

Minireview

Single-cell proteomics using mass spectrometry

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Over the past 2 to 3 years, mass-spectrometry-based single-cell proteomics (SCP) has experienced transformative improvements in microfluidic and robotic sample preparation, innovative MS1- and MS2-based multiplexing strategies, and specialized hardware (e.g., timsTOF Ultra 2, Astral), which have dramatically boosted sensitivity, throughput, and proteome coverage from picogram-level protein inputs. Concurrently, tailored computational workflows that encompass normalization, imputation, and no-code platforms have addressed pervasive missing data challenges and standardized analyses, collectively enabling high-throughput, reproducible profiling of cellular heterogeneity. This minireview summarizes the latest progress in SCP technology and software solutions, highlighting how the closer integration of analytical, computational, and experimental strategies will facilitate a deeper and broader coverage of single-cell proteomes.

INTRODUCTION

Bulk proteomics has been crucial for understanding diseases, but it simplifies our view of biological systems by averaging single-cell protein quantities across thousands of cells. Single-cell proteomics (SCP) provides a comprehensive view of heterogeneous cell states and functions, such as cell proportions in tissues.^{1,2} However, SCP is substantially more challenging because of the minuscule protein quantities per cell, the vast range of protein abundance, and the fact that proteins cannot be amplified as done with RNA in transcriptomics.¹ This creates challenges in protein identification and quantification, and computational workflows may not be optimized for the low-signal, sparse nature of SCP data.² The need for highly sensitive detection and reproducibility in SCP is essential. In SCP, bottom-up mass spectrometry (MS) is preferred because of its significantly greater sensitivity and broader proteome coverage compared to top-down.¹ Several prior reviews have covered SCP more broadly,^{1,3–9} and recent work has pushed technology to understand heterogeneous drug responses.¹⁰ Here, we provide a brief overview of recent advances in bottom-up SCP sample preparation, data acquisition, and computational tools, with a focus on the last 2–3 years.

A general workflow for SCP is shown in Figure 1. A population of dissociated cells is obtained from the biological system of interest. Those cells are sorted into single cells using various sorters and various substrates. Cells are lysed mechanically or chemically before proteolysis with trypsin to generate peptides. Optionally, peptides are covalently tagged with amine-reactive multiplexing reagents, which enable the pooling of multiple cells. The single cells or pools of cells are then analyzed by liquid chromatography coupled to mass spectrometry to detect and quantify peptides, which are used to infer protein quantities.

Relevant to many advances discussed in this review, there are two competing data collection strategies for single-cell proteomics: data-independent acquisition (DIA) and data-dependent

acquisition (DDA). These two distinct MS approaches differ primarily in their data acquisition and quantification strategies. DDA is most often paired with chemical labeling by tandem mass tags (TMTs), in which peptides from multiple samples (i.e., individual single cells) are tagged with mass-encoded reporter ions and pooled for simultaneous analysis in a single MS run¹¹ (Figure 2A). Quantification in DDA-TMT is achieved by measuring the relative intensities of these reporter ions in MS2 or MS3 scans, allowing for multiplexed sample processing with up to 35 channels in modern workflows. In contrast, DIA is most often paired with label-free quantification (DIA-LFQ), which systematically fragments all precursor ions within a specified mass range, capturing comprehensive MS/MS spectra in a single run¹² (Figure 2B). This approach enables continuous and unbiased peptide sampling, reducing the stochastic nature of measurements from DDA and enhancing quantitative reproducibility. While DIA-LFQ directly measures peptide abundances from each sample, DDA-TMT relies on comparative quantification across multiplexed samples, requiring additional computational corrections to match signals.

DIA-LFQ and DDA-TMT have their own strengths and weaknesses when applied to SCP. DDA-TMT excels in throughput by allowing multiple single cells to be analyzed in parallel within a single LC-MS run, reducing instrument time per sample. Additionally, the use of a carrier proteome, where an excess amount of pooled protein is included in one TMT channel, enhances peptide identification for low-abundance proteins. However, DDA-TMT suffers from ratio compression and co-isolation interference, where peptides from different samples share precursor ions and fragment together, distorting quantification and reducing dynamic range. Furthermore, ion suppression effects from highly multiplexed samples, especially in the presence of a carrier, can hinder the detection of low-abundance proteins, limiting sensitivity. TMT may also experience issues with missing values across conditions due to the stochasticity of peptide fragmentation across TMT batches. This problem is



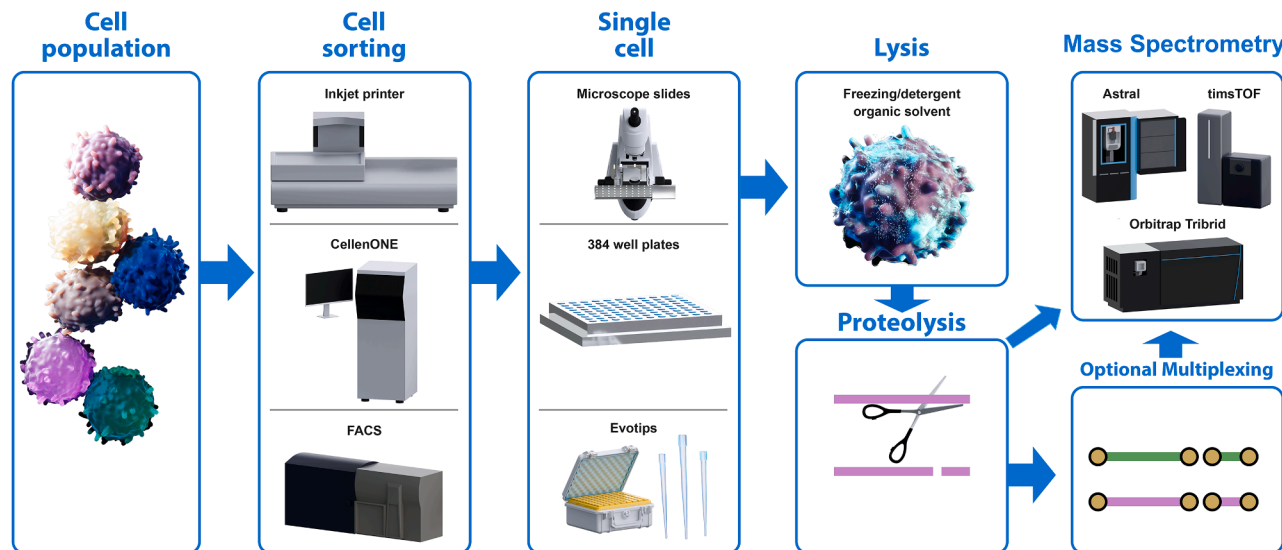


Figure 1. General workflow for SCP-MS highlighting the choices for cell sorting, cell substrates, multiplexing, and data collection

alleviated mainly with prioritized SCoPE (pSCoPE),¹³ where peptides of interest are preferentially fragmented across batches. DIA-LFQ, on the other hand, avoids these issues by independently measuring the peptide abundances of each sample, thereby eliminating inter-sample interference. This leads to more accurate, complete, and reproducible quantification; a wider dynamic range; and improved sensitivity for low-copy proteins. However, DIA-LFQ usually requires a separate LC-MS run for each cell (or, more recently, a small pool of cells), resulting in lower throughput in terms of cells per unit of time compared to TMT. Nevertheless, recent advances in instrumentation, MS1-based multiplexing,^{14,15} and data analysis pipelines have significantly increased the scalability of DIA-LFQ, making it a powerful alternative for unbiased SCP. Ultimately, the choice between DIA-LFQ and DDA-TMT depends on the trade-off between throughput and quantitative accuracy, with DDA-TMT excelling in high-throughput settings, where multiplexing is crucial. In contrast, DIA-LFQ is increasingly favored for its superior quantification, sensitivity, and dynamic range.

A broad survey of SCP figures of merit reveals wide variation in performance across recent studies. We tabulated the number of proteins quantified per minute, proteins quantified per cell, corrected run time in minutes/cell (lower is better), and the data acquisition type (Figure 3; Table S1). We found that DDA-TMT methods consistently quantify the most proteins per minute, owing to their multiplexing of multiple cells per injection, with the 32-plex TMT leading. Accordingly, TMT also produces the lowest analysis time per cell. The most proteins per cell (*x* axis) were found in studies using the Astral, where over 5,000 proteins could be detected. Most studies used DIA methods. Future studies should focus on filling in the upper-right quadrant with high protein content per cell and high protein quantification per minute. In agreement with a recent perspective, we propose that the main limitation requiring further advances is the need for higher throughput to better sample cell populations.¹⁶

SAMPLE PREPARATION

The tiny amount of protein and the need for thousands of samples per experiment both lead to unique sample preparation challenges. A typical proteomics experiment aims to inject 1 μ g of total peptide into the LC-MS system. The average single mammalian cell contains 5,000-fold less protein, about 200 pg. Adapting sample preparation approaches designed for microgram-scale quantities to the $\sim$ 200 pg range is a substantial challenge that continues to be a significant research focus in the SCP field. For example, at these ultra-low sample amounts, surface absorption to pipette tips and tube walls can cause noticeable losses in observable proteomic depth.

When considering sample preparation, it is also essential to consider experimental designs to avoid technical and batch artifacts, such that biological effects can be distinguished with confidence. In plate-based workflows, all batches should contain all biological conditions. Incorporating a consistent reference sample across batches helps to control for signal shifts, especially if the reference closely resembles the study samples and includes all cell types of interest.^{8,17} For inter-group or longitudinal comparisons, distributing samples from all biological groups randomly and uniformly across batches can further minimize batch-related variability. If this randomization was not done, one should include batch as a variable in the data interpretation models.

There are many cell sorting options to consider. While a traditional fluorescence-activated cell sorter has been successfully used in many SCP studies, the CellenONE^{18–21} has become a common choice for SCP. Nano-proteomic sample preparation (nPOP)¹⁸ leverages the flexibility of the CellenONE with fluorocarbon-coated microscope glass slides as a surface for any sample layout. Nanoliter droplets of DMSO are deposited onto the slide, followed by a single cell, then the digestion mixture, and, optionally, a multiplexing label. A larger droplet is then used to collect any cell droplets of interest and move them to

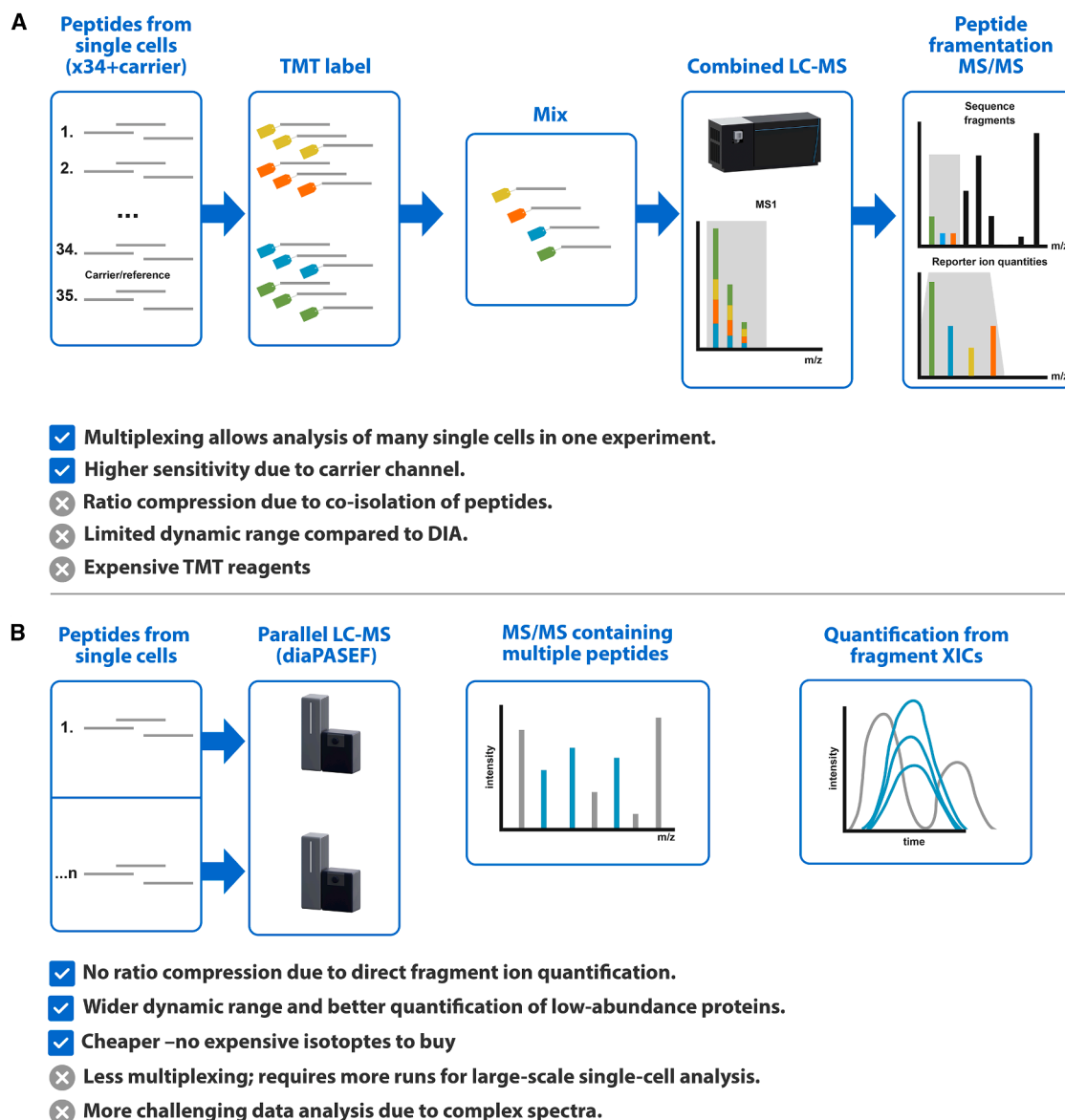


Figure 2. Comparing the two main competing SCP-MS strategies with DDA-TMT and DIA-LFQ

an autosampler-compatible 384-well plate. This approach was used to explore functional protein covariation across single cells related to the cell cycle.²² Another solution adapts an inkjet printer for single-cell and reagent dispensing in 384-well plates.^{23,24} This approach can offer considerable cost savings while still providing fast and reproducible sorting, achieving up to 2,600 protein identifications with LFQ. Although cells should be sorted fresh whenever possible, cell refrigeration or freezing enables SCP analysis of stored cells.¹⁹

The chemical choices for lysis and proteolysis solutions also require unique considerations for SCP. An extensive exploration of one-pot sample preparation and data collection was recently performed by Matzinger et al.²⁵ This work is an essential read for new SCP practitioners, as it covers nearly all aspects of sample

preparation and presents a simple protocol for single-cell sample preparation in 384-well plates. The authors report optimal isolation parameters for cellenONE, choice of trypsin, and various data analysis options. Other best practices include minimizing sample transfers (even a single transfer can reduce protein identification by 50%), keeping samples hydrated with DMSO prior to placing in the autosampler, adding trypsin in two boluses rather than a single addition, and using short 5.5 cm micro-pillar array columns (μ PAC) rather than packed beds. It's also important to note that for SCP, generally the traditional proteomic sample preparation workflow with sequential lysis, reduction, and alkylation of cysteines before proteolysis is not followed, and one-pot protocols that omit reduction and alkylation are favored.²⁶

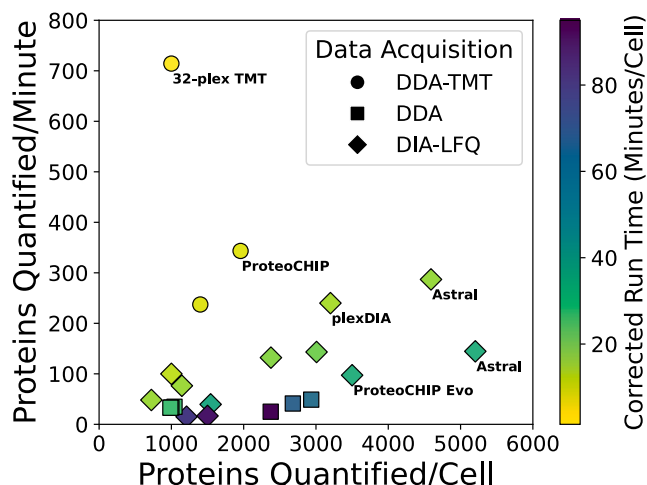


Figure 3. A survey of SCP figures of merit across studies from the last 2–3 years

Proteins quantified per minute (y axis) are plotted against proteins quantified per cell (x axis). The corrected run time, in minutes, for data collection per cell is displayed using color mapping, with the marker shape indicating the acquisition type. Selected points are labeled based on the highest performance in either x or y dimensions. “DDA” refers to DDA without TMT.

Given the importance of minimizing surface contact during SCP sample preparation, several studies have explored non-traditional sample preparation ideas. Two recent concepts utilize the Evosep One tips for sample preparation, specifically disposable precolumns. First, proteoCHIP,²⁷ which is a collection of nanowells covered with oil, enables cell dispensing, nanoliter lysis, digestion, and TMT labeling. The cells in one group are pooled with a funnel device by centrifugation and then directly placed into the autosampler. This enabled the identification of nearly 2,000 protein groups per TMT plex injection using a 20× carrier. This approach substantially reduces the amount of manual manipulation required for single-cell TMT experiments at the added expense of additional consumables. A subsequent study described proteoCHIP EVO. This device arranges single cells on pedestals such that they can be directly centrifuged into a 96-tip rack of Evosep One tips for analysis.²⁸ The authors found that this direct loading approach resulted in about twice as many protein identifications compared to manually transferring cells to an autosampler vial. However, these results may be confounded by losses to pipette tip surfaces solely during the sample transfer process (as opposed to directly injecting for a well plate), which is known to cause the loss of nearly half of proteins.²⁵ Importantly, while Evotips add sample preparation expense, they may present a return on cost investment, as these protect the expensive analytical column, which may otherwise degrade over thousands of single-cell injections.

In line with limiting sample contact with surfaces, microfluidics is a versatile, multidisciplinary technology that enables precise manipulation, observation, and processing of fluid samples ranging from nanoliters to femtoliters into well-defined compartments.²⁹ A recent report described using a microfluidic-liquid-handling robot for cell sorting and sample preparation directly in autosampler vial inserts. The authors achieved an average of

over 3,000 protein groups in HeLa and A549 cells using DIA and matching between runs (MBR). A benefit of this approach is that the cell-sampling robot can sample specific single cells of interest from a culture plate; for example, the authors demonstrated single-cell sampling of cells that start migration into the cleared zone in a plate scratch assay. Peng and colleagues introduced an all-in-one digital microfluidic pipeline that integrates proteomic sample reduction, alkylation, digestion, isotopic labeling, and analysis.³⁰ Another recent advance demonstrated that cell capture, lysis, digestion, and peptide separation can be carried out in an integrated microfluidic chip.³¹ This approach enabled the quantification of approximately 1,500 proteins from each single cell in a 20-cell parallel chip.

INNOVATIONS IN DATA COLLECTION

One of the early strategies for SCP leveraged the signal stacking of TMT plus a carrier channel to achieve the necessary sensitivity, even while using old MS hardware. This MS2-based quantification has limitations for the large-scale projects required for SCP, where thousands of cells must be analyzed. In many ways, DIA is simpler and provides more complete data across samples. Although multiplexing with DIA has been reported for relative quantification of peptides against heavy signals, it is not widely used.^{32,33} Recently, two studies reported ideas to resurrect 15-year-old MS1-based multiplexing techniques to pair them with DIA for SCP multiplexing. Both approaches allow using one channel as a carrier to boost sensitivity by limiting absorption losses.

There are two approaches for MS1 multiplexing. One approach, plexDIA,¹⁵ uses 3-plex peptide labeling with the original mTRAQ reagent³⁴ to achieve triple the throughput for low sample amounts. The quantitative accuracy was like that of separate DIA injections. From single cells, Derks et al. were able to quantify about 1,000 protein groups from each of the three plexes (up to three single cells per injection) using a timsTOF SCP mass spectrometer and 30-min per injection (10 min per cell). The data completeness using this approach is high at 98% across plexes. This idea was recently scaled further to 9-plex.³⁵ The second approach uses dimethyl labeling³⁴ and is called mDIA.¹⁴ This method could enable simultaneous analysis of up to five cells. Using the 3-plex version with one reference channel and two single cells, the number of protein groups was doubled to a median of 2,377, achieved with a timsTOF SCP at an injection rate of 40 samples per day (80 cells per day).

A challenge with the common SCoPE-MS approach using TMT multiplexing is that it benefits from MS/MS measurement of the same peptides across TMT batches, which is always less than 100% due to the stochasticity of DDA. Prioritized MS overcomes this problem by preferentially fragmenting certain precursor *m/z* species that are known to be of interest *a priori*.¹³ Once the high-priority list is exhausted, lower-priority peptides are then targeted. This approach increased data completeness for the 4,000 high-priority peptides to 72%, compared to 49% without prioritization, while also doubling the number of proteins identified per cell.

A new concept in SCP is called wide window acquisition (WWA) with DDA. This embraces the idea of peptide co-isolation

in the same quadrupole window. This was first reported with low-nanoliter-flow LC.³⁶ Truong et al. found that WWA could identify slightly more proteins than standard DDA or DIA at the expense of quantification reproducibility. In a subsequent paper, WWA was found to increase proteins up to 150% more than narrow window isolation, but results from DIA were not reported for direct comparison.³⁷ Overall, while WWA is an interesting concept that warrants further exploration, based on the presented data in these two papers, current results do not provide a complete benefit over standard DIA.

For DIA, there are avenues to optimize and adapt DIA to single cells, especially given that Orbitrap instruments offer great flexibility in possible scan sequences that achieve variable resolution and measurements per chromatographic peak. A study found many such optimizations.³⁸ First, in contrast to high-load DIA, where smaller windows convey selectivity, more expansive DIA isolation windows improved sensitivity for low input. When coupled with μ PAC columns, this workflow yielded over 1,461 proteins within 20 min of data collection. Adding a high-load library for identification transfer appeared beneficial.

Another DIA idea focused on only MS1 data collection inspired by the accurate mass and time approach is called transferring identifications based on High-Field Asymmetric Waveform Ion Mobility Spectrometry (FAIMS) filtering (TIFF).³⁹ The approach first generates a spectral library using FAIMS fractions, which was found to increase the ion injection times and signal-to-noise ratio. The 3D matching based on retention time, MS1 m/z , and FAIMS mobility then enabled peptide identification. Woo et al. achieved 1,210 protein identifications from HeLa using 80 min total injection times per cell.

LC AND MS INSTRUMENTATION ADVANCES

Optimization of peptide separation and loading is crucial for achieving high-quality and rapid SCP. Trap columns are often used in conjunction with SCP because they can expedite sample introduction and compensate for the lack of offline cleanup typically required for single cells. New ideas involve leveraging tubular open trap columns that bind their analytes to the walls through diffusion, thereby allowing particulate and larger aggregates to flow straight through.⁴⁰ A second idea is the dual-trap single-column (DTSC)⁴¹ approach, where one trap is loaded and washed while the second is eluted to the analytical column.²⁰ Beyond improving or parallelizing sample loading with trap columns, a natural idea is to have multiple peptide-separation columns.⁴² In this approach, one column performs the online separation to the mass spectrometer while another column is being regenerated for the next injection. Webber et al. found that this approach can reduce total analysis time to 7 min per cell, allowing for over 200 cells per day and an average of 621 protein group identifications. Several variations of multiple-column ideas, including the recent idea to stagger column injections for multiplexing in the time domain,⁴³ have been reported.⁴⁴

MS breakthroughs also push SCP forward. The first commercial breakthrough to significantly increase MS sensitivity and enable commercial-scale SCP was the timsTOF. The timsTOF leverages trapped ion mobility spectrometry (TIMS) to preconcentrate peptide ions as they elute from the column while sepa-

rating the previous batch of concentrated ions, increasing signal by about 4-fold⁴⁵ and enabling the detection of 1,000 to 2,000 proteins from single cells. Since then, two updated instruments with higher sensitivity have been released (timsTOF Ultra and Ultra 2). The latter claims the ability to measure over 1,000 proteins from just 25 pg. Another significant advance in instrumentation enabling SCP is the Astral mass analyzer.⁴⁶ This sensitive and fast mass analyzer enables precise peptide analysis with small quadrupole isolation windows. It was found to double the number of peptides compared to the Orbitrap Eclipse, with a sample loading of 1 ng (~31,000 peptides with Astral versus ~16,000 with Eclipse). After further optimization of the scan sequence, ~4,000 proteins could be identified from 250 pg aliquots of HeLa cells, and ~3,500 proteins could be identified from single HEK293 cells, with a total run time of 22.5 min in a 384-well plate format. A subsequent paper suggested that combining the Astral with the proteoCHIP mentioned previously can boost single-cell protein group identifications to over 5,000⁴⁷ and was corroborated by another paper.⁴⁸

SPECTRAL LIBRARY CONSIDERATIONS FOR SCP

There are several challenges present with SCP peptide spectrum matching (PSM) that are not present when dealing with bulk proteomics samples. The quality and comprehensiveness of the library directly influence the accuracy and number of PSMs. Some of these issues relate to the calculation of the false discovery rate, where the number of decoys influences the outcome. Perhaps counterintuitively, if a library is too expansive, then it will increase decoy matches and drive down total identification, whereas if the library contains a smaller set of proteins that better match the true proteins obtainable from that sample, then more proteins can be identified. Another spectral library-related consideration is that, using peptide identifications from DDA, spectra from Orbitraps may have fewer annotated fragment ion peaks and a lower signal-to-noise ratio.² This problem does not appear to be true for timsTOF data.⁴⁹ This suggests that, at least for Orbitrap DIA SCP methods, building libraries from other SCP data may be beneficial. A recent paper described how adding “matching enhancer” injections with slightly higher input material can improve SCP depth by 16% per individual cell.⁵⁰

SCP DATA PROCESSING AND INTERPRETATION RESOURCES

The explosion of SCP has led to a need for SCP data processing tools, including some that are specific to SCP data. Table 1 summarizes those tools.

SCP enables the identification of specific cell populations and provides an understanding of their differentiation trajectories.⁵⁹ After protein quantities are obtained from SCP data, the first step is often to summarize the cell types and their markers using established workflows from single-cell RNA sequencing (scRNA-seq) such as scanpy⁵¹ in Python or Seurat⁶⁰ in R. However, there is no standardized, widely accepted pipeline for SCP data preprocessing, and studies use different analysis methods in varying orders. A unique challenge in SCP is that cells vary in size, leading

Table 1. Single-cell proteomics data analysis tools

Tool name	Purpose
Scanpy ⁵¹	all-purpose single-cell Python package
Seurat ⁵²	all-purpose single-cell R package
PSCS ⁵³	no-code interface to multiple tools
scp ⁵⁴	SCP-specific methods such as precursor rollout to proteins
scplainer ⁵⁵	variance analysis, differential abundance analysis, and component analysis
PIRAT ⁵⁶	statistical imputation for proteomics
PIMMS ⁵⁷	imputation with deep learning
SCeptre ⁵⁸	extends scanpy for SCP data with normalization of TMT data and outlier removal

to substantial differences in total protein content across individual cells. Normalization methods applied in bulk proteomics, such as subtracting the median from log-transformed protein quantities, may be unsuitable when there are vast differences in total protein content across single cells.⁸ Instead, Gatto et al. recommend normalization based on a metric that reflects cell size, such as total protein content or a common reference protein.⁵⁹ The order of processing steps must also be carefully considered to ensure the assumptions of each step are met. For example, a *t* test requires prior batch correction, whereas linear regression can include technical factors like batch as a variable in the model, eliminating the need for prior batch correction.⁵⁹

SCP-specific R packages have been introduced in the past 2 years. One called scp⁵⁴ offers a comprehensive pipeline that includes key steps such as quality control (at both feature and cell levels), data aggregation (PSMs to peptides to proteins), normalization, and batch correction. The scp package supports data input from MaxQuant, Proteome Discoverer, and DIA-NN and requires a sample annotation table for processing. Implementing two different data-processing workflows using scp, SCoPE2 and SCeptre, revealed workflow-dependent differences in clustering, which could lead to varying biological interpretations.⁵⁹ Another package, scplainer⁵⁵ builds on scp capabilities by integrating SCP data-processing steps with linear modeling to facilitate deeper biological insights. There is a need for a community effort to replicate benchmarking experiments across various SCP protocols to better assess the robustness of computational workflows.

Additionally, these tools require some knowledge of programming, which is a barrier for many mass spectrometrists and biologists. The Meyer lab recently published a new platform called the platform for single-cell science (PSCS) that enables no-code, interpretive SCP data analysis.⁵³ This platform is unique in that it enables reproducible analysis through containerization, which is hidden from the user interface. It also allows collaboration where multiple users can be added to a project to run additional analyses. Importantly, it allows interactive data exploration using the CellXGene JavaScript interface in the browser. Finally, it has a unique publication mechanism where users can share their input data of cells and quantified genes; their exact analysis pipeline; the results that were generated, including figures and CellXGene interactive exploration; and the final annotated data

object for those that may want to explore further. Importantly, the publication page displays the exact analysis pipeline for inspection in the no-code interface, and then anyone can clone that pipeline for their own project, which maximizes the reproducibility and transparency of SCP data analysis.

Biological (e.g., vast differences in the number of proteins per cell due to cell size differences) and technical (e.g., presence of batch effects due to the typically large scale of single-cell MS acquisitions⁶¹) factors result in a significantly higher prevalence of missing values in SCP compared to bulk proteomics. In fact, missing 50%–90% of measurements per single cell is common, whereas bulk proteomics typically has a maximum of 50% missing protein quantities per sample.⁶¹ Imputation methods designed for scRNA-seq may not be suitable for SCP because missing values differ fundamentally between the two: zeros in scRNA-seq are often biologically meaningful, while zeros in SCP often result from detection or computational limitations, necessitating tailored imputation strategies for SCP.⁶¹

To handle missing data effectively, imputation methods for low-coverage MS data using LFQ have emerged over the last 2 years. Precursor or peptide imputation under random truncation (PIRAT), for example, addresses challenges in processing protein groups for which only a single peptide is identified.⁵⁶ By leveraging correlations among peptides from the same protein, it handles both random and censored missing values within a single statistical model. Similarly, proteomics imputation modeling mass spectrometry (PIMMS) employs three deep-learning models to impute missing values, collaborative filtering, a denoising autoencoder, and a variational autoencoder.⁵⁷ These three models performed comparably on simulated data, recovering signals across the entire distribution, including low abundance features, and scaled effectively to high-dimensional datasets. Additionally, unlike heuristic methods, these methods produced conservative imputations without biasing values toward detection limits.

SINGLE-CELL MULTI-OMICS INTEGRATION

There is much interest in pairing SCP with other single-cell omics, especially single-cell genomics, which leads to several new analytical challenges. Fulcher et al. show that surface tension can partition single-cell lysis droplets in half, such that two separate plates can be generated for either SCP or scRNA-seq.⁶² This approach, appropriately named nanoSPLITS, enables the quantification of an average of ~6,000 transcripts and 3,000 proteins from a single cell. A long-standing question surrounding proteome/transcriptome integration is whether poor correlation from bulk samples is due to the averaging of many cell types. Here, they found that the correlation from single cells is still low, suggesting that variation is likely due to differing rates of transcription, translation, and protein degradation. Thus, measuring single-cell differences in mRNA and protein could be highly informative to understand cellular regulation.

Other single-cell studies found a low correlation between single-cell proteomes and transcriptomes, indicating that protein levels cannot be reliably predicted from RNA levels even in individual cells.⁴⁵ While transcript expression showed a good correlation between different scRNA-seq technologies, protein measurements diverged significantly from RNA data in

principal-component analysis (PCA) space, and cell-cell correlations were, on average, higher within the proteome compared to the transcriptome. Furthermore, the study observed differences in data completeness, with SCP achieving higher completeness of protein measurements than both SMART-Seq2 and Drop-seq. Analysis of variability revealed that the single-cell transcriptome exhibits significantly higher overall stochasticity compared to the proteome, likely due to shot noise limitations at the RNA level. In contrast, protein variability appears more closely linked to measurement sensitivity. The study emphasizes that direct protein measurements are crucial for a comprehensive understanding of cellular states and the complex interplay between mRNA and protein abundance.

Heterogeneity in macrophages differentiated from monocytes using the U937 cell line were measured by scRNA-seq and SCP in parallel.⁶³ Notably, as illustrated in Figure 6A from Specht et al., SCP was able to sample 10- to 100-fold more copies per gene at the protein level compared to the number of unique barcode reads per mRNA obtained by 10× Genomics. This suggests that proteins are often present at much higher copy numbers per cell than their corresponding transcripts, allowing for more reliable counting statistics in proteomics measurements. Consistent with the papers by Fulcher et al. and Brunner et al., Specht et al. also found that RNA and protein levels in single cells often exhibit distinct patterns, suggesting widespread post-transcriptional regulation. By comparing the correlation structures of RNA and protein data, the study identified groups of genes with either similar or opposite covariation, revealing different underlying regulatory mechanisms. Overall, the findings from this study reinforce the notion that single-cell proteomics and transcriptomics provide complementary and crucial insights for elucidating cellular heterogeneity and the intricate layers of gene regulation.

Hutton et al.⁶³ leveraged the monocyte-to-macrophage data⁶³ and CAPITAL⁶⁴ to perform pseudotime alignment on the combined proteomic and transcriptomic data, which enabled them to investigate the dynamic changes in protein and mRNA levels as cells transition toward a macrophage state, identifying specific transcripts and proteins that exhibited either coordinated or disparate changes along the inferred differentiation trajectory. By demonstrating these capabilities and making the entire analytical pipeline, data, and results publicly available on PSCS⁵³ (<https://pscs.xods.org/p/SzFKQ>), the authors highlighted the platform's potential to enhance the reusability of complex analyses and foster collaboration within the single-cell omics research community.

The integration of single-cell proteomics and transcriptomics recently provided a more complete molecular characterization of aortic cells in a mouse model of aortic aneurysm.⁶⁵ Researchers performed SCP and integrated their data with a previously published scRNA-seq data. Seurat anchor marker analysis revealed concordance in the identification of major cell types but a lower degree of overlap when examining more refined cellular subtypes, highlighting the potential for each omics layer to capture distinct aspects of cellular identity. The study employed LIGER⁶⁶ for multi-omic integration. Despite this integration, the results indicated that the two datasets were primarily aligned at the level of general cell types, with more subtle cell sub-phenotypes not showing clear correspondence across the integrated clusters.

These findings underscore the complexity of integrating single-cell proteomics and transcriptomics data, where major cell identities may be conserved, and the nuanced information captured by each modality can reveal distinct and complementary insights into cellular states and disease-related alterations. The identification of specific multi-omic signatures associated with the Marfan phenotype highlights the potential of such integrated analyses to uncover novel pathways and regulatory mechanisms that may not be apparent from examining either data type in isolation.

GENERAL RECOMMENDATIONS FOR SCP

To strengthen downstream biological interpretations of SCP MS data, whenever possible, data collection should also include all available metadata, such as the location of the cell within the tissue and phenotypic characteristics, including cell images and functional assay results.⁸ Small amounts of bulk cell lysates should be included as positive controls. Dispersing these bulk samples among single-cell samples on the same plate should include assessment of peptide carryover with blank injections. Negative controls, prepared identically to single-cell samples but without any cells, should also be included to detect potential contamination. Due to low protein quantities in single cells, the sample preparation volume should be reduced to the nanoliter range to reduce absorptive losses and to reduce the effect of contaminants present in reagents.^{8,22} Batch sizes should be as large as possible, and experimental designs should include all biological conditions in each batch to ensure detection of biological effects.

CONCLUSIONS

SCP has reached an inflection point where technology, methodology, and computational advancements jointly elevate both the depth and breadth of protein profiling at the single-cell level. The continued evolution of sample handling, ranging from microfluidic devices and inkjet-based cell dispensers to new surface chemistries, has significantly curtailed sample losses. Meanwhile, DIA and multiplexing strategies expedite analysis and improve data completeness, especially when complemented by carefully curated spectral libraries. Emerging hardware innovations, exemplified by the Astral mass analyzer, promise further gains in sensitivity and throughput, propelling SCP beyond proof-of-concept studies into broader biological and clinical applications.

Still, challenges remain. High-throughput and robust reproducibility are essential for capturing the whole gamut of cell states and drawing reliable biological conclusions. Computational pipelines require ongoing refinement to handle the high rate of missing values and to integrate multi-omic data seamlessly. Open-source software packages and no-code platforms facilitate more transparent, reproducible pipelines, but a universal community standard for interpretive analysis has yet to emerge. By coupling state-of-the-art instrumentation with rigorous experimental design, thoughtful data analysis, and a collaborative drive to establish best practices, SCP is

poised to illuminate cellular heterogeneity at a new level of resolution, thereby transforming our understanding of biological complexity.

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AUTHOR CONTRIBUTIONS

The manuscript was written through the contributions of all authors. All authors have given approval to the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

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