forward and side scatter detectors were used for cell identification. Once sorting gates were established, cells were sorted into the RT-PCR tubes using the 1-drop single sort mode. After isolation of the desired number of cells into the RT-PCR tube, the isolated cells were immediately centrifuged at 1000g for 10 min at 4 °C to keep the cells at the bottom of the tube to avoid potential cell loss. The RT-PCR tubes with the isolated cells were stored in a –80 °C freezer until further analysis.

Optimized Single-Tube TFE Digestion Protocol.

For MCF7 cell lysates, starting with 1 $\mu\text{g}/\mu\text{L}$ stock, different concentrations were generated by serial dilution with 10 $\text{ng}/\mu\text{L}$ BSA in 25 mM NH_4HCO_3 . A pipette at the volume range of 0.2–2 μL was used to accurately deliver the desired low volume smaller than 2 μL with pipetting up and down for three times. First, 1 μL of MCF7 cell lysates at the desired concentration was added to the coated RT-PCR tube together with 4 μL of 25 mM NH_4HCO_3 and 0.3 μL of 100 fmol/ μL heavy peptide standards with centrifugation at 5000 rpm for 3 min. Amounts of 9 μL of 100% TFE and 0.6 μL of 50 mM DTT were then added with a final 60% TFE and 2 mM DTT. Samples were centrifuged at 5000 rpm for 5 min, and then gently mixed at 850 rpm for 3 min. The sample were then incubated at 90 °C for 1 h for protein denaturation using the RT-PCR thermocycler (Thermo Fisher Scientific) with the heated lid option followed by cooling to room temperature with centrifugation at 5000 rpm for 3 min. A total of 0.5 μL of 60 mM IAA was added to the RT-PCR tube for a final concentration of ~2 mM. The sample was then incubated in the dark at room temperature for 30 min with gentle shaking at 850 rpm. Prior to the addition of trypsin, the sample was concentrated to a volume of ~4 μL to reduce TFE volume for digestion using a SpeedVac concentrator. An amount of 9 μL of 25 mM NH_4HCO_3 was then added together with 2.25 μL of 100 $\text{ng}/\mu\text{L}$ USB trypsin for a final concentration of ~15 $\text{ng}/\mu\text{L}$. The sample was gently mixed at 850 rpm for 3 min, and then incubated for overnight (~16 h) at 37 °C for digestion. After trypsin digestion 0.5 μL of 5% FA was added to stop the enzymatic reaction. The final sample was centrifuged at 14 000g for 1 h.

For FACS-isolated intact MCF7 cells, the only change was the first step in which 4 μL of 25 mM NH_4HCO_3 , 1 μL of 10 $\text{ng}/\mu\text{L}$ BSA in 25 mM NH_4HCO_3 , and 0.3 μL of 100 fmol/ μL crude heavy peptide standards (total 30 fmol) were added to the sample tube. Amounts of 9 μL of 100% TFE and 0.6 μL of 50 mM DTT were then added with the final 60% TFE and 2 mM DTT. The sample was centrifuged at 5000 rpm for 5 min, and then gently mixed at 850 rpm for 3 min. The sample was sonicated for 1 min, and then placed on ice for 1 min for a total of five cycles.

LC–SRM Analysis.

The selection of surrogate peptides for epidermal growth factor receptor (EGFR) pathway proteins and the SRM assays were described previously.^{42,45} SRM assays for EGFR pathway proteins and the transitions selected are summarized in Tables S1 and S2. High-purity light peptides (>95%) were used to calibrate crude heavy peptide concentrations. The determined copy numbers per cell for EGFR pathway proteins across different human cell lines are listed in Table S3.

Samples were analyzed using a nanoACQUITY UPLC (Waters Corporation, Milford, MA) coupled to a TSQ Vantage triple quadrupole mass spectrometer (Thermo Scientific, San Jose, CA). The UPLC's nanoACQUITY UPLC BEH 1.7 μm C18 column (75 μm i.d. $\times$ 20 cm) was connected to a chemically etched 20 μm i.d. fused-silica electrospray emitter via a stainless metal union. Solvents used were 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in 90% acetonitrile (mobile phase B). An amount of ~ 4 μL out of the total ~ 15 μL peptide sample was directly loaded onto the BEH C18 column from the RT-PCR tube without using a trapping column. Sample loading and separation were performed at a flow rate of 350 and 300 nL/min, respectively. The binary LC gradient was used: 5–20% B in 26 min, 20–25% B in 10 min, 25–40% B in 8 min, 40–95% B in 1 min and at 95% B for 7 min for a total of 52 min, and the analytical column was re-equilibrated at 99.5% A for 8 min. The TSQ Vantage mass spectrometer was operated with ion spray voltages of 2400 ± 100 V, a capillary offset voltage of 35 V, a skimmer offset voltage of -5 V, and a capillary inlet temperature of 220 $^{\circ}\text{C}$. The tube lens voltages were obtained from automatic tuning and calibration without further optimization. The retention time scheduled SRM mode was applied for SRM data collection with the scan window of 6 min. The cycle time was set to 1 s, and the dwell time for each transition was automatically adjusted depending on the number of transitions scanned at different retention time windows. A minimal dwell time 10 ms was used for each SRM transition. All the EGFR pathway proteins were simultaneously monitored in a single LC–SRM analysis.

Data Analysis.

Skyline software was used for all SRM data analysis.⁴⁶ The raw data were initially imported into Skyline software for visualization of chromatograms of target peptides to determine the detectability of target peptides. For each peptide the best transition without matrix interference was used for precise quantification. Two criteria were used to determine the peak detection and integration: (1) same retention time and (2) approximately the same relative SRM peak intensity ratios across multiple transitions between endogenous (light) peptide and heavy peptide internal standards. Standard deviation (SD) and coefficient of variation (CV) were obtained from multiple technical or biological replicates. All the data were manually inspected to ensure correct peak detection and accurate integration. The signal-to-noise ratio (S/N) was calculated by the peak apex intensity over the highest background noise within a retention time region of ± 15 s for the target peptide. The limit of detection (LOD) and the limit of quantification (LOQ) were defined as the lowest concentration points of each target protein at which the S/N ratio of surrogate peptides was at least 3 and 7, respectively. For conservatively determining the LOQ values, in addition to the requirement of S/N ≥ 7 , one additional criteria was applied: surrogate peptide response over different cell numbers must be within the linear dynamic range. All calibration curves were plotted using Microsoft Excel 2007. The RAW data from TSQ Vantage were loaded into Skyline software to display graphs of extracted ion chromatograms (XICs) of multiple transitions of target proteins monitored.

RESULTS

Effective sample processing is one of the major bottlenecks for the MS-based proteomics analysis of low amounts of proteins. To address this issue we have developed a carrier-assisted single-tube TFE digestion method for effective processing of small numbers of cells with minimal sample loss followed by sensitive LC–SRM analysis, termed cLC–SRM (Figure 1A). Prior to cell collection, the RT-PCR tube was pretreated with *S. oneidensis* lysate digests to reduce surface absorption. An amount of 10 ng of BSA (equivalent to ~100 mammalian cells in protein mass) was then added to the coated RT-PCR tube to further prevent adsorption loss, especially when using few cells (e.g., 10 cells). The BSA amount was determined by evaluation of BSA peptide abundance and hydrophobicity distribution across the LC elution profile from tryptic digestion of different amounts of BSA (i.e., 10, 50, 500 ng). Full MS scan analysis indicated that 10 ng of BSA digest generates sufficient peptide peaks across the entire LC profile without potential severe ion suppression (Figure S1). The TFE digestion method was selected because it has been routinely used for digestion of low microgram amounts of proteins.^{47,48} Importantly, it can be performed in a single tube without the need of sample transfer and cleanup to avoid undesired sample loss. EGFR pathway proteins were selected for targeted quantification due to the availability of heavy peptide internal standards with established SRM assays (Tables S1 and S2), as well as the known protein copy number per cell from our recent study (Table S3).⁴³ We thus extensively optimized the single-tube TFE digestion method for effective processing of small numbers of mammalian cells. Denaturing temperature and trypsin amount were found to be two key parameters that most affected digestion efficiency of low protein amounts, whereas the other parameters (e.g., the TFE volume and the concentrations of reducing and alkylation reagents) had negligible effects.

Optimization of Protein Digestion.

Starting with the standard TFE digestion protocol and using a ratio of trypsin to protein (w/w) of 1:50, 10 ng of MCF7 cell lysates was processed and digested in the coated RT-PCR tube with minimal sample dilution (total volume of ~15 μL) (Figure 1B). Following LC–SRM analysis, no SRM signal was detected, even for the most abundant PEBP1 protein. We attributed this to the very low enzyme and protein concentrations (~13 $\text{pg}/\mu\text{L}$ for trypsin and ~667 $\text{pg}/\mu\text{L}$ protein), which would result in a relatively low digestion efficiency as compared to standard solution digestion protocols (10 $\text{ng}/\mu\text{L}$ for trypsin and 1 $\mu\text{g}/\mu\text{L}$ protein).^{49,50} Because of the limited protein concentrations that can be obtained from small numbers of mammalian cells, we felt it necessary to compensate by significantly increasing the trypsin concentration.

A series of trypsin concentrations at 1–20 $\text{ng}/\mu\text{L}$ were evaluated using 10 ng of MCF7 cell lysates. Four surrogate peptides derived from three high-abundance proteins were measured by LC–SRM (Table S4A). As trypsin concentrations increased from 1 to 10 $\text{ng}/\mu\text{L}$, the abundance of all endogenous peptides (i.e., the L/H peak area ratio) increased significantly by at least 6-fold. Increasing trypsin concentration beyond 15 $\text{ng}/\mu\text{L}$ produced variable results and no further significant increase in L/H ratio for any of the tested peptides (Figure 2A). Therefore, trypsin concentration at 15 $\text{ng}/\mu\text{L}$ was selected for the single-tube TFE

digestion method. The decreased abundance for peptide LVVVGAGGVGK at 20 ng/ μ L suggested that ion suppression was occurring, which was supported by global proteomics analysis (Figure S2). Global profiling of 10 ng of MCF7 cell lysate digests at different trypsin concentrations showed that a majority of the highly abundant peptides originated from trypsin autodigestion (Figure S2A). Importantly, elevated trypsin levels also appeared to act as an effective carrier to reduce absorption loss at both the protein and peptide levels because of the total amount of trypsin and its corresponding peptides. When compared to 10 ng of BSA carrier, the total amount of trypsin was ~225 ng (i.e., ~15 ng/ μ L with a total sample volume of ~15 μ L).

Optimizing Denaturing Temperature.

Another important factor to affect digestion efficiency is protein denaturation. For standard TFE digestion protocols, proteins are incubated at 60 °C.^{47,48} However, 60 °C did not appear to be adequate for denaturing the low amounts of proteins in our samples (Figure 2B). In an attempt to further improve trypsin digestion efficiency, three denaturing temperatures (60 °C, 75 °C, 90 °C) were evaluated at a trypsin concentration of 15 ng/ μ L. Protein denaturation was performed in a PCR thermocycler with a heated lid option. Comparative analysis of the L/H area ratios for the three temperatures showed that increasing denaturing temperature improved the digestion efficiency (Figure 2B). When compared to the standard 60 °C, 90 °C improved L/H area ratios by at least 7-fold. Thus, 90 °C was selected as the optimal denaturing temperature.

Sensitivity and Reproducibility of cLC–SRM.

The performance of cLC–SRM on small amounts of cell lysates was evaluated by targeted quantification of 10 EGFR pathway proteins spanning a wide dynamic concentration range (Table S4B). We used pooled MCF7 cell lysates to eliminate variations due to stochastic sampling of small numbers of intact cells. Different amounts of MCF7 cell lysates with 0.5–20 ng of total proteins (equivalent to 5–200 cells) were processed with the optimized single-tube TFE digestion method followed by LC–SRM analysis. Multiple SRM technical replicates were performed for each data point (i.e., three replicates for 0–1 ng and two replicates for 2–20 ng) (Table S5). The obtained LODs and LOQs were used to evaluate the detection sensitivity. As shown in Figure 3A, XICs clearly shows transitions for ATADDELSFK derived from GRB2 present at ~220 000 copies per MCF7 cell. cLC–SRM was able to reproducibly quantify endogenous ATADDELSFK down to five MCF7 cell equivalents with an S/N ratio of 14 and ~1800 zmol of quantification sensitivity (Table 1 and Table S6). Furthermore, moderate-abundance PTPN11 at ~54 000 copies per cell was detected in 10 cell equivalents with an S/N ratio of 6 and ~900 zmol of detection sensitivity (Table 1 and Table S6). Sensitivity was further confirmed by confident detection of multiple endogenous peptides from other low-abundance EGFR pathway proteins in 20 MCF7 cell equivalents (e.g., FYGAEIVSALDYLHSEK derived from AKT1 at ~4000 copies per cell with S/N ratio of 5 and ~120 zmol of detection sensitivity, and SFEDLTDHPVTR derived from ADAM17 at ~10 000 copies per cell with S/N ratio of 3 and ~300 zmol of detection sensitivity) (Table 1 and Table S6). The resultant calibration curves displayed excellent linearity over a wide dynamic range of MCF7 cell equivalents (Figure 3, parts B and C, and Figures S3–S11) with LOQs of ~5–10 cells for high-abundance proteins, ~10–20 cells for

moderate-abundance proteins, and ~50–200 cells for low-abundance proteins (Table 1). All these results suggest that cLC–SRM can provide ~100–4000 zmol of detection sensitivity and ~300–7000 zmol of quantification sensitivity.

The median SRM technical CV for all target peptides across all the data points was ~9% (Table S5), consistent with the technical reproducibility of well-characterized LC–SRM with CV below 10%.^{42,45,51–54} The overall reproducibility of cLC–SRM was evaluated by targeted analysis of three processing replicates for 12 surrogate peptides derived from low- to high-abundance EGFR pathway proteins in 50 MCF7 cell equivalents (Table S7). The processing CVs ranged from 1.4% to 28.7% with the median value of 11.8%, suggesting high processing reproducibility of cLC–SRM. As expected, low- and moderate-abundance proteins have higher CVs than high-abundance proteins, which can be largely attributed to variations in SRM detection sensitivity.

Quantification of EGFR Pathway Proteins in FACS-Isolated MCF7 Cells.

With the demonstration of technique robustness using cell lysates, we next applied cLC–SRM to measure EGFR pathway proteins in 10 and 100 intact MCF7 cells isolated by FACS. Using our previously established SRM assays⁴³ (Tables S1 and S2), 32 core EGFR pathway proteins and feedback regulators with a wide dynamic concentration range (Table S3) were measured using 10 and 100 intact MCF7 cells with two biological replicates per each to evaluate practical applicability. Three technical replicates per biological replicate were performed to evaluate the overall performance of cLC–SRM for quantitative analysis of intact cells (Table S8). cLC–SRM enabled detection of high- and moderate-abundance proteins in 10 intact MCF7 cells with the median CV of 13.5% (Figures 4 and S12, and Tables 2 and S8). Even low-abundance AKT1 at ~3700 copies per cell was also detected with the average S/N ratio of 7 (Table S9), consistent with cLC–SRM measurements of MCF7 cell lysates (Figure S11). When the cell number was increased to 100, the endogenous SRM signal of AKT1 peptide was increased accordingly with the average S/N ratio of 28 (Table S9). A majority of previously identified important EGFR pathway proteins (22 out of the total 32 proteins)⁴³ were reliably detected and quantified by cLC–SRM with the median CV of 12.7%, with the exception of extremely low-abundance negative feedback regulators and known low-expression EGFR protein in MCF7 cells (Figure S12 and Table 2). These results suggests that cLC–SRM can be used to study signaling pathways in small numbers of mammalian cells. Significantly, the endogenous peptide from low-abundance SOS1, present at only ~3000 copies per cell, was detected in 100 MCF7 cells with the average S/N ratio of 9 (Table S9), suggesting ~500 zmol of absolute sensitivity of cLC–SRM. This further confirms that cLC–SRM can provide ~100–1000 zmol of detection sensitivity. We also note that the detectability of endogenous peptides shows a strong positive correlation with the total number of protein molecules (Tables 2 and S8).

DISCUSSION

We have recently demonstrated that the addition of exogenous carrier proteins allows small numbers of mammalian cells to be effectively processed using conventional sample preparation protocols with minimal sample loss.⁴¹ However, our previous approach required

high-resolution PRISM to reduce the increased sample dynamic concentration range resulting from the addition of large amounts of carrier protein, thus generating many fraction samples that significantly lowered sample throughput. To alleviate this problem, we have developed a carrier-assisted LC–SRM approach for precise multiplexed analysis of small numbers of mammalian cells. When compared to cPRISM–SRM, cLC–SRM has 3–5-fold lower sensitivity for protein quantification.⁴¹ cLC–SRM capitalizes on using optimized single-tube TFE digestion and a combination of low amounts of carrier protein and high trypsin concentration for effectively processing small numbers of cells while retaining the ability to perform sensitive SRM for protein quantification.

The optimized TFE digestion method was demonstrated to effectively prevent sample losses at both the protein and peptide levels (Figure 1). First, it utilizes relatively small volumes (~15 μL) without sample transfer and cleanup to avoid sample loss. Second, to significantly reduce surface absorption, the *S. oneidensis*-coated RT-PCR tubes were used together with small amounts of BSA (i.e., 10 ng) and relatively high amounts of trypsin (~225 ng). The digested BSA and trypsin peptides as well as heavy peptide standards help prevent sample loss at the peptide level (e.g., peptide losses during LC separation). Importantly, high concentrations of trypsin increased protein digestion efficiency when small numbers of cells were used. Formation of the enzymatic digestion complex is very rapid with the rate constant K ($K = [\text{enzyme-protein}]/[\text{enzyme}][\text{protein}]$) being the limiting factor.⁵⁵ Thus, digestion efficiency is primarily determined by protein and enzyme concentrations. For small numbers of mammalian cells (e.g., 100 cells equivalent to 10 ng) processed in a volume of ~15 μL , the protein concentration is less than 1 ng/ μL , which is ~1000-fold lower than typical for bulk cell digestion (~1 $\mu\text{g}/\mu\text{L}$).^{49,50,56} Thus, significantly increasing trypsin concentrations is required for rapid and efficient digestion. Our optimized TFE digestion protocol has a trypsin to protein ratio of ~20:1 (w/w), which is ~1000-fold higher than standard trypsin digestion protocols (i.e., ~1:50).^{42,57-59} This trypsin concentration significantly improved digestion efficiency with negligible effects on targeted MS quantification. However, we did observe that the increased trypsin amount had a disproportionate effect on data-dependent acquisition-based global proteomics profiling because the highly abundant trypsin and BSA peptides are much more likely to be selected for MS/MS sequencing (Figure S2). Thus, global proteomics measurements do not benefit from the high trypsin concentration used here for targeted proteomics. Protein denaturing temperature was also found to be another important factor affecting digestion efficiency. Because TFE is a volatile solvent with boiling point of 74 °C, at the optimal denaturing temperature (90 °C) a PCR thermocycler rather than other heating devices⁵⁰ (e.g., the Eppendorf thermomixer) was used to maintain a good cap seal so as to avoid TFE loss. In addition, prior to the addition of trypsin, it was necessary to reduce the TFE levels to ~10% for effective trypsin digestion. To avoid drying out samples with unrecoverable loss, it was necessary to frequently check the sample volume (~4 μL of the remaining volume) during SpeedVac concentrating.

In terms of overall analytical performance, cLC–SRM was found to provide high sensitivity, reproducibility, and good multiplexing capability. The median CV for processing small numbers of cells, including sample preparation, was 11.8%, which is consistent with that of ~10% obtained by LC–SRM in technical replicates.^{42,45} With peptide retention time

scheduling and the use of advanced triple quadrupole mass spectrometers, cLC–SRM should enable simultaneous quantification of up to 1000 target peptides (i.e., ~500 proteins assuming that each protein has two surrogate peptides) in a single LC–SRM analysis.

Using standard LC–MS instruments, cLC–SRM was demonstrated to enable detection of most moderate-abundance proteins at ~30 000 copies per cell in 10 MCF7 cells and even lower abundance proteins (e.g., SOS1 at ~3000 copies per cell) in 100 MCF7 cells (Figure S12 and Table S8). The previously reported low-abundance AKT1 could also be detected and quantified in 10 MCF7 cells. Furthermore, unlike immunoassays that require significant amounts of development time and rely on the availability of high-quality antibodies,^{13,15} cLC–SRM is relatively easy to implement with commercially available platforms, and should be useful for precise quantification of important proteins in small subpopulations of cells (e.g., fine-needle aspirants from minimally invasive biopsies) that cannot be readily accessed by targeted antibody-based immunoassays.

We note that cLC–SRM still cannot provide sufficient sensitivity to detect and quantify the majority of proteins in a single mammalian cell. Significantly enhancing the detection sensitivity of cLC–SRM is necessary for moving toward targeted single-cell proteomics. The LC–MS sensitivity increases almost linearly with decreasing the LC flow rate, and is thus inversely proportional to the square of the LC column inner diameter.^{60,61} When compared to ultrasensitive LC–MS platforms (1–10 zmol sensitivity with an ultralow flow rate of 10–20 nL/min),^{22,25} current cLC–SRM uses the standard nanoLC flow rate of ~300 nL/min with ~100–1000 zmol sensitivity. Therefore, further enhancing the detection sensitivity of cLC–SRM could be achieved by implementation of ultralow-flow capillary electrophoresis^{24,25} or LC^{22,23} with maintaining similar separation efficiency when integrated with a high-efficiency ion source and ion transmission interface.^{62,63} In addition, in our current LC–MS settings, the maximum loading volume is ~4 μ L with a 5 μ L loop, and only approximately one-third of the total amount of samples was used for LC–SRM analysis. Our next step is to replace the current 5 μ L loop with 20 μ L loop for loading nearly all the samples to the LC column. We anticipate that >10-fold improvement in sensitivity can be achieved (>5-fold from the ultralow-flow separation and ~2-fold from the full loop loading), which may allow cLC–SRM for quantification of proteins at 30 000 copies per cell in a single mammalian cell. Such levels of improvement could enable quantifying ~35% human proteome in a single mammalian cell (i.e., ~5200 out of ~15 000 proteins in a single HeLa cell).²⁷

CONCLUSION

We have developed a simple carrier-assisted targeted proteomics approach for enabling effective sample processing and multiplexed quantification of proteins in very low numbers of mammalian cells. Using a standard LC–MS platform it can reproducibly detect target proteins at 30 000 and >3000 copies per cell in 10 and 100 mammalian cells, respectively. Such sensitivity is sufficient for quantification of the majority of important pathway proteins in small subpopulations of cells or mass-limited clinical specimens. Furthermore, this method can be easily implemented in any MS and proteomics laboratories at no additional

cost for instrument or reagents. We anticipate that it can be broadly applied to biomedical research and systems biology with the potential of facilitating precision medicine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Schematic diagram of the cLC-SRM workflow. Small numbers of cells are collected into a RT-PCR tube precoated with *S. oneidensis* lysate digests. Exogenous proteins (i.e., ~10 ng of BSA and excessive trypsin) as well as heavy peptide standards added to the RT-PCR tube are used as a carrier to prevent sample loss. An optimized single-tube TFE digestion method is used for sample processing to generate tryptic peptides. Sensitive LC-SRM is then used for multiplexed quantification of target proteins. Skyline software is used for SRM data analysis. (B) Single-tube TFE digestion. A small number of cells are processed with minimal sample dilution in a processing volume of ~15 µL without sample transfer and cleanup. Prior to LC-SRM analysis, the cap of the RT-PCR tube is removed and the tube is then inserted into a sample vial to avoid sample transfer loss.

Figure 2.

Optimization of trypsin digestion efficiency. (A) Trypsin amount: ~10 ng of MCF7 cell lysates were processed with the standard TFE digestion protocol using different concentrations of trypsin (1, 2.5, 5, 10, 15, 20 ng/μL). (B) Denaturing temperature: ~10 ng of MCF7 cell lysates were processed at different denaturing temperatures (60, 75, 90 °C) with 15 ng/μL of trypsin. Three high-abundance proteins (PEBP1, H/K/NRAS, and NRAS) and five high-abundance proteins (PEBP1, H/K/NRAS, K/NRAS, NRAS, and GRB2) were measured to optimize the trypsin amount and the denaturing temperature, respectively. Two processing replicates were used for each condition with the addition of ~100 fmol of heavy peptide standards per replicate. Average L/H ratios were used for plotting.

Figure 3.

Sensitivity and accuracy of cLC-SRM for protein quantification in small amounts of MCF7 cell lysates. (A) Extracted ion chromatograms (XICs) of transitions monitored for ATADDELSFK derived from GRB2 at different numbers of MCF7 cell equivalents: 548.8/924.4 (blue), 548.8/853.4 (purple), 548.8/738.4 (chestnut). Calibration curves for quantifying high-abundance GRB2 (B) and moderate-abundance PTPN11 (C) with the use of the best responsive interference-free transitions, ATADDELSFK (548.8/924.4) for GRB2 and SNPGDFTLSVR (596.9/496.3) for PTPN11. Three and two SRM replicates were performed for 0–10 and 20–200 MCF7 cell equivalents, respectively.

Figure 4.

Comparison of SRM signal between FACS-isolated 10 and 100 intact MCF7 cells measured by cLC-SRM. Each sample consists of two biological replicates with the addition of ~30 fmol of heavy peptide standards per replicate. XICs of transitions monitored for ATADDELSFK derived from GRB2: 548.8/924.4 (blue), 548.8/853.4 (purple), 548.8/738.4 (chestnut); XICs of transitions monitored for FYGAEIVSALDYLHSEK derived from AKT1: 648.0/897.9 (blue), 648.0/816.4 (purple), 648.0/283.1 (chestnut); XICs of transitions monitored for LPSADVYR derived from SOS1: 460.7/807.4 (blue), 460.7/710.3 (purple), 460.7/404.2 (chestnut).

Table 1.

Limits of Detection (LODs), Limits of Quantification (LOQs), and Absolute Sensitivity of cLC-SRM for Selected High-, Moderate-, and Low-Abundance EGFR Pathway Proteins

gene	estimated protein copies per cell ^a	surrogate peptide	detection sensitivity			
			LOD (cell)	LOQ (cell)	LOD (zmol)	LOQ (zmol)
PEBP1	1 245 120	VLTPQVK	<5	<5	<2940	<2940
H/K/NRAS	227 345	LVVVGAGVGK	<5	10	<2363	4726
K/NRAS	<227 345	SYGIPHETSAK	<5	10	<1888	3776
NRAS	224 892	SFADINLYR	10	20	3736	7472
GRB2	219 449	ATADDELSFK	<5	<5	<1822	<1822
MAP2K2	76 418	ISELGAGNGGVVTK	10	10	1269	1269
PTPN11	54 124	SNPGDFTLSVR	10	20	899	1798
ADAM17	9467	SFEDLTDHPVTR	20	200	314	3146
CBL	9404	VPVSA PSSDPWTGR	100	200	1562	3124
AKT1	3683	FYGAEIVSALDYLHSEK	20	50	122	306

^aOur recent study for absolute quantification of EGFR pathway proteins (Table S3).

Table 2.

Endogenous Detectability of Core EGFR Pathway Proteins and Feedback Regulators in FACS-Isolated 10 and 100 MCF7 Cells Measured by cLC-SRM

gene	estimated protein copies per cell ^a	surrogate peptide	endogenous detectability	
			10 cells	100 cells
PEBP1	1 245 120	VLTPTQVK	yes	yes
H/K/NRAS	227 345	LVVVGAGGVGK	yes	yes
K/NRAS	<227 345	SYGIPPIETSAK	yes	yes
NRAS	224 892	SFADINLYR	yes	yes
GRB2	219 449	ATADDELSFK	yes	yes
MAP2K2	76 418	ISELGAGNGGVVTK	^b yes	yes
MAPK1	67 349	NYLLSLPHK	yes	yes
PTPN11	54 124	SNPGDFTLSVR	yes	yes
MAP2K1	47 289	ISELGAGNGGVVFK	yes	yes
PDPK1	32 098	QLLLTEGPHLYYVDPVVK	yes	yes
PIK3R2	29 065	ALGATFGPLLR	yes	yes
SHC1	27 489	EAISLVCEAVPGAK	^b yes	yes
ARAF	25 284	TQHCDPEHPPFPAPAINAPLQR	yes	yes
RASA1	15 312	LLAITELLQVK	yes	yes
RAF1	14 521	SNNIFLHEGLTVK	yes	yes
PIK3R1	13 233	TAIEAFNETIK	yes	yes
ADAM17	9467	SFEDLTDHPVTR	^b yes	yes
CBL	9404	GTEPIVVDPDPR	yes	yes
SHOC2	8615	LDSLTTLYL	^b yes	yes
AKT1	3683	FYGAEIVSALDYLHSEK	yes	yes
SOS1	3379	LPSADVYR	yes	yes
GAB1	2867	SNTISTVDLNK	yes	yes

^aOur recent study for absolute quantification of EGFR pathway proteins (Table S3).

^bEndogenous peptides can be detected but are not quantifiable.