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Advances in microscale separations towards nanoproteomics applications

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Abstract

Microscale separation (e.g., liquid chromatography or capillary electrophoresis) coupled with mass spectrometry (MS) has become the primary tool for advanced proteomics, an indispensable technology for gaining understanding of complex biological processes. In recent decades significant advances have been achieved in MS-based proteomics. However, the current proteomics platforms still face an analytical challenge in overall sensitivity towards nanoproteomics applications for starting materials of less than 1 μg total proteins (e.g., cellular heterogeneity in tissue pathologies). Herein, we review recent advances in microscale separation techniques and integrated sample processing strategies that improve the overall sensitivity and proteome coverage of the proteomics workflow, and their contributions towards nanoproteomics applications.

Keywords

Microscale separations; Nanoproteomics; NanoLC; Capillary electrophoresis; Mass spectrometry

1. Introduction

Proteins are the workhorses of the cell, impacting nearly all aspects of cellular processes. The proteome by its nature is dynamic, and the acute state of the proteome (i.e., the proteotype) depends on both the genotype and external perturbations [1,2]. Therefore, quantitative analysis of the dynamics of the proteome including post-translational modifications (PTMs) and its connection to phenotypes (e.g., diseases) has become indispensable in biological and clinical research [3–5]. Recent advances in mass spectrometry (MS)-based proteomics for both global deep-profiling of the proteome and selected types of PTMs (e.g., phosphorylation) [6–8] and targeted quantification of proteins from specific signaling path-ways [9,10] have greatly expanded our capabilities in performing proteogenomics and systems biology studies for gaining detailed mechanistic insights into physiological and pathological processes.

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Conflict of interest
None.

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During the past decade, major advances have been achieved in nearly all areas of the proteomics workflow such as high resolution microscale chromatographic separations, mass spectrometry instrumentation, and bioinformatics data analysis tools to enable large-scale proteome interrogation [11]. Current state-of-the-art MS-based proteomics platforms can afford deep coverage for both the global proteome and selected PTMs in cell or tissue samples. For example, recent studies have reported the identification or quantification of ~10,000 proteins [6,7], >20,000 phosphorylation [12,13] and >15,000 ubiquitination sites [12,14].

Despite recent advances in improving overall proteome coverage, the current proteomics workflows typically require relatively large amounts of starting materials on the order of millions of cells or 100 s μg of proteins, which excludes many important biological and biomedical applications. The ability to effectively analyze extremely small amounts of protein samples (e.g., nanograms of proteins) from cells or tissues by MS is one of the most significant challenges for current MS-proteomics. Herein, we define sample amounts with less than 1 μg of total protein as "nanoscale" and proteomics analyses of these nanoscale samples as "nanoproteomics" (Fig. 1). The biomedical need for nanoproteomics technologies are compelling, including the analyses of tissue substructures, cellular microenvironments of disease pathologies, rare or small subpopulations of cells, extracellular vesicles, as well as single cell resolution profiling (Fig. 1). Some of these sample types are readily produced by existing technologies such as fluorescence activated cell sorting(FACS) [15], laser capture dissection (LCM) [16,17], and exosome isolation techniques [18]. Moreover, single cell resolution genomics technologies, such as single-cell genomic sequencing [19] and single-cell transcriptomic profiling (RNA-Seq) [20,21], have been making tremendous impact in biological research. However, the current state-of-the-art in MS-based proteomics still falls far short of the sensitivity required for single cell analyses.

Considerable efforts have been devoted to enhance the overall sensitivity of MS-based proteomics workflow towards enabling analysis of small samples, including the front-end sample processing, microscale separations, and MS instrumentation. Herein, we review recent advances in microscale separation, as well as nanoscale sample processing systems for proteomics analysis. Our focus will be on bottom-up proteomics, and the other important advances in top-down proteomics (measurement of intact proteins) [22] are not covered here.

2. Factors governing overall MS-based proteomics sensitivity

Fig. 2 illustrates a typical MS-based proteomics workflow for protein identification in biological samples. Conceptually, the overall analytical sensitivity of MS-based proteomics depends on the following aspects: a) the efficiency and recovery of front-end sample processing (e.g., protein extraction and protein digestion) and the degree of reducing sample complexity by extensive fractionation and/or enrichment; b) the resolving power of chromatographic or electrophoretic separations when coupled with electrospray ionization (ESI)-MS online in order to achieve deep proteome coverage; and c) the overall sensitivity

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of MS platforms including ESI and ion transmission efficiency, the resolving power of mass analyzers, and the sensitivity of MS detector.

The advances in MS instrumentation during the past decade are perhaps the most significant factor for achieving considerably greater overall sensitivity. Indeed, we have witnessed tremendous technological advancements in MS instrumentation including advanced ion optics (e.g., electrodynamic ion funnel [23]), and the ever increasing scanning speed, resolution, mass accuracy, sensitivity, dynamic range, and the multi-mode fragmentation technologies to facilitate much faster, more accurate, and more sensitive peptide identification and quantification [4,24–26]. Similarly, liquid-phase separations including liquid chromatography (LC) and capillary electrophoresis (CE) systems have become much more robust, reproducible, and sensitive through the use of smaller inner diameter (i.d.) LC columns and operating ESI at very low flow rates [27–30]. In general, the analytical sensitivity of nanoLC-MS and CE-MS is sufficient to detect peptides from a sample with mass equivalent to a single mammalian cell [29,30].

It has become clear that the major bottleneck for applying current MS-based proteomics platforms to nanoscale samples is the front-end sample processing step, where significant sample loss occurs. Further advances in microscale separation-based developments enabling online digestion/separation or miniaturized sample handling hold the promise to make significant contributions in addressing this bottleneck. In the following sections, we review recent advances in individual components of the nanoproteomics workflow (Fig. 3), including LC or CE-MS platforms and front-end microscale sample processing strategies that aim at enhancing the overall sensitivity and proteome coverage towards nanoproteomics applications. We also highlight some recent biological applications enabled by nanoproteomics technologies.

3. LC or CE for nanoproteomics

Reversed phase (RP) LC, either in packed columns or monolithic columns, is the most widely used LC separation for bottom-up proteomics due to its relatively high resolving power and ESI friendly mobile phases. CE has also emerged as a powerful separation technique when directly coupled with ESI-MS for proteomic research. One of the foundational discoveries in ESI-MS was that the ESI 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 μl/min) [27,28,31]. This discovery set the general direction of recent LC and CE developments by pushing the limits of operation to nanoESI regimes and at the same time to enhance the overall resolving power of the separations in order to achieve highly sensitive measurements with good proteome coverage. Given that ESI-MS is generally considered as a concentration dependent detector, lower flow rates through a narrower i.d. column separation not only generate higher concentration, but also higher ionization efficiency, and thus higher signal intensity for individual analytes. Herein, we discuss the advances of LC and CE for the analysis of nanoscale samples. The performance metrics for selected LC and CE-MS platforms, including separaration paramters, type of sample, and identification numbers, are summarized in Table 1. Other detailed parameters not listed can be found in the References.

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3.1. LC

The overall performance of LC-ESI in terms of sensitivity and proteome coverage will depend on the i.d. of the capillary column, which determines the optimal flow rate, and the resolving power or peak capacity of LC separations. An early study by Shen *et al.* has demonstrated that peak capacities close to ~10^3 (Table 1) can be achieved independent of the column i.d. (15–75 μm) by using 87 cm long columns packed with 3 μm porous particles [32]. LC-ESI-MS sensitivity was significantly increased when the column i.d. decreased from 75 to 15 μm to enable ESI at lower flow rates. When analyzing ~100 ng of a yeast protein digest, the number of detected species was increased from 7 to 1345. In another study with a 15 μm × 80 cm column, a detection limit of 10 zmol for BSA peptide and identification of high abundance proteins from as low as 0.5 pg of bacterium digests were reported [29].

To date, most proteomics applications have been limited to 50–75 μm i.d. capillary columns when commercially available LC systems are used, due to their advantage in operation robustness. These platforms have been applied to nanoscale samples. For instance, Karger *et al.*, reported an early illustration of LC-MS in nanoproteomics in analyzing ~10,000 breast cancer cells isolated by laser capture microdissection (LCM) using a 75 μm × 15 cm column packed with 3 μm particles on a first generation LCQ ion trap platform, leading to identification of 76 proteins [33]. More recently, Waanders *et al.* reported the identification of ~2000 proteins from single pancreatic islets (2000–4000 cells) using a 50 μm i.d. column packed with 3 μm particles and a long gradient (4-h) gradient separations with ~50 nL/min flowrate on an Orbitrap platform [34].

The sensitivity of 50–75 μm i.d. packed columns seems to be sufficient for analyzing small number of cells, but the achieved proteome coverage typically drops significantly when the starting sample amounts decrease [35,36]. Thus, more sensitive microscale separations are critical for achieving comprehensive proteome coverage of nanogram samples (Table 1). However, there are tremendous challenges for applying packed columns with i.d. smaller than 50 μm including the difficulty in packing, the requirement of ultra-high pressure pump, and limited robustness. Therefore, the development of alternative LC column technologies (e.g., monolithic columns) is an attractive solution.

Monolithic columns can be prepared with either in situ polymerization [37] or a sol-gel process involving the hydrolysis and polycondensation of alkoxysilanes [38]. Due to the small-sized skeletons and relatively large pores in monolithic columns, they can be manufactured in very small i.d. (e.g., 10 μm) with extended length (up to 8 m) and operated at the optimal nanospray ESI flow rates of 20 nL/min for achieving the optimal ESI-MS sensitivity. Luo *et al.* reported an early demonstration of attomole detection limit of peptide on a 20 μm × 70 cm silica based monolithic column at a flow rate of 40 nL/min when combined with an online SPE column and ESI-MS detection [39]. Follow-up studies by the same group and others have demonstrated the feasibility to identify thousands of proteins from sub-μg of tryptic digests on silica-based monolithic columns [40–42].

Another interesting contribution is the development of polymer monolithic porous layer open tubular (PLOT) columns for ultrasensitive proteomics analyses [43,44]. The PLOT

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columns displayed good reproducibility in retention times (3% RSD), detection limits of attomole to sub-attomole, and a peak capacity of 400. Rogeberg *et al.* compared the performance of silica-based and polymer-based monolithic columns and discovered a peak capacity of 950 from a 10 μm × 8 m PS-DVB PLOT column, which meets or surpasses peak capacities of previously reported using packed nanoLC or long silica monolithic columns [45]. With the PLOT column, about 500 and 1200 proteins were identified from an extract corresponding to 1000 and 10,000 cells, respectively [45].

3.2. CE

The potential of CE as a highly sensitive and efficient separation technique is demonstrated by its reputation in deciphering the human genome, but it has not gained as much attention as LC in the field of proteomics. However, some recent developments have made CE-MS based proteomics more promising by allowing for effectively interfacing of CE with MS for peptide detection and high resolution peptide separations.

Although the first sheath-liquid CE-MS interface was developed several decades ago [46], the sheath liquid causes significant dilution of analytes eluted from capillary [47]. Because ESI-MS is a concentration dependent detector, minimal or no sheath-liquid is preferred for high sensitivity CE-ESI and such hyphenation interfaces have been developed in the past decade [48–51]. One successful type of interface that decreases the sheath liquid dilution effect in recent proteomics applications is the nanospray sheath-flow liquid interface, developed by several groups with slightly different ways to pump sheath liquid [48,50,52]. This type of interface has been used for proteomics analyses in both global discovery and targeted quantitation modes [52–60]. Detection limits as low as zmol of protein digest was achieved using a targeted method [53,55]. A high peak capacity of 300 with this interface was reported with a 90 cm length linear polyacrylamide coated capillary with a separation window of 90 min [58]. A single shot CE-MS analysis of 400 ng of HeLa cell lysate digests has resulted in identification of ~10,000 peptides and 2100 proteins, which is approximately 2.5-fold lower than the number from nanoLC-MS using a 300 ng sample. There is 70% overlap of the identified peptides between the CE and LC methods, but CE tends to identify larger peptides than LC [57,58]. More recently, a number of groups developed sheathless CE-MS interfaces where the electroosmotic flow from capillary is the only source of nanospray [49,51].

3.3. Multidimensional separations

Besides the absolute sensitivity, the overall proteome coverage is a critical metric of performance of any proteomics workflow. The resolving power of separations is essential for achieving deep proteome coverage in proteomics analyses. Towards this direction, multidimensional separation platforms have been developed to provide higher peak capacity and applied to achieve deep proteome coverage in complex biological samples. Such multidimensional separation strategies can be operated in either offline or online modes [61–63]. In the offline mode, the fractions from the first dimension separations are typically collected through fraction collectors for later subsequent analyses. For online modes, the eluent fraction from the first dimension will be directly delivered to subsequent dimensions of separations for analysis in a single online system. When compared to online systems, the

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main limitation of offline multidimensional separations is the potential of sample loss during offline fractionation. On the other hand, a number of studies reported the development and application of online 2D or 3D LC-MS platforms for the analysis of relatively small amounts of digested samples [64–68]. To achieve high efficiency, the small i.d. analytical columns (e.g., 25 μm i.d. × 100 cm) with integrated emitters operating at flow rates of ~10nL/min were typically applied for last dimensional LC-MS, whereas the other dimensions were operated at either 1 or 2 μL/min [64,67]. The peak capacity was enhanced from 750 to ~13,000 for 1D to 3D separation systems. Using their latest 3D separation platform, the interference was largely removed and the quantitation ratios were only 8% compressed compared with 40% using the standard 1D LC-MS/MS [66,68].

Integrated biphasic or multi-phasic columns are also interesting alternatives to achieve the effect of multi-dimensional separations due to their simple configurations with minimum sample exposure surface. The original biphasic multidimensional separation strategy was reported in 2001 by Washburn *et al.* [63]. Since then, many improvements have been reported [69,70]. For example, Luo *et al.* demonstrated an integrated triphasic (RP/SCXμ-SPE) trapping column connected to a 10 μm × 3.2 m PLOT analytical column [71]. ~850 proteins were identified from an injection amount equivalent to 1200 cells (~500 ng) from a cervical cancer cell line.

As summarized in Table 1, recent advances in LC or CE-MS clearly make it possible to identify and quantify thousands of protein from sub-μg sample digests. Given this highly sensitive LC or CE-MS platforms being available, the efficiency in microscale front-end sample processing become an extremely critical component for achieving the success of the overall proteomics workflow.

4. Front-end microscale sample processing

As a critical component in the overall workflow, there has been a significant interest in developing microscale sample processing techniques to minimize sample loss and increase processing efficiency. In principle, this would involve processing with minimized liquid volumes and transfer steps. These efforts generally employ two main approaches to reduce sample losses and enhance processing efficiency: single-tube preparation techniques or integrated online processing systems.

The single tube techniques appear to be most straightforward by utilizing volatile solvents or acid-cleavable detergents for protein denaturation and performing all sample manipulations within a single tube to minimize loss [72–76]. This approach has been applied for proteomics analyses of LCM tissue samples [74,75,77]. However, it still falls short of effective processing for nanograms of proteins primarily due to low digestion efficiency when protein and enzyme concentrations get extremely low.

Alternatively, online processing systems integrating with microscale separations become more attractive. Proteomic reactors have been reported by employing a solid phase support to immobilize proteins and/or peptide to allow relatively rapid digestion and buffer exchange without sample transfers compared to traditional in-solution digest [78–80]. However, most

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proteomic reactor approaches still require many manual sample handling steps and long preparation times [81,82]. Online digestion systems were developed by adapting concepts from microscale separations to carry out proteomics digestion within enclosed systems, i.e. fused silica capillaries, controlled by chromatography pumps and fluidic systems [83–85]. Most commonly, immobilized enzyme reactors (IMER) in a micro-column format are used to achieve rapid protein digestions and eliminate the need for frequent additions of fresh protease [86–92]. Chip-based IMER [93] or tip-based nanoreactors [94] with comparable digestion efficiency were also developed and could be potentially automated and integrated for multiple functions. In general, IMER-based device offers much faster digestion time ranging from several seconds to less than 1 h compared to that of in solution digestion (typically >3 h) [87,92].

Recently, our group developed a simplified nanoproteomics platform (SNaPP) that integrated an online IMER column for trypsin digestion and solid phase extraction directly coupled to LC-MS (Fig. 4A) [17,91]. The SNaPP system created a simple, sensitive, robust, and reproducible nanoproteomics platform. As a pilot study using this system, 348 proteins were identified from ~20 mouse blastocysts, equal to 100–200 ng samples [91]. More recently, >3400 proteins were identified by SNaPP from only 4000 lung cells isolated by LCM [17]. In another example, Tian *et al.* integrated a monolithic SCX column-based proteomic reactor that serves for both protein capture/reduction/alkylation/digestion reactor and the first dimensional SCX separation followed by LC-MS (Fig. 4B) [95]. This platform was applied to analyze 500–50,000 human embroyonic stem cells and the identified protein numbers were increased from 69 to 2281 linearly. In an independent study, Zhang *et al.* reported an integrated proteome analysis device (iPAD) that incorporated a direct injection capillary column based sample loop (100 μm i.d., 40 cm long) and a C8 trap column (75 μm i.d., 10 cm long) and an online nanoLC analytical column (75 μm i.d. C18) (Fig. 4C) [96]. This system allowed direct flow injection of cells in trypsin solution and was able to identify an average 635 proteins from ~100 cells [96]. These examples of integrated online processing systems provided excellent illustrations on the promising aspects of integrating different components into a system for more effective sample processing with a minimal sample loss as well as multidimensional separations towards nanoproteomics applications.

5. Highlights of nanoproteomics applications

Nanoproteomics is an evolving technological capability for enabling analysis of cellular heterogeneity, tissue substructures, and other nanoscale biological or clinical samples at the proteome level when coupled with cell isolation techniques (e.g., LCM [97] and FACS [98]). While we recognized that most of the reports on nanoproteomics were focused on method development or proof-of-concept demonstrations, in this section we highlight some studies to illustrate the potential of biological applications enabled by nanoproteomics.

One of the early interesting nanoproteomics applications was the ability to perform single pancreatic islet proteome profiling by 1D-LC-MS/MS from Waanders et al. [34]. The study utilized a custom RePlay setup which employs a split flow design to create technical replicates and utilizes narrow diameter (50 μm) LC columns to achieve high sensitivity and they were able to quantitatively compare the proteomes of single islets (a type of micro-

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organ with 2000–4000 cells) with and without glucose treatments. A total of 1482 proteins were quantified with ~140 proteins displaying statistically significant alterations in protein abundances upon glucose stimulation. A general up-regulation of glycolysis, the TCA cycle and ATP translocation was observed [34]. This study illustrates the potential of applying similar nanoproteomics approaches to investigate islet heterogeneity in type 1 or type 2 diabetes [99].

Our group has recently coupled LCM with the SNaPP system to perform spatially resolved quantitative proteome profiling of microdissected lung alveolar tissue that was equivalent to only 4000 cells [17]. A depth of >3400 proteins was achieved on this platform with 2-fold lower quantification variance and 5-fold faster sample processing time when compared to offline sample processing. This study revealed seven defined modules of coordinated transcription factor-signaling molecule expression patterns and suggested the importance of epigenetic regulation in preferentially fine-tuning early processes in lung development. It also demonstrated the unique value of nanoproteomics in providing mechanistic or molecular basis of tissue heterogeneity and dynamic phenotypes.

Another interesting study is the application of a three-dimensional capillary or nanoLC platform to achieve deep proteome coverage of murine embryonic stem cells using only low μg of total cell lysate (12.6 μg) [68]. This study unambiguously quantified 11,352 protein groups that covered ~70% of Swiss-Prot and revealed protein regulation across the full detectable range of high-throughput gene expression and protein translation. We should note that the starting amount of materials was still one order of magnitude higher than the defined upper limit of the nanoproteomics domain. Nevertheless, this does illustrate the potential to achieve deep proteome coverage of nanoproteomics with further advances in sensitivity.

6. Conclusions and perspectives

Tremendous advances in LC- and CE-MS platforms have been achieved in terms of overall sensitivity, proteome coverage, reproducibility, and quantification for global proteome analyses. The absolute sensitivity for LC-MS and CE-MS operating in the nanoflow regime is sufficient for analyzing low ng protein samples or small numbers of cells, and potentially even for single mammalian cells [29,30]. The main bottleneck for the overall sensitivity lies in the sample losses and efficiency in the front-end sample processing. Recent developments in integrated online separation systems for microscale sample processing have demonstrated to be highly promising towards nanoproteomics analyses of small populations of cells [17,91,95,100]. While the current sensitivity of proteomics platforms still falls short of single cell analyses, continuous developments in the front-end integrated sample preparation techniques perhaps will be the most likely path in achieving such level of sensitivity. For instance, automated nanoliter sample handling technology is one potential capability for performing proteomic sample handling in nanoliter volumes by utilizing special patterned surfaces to perform sample manipulations in nanodroplets [101].

While our discussion has been mainly focused on global profiling of protein abundances of nanoscale samples, a uniquely important aspect of proteomics is PTMs, due to their fundamental roles in signal transduction and protein functional regulation. However, the

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sensitivity challenge for most types of PTM analyses is much more significant compared to global proteome profiling due to sub-stoichiometric and low-abundance nature of PTMs. Using phosphoproteomics as an example, typically hundreds of micrograms of peptides are required even when the most advanced nanoLC-MS platforms were used [102,103]. Recently, online integration of TiO_2 enrichment with nanoLC-MS [104,105] or orthogonal 3D separations [64] have significantly improved the overall sensitivity of the phosphoproteomics workflow. However, the current state-of-the-art of phosphoproteomics still falls far short of analyzing nanogram-scale samples [103].

Based on the current performance status of MS-based proteomics, significant advances are still required for effective analyses of both the proteome and multiple types of PTMs in nanoscale samples or even at the single cell resolution in a high throughput manner. Most likely, further breakthroughs in all aspects of the proteomics workflow including MS instrumentation, microscale separations, and front-end sample processing are needed to achieve this long-term goal. With current pace of technological advances in the proteomics field, it is highly likely that nanoproteomics will soon become an indispensable technology for molecular characterization of clinical tissues in biomedical applications. While nanoproteomics is mainly recognized as a discovery technology, it is likely to make an impact on the clinics in facilitating more accurate molecular diagnosis/prognosis provided that further advances in robustness, automation, and throughput are achieved.

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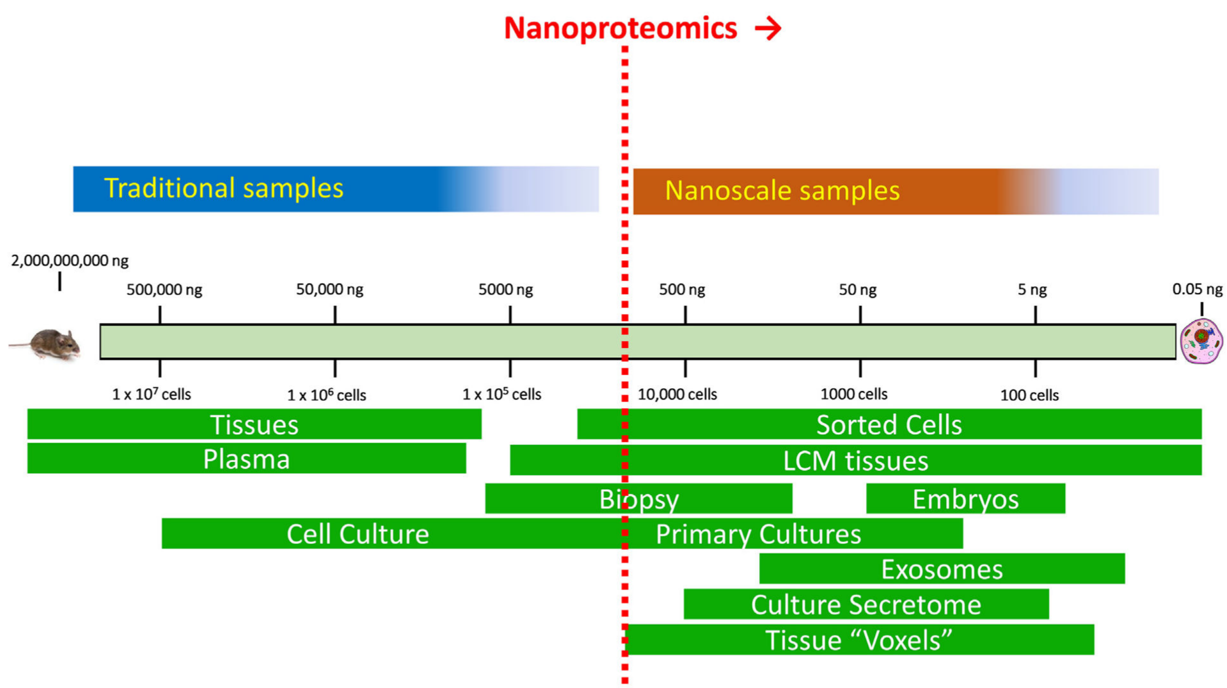

Fig. 1.

An illustration of traditional and nanoproteomics domains. The nanoproteomics is defined for dealing with samples containing <1 μg total protein in starting material.

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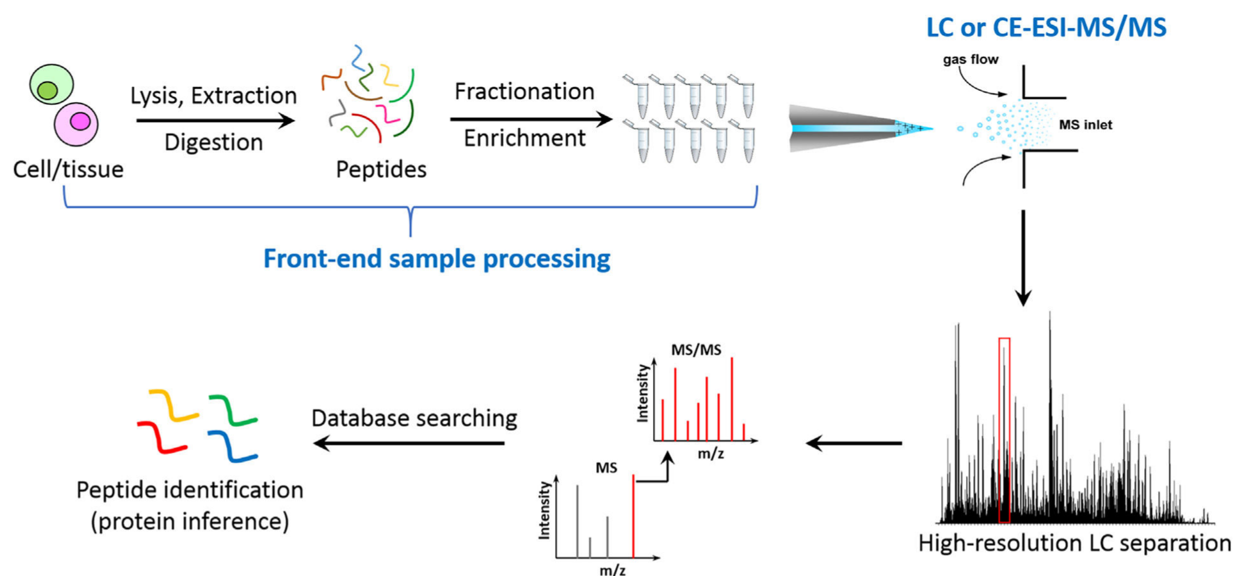

Fig. 2.

A general workflow of bottom-up MS-based proteomics. It typically starts from cell lysis, protein extraction, and proteolytic digestion to peptide mixtures, followed up by fractionation or enrichment procedures and LC or CE-MS/MS analyses. The experimental spectrum is matched with database to identify peptides or contaminants.

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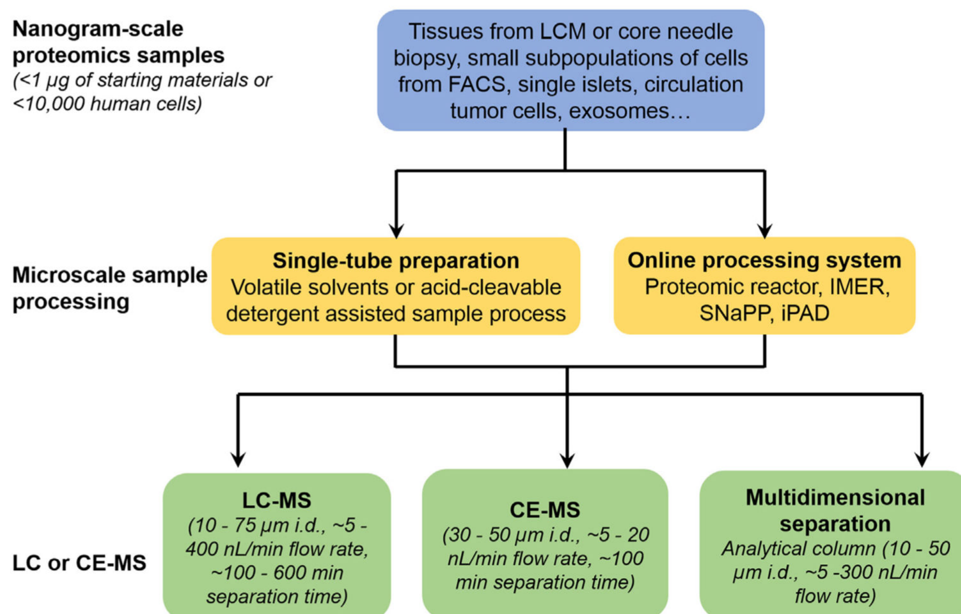

Fig. 3.

An overview of techniques adopted in different components of MS-based nanoproteomics. Generally, nanogram-scale samples are obtained from different sources, and are processed using microscale sample processing techniques such as single-tube, or online processing systems to reduce sample transfer steps for minimum sample loss. Then the digested peptides are subjected to highly sensitive nanoLC-MS or CE-MS analysis. In some cases, multidimensional separation is utilized to increase overall proteome coverage.

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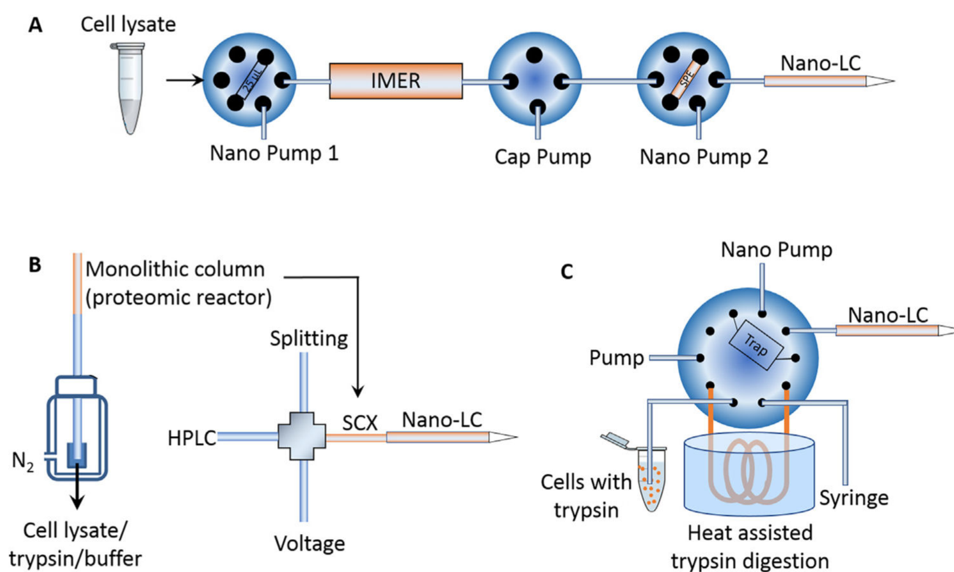

Fig. 4.

Schematics of online integrated sample processing techniques. (A) SNaPP system for global proteomics of nanogram samples. Adpated from Ref [91]. (B) A monolithic column was used as a proteomic reactor and then assembled as the first dimensional SCX separation column. Adapted from Ref [95]. (C) iPAD system for living cell proteome profiling. Adapted with permission from Ref [100]. Copyright (2015) American Chemical Society.

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Table 1

Microscale separation platforms and their performance in nanoproteomics*.

Column type	L(cm)	i.d. (μm)	F (nL/min)	Sample size	Cp	# proteins	Detection limit	Ref
Packed	87	15–75	20–400	0.1 μg yeast digest	~1000	1345–7 peptides		[32]
LC	10	50	500	1 μg yeast digest	~100	288–492		[106]
	85	15	20	0.5 pg-10 ng bacterium digest	~1000	3–872	75 zmol	[29]
	15	75	200	10,000 LCM breast cells		76		[33]
	25	75	400	~15,000 LCM pancreatic cells		900–1300		[107]
	10	100	700	0.2–0.8 μg single blastomeres		644–1466		[35]
	10	75	300	500–5000 MCF7 cells		167–619		[36]
	10	50	50	single islets (2000–4000 cells)		2013		[34]
	20	75	300	2000 kidney cells (Spintip-based)		1270 (duplicates)		[108]
	12	75	200	100,000 cancer cells (Direct lysis-digest)		2987 phosphosites		[109]
	75	50	300	4000 LCM lung cells (SNaPP)		3446		[17]
			300	100–500 cells (iPAD)		635–1060	200 zmol	[100]
	100	25	5	0.2 μg murine cell digest	~750	3243		[66]
Monolithic	70	20	40	0.5–100 ng bacterium digest	~420	18–217	15 amol	[39]
LC	25	10	10	0.1 μg bacterium digest		1332	5 amol	[42]
	60	75	100–300	0.5 μg yeast digest	~313	1323		[40]
	200	100	500	1 μg HeLa cell digest	~360	2605		[110]
	420	10	20	0.05 μg microbe digest fraction	~400	566	<amol	[44]
	320	10	20	45 ng protein (equivalent to 350 cells)		343		[43]
	320(2D)	10	20	500 ng cancer cell digest		850		[71]
	800	10	40	1000–10,000 tumor cell digest		456–1187		[45]
	500	10	40	0.5 μg cancer cell digest (OTER)		1462		[88]
CE	60	50		6ng rat testis H1 histones digest		135 peptides	<30 amol	[111]
	90	50		400 ng HeLa cell digest	~300	2100		[58]
	50	50		tumor cell digest (1 μL)		112	7 fmol	[54]
	60	50		1–100ng bacterium digest		142–312		[57]
	60	50		<1 μg bacterium digest		871		[56]
	100	20		80 ng cancer cell digest		283	<2 amol	[59]
	60	50		20–40 ng bacterium digest		179–199		[60]
	90	30		5–100 ng microbe digest		370–548 (duplicates)		[112]

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Column type	L(cm)	i.d. (μm)	F (nL/min)	Sample size	Cp	# proteins	Detection limit	Ref
	40	10	20	400 fg–84 pg bacterium digest		4–162 (duplicates)		[30]
	95 (2D)	50		50 ng frog egg digest		330		[113]
	95 (2D)	50		5.5 ng bacterium digest		145		[113]

*L: column length; i.d.: column inner diameter; F: flow rate; Cp: peak capacity.