Recent advances in the field of single-cell proteomics

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A B S T R A C T

The field of single-cell omics is rapidly progressing. Although DNA and RNA sequencing-based methods have dominated the field to date, global proteome profiling has also entered the main stage. Single-cell proteomics was facilitated by advancements in different aspects of mass spectrometry (MS)-based proteomics, such as instrument design, sample preparation, chromatography and ion mobility. Single-cell proteomics by mass spectrometry (scp-MS) has moved beyond being a mere technical development, and is now able to deliver actual biological application and has been successfully applied to characterize different cell states. Here, we review some key developments of scp-MS, provide a background to the field, discuss the various available methods and foresee possible future directions.

Introduction

Multicellular organisms are comprised of specialized tissue that is formed from numerous cell types organized into complex hierarchies that carry out vital physiological functions. The distinct functional and morphological features of each cell type are dependent on a complex interplay between the genome, transcriptome, proteome and other regulatory molecules that constitute the cell. The spatiotemporal expression of different molecules that are required to generate a specific cell type, is orchestrated by the genetic program found in every cell. However, loss of genome integrity in the form of genetic mutations can disrupt this complicated process and lead to the development of cancer. Malignant transformation can induce alterations in the cellular proteome, changing cell morphology and function, and in turn disrupting the cellular hierarchies within tissues and creating a microenvironment that is composed of distinct subpopulations of both healthy and cancerous cell types [36]. However, the altered cell population dynamics, and shifts in cell-type specific proteomes remain obscured with population-based techniques, as bulk methods provide merely an aggregated view of all the cells that comprise a population (Fig. 1). Especially in light of cancer stem cells [8], if we are to define pure cancer stem cell proteomes, and discover the protein signalling networks underlying their biological phenotypes, we absolutely rely on approaches with single-cell resolution. It has become increasingly clear that distinct cell states can respond differently to the same extrinsic signal and that the response can even vary even within the same cell type [2,28,30, 101]. Accordingly, the rise of technologies that allow genome, transcriptome, proteome and metabolome profiling at the single-cell level, has transformed our ability to study cell development and disease pathology, by providing unprecedented insight into these mechanisms [23,28,49].

The demand and emergence of single-cell proteomics

Transcriptomics has so far taken central stage in the field of single-cell research, where transcript abundances are routinely used as a surrogate for protein abundances. While gene signatures are of great value [25,46,58,60,80], finding novel key biological regulators ultimately should involve protein-centric approaches for several reasons. Not only are cell fate decisions largely driven by protein networks [29,38,69,81], accumulating evidence is demonstrating a discordance between mRNA and protein abundances both on population and single-cell level [12,32, 33,42,53,61,74,90,94,100], bringing into question the validity of using mRNA-levels as a proxy for protein expression. Furthermore, sequencing-based techniques are not inherently able to capture protein post-translational modifications, which can be crucial for their function [66]. However, it is now possible to quantify limited numbers of proteins and protein PTMs with targeted antibodies conjugated with DNA barcodes [77]. As proteins are the effector molecules of the cell, there is a rising demand for techniques that allow the direct quantification of global single-cell proteomes, that together with other single-cell modalities could provide direct molecular inference of cell states.

Single-cell proteomics techniques

The human genome contains ~20,000 protein coding genes. However, proteins can be translated from different splice variants and contain post-translational modifications (PTMs), such as

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phosphorylation or glycosylation. Considering this, the number of unique proteoforms that comprise the proteome can range in the billion [1]. Since cells do not express all proteins at the same time, so far the detectable proteome in cancer cells seems to be limited to ~12,000 proteins [6,61]. Multiple techniques that allow the analysis of proteins at single-cell resolution have emerged. These assays can be categorized based on the protein detection principle and are discussed in more detail below.

Antibody and tag-based single-cell proteomics approaches

The most widely used approaches to quantify protein abundance at the single-cell level to date rely on antibody-based detection or attaching a fluorescent probe to the terminus of the protein of interest with genetic engineering [45]. Once labelled, the protein of interest can be tracked with techniques that inherently have single-cell resolution, such as immunofluorescent (IF) microscopy or fluorescence-activated cell sorting (FACS) [53,64]. Cytometry by time of flight (CyTOF), further enhances the number of antibodies that can be quantified in parallel by combining the principles of flow-cytometry with MS [4,84]. Furthermore, sequencing-based techniques that utilize protein targeting antibodies fused to DNA oligonucleotides, allows the characterization of multiple modalities such from individual cells [14,57,77]. While these techniques allow the processing of thousands of cells, their ability to quantify only dozens to hundreds of proteins limits their utility in hypothesis-free, global data-driven biological interrogations. Furthermore, these assays are dependent on the availability of high-quality antibodies, which is especially important when protein PTMs are of interest, and inherently challenges aspects such as absolute quantitation (i.e. protein copy numbers) of protein molecules within the cell. Nevertheless, they represent a valuable tool for validating more targeted hypotheses.

MS based single-cell proteomics

Protein quantification with MS

MS can directly measure abundances of both protein and their PTMs. To facilitate proteome quantification, proteins are extracted from cells and digested with sequence specific proteases, such as trypsin. Resulting peptides are subsequently desalted to remove all MS-incompatible solvent constituents and separated based on their hydrophobic properties with High Performance Liquid Chromatography (HPLC). During this separation, the eluting peptides are introduced into the instrument via electrospray ionization, and the MS spectra of the consecutively eluting peptides are acquired in real-time. This type of measurement is commonly referred to as LC-MS. Two types of MS spectra are recorded for the eluting peptides; the MS1 spectra with contain information of the intact peptides (precursors) and MS2 (MS/MS) spectra, which acquire the mass information of peptide fragments [65]. Both peptide precursors and fragments give rise to peaks at a specific mass-to-charge (m/z) value, which can be used to identify exact peptide sequences. Peptide abundances can then be extracted from either the MS1 or MS2 peak intensities and rolled up to infer protein abundance [39].

Peptide spectra are typically collected in either data dependent (DDA) or independent (DIA) acquisition modes [10,11,34,39,51,97]. During DDA, single peptide precursor ions are selected for fragmentation by isolating a narrow m/z range surrounding the most intense ions (Fig. 2A). Because of this, information is gathered only for the most intense peptide precursor ions, and heavily biased by chromatography conditions which can vary from run-to-run. In contrast, during DIA, wider isolation segments are used to systematically fragment all ions within selected m/z ranges. This leads to the co-isolation and fragmentation of multiple peptide precursor ions, generating more chimeric spectra that theoretically contain information on all the peptides present in the sample [11]. Overall, DIA generated spectra have a higher spectral complexity, which poses a challenge to peptide identification. Extensive efforts have led to the development of powerful computational approaches that can handle this type of data. The newest generation of algorithms have propelled DIA based workflows to a level surpassing DDA in both proteome depth and data completeness [20,21,51,85].

Label-free and reporter-based peptide quantification are most frequently used. With label-free quantification, digested peptides are simply injected into the instrument and peptide abundance is estimated by generating an extracted ion chromatogram (XIC) from the peptide precursor or fragment spectra profile (Fig. 2B). Only DIA based acquisition commonly performs chromatogram extraction on both MS1 and MS2 levels, while DDA is limited to MS1. Reporter based quantification is generally employed when samples are multiplexed, to reduce instrument time and minimize between-run variation. Peptides from different samples are labelled with isobaric tags, such as tandem mass tags (TMT) [83], pooled together and analyzed in a single run. Individual peptides from the pooled samples appear as single indistinguishable MS1 peaks,
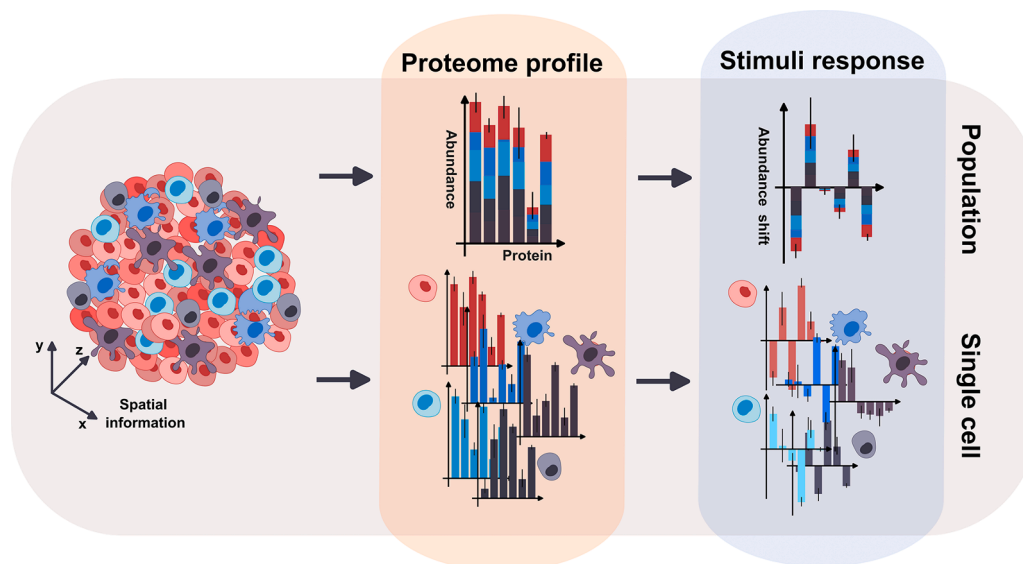

Fig. 1. Population vs. single-cell tumor proteome resolution. Tumors are comprised of multiple different cell types that have unique proteome profiles and responses to different stimuli. Population (bulk) based techniques only capture an average view and often preclude the identification of distinct cell-state driven events and their phenotypes.

Fig. 2. Illustration of different types of data acquisition and quantification methods. (**A**) During data dependent acquisition (DDA), single precursors that have the highest intensity are isolated (yellow arrows), and subsequently undergo fragmentation. In data independent acquisition (DIA), wide isolation windows (red bars) are used to systematically isolate and fragment the whole m/z range of interest. Relative to DDA, the subsequently generated MS2 spectra contain fragment information from multiple precursors and are more convoluted. (**B**) Extraction of intensity chromatograms (XIC) in label-free quantification (LFQ). XIC can generate an intensity peak from precursor (MS1) or fragment (MS2) intensities in DIA, while only precursor information is available in DDA. (**C**) Peptides arising from different samples can be labelled with isobaric tandem mass tags (TMT). This allows for samples to be pooled and analyzed in a single run. The labelled peptides give rise to a single MS1 peak. Upon fragmentation, three categories of ions are generated: (1) reporter ions that are used to quantification, (2) peptide fragment ions that are used for identification and (3) complementary ions that contain the remaining TMT tag coupled with the unfragmented peptide precursor. While only three channels are illustrated for simplicity, the latest TMTPro reagent allows multiplexing up to 18 samples. (**D**) In the SCoPE-MS approach, each TMT channel is used to label the peptide that are derived from one cell. One channel is reserved for the carrier, which is comprised of 100-200 single cells. This boosts the precursor and fragment ion intensities, increasing the probability of selection and identification of the peptide, while retaining single-cell resolution through distinct reporter ions.

resulting in higher signal intensity (Fig. 2C). However, once the peptide is fragmented, unique reporter ions present on the TMT tags are cleaved off and their intensities are used to infer the peptide abundance of each individual sample in the multiplexed pool (Fig. 2C). Both approaches have certain benefits and drawbacks when applied to single-cell input, which are discussed in more detail below.

Making single-cell proteomics a reality

Mass-spectrometry analysis of proteins generally requires >100ng of input material. However, one mammalian cell is estimated to contain only 100-200pg of protein [92]. Unlike for genome sequencing-based methods, there is currently no known way to artificially amplify the amount of protein in a cell, making it essential to minimize sample loss during processing. The median protein molecule number, contained in one cell, in theory should be sufficiently high for detection in a mass spectrometer [74,92], however successfully transferring every molecule into the mass spectrometer presents many challenges.

The first successful method for scp-MS profiling using LC-MS, termed SCoPE-MS (Fig. 2D), was facilitated by reducing the sample preparation

volume to low microliter scale and employing a carrier channel, consisting of 200 cells to ensure sufficient fragment ion intensity for peptide identification [13]. A second major breakthrough in the field was achieved by further minimizing the sample processing volume with the use of nanodrop-based sample preparation in combination with custom fabricated nanowell chips [102]. So far, four different sitting-droplet-based methods have been reported, nanodroplet Processing in One pot for Trace Samples (nanoPOTS), oil-air-droplet (OAD) chip, proteoCHIP and nano-ProteOmic sample Preparation (nPOP) [17, 47,48,95,102,103]. Three of these methods now utilize the cellenONE® liquid handling system for single-cell isolation and carrying out all the subsequent processing step in a low nanoliter volume, which drastically limits surface interaction and protein loss. Microfluidics devices that can streamline both cell isolation and processing for mass spectrometry have also been developed [31,72], and will be briefly discussed further below. Alternatively, use of FACS and low-protein binding microwell-plates with working volumes < 2uL showed comparable levels of identification depth as nanodroplet-based approaches [12,27,71,74], which is an attractive approach as the required instrumentation is accessible in most

standard lab settings. Additionally, FACS indexing can be used to measure cell surface markers and size during cell depositing, which aids downstream data analysis by e.g. pre-defining common cell populations, and establishing novel links between intracellular proteomes and extra-cellular protein expression [71]. Nevertheless, the benefits of nanolitre volumes remain undeniable as their enhanced signal-to-noise ratios allow identification of single-cell proteomes on MS instruments that have been around for more than a decade [13,22]

The third breakthrough came from coupling of ion mobility technology in the form of either trapped ion mobility (TIMS, Bruker instrumentation) or Field Asymmetric Ion Mobility Spectrometry (FAIMSPro, Thermo instrumentation) [5,73]. During LC-MS analysis, peptide ions are generally presented as multi-charged species, whereas chemical contaminants are specifically singly charged. To avoid contaminant analysis, typically only multiply-charged ions are selected for analysis. However, singly-charged species can be co-isolated with the peptides of interest and hamper identification. This issue becomes exceptionally acute when single-cell digests are measured, as single-cell spectra inherently have low signal signal-to-noise (S/N) ratios. FAIMS functions as a purifying module blocking the transmission of singly charged ions into the instrument, while allowing multiply charged ions to be transmitted [5,7,37]. Accordingly, FAIMS has shown to significantly increase the S/N ratio of single-cell spectra and boost identification [16]. Utilizing different compensation voltages (CV), FAIMS can perform real-time gas-phase fractionation of incoming peptides, which has been a valuable feature for scp-MS [27,71,96]. The use of multi-CV libraries generated from 50 to 100 cells almost doubled the proteome depth of single cells level input, attributed to lower background ion levels and enhanced ion injection times [96].

Additionally to filtering out contaminant ions, the TIMS module serves as an extra separation dimension as the peptide ions are trapped in the device depending on their mass and charge ratio. The captured ions can then be sequentially released based on their mobility properties [54,55,73]. A dual TIMS device is used to first accumulate and separate ions in the first tunnel, to then sequentially release those ions one-by-one from the second tunnel, to be analyzed with time-of-flight (TOF) spectral acquisition. To accommodate this advance Parallel accumulation−serial fragmentation (PASEF) method was developed that allows faster scanning speed by utilization the compressed peptide ion pockets in the TIMS device [54,55]. Recently, timsTOF based method have shown the capacity to quantify thousands of proteins from single-cell and ultra low-input samples [12,21]

Data acquisition and quantification method

Current scp-MS methods can be divided into two major categories depending on the chosen type of quantification. In label-free quantification (LFQ) workflows, each single cell is treated as a sample and quantification is carried out at either precursor or fragment level. Current state-of-the-art label-free quantification methods can consistently identify ~1000-2000 protein groups when using DDA [16,26], and the 2000-proteins-per-cell coverage has also been breached with the use of diaPASEF, albeit with use of a higher-load spectral library [12,55]. Label-free approaches benefit from higher quantitative accuracy than isobaric tag-based ones, but current iterations suffer from limited throughput of 20-40 single-cells per day, making studies on a similar scale as transcriptomics virtually intractable due to prohibitive data acquisition times. Efforts are underway however to alleviate these throughput issues [22,91].

In contrast, in isobaric tag-based workflows, peptides arising from a single cell are labelled with a specific isobaric TMT tags and pooled with differentially labelled peptides derived from 15 other cells generating a multiplexed samples of 16 cells. One channel is usually reserved for a "carrier" sample, comprised of 100-200 cells, that match the experimental set-up in terms of cell populations to boost peptide identification [13,15,71]. With nanoliter sample preparation, it is even possible to carry out the workflow without the use of a carrier channel [17]. The

carrier sample boosts not only the peptide precursor signal, increasing the probability of selection for MS2 analysis, but the increased signal intensity of fragment ions also leads to a greater proportion of successful MS2 sequencing. The use of 100-fold higher abundance sample within the same multiplex has sparked concerns about quantification biases as a result. Accordingly, multiple studies have evaluated how different amounts of carrier samples affect the quantification of scp-MS [15,19, 27,98]. Excluding the carrier TMT channel with the use of a linear ion trap was suggested as a possible work-around, but as the quantification bias is minimal at or below 200-cell carriers, the impact of such an adaptation is minor [15]. Isobaric tag-based approaches can quantify 1000-1500 protein groups per cell and process >100 cells per day, and incorporating real-time search approaches such as RETICLE or pSCoPE have shown substantial potential [27,40,71].

TMT multiplexing based approaches currently seem to outcompete LFQ ones in both identification depth and throughput per unit time. However, label-free quantification does not suffer from the same biases as reporter-based quantification, and can potentially provide more accurate peptide abundance measurements. The highest proteome depth of ultra-low input material has been reported on TOF-based instruments in combination with TIMS [12,21,59], although exciting results for LFQ-based approaches that leverage the linear ion trap (LIT) for MS2 fragment measurements have also been reported. LIT based acquisition could outcompete orbitrap based ones for low-input samples [9,62] and one study was able to quantify ~ 3000 proteins groups at the single-cell level with the use of a spectral library generated from 11 cells. [26]. While the LIT is yet to be applied to multiplexed single-cell workflows, we anticipate this mass analyzer to significantly propel alternative mass analyzer single-cell multiplexed-based methods forward in the near future.

Novel multiplexing and corresponding data acquisition methods

Label-free scp-MS can utilize both DDA and DIA based workflows, however the nature of the TMT labelling approach precludes the use of DIA. If two peptide precursor ions are isolated together in a single isolation window, the reporter ions arising from both precursor species will be indistinguishable hindering accurate peptide abundance quantification (Fig. 3A). Other multiplexing approaches such as iTRAQ or EASI-tags [56,89] utilize an analogous approach, hence their application for DIA is also limited. Despite these compatibility issues, a workflow combining DIA with TMT multiplexing has been reported [19]. TMT-DIA (Fig. 3A) improved the precision and reduced the number of missing values while maintaining the TMT multiplexing benefits, albeit not directly resolving the co-isolation issue [18,19]. Interestingly, apart from the reporter ions, TMT-labelled precursor fragmentation also generates complementary ions [41], which are comprised of the remaining TMT tag fragment attached to the unfragmented peptide (Fig. 2C). Exploiting the complement ions for quantification in another study increased the quantified proteome depth and accuracy, relative to conventional reporter ion based quantification [41]. As the precursor-specific complementary ions can be distinguished in the case of co-isolation, this property could be exploited to further develop multiplexed DIA workflows. Although, the large m/z value of the ions might cause issues resolution-wise for high-load samples, potentially phi-SDM [35] or other advanced mathematical transformation will be required for proper resolution. Luckily, the high injection times needed for scp-MS, concurrent with the ability to read out fragment ions at very high orbitrap resolutions [27,71], might provide enough resolving power to properly discriminate the complementary ions.

Tags that are specifically tailored for DIA type analyses have also been synthesized [105]. Ac-IP tags function as precursor coupled-reporters, where the labelled peptide precursors generate MS1 peaks with 1 Da difference, but after fragmentation, yield identical MS2 fragments [105]. Applied to scp-MS, this should not only provide higher throughput, but also increase proteome coverage due to the boosted MS2 signal, similarly to TMT. Alternatively, the Slalov lab has shown
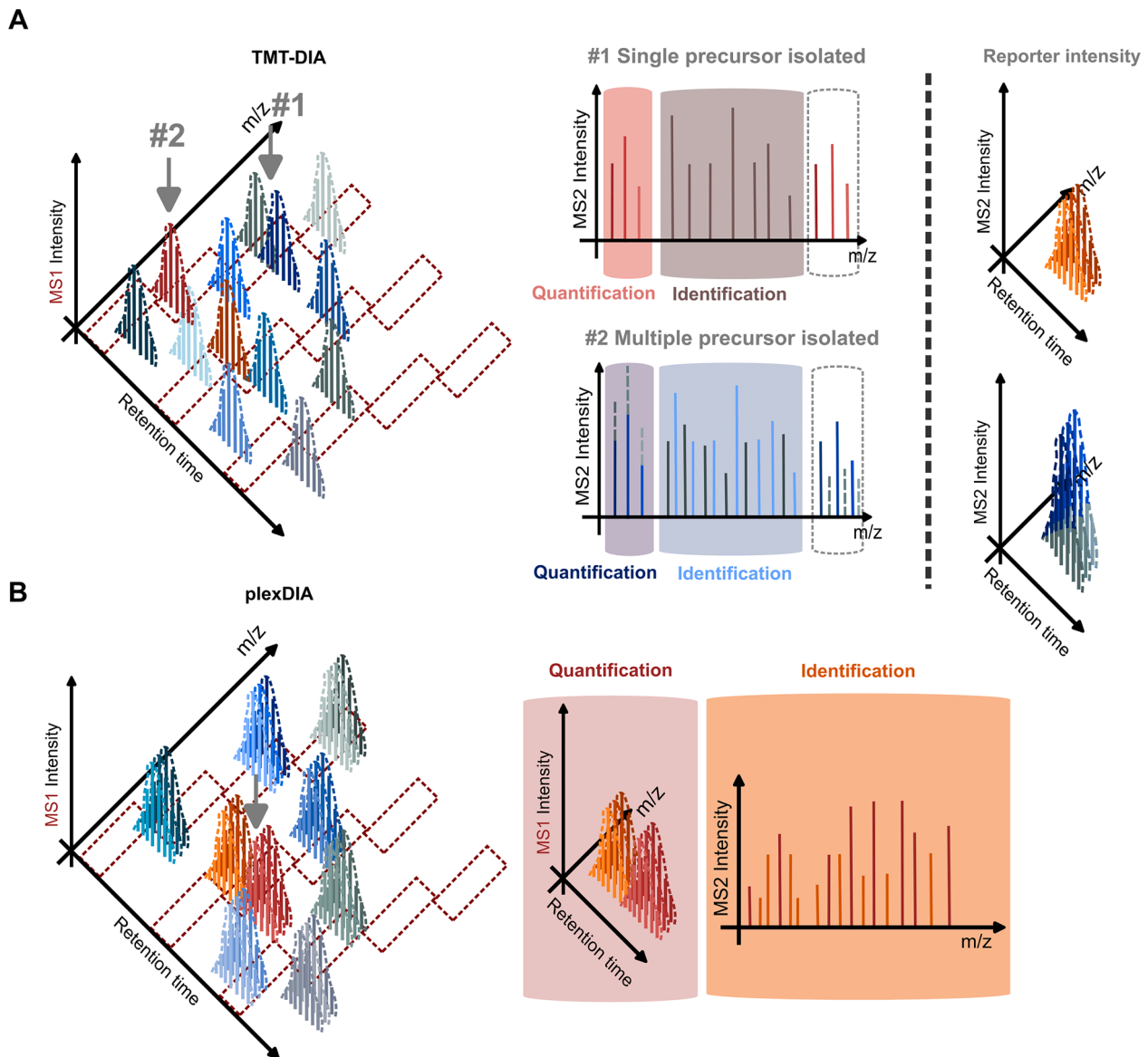
Fig. 3. Data independent acquisition multiplexing approaches. (A) Outline of the TMT-DIA approach, where TMT labelling is used to multiplex the samples. Similarly, to LFQ-DIA, XIC can be generated, however reporters arising from different precursors if co-isolated cannot be distinguished, which could potentially generate biases in the quantification. Using the complementary ions as outlined by the dashed square, should in theory overcome this limitation. **(B)** Outline of the plexDIA approach, in contrast to TMT-DIA, MS1 based quantification is used by employing mTRAQ, which give rise to precursor ions with 4 Da offset. XIC can then be generated for each channel separately on both MS1- and MS2-level, and MS2 fragment information is used for peptide identification.

proof-of-concept for multiplexed DIA, termed plexDIA (Fig. 3B), by employing an mTRAQ triplex relying on MS1 based quantification exploiting the benefits of DIA to improve the obtained scp-MS data quality [22]. Compared to TMT-DDA approaches, there is a small sacrifice in terms of proteome depth and throughput, but with the benefit of fewer missing values between runs. While the latest TMTPro reagent allows multiplexing up to 18 samples, the limit of DIA compatible multiplexing remains to be determined. Nevertheless, even with these potential limitations, it will be exciting to see their further application in scp-MS, as they are expected to continue improving multiplexed DIA based scp-MS workflows.

Advances in chromatography

Advances in chromatography play a vital role in furthering the field of scp-MS. Using narrower columns that allow lower flowrates can facilitate higher sensitivity, by increasing eluting peptide concentration and ionization efficiency. Such narrow columns are difficult to

manufacture reproducibly and have diminished resolution capacity, but this drawback seems to be more than compensated for by the increased ion flux. Despite these obstacles, nanoBore columns were successfully manufactured and applied for single-cell input (Cong et al., 2020). Decreasing the column width lead to a considerable increase in both peptide and protein identification. Furthermore, the development of a novel type of micropillar array-based column (uPAC) is paving the way for higher proteome quantification depth [75]. The nonporous nature of the 'limited sample' version of this column has low binding capacity, making it extremely well suited for low-input proteomics. [76]. These columns display improvements in peak width and peptide retention time robustness, and crucially, relative to standard C18 based columns, the uPAC almost doubled the number of quantified proteins from low-input samples ([75], and the standard 50cm uPAC has been successfully used for scp-MS [27,78].

The LC systems themselves have not remained exempt from the drive to improve scp-MS proteome coverage and throughput. The Evosep

platform has developed robust chromatographic method call Whisper™, which is specifically aimed at clinical and single-cell proteomics [3]. In the system, custom StageTips that are routinely employed for sample clean up after digestion [67] are used as disposable trap columns, and separate peptides from contaminants present in the sample. The disposable trap synergizes well with current scp-MS workflows, which do not allow offline sample clean-up due to material loss concerns. The Whisper chromatographic methods, reducing flowrates down to 100nl/min, can process 20 or 40 samples per day, and have been successfully used for proteome profiling of single-cell and low-input (<10ng) samples [12,62]. Furthermore, dual-column LC methods have been proposed that can more effectively utilize mass spectrometry time by increasing sample throughput [91]. These new developments hold a lot of promise and will undoubtedly affect their future application in the scp-MS field.

Alternative exploratory microfluidics platforms for sample preparation

Droplet-based sample processing with the use of tailored microfluidics devices had a transformative effect on the field of single-cell transcriptomics as it alleviated substantial issues such as throughput and technical variation. Microfluidic platforms can encapsulate single cells in droplets that serve both as fluidic cell carriers and reaction chambers, and can be merged with other droplets containing reagents needed for subsequent sample processing. While microfluidics devices are routinely used in the field of transcriptomics, their application for proteomics is only just emerging. A device that can capture single cells and carry out all the required processing steps for mass spectrometry analysis has recently been manufactured [31]. Digital microfluidics systems, where dispensed droplets are manipulated with insulated electrodes, have also been employed for processing samples with limited cell input, but the complexity of such devices can be prohibitive for wide-spread adoption in non-specialized laboratories. Together these studies represent a big first step in integrating the dynamic field of microfluidics with scp-MS.

Spatial proteomics at single-cell resolution

Most scp-MS studies to date focused either on cells extracted from tissue cultures cultivated in the lab, or on liquid biopsies. Due to the nature of single-cell isolation methods, valuable spatial information is generally lost (Fig. 1). To tackle this, methods leveraging LCM to cut out small segments (20-100 um, 5-10 cells) [63,104], or even single neuronal cells [16], have been successfully employed. More recently, a ground-breaking workflow termed "Deep visual proteomics" combined advanced immunofluorescent imaging and LCM, thereby allowing the LC-MS measurement of pooled single-cell proteomes, while retaining spatial information from formaldehyde-fixed and parafilm embedded (FFPE) samples [59]. While the high-throughput measurement of spatially resolved, true single-cell proteomes remains elusive, the authors demonstrated the vast potential of combining technology with inherent single-cell resolution (i.e. microscopy), advanced machine learning algorithms and ultra-high sensitivity MS. The ability to apply such workflows to FFPE samples opens up major opportunities for analyzing vast primary sample collections stored in biobanks across the globe, and gain deep proteomic insights into disease pathology.

Computational analysis of scp-MS data

Single-cell-based datasets provide a high-resolution view of the different cell states. Most studies aim to identify discrete cell populations, their response to certain stimuli and infer their developmental trajectories [23,43,70,87,88]. However, such statistical inference is complicated by inherent issues present in single-cell datasets. Fundamentally, single-cell measurements have a much higher degree of uncertainty, sparser quantification and capture a smaller fraction of molecules present in the cell, relative to population based techniques [43]. Fortunately, the fields of scp-MS and scRNAseq are plagued by

similar challenges and have complementary downstream analysis goals, with a plethora of successful implementations in the scRNAseq field [52, 79,93]. For scp-MS, only a few dedicated packages have been created so far [71,86]. Potentially, tools tailored for scRNAseq analysis could be repurposed for analysis of scp-MS data.

Integrating the different single-cell modalities can provide a more comprehensive picture of cell states. For example, spatial transcriptomics can measure transcript abundance while preserving the cells native environment information but suffer from low coverage depth. On the other hand, scRNAseq measurements are oblivious to the native environment, but can quantify the transcriptome with much higher depth [50]. Integrating both datasets would provide a higher-resolution image of the inner workings of the present discrete cell subpopulations [24,79]. Cell mapping between the different methods is based on the quantified transcripts, which should be linearly correlated to a high degree. Linking appropriate single-cell transcriptomes and proteomes might be more challenging, as the correlation between protein and transcript abundances is more complicated [12,53]. A temporal factor might play a vital role in defining the transcript and protein correlation; if true, an RNA velocity type approach could be used to model cell state trajectories [44,53]. Ideally, techniques that capture different modalities, such as spatial information, transcriptome and proteome, from the same individual cells are needed. A recent pre-print has already indicated the ability to analyse both transcriptome and proteome from the same cell [26]. If widely applicable, it holds the potential to extend current multimodal single-cell approaches into the proteome sphere, taking a huge step forward towards complete multi-omic analysis of a single cell.

Outlook

The field of single-cell omics has made great strides forward and dedicated efforts from multiple groups are rapidly advancing progress. Transcriptomics in combination with machine learning has been used to systematically identify and categorize all the cell types that comprise the human body, address heterogenous response of cancer patients to different treatments and predict potential sensitivities [2,28,30,68,82, 99]. Although scp-MS is a late comer to the field, it is developing at a dizzying rate and holds the potential answer to key questions in disease pathology and development. In a biological setting, scp-MS has been applied to investigate cell cycle dependent heterogeneity [12,22] and characterization of a heterogeneous acute myeloid leukemia hierarchy, where a possible new cell differentiation path was found [71]. As both the proteome depth and throughput of scp-MS is constantly improving, it will be exciting to see its application to pivotal aspects of cell development and disease. Together with DNA and RNA sequencing, single-cell proteome profiling can yield unprecedented information, characterizing the central dogma of biology (DNA to RNA to Protein). Although further technical advancement is needed to achieve this, one can only imagine what remarkable insight we can gain on the inner workings of cells by integrating these different data sources, from the very same cell.

CRediT authorship contribution statement

Valdemaras Petrosius: Conceptualization, Writing – original draft, Visualization. **Erwin M. Schoof:** Conceptualization, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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