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Supporting Information

Proteomic Analysis of Single Mammalian Cells Enabled by Microfluidic Nanodroplet Sample Preparation and Ultrasensitive NanoLC-MS

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Experimental Section

Reagents and Materials

Dithiothreitol (DTT), iodoacetamide (IAA) and Oregon Green™ 488 Carboxylic Acid Diacetate, Succinimidyl Ester were purchased from ThermoFisher Scientific (Waltham, USA) and their working solutions were freshly prepared before use. n-dodecyl-β-D-maltoside (DDM) was obtained from Sigma-Aldrich (St. Louis, USA) and prepared in 50 mM ammonium bicarbonate buffer (pH 8.0) with a concentration of 0.2% (w/w), and stored in -20 °C. Trypsin (MS grade) and Lys C (MS grade) were products of Promega (Madison, USA). Deionized water (18.2 MΩ) produced from a Barnstead Nanopure Infinity system (Los Angeles, USA) was used throughout.

NanoPOTS Chip Fabrication

An array of 5 × 13 nanowells with diameters of 1 mm and on-center spacing of 2.25 mm was designed and fabricated on a standard microscopic slide pre-coated with chromium and photoresist (25 mm × 75 mm, 1 mm in thickness, soda lime, Telic, Valencia, USA).[1–3] The nanowell surface was retained its native hydrophilicity while the surrounding chip surface was treated to be hydrophobic with a solution containing 2% (v/v) heptadecafluoro-1,1,2,2-tetrahydrodecyl)dimethylchlorosilane (PFDS) in 2,2,4-trimethylpentane. A glass spacer was fabricated by laser-milling a glass slide to remove the central area and leave a 5-mm-wide frame. The glass spacer was glued to the nanowell chip using silicon adhesive. A cover plate was fabricated by spin coating a layer of Sylgard 184 (Dow Corning) base and curing reagent (10:1, v/v) on a microscopic slide at a spin speed of 500 rpm for 30 s followed by 3000 rpm for 5 min. The PDMS layer was cured overnight at 70 °C. The cover plate can reversibly seal the nanowell chip via the spacer to minimize evaporation during reaction incubation procedures.

Cell Culture

HeLa cell lines were purchased from ATCC (Manassas, USA). Growth media was Minimum Essential Medium (GIBCO, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS, GIBCO) and 1× penicillin streptomycin (ATCC). The cells were split every 3 days and cultured at 37 °C and 5% CO_2.

Cell preparation and FACS sorting

To evaluate the FACS sorting efficiency, cultured HeLa cells were stained with Oregon Green™ 488 Carboxylic Acid Diacetate, Succinimidyl Ester, which is catalysed by intracellular esterases to yield a highly fluorescent dye. Cells were harvested and washed three times in 10 mL PBS buffer to remove protein contamination from the culture media. Cell suspension was incubated with 10-μM Oregon Green™ 488 dye

at 37 °C for 30 min. The residual dye was removed with three rinses with PBS buffer. The cell concentration was counted and adjusted to 1×10^5/mL.

For proteomic analysis, HeLa cells were collected, washed, and the concentration was adjusted as described above but without staining steps.

Lung cells

Cryopreserved cells from enzyme-mediated lung tissue digest were thawed and counted. Cells were incubated in 4% normal mouse serum (Sigma: M5905) solution in PBS + 2% BSA (5 µl per 1 million cells) to block non-specific antibody staining. A staining cocktail consisting of 1:50 Podoplanin-AF647 (Biolegend: 337008), 1:50 CD31-BV605 (BD: 562855), 1:50 CD326-PE (eBiosceince: 12-9326-42), 1:50 CD45-V450 (BD: 560367), CD144-FITC (BD: 560411), and 1:800 CD235a (BD: 559944) was added to cells (5 µl per 1 million cells) for 80 min on ice in the dark. Cells were washed twice with PBS + 2% BSA and then filtered. Cells were then sorted using a FACSAria II (BD) instrument. We isolated CD45+ mixed immune cells, CD31/144+ endothelial and CD326+ epithelial cells via positive selection. Mesenchymal cells were remaining cells isolated after negative selection. Following sort, the epithelial and mesenchymal cells were centrifuged. The pellet was resuspended in 500 microliters of freezing media (90% FBS + 10% DMSO) and transferred to cryovials. The cryovials were then placed in a Mr. Frosty (ThermoFisher) and frozen using a -80°C freezer. Sample was then shipped on dry ice to PNNL for analysis.

A fluorescence activated cell sorter (Influx, BD Biosciences, San Jose, USA) was employed for collecting cells into nanowells. Before experiments, the cell sorter was trained to fit in the format of nanowell chip. After alignment, fluorescent beads (Spherotech) were sorted at specific beads number to confirm targeting and optimize cell sorter parameters. For stained HeLa cells, forward and side scatter were used to gate out cellular debris and fluorescence (excited at 488 nm and emitted at 520/15 nm) was used to select cells. For unstained HeLa cells, forward and side scatter were used for cell selection. The lung primary cells were stained with a membrane impermeant dye propidium iodide (91 mg/mL, 5 min) to detect dead cells. Sort gates were set to exclude the dead cell population from being enriched. The cells were sorted based on fluorescence excited at 520 nm and emitted at 585/29 nm. After FACS sorting, the nanoPOTS chip was sealed with cover plate and stored in −20 °C until sample preparation.

Proteomic sample preparation in nanodroplets

All reagents were dispensed into nanowells using a home-built robotic liquid handling system with sub-nanoliter dispensing resolution.[1,2,4] Single-pot sample preparation using a MS-compatible surfactant (DDM) were employed. Briefly, 100 nL of 0.2% DDM surfactant containing 5 mM DTT in 0.5× PBS and 25 mM ABC buffer was added to a nanowell containing one or more FACS-sorted cells. Cell lysis, protein extraction and reduction were performed by incubating the chip at 70 °C for 1 h. Next, the extracted proteins were

alkylated by dispensing 50 nL of 30 mM IAA in 50 mM ABC buffer and then incubating the chip in the dark at 37 °C for 30 min. Two–step enzymatic digestion using Lys-C and trypsin were carried out by dispensing 50 nL of 5 ng/μL enzyme solutions in 50 mM ABC buffer, and incubating for 4 h and 6 h, respectively. Finally, the droplet sample was collected as a plug into a fused-silica capillary (200 μm i.d., 5 cm long). Each nanowell was washed twice with 200 nL of 0.1% formic acid in water (LC Buffer A) to improve sample recovery of the nanoliter-scale sample. The sample collection capillaries were sealed on both ends with Parafilm and stored at -70 °C until use.

Protein identification with LC-MS/MS

The sample was first loaded and purified using a capillary solid phase extraction (SPE) column (75-μm-i.d., 3 μm and 300 Å pore size C18 particles, Phenomenex, Torrance, USA). After loading, the SPE column was connected to a 50-cm, 30-μm-i.d. LC column (packed with the same particles[1,5]. A Dionex UltiMate NCP-3200RS was used to deliver mobile phase to the column at a flow rate of 60 nL/min. The peptides were separated with a linear 60-min gradient from 8–22% Buffer B (0.1% formic acid in acetonitrile), followed by a 10-min increase to 45%, 5-min to 90% for column wash, and finally to 2% for column equilibration for 15 min.

All data were acquired using an Orbitrap Fusion Lumos Tribrid mass spectrometer (ThermoFisher). The separated peptides were ionized at a spray voltage of 2 kV and the ions were collected into an ion transfer capillary set at 250 °C. The RF lens was set at 30%. The MS^1 scan was set at a mass range from 375 to 1575, a scan resolution of 120 k, an AGC target of 3E6, and a maximum injection time of 246 ms. Precursor ions with charges of +2 to +7 and intensities >8, 000 were selected for MS/MS sequencing. Precursor ions were isolated with an *m/z* window of 2 and fragmented by high energy dissociation (HCD) set at 30%. Repeat sampling were reduced with an exclusion duration of 40 s and *m/z* tolerance of ±10 ppm. MS/MS scan was carried out in the Orbitrap with an AGC target of 5E4. The MS/MS scan resolutions and maximum injection times were set as 240k and 502 ms, 120k and 246 ms, and 50 k and 86 ms for samples containing <10 cells, 20 cells, and >20cells, respectively.

Data analysis

MaxQuant (version 1.5.3.30) was employed for database searching and label-free protein quantification[6]. All MS/MS spectra were searched against the UniProtKB/Swiss-Prot human database (Downloaded in 12/29/2016 containing 20,129 reviewed sequences). Carbamidomethylation was selected as fixed modification, and n-terminal protein acetylation and methionine oxidation were set as variable modifications. Peptides were limited to contain >6 amino acids and the peptide masses were below 4600 Da. Two missed cleavages were allowed for each peptide. Both peptides and proteins were filtered with a false discovery rate (FDR) of 0.01. Match Between Runs algorithm was activated where specified to improve proteome coverage with an alignment window of 15 min and a match time window of 0.5 min. iBAQ protein intensities were

used for quantification. The output tables were processed and visualized in R. The iBAQ intensities were log2 transformed and median centered. Only the proteins with iBAQ intensities in 2/3 replicates in a given condition were conserved for quantification. Missing values were imputed using the method recently described by Cox and collaborators,[7] briefly missing values were replaced by random values with a distribution down-shifted of 1.8 standard deviation (compared to the normal distribution of the filled values) and distribution width of 0.5. FactomineR package[8] was used to perform the PCA and the aov() function of the 'stat' R package was used for the analysis of variance.

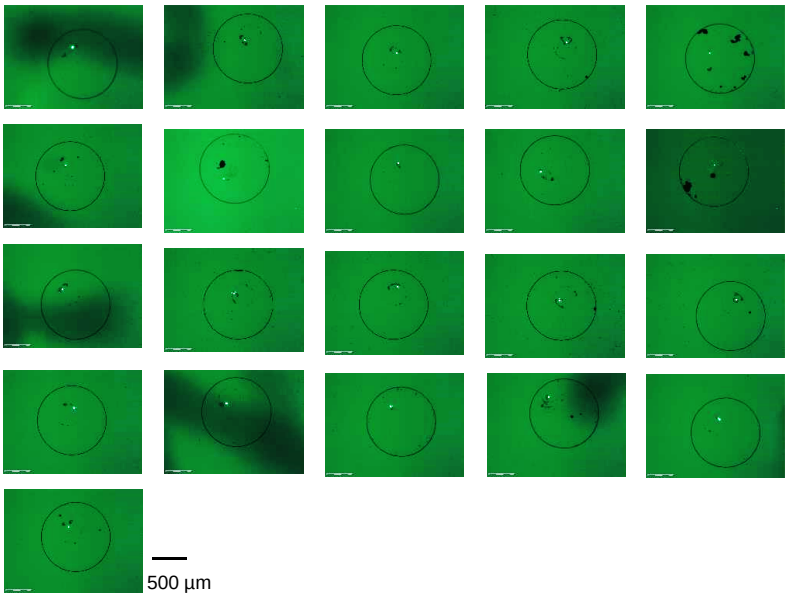

Figure S1. Evaluation of the FACS sorting efficiency for single cells by sequentially sorting 21 cells into nanowells. All the cells were successfully collected into nanowells.

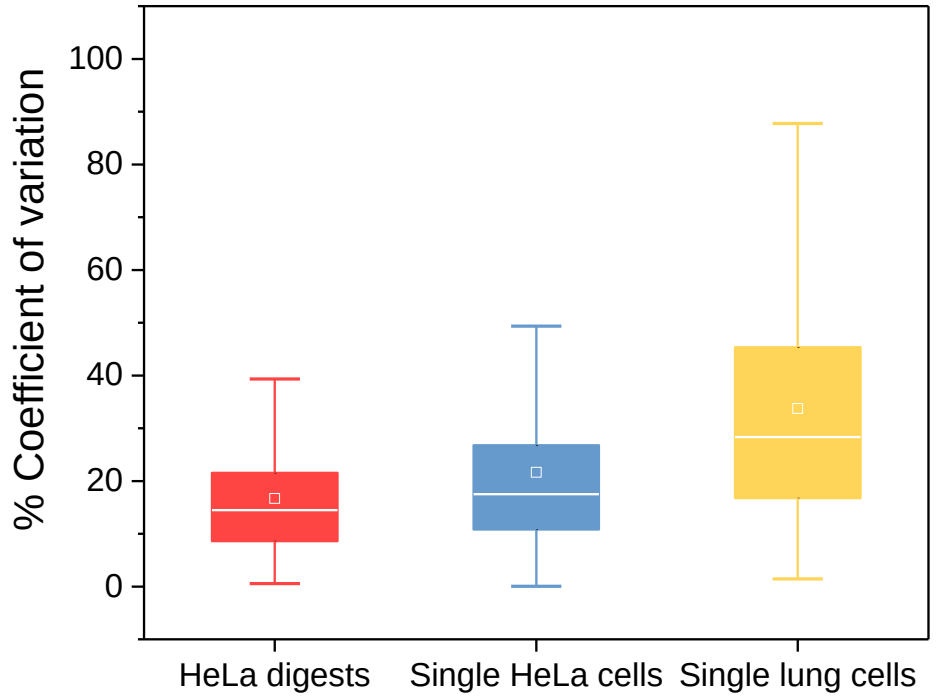

Figure S2. Evaluation of technical and biological variation for the single cell analysis platform. To evaluate technical variation for the whole procedure, HeLa digest samples were prepared in large scale (20 µg) and diluted to 2.5 ng/µL. 200 nL of diluted HeLa digests (0.5 ng total protein) were deposited on nanowells, incubated overnight, and then collected for LC-MS measurement. Three single cultured HeLa cells and six single lung cells (3 epithelial cells and 3 mesenchymal cells) were used to evaluate the capability to measure biological variation. Coefficients of variations (CVs) were defined as the standard deviation of LFQ intensities divided by the mean intensity across the processing replicates.

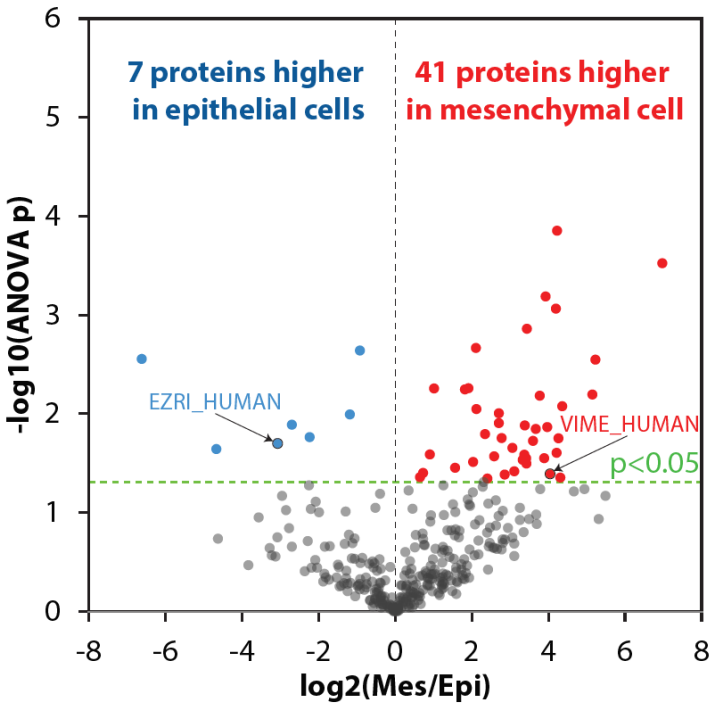

Figure S3. Volcano Plot of differentially expressed proteins of epithelial (Epi) and mesenchymal (Mes) cells from human lung.

Supplementary References

[1] Y. Zhu, P. D. Piehowski, R. Zhao, J. Chen, Y. Shen, R. J. Moore, A. K. Shukla, P. A. Vladislav, M. L. Campbell-Thompson, C. E. Mathews, et al., *Nat. Commun.* **2018**, *9*, 882.

[2] W. W. Liu, Y. Zhu, Q. Fang, *Anal. Chem.* **2017**, *89*, 6678–6685.

[3] W. W. Liu, Y. Zhu, Y. M. Feng, J. Fang, Q. Fang, *Anal. Chem.* **2017**, *89*, 822–829.

[4] Y. Zhu, L.-N. Zhu, R. Guo, H.-J. Cui, S. Ye, Q. Fang, *Sci. Rep.* **2014**, *4*, 5046.

[5] Y. Zhu, R. Zhao, P. D. Piehowski, R. J. Moore, S. Lim, V. J. Orphan, L. Paša-Tolić, W. J. Qian, R. D. Smith, R. T. Kelly, *Int. J. Mass Spectrom.* **2017**, DOI:10.1016/j.ijms.2017.08.016.

[6] S. Tyanova, T. Temu, J. Cox, *Nat. Protoc.* **2016**, *11*, 2301–2319.

[7] S. Tyanova, T. Temu, P. Sinitcyn, A. Carlson, M. Y. Hein, T. Geiger, M. Mann, J. Cox, *Nat. Methods* **2016**, *13*, 731–740.

[8] S. Lê, J. Josse, F. Husson, *J. Stat. Softw.* **2008**, *25*, 1–18.