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Separation methods in single-cell proteomics: RPLC or CE?

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Abstract

Cellular heterogeneity is commonly investigated using single-cell genomics and transcriptomics to investigate biological questions such as disease mechanism, therapeutic screening, and genomic and transcriptomic diversity between cellular populations and subpopulations at the cellular level. Single-cell mass spectrometry (MS)-based proteomics enables the high-throughput examination of protein expression at the single-cell level with wide applicability, and with spatial and temporal resolution, applicable to the study of cellular development, disease, effect of treatment, etc. The study of single-cell proteomics has lagged behind genomics and transcriptomics largely because proteins from single-cell samples cannot be amplified as DNA and RNA can using well established techniques such as PCR. Therefore, analytical methods must be robust, reproducible, and sensitive enough to detect the very small amount of protein within a single cell. To this end, nearly every step of the proteomics process has been extensively altered and improved to facilitate the proteomics analysis of single cells including cell counting and sorting, lysis, protein digestion, sample cleanup, separation, MS data acquisition, and data analysis. Here, we have reviewed recent advances in single-cell protein separation using nano reversed phase liquid chromatography (nRPLC) and capillary electrophoresis (CE) to inform application driven selection of separation techniques in the laboratory setting.

Keywords

Single-cell proteomics; reversed-phase liquid chromatography; capillary electrophoresis mass spectrometry

Introduction

Single-cell genomics and transcriptomics has enlightened the scientific community to the genetic variation between individuals, within organ tissue, and as a result of disease states.[1] The further development of mass spectrometry (MS)-based single-cell bioanalysis (*e.g.,* proteomics and metabolomics) techniques holds even more promise for the opportunity to interrogate biological phenotypes at the single-cell level. Standard bioanalytical methods are designed to analyze cellular biomolecules from lysates made from blending thousands

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of cells to interrogate average cellular expression.[2] These bulk cell analysis techniques can obscure the cellular variation of unique cellular phenotypes as well as their responses to environmental changes, disease progression, and therapeutic treatment.

The advantage that the development of single-cell proteomics techniques will afford is the ability to analyze phenotypical cellular variation with spatial and temporal resolution. Spatial resolution of cellular protein expression is valuable to the study of tissue morphologies,[3] localization of diseased tissues, and observation of the effect of environmental or therapeutic treatment on diseased *vs.* healthy tissue.[4] Furthermore, temporal resolution of single-cell proteomics has the potential to track cellular development,[5] disease progression,[6] and observe response to stimuli such as disease treatment.[7] However, analysis of the extremely limited sample available in a single cell is challenging for a myriad of reasons including low amounts of analyte with high dynamic range, inefficient small volume sample preparation and handling techniques, inadequate separation methods, and low throughput. These challenges required the overhaul of nearly every step of the MS-based proteomics methodology.

The state of single-cell proteomics has been recently reviewed with respect to current technologies.[8–11] Additionally, an excellent review that discusses sample preparation, separation, and MS analysis of mass-limited samples indicates potential application to single-cell proteomics.[12] This perspective will discuss developments and applications of single-cell MS-based proteomics with a particular focus on the separation methodology (*i.e.*, nano reversed phase liquid chromatography (nRPLC) and capillary electrophoresis (CE)) (Figure 1 and Table 1). Furthermore, we will discuss the future of this field and how further improvements and developments with regard to sample preparation and separation may impact the feasibility of single-cell proteomics analysis.

Single-cell Proteomics Sample Preparation

Early demonstrations of single-cell MS proteomics analysis were performed on single red blood cells to observe hemoglobin in healthy cells and those effected by sickle cell disease using matrix assisted laser desorption/ionization (MALDI). High-throughput proteomics workflows have since been applied to deepen proteome coverage of single cells. Protein preparation for high-throughput proteomics generally consist of a series of steps including bulk cell lysis, protein extraction, proteolysis (denaturation, reduction, alkylation, and digestion), desalting, separation, MS data acquisition, and data processing.[2] Efforts have been made to adapt existing bulk cell lysis and protein extraction techniques (i.e., tube-based methods that are conducted in Eppendorf tubes) to processing limited mass samples and miniaturization of high-throughput proteomics workflow has been demonstrated to outperform traditional workflows for mass limited samples.[13–15] Some of the first iterations of high-throughput proteomics analysis to single cells were performed on Frog (*Xenopus laevis*) embryos[16–19] which contain relatively large cells with high protein content (approximately 1 μg of yolk-free protein at the 16-cell stage that decreases as embryonic development proceeds to higher number cell stages[20–21]). These experiments utilized dissection to isolate cells and a miniaturized, tube-based, bottom-up workflow for lysis, protein extraction, and digestion. Additionally, more recent proteomics analysis of

these relatively large cells has been performed via the direct penetration of the cells to aspirate cytosol for further downstream processing.[20, 22] Single Cell ProtEomics by Mass Spectrometry (SCoPE-MS) was also developed initially as a tube-based method in which cells are lysed by sonication to avoid the addition of non-MS compatible reagents.[23–24] Samples were then TMT labeled and combined with a carrier proteome to increase identification numbers.

Tube-based sample preparation typically used for bulk cell sample preparation has been demonstrated to cause sample loss due to adhesion to surfaces, desalting, and speed-vac drying which disproportionately effect low sample quantities.[25] Nonspecific adsorption has even been recently reviewed in the literature and was discussed as a primary inhibitor to application of single-cell proteomics analysis.[26] Thus, application of typical bottom-up sample preparation techniques are not suitable for extremely small sample amounts in human somatic cells, (*e.g.,* hundreds of picograms).[25, 27] Furthermore, consistent sample processing on such a small scale is susceptible to human error when conducted by hand and can be time consuming leading to low throughput. Therefore, as an alternative to tube-based sample processing, ultra-low-volume (*e.g.*, nL range) sample preparation techniques have been developed to enable processing of single-cell analytes with reduced sample loss.

One of the most promising areas of advancement for single-cell proteomics is droplet-based sample preparation including Oil-Air-Droplet (OAD)[28], nanodroplet Processing in One pot for Trace Samples (nanoPOTS)[29], automated Preparation in One pot for Trace Samples (autoPOTS)[30], nested nanoPOTS (N2)[31], and nano-ProteOmic sample Preparation (nPOP).[32] These methods utilize automated sample handling platforms for droplet-based cell lysis and digestion in small sample volumes (approximately 200 and 550 nL for nano/autoPOTS and OAD, respectively). The N2 chip and nPOP method further scale down the sample volume to 30 nL[31] and 20 nL[32], respectively. m(n)POP was further integrated into the SCoPE-MS workflow resulting in SCoPE-2.[33] As an alternative to the droplet sample preparation approach, the Zhang lab introduced the integrated Proteomic Analysis Device for single-cell proteomics (iPAD-1).[34] Unlike the droplet-based approaches, the iPAD-1 directly aspirates a single cell into a capillary and performs in-capillary lysis and protein digestion in 2 nL volume and the whole volume can then be loaded for sample analysis.

In addition to the sample preparation methods for single cells discussed, some new techniques have emerged that have not yet been applied to single-cell proteomics but demonstrate promise in the analysis of very small numbers of cells. For example, adaptations of the microreactor-based platforms including single-pot solid-phase-enhanced sample preparation (SP3)[35] and filter-aided sample preparation (FASP) method have been introduced by Yang *et. al.* as the nanoparticle-aided nanoreactor for nanoproteomics (Nano3)[36], Zhang *et. al.* as MICRO-FASP[37], and Kostas *et. al.* as on-microsolid-phase extraction tip (OmSET)[38]. These methods show promise as low volume sample preparation methods with limited sample loss for mass limited samples. Additionally, Burns *et. al.* developed a platform that utilizes an automated liquid handler to prepare cells for bottom-up proteomics directly in a 384-well plate used to culture the cells. While this method is not yet directly applicable to single cell analysis, it allows high throughput proteomics for drug screening with minimal sample loss.[39]

To enhance the throughput and precision of these single-cell sample preparation platforms, dedicated platforms have been implemented for cell sorting to isolate single cells onto the sample preparation chips such as fluorescence activated cell sorting (FACS) and CellenONE (SCIENION).[24, 32–33, 40] Additionally, CellenONE can integrate cell sorting and nano-drop based sample prep for a completely automated sample preparation methodology as initially demonstrated by Woo *et. al.*[31]

Decreased volume of sample preparation is preferable to single-cell analysis because the decrease in sample volume limits exposure to surfaces to decrease protein adsorption.[25] Minimal protein loss is critical to single-cell analysis in which the protein is very limited. Furthermore, automation of the sample preparation platforms, particularly for exceedingly small volumes of sample, increases sample throughput and limits sample to sample variation.

Nanoscale Reversed phase liquid chromatography (nRPLC)

For high-throughput proteomics of biological samples, high sample complexity and dynamic range complicates MS spectra obscuring peptide identifications. Single-cell proteomics is further complicated due to the small amount of protein contained within a single cell as most high-resolution mass spectrometers need many copies of a protein for fragmentation and ionization to result in identification of peptides. Separation has been implemented to decrease sample complexity and concentrate peptides prior to MS analysis to increase proteome coverage, particularly for low abundance proteins. nRPLC is the most popularly implemented separation technique for single-cell analysis because it is highly sensitive with high resolution and can utilize MS compatible buffers to facilitate direct coupling with MS via nanoESI interfaces.

After single-cell sample preparation (either tube-based or droplet-based) that typically incorporate one of two types of quantitation, label-free or isobaric chemical tag labeling, the sample volume is commonly scaled up from nL to μL and transferred to a tube for nRPLC sample injection. Online solid-phase extraction columns for concentration and/or sample cleanup increase the separation efficiency and sensitivity followed by application of the LC gradient for separation and elution.

Label-free quantitation is a simple method for relative quantitation of single-cell proteins that benefits from limited sample preparation, low cost, and wide applicability (not limited to lab cultured samples like isotopic labeling).[41] Notably, label-free quantitation and data independent acquisition were applied to the analysis of 10 single human lung adenocarcinoma (PC-9) cells (processed using a chip-based sample preparation method, SciProChip) for average identification of approximately 1500 protein groups/cell.[42]

Generally speaking, the primary drawback of label-free quantitation (aside from run-to-run variation) is limited throughput. To increase throughput, the Kelly group developed an LC configuration that utilized two parallel subsystems to alternate sample separation and data acquisition with the support functions of the system such as sample loading, desalting, and regeneration to maximize instrument utilization.[43] With this platform, they

identified ~1000 protein groups/cell using a 30-minute gradient resulting in the capability to analyze 48 samples per day. Additionally, Williams *et. al.* developed an autosampler for the nanoPOTS system to allow automated sampling of single cells to further increase throughput.[4443] Another drawback of single-cell label-free quantitation is the prevalence of missed identifications between runs due to inadequate MS peptide identification based on MS^n fragmentation.[45] The number of quantifiable proteins from label-free datasets can be increased when protein libraries from larger numbers of cells are combined using accurate mass and time tag (AMT)[46] comparisons or algorithms such as MaxQuant's Match-Between-Runs (MBR).[47–48]

Generally, the advantage of isobaric chemical tag labeling (tandem-mass-tag, TMT; or isobaric tags for relative and absolute quantitation, iTRAQ) is multiplexing to increase throughput and eliminate run-to-run variation.[49] For single-cell proteomics, isobaric chemical tag labeling also has the advantage of increasing the total amount of protein that can be injected to boost signal and increase the number of identified proteins. To this end a series of techniques including the SCoPE[23–24, 33, 50] and nanoPOTS[29, 31, 44] related methods and Improved Boosting to Amplify Signal with Isobaric Labeling (iBASIL)[51–52] that utilize carrier proteomes or booster channels, samples that are labeled that contain higher masses of proteins than the single cell, are multiplexed with single-cell samples to increase the amount of protein injected for nRPLC analysis.[51, 53] In one study, the iBASIL approach led to the identification and quantification of 1500 proteins from 3 different myeloid leukemia cell lines.[51] While TMT labeling with carrier or boosting proteomes increase the number of identified protein groups, the accuracy of the quantitation can be effected by precursor co-isolation[54], isotopic impurities[55], and batch effects[56]. Specific to single-cell analysis using carrier proteomes, quantitative accuracy was dependent on the ratio of carrier to sample which limits the amount of protein that can be added for the carrier/booster.[57] Overall, the advantages of nRPLC single-cell analysis include the ability to automate the sample preparation and analysis platform for decreased sample variation and the ability of nRPLC to incorporate online SPE sample cleanup and concentration for more sensitive detection.

To improve the proteome coverage, miniaturization of nRPLC column is used as a strategy to increase the efficiency of nRPLC separation and ESI-MS sensitivity for single-cell level peptide analysis. Shen and co-workers detected ~190-fold more mass features upon decreasing self-packed LC column inner diameter from 74.5 μm to 14.5 μm using 100 ng yeast tryptic digest.[58] Zhu and co-workers demonstrated a 32% increase in peptide identifications using 30-μm-i.d. columns compared with standard 75-μm-i.d. columns using 10 ng tryptic peptides.[59] Recently, Cong and co-workers achieved a ~41% increase in protein group identification using an ultranarrow-bore (20 μm i.d.) LC column compared with a 30 μm i.d. column for nanoPOTS-prepared single *HeLa* cells.[60] Overall, decreasing the inner diameter of the columns can increase protein identification; however, the improvement is only incremental and the relatively large sample volume and dilutions required for nRPLC analysis can still lead to sample loss and decreased sensitivity. Application of methods beyond the packed LC column, such as open tubular[61] or micropillar array columns[62], may also be explored for increased nRPLC sensitivity.

Capillary Electrophoresis (CE)

Capillary electrophoresis (CE) coupled with mass spectrometry is advantageous for the analysis of single cells due to the highly efficient separation[63] of extremely small (low nL) sample volumes[64] with ultra-low detection limit (zmol)[65]. However, CE separation has been less frequently implemented for single-cell proteomics than nRPLC, with the most popular targets being large, nonhuman cell types such as mouse neurons[22] and embryonic cells from frog[16–18, 20] and zebrafish[20]. Typically, sample preparation for single-cell CE-MS analysis was done via dissection[66] to isolate single cells into tubes followed by a miniaturized version of the traditional bottom-up proteomics sample preparation workflow[2] to minimize sample loss. Subsequently, a portion of the extracted proteins were injected for CE-MS analysis, and 3–4 technical replicates were performed. Further adaptation of these techniques led to direct sampling of cytoplasm from living cells followed by protein digestion and CE-MS analysis.[20]

Using label-free CE-MS methods, the Nemes group analyzed 16 ng of cytoplasmic protein digest from biological triplicate runs of three different Frog embryo cells resulting in the identification of 438 protein groups.[17] Application of TMT labeling to the same system allowed for the identification of 1709 protein groups from 3 biological replicates using 20 ng of protein.[16] As an impressive application of this technology, the Nemes group sampled cytoplasm directly from living *Xenopus laevis* embryos and zebrafish embryos at different development stages to compare protein expression throughout development.[20] They were able to identify 750–800 protein groups from 5 ng of protein in 16-cell *Xenopus laevis* embryo using label-free quantitation.[20] In what is, to our knowledge, the smallest amount of protein analyzed by CE-MS single-cell proteomics to date, the Nemes group analyzed 1 pg of protein digest from single mouse neurons to quantify 157 proteins using TMT labeling and a carrier proteome.[22]

Overall, CE-MS has been demonstrated to be a powerful method for single-cell proteomics. Label-free and isobaric chemical tag labeling techniques have resulted in identification of hundreds to more than 1000 protein groups utilizing low nanogram levels of sample performed primarily with tube-based sample preparation methods (Table 1). While application of CE separation to human single cells has lagged behind LC-based single cell analysis, CE-MS/MS analysis has been applied to the analysis mass limited human lysate. For example, Johnson *et. al.* identified 744±127 proteins from 1-cell equivalents of *HeLa* lysate using their CE-MS/MS platform.[67] Application of droplet-based sample preparation methods may further amplify the number of identifications and allow further application of CE separation to human cell lines.

Some common drawbacks associated with CE separation are nonspecific sample adsorption to the bare CE capillary column and unstable electroosmotic flow (EOF) resulting in reduced separation efficiency.[68] Application of coatings including covalently bound chemical coatings such as linear polyacrylamide[69] or polyethyleneimine[70] not only decrease nonspecific sample adsorption but also increase separation efficiency by adjusting EOF; coatings used for CE separation of peptides have been reviewed in the literature.[71] Another challenge associated with single-cell CE proteomics is manipulating and loading the

exceedingly small sample volumes associated with the small-scale sample preparation. CE can tolerate extremely low injection volumes from low nL to pL so it is not necessary to dilute single-cell samples to high volumes as is generally done with the droplet-based methods[21]; however, pressure-based sample injection and micropipettes can be limiting regarding the sample volume they are capable of manipulating. Methods such as sample stacking[72] or SPME[73–74] have been used to increase the sample loading capacity for CE separation and, while these methods have not yet been applied to single cells, they have proven to be useful for single-cell amounts of protein digests and would be valuable to single-cell applications. Furthermore, as an alternative to micropipette-based CE sampling, a microsampling device, Spray-capillary, has been developed that uses the pressure differential from generation of ESI as the driving force for tunable and quantitative ultra-low volume sample injection (*e.g.*, as low as 15 pL/s).[75] Furthermore, the device can be used directly for CE separation with no additional sample handling steps.[76] Coupling of the spray-capillary CE-MS platform with droplet-based sample preparation without excessive dilution could enable injection of contents from a single cell with minimal sample loss. Since the sample loading requirement for CE-MS analysis is very low, the spray-capillary could also be further applied for multidimensional, single-cell analysis coupled with nRPLC fractionation for deeper single-cell proteome coverage.

Conclusions and Final Perspectives

nRPLC workflows are advantageous due to the SPE loading and high-resolution separation of peptides; CE separation benefits from low detection limit and low mass/volume sample consumption. NanoRPLC and CE can both utilize MS compatible solvent systems for direct coupling with ESI-MS techniques. In both methods, isobaric chemical tag labeling based single-cell sample preparation techniques can be adapted for increased sample injection resulting in higher proteome coverage, increased throughput, and decreased sample-to-sample variation.[57, 77] Further optimization of nRPLC columns such as utilizing monolithic columns or nano-open-tubular columns[61] can improve the separation efficiency and ESI-MS sensitivity for improving single-cell proteome coverage.[58–60, 78] Separation efficiency of CE separation for single-cell proteomics can also be improved by the application of column coatings such as linear polyacrylamide and polyethylenimine[69–70] or using online SPME trapping prior to CE separation[74]. Sample volume (nL level) used for most droplet and tube-based single-cell methods and a lack of ultra-low-volume sample handling methods limit the application of CE due restrictions on sample injection volume.[79] Improvements to ultra-low-volume sample handling techniques and further minimization of single-cell sample preparation volume to limit sample loss may broaden the applications of CE separation for more diverse single-cell proteomics. Thus, both nRPLC and CE have room for improvement as they are applied to single-cell proteomics and selection of these methods is largely application driven and will be dependent on future improvements to sample preparation and handling methods as well as optimization of the separation methods themselves.

Aside from improvements to liquid-phase separation, innovative gas phase separation techniques have been applied to single-cell MS analysis to increase instrumental sensitivity including implementation of High field asymmetric waveform ion mobility spectrometry (FAIMS). FAIMS can be applied to high resolution mass spectrometers to filter out chemical

noise and interfering ions to improve dynamic range and detection limits.[80] Since these instruments have a maximum charge capacity, the presence of +1 charged contaminant species can lead to signal suppression. The first report of the application of FAIMS to single-cell proteomics utilized FAIMS to remove singly charged species and reported the identification of 1056 protein groups from 2912 peptides which demonstrated a 2.3 and 2.0-fold improvement, respectively, over the same analytical setup without FAIMS.[81] As a further implementation of FAIMS filtering applied to single-cell proteomics, Transferring Identification based on FAIMS Filtering (TIFF) creates a library of peptides via the repetitive analysis of larger sample amounts using varying FAIMS compensation voltages (CV).[82] This library is integrated with precursor mass and elution time as in an AMT or MBR approach for MS1 level matching to single-cell spectra to decrease false discovery rates. TIFF, using a library created using 4 CVs, was applied to the analysis of single *HeLa* cells and increased the number of proteins identified from an average of 209 to 1,212/cell.[82]

Overall, the application of nRPLC or CE separation methods to single-cell proteomics is application dependent and requires deep consideration of relevant available methods for successful analysis. While it seems unlikely that any particular method or platform will emerge as vastly superior, the work done in this field has made impressive strides toward the realization of comprehensive single-cell proteomics; perhaps the implementation of novel technologies or combination platforms as discussed here will give rise to new heights in single-cell proteomics.

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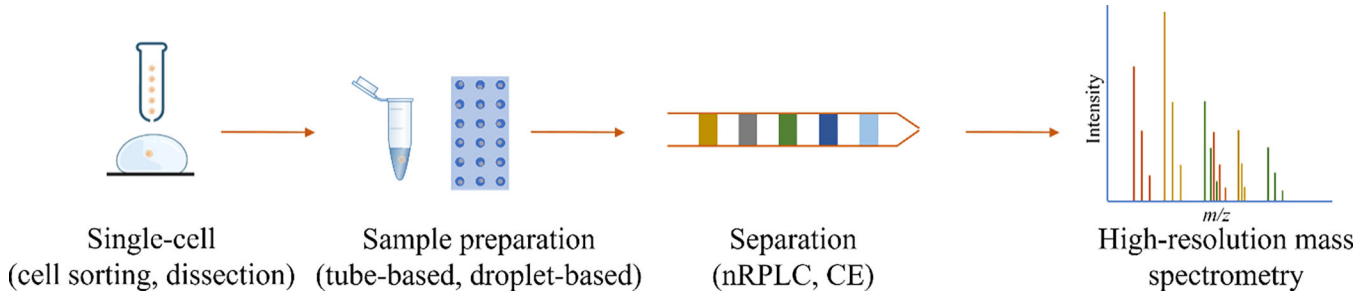

Figure 1:
General workflow for MS-based single-cell proteomics.

Table 1:

Comparison of single-cell applications including selected publications that utilize single-cell sample preparation methods.

Quantification Method	Separation	Cell type	Sample handling	Sample loading amount	CE/LC column	MS	Protein ID count
	CE	Neurons (C57Bl6/J mice)	Tube-based	0.001 ng (1 pg, ~0.25% of the total cellular proteome) + 0.1 ng carrier/ boosting peptides	100 cm fused silica capillary (90/20 μm o.d./ i.d.)	Q Exactive Plus MS	157[22]
		Murine (C10, epithelial cells; RAW, macrophage cells; SVEC, endothelial cells)	Droplet-based	1 cell + l50 ng carrier/boosting peptides	In-house packed 50 cm C18 column (30 μm i.d., 50 nL/min)	Fusion Lumos Tribrid MS	1408[53]
		Acute myeloid leukemia (AML) cell (MOLM-14, K562, and CMK)	Droplet-based	1 cell + 10 ng carrier/boosting peptides	In-house packed 30 cm C18 column (50 μm i.d., 100 nL/min)	Fusion Lumos Tribrid MS	1926[51]
		Acute myeloid leukemia (AML) cell (MOLM-14, K562, and CMK)	Droplet-based	1 cell + 10 ng carrier/boosting peptides	In-house packed 60 cm C18 column (50 μm i.d., 150 nL/min)	Fusion Lumos Tribrid MS	~1800[44]
		OCI-AML8227 cell	Droplet-based	1 cell + 500 cell digest as carrier/ boosting channel	A 15 cm C18 column (ThermoFisher EasySpray ES804A, 100 nL/min)	Exploris480 with FAIMS Pro	~1000[40]
		Murine (C10, epithelial cells; RAW, macrophage cells; SVEC, endothelial cells)	Droplet-based	1 cell + 10 ng carrier/boosting peptides	A 25 cm C18 column (Waters, 50 μm i.d., 100 nL/min)	Q Exactive Plus MS	~1500[31]
Isobaric labeling with carrier/boosting	**RPLC**	U-937 (monocytes) cell	Tube-based	1 cell + 200 cell digest as carrier/ boosting channel	25 cm C18 column (AUR2-25075C18A, 200 nL/min)	Q Exactive MS	~1000[50]
Isobaric labeling without carrier/ boosting	**CE**	Frog (Xenopus) Embryo	Tube-based	20 ng	85-cm fused silica capillary (110/40 μm o.d./ i.d.)	Orbitrap Fusion Tribrid MS	500–800[16]
		Frog (Xenopus) Embryo	Tube-based	16 ng	85 cm fused silica capillary (110/40 μm o.d./ i.d.)	Orbitrap Fusion Tribrid MS	438[17]
		Frog (Xenopus) Embryo	Tube-based	~5 ng	90 cm fused silica capillary (110/40 μm o.d./ i.d.)	Q Exactive Plus MS	~750–800[20]

Quantification Method	Separation	Cell type	Sample handling	Sample loading amount	CE/LC column	MS	Protein ID count
	CE	Frog (Xenopus) Embryo	Tube-based	~5 ng	90 cm fused silica capillary (110/40 μm o.d./ i.d.)	Q Exactive Plus MS	722[83]
Label free quantification	**RPLC**	Human Oocytes	Tube-based	1 cell (~100 pg)	20 cm C18 column (nanoAcquity, 300 nL/ min)	Orbitrap Velos Pro MS	~450[84]
		Frog (Xenopus) Embryo	Tube-based	200–800 ng	10 cm C18 column (Waters, 100 μm i.d., 700 nL/min)	Q-Exactive HF MS	644–1466[18]
		HeLa	Droplet-based	1 cell (~0.15 ng)	50 cm C18 column (30 μm i.d., 60 nL/min)	Orbitrap Fusion Lumos Tribrid MS	669[85]
		HeLa	Droplet-based	10 cells (~1.5 ng)	In-house packed 70 cm C18 column (30 μm i.d., 60 nL/min)	Orbitrap Fusion Lumos Tribrid MS	~3000[29]
		Frog (Xenopus) Embryo	Tube-based	~40ng	In-house packed 20 cm C18 column (75 μm i.d., 300 nL/min)	Q-Exactive HF-X Hybrid Quadrupole-Orbitrap MS	~1650[21]
		Chick utricle E15 (Hair cell)	Droplet-based	1 cell	In-house packed 50 cm C18 column (30 μm i.d., 60 nL/min)	Orbitrap Fusion Lumos Tribrid MS	50–75[86]
		HeLa	Droplet-based	1 cell (~0.15 ng)	In-house packed 50 cm C18 column (20 μm i.d., 20 nL/min)	Orbitrap Eclipse Tribrid MS	1056[81]
		MCF10A (MCF7) breast cancer cell	Tube-based	1 cell	In-house packed 70 cm C18 column (50 μm i.d., 150 nL/min)	Q Exactive Plus MS	384[87]
		HeLa	Droplet-based	1 cell	In-house packed 45 cm C18 column (75 μm i.d., 300 nL/min)	Trapped ion mobility spectrometry quadrupole time-of-flight MS	1279[88]
		Human lung adenocarcinoma cell (PC-9)	Chip-based	1 cell	A 25 cm C18 column (Waters, nanoEase, 75 μm i.d., 300 nL/min)	Orbitrap Eclipse MS	~1500[42]
		HeLa	Droplet-based	1 cell	In-house packed 10 cm C18 column (50 μm i.d., 80 nL/min)	Orbitrap Exploris 480 MS	986[43]
Label free quantification	**RPLC**	Hela	Droplet-based	1 cell	In-house packed 25 cm C18 column (50 μm i.d., 100 nL/min)	Orbitrap Fusion Lumos Tribrid MS with FAIMS pro	1212[82]

Quantification Method	Separation	Cell type	Sample handling	Sample loading amount	CE/LC column	MS	Protein ID count
		Frog (Xenopus) Embryo	Tube-based	~200 ng	In-house packed C18 column (75 μm i.d., 315 nL/min)	Orbitrap hybrid Fusion Lumos MS	3468[89]