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Nanoproteomics comes of age

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Abstract

Introduction: Nanoproteomics, which is defined as quantitative proteome profiling of small populations of cells (<5000 cells), can reveal critical information related to rare cell populations, hard-to-obtain clinical specimens, and the cellular heterogeneity of pathological tissues.

Areas covered: We present a brief review of the recent technological advances in nanoproteomics. These advances include new technologies or approaches covering major areas of proteomics workflow ranging from sample isolation, sample processing, high-resolution separations, to MS instrumentation.

Expert commentary: We comment on the current state of nanoproteomics and discuss perspectives on both future technological directions and potential enabling applications.

Keywords

Nanoproteomics; small cell populations; single cell proteomics; spatially resolved; nanoPOTS; laser capture microdissection

1. Introduction

Mass spectrometry (MS)-based proteomics has become the method of choice to identify proteins and quantify the dynamic changes in the levels of protein expression and post-translational modifications (PTM) in complex biological systems in a global fashion [1]. Fueled by rapid technological advances in both instrumentation and informatics during the past decade, MS-based proteomics has enabled deep quantitative profiling of ~10,000 proteins for human tissues and has greatly expanded our understanding of signaling, regulatory, and metabolic pathways [2]. However, most proteomics studies to date are based

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on bulk analyses of cell lysates or tissue homogenates from large populations of cells (e.g., >1 million cells), thus masking critical information related to cell-type specificity and cellular heterogeneity of cells and biological tissues. The applications of MS-based proteomics to rare cell populations—such as circulating tumor cells, fine needle aspiration biopsies, and substructures within heterogeneous tissues—has been largely restricted.

During the past decade, substantial technological advances have been made towards enabling a “nanoproteomics” capability, i.e., proteomics analyses of small cell populations. The term “nanoproteomics” was recently defined as proteomics analysis of small cell populations ranging from single cells to populations of cells that will result in a total protein amount less than 1 µg [3,4]. Assuming a typical mammalian cell containing ~0.2 ng of proteins, nanoproteomics deals with any types of samples equivalent to <5,000 mammalian cells. Since the first several reports nearly a decade ago [5,6], nanoproteomics has undergone significant advances from sample isolation and processing, separations, MS detection, to data analysis. Confident identification and quantification of over 3000 proteins was recently demonstrated for as few as 10 mammalian cells containing <2 ng of protein [7]. Single cell proteomics with a coverage of ~700 proteins has also been reported utilizing a similar approach [8]. These advances demonstrate a rapid maturation of nanoproteomics technologies, which are now poised to transform biological and biomedical research. Herein, we present a brief appraisal of the state of nanoproteomics, discuss the remaining technological challenges, and offer future perspectives on both technological advances and potential applications.

2. Challenges in nanoproteomics

Unlike genomics and transcriptomics, there is no whole-proteome amplification approach available for proteins. Thus, one of the most critical factors for nanoproteomics is the overall achievable sensitivity of the proteomics workflow, which in turn depends on two main factors: (1) the efficiency and recovery of proteomic sample processing and (2) the sensitivity along with spectrum-acquisition speed of the MS platform. In a typical bottom-up proteomics workflow (Figure 1), proteins are extracted from cells, reduced, alkylated, and finally digested into peptides. During this process, non-specific sample losses to surfaces such as container walls significantly limit the overall sensitivity for nanoproteomics although such losses are rarely detrimental for large sample inputs. To achieve an in-depth proteome coverage, the sensitivity of the typical liquid chromatography (LC)-MS platform is also a limiting factor, especially for single cell proteomics. In addition to proteome coverage, the reproducibility and robustness of analyses between replicates is also critical for reliable quantification. Finally, to gain understanding of cellular heterogeneity at single cell or near single cell levels, the analysis of a larger number of samples is often required, and the sample throughput of the nanoproteomics platform is also a significant challenge. For example, single cell omics studies commonly analyze hundreds to thousands of cells to reveal cell subtypes by statistical analysis. Spatially resolved proteome mapping of heterogeneous tissue sections require analysis of hundreds of spots with high spatial resolution. However, the current LC-MS/MS platform (with a typical 10–25 cm long LC column) typically requires at least 1 hour to analyze a single sample. This throughput is insufficient for large scale nanoproteomics studies.

3. Advances in analytical platforms

Protein digests are typically analyzed by LC or CE coupled with MS through an electrospray ionization (ESI) interface. Advances in the sensitivity of LC or CE-MS platforms are critical in the overall performance of nanoproteomics.

The sensitivity of LC-MS platform generally depends on the LC flow rate, the resolution of the separation, and the ionization efficiency of an ESI source in addition to MS instrumentation itself. One of the foundational discoveries in ESI-MS was that ionization efficiency can be dramatically enhanced by nearly ~100-fold when ESI was operated at low flow rate (20–40 nL/min) (i.e., nanoESI) compared to conventional flow rate (>1 μ L/min)) [9–11]. To enable nanoflow LC without compromising separation resolution, the inner diameter (*i.d.*) of the LC column is often decreased to maintain optimal linear flow rate. In 2004, Shen *et.al.* [12] demonstrated that sensitivity of proteomic analyses can be significantly improved by decreasing column *i.d.* from 75 μ m to 15 μ m, corresponding to reducing flow rates from 300 nL/min to 20 nL/min. A detection limit of 10 zmol (~6000 molecules) was reported using BSA tryptic digest as a model sample. In a more recent study, we demonstrated a coverage of 965 proteins from 500 pg bacterial digest using a 30- μ m LC column coupled to an LTQ-Orbitrap MS [13]. To mitigate the challenge of packing small *i.d.* columns, Ivanov and coworkers [14] developed porous layer open tubular (PLOT) columns with an *i.d.* of 10 μ m and a flow rate of 20 nL/min. When coupled to a Q-Exactive MS, the PLOT column led to detection limits of 10 zmol for a standard digested protein mix. In addition to LC-MS, capillary zone electrophoresis (CZE)-MS has been employed as an alternative method for ultrasensitive detection. Sun *et.al.* [15] developed an electrokinetically pumped sheath-flow interface to couple high resolution CZE separation with ESI-MS for proteomics studies. The new interface efficiently minimized sample dilution and led to identification of >60 proteins from just 400 fg of protein digest, corresponding to a detection limit of 1 zmol.

In addition to the progress in separations and ESI, dramatic advances in MS instrumentation during the past decade has been an essential factor for enabling nanoproteomics. One of the most significant advances in MS instrumentation was the invention and commercialization of the Orbitrap analyzer [16]. Orbitrap detect ions by measuring image current and then employing Fourier transformation for data processing, which is similar to Fourier-transform ion cyclotron resonance (FT-ICR) mass analyzer. However, Orbitrap utilizes electrical fields to manipulate ions rather than magnetic fields as with FT-ICR, dramatically reducing the size and cost of the instrument, leading to widespread adoption by the proteomics community. The detection limit of Orbitrap is determined by internal preamplifier noise, which is approximate 2–4 elementary charges in a 1 s acquisition. Such low noise level has made possible single molecule detection of intact proteins, including myoglobin [17] and *E. coli* GroEL [18]. In addition to the mass analyzer, front-end ion transmission optics have also been substantially improved. One of the most important improvements is the development of the ion funnel [19], which sought to efficiently transfer ions from the first vacuum stage to the high vacuum of the ion detector. The ion funnel employs a stacked radio frequency ring electrode arrangement in a funnel shape to focus ions. Nearly 1 order of magnitude gain in sensitivity relative to a standard skimmer device was achieved [20]. When

combined with a low-flow sub-ambient pressure ionization source (SPIN) [21], 50% of the analyte ions initially in the solution can be delivered into high vacuum mass detector [22], representing the highest overall ion utilization efficiency yet achieved.

Finally, advances in data analysis algorithms provide another avenue to improve the sensitivity of proteome identification and quantitation. In a typical proteomics workflow, peptides are identified by tandem MS spectra, where individual peptides are isolated and dissociated, followed by fragment measurement and database searching. However, the stochastic sampling nature limits the ability to achieve broad proteome coverage because many detectable molecules may never be sampled for fragmentation due to the overall low sampling efficiency for fragmentation. Several advances have been made to address this limitation through informatics algorithms. For example, Smith and coworkers [23] initially reported the accurate mass and time (AMT) tag concept that peptides can be identified by matching accurately measured masses and retention times to a pre-established peptide AMT database. The database was typically generated by two-dimensional LC-MS/MS analyses of the proteome samples. This concept has been further adopted into the more broadly disseminated MaxQuant software with integrated match between runs (MBR) algorithm [24] as well as the recently reported IonStar approach [25]. Our recent results demonstrated ~1000 proteins can be identified from 0.5 ng tryptic digest of *S. oneidensis* using MBR algorithm [13].

Taken together, these advances suggest that current analytical sensitivity of LC-MS or CE-MS platforms is sufficient to make proteome-level measurements even in single mammalian cells containing subnanogram amounts of protein.

4. Minimizing loss during proteomic sample processing

With the great progress in advancing analytical sensitivity, the next grand challenge is how to maximize sample recovery of proteomic sample processing. Initial efforts suggested that MS incompatible surfactants, acetone precipitation, and multi-step transfers should be avoided to reduce the sample losses to vessel surfaces or cleanup steps [5,6]. Additionally, sufficient protein and enzyme concentrations are required in order to achieve efficient tryptic digestion. Considerable efforts have been recently devoted into developing new sample preparation systems for nanoproteomics, including online processing systems [26–31], single tube preparation systems [6,14,32,33], and a microfluidic nanodroplet processing system (Table 1) [7,8,34].

Two types of online proteomic processing systems have been developed. The first is based on a strong cation exchange (SCX) capillary column, termed as a proteomic reactor [26]. Cell lysates were directly loaded, and proteins were trapped in the SCX capillary. Protein denaturation, alkylation, and tryptic digestion were performed by serially introducing reagents into the SCX column [26,27]. Proteome profiling of 17 and 68 proteins from 300 mouse P19 testicular cancer cells [26] and 500 human embryonic stem cells [27] were reported, respectively. To improve the sample throughput of the proteomic reactor approach, Tian and coworkers [28] prepared the SCX columns in pipette tips and introduced reagents by centrifugation, allowing multiple samples to be processed in parallel. The second type of

online processing system was based on an immobilized enzyme reactor (IMER), where proteins were directly loaded and digested in an IMER column [29]. Huang *et.al.* [30] reported a simplified nano-proteomics platform (SNaPP), which consisted of an online IMER digestion system and a solid phase extraction (SPE) column coupled with a nanoLC separation system. SNaPP was applied for proteome profiling for as few as 500 mouse lung alveoli cells isolated by laser capture microdissection (LCM) [31]. Although online processing systems have greatly increased the preparation efficiency and simplified experimental operations, sample loss on the surfaces of SCX or IMER beads is still a significant factor precluding the effective analysis of smaller cell population (e.g., <500 cells).

Single tube preparation is an interesting concept to minimize surface exposure by performing all procedures in a single centrifugation tube or vial. Low binding tubes or plates (e.g., Eppendorf Protein LoBind) were commonly used. To facilitate efficient cell lysis and protein extraction, numerous modified procedures and alternative surfactants have been introduced. For instance, MS and trypsin-compatible surfactants—such as RapiGest, ProteaseMAX, PPS Silent Surfactant, and sodium deoxycholate—were introduced to replace sodium dodecyl sulfate and avoid extra cleanup steps. Waanders *et. al.* [32] described a PPS Silent Surfactant-based workflow for proteomic profiling of single pancreatic islets containing 2,000–4,000 cells. To avoid the use of detergent, Wang *et al.* [6] reported a trifluoroethanol (TFE)-based approach where the volatile organic solvent TFE facilitates efficient protein extraction and denaturation from cells or tissues. This TFE approach was demonstrated for proteome profiling of 5000 MCF-7 cells. The Ivanov group [14] introduced focused ultrasonication for cell lysis and protein extraction. When coupling with ultrasensitive PLOT LC, 2512 proteins were identified by injecting an aliquot equivalent to 122 cells taken from a single tube preparation of 1000 MCF-7 cells. The focused ultrasonication approach was applied to measure protein expression in single mammalian cells [35]. To improve proteome coverage of single cell samples, a signal boost concept was introduced, where one tandem mass tag (TMT) labeled channel that contains a large amount of reference sample served as a signal boost to trigger MS/MS and to enable detect low abundance species in other single cell channels [35,36]. A total of 767 proteins were quantified across a group of 24 single cells, which could be clustered by cell types in a two-dimensional principal component analysis plot. Chen *et. al.* [33] developed a single tube preparation system based on on-valve tryptic digestion, termed the integrated proteome analysis device (iPAD). Living cells were injected into an on-valve sample loop together with trypsin solution. Cells were lysed, and proteins were digested by heating the loop at an elevated temperature of 50 °C. Generated peptides were purified by online SPE column and identified by LC-MS. An average of 635 proteins were identified from 100 DLD cancer cells. The Krijgsveld group [37,38] developed a single-pot, solid-phase-enhanced sample preparation (SP3) method, where hydrophilic paramagnetic beads were used to trap proteins and peptides during multiple-step sample processing. A unique advantage of the SP3 method is that it allows the use of strong detergents for protein extraction and organic solvent for sample clean-up. The performance of SP3 for nanoproteomics was evaluated in the analysis of single *Drosophila* embryo, and 2937 proteins were identified [37]. In the above-mentioned single tube approaches, low reaction volumes from 3.2 µL to 25 µL were

commonly used to minimize total surface exposure, which suggested a new direction to improve sample recovery.

Despite of all these advances in minimizing sample loss during processing, it is still challenging for effective processing of very small cell populations (e.g., <100 cells) without significant loss. We recently achieved a major breakthrough in developing a microfluidics-based sample preparation system, termed as nanodroplet Processing in One-pot for Trace Samples (nanoPOTS) [7]. NanoPOTS significantly reduced adsorptive sample losses to surfaces by minimizing processing volumes to <200 nL in microfabricated nanowells with diameter of only 1 mm. Compared with typical 100-μL sample preparation in microcentrifuge tubes, nanoPOTS reduced total surface area exposure by 99.5% [7]. Meanwhile, nanoliter reaction volumes increased both concentrations of proteins and trypsin, thus greatly enhancing enzymatic digestion kinetics. When coupled with an ultrasensitive LC-MS system (30-μm-*i.d.* nanoLC, Orbitrap Fusion Lumos), nanoPOTS enabled quantitative profiling of ~3000 proteins from 10 HeLa cells by incorporating the MBR algorithm of MaxQuant and ~2400 proteins from single human pancreatic islet thin sections isolated from clinical specimen from human donors [7]. In a follow-up study, we further demonstrated the identification of 770 proteins from single HeLa cells using this system [8].

5. Sample isolation techniques for nanoproteomics

To enable broad applications of nanoproteomics, successful integration of sample isolation techniques with nanoproteomics workflows will be critical. Currently, there are two commonly used sample isolation techniques for small cell populations: fluorescence activated cell sorting (FACS) and LCM, which have been coupled effectively to nanoproteomics. In a FACS system, cells can be precisely counted and collected for quantitative analysis, including single cell analysis. We have demonstrated FACS can precisely isolate single mammalian cells and minimize protein contamination for highly sensitive nanoPOTS-based proteomic analysis [8]. The FACS-based sample isolation method has also been employed to purify small numbers of colon stem cells from mouse intestines for proteomic characterization [39]. Using only 5000 sorted cells, the authors observed 21% of stem cell-specific genes could be detected at protein level. Despite the advantages, FACS has inherent limitations for analysis of solid tissues because enzymatic dissociation step is required to generate sortable individual cells, thus inevitably losing the spatial information of the tissue sample. Thus, critical molecular information relating to the tissue microenvironment, heterogeneity, and cell-to-cell interactions is mostly lost. Moreover, the cell proteome may alter during the tissue dissociation steps, thus not actually reflecting the *in situ* proteome.

LCM offers a particularly useful alternative for *in situ* and spatially resolved analyses of tissue samples [40]. By combining optical microscopy with laser ablation, it enables the precise isolation of specific cell types or tissue microstructures based on morphology, autofluorescence, antibody staining, or MS imaging. McDonnell and coworkers [41] employed LCM to isolate mouse kidney cortex and medulla microdissections as small as 0.5 mm². A total of 3440 proteins were identified, and statistically significant protein expression

differences were revealed. We also coupled LCM with nanoPOTS to analyze single human islet sections obtained from a non-diabetic donor and a type 1 diabetes (T1D) donor [7]. Furthermore, automated sample transfer of LCM isolates to nanoPOTS was achieved through prepopulating nanowells with dimethyl sulfoxide (DMSO), and spatially resolved proteome mapping of rat brain cortex tissue was demonstrated [34].

In addition to LCM, in situ protein digestion approaches have also been developed for spatially resolved proteomic analysis of tissue sections [42,43]. In these approaches, small trypsin droplets [42] or hydrogels [43] were directly applied on tissue sections to digest proteins into peptides. The peptide samples were either directly injected into LC or recovered for subsequent analysis. Approximately 500–600 proteins could be identified by sampling small tissue areas with diameters of 250–260 μm .

6. Expert commentary

An effective nanoproteomics and single cell proteomics capability holds tremendous potential for biomedical research by enabling in-depth characterization of cellular heterogeneity, spatially resolved proteome mapping of pathological tissues (i.e., highly multiplexed molecular pathology), the generation of foundational protein atlas for organs and tissues, and the discovery of cell subtypes and novel signatures for specific cell populations. While other single cell technologies have already made significant strides into revealing novel molecular information about the great heterogeneity of cell populations and rare cells [44,45], nanoproteomics for a long time has been considered infeasible for practical applications. However, the aspiration of nanoproteomics down to even the single cell level has come a long way built upon the dramatic advances in nearly all facets of the overall proteomics workflow. The state of nanoproteomics is now a reality for enabling effective quantitative analyses of very few cells (e.g., 10–100 cells) with technologies such as nanoPOTS coupled with advanced LC-MS platforms [7]. The current nanoproteomics technologies are poised to enable broad biomedical applications.

Despite the promising aspects of nanoproteomics, there are still a number of challenges that need to be addressed in the near future in order to realize the full impact of nanoproteomics or single cell proteomics capabilities. First, the absolute sensitivity of the current workflows is still insufficient to obtain in-depth coverage for single cell proteomics although prototype measurements of single cells have been reported [8]. It is possible that the continued advances in MS instrumentation would provide further enhancement in sensitivity needed for single cell proteomics. Second, the current sample throughput for nanoproteomics is a major limitation since a typical LC-MS/MS analysis takes at least 1 hour. Much higher throughput will be necessary for single cell proteomics or other applications involving spatially resolved proteome mapping or imaging of heterogeneous tissues. There are several potential avenues to improve the overall sample throughput. First, the concept of multiplexing labeling, such as TMT-based isobaric labeling or even hyperplexing (e.g., 50- or 100-plex) labeling, might greatly enhance the overall throughput for nanoproteomics or single cell proteomics; however, the accuracy of quantification of such methods will be an important concern due to the potential co-eluting peptide species within the same mass isolation window for MS/MS fragmentation. Second, the continued advances in MS

acquisition speed in the newer generation of instrumentation could allow much shorter LC separations to be employed (e.g., 5–10 min). Finally, advances in ultrafast gas phase separations such as structure of lossless ion manipulation (SLIM)-based ion mobility spectrometry may enable much faster separations in nanoproteomics applications [46].

Another important aspect of future nanoproteomics is the potential of performing top-down proteomics at the near single cell level. Most current technological advances are focused on bottom-up nanoproteomics; the extension of nanoproteomics to top-down and PTM measurements still lags significantly behind. In principle, top-down proteomics directly analyzes intact proteins, allowing much better characterization of proteoforms and combinatorial PTMs. Protein PTMs (e.g., phosphorylation, acetylation, or glycosylation) are highly significant in the regulation of protein function and are involved in a large variety of biological processes such as enzymatic activity, signal transduction, and gene expression regulation. However, top-down and PTM proteomics pose even greater analytical challenges for nanoscale samples than typically global bottom-up proteomics. For example, compared with digested peptides, over 100-time signal reduction is commonly observed for intact proteins due to low ionization and transmission efficiency in ESI source, multiple-charged precursors, and complex proteoforms [47]. In PTM proteomics, modified peptides usually presented in low abundance and low stoichiometry together. Additional enrichment steps are required to purify PTM peptides for MS detection. Low sample recovery was observed in these enrichment steps for nanoscale samples [48]. Despite these challenges, given the great importance of top-down and PTM proteomics, increasing research efforts and more technological advances are anticipated for these areas.

7. Five-year view

Nanoproteomics is still in its early stage of enabling broad biomedical applications. In the next 5 years, we anticipate both the continued technological advances in this field, but also much broader biological and biomedical applications in the areas of nanoproteomics for analyzing small cell populations, single cells, and spatially resolved proteome mapping of tissues of both normal and disease subjects. In the technological aspects, substantial progress in both sensitivity and throughput is anticipated for the nanoproteomics workflow, and true single cell proteomics technologies may become reality, similar to those in single cell RNA sequencing. We anticipate more technical developments will be focused on top-down and PTM proteomics to improve the overall sensitivity of intact proteins and modified peptides. We would anticipate that vast amounts of detailed novel proteomic information regarding cellular heterogeneity, subpopulations of cells, and spatial organization will be revealed by the enabling technologies. The integration of proteome, genome, and transcriptome data will allow us to gain much better understanding of the molecular underpinning of cellular heterogeneity in normal physiology and pathophysiology. Finally, we anticipate the nanoproteomics capability will be extended to analyzing cell-type-specific and spatially resolved PTMs across heterogeneous tissue to advance our knowledge of signal transduction pathways.

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Key Issues

- The sample recovery during processing and the sensitivity of LC or CE-MS platforms are the two most critical factors for nanoproteomics.
- Substantial advances have been made in analytical platforms, and the current advanced LC or CE-MS platforms have sufficient sensitivity for single cells.
- The main bottleneck for nanoproteomics is actually the loss of proteins or peptides on the various vessel surfaces during sample processing.
- The development of the NanoPOTS platform represents a major breakthrough in processing extremely small numbers of cells or single cells by achieving high processing efficiency and recovery through the nanodroplet-based procedure.
- Future advances in sensitivity and throughput will make nanoproteomics or single cell proteomics a much more powerful technology for enabling broad biomedical applications.

Figure 1. Typical workflow for bottom-up nanoproteomics.

Briefly, specified cell population is isolated from tissues or cultured cells. Proteins are extracted and digested by proteolytic enzymes (e.g., trypsin) into peptides. The digested samples are analyzed by nanoflow LC or capillary electrophoresis (CE) coupled with MS through electrospray ionization. Peptide sequences are identified from MS/MS spectra through database searching, and MS intensity information is typically utilized for protein quantification.

Table 1. A summary of nanoproteomics performance with different sample preparation methods along with LC-MS platforms

Sample preparation method	Analytical systems	Minimal sample size	Protein identifications	Publication year and Reference
Acetone precipitation	75 $\mu\text{m} \times 100\text{ mm}$ C18 LC, QTOF Premier MS	500 MCF-7 cells	187	2010, [5]
Proteomic reactor	75 $\mu\text{m} \times 50\text{ mm}$ C18 LC, LTQ ion trap MS	300 P19 testicular cancer cells	17	2006, [26]
Proteomic reactor	50 $\mu\text{m} \times 200\text{ mm}$ C18 LC, LTQ-Orbitrap XL MS	500 embryonic stem cells	68	2011, [27]
Spintrap-based proteomic reactor	75 $\mu\text{m} \times 200\text{ mm}$ C18 LC, Orbitrap Fusion MS	2000 HEK 293 cells	1270	2016, [28]
Immobilized enzyme reactor	50 $\mu\text{m} \times 750\text{ mm}$ C18 LC, Q-Exactive plus MS	500 mouse lung alveoli cells	652	2016, [31]
PPS Silent Surfactant-based single tube preparation	40 $\mu\text{m} \times 220\text{ mm}$ C18 LC, LTQ-Orbitrap MS	single pancreatic islets (~2000 cells)	2013	2009, [32]
TFE-based single tube preparation	150 $\mu\text{m} \times 650\text{ mm}$ C18 LC, 11 T FTICR MS	5000 MCF-7	224	2005, [6]
Focused ultrasonication-based single tube preparation	10 $\mu\text{m} \times 400\text{ cm}$ C18 PLOT LC, Q-Exactive MS	1000 MCF-7	2512	2015, [14]
On-valve single tube digestion system	75 $\mu\text{m} \times 100\text{ mm}$ C18 LC, Q-Exactive MS	100 DLD cells	635	2015, [33]
Single-pot solid-phase-enhanced sample preparation	75 $\mu\text{m} \times 300\text{ mm}$ C18 LC, Q-Exactive MS	Single Drosophila embryo (~200 ng total proteins)	2937	2014, [37]
Nanodroplet-based preparation system	30 $\mu\text{m} \times 600\text{ mm}$ C18 LC, Fusion Lumos MS	10 HeLa cells	3092	2018, [7]
LCM and nanodroplet-based preparation	30 $\mu\text{m} \times 600\text{ mm}$ C18 LC, Fusion Lumos MS	~100 cells from single pancreatic islet section	2676	2018, [7]
FACS and nanodroplet-based preparation system	30 $\mu\text{m} \times 600\text{ mm}$ C18 LC, Fusion Lumos MS	1 HeLa cells	669	2018, [8]
LCM and urea-based single tube preparation	75 $\mu\text{m} \times 500\text{ mm}$ C18 LC, Orbitrap Fusion MS	0.5 mm^2 mouse kidney tissue	3440	2016, [41]
in situ droplet digestion	50 $\mu\text{m} \times 150\text{ mm}$ C18 LC, Q-Exactive MS	0.25 mm in diameter of rat brain tissue	515	2017, [42]
in situ hydrogel digestion	75 $\mu\text{m} \times 250\text{ mm}$ C18 LC, Orbitrap Fusion MS	0.26 mm in diameter of rat liver tissue	671	2017, [43]