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Droplet-Based Microfluidics with Mass Spectrometry for Microproteomics

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ABSTRACT

Microproteomics, the profiling of protein expressions in small cell populations or individual cells, is essential for understanding complex biological systems. However, sample loss and insufficient sensitivity of analytical techniques pose severe challenges to this field. Microfluidics, particularly droplet-based microfluidics, provides an ideal approach by enabling miniaturized and integrated workflows to process samples and offers several advantages, including reduced sample loss, low reagent consumption, faster reaction times, and improved throughput. Droplet-based microfluidics manipulates droplets of fluids to function as discrete reaction units, enabling complex chemical reactions and biological workflows in a miniaturized setting. This article discusses a variety of on-chip functions of droplet-based microfluidics, including cell sorting, cell culture, and sample processing. We then highlight recent advances in the mass spectrometry (MS)-based analysis of single cells using droplet-based microfluidic platforms, including digital microfluidics (DMF). Finally, we review the integrated DMF–MS systems that enable automated and parallel proteomic profiling of single cells with high sensitivity and discuss the applications of the technology and its future perspectives.

1. Introduction

Multicellular organisms consist of diverse cell types with distinct functions and phenotypic profiles. Cellular heterogeneity contributes to the functionality and adaptability of complex biological systems. Understanding cellular heterogeneity is critical to decipher cell subpopulations, cellular differentiation, and effects of the microenvironment [1,2]. With recent advances in genetic amplifications, single-cell RNA sequencing has enabled the profiling of gene expression patterns from a single cell [3–5]. However, accumulating studies have revealed that RNA abundance poorly correlates with protein levels, suggesting that transcriptomic profiles only show a partial picture of the phenotypic feature of a cell [6,7]. To illustrate the missing inference during genetic translation and comprehensively understand the cellular heterogeneity in proteins, single-cell proteomic analysis is highly desirable and has become a popular research topic in recent years. Proteomic measurements largely rely on the instrumental sensitivity and technical workflow because amplification approaches are not applicable for proteins. Current profiling of proteins in single cells often uses targeted methods to analyze molecules based on labeled antibodies [8,9], such as mass cytometry [10,11], single-cell barcode chips

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[12,13], and single-cell western blotting [14–16]. Although only targeted proteins, typically less than 100 proteins, can be profiled or quantified at a time, the advancements in mass spectrometry (MS) technology are rapidly expanding this limit.

MS is a well-regarded, powerful tool for protein analysis, with high sensitivity, specificity, and accuracy for both qualitative and quantitative studies [17,18]. However, conventional proteomics approaches encounter challenges in analyzing small cell populations due to the substantial sample loss in the processing steps. Efforts have been devoted to reducing the sample loss by blocking nonspecific adsorptions, miniaturizing the sampling platform, and optimizing the streamlined workflow [19,20]. Microfluidic approaches have emerged as ideal options for such endeavors, as they enable further reductions in sample loss and improved integration of sample preparation processes [21].

In the past several decades, microfluidics techniques have significantly benefited from advancements in micro-electro-mechanical systems (MEMS) technology, which encompass many processes designed to create intricate microscale fluidic channels and structures. Photolithography in MEMS etches patterns onto a substrate using ultraviolet light, which is ideal for achieving the precision required for microfluidic devices. Soft lithography often uses materials such as polydimethylsiloxane to replicate these patterns into functional devices. Other fabrication approaches include hot embossing and injection molding, which are excellent for scaling production. Additionally, advances in three-dimensional (3D) printing are providing a foundation for rapid prototyping and complex geometries. These techniques are critical for integrating microfluidic devices with electronic and mechanical elements, which facilitates the development of complex systems that are applicable in diverse fields, including biological assays and chemical synthesis [22].

Microfluidics offers precise fluid manipulation and the ability to integrate multiple analytical steps on a single chip. Several advantages of microfluidics are apparent, including reduced sample and reagent consumption, faster reaction times, and improved throughput [23]. The miniaturization of the reaction volumes in microfluidics concentrates reagents and minimizes surface adsorption, leading to efficient reactions with short durations. Moreover, performing sequential manipulations on a single chip eliminates the requirement of sample transfer between containers, which reduces the possibility of sample loss during the process. Additionally, parallel manipulation simultaneously facilitates high-throughput processing of individual cells and improves the analysis efficiency. Droplet-based microfluidics is an important technology that can manipulate discrete droplets of fluids ranging from a few femtoliters to microliters within microscale compartments [23,24]. Droplet-based microfluidic systems provide accurate control over the droplet size, location, and content. These droplets function as discrete reaction units, providing an ideal platform to study and perform complex chemical reactions and biological workflows [24–26]. Droplet-based microfluidics has found diverse applications in proteomics fields, including extraction of analytes from single cells, precipitation-based extraction of proteins, immunodepletion, immunoenrichment, protein digestion, and mass-tag labeling. Its successful integration with MS has also been applied for single-cell proteomics (SCP) research [27,28].

In this article, as summarized in Fig. 1, we first introduce droplet-based microfluidics and its functions in biological analysis, including cell culture, isolation of single cells, cell manipulations, and sample pretreatment and enrichment. We then extensively discuss recent advancements in MS-based single-cell proteomic analysis, focusing on a variety of droplet microfluidics techniques, including digital microfluidics (DMF). The article also highlights versatile droplet-based microfluidics that can be integrated with MS systems for proteomic analysis of limited input samples. Furthermore, we summarize the current applications of microproteomics in biological research and conclude with a discussion of the challenges and future perspectives on advancing integrated microfluidics-MS strategies for biological studies and clinical applications.

2. Droplet-based microfluidics and its functions in biological analysis

2.1. Droplet generation in microfluidics and main platforms

In droplet-based microfluidics, droplets are typically formed when a shear force is applied to a liquid–liquid interface. The force can be exerted through either passive control of the hydrodynamic flow or active control of the external power supply [29]. As shown in Figs. 2(a)–(d) [30,31], in passively driven methods, two immiscible fluids meet at a junction to form an interface, and the microchannel junction geometry defines the fluid fields to ensure droplet breakup. The interfacial tension between the fluids and channel walls, along with the viscous forces, dictates the discrete and continuous phases of the fluids. This allows one fluid, usually the carrier fluid, to preferentially wet the channel surface, while the discrete phase remains isolated from the channel surface due to a thin layer of carrier fluid in between [30]. Passive forces to induce droplet flow are most commonly generated by T-junction [32,33], Y-junction [34], co-flow [35], flow focusing [36,37] and cross-focusing flow [38]. In all cases, fluid behaviors are characterized by dimensionless parameters that are relevant to the fluid properties, flow conditions, and device geometry [26]. For example, the Sequential Operation Droplet Array (SODA) system, developed by Zhu et al. [39], is a classic droplet microfluidic platform that uses capillary probes for precise liquid metering and handling, microdroplet array chips for systematic droplet loading, and a sophisticated control unit for accurate positioning. This system can flexibly manipulate droplets ranging from nanoliters to picoliters, facilitating complex droplet management tasks that are critical for single-cell assays and high-throughput drug screening. Based on the SODA system, several nanoliter-scale droplet microfluidic systems have been developed for SCP. Typical ones include the nanodroplet processing in one pot for trace samples (nanoPOTS) platform [40]. This system enables the processing and preparation of trace biological samples within nanoliter-sized droplets, significantly enhancing the detection of low-abundance biomolecules for MS analysis.

In the context of actively driven microfluidics (such as the DMF technology based on the electrowetting-on-dielectric (EWOD) [41–43] effect, see Figs. 2(e) and (f)), external forces directly activate or assist to generate droplets [31]. Depending on the energy sources, droplet microfluidic systems can be classified into electrical [31,44,45], thermal [46,47], magnetic [48], and mechanically activated methods [49]. Additionally, droplets can be generated through multiple emulsions using multiphase structures, such as cylindrical glass capillaries and square glass tubes, which work jointly to form the droplets [50]. Droplets can function as individual microreactors for biochemical analysis and need to be precisely manipulated following their generation. These manipulations, which include sorting, trapping, mixing, splitting, and merging, facilitate the implementation of desired laboratory-on-a-chip applications. For instance, sorting involves rapid assessment of the content encapsulated in the droplet and triggering flow perturbations to direct droplets of interest away from the primary flow. Dielectrophoretic, acoustic, and mechanical sorters are widely used to control droplet movement and sort droplets. Additionally, various functional components, such as droplet traps [51], synchronizers, diluters [52], and mixers [53], have been developed and play important roles in various experimental workflows. With the advancement in microfabrication, compact and versatile dro-

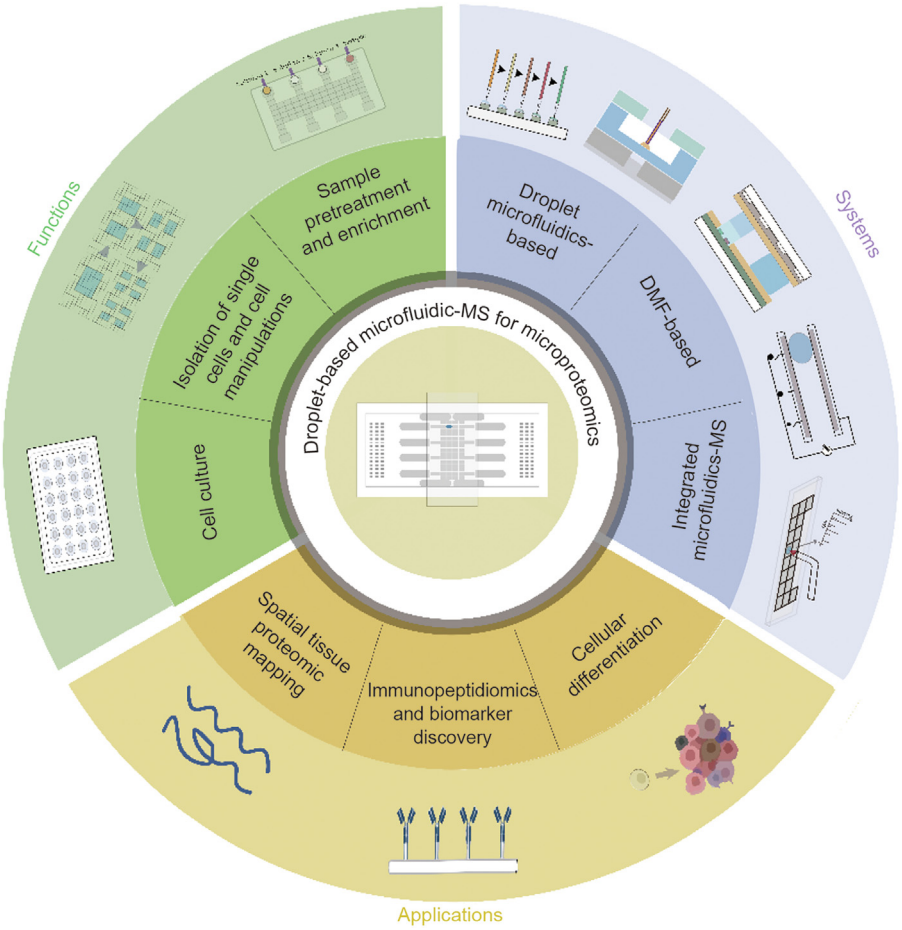

Fig. 1. Schematic illustration of three aspects of droplet-based microfluidics-MS, including functions, systems, and applications. DMF: digital microfluidics.

plet microfluidic chips with multiple and integrated functions show great potential to probe complex biological systems, providing functions including cell culture, cell sorting, and sample preparation, which will be introduced in detail in the following sections. In particular, DMF has emerged as an innovative technique for the precise and flexible manipulation of droplets or particles within the picoliter to nanoliter range [54–57]. By using an array of electrodes, DMF enables programmable control over individual droplets [58].

Both DMF and flow-based droplet microfluidics are flexible methods for manipulating microscale to nanoscale fluid droplets. DMF operates based on the EWOD principle to handle relatively large droplets at the microliter to nanoliter scale, offering precise control for complex, multi-step assays with a throughput defined by the electrode array configuration. In contrast, flow-based systems generate smaller, highly uniform droplets ranging from nanoliters to picoliters at high frequencies using passive control of the hydrodynamic flow through mechanisms such as T-junctions and flow focusing. They typically support simpler assays due to the linear nature of the droplet flow. Therefore, the selection between DMF and flow-based droplet microfluidics depends on the required balance between assay complexity and throughput.

2.2. Cell culture

In-vitro cell culture methods often maintain a large population of cells, which can obscure the effect of microenvironmental factors and the inherent cellular diversity. Conventional *in-vitro* cell culture requires laborious, experienced, and tedious liquid handling in culture flasks to supply the necessary nutrients for cell growth. The emerging microfluidic systems revolutionize traditional protocols by automating the handling process, such as precise feeding at controlled rates, which is rather difficult to achieve using conventional methods. In droplet-based microfluidic systems, cells are encapsulated in individual droplet compartments, while nutrients and growth factors are supplied through the continuous phase of the culture medium. This approach efficiently reduces the chance of cell contamination and facilitates air and nutrient exchange. Both cells and microorganisms can be cultured in droplet-based microfluidic chips with the viability/state easily monitored and evaluated [30,59–61]. This approach further involves low reagent usage and enables high throughput screening of heterogenous cells. For instance, Mazutis et al. [62] developed a modular droplet microfluidic system for high-throughput cell screening, as shown in Fig. 3(a). Individual cells were encapsulated in picoliter droplets along with reagents, incubated off-chip, and reinjected for downstream sorting and analysis. As an example, hybridoma cells were screened for antibody secretion by co-encapsulating with beads to trigger fluorescence signals. The microfluidic approach provides highly efficient flexible liquid handling to culture and sort single cells. However, studies have suggested that cells cultured in small droplets tend to show delayed cell cycle progression, slow proliferation, and altered morphologies [63]. Thus, various methods have been studied to improve the droplet biocompatibility and enhance the cellular vitality.

Hydrogel droplets provide a natural biomimetic 3D environment, mimicking the extracellular matrix for cells [64]. Tiemeijer et al. [65] integrated thermo-reversible polyisocyanide hydrogels into droplet microfluidics to enable the high-throughput culture and analysis of individual adherent immune cells, as shown in

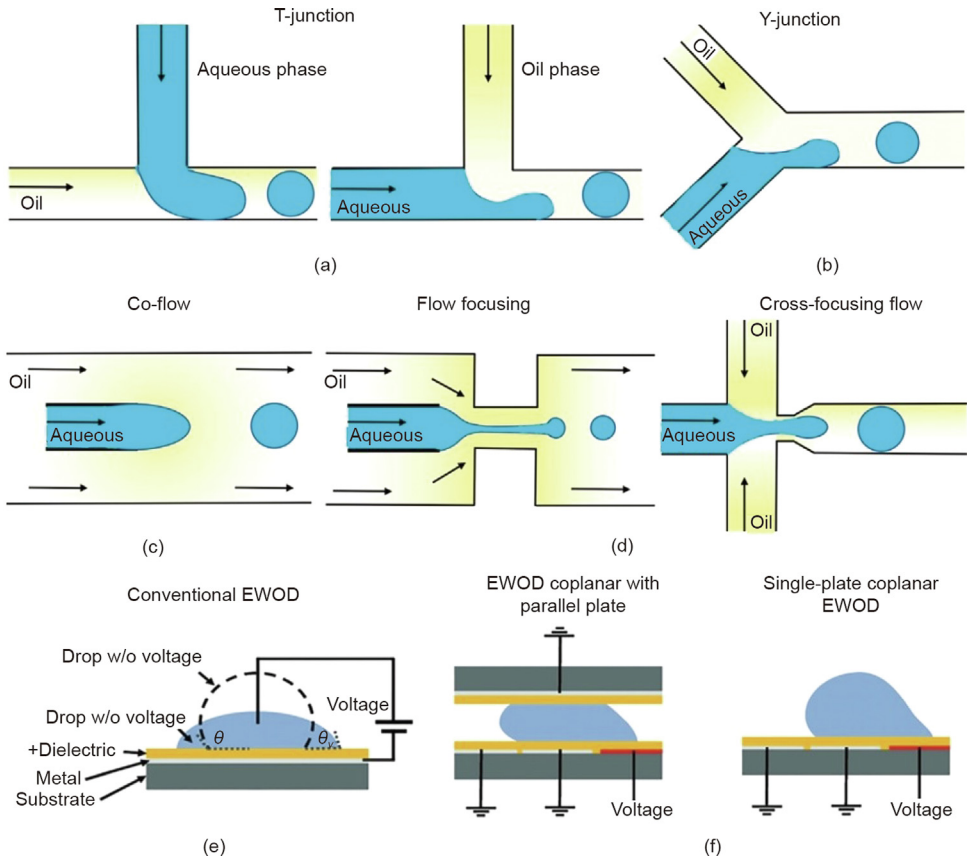

Fig. 2. Microfluidic strategies for droplet generation, including (a) T-junction, (b) Y-junction, (c) co-flow, (d) flow focusing and cross-focusing flow, and (e, f) electrowetting-on-dielectric (EWOD). θ: the contact angle, defined as the angle formed between a liquid droplet and a solid surface. The definitions of all the abbreviations in the figure could be find in the cited references. Reproduced from Refs. [30,31] with permission.

Fig. 3(b). The hydrogel droplets allow gentle encapsulation of cells while providing a 3D matrix for adhesion and growth under controlled conditions. The thermo-responsive gelation of polyisocyanide facilitates droplet formation and retrieval of cells for downstream assays. Moreover, using functionalized gels has shown improved cell viability and enhanced anti-inflammatory polarization. Employing this platform for the analysis of macrophage polarization has revealed a subset of distinct cell phenotypes and persistent pro-inflammatory polarization heterogeneity that remained hidden in bulk cultures. Moreover, Lin et al. [66] reported a microfluidic system for the high-throughput 3D culture of individual cancer cells to selectively expand colorectal cancer stem cells (CSCs), as illustrated in Fig. 3(c). The system encapsulates cells in monodisperse alginate hydrogel microdroplets. The hydrogel provides a 3D matrix for cells to proliferate while preventing aggregation. Non-adhesive hydrogels mimic starvation to induce anoikis of non-CSCs. Subsequent decapsulation through alginate liquefaction allows the recovery of viable spheres or CSCs for downstream assays such as RNA-sequencing. This platform successfully combines high-throughput single-cell 3D culture with controlled microenvironments for selective CSC expansion and drug discovery. Overall, hydrogel droplet microfluidics approaches provide a biomimetic 3D culture matrix with natural biocompatibility and flexible phase control, making them promising platforms for the investigation of cellular functions.

2.3. Cell sorting and manipulation

Apart from cell culture, the ability to isolate and manipulate single cells at the microscale is another advantage of droplet-based microfluidic technology in the field of biological analysis. Microstructure trapping techniques, such as hydrodynamic droplet and microwell systems, leverage the inherent properties of microfluidic structures to confine and immobilize targeted cells. The approaches facilitate efficient cell trapping and isolation by precisely controlling the fluid flow and channel geometries. In contrast, external force-based trapping methods, such as dielectrophoresis, optical tweezers, and acoustic tweezers, leverage external forces to capture and manipulate cells. These approaches greatly improve the efficiency of cell sorting by controlling their movement and position [67,68]. For example, Khajvand et al. [69] created an integrated microchip for multiplexed cellular protein secretion assays at the single-cell level. This device generates a static droplet to trap single cells, perform antibody assays, and identify specific secreted proteins from the cell. It mainly consists of a microfluidic chip featuring an array of droplet chambers and a glass substrate with four different antibodies to capture the proteins. Fig. 4(a) illustrates the workflow, in which individual cells are trapped and encapsulated into droplets using compressed air. The cells in the droplet secrete proteins that are captured by the antibodies and analyzed using a fluorescence-based enzyme-linked immunosorbent assay.

Moreover, Hu et al. [70] presented an active-matrix (AM)–DMF platform based on thin-film transistor technology [71,72] for the manipulation of single cells using picoliter droplets. The thin-film transistor technology allows the fabrication of large arrays of individually addressable small-sized electrodes that can simultaneously manipulate massive amount of nanoliter to picoliter droplets. This unique function enables the automated sorting and moving of single cells using the droplets as traps and containers. Multiple droplets with single cells encapsulated in them could be generated within 10 s without the need for prefabricated physical

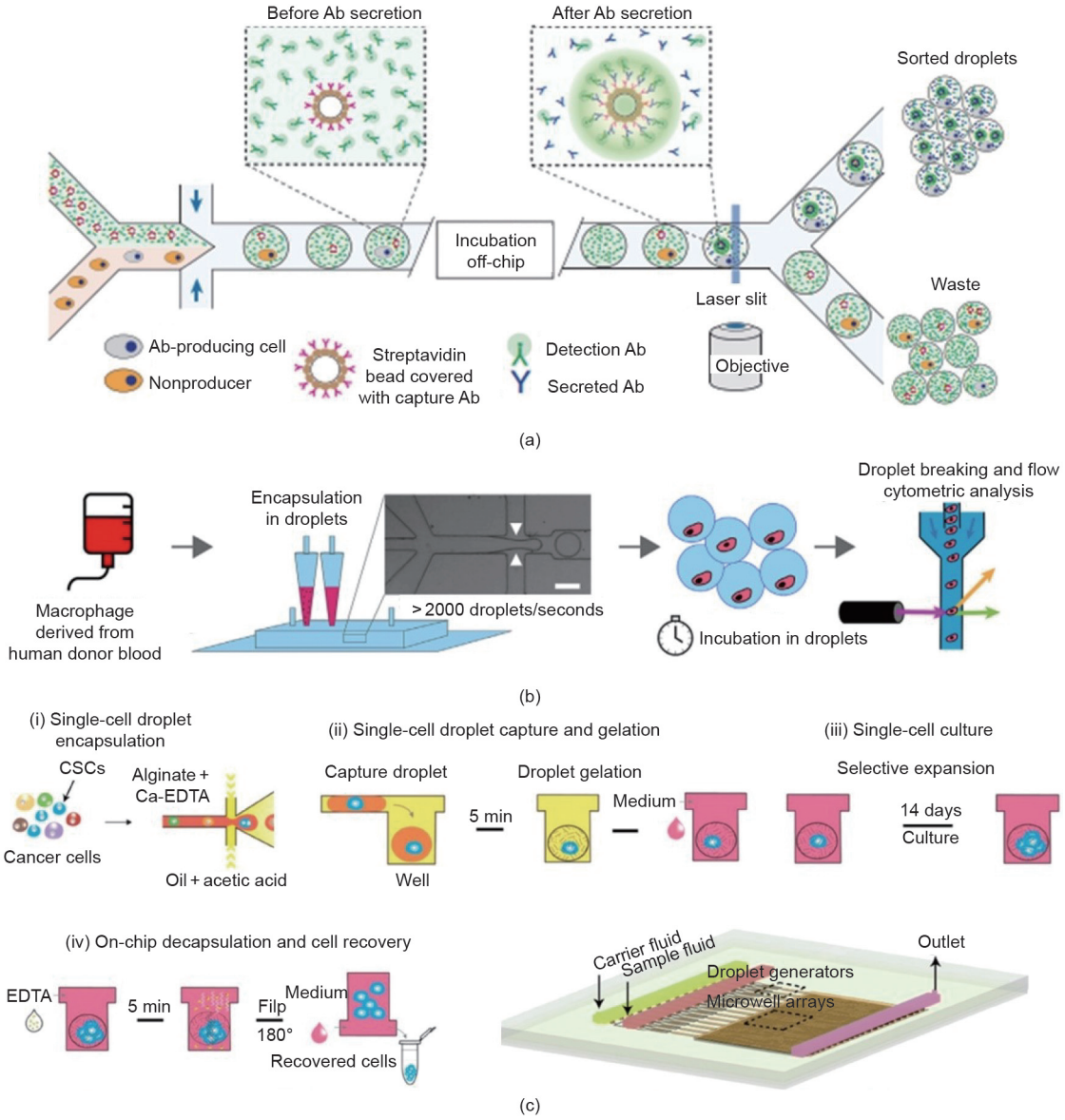

Fig. 3. On-chip cell culture. (a) Design of droplet microfluidics involves encapsulating a mixed cell suspension containing two cell types, one secreting antibodies (Ab) (gray) and the other not (orange), along with Ab-coated beads, within droplets formed using fluorinated oil. After off-chip incubation, droplets containing Ab-secreting cells and fluorescent beads are collected, while droplets with non-secreting cells or no bead are discarded. Reproduced from Ref. [62] with permission. (b) Schematic workflow of single-cell culture and the single-cell analysis of primary macrophages in a droplet microfluidic chip; white arrows highlight the oil cuts of droplets, and the scale bar is 50 μm. Reproduced from Ref. [65] with permission. (c) Illustration of encapsulation and decapsulation of single-cell alginate microgel array. The inset shows the structure of multilayered microfluidic chip. EDTA: ethylenediaminetetraacetic acid. Reproduced from Ref. [66] with permission.

microstructures, as shown in Fig. 4(b). More importantly, the single cells inside the droplets remained viable and were successfully cultured on-chip .

Other kinds of droplet microfluidics approaches exist for on-chip cell encapsulation, cell translation, and cell extraction [67,73]. For instance, Samlali et al. [74] developed a hybrid microfluidic device consisting of a droplet generator and a droplet array. This device allows cells to be manipulated through electrodes and the encapsulation of single cells within picoliter droplets. As illustrated in Fig. 4(c), both *in situ* droplet generation and cell manipulation were achieved, which are useful for monoclonal cell line development. In addition, Jing et al. [75] reported the use of a droplet jetting generator to encapsulate individual cancer cells in picoliter droplets and sort the droplets using a size-based deterministic lateral displacement (DLD) channel. The single cell encapsulated in the droplet was then analyzed using single-cell protease assays to measure the secretions of heterogenous matrix metalloproteinase. This platform offered a high single-cell encapsulation rate of 78%, enabling direct single-cell enzymatic activity analysis without immunestaining and active sorting, which was very useful for the research of precision medicine and tumor therapeutics. Overall, droplet-based microfluidic chips are valuable for cell isolation and manipulation, enabling high-throughput cell analysis, real-time monitoring, and the exploration of dynamic cellular functions with high sensitivity and resolution [73,76].

2.4. Sample pretreatment and enrichment

The way biological samples are handled directly influences the analysis results. Currently, there is an urgent demand for automated, highly precise, and high-throughput sample handling systems. Droplet-based microfluidics plays an important role in sample pretreatment and enrichment, enabling automated sample preparation with reduced sample loss for biomolecular analysis [77–79]. For

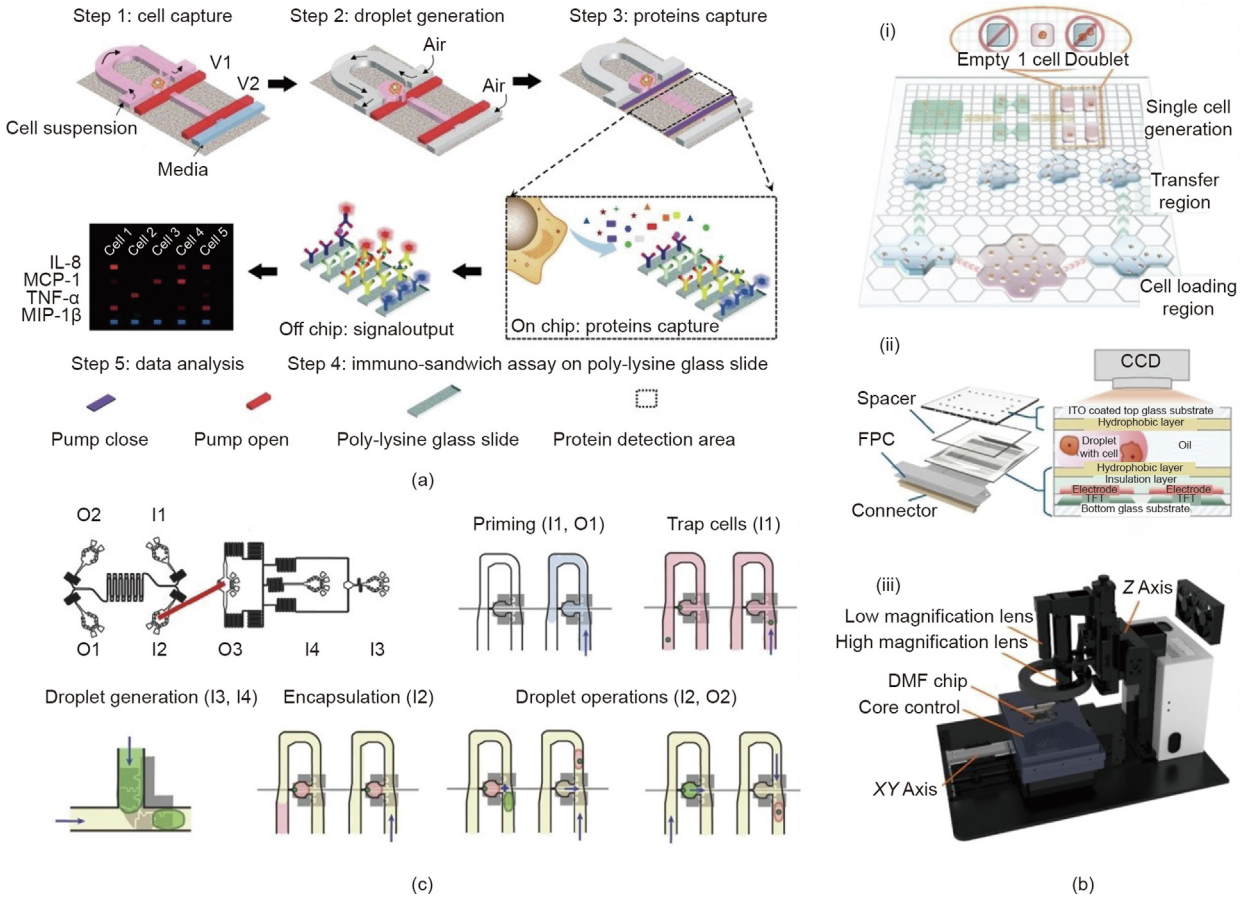

Fig. 4. Isolation of single cells. (a) Schematic workflow of the integrated platform: single cells are isolated into 100 individual picochambers with closed microvalves that are pre-labeled with an array of Ab barcodes. Following 4 h of incubation, the captured proteins secreted by individual cells are determined by fluorescence sandwich enzyme-linked immunosorbent assay. V1, V2: horizontal blocking microvalve; IL-8: interleukin-8; MCP-1:monocyte chemoattractant protein-1; TNF-α: tumor necrosis factor-α; MIP-1β: macrophage inflammatory protein-1β. Reproduced from Ref. [69] with permission. (b) AM–DMF chip for single-cell generation and manipulation: (i) schematic diagram of the functional workflow, (ii) structure and cross-sectional view of the AM–DMF chip, and (iii) control, movement, and imaging system of the AM–DMF chip in a Boxmini system. CCD: charge-coupled device; FPC: flexible printed circuit; ITO: indium tin oxide; TFT: thin-film transistor. Reproduced from Ref. [70] with permission. (c) Workflow of the hybrid microfluidic device: chip is primed with Pluronics F-127, then cells are trapped, and droplets are generated around the trapped cells by flowing oil with fluorosurfactant. The cell-containing droplets flow through a bridge, followed by droplet manipulation. I1–I4: inlet; O1–O3: outlet. Reproduced from Ref. [74] with permission.

example, a digital microfluidic platform for the processing and extraction of estradiol samples was reported [80]. As shown in Fig. 5(a), the chip incorporates five reservoir electrodes, with four of them dedicated to raw samples, lysing solvent, polar extraction solvent, and nonpolar extraction solvent. The fifth electrode reservoir serves as a collection point for the processed sample. Movement and transport of individual droplets is precisely regulated by applying bias voltages on specific electrodes. Improved reproducibility and accuracy of the sample extraction were achieved using this chip. Furthermore, in the integrated microfluidic chip shown in Fig. 5(b) [81], inkjet cell printing is used to encapsulate single cells in a droplet, which are then transferred into a dielectrophoretic channel. The captured single cells are manipulated into a confined region within the droplet and then separated through a Y-shaped structure to remove the interfering culture medium. The resulting smaller droplets, each with a single cell, are transported to an interface and incorporated for MS analysis, enabling automated single-cell encapsulation and profiling.

Various droplet microfluidic devices have demonstrated their usefulness in biological analysis. However, there is a need for a versatile platform that can adapt to different applications. Zhu et al. [39] introduced the SODA system, offering automated and flexible manipulation of nanoliter to picoliter droplets. Fig. 5(c) illustrates a SODA device that comprises capillary probes, microdroplet array chips, and a movement control unit. Through the joint operations of aspirating–depositing–moving (ADM), the SODA system enables complex droplet handling tasks such as assembling, fusion, transferring, addressing, and capture. A practical application is demonstrated in single-cell reverse-transcription quantitative polymerase chain reaction (PCR) assays, where the SODA system efficiently executes sequential sample processing steps, including single-cell encapsulation, thermal lysis, reverse transcription, and detection. For drug screening, the SODA system can be configured into a high-density nanoliter-scale droplet array specifically for enzyme assays. This setup automates the multiple steps required for enzyme inhibition assays, such as adding the enzyme solution, conducting the incubation, and introducing the substrate. As a result, it significantly reduces both sample and reagent consumption. The powerful functions of the SODA system open new possibilities for ultralow sample handling and precise analysis of small-volume samples [82,83].

3. Droplet-based microfluidics in combination with MS

3.1. Advancements in MS techniques for microproteomics

Since a SCP technology was published in *Nature Methods* [84], there has been a rapid growth in the development of new tech-

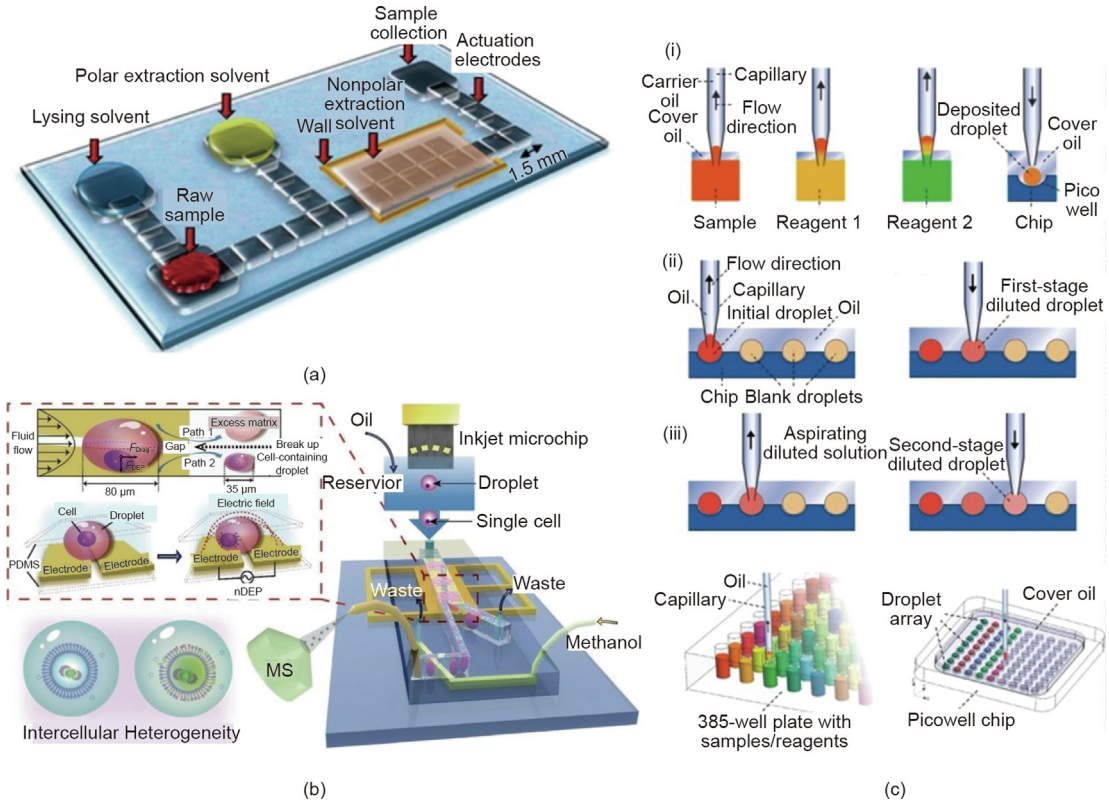

Fig. 5. Sample pretreatment designs. (a) Schematic of the DMF device that includes sample and solvent reservoirs and the liquid–liquid extraction zone. Reproduced from Ref. [80] with permission. (b) Integrated microfluidic platform for online live single-cell lipid profiling. PDMS: polydimethylsiloxane; nDEP: negative dielectrophoresis. Reproduced from Ref. [81] with permission. (c) Illustration of (i) the working principle for droplet array generation and (ii) serial dilution in droplets, and (iii) setup of the SODA system. Reproduced from Ref. [39] with permission.

niques to advance this emerging field. These methods can be broadly categorized into two primary strategies: ① enhancing the protein coverage of individual cells by reducing sample loss or increasing instrumental sensitivity, and ② improving the throughput by leveraging parallelization, automation, and advanced microfluidics to efficiently analyze thousands of individual cells in parallel.

In reducing sample losses, a series of proteomic platforms have been developed for characterizing proteins from small samples (e.g., < 1000 cells) by integrating cell lysis, digestion, or detergent treatment in a compact and miniaturized setting to reduce the loss of sample during the transfer process. Typical systems include the integrated proteome analysis device (iPAD)-100 [85], single-pot solid-phase-enhanced sample-preparation (SP3) [86], miniaturized filter-aided sample preparation [87], angled-shape tip reactor [88], simple and integrated spintip-based proteomics technology [89], and automated preparation in one pot for trace samples (autoPOTS) [90]. A water-droplet-in-oil digestion (WinO) method has been introduced to process cells in a water droplet suspended in ethyl acetate oil by minimizing surface contact and enhancing digestion. This method improves protein and peptide recovery approximately 10-fold compared to conventional in-solution digestion methods [91].

The instrumental sensitivity for proteomic analysis is determined by both the separation step and the mass analyzer. Zhu et al. [92] demonstrated that liquid chromatography (LC) separation using a narrow nanocolumn for low-flow-rate elution can significantly increase the signal, which results in improved proteome coverage. The coupling of ion mobility separation provides additional structural information of the collision cross section, facilitating protein identification [93]. Strategies to optimize MS parameters, such as increasing automatic gain control (AGC) and extending ion injection times, also improve the final results [94]. Moreover, the integration of two advanced technologies, trapped ion mobility spectrometry–time of flight (TIMS–TOF) [95] and high-field asymmetric waveform ion mobility spectrometry (FAIMS) [96], has increased the identification capacity to reveal cellular heterogeneity by incorporating ultrahigh-resolution separations. Novel data acquisition strategies, such as data-independent acquisition, deep-learning-based retention time prediction, and MS1-only acquisition [97], have also been reported for rapid and deep proteomic profiling of small samples with limited quantities. An integrated approach such as an ultrasensitive MS-based workflow for true SCP (T-SCP) combines miniaturized sample preparation, very-low-flow chromatography, and a new trapped ion mobility mass spectrometer with high sensitivity. This platform enables robust quantification of over 1000 proteins per single cell as well as the analysis of biological responses to drug perturbation by profiling over 400 single cells. The resulting proteomics data showed a stable "core proteome" across cells despite perturbations, in contrast to the greater variability observed in single-cell transcriptomes [95].

Typical approaches for enhancing proteomic throughput include multiplexed Single-Cell ProtEomics (SCoPE) methods [98–101]. Budnik et al. [98] and Petelski et al. [100] developed a strategy using tandem mass tags (TMT) to label peptides from single cells, along with a larger "carrier" proteome to boost identification. These labeled peptides are then pooled and analyzed by LC–MS/MS. The carrier proteome enhances the peptide identification, while the TMTs enable relative quantification of proteins across single cells. Merging multiple samples further reduces the risk of sample loss during the detection process. Based on these advanced

features, this TMT-carrier approach has been successfully applied for quantification of phosphoproteins and *N*-glycopeptides from individual cells [94,102].

3.2. Droplet microfluidics based on independent dispensing techniques

Independent dispensing techniques are methods that employ automated, modular units, such as syringe pumps, droplet microfluidics, and acoustic droplet ejection, to precisely dispense fluids in volumes ranging from nanoliters to microliters. These techniques allow for accurate and contamination-free liquid handling, which is essential for many scientific and industrial applications. Droplet microfluidics offers competing capabilities in miniaturization and integration to promote proteomic analysis of limited input samples from individual cells in a highly parallel manner. In recent years, droplet-based microfluidic techniques have opened new possibilities by enabling the manipulation of cell-encapsulated droplets with high precision and throughput. This innovative approach offers exceptional control over the reaction conditions, enhanced sensitivity, and reduced sample loss. A typical example is the nanoPOTS [40] and its versatile versions. By reducing the sampling volume to < 200 nL, surface-adsorption-induced sample loss is minimized and an increased rate of enzymatic digestion is achieved. In combination with LC–MS, the system enables > 3000 proteins to be consistently identified from ~10 cells by incorporating the Match Between Runs algorithm, as shown in Figs. 6(a) and (b). Moreover, Zhu et al. [103] used fluorescence-activated cell sorting (FACS) to deposit cells into the defined nanoPOTS chip, and an average of 670 protein groups were identified from single HeLa cells using nanoLC–MS. This method also enables differentiation of human lung primary cells based on proteome expressions.

One of the versatile versions of nanoPOTS is the protein quantification platform, which is based on an improved boosting to amplify signal with isobaric labeling (iBASIL) strategy with nanoPOTS [94], as illustrated in Fig. 6(c). This strategy allows for the precise quantification of 1500 protein groups from FACS-sorted single cells and enables the boosting-to-sample (B/S) ratio of 100 used for implementing iBASIL for single-cell proteomic analysis. Higher B/S ratios may result in enhanced proteome coverage, but with a trade-off of quantifiable proteins loss, as shown in Fig. 6(d). Furthermore, Dou et al. [104] integrated microfluidic nanodroplets with TMT isobaric labeling to probe proteomes of single cells. Improved throughput was acquired with multiplex analysis, and this strategy enabled over 2300 proteins to be identified from 72 individual murine cells within two days. Another type of nanoPOTS is an automated proteomic imaging platform [105] with laser capture microdissection (LCM), an automated sample transport device, and nanoPOTS integrated. Fig. 6(e) illustrates the typical workflow. Over 2000 proteins were quantitatively mapped at a spatial resolution of 100 μm in a mouse uterus tissue section, as presented in Fig. 6(f). The system allows for visualizing cell-type-specific proteome alterations, providing in-depth insights into the spatial distribution and heterogeneity of uterine proteins.

Furthermore, a nested nanoPOTS (N2) chip [106] was proposed for high-throughput and streamlined SCP. The N2 chip reduces the sampling volume to < 30 nL, enhances the chip capacity to 240 cells, and simplifies the TMT labeling step. Fig. 6(g-i) shows the chip structure with nine nanowells nested and circled with a hydrophilic ring for TMT. The SCP workflow is shown in Fig. 6(g-ii). The platform has been shown to have good sensitivity for quantifying ~1500 protein groups from three different cell lines, and the protein distribution of identified proteins is displayed in Fig. 6(g-iii). This platform shows great potential in probing tumor heterogeneity and cellular differentiation. Additionally, a recently developed dual-column nanoflow LC system enables the analysis of over 200 single cancer cells per day using the nanoPOTS workflow [107]. This system multiplexes sample loading, online desalting, and column regeneration between two parallel subsystems. Using optimized settings and MS1-based feature mapping, this platform identified ~660 proteins per cell in 15 min cycles and ~990 proteins per cell in 30 min cycles. The platform demonstrates the potential to analyze hundreds of single cells per day with wide proteome coverage.

Another SODA-based microfluidic device was a nanoliter-droplet sandwich-type microchip, the oil–air–droplet (OAD) chip [108], which provides an *in situ* microreactor for multi-step pretreatment. Air is introduced to isolate the droplet from the covered oil, and the oil layer forms a closed chamber to protect the sample droplet from evaporation. The captured cells are lysed, and the released proteins are then reduced, alkylated, and digested in the droplet, as shown in Figs. 7(a)–(c). By integrating with LC–MS, this system allows proteomic profiling of small cell populations with minimized sample loss and high efficiency. Over 355 protein groups have been identified from a single mouse oocyte.

These droplet-based microfluidic platforms in combination with MS have significantly advanced SCP. Despite proteins being building blocks of biological systems, post-translational modifications and metabolites also play a crucial role in regulating molecular functions and signaling pathways. Recently, a microwell-chip-based strategy [109] was reported for the simultaneous characterization of proteins and metabolites, as illustrated in Fig. 7(d). The microwell inner surface was modified to sequentially extract metabolites and proteins. With the data-independent acquisition (DIA)-MS approach employed, the platform enables the identification of > 1200 proteins, > 130 putatively annotated metabolites, and large-scale phosphorylation data sets. Overall, droplet-based microfluidic platforms facilitate multi-step sample preparation in a single microchip and a decreased sampling volume, resulting in reduced sample loss, high sensitivity, and enhanced throughput for in-depth proteomic profiling.

3.3. DMF

Versatile DMF platforms have been developed to enable processes such as monitoring rapid chemical reactions [110], protein extraction and purification [77], and proteomic sample preparation for MS [111,112]. For example, Jebrail et al. [113] demonstrated dried blood spot analysis by integrating DMF with microchannel nano-electrospray-ionization (nanoESI) to quantify extracted amino acids. They have investigated both off-line and in-line DMF integration with MS, establishing foundations for further progress in biological studies [114–116].

DMF has been used in recent years to prepare samples for MS-based proteomics with small amounts of input samples. For instance, Leipert et al. [117] demonstrated the first DMF strategy for proteome sample preparation and established a standard workflow. An SP3 step is introduced to remove polymeric detergents through magnetic beads for protein clean-up, as shown in Fig. 8(a). A detergent–buffer system is then optimized to be fully compatible with DMF chips as well as downstream digestion and LC–MS analysis (Fig. 8(b)) [117]. The DMF-SP3 method, in combination with MS, allowed for ~1200 proteins to be identified from 100 Jurkat T cells, demonstrating good sensitivity in analyzing limited biological materials. In addition, a DMF microproteomics workflow has been reported for analyzing tissue biopsies, suggesting the potential use of DMF for clinical applications [118].

Furthermore, the quantification capability of the DMF-SP3-MS was investigated. To implement isobaric peptide labeling for multiplexed quantitation, various detergents were tested and the maltoside-based detergent 3-dodecyloxypropyl-1-β-*D*-maltopyranoside (DDOPM) was determined to be the ideal one to

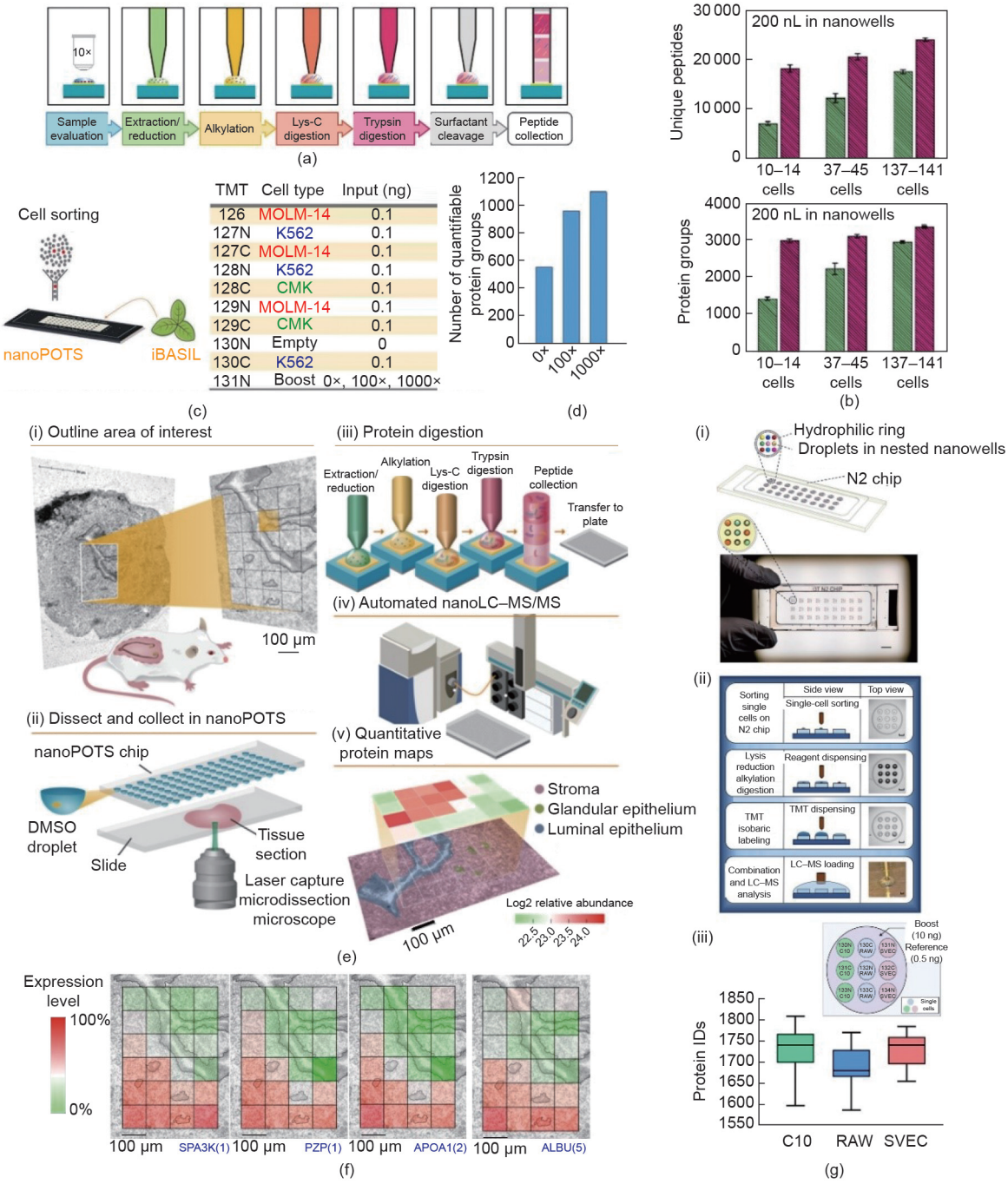

Fig. 6. Droplet microfluidic-based system for proteomic MS and imaging. (a) One-pot protocol for proteomic sample preparation with nanoPOTS. Lys-C: endoproteinase Lys-C. (b) Numbers of unique peptides and protein groups identified from different cell loadings determined in triplicate. Reproduced from Ref. [40] with permission. (c) Schematic and TMT channels for the nanoPOTS-iBASIL analysis of FACS-isolated single-cell samples (K562, MOLM-14, and CMK cells). MOLM-14: acute myeloid leukemia cell line; CMK: chronic myeloid leukemia cell line. (d) Numbers of quantified proteins for the quantitative SCP analysis using three different boosting ratios (no boosting, 100×, and 1000×). Reproduced from Ref. [94] with permission. (e) Schematic workflow of spatially resolved proteomics using the nanoPOTS imaging platform. DMSO: dimethyl sulfoxide. (f) Distribution of serine protease inhibitor A3K (SPA3K), pregnancy zone protein (PZP), apolipoprotein A-I (APOA1), and serum albumin (ALBU) in stromal-dominant uterine section. Reproduced from Ref. [105] with permission. (g) Illustration of (i) the structural design and (ii) SCP workflow of the nested nanoPOTS (N2) chip and (iii) the distributions of the protein identification numbers (n = 36 single cells for each cell type). The inset shows the single-cell isolation and TMT labeling on the N2 chip. C10: mouse embryonic fibroblasts; RAW: murine macrophage cell line; SVEC: murine lymphatic endothelial cell Line; IDs: identifiers. Reproduced from Ref. [106] with permission.

facilitate droplet movement and TMT labeling with minimized interference to peptide detection, as shown in Fig. 8(c) [119]. This is the first on-chip isobaric-labeling-based quantitative proteomics approach, enabling the identification of differentiated proteins expressed upon drug treatment. Another quantitative proteome analysis was conducted by coupling DMF–MS with FAIMS, as shown by the DMF workflow in Fig. 8(d) [120]. The system enables quantification of proteins from a single *Caenorhabditis elegans* cell. Moreover, a facile one-pot sample preparation strategy was recently proposed based on DMF-SP3. By eluting intact proteoform with 40% formic acid (FA), top-down proteomic analysis of single *Caenorhabditis elegans* nematodes was performed, and the quantification results of differentiated nematode proteins were indistinguishable from those of bottom-up methods, as presented in Fig. 8(e) [121].

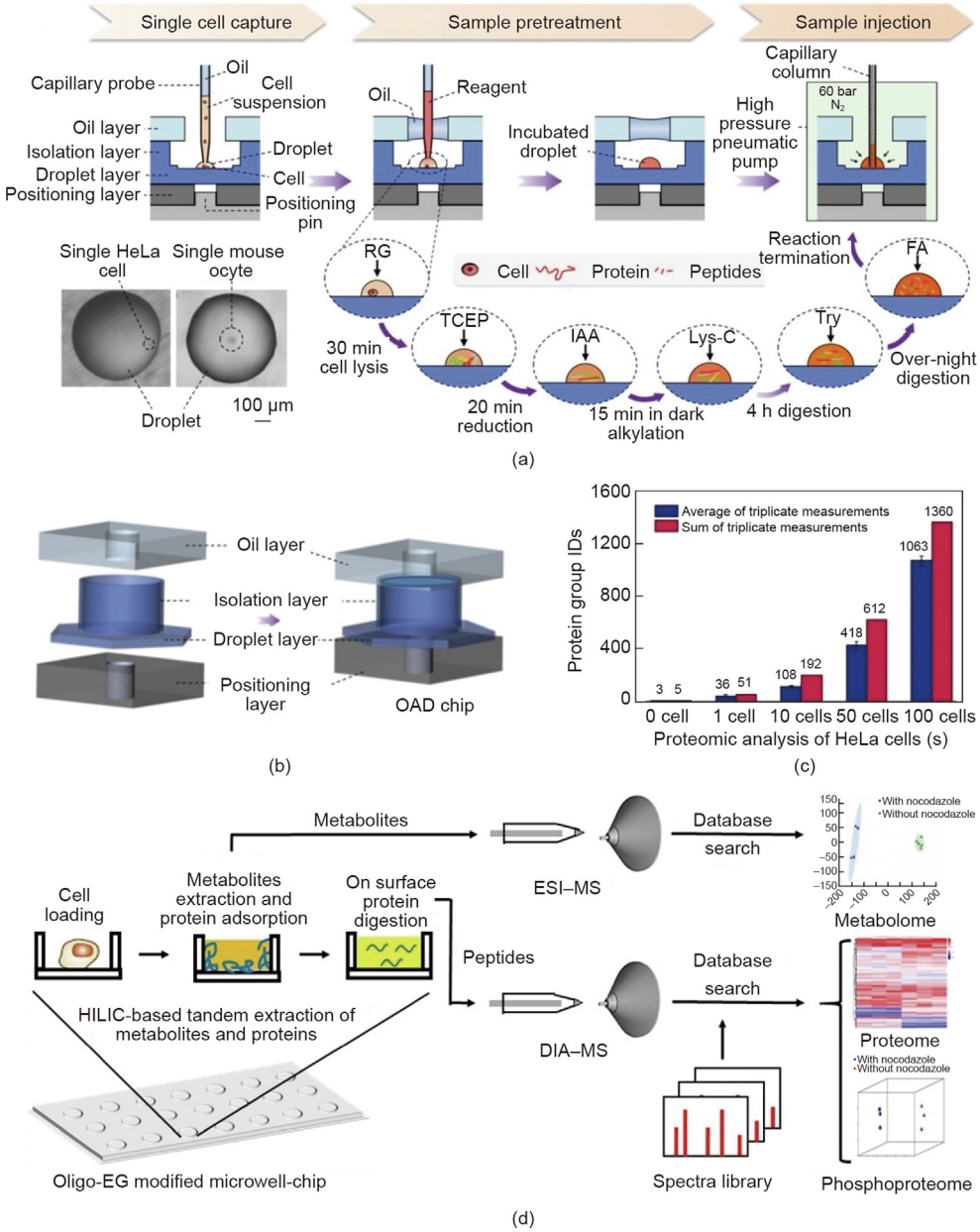

Fig. 7. (a) Schematic workflow of sample pretreatment and injection for single-cell proteomic analysis using the nanoliter-scale OAD chip. RG: RapiGest; TCEP: tris(2-carboxyethyl)phosphine; IAA: iodoacetamide; Try: trypsin; FA: formic acid. (b) Structure of the OAD chip. (c) Number of identified proteins from samples containing 100 ± 10, 50 ± 5, 10, and 1 HeLa cells. Reproduced from Ref. [108] with permission. (d) Schematic overview of the hydrophilic interaction LC (HILIC)-based tandem extraction of metabolites and proteins, and the strategy for multiomics analysis of single cells. Oligo-EG: 2-[methoxy(polyethyleneoxy)propyl]trimethoxysilane; ESI: electrospray ionization. Reproduced from Ref. [109] with permission.

As DMF shows advantages of precise droplet manipulation, high throughput, and miniaturization, a digital microfluidic isolation of single cells for omics (DISCO) platform was developed [122]. It integrates DMF, laser cell lysis, and artificial-intelligence-based image segmentation and recognition for the selection of cells of interest, followed by multi-omics analysis using a different strategy. The DMF platform, illustrated in Figs. 9(a)–(d), enables various processing steps, such as cell lysis and cell content collection. In combination with LC–MS, ~699 proteins were identified from five U87 cells and ~427 proteins were identified from a single U87 cell, which were comparable results to those of reported SCP methods at the time. More recently, AM–DMF has been employed for integrated sample processing for MS, with over 2200 proteins identified from single HeLa cells, thereby providing an automated and sensitive platform for microproteomics [123].

Therefore, DMF devices offer an automated platform for highly sensitive proteomic profiling of small cell populations, advancing our understanding of cellular heterogeneity and uncovering novel insights into complex biological systems.

4. Integrated droplet-based microfluidics-MS system

It is crucial to integrate optimized microfluidic workflows with sensitive MS instrumentation to fully realize the potential of microfluidics for proteomics. The core of this integration is the compartmentalization of samples within droplets, which act as individual micro-reactors, and their subsequent introduction into a mass spectrometer using methods such as electrospray ionization. This not only reduces sample losses during processing and

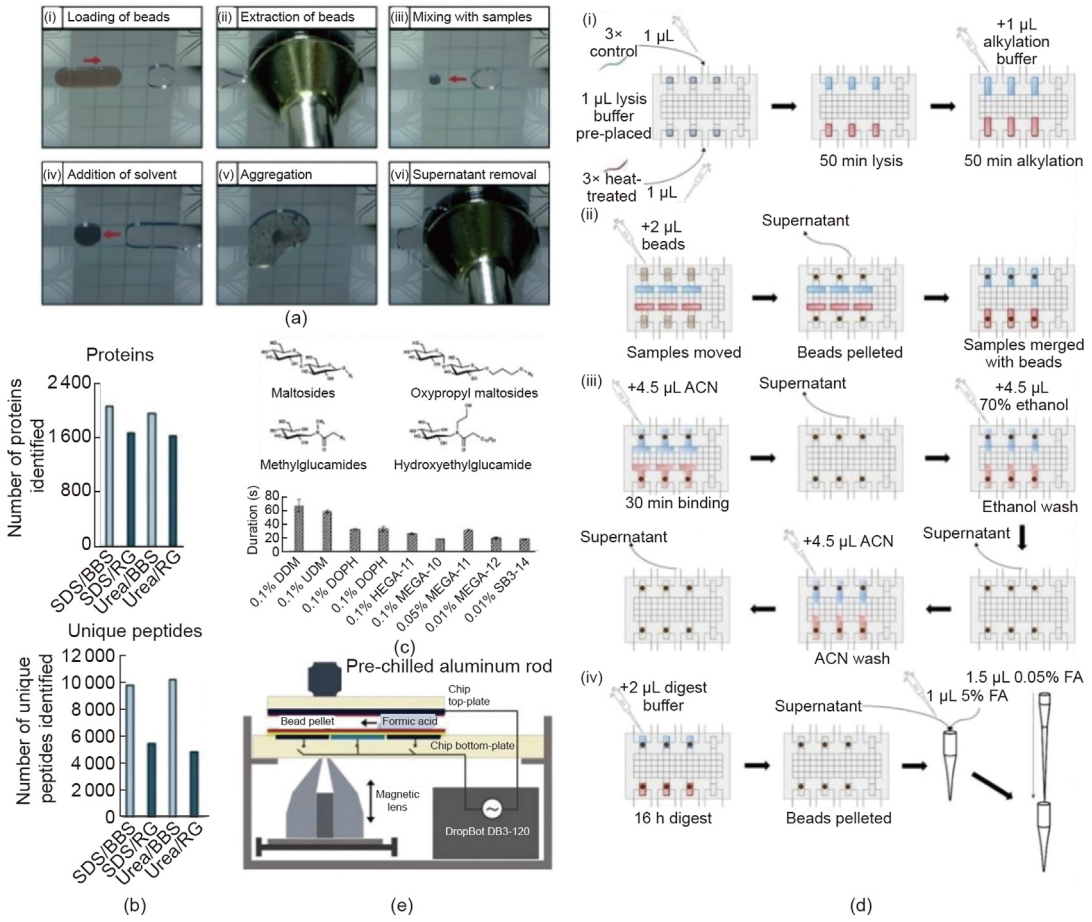

Fig. 8. (a) Sample clean-up by DMF-SP3 using a permanent magnet. (b) Comparison of SDS or urea for on-chip lysis of ~500 Jurkat T cells and RG or BBS for protein digestion after DMF-SP3 cleanup. SDS: sodium dodecyl sulfate; BBS: borate buffer solution. Reproduced from Ref. [117] with permission. (c) Nonionic detergents tested and movement duration of droplets of aqueous detergent solutions on a DMF device tracked by capacitance sensing. DDM: dodecyl β-D-maltoside; UDM: undecyl β-D-maltoside; DOPH: 1,3-diphenyl-2-pyrazoline; HEGA-11: hydroxyethyl glycol amide 11; MEGA: maltoside ethylene glycol amide; SB3-14: sodium butyl-3-sulfonate 14. Reproduced from Ref. [119] with permission. (d) Schematic diagram of the DMF workflow: (i) loading of worms, lysis, and alkylation, (ii) addition of SP3 beads and merging with samples, (iii) SP3 procedure with subsequent washing steps, and (iv) on-chip digestion and subsequent sample retrieval. ACN: acetonitrile. Reproduced from Ref. [120] with permission. (e) DMF setup used for top down-SP3 elution of intact proteins. Reproduced from Ref. [121] with permission.

prevents cross-contamination between samples but also enables high-throughput analysis by allowing the simultaneous processing of multiple samples. Recently, efforts have been devoted to developing integrated DMF–MS systems, and several advanced platforms have emerged with exceptional performances.

A typical example is the all-in-one DMF pipeline proposed by Peng et al. [124]. As shown in Figs. 10(a) and (b), the system features end-to-end automation, which not only allows entire sample processing steps to be completed, including protein alkylation, reduction, and digestion but also incorporates an automated sampling interface directly connected to the LC system. Quantification of trace samples is achieved through dimethyl labeling. As shown in Fig. 10(c), two model breast cancer cell lines were labeled with different isotopic tags, and a total of 973 proteins were identified from the cells. The proteome profiles also corresponded to several known proteins and pathways in cancer cells with association to hormone responses and cell morphologies. The platform has demonstrated good capabilities in automated sampling and proteomic analysis.

A recent online DMF–MS system was developed by coupling DMF with MS through an on-chip microspray hole (DMF-μSH-MS) [125], as shown in Fig. 10(d). This unique electrostatic spray ionization can ionize a small portion of the droplet through the microhole, and the corresponding mass spectrum reveals the chemical contents. This facilitates the time-dependent monitoring of chemical reactions, as the starting material and product are sprayed separately for tracing. Fig. 10(e) presents the mass spectra of cytochrome c and its tryptic digests analyzed using DMF-μSH-MS. Integrated DMF–MS platforms avoid sample transfer with reduced sample loss or contamination and enable direct analysis of droplet samples, opening new possibilities for proteomic profiling of trace samples as well as studying rapid chemical reactions.

Furthermore, the development of a nanoPOTS autosampler, which automates the direct injection of nanodroplets into LC–MS systems, has been reported [126]. This advancement enables the fully automated, high-throughput LC–MS analysis of SCP, which facilitates the detailed quantitative study of cells, including those from acute myeloid leukemia. Moreover, an integrated microfluidic platform, proteoCHIP, has been recently developed for the automated and high-throughput preparation of single-cell proteomic samples for multiplexed quantitative analysis [127]. This microchip enables the parallel processing of up to 592 individual cells utilizing a robotic picoliter liquid-dispensing handler. Cells are directly digested on the chip, labeled with TMT for multiplexing, and seamlessly integrated with an LC–MS autosampler, thus eliminating the need for manual transfers. This system offers an efficient and automated approach for in-depth single-cell proteomic profiling . Alternative sample processing systems also include iProChip and SciProChip, two highly applicable platforms for proteomic profiling across varying cell quantities [128].

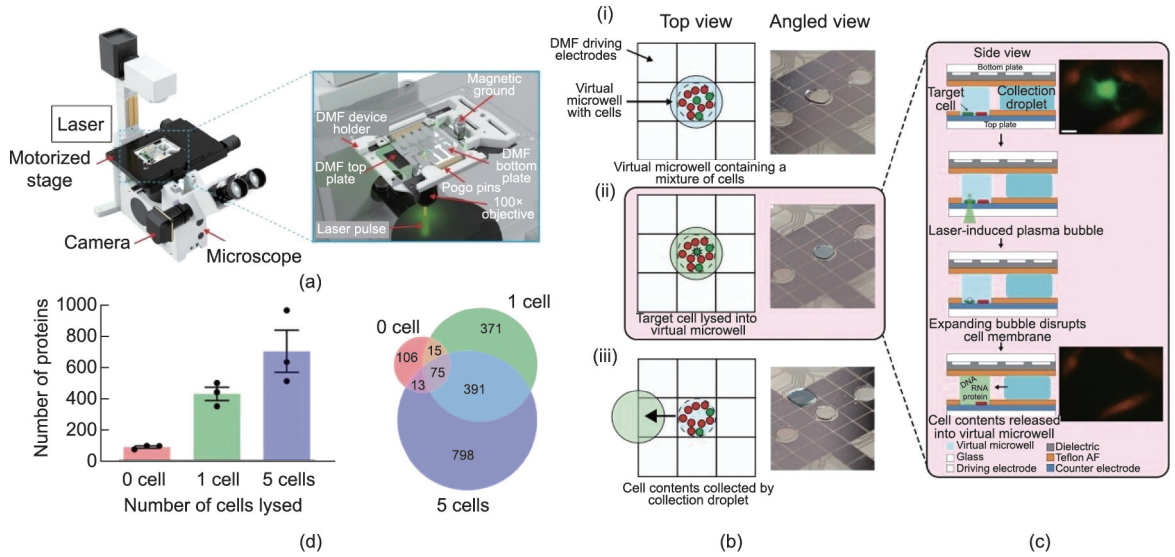

Fig. 9. DISCO platform. (a) Illustration of the platform used for DISCO. (b) Top-view schematics (left) and angled-view photographs (right) of a digital microfluidic device at various stages of processing: (i) mixture of adherent cells (red and green) with a collection droplet (blue), (ii) a single green cell is targeted for laser lysis and collected into a droplet, and (iii) collected droplet (green) is queued for omics analysis. (c) Side-view schematic (left) showing two adherent cells cultured on a digital microfluidic device and laser-induced plasma bubble formation and expansion causing cell lysis to release cell contents into a droplet. Fluorescence microscopy images of a coculture of live cells on the DISCO platform before (top) and after (bottom) lysis of the U87 cell in the center. Scale bar: 50 μm. (d) Number of proteins identified from U87 cell lysate droplets containing zero, one, and five laser-lysed cells. Reproduced from Ref. [122] with permission.

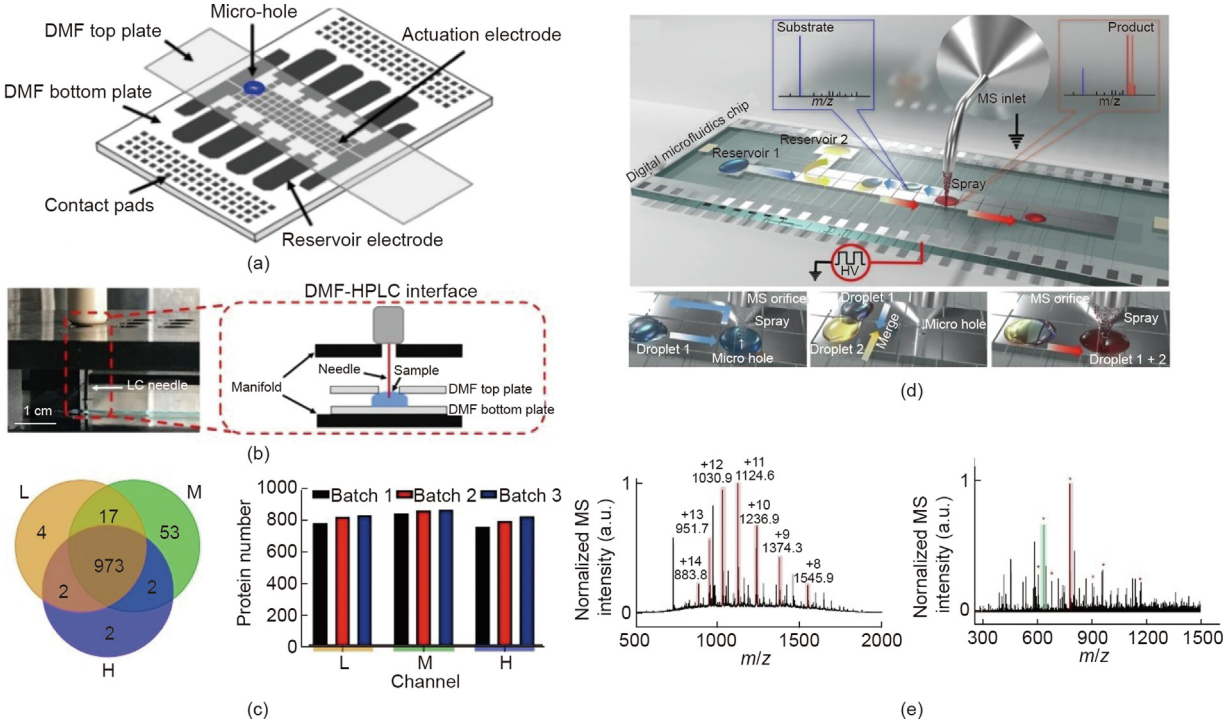

Fig. 10. Integrated microfluidic-MS systems. (a) All-in-one DMF device includes a bottom plate and a top plate featuring a sampling micro-hole. (b) Photograph and schematic (inset) illustration of the sampling process in the DMF-high-performance LC (HPLC) interface. (c) Number of proteins identified from three batches of light (L; MDA-MB-231 cells), medium (M; MCF-7 cells), and heavy (H; MDA-MB-231 cells) channels. Reproduced from Ref. [124] with permission. (d) Schematic illustration of multistage mass spectrometric monitoring of a chemical reaction in DMF via a chip-integrated microspray hole. HV: high voltage. (e) DMF-microspray hole (μSH) mass spectra of cytochrome c and tryptic digest of cytochrome c with all identified peptides marked. *m/z*: mass-to-charge ratio. Reproduced from Ref. [125] with permission.

Therefore, versatile microfluidic devices have demonstrated capabilities in sample pretreatment and enrichment prior to MS. By integrating optimized microfluidics with MS, researchers can gain deep insight into the intricacies of cellular heterogeneity and associated biological processes at the single-cell level. Integrated microfluidics-MS systems greatly facilitate rapid, sensitive in-depth proteomic profiling of limited samples, unlocking new possibilities in cellular biology research.

5. Discussion

With recent advances in microfluidics and MS platforms, microproteomics has exhibited broad applications in biological and clinical studies, such as probing cellular differences, mapping spatial distributions of tissue proteins, and identifying biomarkers for disease diagnosis [129,130]. These achievements have not only expanded our understanding of cellular heterogeneity at the molecular level but also paved the path for more precise and usable approaches in precision medicine.

5.1. Cellular heterogeneity

Emerging microproteomics platforms provide powerful tools to reveal critical insights into tissue heterogeneity at the cellular level. For example, Zhu et al. [40] applied nanoPOTS to analyze individual human pancreatic islet sections that were isolated from clinical tissue samples, comparing islets from a healthy donor to islets from a type-I diabetic donor. The proteomic analysis of 18 single islet sections quantified over 2000 proteins and revealed 304 proteins with significant differential abundance between the diabetic and non-diabetic islets. Key proteins relevant to the type-I diabetes pathology, such as PCSK1 and beta-2-microglobulin, showed marked alterations in abundance between the two donor groups, confirming the loss of beta cells and immune activation in diabetes. Furthermore, Peng et al. [124] applied the integrated all-in-one DMF proteomic workflow to analyze two breast cancer cell lines, MCF-7 and MDA-MB-231, which have different invasion capabilities. They performed quantitative proteomic profiling of the cell lines using dimethyl labeling, and differential protein expression analysis revealed distinctions between the cell lines related to their hormone responses, metabolisms, and morphologies. These findings were consistent with previous reports, and pathway analysis further supported these functional differences. This demonstrated the utility of the integrated DMF platform for cancer subtyping from precious clinical specimens . Additional studies have uncovered distinct proteomic profiles between differentiating embryonic stem cells [98], primary human lung cell subtypes [103], and genetically modified mouse oocytes [131]. These studies offer valuable insights into the molecular basis of tissue heterogeneity and will facilitate future biological and therapeutic research.

5.2. Spatial proteomics

Spatially resolved proteomics preserves anatomical information by correlating proteomic profiles with the cell morphology and position, revealing critical physiologic information obscured in bulk analysis. Microfluidics-based microproteomics approaches have demonstrated their ability to characterize protein distributions in tissue sections with improved resolution. While microfluidics plays a crucial role, LCM is critical for detailed examination of protein expression in tissues. LCM is a precise technique that enables the isolation of specific cells or tissue areas from a heterogeneous sample by utilizing a laser beam under a microscope. This laser beam either excises the targeted cells by cutting around them or activates a transfer film that adheres to the cells, facilitating their removal. Such specificity is crucial for acquiring uncontaminated cell populations for downstream molecular analyses, thereby guaranteeing that the resulting data accurately reflect the targeted cells, free from interference by adjacent tissue [132]. For instance, Zhu et al. [133] integrated LCM with nanoPOTS to map the proteomic profiles of distinct rat brain regions. A coronal section was micro-dissected into isolated specific regions of interest, including the cerebral cortex, corpus callosum, and caudoputamen, and the tissue sections were subsequently captured in nanowells filled with dimethyl sulfoxide. Over 1000 proteins were identified and quantified from the tissue sections with a spatial resolution down to 100 μm in diameter, which corresponded to 10–18 cells. Statistical analysis clearly distinguished the three regions based on distinct proteomic expressions. Each region exhibited a specific subset of highly enriched proteins, reflecting specialized neurological functions. This LCM-nanoPOTS integration significantly advances spatially resolved tissue proteomics through an improved spatial resolution at the single-cell level while achieving wide proteome coverage . Recently, LCM-nanoPOTS has been tailored to characterize the proteomes of regions within different tissue sections, such as breast cancer [42], mouse liver and uterine [105,134], and plant tissues [135,136].

Alternatively, Xu et al. [137] have combined the recently developed spin-tip-based proteomics technology (SISPROT) [89] with LCM for cell-type-specific proteome profiling of clinical tumor samples. Using this approach, the authors profiled proteomes of four colon cancer cell types, including cancer, enterocytes, lymphocytes, and smooth muscle cells from 0.1–5 mm^2 tissue sections with a 10 μm thickness. Approximately 500–5000 proteins were identified for each cell type. They also analyzed the vertical proteome distributions by profiling cell types across consecutive 10-μm-spaced sections, illustrating that most proteins were consistently expressed but some fluctuated across the spatial distance. This proposed LCM-SISPROT approach enables spatial-cell-type proteome profiling of tumor microenvironments from minute clinical specimens. The spatial profiling of the tissue proteome enables detailed investigation of the molecular landscape in both a two-dimensional (2D) and 3D manner. Further integrating spatial proteomics with multi-omics analysis such as transcripts [138] and metabolites [139,140] will potentially provide a multi-modal, systems-level view of tissue functions.

5.3. Biomarker discovery

Advancements in microproteomics have allowed the discovery of molecules that can potentially reflect cellular states, unveil clinical prognostic indicators, and identify therapeutic targets. For example, Stutzmann et al. [130] recently reviewed advances in microfluidics-MS methods for isolating and characterizing immunopeptides. The established PeptiCHIP and CHIP-IP systems that can identify human leukocyte antigen (HLA)-associated peptides from bladder and renal cell tumors [141,142]. They also envisioned that microproteomic platforms, such as nanoPOTS and all-in-one DMF, could be adapted for the quantitative study of HLA peptides. As tumor-specific antigens become increasingly important in cancer immunotherapy, microfluidics-based microproteomics has potential to contribute by enabling in-depth characterization of HLA peptides. This emerging technology is promising for the advancement of personalized cancer therapies (such as chimeric antigen receptor T cell therapy) in the future.

6. Conclusions and future perspectives

Droplet-based microfluidics has emerged as a powerful technology for microproteomics by enabling miniaturized, integrated workflows with exceptional control over reaction conditions and minimal sample loss. In recent years, remarkable progress has been made in developing droplet microfluidic devices, such as nanoPOTS and DMF platforms, that facilitate automated, parallel processing of individual cells for proteomic analysis. Seamless integration of these microfluidic systems with sensitive high-resolution MS provides a compelling approach to achieve deep, quantitative proteome profiling of small cell populations and single cells.

Droplet-based microfluidics combined with MS (Table 1) has demonstrated a variety of biological and clinical applications, ranging from delineating cell heterogeneity and spatial distributions in tissues to biomarker discovery for precision medicine.

Moving forward, further advances in microfluidic and MS technologies will help assist in the development of SCP. Enhancing analytical sensitivity through optimized microfluidic workflows and instrumentation can uncover low-abundance proteins and post-translational modifications that play key signaling roles. Multiplexing strategies that use isobaric labeling can profile thousands of single cells in a single experimental run. Advanced MS detection techniques such as DIA with parallel accumulation serial fragmentation (DIA-PASEF) and scanning sequential window acquisition of all theoretical mass spectra (SWATH) are applied to enhance proteomic identification coverage and throughput [143,144]. Machine learning tools can extract deeper insights from massive proteomic data sets covering many individual cells. In addition, emerging fabrication techniques, such as continuous fluid-assisted etching, have enabled the development of monolithic nanospray ionization emitters integrated with glass microchips, establishing a robust microfluidic chip–MS platform for chemical monitoring and efficient analysis [145]. On-chip derivatization optimized through response surface methodology, combined with solid-phase extraction units, also provides an effective strategy for integrating microfluidic chips with MS [146]. Moreover, translating optimized microfluidic workflows to clinical settings also holds great promise for enhancing diagnostics and treatments based on single-cell proteomic signatures [147]. For example, Chen et al. [89] developed a microcolumn-array microfluidic device that leverages flexible manufacturing technology to efficiently extract and analyze proteins from rare cells at the single-cell level. This device achieves high-sensitivity detection at low sample concentrations, making it suitable for large-scale screening and monitoring in clinical environments . Additionally, Wang et al. [148] designed a microchip

Table 1
Summary of droplet-based microfluidics with MS techniques.

Droplet-based microfluidics format	MS	Cell type	Cell number	Proteins detected per cell	Applications	Ref.
nanoPOTS platform	nanoLC–MS/MS	HeLa cells	10–100	1500–3000 (10–140 cells)	Spatially resolved proteome for clinical tissues	[40]
		HeLa cells and lung primary cells	Single cell	670 (single cell)	Cell type differentiation	[103]
	nanoLC–MS/MS and iBASIL	acute myeloid leukemia cells	Single cell	~2500 (104 single cells), quantification of ~1500 proteins	Cellular heterogeneity	[94]
	nanoLC–MS/MS and TMT	Murine cells (epithelial, immune, and endothelial cells)	Single cell	> 2300 (72 single cells)	Single-cell heterogeneity, quantitative proteome	[104]
	Solid phase extraction (SPE)–LC–MS/MS and imaging MS	Mouse uterine tissue sections	Single tissue section	> 2000 proteins with 100 μm spatial resolution across tissue sections	Proteome-mapping, tissue imaging	[105]
nanoPOTS platform (commercial)	Dual-column nanoLC–MS	HeLa cells and leukemia cells	Single cell	1000 (30 min LC cycles), 660 (15 min cycles)	Cancer type differentiation	[107]
N2 chip	nanoLC–MS/MS and TMT	Murine cells (macrophage, respiratory epithelial, and endothelial cells),	Single cell	~1500 (100 cells)	Cell differentiation, quantitative proteome	[106]
Nanoliter-scale OAD chip	nanoLC–MS/MS	HeLa cells and single mouse oocyte	1–100	> 355 (single mouse oocyte)	Cell heterogeneity	[108]
Microwell chip	nanoLC–MS/MS	Hela cells and HT22 cells	1–50	> 1200	Cellular heterogeneity	[109]
DMF	nanoLC–MS/MS	Mammalian Jurkat T cells	100–500	~2500 (500 cells), ~1200 (100 cells)	Biomedical research, drug development	[117]
	high-performance LC (HPLC)–MS/MS	Tissue biopsies	3 μg tissue biopsy	1969	Size-limited clinical tissue samples	[118]
	LC-MS and TMT	Mammalian Jurkat T cells	25–75	~1815 (75 cells)	Low-cell-number proteomics	[119]
	LC–MS and label-free quantification (LFQ)	*Caenorhabditis elegans*	Single nematode (~959 cells)	~5000 (single nematode)	Model organisms/single-worm study, quantitative proteome	[120]
	LC–MS and top-down quantification	*Caenorhabditis elegans*	single Nematode (~959 cells)	~313 (single nematode)	Top–down proteomics	[121]
	LC–MS/MS	U87 cells	1, 5	~427 (single cell), ~699 (5 cells)	SCP, cellular heterogeneity	[122]
Integrated: DMF	HPLC–MS/MS	Cancer cells (MCF-7 and MDA-MB-231) and human breast tissue lysates	50–100	~973 (cells), ~882 (30 ng tissue lysates)	Cell type differentiation	[124]
Integrated: DMF	ESI–MS	No	No	No	Chemical synthesis, multistep chemical processes	[125]
Integrated: nanoPOTS	LC–MS/MS	MCF10A cells and acute myeloid leukemia cells	Single cell	~2558 (152 cells)	Quantitative SCP, cell type study	[126]
Integrated: nanoPOTS (commercial)	LC–MS/MS and TMT	HeLa cells and HEK293T cells	Single cell	~2600 (170 cells)	Cell type differentiation from organoids	[127]
AM–DMF platform	nanoLC–MS/MS	A549 cells, HEK-293T cells, HepG2 cells, and HeLa cells	Single cell	~2258	Cellular heterogeneity	[123]

capable of multiplex quantitative protein assays in cancer cells, enhancing diagnostic accuracy and supporting personalized treatment strategies. Furthermore, Mou et al. [149] developed a hierarchically structured microchip that can autonomously perform the entire immunoassay process. These innovations successfully integrate microfluidic technology with clinical requirements, significantly improving the efficiency and accuracy of clinical diagnostics and treatments.

Although droplet-based microfluidics combined with MS holds great promise, it still faces challenges, including the need for a novel interface that enables efficient sample transfer and ionization, which are key factors for generating reliable signals in MS. The efficiency of these processes may be influenced by factors such as surface chemistry and ion suppression. Furthermore, the high-throughput potential of microfluidics could be curtailed by the time-intensive separation steps often required in microproteomics. To mitigate this, advanced separation techniques, such as ion mobility separation, could be adopted to facilitate the simultaneous processing of multiple samples. The continued evolution and refinement of droplet-based microfluidic-MS systems are essential to surmount these technical obstacles, thereby unlocking their considerable potential for a wide range of applications in the biological and clinical sciences.

In summary, we presented a comprehensive review of droplet microfluidics, particularly DMF, in conjunction with high-resolution MS for microproteomic analysis. Owing to the collective efforts of researchers worldwide in advancing this technology, we are confident that this emerging field will persist in its growth and continue to provide invaluable insights to help understand the complexities of biological systems.

Acknowledgments

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Compliance with ethics guidelines

Hang Li, Yudan Ma, Rongxin Fu, Jiaxi Peng, Yanbing Zhai, Jinhua Li, Wei Xu, Siyi Hu, Hanbin Ma, Aaron R. Wheeler, and Shuailong Zhang declare that they have no conflict of interest or financial conflicts to disclose.

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