PROTEOMICS – Clinical Applications

WILEY-VCH

Proteomics
Clinical Applications

REVIEW OPEN ACCESS

Advancements in Single-Cell Proteomics and Mass Spectrometry-Based Techniques for Unmasking Cellular Diversity in Triple Negative Breast Cancer

Lakshmi Vineela Nalla[1] | Aarika Kanukolanu[2] | Madhuri Yeduvaka[1] | Siva Nageswara Rao Gajula[2]

[1]Department of Pharmacology, GITAM School of Pharmacy, GITAM (Deemed to be University), Visakhapatnam, Andhra Pradesh, India | [2]Department of Pharmaceutical Analysis, GITAM School of Pharmacy, GITAM (Deemed to be University), Visakhapatnam, Andhra Pradesh, India

Correspondence: Siva Nageswara Rao Gajula (sgajula@gitam.edu; sivapharma.93@gmail.com)

Received: 11 October 2024 | **Revised:** 4 November 2024 | **Accepted:** 8 November 2024

Funding: The authors received no specific funding for this work.

Keywords: intratumoral heterogeneity | mass spectrometry | multi-omics platform | single-cell proteomics | triple-negative breast cancer

ABSTRACT

Background: Triple-negative breast cancer (TNBC) is an aggressive and complex subtype of breast cancer characterized by a lack of targeted treatment options. Intratumoral heterogeneity significantly drives disease progression and complicates therapeutic responses, necessitating advanced analytical approaches to understand its underlying biology. This review aims to explore the advancements in single-cell proteomics and their application in uncovering cellular diversity in TNBC. It highlights innovations in sample preparation, mass spectrometry-based techniques, and the potential for integrating proteomics into multi-omics platforms.

Methods: The review discusses the combination of improved sample preparation methods and cutting-edge mass spectrometry techniques in single-cell proteomics. It emphasizes the challenges associated with protein analysis, such as the inability to amplify proteins akin to transcripts, and examines strategies to overcome these limitations.

Results: Single-cell proteomics provides a direct link to phenotype and cell behavior, complementing transcriptomic approaches and offering new insights into the mechanisms driving TNBC. The integration of advanced techniques has enabled deeper exploration of cellular heterogeneity and disease mechanisms.

Conclusion: Despite the challenges, single-cell proteomics holds immense potential to evolve into a high-throughput and scalable multi-omics platform. Addressing existing hurdles will enable deeper biological insights, ultimately enhancing the diagnosis and treatment of TNBC.

1 | Introduction

Breast cancer (BC) is a heterogeneous disease whose clinical subtypes vary in molecular characteristics, prognosis, and therapeutic responsiveness [1–3]. Genomic research has directed to a more distinct classification of BCs based on their genes and proteins. For hormone receptor-positive cancer, where estrogen (ER-positive) and progesterone (PR-positive) fuel cancer's growth, have BC cells with receptors [4]. In contrast, hormone receptor-negative BC cells lack receptors. These receptor-negative cancers are unhelpful from hormone therapy drugs and tend to grow faster than hormone receptor-positive cancers. BC cells that rely on human epidermal growth factor receptor 2 (HER2) for growth are HER2-positive, and less dependent are HER2-negative [5, 6]. In triple-negative BC, all three protein expression systems are absent [7]. The risk of developing BC increases in women with a BC in family history, lifestyle, diet, and the environment [8]. Heredity-based BC is due to the mutations in abnormal breast

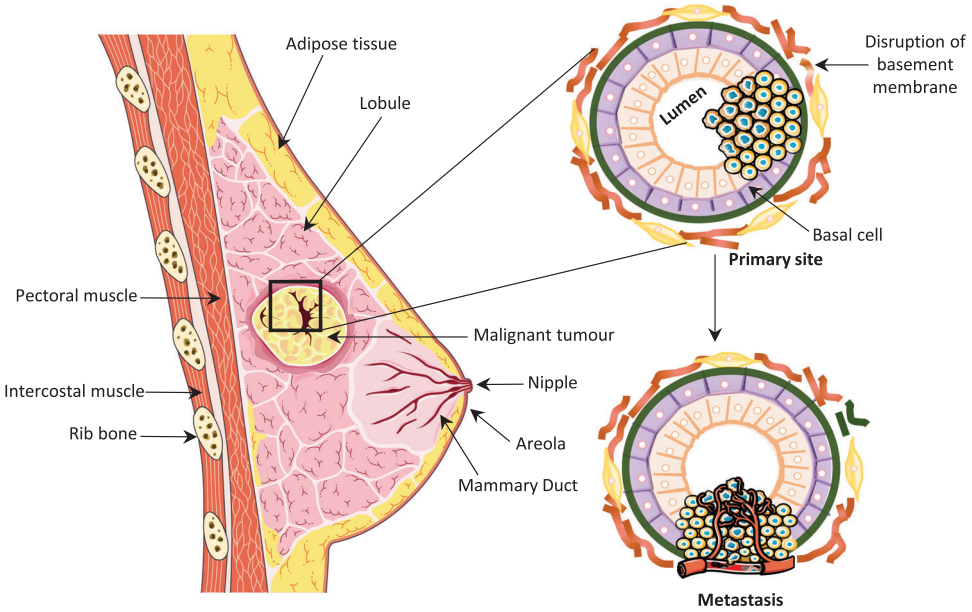

FIGURE 1 | Overview of breast cancer pathogenesis and metastasis. Breast cancer can originate in the cells lining either the mammary ducts (ductal carcinoma) or the lobules (lobular carcinoma), which are the two main types of breast cancer. Cancer cells may invade nearby healthy breast tissue and can spread (metastasize) to lymph nodes, commonly in the underarm area, but also to other lymph nodes or organs. In some cases, breast cancer remains localized within the breast tissue without spreading.

cancer gene 1 (BRCA1) or breast cancer gene 2 (BRAC2) inherited from maternal and paternal relatives [9, 10]. Figure 1 illustrates the anatomy of the breast and the pathophysiology of malignant BC.

Triple-negative breast cancer (TNBC) holds a formidable challenge among these subtypes. Defined by the absence of estrogen receptor (ER), progesterone receptor (PR), and HER2 expression [11], TNBC accounts for approximately 15% of all diagnosed breast cancer cases [12]. TNBC is not only notorious for its aggressive behavior but also for its limited treatment options. The lack of well-defined therapeutic targets, such as hormone receptors or HER2, leaves patients with TNBC facing a more challenging clinical course and limited opportunities for targeted therapy [13, 14]. Extensive research has been devoted to straight out the complex biology of TNBC, with a particular focus on proteomics [15–18]. These unique challenges demand innovative approaches to understanding the disease at a molecular level, such as single-cell proteomics with LC-MS/MS, to discover potential biomarkers, therapeutic targets, and personalized treatment strategies.

Single-cell proteomics is a cutting-edge technique that has the potential to unravel the intricate molecular landscape of TNBC, shedding light on its heterogeneity, resistance mechanisms, and therapeutic vulnerabilities [19]. Unlike traditional proteomic approaches that typically rely on analyzing bulk cell populations, single-cell proteomics dissects the cellular components at the individual cell level. Its significance lies in its ability to uncover heterogeneity, personalize treatment approaches, and illuminate the mechanisms behind TNBC's aggressiveness. On the other hand, mass spectrometry, predominantly liquid chromatography-tandem mass spectrometry (LC-MS/MS), is the most widely used technology in single-cell proteomics. Combining LC's separation capabilities with MS's analytical power provides the most unbiased detailed protein identification and quantification method. Despite being the most powerful technique for protein analysis, its application to single cells has faced several challenges. Recent advancements in simple, multiplexed, automated, and miniaturized sample preparation have facilitated rapid, high-sensitivity analysis. Mass spectrometry can be tailored in various ways to suit specific experimental objectives. Experiments can opt for label-free approaches in single-cell proteomics, where proteins are identified solely based on their mass-to-charge ratios. Alternatively, researchers may employ isobaric labeling methods, which involve comparing the intensities of tagged proteins with those of their untagged counterparts. Once labeled, the protein of interest can be traced using complementary techniques such as immunofluorescent microscopy, fluorescence-activated cell sorting (FACS), or cytometry by time of flight (CyTOF). Moreover, researchers can expand the breadth of their proteomic analyses through multiplexing, which allows simultaneous examination of multiple proteomes across different samples, enhancing the comprehensive understanding of protein dynamics and interactions.

2 | Proteomic Profiling Landscape of Triple Negative Breast Cancer

Proteomics studies that focus on characterizing the complete proteome of specific cells or tissues provide valuable information that complements genomic and transcriptomic breast cancer research [20]. Proteins are inherently more complex and dynamic than genes and closely reflect biological functions. Limitations in bioinformatics' ability to predict gene product presence and function underscore the need for protein analysis [20]. Humans are estimated to produce between 500,000 and 1,000,000 protein isoforms from approximately 20,000 protein-coding genes [21, 22]. This is possible because a single gene can

produce multiple distinct proteins through alternative splicing of pre-mRNA transcripts and various posttranslational modifications (PTMs). The development of advanced proteomics technologies has enabled the exploration of protein abundance, protein–protein interactions, PTMs, and ultimately protein function.

Kennedy et al. conducted a proteomics-based analysis that quantitatively characterized 319 proteins with differential expression across 30 distinct human breast cancer cell lines. This proteomic data was instrumental in distinguishing molecular subtypes of breast cancer and identified 118 proteins with varying expression levels between basal-like and luminal cancers. Further analysis revealed that 30 of these proteins had corresponding genomic data, but only 10 showed significant concordance with mRNA levels [23]. In another study, Liu et al. utilized a label-free proteomic approach to analyze 126 frozen primary tumor samples of TNBC, dividing them into training and testing sets [24]. They identified an 11-protein signature associated with clinical outcomes (CMPK1, AIFM1, FTH1, EML4, GANAB, CTNNA1, AP1G1, STX12, AP1M1, CAPZB, and MTHFD1). The expression patterns of these signature proteins in the training set could accurately predict clinical outcomes in the testing set of TNBC with high sensitivity and specificity. Of these 11 proteins, 10 were upregulated (while MTHFD1 was downregulated) in patients with favorable prognoses. Three upregulated proteins were linked to immunomodulation and apoptosis pathways, whereas MTHFD1, associated with poor prognosis, is involved in nucleotide and noncoding RNA metabolism. Liu et al. demonstrated that over 60% of TNBC patients who received adjuvant chemotherapy based on the conventional St. Gallen [25] and NIH [26] criteria were unnecessarily treated, according to the 11-protein signature identified in their study. A subsequent proteomics study further validated FTH1, an immunomodulatory molecule that enhances CD8+ T cells in the tumor area, as a potential therapeutic target in TNBC [27].

The initial comprehensive proteomic analysis of TNBC identified 12,000 distinct proteins, revealing expression patterns that distinguish between TNBC subtypes. This study also illuminated specific signaling pathways in TNBC associated with metastasis, adhesion, and angiogenesis [28]. Using an iTRAQ labeling-based proteomic approach, distinct expression signatures were uncovered for three proteins named desmoplakin (DP), thrombospondin-1 (TSP1), and tryptophanyl-tRNA synthetase (TrpRS), that differentiate relapse-prone from non-relapse TNBC tumors [29]. Previous studies have noted DP's overexpression in luminal breast cells and TSP1's roles in metastasis and angiogenesis in breast cancer cells [30, 31]. TrpRS, known for its involvement in protein synthesis, translation, and angiogenic signaling, was also highlighted [32]. Overexpression of DP and TSP1 significantly impacted disease-free survival and increased recurrence risk in TNBC patients, whereas TrpRS overexpression correlated with improved disease-free survival and reduced recurrence risk [29]. In another iTRAQ labeling-based proteomics investigation, several factors strongly associated with molecular subtypes of breast cancer were identified, including fibronectin, alpha-2-macroglobulin (A2M), complement component-4-binding protein alpha (C4BPA), and complement factor-B. Notably, A2M, an antiprotease and large plasma protein, exhibited genetic alterations and differential expression in plasma and tissue samples from TNBC patients [33].

Label-free proteomics strategies have been extensively applied in breast cancer research. For instance, a label-free shotgun proteomic approach identified a signature of 21 proteins in a High Mobility Group A1 (HMG1) silenced TNBC cell line, correlating with poor prognosis [34]. A specific subset of three proteins named kinesin family member C1 (KIFC1), thyroid hormone receptor-interacting protein 13 (TRIP13), and leucine-rich repeat containing 59 (LRRC59) are associated with HMG1 was identified as novel in TNBC, influencing cell motility [34]. In another study using label-based proteomics, PTPN12, a tyrosine phosphatase, was found to suppress cellular transformation and metastasis in TNBC cells [35]. Furthermore, a systematic 2D gel-based proteomic analysis of TNBC tissue biopsies revealed Mage-A4, a member of the Mage-A family, as a distinctive biomarker for TNBC and HER2-positive patients [36]. In addition, label-free proteomics identified Iroquois homeobox protein 1 (IRX1), involved in metanephric nephron development, as a potential plasma biomarker for TNBC [37].

Another label-free proteomic study focused on TNBC identified a protein signature of 30 proteins linked to drug resistance and poor patient survival [38]. Notably, HSP70 kDa-8, periostin, RhoA, actinin alpha 4, cathepsin D, preproprotein, and annexin 1 exhibited high expression levels in TNBC tumors resistant to neoadjuvant chemotherapy. In addition, ALDH1A1, complement component 1 inhibitor, and G3BP (also known as 90-kDa Mac-2-binding protein) were identified as associated with specific TNBC subtypes, with G3BP's connection to TNBC being previously unrecognized. Furthermore, a systematic phosphoproteomic analysis of TNBC tissues revealed six proteins highly phosphorylated in primary TNBC tumors and five distinct proteins at metastatic sites [39]. This finding suggests that targeted dephosphorylation of these proteins could potentially inhibit cancer progression and mitigate the metastatic capability of TNBC cells.

A recent study identified distinct proteome expression profiles associated with two subclasses of TNBC breast cancer, basal A and B, using comprehensive proteomics analysis of breast cancer cells [40]. It was observed that kinases and proteases exhibited unique expression patterns within these subclasses. Detailed analyses of protein–protein interactions and co-regulation networks involving these kinases and proteases revealed disrupted pathways and potential therapeutic targets specific to each TNBC subclass. Specifically, the study pinpointed AXL, PEAK1, and TGFBR2 kinases, along with FAP, UCHL1, and MMP2/14 proteases, as specific targets for the basal B subclass, which is characterized by more aggressive TNBC cell lines. These findings underscore the complexity of TNBC and emphasize the importance of targeting subclass-specific mechanisms rather than adopting a universal approach for TNBC therapy [40].

Collectively, these studies underscore the significance of proteomics-based research and emphasize the necessity for precise proteomic signatures to unravel the complexity of TNBC. A well-defined molecular signature is crucial for guiding targeted therapeutic strategies against TNBC. Quantitative functional proteomic methods are essential in identifying characteristic protein-protein interaction networks and specific protein

signatures. These approaches are pivotal in identifying critical regulators of TNBC that facilitate the development of predictive, diagnostic, and therapeutic applications.

3 | Significance of Single-Cell Proteomics

Single-cell proteomics stands poised to advance the field of single-cell biology significantly. Proteomic variances remain relatively stable, unlike transcriptomic differences, which can vary widely between biological states [41]. A recent comparative study of single-cell RNA and proteome levels underscored this stability in proteomics, highlighting the intricate regulation of translation at the single-cell level [42]. To gain a comprehensive understanding of translational regulation, particularly amongst cellular diversity in diseases, single-cell proteomics complements single-cell RNA-seq effectively. Bulk proteomics, which averages protein expression across thousands of cells, fails to capture crucial differences between individual cells due to cellular heterogeneity. This limitation emphasizes single-cell proteomics' critical role in presenting cell-to-cell variations essential for precise disease biology comprehension. Moreover, single-cell proteomics uniquely enables the dynamic study of protein expression changes in live cells, contrasting with fixed and processed samples used in bulk proteomics. Notably, single-cell proteomics' sensitivity also facilitates the analysis of rare cell populations, such as cancer stem cells, which are often overlooked by conventional proteomic approaches. Thus, single-cell proteomics is indispensable for exploring rare cells' intricated roles in disease pathogenesis.

Single-cell proteomics now offers high-resolution data at the proteome level and provides insights into PTMs, which are beyond the reach of transcriptomic analysis. This single-cell proteomics data is essential for studying early cell-signaling events and changes resulting from environmental fluctuations due to drug interventions or disease influences. While bulk proteomics techniques have been used to explore cell-signaling dynamics, their results are limited as they average signals from heterogeneous cell populations. Single-cell proteomics enables us to address critical biological questions about signaling mechanisms based on protein binding, modifications, and degradation areas that bulk proteomics has struggled to explore. The regulation of protein abundance and activity through degradation and PTMs cannot be inferred from genomic and transcriptomic data. Furthermore, transcriptomic and genomic sequencing do not provide insights into protein–protein interactions and protein localization, which are crucial for understanding many signaling pathways [43].

Single-cell proteomics delivers detailed insights, unlike conventional laboratory methods that provide generalized perspectives on protein interactions within average cell populations. This technology enables researchers to discern specific cellular responses and untangle molecular-level pathways and processes with unprecedented precision. Such accuracy is crucial in studying heterogeneous tissues where protein reactions can significantly differ among cells. The single-cell proteomics approach promises to be a cornerstone in our efforts to combat this challenging form of breast cancer. This single-cell proteomics enables researchers to investigate tumor heterogeneity at the protein level, uncovering details about distinct cellular subsets that may contribute to tumor growth, metastasis, or therapy resistance, which have historically been challenging to identify, which could be crucial in understanding TNBC's complexity and uncovering therapeutic opportunities [19]. This level of precision is critical in studying heterogeneous tissues, where protein responses frequently differ between individual cells. Single-cell proteomics is becoming increasingly valuable in drug development, as proteins are the primary targets for up to 95% of drugs. Traditional methods like high-throughput screening (HTS) generally assume uniform behavior across all cells when evaluating potential new drugs and their mechanisms of action. In contrast, single-cell proteomics can reveal individual cell-level responses, providing a more detailed understanding of off-target effects, dose responses, and other critical factors. This approach also speeds up the discovery of novel biomarkers, improving early cancer detection through diagnostics.

Single-cell proteomics provides an advanced analytical approach that allows for the high-resolution analysis of protein expression and signaling pathways within individual cancer cells. By capturing the proteomic profile of single cells, this technique enables us to dissect the cellular diversity within TNBC tumors, identifying distinct cell populations that may contribute to drug resistance, immune evasion, and tumor progression [44]. Importantly, single-cell proteomics can uncover rare or minor cell subpopulations that might drive disease recurrence or metastasis, which bulk analyses often overlook [45]. This makes it an invaluable tool in uncovering potential therapeutic targets within TNBC's heterogeneous cellular environment, providing insights that could guide the development of more targeted and effective treatments for this challenging breast cancer subtype.

4 | Advancements in Single-Cell Sample Preparation

Single-cell proteomics aims to identify and quantify the proteome of individual cells using LC-MS/MS technology. Figure 2 illustrates the steps involved in the single-cell proteomics process. An appropriate workflow depends on budget, available instruments and consumables, and specific research objectives. This section provides an overview of advanced workflows to offer insights for future single-cell proteomics experiments.

4.1 | Single-Cell Isolation

Sample preparation is crucial for single-cell proteomics. Researchers have been working to miniaturize reaction volumes from micro- to nanoliters, which reduces peptide adsorption to vessel surfaces, improves reaction efficiency, and lowers reagent consumption [46]. Simultaneously, efforts are being made to increase batch sizes to minimize background noise by reducing batch effects. Single-cell isolation in single-cell proteomics can be achieved through various methods, including (i) manual techniques such as (a) capillary probes and (b) laser capture microdissection (LCM), and (ii) automated methods like (c) FACS and (d) imaging-based cell sorting. Table 1 provides the various techniques for isolating single cells from the tumor tissue.

TABLE 1 | Tumor cell isolation techniques in single-cell proteomics.

Cell isolation technique	Platform/method	Key feature	Cell type	Preparation	Reference
Capillary probe	Single-cell transfer	- Microscopic, hollow needles or glass probes - Single-cell aspiration directly from tissues or culture	CCRF-CEM cells	The CCRF-CEM cells are cultured in RPMI 1640 medium with fetal bovine serum and antibiotics, then isolated using biotinylated aptamer Sgc8 for spiking experiments.	[47]
Laser capture microdissection	Microscopy-based	- High specificity - Minimal contamination	Breast tissue specimens	Breast tumor tissues were sectioned at 8 μm and stained with hematoxylin and eosin.	[48]
Fluorescence-activated cell sorting	Flow cytometry	- Live cell sorting - High purity and yield	Pluripotent stem cells	Cells are stained with various fluorescent dyes to distinguish between different populations.	[49]
Imaging-based cell sorting		- High-resolution imaging for cell identification - Fluorescence labeling - Morphometric feature extraction	Circulating tumor cells (CTCs)	CTCs were cultured and stained using various dyes, including CellTracker Green for cytoplasm, Hoechst 33342 for nuclei, and Mito-Tracker DeepRed for mitochondria for imaging-based analysis	[50]
Microfluidic devices	Lab-on-a-chip	- High-throughput - Precise fluid control	PC-9 cells, MEC-1 cells	The microfluidic chip isolates 1–100 cells, traps them in chambers, lyses, digests, and alkylates the proteins.	[51]
Mechanical disruption	Bead mill, homogenizer	- Simple and effective - Fast	Breast cancer cell lines—MCF-7, T-47D MCF-7 cells	Cell pellets were exposed to focused ultrasound at different intensity levels (H4 and H5). Centrifuged, filtered, and ultrafiltered to isolate small extracellular vesicles	[52]
Multi-well plates	Automated liquid handling	- Physical separation of individual cells into wells - Automated cell deposition - Compatible with various assays	Cancer stem cells	Cancer cells were seeded into agarose micro multi-well dishes. These dishes were prepared using rubber micro-molds to form spheroids in a 3D environment.	[53]

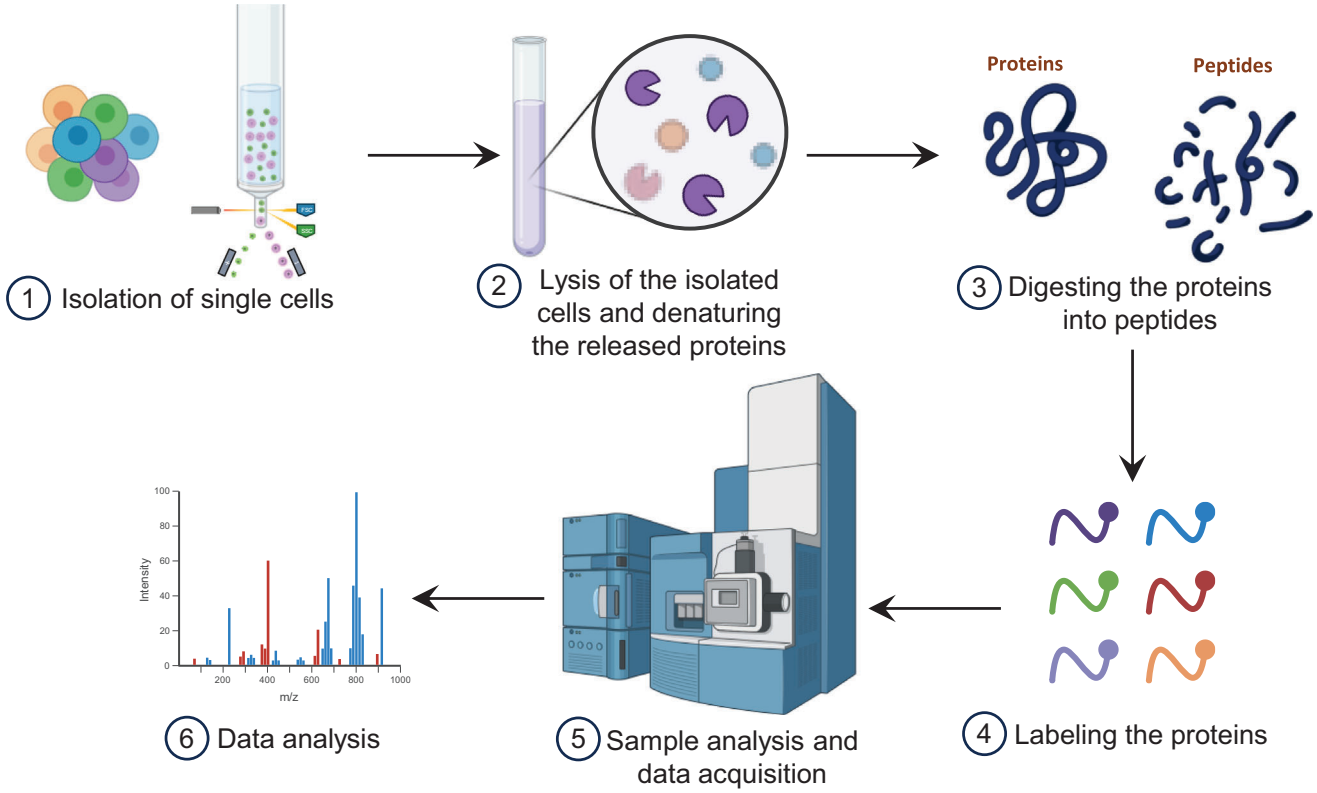

FIGURE 2 | General overview of the single-cell proteomics workflow. The process begins with the isolation of individual cells using either manual or automated techniques. This is followed by protein extraction from the isolated cells using mass spectrometry-compatible lysis buffers or mechanical methods. The extracted proteins are then digested into peptides, which can be labeled or analyzed using a label-free approach. The peptides are separated using liquid chromatography and analyzed by high-resolution mass spectrometry. Finally, data analysis is performed to interpret protein expression profiles, providing insights into cellular heterogeneity and disease mechanisms, such as those seen in triple-negative breast cancer.

4.1.1 | Capillary Probe

A capillary probe can manually pick up individual cells from a solution using microcapillary pipettes, typically under a microscope [46, 54, 55]. This labor-intensive method requires precise selection and pipetting by hand or robotic arm. Despite its laborious nature, it has several advantages: it preserves spatial information, is the fastest method for handling small sample sizes (1–10 cells), and is particularly useful when cell states are visible under a standard light microscope [56]. This technique is ideal for isolating valuable samples such as blastomeres and oocytes and distinguishing between migrating and non-migrating cells in scratch assays [55].

4.1.2 | LCM

LCM isolates regions of cells from a polyethylene naphthalate (PEN) membrane slide using infrared radiation under a microscope [57]. LCM operates in two ways: (i) capture or (ii) gravity, generally employing an IR or UV laser to target tissue regions of interest on the PEN slide [58]. Like the capillary probe method, LCM preserves spatial information and is equally labor-intensive, requiring manual cell picking. However, LCM offers additional advantages, such as isolating fluorescence-tagged cells when connected to a fluorescence microscope and directly isolating single cells from histologically stained solid tumors [59]. The drawbacks include high costs due to the need for specific instruments and consumables and additional steps for locating samples on slides using micro-sectioning for solid samples and cytospin for liquid samples [57]. Therefore, LCM is particularly recommended for histopathologically annotated tissue samples.

4.1.3 | FACS

FACS sorts cellular solutions into single cells using a fluidics system and filters them based on optical properties, such as light scattering and fluorescence [60–63]. This allows for preliminary filtering based on cellular markers before proceeding to single-cell proteomics for more detailed analysis. FACS is widely accessible compared to other single-cell isolation methods and offers a high level of automation, from single-cell isolation to cell dispensing [64]. Consequently, FACS has become the most popular method for integrating single-cell omics analysis, with or without preliminary optical filtering [65].

4.1.4 | Imaging-Based Cell Sorter

Recent single-cell proteomics methods have started incorporating advanced cell sorters, such as the imaging-based cellenONE cell sorter [66]. This device operates similarly to FACS but

includes dispensers equipped with imaging devices, ensuring high-confidence single-cell dispensing. The cellenONE also offers flexibility in dispensing arrangements, compatible with standard microwell plates and customized chips like nested Nanodroplet Processing in One pot for Trace Samples (nanoPOTS) and nanoProteOmic sample Preparation (nPOP) glass slides [66, 67]. However, a drawback of this method is its lower accessibility. Besides, NanoPOTS is a microfluidic sample-preparation platform initially designed to analyze samples containing as few as ten cells [68] and has since been adapted for single-cell analysis [42]. This method significantly reduces the contact area between samples and the glass surface used for sample preparation, including lysis and digestion. This innovation has inspired other techniques to minimize sample volume and surface contact to enhance sensitivity [46, 66]. In the nPOP method, piezo acoustic dispensing is employed to isolate individual cells into 300 pL volumes. All subsequent sample preparation steps, including cell lysis, digestion, and labeling, are conducted in small droplets on fluorocarbon-coated slides, with each step being performed in 20 nL volumes.

4.2 | Cell Lysis and Denaturation

Sample preparation for single-cell proteomics is generally optimized to reduce sample clean-up and desalting steps to minimize sample loss. Instead of using conventional mass spectrometry (MS)-incompatible lysis buffers like urea, researchers have shifted to MS-compatible lysis buffers, such as MS-compatible detergents (e.g., RapiGest, trifluoroethanol, and n-dodecyl-β-D-maltoside and solvents like dimethyl sulfoxide [DMSO]) [51, 66, 61, 67]. Using MS-compatible lysis buffers eliminates the need for extensive cleaning steps like StageTips, which can lead to sample loss due to excessive liquid transfers between vessels [69]. These detergents have long been used in bulk proteomics and have demonstrated improvements in digestion efficiency and peptide recovery [70–72]. High concentrations of DMSO (90%–100%) as a cell lysis buffer have been pioneered in single-cell proteomics by the nPOP workflow, showing extraction efficiency comparable to the traditional urea method [66]. DMSO is particularly compatible with glass slide reaction platforms due to its ability to form proper droplets, unlike other reagents [66].

In addition, some workflows propose eliminating lysis buffers, using mechanical methods to lyse cells and produce even cleaner lysates than chemical lysis. For example, ultrasonication [54], freeze-thaw cycles [73], or a combination of both [60] can perform cell lysis without surfactants. These mechanical methods have been used since the early days of proteomics in the 20th century and continue to be employed in bulk proteomics [74]. However, these methods are more aggressive than chemical lysis, potentially degrading proteins and requiring less accessible equipment like an ultrasonicator [75, 76].

4.3 | Sample Processing Reaction Platforms

Researchers have developed "one-pot" reaction platforms that allow single-cell proteomics sample preparation from cell lysis to protein labeling in a single vessel to minimize sample loss due to adsorption to vessel surfaces. The currently available single-cell proteomics reaction platforms, which vary in reaction volume from larger to smaller, can be categorized into capillary probes, multi-well plates, microfabricated chips, and glass slides.

4.3.1 | Capillary Probes

This reaction platform simplifies sample preparation by conducting it directly in vials or capillary probes. For example, single cells and reagents such as lysis buffer and digestion mix can be injected into HPLC injection vials, allowing the reactions to occur in the same location [73]. This setup seamlessly integrates with LC-MS/MS, as the vial contents can be directly processed using an autosampler. Additionally, another workflow involves performing cell lysis and digestion steps within the same microcapillary after extracting the single cell from the cell solution [54]. Although these methods are highly labor-intensive, they are ideal for valuable samples since they minimize the need for transferring samples between vessels, thereby reducing the risk of sample loss due to adsorption.

4.3.2 | Multi-Well Plates

PCR well-plates are widely used in laboratories due to their prevalence, low cost, and multiplexing capabilities [60]. Consequently, many single-cell proteomics workflows have been developed around them. Some workflows initially relying on custom platforms have also created alternative versions using PCR well-plates to increase accessibility [77]. The primary drawback of multi-well plates is their limited customizability. However, they are compatible with many existing automated liquid handlers, making automation less challenging. Additionally, PCR well-plates can be easily integrated with the freeze-heat cell lysis method using a PCR thermocycler [60].

4.3.3 | Microfabricated Chips

Several research groups utilize customized microfabricated chips tailored for specific single-cell proteomics workflows. The current trend in single-cell proteomics workflow development emphasizes conducting reactions within individual droplets to minimize sample loss through reduced contact with vessel surfaces. For instance, R.T. Kelly's lab micro-etched nano-wells onto glass slides with hydrophilic interiors and hydrophobic exteriors to stabilize droplet shape and position. These nano-wells are arranged in spatially separated clusters to facilitate intra-cluster pooling for multiplexing experiments [46, 67]. In addition, some workflows incorporate an external oil layer on the chip to prevent sample evaporation. Hartlmayr et al. and Li et al. structured their chips to accommodate this oil layer, either in direct contact with the sample or not [46, 78]. Gebreyesus et al. and Wang et al. customized their chips further by integrating them with home-built liquid handling systems. Their designs range from simple arrays of vials to complex structures with interconnected chambers, each tailored for different stages of sample preparation [51].

4.3.4 | Glass Slides

The smooth glass slide, developed by N. Slavov's lab, represents the current state-of-the-art platform for single-cell proteomics. It provides unparalleled flexibility in arranging sample droplets across a two-dimensional plane [66]. One method for sample pooling involves initially organizing the droplets into clusters and then introducing a larger droplet to cover and collect these clusters [66]. This platform supports parallel preparation of up to 2000 samples per slide, significantly reducing background noise caused by batch-to-batch variations.

5 | Recent Advancements in the LC-MS/MS–Based Single-Cell Proteomics

The rapid development of mass spectrometry-based technologies, with improvements in mass accuracy, sequencing speed, resolution, power, and cost-effectiveness, presents a unique opportunity for accurate and rapid profiling of cancer metabolomics [79] and proteomes [80]. Advanced mass spectrometers such as time of flight, Orbitrap, or Fourier transform ion cyclotron resonance, are known for their high resolution, sensitivity, and sub-ppm mass accuracy [81]. The features ideally suited for shotgun proteomics approaches that aim to quantify hundreds to thousands of proteins in a biological sample [82, 83]. Liquid chromatography-tandem mass Spectrometry (LC-MS/MS) stands at the forefront of modern proteomic analysis, offering powerful tools and techniques for precisely characterizing proteins [84]. In TNBC research and single-cell proteomics context, LC-MS/MS plays a pivotal role in uncovering the disease's molecular intricacies [85]. Table 2 provides an overview of the breast cancer-related proteins identified and characterized using LC-MS technologies.

5.1 | Label-Free Single-Cell Proteomic Analysis by LC-MS/MS

Label-free analysis is the simplest method to quantify proteins from a single cell using mass spectrometry. A single cell is lysed, proteins are extracted and digested, and the resulting peptide mixture is separated via in-line liquid chromatography coupled with MS analysis [91]. For label-free proteomics, each nanoscale liquid chromatography–MS/MS experiment identifies and quantifies peptides from a single cell, making the optimization of sample preparation, peptide purification and separation, and efficient movement of peptide ions within the mass spectrometer crucial for accurate and comprehensive proteome characterization [60]. Label-free MS analyses can be categorized into two main data-collection methods: data-dependent acquisition and data-independent acquisition [92]

In a data-dependent acquisition experiment, intact peptides are analyzed in a survey scan, and the most abundant peptides are selected for fragmentation and sequencing. Peptides identified in a single-cell mass spectrometry experiment are quantified based on the intact peptide signals in the survey scans [93]. However, the stochastic nature of sampling peptides for fragmentation in data-dependent acquisition can lead to inconsistent peptide identification across multiple experiments [94]. To address this, a data-analysis technique called "match between runs" allows quantifying peptides identified in separate data-dependent acquisition experiments, increasing the number of quantified peptides and proteins. However, it may also increase the likelihood of false positive quantification events [60]. In contrast, independent of a survey scan, data-independent acquisition methods systematically sample all regions within a specified mass range throughout the MS analysis. This approach ensures that all intact peptides are fragmented, resulting in more consistent identification and quantification of peptides and proteins across multiple experiments. A recent landmark single-cell mass spectrometry study using data-independent acquisition quantified the proteomes of single cells at various cell cycle stages, identifying up to 2000 proteins per cell depending on the cell cycle stage [60].

Label-free single-cell proteomic analysis can identify and quantify over 1000 proteins, primarily employing Orbitrap or trapped ion mobility spectrometry time of flight (timsTOF) mass spectrometers [95]. Combining nanoPOTS, ultra-low flow liquid chromatography, and high field asymmetric ion mobility spectrometry (FAIMS) with an Orbitrap Eclipse Tribrid mass spectrometer offers significantly deepened single-cell proteome coverage to 1056 identified proteins on average [96]. Transferring identification based on FAIMS filtering (TIFF) further increased proteome coverage to over 1200 proteins [97]. Data-dependent acquisition is the traditional scan mode in LC-MS/MS, but it suffers from low data completeness in single-cell proteomics. Data-independent acquisition and the SciProChip microfluid chip have emerged as solutions, resulting in an average identification of around 1500 proteins from single cells with minimal missing values [19]. In timsTOF single-cell proteomic, the diaPASEF (parallel accumulation-serial fragmentation combined with data-independent acquisition) scan mode maximizes quantifiable proteins (up to 2083) per single HeLa cell with high completeness [60, 98]. Despite challenges, label-free single-cell proteomic analysis has yielded insights into critical cellular processes and potential applications in cancer research and drug treatment. In addition, providing quantitative data on the proteins within single cells empowers researchers to understand the heterogeneity within TNBC, identify potential biomarkers and therapeutic targets, and ultimately contribute to more personalized and effective treatment strategies.

5.2 | Tandem Mass Tags Multiplexing Based Single-Cell Proteomic Analysis by LC-MS/MS

To attain higher throughput in single-cell mass spectrometry, researchers can employ a method known as isobaric labeling, which enables multiplexed quantitative analysis. Tandem mass tags (TMT) are isobaric chemicals used for precise and multiplexed measurement of peptides and proteins using tandem mass spectrometry analysis [99]. TMT-based multiplexing is particularly valuable in large-scale proteomics at the single-cell level, which requires high sensitivity and throughput. This concept is similar to DNA barcodes in next-generation sequencing, albeit with mass-based identifiers. The existing range of mass barcodes for single-cell mass spectrometry restricts simultaneous detection to 18 samples [100]. Hence, the chemical composition constraints enable effective multiplexing, typically involving heavy isotopes like ^{13}C and ^{15}N [101]. Although alternative chemical tags have been proposed, challenges in finding compatible

TABLE 2 | LC-MS–based analysis of breast cancer-associated proteins.

Biomarker	Type of study	Chromatographic condition	Ionization source	Analyzer	Observation	References
Lipocalin-1, SMR-3B, and plastin-2	Clinical saliva	Column: C18, 50 cm × 3.0 μm Peptides were eluted with a 0%–40% gradient of buffer B (80% acetonitrile, 0.1% formic acid) at 300 nL/min) LC gradients were run for 100 min	NanoESI	Orbitrap	Upregulation of lipocalin-1 and SMR-3B, and downregulation of Plastin-2 in disease.	[16]
HER2, hormone receptors, Ki-67, or inflammation-related proteins	Clinical breast tumor tissue	Column: C18 (50 cm × 75 μm, 2 μm) Mobile phase A (0.1% formic acid, 2% acetonitrile) and mobile phase B (0.1% formic acid in acetonitrile) Flow rate of 250 nL/min	ESI	QqQ	Quantitative information on 185 proteins from three groups of breast tumors: triple-negative, HER2-overexpressing, and luminal A. In addition to markers such as HER2 or hormone receptors, we identified several proteins expressed differentially in triple-negative breast cancer samples.	[86]
RhoGDI-2, HSP27, SOD1, DJ1, UBE2N, PSME1, FTL, SH3BGRL, and eIF5A-1	Clinical	Column: C18, (1.7 μm, 75 μm × 100 mm	NanoESI	Q-TRAP	Deregulated proteins in this aggressive breast cancer subtype, namely DB1, COX5, and MTPN. In addition, RhoGDI-2 overexpression in breast cancer subtypes is associated with inhibiting the intrinsic pathway of apoptosis through caspase-3 and -9 modulation.	[18]
CMPK1, AIFM1, FTH1, MTHFD1, EML4, GANAB, CTNNA1, AP1G1, STX12, AP1M1, and CAPZB	Clinical	Mobile phase A: water; mobile phase B: acetonitrile) in a 3-μmm C18 silica-packed 50-cm capillary column with a 75-μm inner diameter	ESI	LTQ-Orbitrap-XL	Developed a distant metastasis with high sensitivity, specificity, positive predictive value, and negative predictive value (82.3%–98.9%).	[24]
TrpRS, DP, TSP, and IDH	Clinical	C18, (PepMap100, 3 m, 100 A, 75 m id 15 cm, Dionex) at 300 nL/min flow rate. Buffer A was 2% ACN in water with 0.05% TFA, and buffer B was 80% ACN in water with 0.04% TFA.	nano-LC	MALDI TOF/TOF	These four proteins are overexpressed	[29]

(Continues)

TABLE 2 | (Continued)

Biomarker	Type of study	Chromatographic condition	Ionization source	Analyzer	Observation	References
Phosphorylated proteins	Clinical	C18 capillary column. An Eksigent NanoLC (Dublin, CA) delivered a 90 min gradient (2–50% B) at 400 nL/min (solution A: 0.5% formic acid in 2% aqueous methanol; solution B: 0.5% formic acid in 98% aqueous methanol)	ESI	Linear quadrupole ion trap	Five proteins were highly phosphorylated in the metastatic site, whereas six were highly phosphorylated in the cancer site of the TNBC patient.	[39]
59 urinary proteins	Clinical	C18 pre-column (500 μm × 2 mm) with Buffer A at 10 μL/min (2% ACN and 0.01% Heptafluorobutyric Acid (HFBA) in water). After a 4-min wash, the pre-column was switched (Valco 10 port valve, Dionex) into line with a fritless nano column (75 μm diameter × 12 cm) containing reverse phase C18 media (3 μm, 200Å Magic, Michrom Bio-resources). Peptides were eluted using a linear gradient of Buffer A to Buffer B (98% ACN, 0.01% HFBA in water) at 250 nL/min over 60 min.	ESI	Orbitrap	In breast cancer patients compared to the normal control subjects. Thirty-six urinary proteins were exclusively found in specific breast cancer stages, with 24 increasing and 12 decreasing in their abundance. Among the 59 significant urinary proteins identified, 13 novel up-regulated proteins were revealed that may be used to detect breast cancer.	[87]

(Continues)

TABLE 2 | (Continued)

Biomarker	Type of study	Chromatographic condition	Ionization source	Analyzer	Observation	References
250 proteins	In vitro	C18, (0.15 mm × 150 mm, VC-10-C18-150; Micro-TechScientific, Vista, CA) using a binary solvent system made up of 98.8% water, 1% acetonitrile, and 0.2% formic acid (solvent A), and 94.8% acetonitrile, 5% water, and 0.2% formic acid (solvent B)	Nano-ESI	Q-TOF	This analysis of an isogenic series of cell lines suggests the potential usefulness of the secretome for identifying prospective markers for the early detection and aggressiveness/progression of cancer.	[88]
N-glycosylated membrane proteins	In vitro	C18, (15 cm × 75 μM diameter, 5 μm particle size) acetonitrile/water/formic acid, 98.9/1/0.1 Flow rate: 300 nL/min	Nano-ESI	LTQ Orbitrap	MCF-7 had high quantities of keratin 8 and 18, solute carrier protein 3, HSP 27, and ErbB-binding protein, while MDA-MB-453 had high quantities of enolase 1, nucleolin, RAB1B Ras oncogene, stomatin, filamin A, valosin, and cytokeratin 7. MDA-MB-468 cells had high quantities of EGFR, CD44, filamin A, progesterone receptor component 1, and valosin. All three cancer cell lines also share high expression of STIP1, keratin 19, chaperonin, and HSPalpha.	[89]
Serum protein	Clinical	NA	SELDI	TOF	Apolipoprotein C-I and C3a des-arginine anaphylatoxin (C3adesArg) were higher in pre-diagnostic breast cancer serum. With 2D-nanoLC-MS/MS, afamin, apolipoprotein E, and isoform 1 of inter-alpha trypsin inhibitor heavy chain H4 (ITIH4) were found to be higher in pre-diagnostic breast cancer.	[90]

Abbreviations: AIFM1, apoptosis-inducing factor 1, mitochondrial; AP1G1, AP-1 complex subunit gamma-1; AP1M1, AP-1 complex subunit mu-1; CAPZB, F-actin-capping protein subunit beta; CMPK1, UMP-CMP kinase; CTNNA1, Catenin alpha-1; DJ1, deglycase DJ-1; DP, desmoplakin; eIF5A-1, eukaryotic initiation factor 5A; EML4, echinoderm microtubule-associated protein-like 4; ESI, electrospray ionization; FTH1, ferritin heavy chain; FTL, ferritin light chains; GANAB, neutral alpha-glucosidase AB; GASP-1, G-protein coupled receptor-associated sorting; HSP27, heat shock protein 27; IDH, isocitrate dehydrogenase; QqQ, triple-quadrupole; LTQ, linear ion trap quadrupole; MTHFD1, C-1-tetrahydrofolate synthase, cytoplasmic; PSME1, proteasome activator subunit 1; Q TRAP, quadrupole ion trap; RhoGDI-2, Rho GDP dissociation inhibitor 2; SELDI, surface enhanced laser desorption/ionization; SH3BGRL, SH3 domain binding glutamic acid rich protein; SMR3B, submaxillary androgen-regulated protein 3B; SOD1, superoxide dismutase 1; STX12, Syntaxin-12; TOF, time-of-flight; TrpRS, tryptophanyl-tRNA synthetase; TSP1, thrombospondin-1; UBE2N, Ubiquitin-conjugating enzyme E2 N.

structures restrict the maximum number of samples that can be concurrently analyzed [102]. With the current technology, multiplexed single-cell mass spectrometry allows for the simultaneous analysis of up to 18 single-cell proteomes. For instance, if analyzed under standard conditions (90 min per sample), this could potentially process 288 single-cell proteomes in a day. Throughput enhancement could be achieved by reducing analysis times, expanding multiplexing capabilities, or adopting dual-column configurations. Even with existing capabilities, multiplexed single-cell mass spectrometry significantly reduces the time required to analyze 1000 cells to a matter of days [100] compared to the weeks needed for label-free single-cell mass spectrometry methods.

Beyond increasing throughput, multiplexed quantitative analysis facilitates new experimental designs that enhance proteomic depth by incorporating a carrier proteome [103]. This carrier proteome, containing protein amounts equivalent to 20–500 times that of a single cell, helps optimize experimental parameters for detecting, identifying, and quantifying proteins that might otherwise go undetected [104]. The concept was first applied in single-cell mass spectrometry experiments with the advent of single-cell proteomics by mass spectrometry method [103, 104]. Subsequently, various studies have aimed to establish the maximum carrier proteome limit that can be used without causing negative effects [105, 106]. Recent single-cell mass spectrometry experiments generally set this upper limit at 200× for single-cell quantification using single-cell proteomics by mass spectrometry. Optimized multiplexed approaches have achieved label-free quantification levels, enabling the quantification of approximately 1000–1500 proteins per cell and characterizing over 250 cells per day [105, 100].

Various sample preparation methods have been proposed to confidently analyze thousands of proteins in a single cell to reduce sample loss and enhance sensitivity. These methods include using organic co-solvents instead of detergents [107], eliminating the need for cleaning steps, and utilizing simplified platforms like nanoPOTS to process small cell populations efficiently. A pioneering approach called single-cell proteomics by mass spectrometry involves labeling carrier cells with TMT and analyzing them alongside individual TMT-labeled single cells. This method increases sensitivity and reduces missing data during analysis [69]. Single-cell proteomics by mass spectrometry has quantified over a thousand proteins in single cells, aiding the identification of different cell types and studying the relationship between mRNA and protein levels. An updated version, single-cell proteomics, improves sample preparation and data analysis, quantifying thousands of proteins in single monocytes and macrophages [100]. NanoPOTS is another approach that combines TMT labeling to enhance processing efficiency and throughput for single-cell samples [68]. It utilizes a chip-based platform for preparing small cell populations with minimal sample volume, significantly improving sensitivity and sample recovery. This method has identified thousands of proteins from as few as 10 cells, a significant advancement in single-cell proteomics. Nested nanoPOTS further enhances isobaric-labeling-based single-cell proteomics, offering increased quantification and analysis of more single cells [67]. Despite its benefits, nanoPOTS does require specialized equipment and manual operation, which limits its widespread adoption. Nevertheless, it presents a promising platform for high-sensitivity single-cell proteomics, with potential applications in various studies, including circulating tumor cells, stem cell development, cellular heterogeneity, and disease biomarkers. Ongoing developments aim to address its limitations and make it more accessible for broader use in single-cell omics research.

Multiplexed proteomics using isobaric labels offers the highest throughput for single-cell mass spectrometry, but it faces challenges due to ratio compression due to precursor interference [105]. When selecting precursor peptides labeled with isobaric tags for fragmentation and quantification, other peptides are often co-isolated with the peptide of interest. The mass barcodes from these additional peptides are indistinguishable from those of the target peptide. Because most proteins in an experiment show no change, they contribute an equal signal across all mass barcodes. This can cause a tenfold change in the target peptide to appear as only fourfold [108]. To address this issue, gas-phase purification techniques like proton-transfer reactions or synchronous precursor selection MS^3 are used. These methods reduce the impact of precursor interference so that a tenfold change appears closer to an eightfold change [109, 108]. However, while they improve quantitative accuracy, they also lower the signal for mass barcodes, potentially resulting in fewer protein-quantification events. Given the challenges associated with isobaric labels, researchers have turned to multiplexed data-independent acquisition approaches to balance single-cell throughput and quantitative accuracy. Unlike traditional data-independent acquisition, multiplexed data-independent acquisition employs non-isobaric chemical tags or heavy amino acids to generate two to three precursor signals for each peptide sequence [110, 111]. This method offers higher throughput than label-free techniques and greater quantitative accuracy than multiplexed experiments using isobaric labels.

A notable multiplexed data-independent acquisition approach, plex data-independent acquisition, has successfully analyzed three single-cell proteomes simultaneously, quantifying approximately 1000 proteins per cell [112]. This method requires 30 min of analysis time (about 10 min per cell), enabling the analysis of 144 cells per day. Encouragingly, the study demonstrated quantitative accuracy comparable to label-free quantitation and successfully analyzed different cell cycle states and multiple cell lines. Future advancements may extend the multiplexing capacity of plex data-independent acquisition and reduce the analysis time per cell. In addition, a new approach has been proposed combining data-independent acquisition and isobaric labels, achieving the highest multiplexing in a data-independent acquisition experiment to date [113]. However, this method relies on mass barcodes and uses a large precursor isolation window, which may exacerbate precursor interference challenges, complicating accurate quantification. Though applying multiplexed data-independent acquisition to single-cell mass spectrometry is a recent development, it holds promise as a broadly attractive approach for future experiments.

To further enhance the single-cell proteomic profiling in the context of cancer heterogeneity, imaging mass cytometry (IMC) offers a significant advancement, allowing spatially resolved phenotyping of individual cells within tissue samples. IMC enables multiplexed imaging by using metal-tagged antibodies,

allowing simultaneous visualization of multiple markers in a single scan. This technology has been successfully applied to breast cancer studies by Raza Ali et al., who combined IMC with multiplatform genomics to define the phenogenomic landscape of breast tumors. This approach revealed detailed cellular compositions and interactions across breast cancer subtypes, providing insights into the tumor microenvironment and its link to genomic alterations, as well as potential implications for prognosis and therapeutic response [114].

6 | Comparative Analysis of Alternative Technologies

While mass spectrometry (MS)–based single-cell proteomics is a powerful tool for identifying and quantifying proteins in individual cells, it is one of several technologies available for single-cell analysis, each with distinct strengths and limitations. Spatial proteomics and advanced imaging techniques offer unique capabilities by retaining spatial information about protein expression within tissue architecture, which is critical for understanding cellular context and microenvironmental interactions [115, 116]. Unlike MS-based approaches, spatial proteomics can reveal the localization and distribution of proteins in situ, facilitating insights into cell–cell interactions, tissue heterogeneity, and tumor microenvironments in cancer studies, including TNBC [117]. Advanced imaging techniques, such as IMC and multiplexed ion beam imaging (MIBI), provide high-resolution spatial data but are often limited by lower protein coverage and quantification compared to MS [118, 115]. While IMC can simultaneously measure up to 40 proteins within the same tissue section, it may not achieve the depth of proteome coverage available with MS-based techniques, which can identify thousands of proteins. Therefore, MS-based single-cell proteomics remains advantageous for in-depth proteome profiling, although it lacks the spatial resolution offered by imaging techniques.

Single-cell RNA sequencing (scRNA-seq) has also become a widely used technique for understanding cellular heterogeneity and gene expression at the single-cell level [119]. Compared to MS-based proteomics, scRNA-seq allows for high-throughput analysis of transcriptomic profiles across thousands of cells, providing a broader picture of gene expression patterns within heterogeneous cell populations. However, it is important to note that RNA expression does not always correlate directly with protein abundance due to post-transcriptional regulatory mechanisms [43]. MS-based single-cell proteomics, therefore, fills this gap by providing direct protein-level data, which is critical for functional analysis and understanding cellular phenotypes. Each technology offers unique advantages: MS-based proteomics provides depth and accuracy in protein quantification, spatial proteomics enables contextual protein localization, and scRNA-seq offers high-throughput transcriptomic profiling. Combining these approaches, where feasible, may offer the most comprehensive insights into cellular function and heterogeneity, especially for complex diseases like TNBC. Such integrative approaches, for instance, could help to uncover how transcriptomic diversity translates into protein-level heterogeneity within TNBC tumors, enhancing our understanding of disease progression and response to treatment [120].

7 | Clinical Relevance and Practical Applications of Single-Cell Proteomics in TNBC

Single-cell proteomics is an emerging technology with significant clinical implications, especially for challenging cancers like TNBC. As TNBC tumors are known for their molecular heterogeneity, the need for personalized therapeutic approaches has become more urgent. Single-cell proteomics provides an unprecedented level of detail about protein expression within individual cells, allowing for a deeper understanding of the cellular diversity within TNBC and potentially unveiling novel biomarkers and therapeutic targets. One of the primary clinical applications of single-cell proteomics in TNBC lies in its ability to identify biomarkers that can guide treatment selection. Lehmann et al. [2] identified subtypes of TNBC based on protein expression patterns, leading to insights into which patients might respond better to specific chemotherapies. By applying single-cell proteomics, researchers can further refine these classifications, identifying protein signatures associated with drug sensitivity or resistance on a cell-by-cell basis. This capability is especially useful in TNBC, where variability between cells can influence treatment outcomes significantly [44].

Another promising application of single-cell proteomics in TNBC is in guiding immunotherapy. Immunotherapy has shown potential in TNBC due to the presence of immune cell infiltration in some tumors. Single-cell proteomics can help identify immune-related proteins within TNBC tumors, enabling the stratification of patients who may benefit from immune checkpoint inhibitors or other immunotherapies. The presence of specific immune markers in individual TNBC cells can indicate a likely response to immunotherapy, providing clinicians with valuable information to tailor treatments based on a patient's unique tumor biology. In addition, therapeutic resistance remains a major hurdle in TNBC treatment, often leading to relapse and disease progression. Single-cell proteomics enables the identification of resistant cell populations within a tumor, providing insights into the mechanisms by which these cells evade treatment [45]. By isolating and analyzing protein expression in these resistant cells, researchers can identify pathways and molecular targets associated with resistance, paving the way for new combination therapies that could prevent or overcome resistance in TNBC patients.

Case studies underscore the practical utility of single-cell proteomics in real-world clinical settings. In a recent clinical application, single-cell proteomic analysis identified specific protein signatures in TNBC that correlated with a high likelihood of response to neoadjuvant chemotherapy, allowing oncologists to tailor treatment regimens [44]. Another study involves profiling TNBC tumors to identify biomarkers predictive of metastasis, which has proven instrumental in developing early intervention strategies for high-risk patients [45]. These studies highlight how single-cell proteomics can not only inform treatment but also facilitate early detection and monitoring of aggressive TNBC phenotypes. While single-cell proteomics holds immense promise, translating these findings into routine clinical practice will require further development. Standardizing single-cell proteomic workflows and ensuring they are cost-effective and accessible will be essential for broader adoption in clinical settings. Nonetheless, the technology's potential to provide patient-specific insights at

an unprecedented resolution makes it an invaluable tool in TNBC care. By advancing our understanding of TNBC heterogeneity and revealing actionable protein-level biomarkers, single-cell proteomics is positioned to transform the clinical management of TNBC, enabling more effective, personalized treatment strategies [121].

8 | Challenges and Future Perspectives

Single-cell proteomics has emerged as a transformative approach for understanding cellular heterogeneity, especially in complex diseases like TNBC. The TNBC's tumor microenvironment comprises diverse cell types, including cancer-associated fibroblasts, immune cells, and cancer stem cells, each contributing to the cancer's aggressiveness, immune evasion, and resistance to treatment [122]. MS-based techniques and refined sample preparation protocols have propelled the field forward. However, translating these breakthroughs into real-world outcomes presents challenges that must be addressed to ensure broader application in clinical and research settings. The potential for single-cell proteomics to revolutionize TNBC research lies in its ability to unmask cellular diversity at an unprecedented resolution. This could lead to more personalized therapeutic strategies by identifying distinct protein signatures within individual cells, contributing to improved treatment outcomes. However, the translation of these findings into clinical practice remains challenging. High costs, the need for specialized equipment, and the complexity of data analysis are significant barriers. For clinical integration, automation in sample preparation and data analysis is essential, as well as reducing the operational costs of MS instruments. Large-scale clinical studies will be needed to validate the biomarkers identified by these technologies before they can be adopted in routine clinical practice. What prevents widespread adoption is not only the technological limitations but also the need for regulatory approval of proteomic-based diagnostic tools.

One major issue is the variability in protein expression among cells, even within the same population, influenced by factors like cell cycle stage and external stimuli [123]. In addition, PTMs of proteins add complexity to the analysis, necessitating a thorough understanding of cellular biology. One constraint lies in the inability to amplify proteins, unlike DNA or RNA. Consequently, preserving sample integrity and minimizing losses during preparation, handling, and experimental processes become critical concerns. Technological challenges also persist in single-cell proteomics. One of the main challenges in single-cell proteomics is the low signal-to-noise ratio due to the limited amount of protein material in individual cells. Advanced ion mobility techniques, such as trapped ion mobility spectrometry (TIMS) and structures for lossless ion manipulation (SLIM), can significantly improve ion separation and resolution, thereby enhancing the signal-to-noise ratio [124, 125]. A wide dynamic range of protein expression, varying from one to 10 million copies per cell, spanning seven orders of magnitude. Current methods often capture only a fraction of the cell's dynamic protein range, missing many low-abundance proteins called the "dark proteome" [126]. Efforts are underway within the proteomics community and among technology providers to develop advanced techniques that amplify target protein signals while reducing background noise.

Furthermore, integrating multiple workflows and technologies is crucial for a comprehensive understanding of single-cell proteomics. Mass spectrometry-based single-cell proteomics identifies and quantifies proteins at the single-cell level. Still, protein function is also influenced by subcellular localization and the cellular microenvironment, highlighting the need for spatial context through imaging technologies. Combining these workflows can be complex, but advancements in cell-sorting technologies and sample-handling robotics are helping to streamline and enhance analytical capabilities. In-depth single-cell protein analysis requires managing and storing large volumes of data. Ongoing advancements in analytics provide the computational power and sophisticated algorithms necessary to process and interpret this data effectively.

Besides, one of the most significant challenges is sample loss during the isolation and preparation stages, which can impact the accuracy of downstream MS analysis. Improving methods for efficient cell isolation without compromising protein integrity is critical. In addition, increasing the throughput of MS-based techniques while maintaining sensitivity remains a technical hurdle. Current workflows are often time-consuming and require substantial manual intervention, resulting in variability. Advances in microfluidics, automation, and machine learning (ML)-driven data interpretation could resolve many of these issues, enabling more streamlined processes. Microfluidics has revolutionized single-cell analysis by enabling precise sample handling, minimizing sample loss, and allowing high-throughput processing of individual cells. Emerging microfluidic systems, such as nanodroplet-based platforms and microfluidic chips, can facilitate efficient cell lysis, protein extraction, and peptide separation while conserving precious sample material [103, 127]. Nanodroplet platforms, for instance, have been shown to enable single-cell proteomics by encapsulating cells in nanoliter-sized droplets, which reduces sample loss and contamination risk. Additionally, integrated microfluidic chips with automated sample preparation capabilities can streamline the workflow and increase reproducibility, making it feasible to process large numbers of cells with minimal manual intervention. By adopting these improvements, researchers can overcome some of the major bottlenecks in sample handling and enhance the throughput of single-cell proteomics studies. Collaboration between bioinformatics, engineering, and clinical disciplines will also be essential in overcoming these limitations and standardizing protocols.

While integrating single-cell proteomics and LC-MS/MS brings immense promise in the study of TNBC, it is not without its share of challenges and limitations. Recognizing these hurdles is pivotal in advancing the field and realizing the full potential of this ground-breaking approach. One of the most significant challenges in single-cell proteomics is the limited sample availability for analysis. The minuscule volume of proteins within a single cell necessitates highly sensitive techniques like LC-MS/MS. However, this sample scarcity can hinder comprehensive proteomic profiling and make it challenging to gather statistically significant data, especially when dealing with rare cell subpopulations or precious clinical samples. In addition, single-cell proteomics introduces the risk of technical variability, given the minute quantities of proteins involved. Variability can stem from multiple sources, including sample preparation, data acquisition, and data processing. Addressing and minimizing these sources

of variation is an ongoing challenge in the field to ensure the reliability of results.

While single-cell proteomics is undoubtedly a promising area of research, other fields, such as spatial proteomics, are gaining traction. Spatially resolved proteomics allows researchers to map the spatial distribution of proteins within tissues, providing critical insights into the tumor microenvironment. Integrating spatial proteomics with single-cell techniques could create a powerful platform for TNBC research. In addition, advances in deep learning and AI-based approaches for protein identification and quantification could further enhance the precision and speed of data analysis. Despite the allure of these adjacent fields, single-cell proteomics remains at the forefront of proteomic research due to its unparalleled ability to dissect cellular heterogeneity.

Further, single-cell proteomics generates vast datasets, which can be complex and require sophisticated bioinformatics tools for analysis. The challenge lies in extracting meaningful insights from this data, including identifying relevant protein signatures, defining cellular subpopulations, and recognizing key patterns and trends. Developing advanced data analysis, methodologies, and computational algorithms is essential to navigate the intricacies of single-cell proteomic data effectively. Furthermore, while single-cell proteomics aims to uncover the heterogeneity within TNBC, its full resolution remains a challenge. Heterogeneity is confined to protein expression and extends to genetic, epigenetic, and functional differences among cells. Combining multi-omics approaches, such as integrating single-cell genomics with proteomics, can provide a more holistic view of TNBC heterogeneity but presents additional analytical complexities. Single-cell proteomics can elucidate the role of immune cells within TNBC, where immune cell heterogeneity significantly influences the tumor's response to immunotherapy. This approach provides insights into which immune cell subtypes, such as tumor-associated macrophages or regulatory T cells, may be responsible for immune suppression or tumor support, making them potential therapeutic targets [128, 129]. Furthermore, studying cancer stem cells within TNBC using single-cell proteomics may reveal unique protein signatures linked to tumor initiation and metastasis, which could be critical for developing more effective therapies.

ML algorithms are increasingly being utilized to address the challenges of data analysis in single-cell proteomics. ML techniques, such as neural networks and support vector machines, can help differentiate true signals from background noise, improving data quality and enabling the detection of low-abundance proteins. Furthermore, ML algorithms can be employed to identify patterns in complex proteomic datasets, revealing insights into cellular heterogeneity and functional pathways. ML-driven spectral deconvolution algorithms can enhance the identification of peptide spectra in complex mixtures, thus increasing sensitivity and reducing the false discovery rate in single-cell proteomics studies. Implementing these algorithms in data analysis workflows will enable researchers to handle the vast amounts of data generated from single-cell proteomic experiments more effectively.

To bridge the gap between research and clinical settings, single-cell proteomics workflows must become more accessible and user-friendly. Currently, proteomics techniques are often complex, requiring highly specialized skills and sophisticated instrumentation, which limits their adoption in clinical practice. Future advancements should focus on simplifying workflows, from sample preparation to data analysis, enabling broader use in non-research environments [103]. Emerging "plug-and-play" platforms with integrated sample preparation, quantification, and analysis capabilities offer a pathway toward more practical and efficient single-cell proteomics. By developing standardized protocols and user-friendly interfaces, these workflows can be integrated into routine clinical diagnostics, allowing clinicians to harness the power of single-cell proteomics for personalized medicine in cancers such as TNBC.

Another significant future direction is the development of real-time proteomics to inform clinical decisions on the spot. Real-time single-cell proteomics could offer oncologists valuable insights during surgery or treatment planning, enabling rapid decisions based on individual cellular proteomic profiles. Technological advancements in mass spectrometry, including high-speed and high-sensitivity instruments, are making real-time analyses increasingly feasible. Real-time proteomics could, for instance, identify protein markers indicative of aggressive subtypes of TNBC during biopsy analysis, helping tailor treatment plans based on live proteomic data [124]. Although real-time applications are currently in the experimental phase, advances in data acquisition speed, automated sample processing, and rapid data interpretation will make real-time single-cell proteomics a powerful tool in clinical oncology.

In next 5–10 years, the field of single-cell proteomics is expected to evolve into a more accessible and standardized research tool. Refining high-throughput, cost-effective instruments will make these technologies more available to smaller laboratories and clinical settings. Standardized sample preparation and analysis protocols will likely emerge, allowing more reproducible and comparable results across different studies. In addition, real-time single-cell proteomics could become a reality, enabling clinicians to analyze patient samples directly and make more informed decisions on personalized treatments. The convergence of multi-omics approaches will further broaden what can be achieved. Still, it is crucial to ensure these methods are rigorously validated in clinical trials to maintain credibility in therapeutic applications.

9 | Conclusion

Advancements in single-cell proteomics are poised to benefit the healthcare system significantly. Recent estimates indicate that 90% of drugs are only effective for half of the patients, resulting in annual losses of $350 billion in the United States alone [130]. Incorporating single-cell proteomics into medical research promises to enhance diagnostic and treatment capabilities, particularly in cancer. This approach ensures the right treatments are administered to the suitable patients at optimal times. As we stand at the precipice of a new era in understanding and managing TNBC, the promise of single-cell proteomics in LC-MS/MS is indubitable. The future of this dynamic duo holds the potential to shape TNBC research in unprecedented ways, offering insights into emerging technologies and areas of further investigation.

Moreover, improved instrumentation and methodologies are on the horizon, promising even greater sensitivity and throughput. Miniaturization of equipment and more cost-effective solutions will enhance accessibility, allowing a broader spectrum of researchers to harness the power of single-cell proteomics. Notably, integrating multi-omics data, including genomics, transcriptomics, and proteomics, will be a focal point in the future. By combining these diverse datasets, researchers can construct a comprehensive picture of TNBC's complexity, uncovering protein expression patterns and the genetic and functional elements that drive the disease. This holistic approach will provide deeper insights into TNBC's molecular landscape. A bird's eye view is necessary to understand the subcellular distribution of proteins in TNBC cells, which will shed light on their functional roles and their involvement in disease processes. This technology will be instrumental in dissecting the intricacies of TNBC biology. Even though the volume of data generated by single-cell proteomics continues to grow, the future of TNBC research will see an increasing reliance on artificial intelligence (AI) and ML for data analysis. These technologies will aid in the identification of complex patterns, the prediction of patient outcomes, and the discovery of novel therapeutic targets. The ultimate goal of single-cell proteomics in TNBC research is its clinical translation. In the near future, we can anticipate integrating single-cell proteomic data into routine clinical practice. This integration will involve the development of standardized assays, the creation of user-friendly analytical tools for healthcare professionals, and the establishment of robust protocols for patient-specific treatment strategies.

Conflicts of Interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Data Availability Statement

The authors have nothing to report.

References

1. T. Karn, L. Pusztai, U. Holtrich, et al., "Homogeneous Datasets of Triple Negative Breast Cancers Enable the Identification of Novel Prognostic and Predictive Signatures," *PLoS ONE* 6, no. 12 (2011): e28403.

2. B. D. Lehmann, J. A. Bauer, X. Chen, et al., "Identification of Human Triple-Negative Breast Cancer Subtypes and Preclinical Models for Selection of Targeted Therapies," *Journal of Clinical Investigation* 121, no. 7 (2011): 2750–2767.

3. C. M. Perou, T. Sørlie, M. B. Eisen, et al., "Molecular Portraits of Human Breast Tumours," *Nature* 406, no. 6797 (2000): 747–752.

4. L. V. Nalla, K. Kalia, and A. Khairnar, "Self-Renewal Signaling Pathways in Breast Cancer Stem Cells," *International Journal of Biochemistry & Cell Biology* 107 (2019): 140–153.

5. L. V. Nalla and A. Khairnar, "Empagliflozin Mediated miR-128-3p Upregulation Promotes Differentiation of Hypoxic Cancer Stem-Like Cells in Breast Cancer," *European Journal of Pharmacology* 943 (2023): 175565.

6. L. V. Nalla and A. Khairnar, "Empagliflozin Drives Ferroptosis in Anoikis-Resistant Cells by Activating miR-128-3p Dependent Pathway and Inhibiting CD98hc in Breast Cancer," *Free Radical Biology and Medicine* 220 (2024): 288–300.

7. A. G. Waks and E. P. J. J. Winer, "Breast Cancer Treatment: A Review," *Journal of the American Medical Association* 321, no. 3 (2019): 288–300.

8. M. Rihan, L. V. Nalla, A. Dharavath, A. Shard, K. Kalia, and A. Khairnar, "Pyruvate Kinase M2: A Metabolic Bug in Re-Wiring the Tumor Microenvironment," *Cancer Microenvironment* 12, no. 2 (2019): 149–167.

9. L. Da Silva and S. R. J. M. P. Lakhani, "Pathology of Hereditary Breast Cancer," *Modern Pathology* 23, no. 2 (2010): S46–S51.

10. G. N. Sharma, R. Dave, J. Sanadya, P. Sharma, and K. K. Sharma, "Various Types and Management of Breast Cancer: An Overview," *Journal of Advanced Pharmaceutical Technology & Research* 1, no. 2 (2010): 109–126.

11. P. Alluri and L. A. Newman, "Basal-Like and Triple-Negative Breast Cancers: Searching for Positives Among Many Negatives," *Surgical Oncology Clinics* 23, no. 3 (2014): 567–577.

12. W. D. Foulkes, I. E. Smith, and J. S. Reis-Filho, "Triple-Negative Breast Cancer," *New England Journal of Medicine* 363, no. 20 (2010): 1938–1948.

13. N. L. Henry, P. D. Shah, I. Haider, P. E. Freer, R. Jagsi, and M. S. Sabel, "Cancer of the Breast," in *Abeloff's Clinical Oncology* (Elsevier, Philadelphia 2020), 1560–1603. e1512.

14. C. Wang, S. Kar, X. Lai, et al., "Triple Negative Breast Cancer in Asia: An Insider's View," *Cancer Treatment Reviews* 62 (2018): 29–38.

15. D. O. Debets, K. E. Stecker, A. Piskopou, et al., "Deep (phospho) Proteomics Profiling of Pre-Treatment Needle Biopsies Identifies Signatures of Treatment Resistance in HER2+ Breast Cancer," *Cell Reports Medicine* (2023): 101203.

16. K. Giri, S. Maity, and K. Ambatipudi, "Targeted Proteomics Using Parallel Reaction Monitoring Confirms Salivary Proteins Indicative of Metastatic Triple-Negative Breast Cancer," *Journal of Proteomics* 267 (2022): 104701.

17. S. Li, X. Li, S. Yang, et al., "Proteomic Landscape of Exosomes Reveals the Functional Contributions of CD151 in Triple-Negative Breast Cancer," *Molecular & Cellular Proteomics* 20 (2021).

18. M. A. M. Lino, Y. Palacios-Rodríguez, S. Rodríguez-Cuevas, et al., "Comparative Proteomic Profiling of Triple-Negative Breast Cancer Reveals That Up-Regulation of RhoGDI-2 Is Associated to the Inhibition of Caspase 3 and Caspase 9," *Journal of Proteomics* 111 (2014): 198–211.

19. K. Asleh, N. Riaz, and T. O. Nielsen, "Heterogeneity of Triple Negative Breast Cancer: Current Advances in Subtyping and Treatment Implications," *Journal of Experimental & Clinical Cancer Research* 41, no. 1 (2022): 1–26.

20. P. R. Graves and T. A. Haystead, "Molecular Biologist's Guide to Proteomics," *Microbiology and Molecular Biology Reviews* 66, no. 1 (2002): 39–63.

21. I. Ezkurdia, D. Juan, J. M. Rodriguez, et al., "Multiple Evidence Strands Suggest That There May be as Few as 19 000 Human Protein-Coding Genes," *Human Molecular Genetics* 23, no. 22 (2014): 5866–5878.

22. E. Galvão, L. Martins, J. Ibiapina, H. Andrade, and S. Monte, "Breast Cancer Proteomics: A Review for Clinicians," *Journal of Cancer Research and Clinical Oncology* 137 (2011): 915–925.

23. J. J. Kennedy, S. E. Abbatiello, K. Kim, et al., "Demonstrating the Feasibility of Large-Scale Development of Standardized Assays to Quantify human Proteins," *Nature Methods* 11, no. 2 (2014): 149–155.

24. N. Q. Liu, C. Stingl, M. P. Look, et al., "Comparative Proteome Analysis Revealing an 11-Protein Signature for Aggressive Triple-Negative Breast Cancer," *Journal of the National Cancer Institute* 106, no. 2 (2014): djt376.

25. H.-J. Senn, B. Thürlimann, A. Goldhirsch, W. Wood, R. Gelber, and A. Coates, "Comments on the St. Gallen Consensus 2003 on the Primary Therapy of Early Breast Cancer," *Breast* 12, no. 6 (2003): 569–582.

26. National Institutes of Health Consensus Development Panel. "National Institutes of Health Consensus Development Conference

Statement: Adjuvant Therapy for Breast Cancer, November 1–3, 2000," *JNCI Monographs* 2001, no. 30 (2001): 5–15.

27. N. Q. Liu, T. De Marchi, A. M. Timmermans, et al., "Ferritin Heavy Chain in Triple Negative Breast Cancer: A Favorable Prognostic Marker That Relates to a Cluster of Differentiation 8 Positive (CD8+) Effector T-Cell Response," *Molecular & Cellular Proteomics* 13, no. 7 (2014): 1814–1827.

28. R. T. Lawrence, E. M. Perez, D. Hernández, et al., "The Proteomic Landscape of Triple-Negative Breast Cancer," *Cell Reports* 11, no. 4 (2015): 630–644.

29. M. Campone, I. Valo, P. Jézéquel, et al., "Prediction of Recurrence and Survival for Triple-Negative Breast Cancer (TNBC) by a Protein Signature in Tissue Samples," *Molecular & Cellular Proteomics* 14, no. 11 (2015): 2936–2946.

30. S. M. Hyder, Y. Liang, and J. Wu, "Estrogen Regulation of Thrombospondin-1 in Human Breast Cancer Cells," *International Journal of Cancer* 125, no. 5 (2009): 1045–1053.

31. M. Lacroix and G. Leclercq, "Relevance of Breast Cancer Cell Lines as Models for Breast Tumours: An Update," *Breast Cancer Research and Treatment* 83 (2004): 249–289.

32. G. Judes, K. Rifaï, M. Daures, et al., "High-Throughput «Omics» Technologies: New Tools for the Study of Triple-Negative Breast Cancer," *Cancer Letters* 382, no. 1 (2016): 77–85.

33. S. Suman, T. Basak, P. Gupta, et al., "Quantitative Proteomics Revealed Novel Proteins Associated With Molecular Subtypes of Breast Cancer," *Journal of Proteomics* 148 (2016): 183–193.

34. E. Maurizio, J. R. Wiśniewski, Y. Ciani, et al., "Translating Proteomic Into Functional Data: An High Mobility Group A1 (HMGA1) Proteomic Signature Has Prognostic Value in Breast Cancer," *Molecular & Cellular Proteomics* 15, no. 1 (2016): 109–123.

35. T. Sun, N. Aceto, K. L. Meerbrey, et al., "Activation of Multiple Proto-Oncogenic Tyrosine Kinases in Breast Cancer via Loss of the PTPN12 Phosphatase," *Cell* 144, no. 5 (2011): 703–718.

36. T. Cabezon, I. Gromova, P. Gromov, et al., "Proteomic Profiling of Triple-Negative Breast Carcinomas in Combination With a Three-Tier Orthogonal Technology Approach Identifies Mage-A4 as Potential Therapeutic Target in Estrogen Receptor Negative Breast Cancer," *Molecular & Cellular Proteomics* 12, no. 2 (2013): 381–394.

37. S. Corrêa, C. Panis, R. Binato, A. C. Herrera, L. Pizzatti, and E. Abdelhay, "Identifying Potential Markers in Breast Cancer Subtypes Using Plasma Label-Free Proteomics," *Journal of Proteomics* 151 (2017): 33–42.

38. J. He, S. A. Whelan, M. Lu, et al., "Proteomic-Based Biosignatures in Breast Cancer Classification and Prediction of Therapeutic Response," *International Journal of Proteomics* 2011, no. 1 (2011): 896476.

39. S. M. Semaan, X. Wang, P. A. Stewart, A. G. Marshall, and Q.-X. A. Sang, "Differential Phosphopeptide Expression in a Benign Breast Tissue, and Triple-Negative Primary and Metastatic Breast Cancer Tissues From the Same African-American Woman by LC-LTQ/FT-ICR Mass Spectrometry," *Biochemical and Biophysical Research Communications* 412, no. 1 (2011): 127–131.

40. M. Kosok, A. Alli-Shaik, B. H. Bay, and J. Gunaratne, "Comprehensive Proteomic Characterization Reveals Subclass-Specific Molecular Aberrations Within Triple-Negative Breast Cancer," *Iscience* 23, no. 2 (2020): 100868.

41. B. Budnik, J. Straubhaar, J. Neveu, and D. Shvartsman, "In-Depth Analysis of Proteomic and Genomic Fluctuations During the Time Course of human Embryonic Stem Cells Directed Differentiation Into Beta Cells," *Proteomics* 22, no. 9 (2022): 2100265.

42. H. Chung, C. N. Parkhurst, E. M. Magee, et al., "Joint Single-Cell Measurements of Nuclear Proteins and RNA in Vivo," *Nature Methods* 18, no. 10 (2021): 1204–1212.

43. Y. Liu, A. Beyer, and R. Aebersold, "On the Dependency of Cellular Protein Levels on mRNA Abundance," *Cell* 165, no. 3 (2016): 535–550.

44. G. S. Kinker, A. C. Greenwald, R. Tal, et al., "Pan-Cancer Single-Cell RNA-seq Identifies Recurring Programs of Cellular Heterogeneity," *Nature Genetics* 52, no. 11 (2020): 1208–1218.

45. I. Tirosh, B. Izar, S. M. Prakadan, et al., "Dissecting the Multicellular Ecosystem of Metastatic Melanoma by Single-Cell RNA-seq," *Science* 352, no. 6282 (2016): 189–196.

46. C. Ctortecka, D. Hartlmayr, A. Seth, S. Mendjan, G. Tourniaire, and K. Mechtler, "An Automated Workflow for Multiplexed Single-Cell Proteomics Sample Preparation at Unprecedented Sensitivity," *BioRxiv* (2021): 439828.

47. J. Amontree, K. Chen, J. Varillas, and Z. H. Fan, "A Capillary-Force-Driven, Single-Cell Transfer Method for Studying Rare Cells," *Bioengineering* 11, no. 6 (2024): 542.

48. P.-K. Raj-Kumar, X. Lin, T. Liu, et al., "Proteogenomic Characterization of Difficult-to-Treat Breast Cancer With Tumor Cells Enriched Through Laser Microdissection," *Breast Cancer Research* 26, no. 1 (2024): 76.

49. M. Ishida, Y. Kuroki, and K. Agata, "Establishment of a New Method to Isolate Viable X-Ray-Sensitive Cells From Planarian by Fluorescence-Activated Cell Sorting," *Development, Growth & Differentiation* 65, no. 9 (2023): 577–590.

50. M. Kamal, Y. J. Wang, S. Plummer, A. Dickerson, and M. Yu, "An Image-Based Identification of Aggressive Breast Cancer Circulating Tumor Cell Subtypes," *Cancers* 15, no. 10 (2023): 2669.

51. S. T. Gebreyesus, A. A. Siyal, R. B. Kitata, et al., "Streamlined Single-Cell Proteomics by an Integrated Microfluidic Chip and Data-Independent Acquisition Mass Spectrometry," *Nature Communications* 13, no. 1 (2022): 37.

52. H. Y. Murad, R. A. Sabol, J. Nyiramana, et al., "Mechanical Disruption by Focused Ultrasound Re-Sensitizes ER+ Breast Cancer Cells to Hormone Therapy," *Ultrasound in Medicine & Biology* 50, no. 12 (2024): 1885–1892.

53. X. Guo, Y. Chen, W. Ji, X. Chen, C. Li, and R. Ge, "Enrichment of Cancer Stem Cells by Agarose Multi-Well Dishes and 3D Spheroid Culture," *Cell and Tissue Research* 375 (2019): 397–408.

54. X. Shao, X. Wang, S. Guan, et al., "Integrated Proteome Analysis Device for Fast Single-Cell Protein Profiling," *Analytical Chemistry* 90, no. 23 (2018): 14003–14010.

55. Y. Wang, Z.-Y. Guan, S.-W. Shi, et al., "Pick-Up Single-Cell Proteomic Analysis for Quantifying up to 3000 Proteins in a Tumor Cell," *BioRxiv* (2022): 2022.2006. 2028.498038.

56. S. Picelli, O. R. Faridani, Å. K. Björklund, G. Winberg, S. Sagasser, and R. Sandberg, "Full-Length RNA-seq From Single Cells Using Smart-seq2," *Nature Protocols* 9, no. 1 (2014): 171–181.

57. Y. Zhu, J. Podolak, R. Zhao, et al., "Proteome Profiling of 1 to 5 Spiked Circulating Tumor Cells Isolated From Whole Blood Using Immunodensity Enrichment, Laser Capture Microdissection, Nanodroplet Sample Processing, and Ultrasensitive nanoLC–MS," *Analytical Chemistry* 90, no. 20 (2018): 11756–11759.

58. V. Gautam and A. K. Sarkar, "Laser Assisted Microdissection, an Efficient Technique to Understand Tissue Specific Gene Expression Patterns and Functional Genomics in Plants," *Molecular Biotechnology* 57 (2015): 299–308.

59. S. Nichterwitz, G. Chen, J. Aguila Benitez, et al., "Laser Capture Microscopy Coupled With Smart-seq2 for Precise Spatial Transcriptomic Profiling," *Nature Communications* 7, no. 1 (2016): 12139.

60. A. D. Brunner, M. Thielert, C. Vasilopoulou, et al., "Ultra-High Sensitivity Mass Spectrometry Quantifies Single-Cell Proteome Changes Upon Perturbation," *Molecular Systems Biology* 18, no. 3 (2022): e10798.

61. E. M. Schoof, B. Furtwängler, N. Üresin, et al., "Quantitative Single-Cell Proteomics as a Tool to Characterize Cellular Hierarchies," *Nature Communications* 12, no. 1 (2021): 3341.

62. H. Specht, G. Harmange, D. H. Perlman, et al., "Automated Sample Preparation for High-Throughput Single-Cell Proteomics," *BioRxiv* (2018): 399774.

63. Y. Zhu, G. Clair, W. B. Chrisler, et al., "Proteomic Analysis of Single Mammalian Cells Enabled by Microfluidic Nanodroplet Sample Preparation and Ultrasensitive NanoLC-MS," *Angewandte Chemie* 130, no. 38 (2018): 12550–12554.

64. L. Ombrato, E. Nolan, I. Kurelac, et al., "Metastatic-Niche Labelling Reveals Parenchymal Cells With Stem Features," *Nature* 572, no. 7771 (2019): 603–608.

65. C. Ziegenhain, B. Vieth, S. Parekh, et al., "Comparative Analysis of Single-Cell RNA Sequencing Methods," *Molecular Cell* 65, no. 4 (2017): 631–643.e4.

66. A. Leduc, R. G. Huffman, J. Cantlon, S. Khan, and N. Slavov, "Exploring Functional Protein Covariation Across Single Cells Using nPOP," *Genome Biology* 23, no. 1 (2022): 261.

67. J. Woo, S. M. Williams, L. M. Markillie, et al., "High-Throughput and High-Efficiency Sample Preparation for Single-Cell Proteomics Using a Nested Nanowell Chip," *Nature Communications* 12, no. 1 (2021): 6246.

68. Y. Zhu, P. D. Piehowski, R. Zhao, et al., "Nanodroplet Processing Platform for Deep and Quantitative Proteome Profiling of 10–100 Mammalian Cells," *Nature Communications* 9, no. 1 (2018): 882.

69. N. Slavov, "Single-Cell Protein Analysis by Mass Spectrometry," *Current Opinion in Chemical Biology* 60 (2021): 1–9.

70. E. I. Chen, D. Cociorva, J. L. Norris, and J. R. Yates, "Optimization of Mass Spectrometry-Compatible Surfactants for Shotgun Proteomics," *Journal of Proteome Research* 6, no. 7 (2007): 2529–2538.

71. S. Nie, R. O. B. Johnson, Y. Livson, T. Greer, X. Zheng, and N. Li, "Maximizing Hydrophobic Peptide Recovery in Proteomics and Antibody Development Using a Mass Spectrometry Compatible Surfactant," *Analytical Biochemistry* 658 (2022): 114924.

72. E. Patel, M. R. Clench, A. West, P. S. Marshall, N. Marshall, and S. Francese, "Alternative Surfactants for Improved Efficiency of In Situ Tryptic Proteolysis of Fingermarks," *Journal of the American Society for Mass Spectrometry* 26, no. 6 (2015): 862–872.

73. L. Senavirathna, C. Ma, R. Chen, and S. Pan, "Spectral Library-Based Single-Cell Proteomics Resolves Cellular Heterogeneity," *Cells* 11, no. 15 (2022): 2450.

74. B. R. Rushing, M. Schroder, and S. C. Sumner, "Comparison of Lysis and Detachment Sample Preparation Methods for Cultured Triple-Negative Breast Cancer Cells Using UHPLC–HRMS-based Metabolomics," *Metabolites* 12, no. 2 (2022): 168.

75. M. Danaeifar, "New Horizons in Developing Cell Lysis Methods: A Review," *Biotechnology and Bioengineering* 119, no. 11 (2022): 3007–3021.

76. M. Shehadul Islam, A. Aryasomayajula, and P. R. Selvaganapathy, "A Review on Macroscale and Microscale Cell Lysis Methods," *Micromachines* 8, no. 3 (2017): 83.

77. Y. Liang, H. Acor, M. A. McCown, et al., "Fully Automated Sample Processing and Analysis Workflow for Low-Input Proteome Profiling," *Analytical Chemistry* 93, no. 3 (2020): 1658–1666.

78. Z.-Y. Li, M. Huang, X.-K. Wang, et al., "Nanoliter-Scale Oil-Air-Droplet Chip-Based Single Cell Proteomic Analysis," *Analytical Chemistry* 90, no. 8 (2018): 5430–5438.

79. S. N. R. Gajula, D. N. J. Bale, and S. K. Nanjappan, "Analytical and Omics Approaches in the Identification of Oxidative Stress-Induced Cancer Biomarkers," in *Handbook of Oxidative Stress in Cancer: Mechanistic Aspects* (Springer, Singapore, 2020), 1–24.

80. M. Mann and N. L. Kelleher, "Precision Proteomics: The Case for High Resolution and High Mass Accuracy," *Proceedings of the National Academy of Sciences* 105, no. 47 (2008): 18132–18138.

81. S. N. R. Gajula, N. Nadimpalli, and R. Sonti, "Drug Metabolic Stability in Early Drug Discovery to Develop Potential Lead Compounds," *Drug Metabolism Reviews* 53, no. 3 (2021): 459–477.

82. N. Rauniyar, "Parallel Reaction Monitoring: A Targeted Experiment Performed Using High Resolution and High Mass Accuracy Mass Spectrometry," *International Journal of Molecular Sciences* 16, no. 12 (2015): 28566–28581.

83. B. Schilling, B. MacLean, J. M. Held, et al., "Multiplexed, Scheduled, High-Resolution Parallel Reaction Monitoring on a Full Scan QqTOF Instrument With Integrated Data-Dependent and Targeted Mass Spectrometric Workflows," *Analytical Chemistry* 87, no. 20 (2015): 10222–10229.

84. S. N. R. Gajula, A. S. Khairnar, P. Jock, et al., "LC-MS/MS: A Sensitive and Selective Analytical Technique to Detect COVID-19 Protein Biomarkers in the Early Disease Stage," *Expert Review of Proteomics* 20, no. 1-3 (2023): 5–18.

85. S. Lee, H. M. Vu, J.-H. Lee, H. Lim, and M.-S. Kim, "Advances in Mass Spectrometry-Based Single Cell Analysis," *Biology* 12, no. 3 (2023): 395.

86. C. Steiner, P. Lescuyer, P. Cutler, J.-C. Tille, and A. Ducret, "Relative Quantification of Proteins in Formalin-Fixed Paraffin-Embedded Breast Cancer Tissue Using Multiplexed Mass Spectrometry Assays," *Molecular & Cellular Proteomics* 21, no. 11 (2022).

87. J. Beretov, V. C. Wasinger, E. K. Millar, P. Schwartz, P. H. Graham, and Y. Li, "Proteomic Analysis of Urine to Identify Breast Cancer Biomarker Candidates Using a Label-Free LC-MS/MS Approach," *PLoS ONE* 10, no. 11 (2015): e0141876.

88. F. Mbeunkui, B. J. Metge, L. A. Shevde, and L. K. Pannell, "Identification of Differentially Secreted Biomarkers Using LC-MS/MS in Isogenic Cell Lines Representing a Progression of Breast Cancer," *Journal of Proteome Research* 6, no. 8 (2007): 2993–3002.

89. S. A. Whelan, M. Lu, J. He, et al., "Mass Spectrometry (LC-MS/MS) Site-Mapping of N-Glycosylated Membrane Proteins for Breast Cancer Biomarkers," *Journal of Proteome Research* 8, no. 8 (2009): 4151–4160.

90. A. W. Opstal-van Winden, E. J. Krop, M. H. Kåredal, et al., "Searching for Early Breast Cancer Biomarkers by Serum Protein Profiling of Pre-Diagnostic Serum; A Nested Case-Control Study," *BMC Cancer* 11 (2011): 1–11.

91. M. Matzinger, R. L. Mayer, and K. Mechtler, "Label-Free Single Cell Proteomics Utilizing Ultrafast LC and MS Instrumentation: A Valuable Complementary Technique to Multiplexing," *Proteomics* 23, no. 13-14 (2023): 2200162.

92. U. Distler, J. Kuharev, P. Navarro, and S. Tenzer, "Label-Free Quantification in Ion Mobility-Enhanced Data-Independent Acquisition Proteomics," *Nature Protocols* 11, no. 4 (2016): 795–812.

93. H. M. Bennett, W. Stephenson, C. M. Rose, and S. Darmanis, "Single-Cell Proteomics Enabled by Next-Generation Sequencing or Mass Spectrometry," *Nature Methods* 20, no. 3 (2023): 363–374.

94. G. Z. Li, J. P. Vissers, J. C. Silva, D. Golick, M. V. Gorenstein, and S. J. Geromanos, "Database Searching and Accounting of Multiplexed Precursor and Product Ion Spectra From the Data Independent Analysis of Simple and Complex Peptide Mixtures," *Proteomics* 9, no. 6 (2009): 1696–1719.

95. D.-G. Mun, F. A. Bhat, H. Ding, et al., "Optimizing Single Cell Proteomics Using Trapped Ion Mobility Spectrometry for Label-Free Experiments," *Analyst* 148, no. 15 (2023): 3466–3475.

96. Y. Cong, K. Motamedchaboki, S. A. Misal, et al., "Ultrasensitive Single-Cell Proteomics Workflow Identifies >1000 Protein Groups per Mammalian Cell," *Chemical Science* 12, no. 3 (2021): 1001–1006.

97. J. Woo, G. C. Clair, S. M. Williams, et al., "Three-Dimensional Feature Matching Improves Coverage for Single-Cell Proteomics Based on Ion Mobility Filtering," *Cell Systems* 13, no. 5 (2022): 426–434.e4.

98. F. Meier, A.-D. Brunner, M. Frank, et al., "diaPASEF: Parallel Accumulation–Serial Fragmentation Combined With Data-Independent Acquisition," *Nature Methods* 17, no. 12 (2020): 1229–1236.

99. W. E. Jang, J. H. Park, G. Park, et al., "Cntnap2-Dependent Molecular Networks in Autism Spectrum Disorder Revealed through an Integrative Multi-Omics Analysis," *Molecular Psychiatry* 28, no. 2 (2023): 810–821.

100. H. Specht, E. Emmott, A. A. Petelski, et al., "Single-Cell Proteomic and Transcriptomic Analysis of Macrophage Heterogeneity Using SCoPE2," *Genome Biology* 22 (2021b): 1–27.

101. J. Li, J. G. Van Vranken, P. Vaites, et al., "TMTpro Reagents: A Set of Isobaric Labeling Mass Tags Enables Simultaneous Proteome-Wide Measurements Across 16 Samples," *Nature Methods* 17, no. 4 (2020): 399–404.

102. C. R. Braun, G. H. Bird, M. Wühr, et al., "Generation of Multiple Reporter Ions from a Single Isobaric Reagent Increases Multiplexing Capacity for Quantitative Proteomics," *Analytical Chemistry* 87, no. 19 (2015): 9855–9863.

103. B. Budnik, E. Levy, G. Harmange, and N. Slavov, "SCoPE-MS: Mass Spectrometry of Single Mammalian Cells Quantifies Proteome Heterogeneity during Cell Differentiation," *Genome Biology* 19 (2018): 1–12.

104. P. Dwivedi and C. M. Rose, "Understanding the Effect of Carrier Proteomes in Single Cell Proteomic Studies-Key Lessons," *Expert Review of Proteomics* 19, no. 1 (2022): 5–15.

105. R. A. Bradshaw, A. L. Burlingame, S. Carr, and R. Aebersold, "Protein Identification: The Good, the Bad, and the Ugly," *Molecular & Cellular Proteomics* 4, no. 9 (2005): 1221–1222.

106. T. K. Cheung, C.-Y. Lee, F. P. Bayer, A. McCoy, B. Kuster, and C. M. Rose, "Defining the Carrier Proteome Limit for Single-Cell Proteomics," *Nature Methods* 18, no. 1 (2021): 76–83.

107. H. Wang, W.-J. Qian, H. M. Mottaz, et al., "Development and Evaluation of a Micro-and Nanoscale Proteomic Sample Preparation Method," *Journal of Proteome Research* 4, no. 6 (2005): 2397–2403.

108. L. Ting, R. Rad, S. P. Gygi, and W. Haas, "MS3 Eliminates Ratio Distortion in Isobaric Multiplexed Quantitative Proteomics," *Nature Methods* 8, no. 11 (2011): 937–940.

109. G. C. McAlister, D. P. Nusinow, M. P. Jedrychowski, et al., "MultiNotch MS3 Enables Accurate, Sensitive, and Multiplexed Detection of Differential Expression Across Cancer Cell Line Proteomes," *Analytical Chemistry* 86, no. 14 (2014): 7150–7158.

110. J. R. Griffiths, N. Chicooree, Y. Connolly, et al., "Mass Spectral Enhanced Detection of Ubls Using SWATH Acquisition: MEDUSA—Simultaneous Quantification of SUMO and Ubiquitin-Derived Isopeptides," *Journal of the American Society for Mass Spectrometry* 25, no. 5 (2014): 767–777.

111. L. K. Pino, J. Baeza, R. Lauman, B. Schilling, and B. A. Garcia, "Improved SILAC Quantification With Data-Independent Acquisition to Investigate Bortezomib-Induced Protein Degradation," *Journal of Proteome Research* 20, no. 4 (2021): 1918–1927.

112. J. Derks, A. Leduc, G. Wallmann, et al., "Increasing the Throughput of Sensitive Proteomics by plexDIA," *Nature Biotechnology* 41, no. 1 (2023): 50–59.

113. C. Ctortecka, K. Stejskal, G. Krssakova, S. Mendjan, and K. Mechtler, "Quantitative Accuracy and Precision in Multiplexed Single-Cell Proteomics," *Analytical Chemistry* 94, no. 5 (2021): 2434–2443.

114. H. R. Ali, H. W. Jackson, V. R. Zanotelli, et al., "Imaging Mass Cytometry and Multiplatform Genomics Define the Phenogenomic Landscape of Breast Cancer," *Nature Cancer* 1, no. 2 (2020): 163–175.

115. C. Giesen, H. A. Wang, D. Schapiro, et al., "Highly Multiplexed Imaging of Tumor Tissues with Subcellular Resolution by Mass Cytometry," *Nature Methods* 11, no. 4 (2014): 417–422.

116. S. K. Saka, Y. Wang, J. Y. Kishi, et al., "Immuno-SABER Enables Highly Multiplexed and Amplified Protein Imaging in Tissues," *Nature Biotechnology* 37, no. 9 (2019): 1080–1090.

117. M. J. Gerdes, C. J. Sevinsky, A. Sood, et al., "Highly Multiplexed Single-Cell Analysis of Formalin-Fixed, Paraffin-Embedded Cancer Tissue," *Proceedings of the National Academy of Sciences* 110, no. 29 (2013): 11982–11987.

118. M. Angelo, S. C. Bendall, R. Finck, et al., "Multiplexed Ion Beam Imaging of Human Breast Tumors," *Nature Medicine* 20, no. 4 (2014): 436–442.

119. E. Z. Macosko, A. Basu, R. Satija, et al., "Highly Parallel Genome-Wide Expression Profiling of Individual Cells Using Nanoliter Droplets," *Cell* 161, no. 5 (2015): 1202–1214.

120. T. Stuart and R. Satija, "Integrative Single-Cell Analysis," *Nature Reviews Genetics* 20, no. 5 (2019): 257–272.

121. G. Bianchini, J. M. Balko, I. A. Mayer, M. E. Sanders, and L. Gianni, "Triple-Negative Breast Cancer: Challenges and Opportunities of a Heterogeneous Disease," *Nature Reviews Clinical Oncology* 13, no. 11 (2016): 674–690.

122. C. Belli, D. Trapani, G. Viale, et al., "Targeting the Microenvironment in Solid Tumors," *Cancer Treatment Reviews* 65 (2018): 22–32.

123. R. Ahmad and B. Budnik, "A Review of the Current State of Single-Cell Proteomics and Future Perspective," *Analytical and Bioanalytical Chemistry* 415, no. 28 (2023): 6889–6899.

124. F. Meier, A.-D. Brunner, S. Koch, et al., "Online Parallel Accumulation–Serial Fragmentation (PASEF) With a Novel Trapped Ion Mobility Mass Spectrometer," *Molecular & Cellular Proteomics* 17, no. 12 (2018): 2534–2545.

125. K. Michelmann, J. A. Silveira, M. E. Ridgeway, and M. A. Park, "Fundamentals of Trapped Ion Mobility Spectrometry," *Journal of The American Society for Mass Spectrometry* 26, no. 1 (2014): 14–24.

126. C. Redit, S. Cha, and N. Ai, "Single-Cell Proteomics: Challenges and Prospects," *Nature Methods* 20, no. 3 (2023): 317–318.

127. A. C. Nairn and K. R. Williams, *Neuroproteomics* (MDPI, 2020).

128. H. Sadeghi Rad, J. Monkman, M. E. Warkiani, et al., "Understanding the Tumor Microenvironment for Effective Immunotherapy," *Medicinal Research Reviews* 41, no. 3 (2021): 1474–1498.

129. X. Yuan, J. Wang, Y. Huang, D. Shangguan, and P. Zhang, "Single-Cell Profiling to Explore Immunological Heterogeneity of Tumor Microenvironment in Breast Cancer," *Frontiers in Immunology* 12 (2021): 643692.

130. V. Petrosius and E. M. Schoof, "Recent Advances in the Field of Single-Cell Proteomics," *Translational Oncology* 27 (2023): 101556.