An Integrated Platform for Isolation, Processing, and Mass Spectrometry-based Proteomic Profiling of Rare Cells in Whole Blood*

Siyang Li‡§, Brian D. Plouffe‡¶, Arseniy M. Belov‡§, Somak Ray‡, Xianzhe Wang‡§, Shashi K. Murthy‡¶, Barry L. Karger‡§, and Alexander R. Ivanov‡§

Isolation and molecular characterization of rare cells (e.g., circulating tumor and stem cells) within biological fluids and tissues has significant potential in clinical diagnostics and personalized medicine. The present work describes an integrated platform of sample procurement, preparation, and analysis for deep proteomic profiling of rare cells in blood. Microfluidic magnetophoretic isolation of target cells spiked into 1 ml of blood at the level of 1000–2000 cells/ml, followed by focused acoustics-assisted sample preparation has been coupled with one-dimensional PLOT-LC-MS methodology. The resulting zeptomole detection sensitivity enabled identification of ~4000 proteins with injection of the equivalent of only 100–200 cells per analysis. The characterization of rare cells in limited volumes of physiological fluids is shown by the isolation and quantitative proteomic profiling of first MCF-7 cells spiked into whole blood as a model system and then two CD133+ endothelial progenitor and hematopoietic cells in whole blood from volunteers. Molecular & Cellular Proteomics 14: 10.1074/mcp.M114.045724, 1672–1683, 2015.

Rare cells in blood and tissue have been shown to serve as specific indicators of disease status and progression, a source of adult stem cells, and a tool for patient stratification and monitoring. Previous reports (1–4), for example, have shown that the concentration of circulating tumor cells (CTCs) within a cancer patient’s blood can act as a therapeutic monitoring tool (1–4). Additionally, the isolation of adult stem cells provides a needed cell source for tissue engineering and regenerative medicine treatments (5, 6). Finally, separation and genomic analysis of key cell populations from patients allows for targeted treatment regimens (7, 8).

Rare cells in blood or other body fluids represent a particularly challenging problem for discovery proteomic analysis as the volume of the fluid sample is limited and the concentration of cells within that sample is very low. For a blood sample containing rare cells of interest, this low level means capturing a subpopulation of target cells with high recovery and purity from a greatly heterogeneous mixture in only one or a few ml and then performing sample preparation with minimal sample loss. Furthermore, ultra-trace LC-MS needs to be conducted with specially prepared columns with highly sensitive MS, along with advanced data processing. Key to success is the full integration of all the steps in the workflow to achieve the detection level required. The present work combines a series of innovative steps leading to successful discovery proteomic analysis of rare cells.

Consider first rare cell isolation for which several approaches have recently been developed (9, 10). A particularly powerful approach is magnet-activated cell sorting (MACS) where antibody-functionalized magnetic beads are utilized to enrich a subset of cells in a complex sample such as whole blood (10, 11). Although magnet-activated cell sorting-based and other microfluidic approaches of cell separation have recently shown the ability to isolate rare cells (e.g., <10 cells per ml of whole blood) with high levels of purity (>90%) and efficiency (>95%) (12–14), the potential of these systems in enabling downstream molecular analyses has yet to be fully realized. Microfluidic channels, in comparison to traditional magnet-activated cell sorting, allow for improved control of the magnetic field for precise focusing in the microchannels, resulting in higher efficiency, recovery, and purity of isolation.

For proteomic analysis, rare cell isolation is followed by a series of sample preparation steps, for example cell lysis and protein extraction and digestion. Several approaches such as denaturant-assisted lysis, acetone precipitation, filter-aided sample preparation, and monolithic microreactor-based techniques have been developed for processing small amounts of
sample, for example 500–1000 cultured cells (15–17). However, these methodologies only allow identification of a few hundred proteins at these levels. In this work, we describe a sample preparation approach that utilizes novel small volume focused acoustics-assisted cell lysis, followed by low volume serial reduction, proteolytic digestion and ultra-trace LC-MS analysis. Although two-dimensional separations are often used for deep proteomic analysis, limited sample analysis is best conducted by high peak capacity separation in a single dimension, eliminating potential sample losses from the second dimension. Furthermore, it is known that ultra-low mobile phase flow rates (≤20 nL/min) dramatically improve electrospray signals, as a consequence of improved ionization efficiency (18–21). In prior work, we have shown that reduction of the LC column diameter in a high resolution porous layer open tube (PLOT)1 format utilizing ultra-low flow can generate a significant gain in limited sample proteomic profiling capabilities (22). As shown in the current paper, a combination of PLOT-LC with advanced MS instrumentation and data processing can lead to zeptomole detection sensitivity and quantitation. Furthermore, the integration of all the above steps yields thousands of proteins identified and quantitated from a small number of rare cells (less than one thousand) isolated from 1 ml whole blood. The developed technology opens up the possibility of deep proteomic analysis of rare cells in body fluids.

**EXPERIMENTAL PROCEDURES**

**Reagents and Chemicals—**All reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO) at the highest purity unless otherwise stated.

**Cell Handling—**MCF-7 human breast adenocarcinoma cells (ATCC, Manassas, VA) were cultured in 75 cm² tissue culture flasks at 37 °C, 5% CO₂. MCF-7 cells were incubated in Eagle’s Minimum Essential Medium (EMEM; ATCC) supplemented with 10% fetal bovine serum, 100 μg ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin, and 0.01 mg ml⁻¹ bovine insulin. Cells were grown to preconfluence and isolated for experiments by trypsinization using a 0.25% trypsin–EDTA solution. Prior to cell isolation or cell lysis, cells were centrifuged at 200 g for 10 min at 4 °C. The cell culture medium was removed, and MCF-7 cells were washed twice with ice cold 1× Dulbecco’s phosphate-buffered saline (DPBS, Sigma-Aldrich) containing 0.5% EDTA (v/v). The cells were resuspended at a concentration of ~1000 cells/μl, and the cell numbers were counted three times using a hemacytometer and/or flow cytometer (Cell Lab Quanta SC; Beckman Coulter, Brea, CA).

**Microfluidic Device Design and Fabrication—**Microfluidic channels were fabricated as previously described,(23, 24) Wire arrays were designed using PCB123® printed-circuit board design software and ordered from Sunstone Circuits (Mulino, OR). The wire dimensions were set to provide a gap encompassing the width of the microfluidic channel; the height and width of the wires were set to 35 μm and 178 μm, respectively. Teflon-insulated 18 gauge copper wires were soldered to the ends of each of the printed circuit board arrays, and the arrays were connected to a DC power supply (Elenco Electronics XP-4, Wheeling, IL) that provided three fixed-current settings of 0.25 A, 0.50 A, and 1.00 A via standard alligator clip connectors. The PDMS channels and wire arrays were visually aligned.

**Functionalization of Magnetic Beads—**Dynabeads® MyOne™ Carboxylic Acid magnetic particles (Life Technologies, Carlsbad, CA) were modified with antibodies, either against the epithelial cell adhesion molecule (mouse anti-human EpCAM; Santa Cruz Biotechnology, Santa Cruz, CA) or against CD133 (mouse anti-human CD133, Miltenyi Biotec Inc, Auburn, CA) using standard carbodiimide chemistry (25) in ratios suggested by the reagent manufacturer (1:1 molar ratio of beads to protein; Pierce Biotechnology, Rockford, IL).

**Whole Blood Cancer Cell Isolation—Whole blood was drawn from healthy volunteers and collected in EDTA-coated Vacutainer® tubes (Becton Dickinson, Franklin Lakes, NJ). Approval from the Northeastern University Institutional Review Board was obtained for this purpose (NU IRB #: 11–07-19). The location of the interface between the injected blood and buffer was first evaluated (12, 26). A Coulter counter/flow cytometer (Cell Lab Quanta™ SC; Beckman Coulter, Brea, CA) was used to count the number of target (MCF-7) cells versus native polymorphonuclear cells that were separated. A protocol based on the distinct size difference of these two cells, was developed to identify each cell population. The cells were gated by their electronic volume and granularity, and the total number of cells within the recovered suspension was determined. Various concentrations of MCF-7 cells (500–100,000 cells) were spiked into 1 ml of whole blood. Following this, 10 μl of modified antiEpCAM magnetic microbeads was added to 1 ml of unprocessed blood and allowed to incubate for 30 min on a rotary mixer. This experiment was conducted at optimized flow rates, as described in the theory section below. For all MCF-7 experiments, the flow rate of the samples was fixed at 240 μl/min, and a center stream of 1× RBC lysis buffer (Ebioscience Inc., San Diego, CA) flowed at 160 μl/ml. Target and nontarget cells were collected in separate methanol cleaned microcentrifuge tubes.

To establish an accurate gating of MCF-7 for subsequent cell counts, we first analyzed a homogeneous suspension of approx. 100,000 MCF7 cells. To identify the MCF-7 cells in the flow cytometer, we gated the electronic volume (EV) versus side scattering (SS). We also spiked 100,000 MCF-7 cells into whole blood and ran the sample through the flow cytometer to ensure an accurate gating of the target cells. MCF-7 are distinguishably larger than the surrounding blood cells and thus are the only cells located in the gate. The CV for these calibration samples was 1.2–2.6% (n = 15). On the other hand, gating for the EPCs and HSCs could not be based on cultured homogeneous suspensions. Therefore, buffy coat samples (via Ficoll-Paque density gradient centrifugation) were used to establish standard gates. Gates were first generated with unstained samples (EV and SS and CD34+ only staining to obtain the initial gating from the histograms (CV of 7.5%). Also, kinase insert domain-containing receptor (KDR)-only (CV = 5.3%, n = 8) and CD45-only (WBC are CD45+; CV = 15.2%, n = 8) were analyzed for their subsequent histograms. These individual gates then allowed for accurate scatter plots based on three-color staining. From these studies, we can assume the CV was between 5.3 and 15.2% for the EPC and HCS cell counting.

**Isolation of Hematopoietic Stem Cells and Endothelial Progenitor Cells from Whole Blood—**To illustrate the utility of the magnetophoretic design for isolation of rare cells, we extracted hematopoietic stem cell (HSCs) and endothelial progenitor cells (EPCs) from whole blood using antiCD133 functionalized microparticles. Again, whole
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**RESULTS AND DISCUSSION**

**Experimental Platform**—Fig. 1 presents an overview of the platform. For method development, samples consisting of cultured MCF-7 cells spiked into blood at levels of 1000–100,000 MCF-7 cells in 1 ml of whole blood were utilized (Fig. 1A). The magnetophoretic isolation of these cells included incubation of the sample with magnetic beads functionalized with antibodies against EpCAM followed by isolation (12). The collected target cells were rinsed and lysed using focused ultrasonication (Covaris), digested with trypsin and analyzed using PLOT nLC coupled to a Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific) (Fig. 1C). Stable isotope labeled (SIL) peptides were added to the samples after completion of tryptic digestion for targeted quantitative analysis of selected proteins. MS data were processed to enable quantitative proteomic profiling using both label-free and isotope reference-based techniques followed by gene ontology analysis. After development and validation of the platform using MCF-7 cells spiked into whole blood, the optimized workflow was then applied to high specificity isolation from

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**Figure 1** Workflow for target isolation and proteomic profiling of limited amounts of rare cells from biological fluids. A, A model system where cultured MCF-7 cells were counted, spiked into blood, mixed with the antiEpCAM-functionalized magnetic microbeads and captured using a microfluidic magnetophoretic device. B, Blood collected from healthy donors was mixed with antiCD133-functionalized magnetic microbeads. Endothelial progenitor cells and hematopoietic stem cells with bound antiCD133 beads were isolated by the microfluidic device. C, Isolated target cells were lysed with assistance of focused ultrasonication, followed by reduction, alkylation, and enzymatic digestion performed in a single tube. Stable isotope labeled synthetic peptides were added into digested lysates prior to analysis. Aliquots of cell lysates were analyzed using SPE-nLC-PLOT MS. Acquired MS data were submitted for database searching, quantitation, and GO enrichment analysis.
the blood and proteomic characterization of the CD133+ cells from blood (Fig. 1B). A key attribute of this platform is the reduction of the minimum amount of blood necessary to study EPC and HSC and potentially other rare cell populations by at least 1–2 orders of magnitude relative to the current state of the art (27, 28). We first detail the individual steps that lead to the advanced workflow for deep proteomic analysis of rare cells in whole blood.

Ultra-low Flow (ULF) PLOT-nLC-MS/MS—A cornerstone of the platform for deep proteomic profiling of rare cells is the ultra-low flow (ULF) PLOT-nLC column technology. To capture the high sensitivity benefits of ultra-low flow, we used 4 m long 10 μm i.d. poly(styrene-divinylbenzene) (PS-DVB) PLOT columns with −1 μm thickness of the permeable layer (Fig. 2A). It has been previously shown that the PLOT columns offer excellent chromatographic efficiencies (22). The PLOT column was coupled to a monolithic trapping (microSPE) column using a zero dead volume connector. We optimized weight fractions of the PLOT polymerization mixture constituents that resulted in improved column-to-column reproducibility of the PLOT columns and increased hydrophobicity of the monolithic microSPE columns by copolymerizing PS-DVB with 1-decene. Furthermore, the coupling of the PLOT-nLC to an advanced fast duty cycle high resolution mass spectrometer (Q-Exactive) improved the sensitivity and accuracy of quantitation in comparison to our and other previous works (18–21) (see Supplemental Materials).

As a baseline set of experiments to show the potential of the PLOT column-based LC-MS platform for high sensitivity proteomic analysis, the performance of the PLOT column was assessed using a split-injection approach (29). Analyzing an equimolar mixture of digested protein standards (Michrom Bioresources, Auburn, CA, “Bovine 6 Protein Mix,” P/N PTD/00001/63, containing beta lactoglobulin, lactoperoxidase, carbonic anhydrase, glutamate dehydrogenase, alpha casein, and serum albumin) with a nontargeted data dependent data acquisition (DDA) method resulted in detection limits down to 10–50 zmol level (S/N > 5), based on single stage mass spectrometry (MS1) (Fig. 2B), and unambiguous MS/MS fragment matching (Fig. 2C). A linear MS response was recorded over a dynamic range of over four orders of magnitude, ranging from 10 zmol (S/N > 25) to 100 amol (MS1) and higher, with a linear regression $r^2 > 0.99$. The molar peptide amounts per analysis here and below were derived from the molar amounts of digested standards provided by the vendor (Michrom Bioresources). The quantities of the standard were checked by amino acid analysis and comparative LC-MS analysis of the Michrom standard and the same protein digests from other vendors.

As expected, improved levels of detection were found using a targeted approach, parallel reaction monitoring (PRM) with high-energy collisional dissociation (HCD) (30). Detection limits for tryptic peptides were determined to be less than 5 zmol in MS2 (S/N > 25), an ~fivefold increase compared with the DDA scanning mode for MS2 spectra (Fig. 2D). The HCD spectra at 5 zmol levels were still of high quality. Quantitation of targeted ions in the PRM mode was performed by integrating peak areas for the most prominent product ions (e.g. $y^+$, $b^+$, $y'_+$, $y''_+$, and $b''_+$ in the case of peptide ALVYGEATSR, (Fig. 2D)). Excellent linear correlation between sample loads and experimental signal response with $r^2 ≥ 0.99$ for a range of 5–500 zmol was obtained for both parent and fragment ions (Fig. 2D, E). Quantitation was also evaluated by spiking the same equimolar mixture of six digested bovine proteins (Michrom Bioresources, CA) into a complex background of the MCF-7 lysate. Based on monitoring of predominant parent ion–fragment ion transitions for targeted peptides using extracted ion chromatograms for parent ions and resulting fragment ions, and correspondence of their peak profiles, we were able to reliably quantify selected peptides at 50 zmol, and even 10 zmol, using one-dimensional separation of the unfractionated sample and a 40-min long gradient (Fig. 2E and Supplemental Fig. S1). As expected, noisier elution profiles and fewer reliably detectable transitions were observed at lower sample amounts.

To assess the performance of the microSPE-PLOT-nLC-MS platform in proteomic profiling of a limited number of cells, we prepared a lysate of 10 million human MCF-7 cells using conventional lysis and trypsin digestion techniques (see Supplemental Materials). Aliquots corresponding to 500 MCF-7 cells (~50 ng of total protein based on our protein concentration measurements at higher levels and accounting for dilutions, and previous reports(31)) were loaded on the microSPE column and separated using a 4 h long gradient at a flow rate of 20 nL/min. Approximately 3700 unique protein groups and 22,645 unique peptides on average were identified (DDA) in a single analysis, and a combination of five replicate PLOT-nLC-MS runs resulted in identification of 5183 proteins and 41,020 peptides (Fig. 3A). The FDR≤1% was used for all peptide-level identifications. The replicate analyses resulted in an overlap in identification results of ~80%, illustrating the high reproducibility of the analysis (Fig. 3C). The number of IDs decreased when lower amounts of cells were analyzed using the microSPE-PLOT column (Fig. 3B); nevertheless, we were still able to identify 1327 ± 143 and 2026 ± 98 protein groups from injections of 50 ± 3 and 100 ± 5 cells, respectively, ($n = 3$; Fig. 3B, D). The PLOT-nLC-based platform showed at least 4–5 fold improved profiling sensitivity in analysis of similar sample amounts if compared with other state-of-the-art LC/MS methods (15, 17, 32, 33).

Cell Lysis and Protein Digestion for Limited Samples—The above experiments were performed with a starting sample of 10 million MCF-7 cells, injecting the equivalent of the specific low number of cells. Sample preparation of low cell numbers by direct down-scaling of the conventional lysis/digestion protocol led to five to tenfold decrease in the number of proteins identified because of multiple sample transfers and contact-surface adsorption losses. As a result, we developed
Fig. 2. Performance assessment of PLOT nLC-MS. A, Scanning electron microscopy (S.E.M.) images of cross-sections of a 10 μm i.d. PLOT column. A thickness of the porous layer of ~1.2 μm can be seen on the left image and a more detailed morphology of the porous layer could be observed by backscatter S.E. energy (right). B, Extracted ion chromatograms (XICs) of selected peptides from digested bovine protein standards (“Bovine 6 Protein Mix,” Michrom Bioresources, Auburn, CA) for injection levels from 10 zmol to 100 amol acquired using untargeted data-dependent data acquisition (DDA). Peptide ion intensities extracted using 2 ppm mass tolerance showed a linear response to the injected amount with R² values typically of ≥0.95. The following selected peptides are shown in the XIC traces: 1) ALVYGEATSR, CA-II; 2) TPEVDDEALEK, Beta-LG; 3) YSTDVSVDEVK, GDH; 4) VLDALDSIK, CA-II; 5) VLVLDTDYK, Beta-LG; and 6) LVNELTEFAK, BSA. C, MS/MS spectra for ALVYGEATSR (m/z 533.7800) in panel B, demonstrating unambiguous identification even at low zmol levels. D, Targeted analysis of ALVYGEATSR at low zmol levels. E, XICs of selected target precursor ions by parallel product ion monitoring (PRM). XICs for different parent ion – fragment ion transitions are shown in different colors. See text for details.
a new single-tube sample preparation method based on the AFA (Adaptive Focused Acoustics™, Covaris, MA) assisted cell lysis. This technique allowed us to perform cell lysis followed by protein reduction, alkylation and digestion in the same glass microtube with minimal sample dilution, that is, 12–15 μl. The AFA technology is used for extraction of DNA in genomic applications but only previously reported for cell lysis for proteomic profiling (34, 35). We now add to this a key benefit of AFA of conducting cell lysis without chaotropes (e.g. urea, guanidinium chloride) or detergents along the standard steps of protein reduction, alkylation and digestion in the same tube. Eliminating chaotropes and detergents allowed us to avoid sample dilution and additional clean-up procedures, thus significantly improving sample recovery. Furthermore, we conducted cell lysis with the magnetic beads present in the tube, thus removing the step of release of the cells and improving the speed of lysis (2 to 3 min).

We then used the AFA approach to directly lyse 2000 MCF-7 cells (counted by flow cytometry) in a total volume of ~20 μl, followed by reduction, alkylation and in-solution digestion. Proteomic profiling of an aliquot corresponding to 500 cells resulted in identification of 3370 ±119 (n = 3) protein IDs using a 4 h gradient, a number similar to the profiling depth for 10 million MCF-7 cells, again injecting a roughly equivalent of 500 cells. Using the same procedure 2061 ±39 proteins (n = 3) were identified for 100 cells injected, again similar to the number from 10 million cells, whereas the analysis of 50 cells resulted in 1802 ±18 (n = 3) protein groups, a 36% increase in comparison to the sample equivalent to 50 cells from the bulk sample preparation (10 million cells) (supplemental Fig. S2).

**Target Microfluidic Magnetophoretic Cell Isolation Using a Model System**—We combined the above miniaturized approaches with immunoaffinity enrichment of target cells with magnetic beads followed by high specificity microfluidic magnetophoretic isolation of rare cells from biofluids (12). The microfluidic immunomagnetic cell separator device is designed to separate magnetically labeled target cells from two laminar streams adjacent to the walls of a straight channel into a central buffer stream (Fig. 4, supplemental Fig. S3). Post-separation, but before lysis, magnetically-tagged MCF-7 cells separated from blood were counted by flow cytometry via a gate of electronic volume versus side scatter (supplemental Fig. 3).

As a model system, various numbers of MCF-7 cultured cells ranging from 1000 to 10,000 cells were spiked into 1 ml of whole human blood that was then collected in heparin Vacutainers, incubated with magnetic microbeads functional-
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The high specificity of MCF-7 isolation was further assessed by immunoblotting and 1D PAGE using a panel of spiked cells and control blood samples (Fig. 4D and supplemental Fig. 5F). A number of negative control microfluidic cell isolation experiments indicated the absence of immunoblot or gel detectable contamination of isolates from high abundance blood constituents, e.g. plasma proteins, erythrocytes, and leukocytes (Fig. 4D and supplemental Fig. 5F). On the other hand, the isolates of cells spiked at different immunoblot detectable levels (25,000–85,000 cells in 1 ml of blood) showed a proportional response in blot signal and detection of identical to the positive control (neat MCF-7 in DPBS) bands (Fig. 4D).

To assess the level of baseline contamination of the microSPE-PLRT-nLC-MS system and buffers, we injected aliquots of 0.1% aqueous formic acid into the SPE column and ran blanks, prior to analysis of cell lysates, after rinsing the system (Fig. 4E). Two separate quality control experiments
were performed to assess the isolation efficiency and specificity using the PLOT-based nLC-MS platform: identical aliquots of either (1) negative control: whole human blood without added MCF-7 cells or (2) positive control: 2000 counted MCF-7 cells in DPBS processed without passing through the magnetophoretic device. Each aliquot of the AFA-processed and in-solution digested cell lysates, corresponding to 500 cells, was loaded on the SPE and separated by the PLOT column. In the target cell isolation experiments, where ~3000 cells were spiked into 1 ml of blood and ~1800 cells were recovered from the microfluidic device, on average 3126±103 and 5117 protein groups were identified in each analysis of 500 MCF-7 cells and in a total four analyses (two independent isolations analyzed in duplicate), respectively. These numbers and the identified constituents of the MCF-7 proteome were similar to the numbers found in the positive control (neat MCF-7 in DPBS, 3449±204 protein IDs on average and 5223 IDs in total, n = 4) (Fig. 4E, 4F and supplemental Fig. S4A).

Based on our comparative analysis of the above samples, we estimated that only ~3% of proteins identified in MCF-7 cells isolated from the blood corresponded to nonspecifically bound blood proteins, which is in agreement with previous findings where ≥95% purity of isolation was observed (26). The coefficient of variation (CV) of 3% in the number of protein groups identified in the four analyses showed good reproducibility of both cell capture and analysis. We also examined the level of carryover between injections for different amounts of cells (e.g. 50, 100, and 500 cells) and determined that it was negligible (≤1–2% of total protein IDs, see Supplemental Fig. 6A). Additionally, we determined that the presence of the magnetic beads during sample processing aided efficiency as nonfunctionalized beads resulted in a ~20% gain in protein IDs compared with the sample processing without addition of beads to MCF-7 cells.

Further investigation of the differences in the proteome results from the above experiments was performed using comparative Gene Ontology (GO) enrichment analysis. The proteomic profiles of the isolated MCF-7 cells from blood and the negative control isolates from blood showed very significant differences in distributions of the corresponding cellular localization GO terms (supplemental Fig. S4B, S4C). It is noteworthy that many high or moderate abundance plasma proteins, such as immunoglobulins and kallikrein (supplemental Table S1), were unique for the negative control experiment.

For the positive control, where the cultured MCF-7 cells in DPBS were analyzed, the GO profiles were very similar to those in the analysis of the MCF-7 cells isolated from blood from the microfluidic device for both unique and total proteins that verified the high specificity of cell capture (supplemental Fig. S4C).

Proteomic Profiling of Isolated Target Cells in Model Systems—To evaluate the microfluidic platform in efficient isolation of rare cells, we first spiked 1000, 2000, 3000, 5000, and 10,000 MCF-7 cells (counted by flow cytometry, CV ≤2.6% for all MCF-7 cell counts) in 1 ml aliquots of whole blood and enriched using the magnetophoretic device. Approximately 90–95% of the spiked-in cells were isolated by the device and ~50–60% of the cells remained (again by flow cytometry) after concentration and transfer into tubes for AFA-assisted lysis (supplemental Table S1). The cells were processed, as described above, and aliquots corresponding to 1/5th of the resulting digests were subjected to microSPE-PLOT-nLC MS analysis in each replicate. Profiling of a sample aliquot equivalent to 122 cells resulted in identification of 2512 ± 246 protein groups on average and 3752 proteins total in six replicates (two independent isolations analyzed in triplicate) when 1000 cells were spiked in whole blood and ~600 cells recovered (Fig. 4G). The depth of proteomic profiling increased proportionally with the number of injected cells. Ultimately, when ~1440 cells were analyzed, 3402 ± 169 and 5074 protein groups were identified per injection and in total, respectively (Fig. 4G, supplemental Table S2). The number of the identified peptides followed a similar trend (supplemental Fig. S6B).

To assess the quantitative performance, we spiked in 1 ml of blood MCF-7 cells at five different concentration levels (1000 to 10,000 cells) in triplicate (Fig. 5). Isolates from one replicate were used to count cells, and the two remaining replicates were subjected to proteomic profiling in triplicate, where 1/5th of each isolate was injected in each analysis (i.e. 122, 239, 409, 687, and 1440 cells according to the flow cytometry counts). We estimated protein abundance using the iBAQ approach (MaxQuant) (36). Changes in dynamic ranges by proteomic profiling for the five levels of MCF-7 cells showed a log-linear correlation between sample amount and log10 protein intensity with r2 of 0.84 (Fig. 5A). Both isolation and proteomic profiling replicates showed good reproducibility in profiling depths, dynamic ranges of MS signal intensity measurements and distribution of protein abundance values (supplemental Fig. S5).

Isotopically labeled peptides with sequences corresponding to endogenous proteins of different abundances were next spiked in the digested lysates of isolated cells for absolute quantitative analysis (see Fig. 5B shown in dots on protein distribution S-curves). Areas of labeled peptide peaks were used to normalize abundance measurements for peptides of endogenous proteins. As expected, abundance levels of sample peptides increased relative to the labeled standards when the number of cells increased (Fig. 5C, i–iv). Excellent linear correlation (r2 = 0.97–0.99) was achieved within the concentration range of 1000–10,000 cells/ml (Fig. 5C, v–viii).

Proteomic Profiling of Hematopoietic Stem Cells (HSCs) and Endothelial Progenitor Cells (EPCs) Isolated from Whole Blood—Based on the above results, we turned to actual rare cell analysis in whole blood using the developed platform. Specifically, two rare and important CD133+ cell populations in whole blood (found at <1% of the total cell population), namely endothelial progenitor cells (EPCs) and hematopoietic...
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Fig. 5. Quantitative proteomic analysis of isolated MCF-7 cells. A. Box plot distributions of protein abundances (log_{10} iBAQ values) for determined in analyses of different amounts of MCF-7 cells. Cell amounts per injection are shown in the labels on the right. B. Proteins ranked according to their abundance levels (log_{10} iBAQ values) for in proteomes revealed in analyses of 122, 239, 409, 687, and 1440 cells per injection. Synthetic stable isotope labeled peptides spiked in as internal standards. C. (i–iv) Extracted ion chromatograms corresponding to endogenous MCF-7 peptides detected at different cell numbers and spiked SIL internal standards (orange trace); (v–viii) correlation of abundance ratios of peptide peak areas of endogenous (“light”) to SIL (“heavy”) for different cell numbers. In all experiments (A–C), sample aliquots were analyzed in six replicates (two independent isolations in triplicate).

EPCs and HSCs were separately isolated from 1 ml of whole blood drawn from seven healthy donors using magnetic beads against the CD133 antigen. The cells were stained for CD34, KDR, and CD45 and then counted first via a CD34+/H11001/H11002 gate, followed by a dual gating for CD45+/KDR−/CD133− cells (Fig. 6). Cells isolated using the anti-CD133 antibody, that subsequently stain CD34+/CD45−/KDR+, indicate an EPC phenotype, and CD34+/CD45+/KDR− cells are an HSC phenotype (44) (Fig. 6A, 6B). The efficiency of CD133+ cell isolation was determined to be >90%, with >60% cell purity. The specificity of cell isolation was confirmed by immunoblotting (Fig. 6F). Additionally, proteomic analysis revealed the presence of proteins indicative of EPC cells, including caveolin-1, angiopeptin-1 receptor, endothelial nitric oxide synthase and ephrin type-A receptors (45, 46).

Similarly, proteins of a specific extracellular matrix repertoire reported to be indicative of HSCs and other multipotent stem cells (47, 48), including SPARC, integrins alpha-5 and 6, CD44, and ADAM17, among others, were identified in our analysis of the EPC/HSC isolates but not in the MCF-7 isolates.

The number of proteins identified in CD133+ EPC and HSC cells (~2000 proteins identified in ~1000 cells) was somewhat lower than for MCF-7 cells (Fig. 6C), as expected, based on observed cell sizes (Figs. 4C, 6A) (49, 50). Furthermore, the protein content expressed in stem cells is expected to be lower than in cultured cancer cells (51). Interestingly, in two subjects who were known to be smokers, the isolates contained ~20% fewer EPCs and HSCs than those from non-smokers (supplemental Fig. 6B), which is consistent with previously published data (52, 53). CD133+ EPC/HSC cells showed high levels of correlation in their quantitative proteomic profiles (r^2 > 0.8), with the highest similarity observed for cell isolates from the two smokers (Fig. 6D, 6E).

In summary, a combination of the immunoaffinity microfluidic magnetophoretic cell isolation, focused ultrasonication-assisted cell lysis, reduction, alkylation, and digestion, followed by 1D PLOT-nLC-MS profiling and advanced data processing resulted in the identification of ~4000 proteins from the injection of only 100–200 cells per analysis, a level of at least 5–10 times better than reported to date. High specificity of the cell isolation technique in combination with advanced sample procurement and preparation and ultrasensitive PLOT-nLC-MS enabled differential profiling of limited levels of model samples and isolates collected from donors. Furthermore, we reduced the amount of blood necessary to conduct deep proteomic analysis of EPCs and HSCs by at least 1–2 orders of magnitude in comparison to other reported methods (27, 28). These results show the clear potential for in-depth discovery proteomic characterization of circulating...
rare cells for a variety of clinical and biological applications. Both targeted and discovery proteomic profiling of blood (liquid biopsy) or tissue samples using our approach may further enable therapeutic monitoring of individual patients from only small quantities of clinical samples. In addition, proteomic profiling of rare cell populations such as stem and progenitor cells has the potential to provide mechanistic insights into both normal (e.g. hematopoiesis (54)) as well as disease processes (55).

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