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Nanoliter-Scale Oil-Air-Droplet Chip-Based Single Cell Proteomic Analysis

Zi-Yi Li,[†] Min Huang,[∥] Xiu-Kun Wang,[⊥] Ying Zhu,[†] Jin-Song Li,[⊥] Catherine C. L. Wong,*[,‡,§,∥] and Qun Fang*[,†]

[†]Institute of Microanalytical Systems, Chemistry Department and Innovation Center for Cell Signaling Network, Zhejiang University, Hangzhou, 310058, China

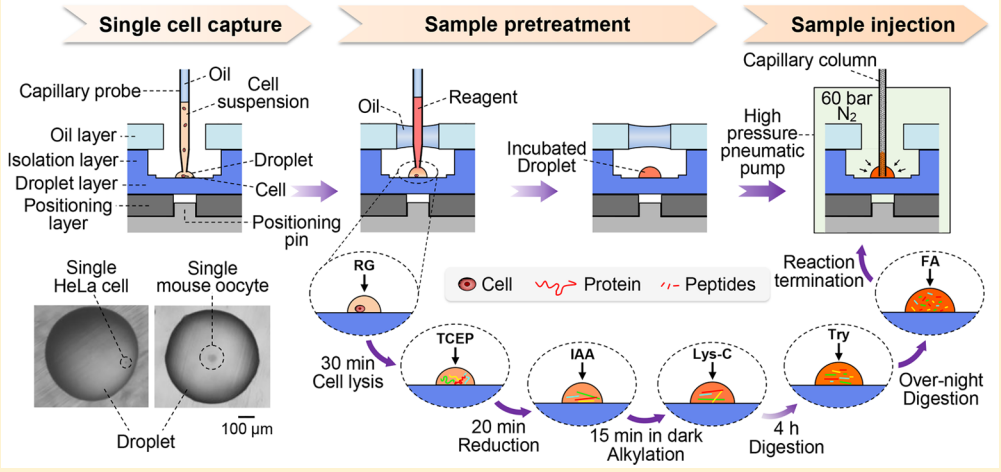
[‡]Center for Precision Medicine Multi-Omics Research, Peking University Health Science Center, Beijing, 100191, China

[§]State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, Beijing, 100191, China

[∥]National Center for Protein Science (Shanghai), Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, 200031, China

[⊥]State Key Laboratory of Cell Biology, Shanghai Key Laboratory of Molecular Andrology, CAS Center for Excellence in Molecular Cell Science, Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, 200031, China

Supporting Information



ABSTRACT: Single cell proteomic analysis provides crucial information on cellular heterogeneity in biological systems. Herein, we describe a nanoliter-scale oil-air-droplet (OAD) chip for achieving multistep complex sample pretreatment and injection for single cell proteomic analysis in the shotgun mode. By using miniaturized stationary droplet microreaction and manipulation techniques, our system allows all sample pretreatment and injection procedures to be performed in a nanoliter-scale droplet with minimum sample loss and a high sample injection efficiency (>99%), thus substantially increasing the analytical sensitivity for single cell samples. We applied the present system in the proteomic analysis of 100 ± 10, 50 ± 5, 10, and 1 HeLa cell(s), and protein IDs of 1360, 612, 192, and 51 were identified, respectively. The OAD chip-based system was further applied in single mouse oocyte analysis, with 355 protein IDs identified at the single oocyte level, which demonstrated its special advantages of high enrichment of sequence coverage, hydrophobic proteins, and enzymatic digestion efficiency over the traditional in-tube system.

The genome-wide deep proteome coverage, benefiting from decades of the fast development of mass spectrometry-based shotgun proteomics technology, has reached the milestone of identifying more than 13 000 human and 10 000 mouse proteins.[1] However, it hits a bottleneck when the protein amounts are limited, especially when it goes to the single cell level. Increasingly, research is focusing on the subtle differences in one molecule that can cause significant variation in cellular behavior;[2] in studying one oocyte to reveal embryonic development;[3] and in clinical research, especially the implication of cancer diagnostics for circulating tumor cells (CTCs).[4] Consequently, single cell proteomics, which provides crucial information on cellular heterogeneity, has reemerged to

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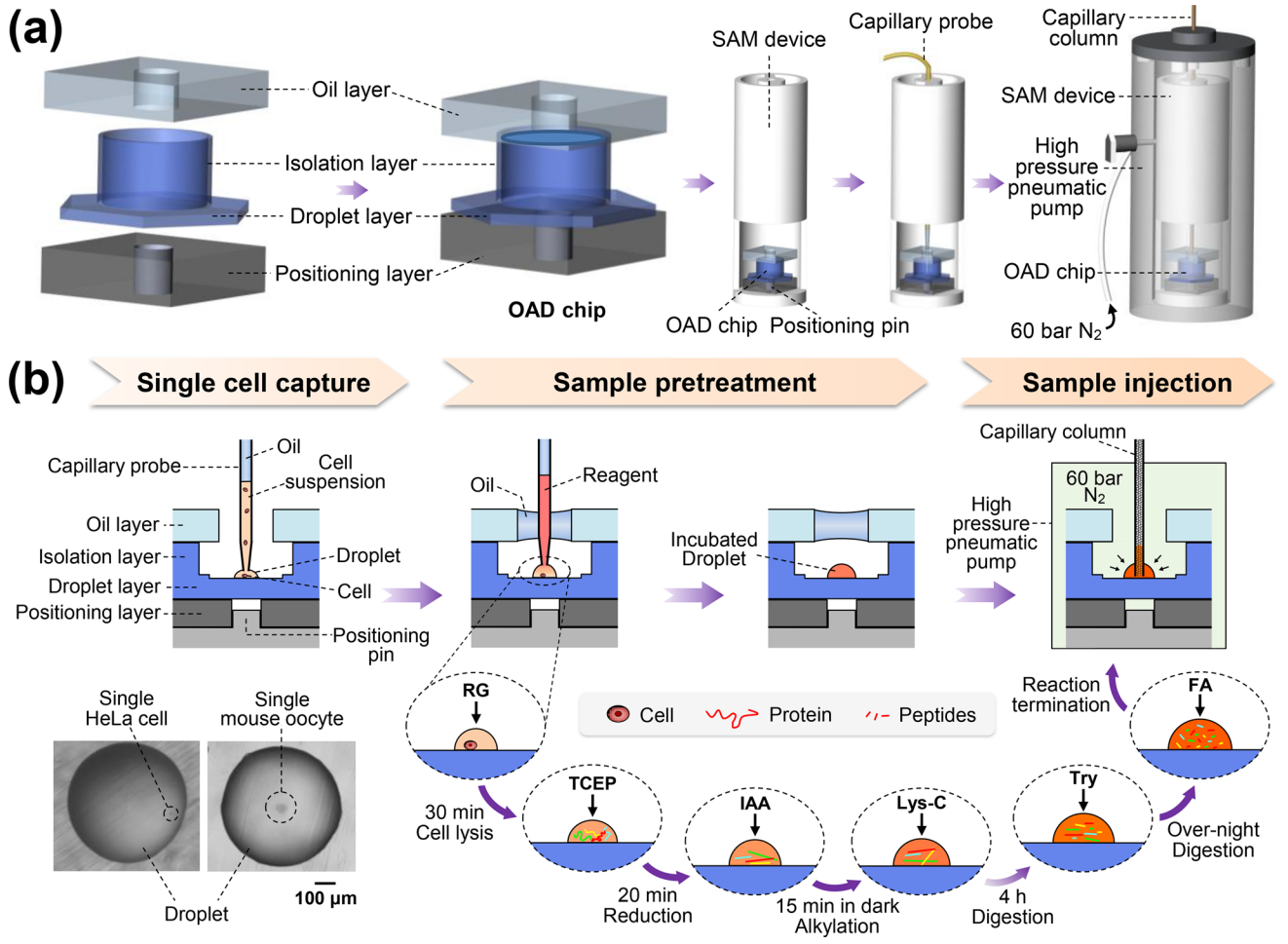

Figure 1. Schematic diagrams of the setup of the nanoliter scale oil-air-droplet (OAD) chip and the self-aligning monolithic (SAM) device (a) and the entire procedure of sample pretreatment and injection for single cell proteomic analysis (b).

become a research hot spot.[5−11] However, the total amount of proteins in a single cell is usually less than 1 ng,[6] which is at least 100 times smaller than the amount used in routine proteome analysis.[12] Thousands of proteins with a high dynamic range, stoichiometry, and copy numbers are in one individual cell, making single cell proteomic analysis by mass spectrometry the long-standing "holy grail" type of challenge for sample pretreatment, separation, and detection.

Recently, the rapid development of mass spectrometers has allowed instrument sensitivity to reach the zeptomole level (i.e., Q-Exactive).[13] Therefore, what matters more in single-cell proteomics is how to handle nanogram amounts of complex protein samples for mass spectrometry analysis. A bottom-up proteomics approach is often used for complex proteomic samples.[14] However, conventional routine protocols require microgram to milligram amounts of protein samples as well as a multitude of pretreatment steps including protein extraction, protein purification, buffer exchange, reduction of disulfide bonds, alkylation of free cysteine residues, and protein digestion.[15,16] Such approaches are not suitable for processing very small amounts of samples such as several cells to a single cell because each step introduces sample loss, which is the issue of sample pretreatment for single cell proteomic analysis.

Currently, many groups have attempted to develop proteomic analysis methods for a small number of cells to progressively approach this goal. Some of them focused on choosing MS-friendly surfactants to improve the processing efficiency,[17] minimizing the number of preparation steps[18−20] and optimizing the chromatographic conditions to increase the sensitivity,[21] while using hundreds to thousands of cells. One of the frequently used strategies for handing smaller numbers of cells is the use of microchannel network-based devices, such microfluidic chips[19] or micro LC injection valves.[20] Chen et al. reported an integrated proteomic analysis device for online injection, in-valve cell lysis and protein digestion, and analysis by nano-LC-MS of 100 living cells with a sample volume of 2 μL.[20] Benefiting from the direct digestion protocol with the sample being transferred only in the connected pipeline, 813 proteins were identified in the proteome of a 100 DLD cells by triplicate analysis. However, this type of microchannel-based strategy still faces challenges in achieving total operation of the complex bottom-up proteomic procedure. Thus, several routine procedures have been omitted to simplify the operations, but this may cause a decrease in the digestion efficiency of proteins. Furthermore, for a microchannel-based device, when the small volume of sample solution passes through the microchannel with a higher specific surface area than routine tubes, evident sample loss may occur due to its adsorption on the channel surface. Another strategy for performing multistep protein pretreatment is the use of stationary reactors such as small test tubes or commercial EP tubes. Li et al. developed a single-tube sample preparation method based on cell lysis assisted by

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acoustics with a volume of 18−20 μL.[21] The treated sample of 2000 MCF-7 cells was first concentrated to 10 μL using a centrifugation concentrator, and then an aliquot corresponding to 50, 100, or 500 cells was loaded onto a monolithic SPE precolumn, and finally injected into a PLOT LC-MS system. Proteins numbering 1802 ± 18 (n = 3) were identified from an aliquot corresponding to 50 cells. However, these tube-based reactors are not suitable for handling nanoliter-scale samples due to their relatively large volumes and lack of liquid handling and evaporation-preventing devices.

In this work, we developed a nanoliter-scale oil-air-droplet (OAD) chip[22] by combining droplet-based microfluidics and conventional shotgun proteomic analysis techniques for achieving multistep complex sample pretreatment and injection for single cell proteomic analysis. In the design of the OAD chip, a stationary nanoliter microreactor mode with an oil-air-droplet sandwich structure was adopted instead of the flow reactor mode for performing multistep sample pretreatment with minimal sample loss. A self-aligning monolithic (SAM) device was also designed to couple with the OAD chip to achieve the accurate manipulation and high-efficiency injection of the nanoliter sample droplets. We evaluated the OAD chip-based system's performance in the proteomic analysis of 100 ± 10, 50 ± 5, 10, and 1 HeLa cell(s). We also further applied the present in-chip system in the proteomic analysis of single mouse oocytes for the first time, which showed a significant advantage in the protein identification number over traditional in-tube systems.

EXPERIMENTAL SECTION

Fabrication of the OAD Chip. The OAD chip (Figure 1a) has a 4-layer cube structure (ca. 7 mm × 7 mm × 7 mm) with a hole in the center of three layers. The components from the top to the bottom are the following: (I) oil layer for holding oil to avoid droplet evaporation; (II) isolation layer for segmenting the droplet with oil; (III) droplet layer for loading the sample droplet; and (IV) positioning layer for chip positioning. We used two types of OAD chips with different combinations of layer materials, a polypropylene (PP) chip and a glass chip. For the PP chip (Figure 2a), the oil layer is made of a 1.6-mm-thick glass plate with a 2-mm-diameter hole fabricated in the center of the plate. The isolation and droplet layers are produced using a lid cut from a 0.6 mL commercial centrifuge tube marked with low binding feature (Axygen, Corning Co., Corning, NY). The inner surface of the center of the lid is used for loading the sample droplet, and the annular ring of the lid is used as the isolation layer to form the oil-air-droplet structure. The positioning layer is produced using a 7 mm × 7 mm poly(methyl methacrylate) (PMMA) plate (2 mm thickness) with a 1.5-mm-diameter hole fabricated in the center of the plate. All of these layers are coaxially aligned and assembled with epoxy glue. For the glass chip (Figure 2b), the difference compared to the PP chip is that the droplet layer is made of a surface-modified glass plate with a 1.6 mm thickness, and the isolation layer is produced using the same type of glass plate with a 3-mm-diameter hole fabricated in the center of the plate. To avoid the cross-contamination between different cell samples, the droplet and isolation layers of the PP chips as well as the droplet layer of the glass chips are disposable. However, the positioning layer and the oil layer of the chips are reusable after thorough cleaning and drying, since these two parts do not directly contact with the droplet samples.

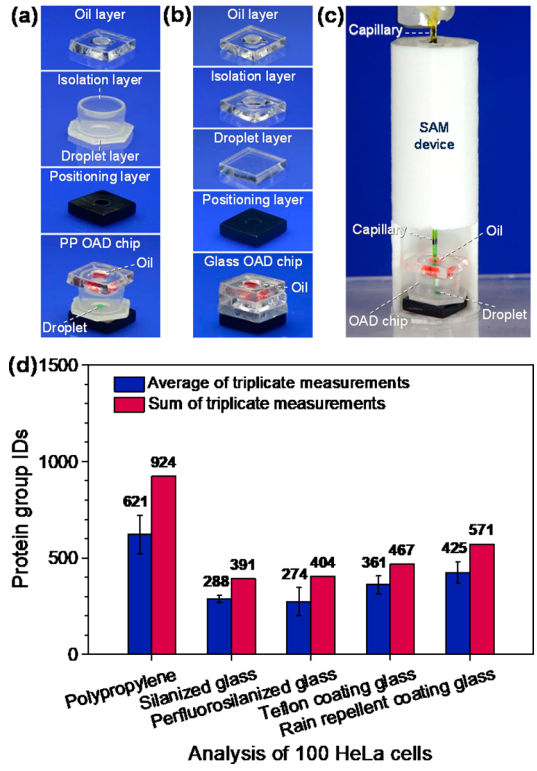

Figure 2. Photographs of the components and assemblies of two types of the OAD chips, polypropylene (PP) chip (a) and glass chip (b). (c) Photograph of an OAD chip installed in a SAM device with a capillary probe inserted through the positioning channel of the SAM device and the cover oil above the droplet in the chip. (d) Proteomic analysis of samples containing 100 HeLa cells using the OAD chips with different droplet layer materials and surface modification methods. The result shows significant difference between the results of the PP chip and the four types of glass chips (P < 0.05, t test). Conditions: lysis buffer: 200 mM guanidine hydrochloride; digestion reagent: trypsin at 1:50 enzyme to protein ratio; LC separation: 15-cm-long column with a 90 min gradient duration.

Setup of the OAD Chip-Based Proteomic Analysis System. For manipulating the cell sample droplet in the OAD chip, we used the droplet manipulation mode of the sequential operation droplet array (SODA) previously reported by the authors' group.[23] A 6-cm-long fused silica capillary with a tapered tip (530 μm i.d., 690 μm o.d.; tip size: 120 μm i.d., 150 μm o.d., Refined Chromatography Co., Yongnian, China) connected with a syringe pump (PHD2000, Harvard Apparatus, Holliston, MA) was used as the probe for droplet manipulation in the OAD chip (Figure 1a). The inner and outer surfaces of the capillary probe were silanized with 1% 1*H*,1*H*,2*H*,2*H*-perfluorodecyltrichlorosilane (Alfa Aesar, Ward Hill, MSA) in isooctane (v/v) before use.

A self-aligning monolithic (SAM) device (Figure 1a) produced by a 3D printer (Fengyi Automation Co., Foshan, China) has a cylinder structure with 34 mm in height and 13 mm in diameter. In the SAM device, a 1.5-mm-diameter and 20-mm-high center channel was produced for positioning the capillary probe or capillary LC column. A 1-mm-high and 1.5-mm-diameter positioning pillar was also produced on the center of the bottom of the SAM device for fixing the OAD chip by inserting the pillar into the hole of the positioning layer of the OAD chip. The SAM device was used to automatically achieve the coaxial alignment of the capillary probe/capillary

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LC column to the OAD chip during the droplet manipulation/sample injection process. For droplet manipulation operation, the SAM device was installed on a manual *x*-*y*-*z* translation stage, and a stereo microscope (Supereyes Co., Shenzhen, China) was used for observing the droplet in the OAD chip.

Home-packed 15 cm × 50 μm C18 columns (stationary phase: Synergi, 2.5 μm, 100 Å, Phenomenex, Torrance, CA) with a pulled tip served as the MS emitter (outer tip diameter, ca. 20 μm; inner tip diameter, ca. 5 μm; interface dead volume, <30 pL) were used for the LC separation of in-droplet peptides generated from the cell extracts.[24] A commercial pneumatic high-pressure pump (PC77, Next Advance Co., Averill, NY) was coupled with the OAD chip and SAM device to perform droplet sample injection into the capillary LC column (Figure 1a). The LC-MS/MS analysis was accomplished with an Agilent 1200 HPLC system (Agilent, Palo Alto, CA) coupled with an Orbitrap Elite mass spectrometer (Thermo-Fisher, San Jose, CA).

Procedures. *Proteomic Sample Pretreatment.* A schematic workflow of the OAD chip method for multistep cell sample pretreatment in single cell proteomic analysis is shown in Figure 1b. Before use, the capillary probe and syringe were filled with degassed water and the capillary tip was filled with 1 μL of fluorinated oil FC40 (3M, Saint Paul, MN) to segment the aspirated solution from the water. For forming cell sample droplets, 1 μL of cell suspension with an appropriate cell density was first aspirated into the capillary probe. The capillary probe was inserted into the positioning channel of the SAM device with a distance of 500 μm between the capillary tip and the sample layer of the OAD chip. Then, 100 nL of cell suspension was deposited on the droplet layer center of the OAD chip to form a cell sample droplet. Cell samples, including HeLa cells and mouse oocytes, were prepared according to the standard procedure (see details in the Supporting Information). Droplets encapsulating 0 cells (as blank control), 1 cell, and 10 cells were generated using the cell counting method with cell suspensions of 10^4, 10^4, and 10^5 cells/mL densities, respectively, and droplets containing 50 ± 5 and 100 ± 10 cells were formed by adjusting the cell suspension densities to 5 × 10^5 and 10^6 cells/mL, respectively. With the conditions of 10^4 cells/mL density and 100 nL droplet volume, the success rate of obtaining single-cell droplets was around 30%. After counting the in-droplet cell number with a microscope (Optika, Ponteranica, Italy), 5 μL of oil was added into the hole of the chip oil layer with a 10 μL pipet to avoid droplet evaporation. The oil was attached to the sidewall of the hole by surface tension and did not come into contact with the sample droplet. In the proteomic analysis of single oocytes, a mouth pipet (tip size: 200 μm i.d., 250 μm o.d.) was adopted to capture one oocyte from the prepared oocyte suspension and deposit it on the chip droplet layer. For the control experiment, an equal volume of the suspension solution without oocyte was used as the blank sample.

After that, to add reagents into the cell sample droplet for the subsequent proteomic sample pretreatment, a similar droplet handling method for forming cell sample droplets as described above was used, by allowing the capillary probe filled with reagent first to insert into the positioning channel of the SAM device, then to pass through the cover oil, and finally to deposit definite volume of reagent into the cell sample droplet (Figure 2c) under the semicontact depositing mode.[25] First, 100 nL of 1% (w/v) RapiGest[26] (Waters, Milford, MA) in 250 mM NH_4HCO_3 solution was added into the sample droplet, followed by ultrasound treatment for 10 min and incubation for 30 min to lyse the cell(s). Second, protein reduction was performed by adding 50 nL of 25 mM tris(2-carboxyethyl)-phosphine (TCEP) to the droplet and incubating the droplet for 20 min. Third, protein alkylation was conducted by adding 50 nL of 150 mM iodoacetamide (IAA) to the droplet and allowing it to react for 15 min in the dark at room temperature. Fourth, the in-droplet proteins were digested by adding 100 nL of Endroproteinase Lys-C with an enzyme/substrate ratio of 1:50 (w/w) and a reaction time of 4 h. Then, 100 nL of trypsin was added with an enzyme/substrate ratio of 1:20 (w/w), and the reaction was left to proceed overnight at room temperature. Finally, 50 nL of 50% formic acid (FA) was added to the droplet, and the droplet was incubated for 30 min to terminate the reaction. The final volume of the sample droplet was ca. 550 nL. For eliminating cross-contamination in handling different solutions, the capillary probe was washed three times by ethanol (95%) and water, respectively, before handling a new reagent.

Besides the in-droplet experiments, a comparison experiment was also performed by using a previously reported conventional tube-based method[19−21,27] for proteomic analysis of a limited number of cells to analyze the same sample of 100 HeLa cells. These traditional methods usually used centrifuge tubes as reactors for the sample pretreatment with relatively large reaction volumes in the range of tens of microliters. Moreover, two traditional tube-based methods[17,19−21,27] for a limited number of cell samples were also used to analyze the mouse oocyte sample (see Figure S1).

Injection of a Droplet Sample. For droplet injection, to avoid the oil being introduced into the column, first, the oil in the oil layer of the OAD chip was removed by using a small piece of a dust-free paper to suck the oil away from the chip hole before the sample injection operation. Then, the SAM device with the OAD chip was inserted into the tank of the high-pressure pump. The capillary LC column was inserted through the central channel of the SAM device and into the sample droplet with its inlet end touching the sample layer surface of the OAD chip and its outlet end leaving outside the tank. The droplet sample injection was achieved by applying a pneumatic pressure of 60 bar (N_2) to the closed pump tank for 3 min to drive the droplet solution into the capillary LC column (Figure S2). After that, a desalting operation was carried out by flushing the column with 0.1% formic acid in water at 200 nL/min for 1 h by the LC system.

LC-MS/MS Analysis. After the preparation of a new LC column, a model sample of *E. coli* tryptic digest was used to precondition and saturate the newly packed column, as well as evaluate the column separation performance. The *E. coli* system was adopted due to its properties of nonmammalian species and high protein complexity. After the analysis of the *E. coli* digest sample with the same program as for HeLa cells and oocyte samples, the column was washed with water and ACN for 2 h. Before analyzing each actual sample, the column was prewashed with the mobile phase A (0.1% formic acid in water) and B (0.1% formic acid in ACN) at a 90 min gradient (0−5 min, 0% B; 5−65 min, 0−45% B; 65−70 min, 45−80% B; 70−80 min, 80% B; and 80−90 min, 0% B) to avoid cross contamination.

In the analysis of HeLa cells and oocyte samples, peptide separation was performed at a flow rate of 200 nL/min with mobile phase A (0.1% formic acid in water) and B (0.1% formic acid in ACN) along with a 360 min gradient as follows: 0−5

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min, 0% B; 5−205 min, 0−20% B; 205−325 min, 20−40% B; 325−340 min, 80% B; and 340−360 min, 0% B.

Peptides eluted from the LC column were directly electrosprayed into the mass spectrometer with a 2 kV spray voltage. Top-20 data dependent acquisition method was used. Full MS spectra were acquired in the Orbitrap analyzer over a range of m/z 300−1800 with resolution set at 60 000, followed by 20 MS/MS events in the linear ion trap (LTQ) using collision-induced dissociation (CID) fragmentation at a normalized collision energy of 35%, sequentially generating the first to the twentieth most intense ions selected from the full MS spectrum.

Data Analysis. Tandem mass spectra of the raw data were searched against the UniProt database (UniProt_Human_09−19−2016 for the HeLa cell, UniProt_Mouse_10−13−2016 for the mouse oocyte) on the Integrated Proteomics Pipeline (IP2) platform (Integrated Proteomics Applications, Inc.).[28] ProLuCID algorithm was employed for the database search with the following parameters: two ends specificity of trypsin, up to two maximum missed cleavage sites, 50 ppm and 0.8 Da mass tolerance for precursor and fragment ions, a static modification of 57.0215 on cysteine, and a differential modification of 15.9945 on methionine. The final results are sorted by XCorr. ProLuCID search results were then filtered with DTASelect 2.0 using XCorr and DeltaCN at a false positive rate of 0.1% (−pfp 0.001) estimated by number of reverse hits. Only fully tryptic (−y 2) peptides (−m 1) were accepted. Only peptides with a minimum of 6 amino acids in length were considered for identification. The minimum number of peptides to identify a protein was set to 1(−p 1). The final proteins all passed these filtering criteria.

RESULTS AND DISCUSSION

System Design. The objective of the present work was to develop an effective system for performing sample pretreatment and injection for single cell proteomic analysis with the requirements of multistep complex operation, small reactor volume, and low sample loss. In the design of the OAD chip, to reduce the sample loss by having adsorption on the reactor surface and sample transfer be as small as possible, an in situ stationary microreactor was adopted for performing multistep sample pretreatment instead of a flow reactor to ensure that the sample droplet has the smallest possible contact area on the chip surface. In addition, a small droplet volume in the nanoliter range was used to further reduce the droplet contact area on the chip. In the previous tube-based systems for a small number of cells,[17,18,21] sample reaction volumes in the 10−20 μL range are commonly used, with contact areas higher than 18−30 mm^2 in the tube. In the present device, a final droplet volume of ∼550 nL was adopted with a contact area of <1.3 mm^2, which is ca. 1/15th of a 10 μL droplet.

In a typical droplet-based microfluidic system, small volume droplets are usually covered or enclosed by oil to avoid droplet evaporation.[23,29,30] However, for in-droplet sample pretreatment in proteomic analysis, such an arrangement may lead to the loss of in-droplet lipophilic peptide components due to their dissolution in the oil. Moreover, the oil is LC and mass spectrometry incompatible. At the beginning of chip design, we also tried to seal the device with a cap. However, the repeated operation of fitting and removing the cap during the multistep sample pretreatment process would cause the obvious evaporation of the nanoliter-scale droplet, as well as the position excursion of the sample droplet on the droplet layer of the chip, resulting in the difficulty or even failure in subsequent operations. In the present system, we used an oil-air-droplet sandwich design adopting air to separate the droplet and the cover oil in the droplet chip to avoid the droplet directly contacting the cover oil (Figure 1b). Oil is held in the hole of the oil layer by surface tension, and a small distance between the oil and droplet is produced to form a closed small chamber to protect the droplet from evaporation. Data shows 5 μL of mineral oil filling in the oil layer could ensure the volume loss of a 500 nL droplet by evaporation to be less than 10% in 10 h at 25 °C (Figure S3), which is sufficient to support the entire sample pretreatment process prior to LC-MS/MS analysis. While protecting the droplet from obvious evaporation in the prolonged reaction process, the OAD chip completely eliminates the possibility of the dissolution of the in-droplet lipophilic peptide components in the cover oil and the interference of the oil for droplet sample injection into the LC column. With such a design, the droplet device also acquired a semiopen feature, which facilitated the capillary probe to sequentially add different nanoliter-scale reagents required for proteomic analysis into the droplet as well as sampling from the droplet for sample injection into the LC column by removing the layer of oil.

For forming cell sample droplets and adding different reagents to the droplets, we used the droplet manipulation mode of the sequential operation droplet array (SODA) previously reported by the authors' group, which has been applied in nanoliter-to-picoliter high throughput screening,[23,25,29] single cell microRNA assay,[31] and digital PCR.[32] In the present work, to facilitate droplet manipulation, we designed a SAM device that has the functions of fixing the OAD chip center in the middle of the SAM device precisely by its positioning pillar and positioning the capillary probe vertically through the SAM device's center channel. With the aid of the SAM device, the cell sample droplet could be accurately deposited on the center of the chip droplet layer, and different reagent solutions could also be readily added into the sample droplet in sequence, due to the device's self-aligning feature, to achieve a series of operations of in-droplet cell lysis, protein reduction, alkylation, and digestion. Such a design significantly enhances the ease and efficiency of system building and operation.

Another challenge for single cell proteomic analysis is how to inject the pretreated nanoliter-scale sample droplet into the capillary LC column with the lowest sample loss. Some special injection valves may be able to achieve small injection volumes in the nanoliter range, such as the Valco 4-nL injection valve. However, these valves also suffer from the risk of sample loss during the process of transferring sample solution from the container to the valve, i.e., analytes may be absorbed on the surface of the connection tubing. We had tried to use a traditional pressure loading method and a commercial pneumatic high-pressure pump to perform nanoliter-scale droplet injection into a capillary LC column, which are commonly employed for filling capillary LC columns[33] or direct sample injection into capillary LC columns without the need for an injection valve.[24] However, such a device could not achieve nanoliter-scale droplet injection since the capillary LC column could not effectively align and insert into the small droplet on the chip placed in the opaque and closed pneumatic tank of the pump. To overcome this problem, another function of the SAM device was exploited by using it as a sample holder that can be well matched for total insertion into a high-pressure

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pump for sample loading onto a capillary LC column. With its help, direct nanoliter volume sample injection into the LC column from the chip could be conveniently achieved, completely avoiding the sample loss from intermediate transferring, such as passing through several valves or channels in other methods.[17−21] A sample droplet with a volume of 550 nL was able to be loaded onto the 15-cm-long packed capillary C18 column in 3 min with an injection percentage of >99% (see Supporting Information).

Optimization of the OAD Chip-Based System. We have optimized the OAD chip-based system with regard to the chip materials, surface modification methods, protein digestion, and LC separation conditions (Figure 2d and Figures S3−S7) using a model sample containing 100 HeLa cells.

In the building of the OAD chips, we used three types of commonly used material, PMMA, polydimethylsiloxane (PDMS), and glass plates to produce the oil and isolation layers of the OAD chip. We evaluated the air-impermeability of the three types of chips by measuring the volume change of in-chip droplets during prolonged incubation periods. The glass chip exhibited the lowest droplet evaporation rate of 4.7 nL/h at 25 °C (Figure S3), and it was chosen as the chip oil and isolation layer material.

For the optimization of the material and surface modification method for the chip droplet layer, we tested the performance of the PP droplet layer made from a commercial centrifuge tube (PP chip) and glass layers (glass chip) with four different surface modifications including silanization with octadecyltrichlorosilane,[25] perfluorosilanization with 1*H*,1*H*,2*H*,2*H*-perfluorodecyltrichlorosilane,[34] Teflon-AF coating,[35] and Rain Repellent coating (PPG Industries, Pittsburgh, PA). In the analysis of samples containing 100 HeLa cells, the PP chips showed the highest identified protein IDs compared to those of the different glass chips ($P < 0.05$, t test) (Figure 2d), which could be attributed to their feature of low surface binding. Among the glass chips, the chips with Rain Repellent coating exhibited the largest protein IDs, but they were still lower than those of the PP chips (Figure 2d). These results further demonstrated the importance of reducing sample adsorption on the reactor surface for the analysis of a limited number of cells.

We also optimized the conditions of cell lysis, protein digestion, gradient duration time, and LC column length using samples containing 100 HeLa cells. Based on the results (Figures S4−S7), the RapiGest reagent was chosen for cell lysis, a trypsin-to-protein ratio of 1:20, and the Lys-C/Trypsin method[36] were used to obtain higher digestion efficiency, and the 15 cm columns with a LC gradient duration of 6 h were adopted in the LC separation of a small number of HeLa cells and single oocytes.

Performance of the OAD Chip-Based System. To evaluate the performance of our OAD chip-based system, we applied it in the proteomic analysis of samples containing different numbers of HeLa cells. Under the optimized conditions, we identified 1360, 612, 192, and 51 protein IDs for 100 ± 10, 50 ± 5, 10, and 1 HeLa cell(s), respectively, summing triplicate runs of each (Figure 3 and Table S1). The results of blank samples containing 0 cell show that less than 5 protein groups were identified in the blank runs, demonstrating that there is no obvious interference from the extracellular proteins in the cell suspensions to the cell analysis results. Compared with the experiment using the traditional in-tube sample pretreatment method with a result of 715 proteins identified from 100 HeLa cells (Figure 4a and Table S2), the

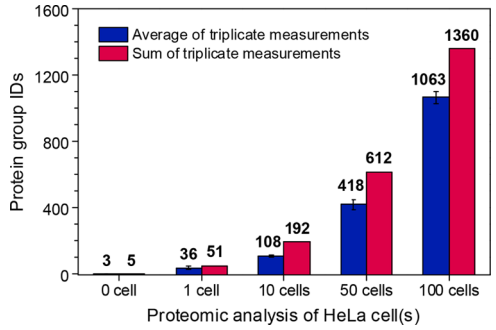

Figure 3. Proteomic analysis results of samples containing 100 ± 10, 50 ± 5, 10, and 1 HeLa cell(s) with the OAD chip-based system. Conditions: lysis buffer, 1% (w/v) Rapigest; digestion reagent, trypsin at 1:20 enzyme to protein ratio combined with Lys-C; LC separation, 15-cm-long column with a 360 min gradient duration.

protein IDs of our in-chip method exhibited approximately a significant 90% increase ($P < 0.01$, t test). We evaluated the properties, including sequence coverage, molecular weight (MW), pI value, hydrophobicity, and cell copy number, of the proteins identified by the in-chip method and compared them with those of the in-tube method. Normal bell-shaped distributions of the MW and pI indicated that the present system performs as good as the traditional systems (Figure S8). We also compared the data of 1360 identified proteins in our system with the quantitative result previously reported by Mann et al.,[37] in which 9667 proteins were identified from the HeLa cell line. Among the 1360 proteins identified in 100 HeLa cell samples by the OAD chip system, 1329 proteins were also observed in the list of the 9667 proteins with a dynamic range across 6 orders of magnitude. Moreover, the system brought us not only more protein IDs but also three unique valuable advantages in terms of sequence coverage, hydrophobicity, and unique protein candidates. Figure 4b shows the distributions of sequence coverage (namely the ratio of the amino acid number of the identified peptides to the total number of the amino acids of a protein) of the 480 proteins identified in both the chip and tube-based systems. We obtained more protein IDs with higher protein sequence coverage (>10%) in the in-chip system, while the traditional in-tube system caught more proteins with lower coverage (≤10%). In addition, many more hydrophobic proteins were obtained in our system (Figure 4c). The last special feature of the present system is that we constantly observed that a certain number of proteins were always exclusively identified, which have never been shown with the in-tube system (Figure 4a). We identified a total of 1595 proteins from 100 HeLa cells combining both systems; however, 55% of proteins were identified only in the OAD chips, while only 15% were identified only in the tubes. These features demonstrate that our system is more suitable for handling samples of a limited number of cells. We proposed that these effects might be caused by the minimal sample loss from adsorption on the reactor surface and the transfer of sample as well as the high enzymatic digestion efficiency due to the low dilution of sample and reagents and short molecular diffusion distance in the constrained in-droplet microenvironment provided by the OAD chip compared to the traditional in-tube systems with larger reactor volumes. Gene ontology (GO) analysis (Figure S9) of the exclusively identified proteins in the OAD chip showed that 90% of the proteins were involved in cellular component organization or biogenesis and

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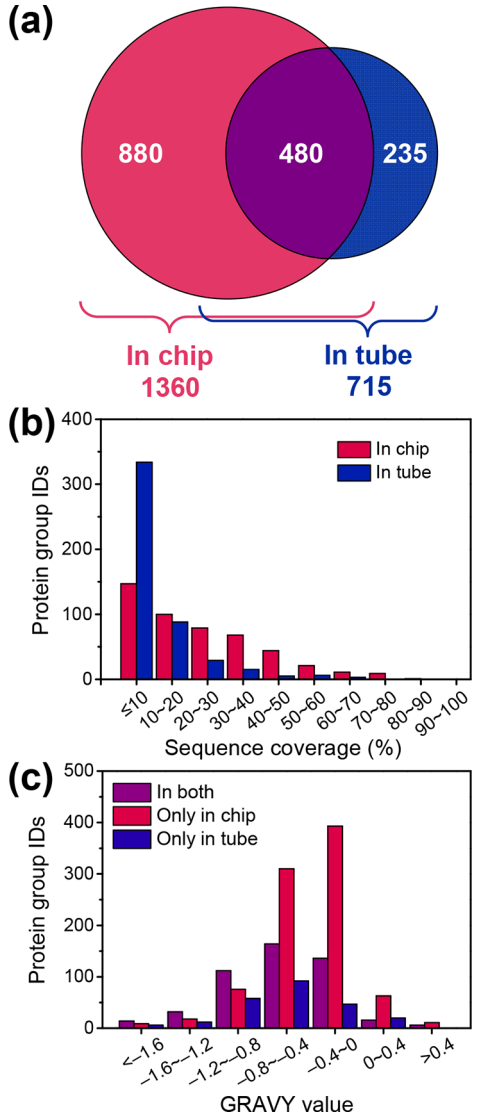

Figure 4. Comparison of proteomic analysis results of samples containing 100 HeLa cells with the OAD chip-based and tube-based systems, including the Venn diagram of the identified protein group number (a) from a sum of triplicate analysis and categorized according to the distributions of the sequence coverage (b) of the 480 protein groups both identified in the two different systems, and the grand average of hydropathy (GRAVY) values (c) of the 1595 protein groups identified by the two systems. Conditions are as in Figure 3.

cellular metabolic processes. With regards to molecular function, over 90% have protein and RNA binding functions, which in agreement with the same percentages of membrane-bounded organelle, vesicle, and intracellular components. This provided additional evidence of the side consequence of the enrichment effect of hydrophobic proteins by the OAD chip-based system.

Proteomic Analysis of Single Mouse Oocytes. To further demonstrate the potential of the OAD chip-based system in single cell proteomic analysis, we applied it in the proteomic analysis of single mouse oocytes. The understanding of oocyte proteome composition and diversity during maturation is vital for developmental and evolutional biology. Several groups are studying *Xenopus laevis*[27,38] or mammalian oocytes[3] using a single-cell approach. Among the three types of oocyte models, mouse oocytes have the smallest size, where one contains approximately 20 ng of protein,[39] while a human oocyte contains approximately 100 ng of protein and the size of a frog oocyte is usually 1000-fold larger than the human one. We herein applied our OAD chip pipeline for analyzing one single mouse oocyte and identified 355 proteins summing triplicate measurements (Figure 5 and Table S3a), which has

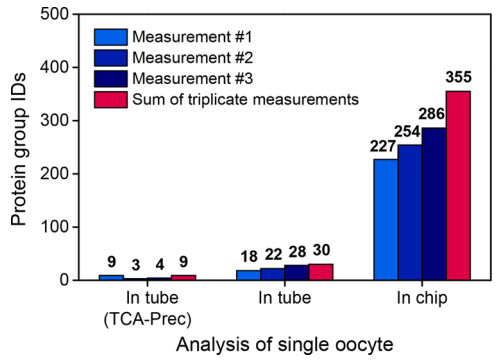

Figure 5. Proteomic analysis of single oocytes using the tube-based system with and without TCA precipitation treatment and the OAD chip-based system. Each of the blue bars shows the result of a single measurement, and the red bars represent the sum of results of triplicate analysis of three single oocytes. There are significant differences between the results of the three methods ($P < 0.01$, t test). Conditions are as in Figure 3.

never been reported before. The number of protein IDs is quite high considering the very low amount of starting material of 20 ng. In the blank runs of suspensions without oocyte, only 5 ± 1 ($n = 3$) protein groups were identified. In a previous study,[3] ~ 450 proteins were able to be consistently identified in one human oocyte, whose size is five times larger than a mouse oocyte, which yields approximately 100 ng. Using the in-tube method for comparison, we only identified 36 proteins (Table S3b and c), among which only 9 proteins were exclusively from the traditional sample handling system. Compared with the results of 100 HeLa cells, our OAD chip-based system exhibited many more protein IDs compared to the in-tube system in single oocyte analysis, which demonstrated that it is more suitable for handling single-cell samples. Compared with the microliter-scale reactors used in the tube-based systems, the droplet reactors in the nanoliter range could evidently increase the in-droplet protein concentration and thus shorten the molecular diffusion distance within the reactor, which would lead to the increase of enzymatic digestion efficiency. Such an enhancement effect of reaction efficiency in constrained in-droplet microenvironment has been frequently reported in droplet-based systems.[40,41] In addition, the decrease of reactor volumes could also reduce the contact area of reaction solution with the reactor wall, and thus minimize the protein loss by the adsorption on the reactor wall. Additionally, 328 proteins were uniquely obtained, and most of them were oocyte-specific proteins with important biological functions related to transport, oxidation−reduction process, sexual reproduction, fertilization, homeostasis, binding, and endocytosis. Table S4 lists the corresponding gene names and protein numbers of the GO analysis results of the oocyte proteins identified with the present system. Many oocyte-specific proteins were identified including the following: Nlrp14,[42] Eif4e1b,[43] and Zar1,[44] which are important for oocyte-to-embryo transition. Several proteins involved in early embryonic development and embryogenesis, such as Ovgp1[45] (oviduct-specific glycoprotein) were observed.

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Zona pellucida sperm-binding proteins 1, 2, and 3 (Zp1, Zp2, and Zp3)[39] and Pabpc1,[46] which are essential for fertility, maternal effect, and maturation, were also identified.

Due to the special advantage of the enrichment of hydrophobic proteins, several vital proteins were identified with confidence including the following: transmembrane proteins (Tmem14c) and transcriptions factors and regulators (Chchd3, Mphosph6, and Ptges3). There were some other interesting targets in our results such as sperm proteins (Nasp and Paox) and cancer-associated proteins (AnxA6, Kpna2, Cct6a, and Pcbp2),[47] leaving us a reason to explore more in the future. Our OAD chip-based system demonstrated its substantial capability and essentiality, as it gave the best result compared to the other methods for single oocyte proteomic analysis. Results show that the protein number drastically increased, but, due to the unique feature of the OAD chip, the hydrophobic protein number also increased due to the use of the stationary droplet microreactor and high in-droplet enzymatic digestion efficiency. Many proteins with important properties were observed, which gave researchers a great resource to do further exploration on oocyte biology.

CONCLUSION

In summary, we established a miniaturized nanoliter-droplet sandwich-type microchip device, an OAD chip, integrated with an optimized shotgun approach to perform sample treatment and LC-MS/MS analysis for samples with a limited number of cells. The entire system substantially shows its features of multistep complex operation, low sample loss, high efficiency, and easy system building and use, thus specifically favoring single cell proteomic analysis. The system allows all operation procedures to be performed in a nanoliter-scale droplet with minimum sample loss and a high sample injection efficiency (99%), thus apparently increasing the analysis sensitivity for a small number of cells. Such an operational mode also demonstrated high reproducibility and reliability. The cut-down traditional sample treatment procedures gave the benefit of simplifying the operation yet retaining the same good result. Finally, the OAD chip-based system demonstrated three special advantages compared to the traditional in-tube systems when handling samples with a limited number of cells: (1) enrichment of sequence coverage, (2) enrichment of hydrophobic proteins, and (3) enrichment of the enzymatic digestion efficiency, thus identifying unique proteins. These special features were especially exhibited in single mouse oocyte analysis. We identified 355 proteins at the single mouse oocyte level. To the best of our knowledge, there is no report of the proteomic analysis of a single mouse oocyte as the starting material, and it opened up a new source for oocyte biology. This system has shown potential as a very encouraging starting prototype for single-cell proteomic analysis and is being further modified by merging it together with a porous layer open tubular (PLOT) column, capillary electrophoresis, and a more sensitive mass spectrometer to further enhance the sensitivity and deep proteome mining. We envision that it will be widely applied in different areas such elucidating the heterogeneities of cells and their underlying cause or clinical research, such as profiling a single cell in circulating tumor cells (CTCs), quality control for in vitro fertilization (IVF), or other disease diagnosis and prognosis.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.8b00661.

Experimental Section; Results and Discussion (PDF)

AUTHOR INFORMATION

Corresponding Authors

*E-mail: fangqun@zju.edu.cn.
*E-mail: catherine_wong@bjmu.edu.cn.

ORCID

Catherine C. L. Wong: 0000-0001-7270-980X
Qun Fang: 0000-0002-6250-252X

Notes

The authors declare no competing financial interest.

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