

Review

Proteomics approaches to characterize the immune responses in cancer



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ABSTRACT

Despite the dynamic development of cancer research, annually millions of people die of cancer. The human immune system is the major 'guard' against tumor development. Unfortunately, cancer cells have the ability to evade the immune system and continue to grow. The proper understanding of the intricate immune response in tumorigenesis remains the holy grail of cancer immunology and designing effective immunotherapy. To decode the immune responses in cancer, in recent years, proteomics studies have received considerable attention. Proteomics studies focus on the detection and quantification of proteins, which are the effectors of biological functions, and as such, are proven to reflect the cell state more accurately, in comparison to genomic or transcriptomic studies. In this review, we discuss the proteomics studies applied to characterize the immune responses in cancer and tumor immune microenvironment heterogeneity. Further, we describe emerging single-cell proteomics approaches that have the potential to be applied in cancer immunity studies.

1. Introduction

In 2020, according to the International Agency for Research on Cancer, over 19 million new cases and 10 million deaths caused by cancer were estimated to occur worldwide. Breast, lung, and colorectal cancer (CRC) were assigned as the most commonly occurring types of

cancer [1]. There are many known risk factors of cancer, both independent from lifestyle e.g., genetic predisposition or random DNA mutation, and lifestyle dependent such as tobacco smoking habits, lack of exercise and obesity, exposure to radiation, or poor diet [2]. Despite some differences in the mortality rate due to cancer between developed and developing countries, undeniably this issue concerns the global

Abbreviations: 2-DE, two-dimensional electrophoresis; ABCD, antibody barcoding with cleavable DNA; ALN, axillary lymph node; AML, acute myeloid leukemia; APC, antigen presenting cell; ARG2, arginase-2; BAFF, B-cell activating factor; CAC, colitis-associated cancer; CCL, C-C motif chemokine; CD, cluster of differentiation; CITE-seq, cellular indexing of transcriptomes and epitopes by sequencing; CODEX, CO-Detection by indEXing; CRC, colorectal cancer; CTLA-4, cytotoxic T cell antigen-4; CyTOF, cytometry by time-of-flight; EV, extracellular vesicle; ESI, electrospray ionization; FASLG, FAS ligand; FC, flow cytometry; FFPE, formalin-fixed paraffin-embedded; FoxP3, forkhead box P3; FSFC, full spectrum flow cytometry; GAM, glioma-associated macrophages; GBM, glioblastoma; IC, immune checkpoints; IFN- γ , interferon- γ ; IgE, immunoglobulin E; IHC, immunohistochemistry; IL, interleukin; ILC, innate lymphoid cell; IMC, imaging mass cytometry; LAG-3, lymphocyte activation gene-3; LAP, latent-associated peptide; LCM, laser-capture microdissection; LC-MS/MS, tandem mass spectrometry coupled with liquid chromatography; mAb, monoclonal antibody; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MC, mass cytometry; MELC, Multi-Epitope Ligand Cartography; MHC, major histocompatibility complex; MIBI, multiplexed ion beam imaging; mIF, multiplexed Immunofluorescence; MIST, multiplexed in situ targeting; mPOP, minimal ProteOmic sample Preparation; MS, mass spectrometry; MSI, microsatellite instability; nanoPOTS, nanodroplet processing in one pot for trace samples; NAPP, Nucleic Acid Programmable Protein Array; NF- κ B, nuclear factor-kappa B; NGS, next-generation sequencing; NK, natural killer; PBMC, peripheral blood mononuclear cell; PD-1, programmed cell death protein 1; PD-L1, programmed cell death ligand 1; PEA, Proximity Extension Assay; PLAYR, Proximity Ligation Assay for RNA; PTM, posttranslational modification; REAP-seq, RNA expression and protein sequencing; RPPA, Reverse Phase Protein Arrays; SAA, serum amyloid A; scATAC-seq, single-cell sequencing assay for transposase-accessible chromatin; SCBC, single-cell barcode chip; SCOPE-MS, Single Cell Proteomics by mass spectrometry; SIRT, sirtuin; SISPROT, simple and integrated spin tip-based proteomics technology; SN, Sentinel Node; SUGAR-seq, SUrface-protein Glycan And RNA-seq; TAM, tumor-associated macrophage; TGF, tumor growth factor; Th, Helper T cells; TIL, tumor-infiltrating lymphocyte; Tim-3, T cell immunoglobulin and mucin-domain containing-3; TME, tumor microenvironment; Treg, T regulatory lymphocyte.

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population [1]. For some types of cancers, inflammation is associated with tumor development, either as a cause or a consequence of ongoing tumor growth. Regardless of the origin, the inflammation and immune cells in the tumor microenvironment (TME) play an important role in cancer development [3,4]. Helper T (Th) cells, essential moderators of the immune response, exhibit a dual role in cancer progression and immunity. The cluster of differentiation (CD)4+ T cells orchestrate immune responses against tumors and can differentiate into different subsets within TME [5]. Th1 lymphocytes, as the main producers of interferon- γ (IFN- γ), play the major role in anti-tumor response by activating innate immune cells such as macrophages and natural killer (NK) cells, promoting proinflammatory phenotype of macrophages, and inducing expression of major histocompatibility complex (MHC) class II on the surface of antigen-presenting cells (APCs). In addition, Th1, via the production of IFN- γ , induce the differentiation of cytotoxic CD8+ T cells and inhibit T regulatory lymphocytes (Tregs) function [6]. Th2 lymphocytes are the key players in host immunity and tissue repair signaling. Signatory cytokines produced by Th2 cells, interleukin (IL)-4, IL-5, IL-9, and IL-13, participate in B cell proliferation and immunoglobulin E (IgE) production. They are also associated with the pathological states of chronic inflammation e.g., asthma [7]. Their role in cancer clearance has been linked with the recruitment of eosinophils, neutrophils, and macrophages at tumor sites via IL-4 signaling [8].

Another subset of CD4+ T cells, Th17, are the main producers of IL-17 and play a key role in the host defense against pathogens, especially in the gut [9]. Th17 cells have been linked with the induction of a protumor environment [10], however, preclinical and clinical studies demonstrate that Th17 cells contribute to the recruitment of effector cells such as neutrophils to TME [11]. Therefore, the role of Th17 in cancer progression remains controversial and requires further studies [12]. On the other hand, Treg cells are a subpopulation of T cells that are engaged in sustaining immunological self-tolerance and homeostasis. They can suppress and downregulate the immune response, as such, they participate in promoting the tumor favorable conditions [9,13]. Moreover, Treg cells' phenotypic plasticity facilitates the conversion to different subsets with superior immunosuppressive activity such as IL-17 producing Treg and latent-associated peptide (LAP)+ Treg cells [14]. More recently, other novel T cell subsets such as Th9, Th22, and follicular Th cells have been suggested to affect the TME with controversial effects, regarding their anti-tumor or protumor activity [15,16]. Despite the great advance in cancer immunology in the last few years, a better understanding of the TME heterogeneity and the complexity of immune cell interactions is needed.

Cancer immunotherapy with monoclonal antibodies (mAbs) that block the interaction of programmed cell death protein 1 (PD-1) with its ligand PD-L1 has shown clinical response in a wide range of solid and hematological cancers [17]. However, only a minority of patients exhibit dramatic positive responses. The low response rate can be linked to other immunosuppressive mechanisms and an array of factors affecting immunotherapy effectiveness such as tumor genomic instability, immune phenotype, level of inflammation, microbiome, T cell memory, or even sunlight exposure [18]. Therefore, a comprehensive understanding of the role of T cells in TME is needed to discover novel targets and biomarkers for the effective treatment of cancer.

High-dimensional and high-throughput techniques are promising tools in unraveling this issue [19]. Omics-based strategies such as transcriptomics have been applied to uncover the immune surveillance mechanisms and immune profiling in various cancer types [20–23]. However, the knowledge about the mechanism of gene regulation at the posttranscriptional, translational, and posttranslational levels is still limited. Poor levels of concordance between changes in protein abundance and mRNA expression have been reported, especially in CD4+ T cells [24,25]. Therefore, with steady progress in proteomics technology, proteomics analyses can provide a more comprehensive view of T cells' fate in cancer progression through simultaneous detection, identification, and quantification of thousands of proteins in a single study. In

particular, tandem mass spectrometry (MS) coupled with liquid chromatography (LC-MS/MS) provides an integrated system for proteomics analysis with improved sensitivity and moderate throughput [26,27].

Nowadays, two basic proteomics strategies are commonly used in cancer research: MS-based and antibody-based. Bottom-up proteomics is currently the predominant MS-based strategy, which is applied to discovery research aiming at the deep identification of a given proteome in an exploratory and unbiased manner. In contrast, antibody-based strategies are widely used in targeted approaches, which can detect pre-selected proteins from a given sample, ideally, with high sensitivity, selectivity, quantitative accuracy, and reproducibility. However, antibody-based approaches are limited by the number of proteins that can be detected simultaneously and the availability of antibodies. MS-based strategies can potentially detect hundreds or thousands of proteins to establish novel biomarkers, potential drug targets, and other research efforts [28]. So far, neither of the two strategies has achieved the detection of the whole proteome. In this review, we focus on different proteomics approaches, including antibody-based and MS-based strategies, for immune characterization of cancer states with an emphasis on CD4+ T cells. Finally, we will present novel single-cell proteomics approaches with great potential in cancer immunology.

2. A brief overview of proteomics

Proteomics is a large-scale analysis of the sum of proteins from an organism, tissue, cell, or biofluid [29]. Clinical proteomics aims at understanding how their abundance, expression, localization, post-translational modifications (PTMs), and molecular interactions cause disease to improve patient care [30]. Various protein identification techniques have been applied to study proteins involved in cancer formation and progression such as flow cytometry (FC), mass cytometry (MC or CyTOF; cytometry by time-of-flight) [31,32], and immunohistochemistry (IHC) [33]. However, these strategies are limited by their multiplexing capacity and the availability and quality of specific antibodies [27].

Bottom-up proteomics is currently a predominant strategy that utilizes protein digestion before MS analysis. The general sample preparation workflow in bottom-up proteomics (Fig. 1) consists of protein extraction, solubilization with detergents, reduction of disulfide bonds, alkylation of free cysteines, and lastly enzymatic digestion (normally trypsin) conducted in-solution or filter-aided. Then, obtained peptides are desalted with reversed phase C18 tips [34,35]. This workflow can be combined with fractionation steps at protein and peptide levels with different biochemical approaches such as two-dimensional electrophoresis (2-DE), strong cation exchange, or enrichment of peptides with PTMs (e.g., phosphorylation, acetylation, glycosylation) [36]. The resulting mixtures of peptides are identified and quantified in the mass spectrometer by the analysis of mass-to-charge ratios of molecular ions.

LC-MS/MS has revolutionized proteomics because of the great advances in reproducibility, high resolution, high mass accuracy, improvement of scanning modes, and excellent sensitivity. The combination of nano-LC technology or capillary electrophoresis with electrospray ionization (ESI) enables the identification and quantification of thousands of proteins from one single injection in high-resolution mass spectrometers [27,37,38]. This progress in clinical proteomics accelerates the study of the underlying mechanisms of cancer as well as biomarkers discovery and, at the same time, improves diagnostic, prediction, prognostic, and monitoring efficacy of novel immunotherapies [26,39,40].

MS Imaging is a cutting-edge technology that incorporates matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) with micrometer laser beams that shed on frozen or Formalin Fixed Paraffin-Embedded (FFPE) tissue samples. Each laser-excited spot generates ionized proteins/peptides which are generally identified by MALDI-TOF. Thus, tissue images are generated via a raster scan in which each spot is associated with its mass spectrum, providing the spatial

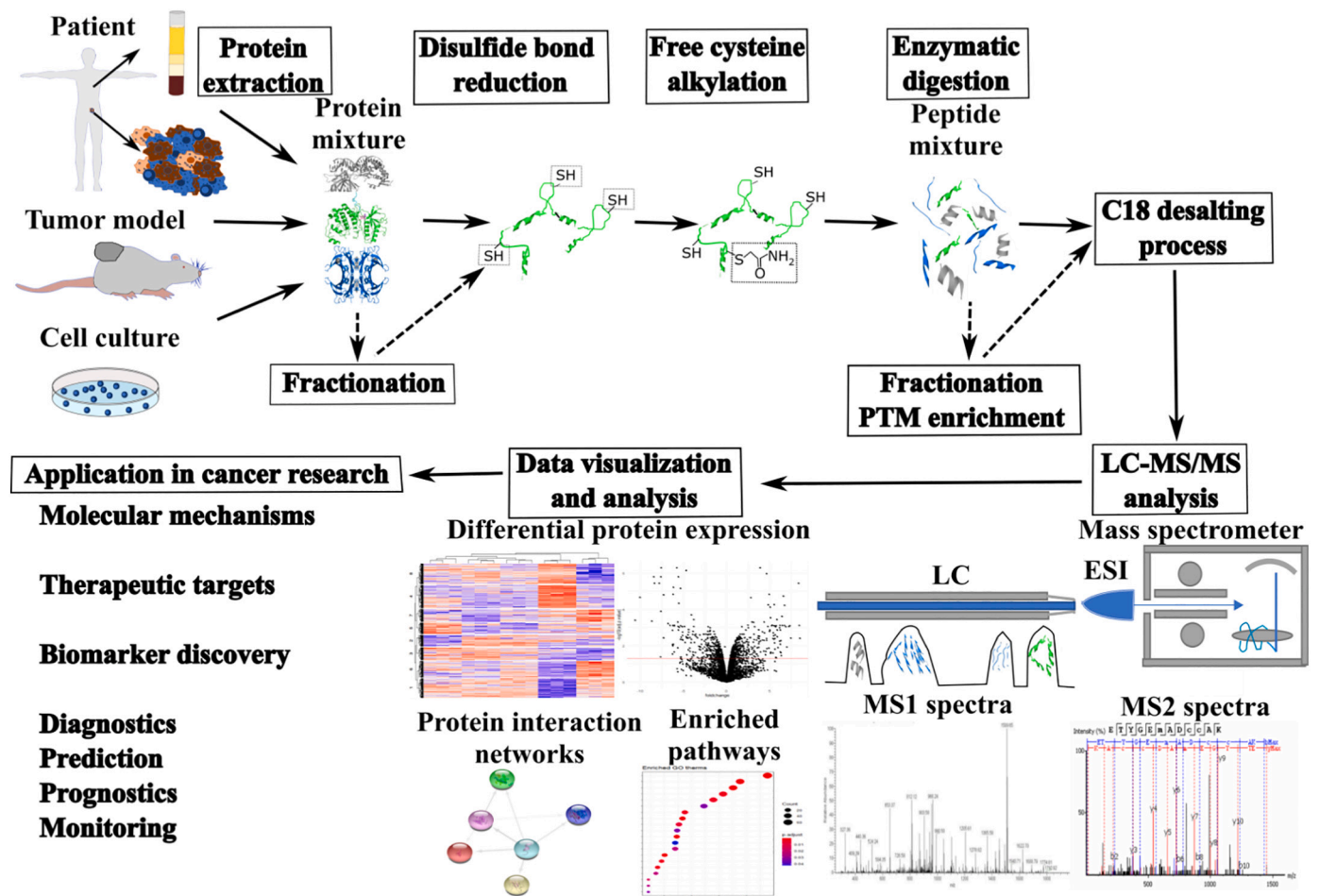


Fig. 1. Bottom-up proteomics workflow. Protein mixtures are extracted from patient samples, tumor model samples, or cell culture. Proteins are solubilized, disulfide bonds are reduced, free cysteines are alkylated, and proteins are digested with enzymes. Alternatively, proteins and peptides can be fractionated or enriched in posttranslational modifications (PTMs). Peptide mixture is desalted with reversed phase C18 tips and prepared for tandem mass spectrometry coupled with liquid chromatography (LC-MS/MS) analysis. LC separates peptides that are ionized by electrospray ionization (ESI) and analyzed in the mass spectrometer, generating MS1 and MS2 spectra. Data visualization and analysis allow the identification and quantification of differentially expressed proteins as well as the identification of enriched pathways and protein interaction networks. Proteomics analysis has several applications in cancer research such as the discovery of underlying molecular mechanisms, therapeutic targets, and biomarkers as well as improvement of diagnostics, prediction, prognostic, and therapy monitoring.

distribution and relative abundance of the analytes over the entire tissue section [41]. MS Imaging is mostly non-destructive and can be combined with histological staining to study regions of interest or digital PCR [42,43]. This technique can resolve the complexity of spatial protein patterns and other biomolecules (lipids, glycans, and metabolites) within the TME in an untargeted manner [44–47]. Interestingly, recent technical advances in laser resolution enable the measurement of analytes at the single-cell level [48]. However, its wider application is currently limited by the required heavy instrumentation, non-standardized workflows, and its suboptimal quantification capability [49].

Another approach is top-down proteomics that identifies intact proteins by the combination of different protein separation techniques with LC-MS/MS, where the proteins are ionized and subsequently fragmented. However, the sensitivity is about 100-fold lower than bottom-up proteomics with lesser proteomic coverage and throughput due to its lower efficiency to fragment intact proteins [50,51].

3. MS-based proteomics approaches applied to study immune responses in cancer

Upregulation of immune checkpoints (IC) such as cytotoxic T cell antigen-4 (CTLA-4) and PD-1 molecules within the TME is considered as

the major immunosuppressive mechanism that inhibits effector T cell functions [52]. Apart from that, the TME is enriched in soluble factors such as tumor growth factor- β (TGF- β), IL-10, and CD73-derived adenosine which potently suppress T cell anti-tumor functions and promote the conversion of naïve CD4+ T cells into Tregs [53,54]. Moreover, metabolic restriction of T cells by nutrient competition from tumor cells inhibits effector T cell anti-tumor functions [55]. MS-based discovery proteomics can contribute to elucidating the most relevant proteins, molecular mechanisms, and pathways involved in immunosuppression, which will lead to the identification of novel targets for potential immunotherapy. This section describes various MS-based proteomics approaches and their application in the analysis of the immune responses in cancer by characterization of T cells, the tumor-infiltrating lymphocytes (TILs) as well as biofluids in mice models and clinics.

3.1. The potential of MS-based proteomics approaches in preclinical cancer model studies for discovery research

Preclinical studies in mice models are an essential milestone towards novel therapeutic strategies in humans as well as to uncover molecular mechanisms involved in the disease progression. Despite the great potential of proteomics to discover novel therapeutic targets, proteomics analysis has not been broadly applied in mice models in the research

field of cancer immunology. Interestingly, a few bottom-up proteomics studies exemplify its ability to characterize T cells originating from spleen and lymph nodes in cancer mice models, providing novel insights in this field. For instance, proteomics analysis of T cells in a mice model of colitis-associated colorectal cancer (CAC) demonstrated that sirtuin 5 (SIRT5) downregulates numerous proteins related to the T cell receptor signaling pathway and enhances immunosuppressive Treg cell differentiation. However, further studies are needed to evaluate the broader role of SIRT5 in cancer immunotherapy. In addition, bottom-up proteomics analysis can be applied to reveal PTMs involved in tumor immunosuppression. MS-based proteomic analysis of SIRT2-immunoprecipitated proteins and acetyl-lysine peptides demonstrated that SIRT2 suppresses key metabolic enzymes by deacetylation in T cells, promoting a T cell exhausted phenotype. These findings were validated in melanoma and lung cancer mice models as well as in vitro in T cells originating from healthy donors and TILs isolated from non-small cell lung cancer patients, which revealed that pharmacologic inhibition of SIRT2 can enhance cancer immunotherapies [56]. Interestingly, the sirtuins family has been associated with cancer progression and metastasis through different mechanisms [57–59]. Application of bottom-up proteomics in an arginase 2 (*Arg2*^{-/-}) T-cell-specific knock-out in CRC and melanoma xenograft models discovered the immunosuppressive function of mitochondrial ARG2 in CD8⁺ T cells. *Arg2*-deficient CD8⁺ T cells were synergized with PD-1 blockade, unveiling the potential application of ARG2 inhibition as novel immunotherapy [60]. Bottom-up proteomics has also been applied to study the immune response to treatment in a breast cancer mice model. Shotgun MS analysis of mice serum revealed that cryo-thermal therapy induces acute phase response with IL-6 activation, promoting Th1 anti-tumor activity [61].

Application of shotgun proteomics in hyperactive platelets derived from CAC mice revealed an increased level of protumor serum amyloid A (SAA) proteins, suggesting a novel target to treat CAC patients at early clinical stages, or even to prevent cancer development [62]. Also, bottom-up proteomics analyzed extracellular vesicles (EVs) from tumor-associated macrophages (TAMs) derived from a CRC mouse model. Surprisingly, TAM-EVs possessed a proteomic signature that was associated with inflammation and immune response through Th1/M1 macrophage polarization [63]. Both studies show the broad application of MS-based proteomics in the analysis of innate immune cells which influence the cancer immune response.

The abovementioned studies show the potential application of MS-based proteomics in preclinical cancer mice models to understand molecular mechanisms involved in immunosuppression, in the studies on the effect of therapies at the protein level as well as in the discovery of novel therapeutic targets for immunotherapy. However, instead of inferring their activity from peripheral blood, further proteomics analysis of TILs will provide more valuable information of T cell functions within the TME.

3.2. MS-based proteomics application in clinical studies to characterize cancer immune responses

The advancement of shotgun MS-proteomics enables better characterization of TILs in clinical samples. First step towards this goal was the development of the simple and integrated spin tip-based proteomics technology (termed SISPROT) combined with laser-capture microdissection technology (LCM) [64]. LCM-SISPROT provided spatial proteome profiling of cancer cells, enterocytes, lymphocytes, and smooth muscle cells of both normal and CRC tissue obtained from the same patient. Each cell type possessed an individual proteomic signature such as immune processes enrichment in lymphocytes. Interestingly, the spatial proteomic composition from the same cell type showed expression fluctuations across micrometer spatial distance which highlights the heterogeneity of TME [64]. This proof-of-concept study demonstrates the technical advancement towards high-throughput proteomics characterization of TILs. The next step is the application of LCM

combined with shotgun proteomics in studies of clinical importance. For instance, this approach has been recently applied to compare the proteomes of microdissected TILs from 3 metastatic melanoma patient samples (IFN- γ -high, lymphocyte activation gene-3 (LAG-3)-high, and none), showing that only the IFN- γ -high sample was enriched in different inflammatory pathways [65].

It is well known that tumor-secreted factors and exosomes enrich immunosuppressive cells within the tumor-draining lymph nodes, leading to defective local T cell priming [66,67]. Further characterization of the tumor-draining lymph node cellular and protein composition is needed to release T cell inhibition and to develop potential immunotherapy. MS-based proteomics has been recently applied to characterize the pathophysiology of perfused breast cancer patient-derived axillary lymph nodes (ALNs) sustained ex vivo using normothermic perfusion [68]. Neutrophil degranulation and extracellular matrix degradation pathways were enriched in metastatic ALNs compared to reactive ALNs. Similar results of enriched pathways were observed in metastatic lymph nodes from pancreatic ductal adenocarcinoma and prostate cancer [69,70]. These studies demonstrate that MS-based proteomics is a powerful tool to characterize biofluids such as perfusates from tissue, facilitating the protein characterization of lymph nodes. MS-based shotgun proteomics analysis has also been applied to study the cellular composition of tumor-draining lymph nodes, such as Treg cells from Sentinel Nodes (SN) compared to non-SN Tregs in bladder cancer patients [71]. It was found that SN-resident Tregs were enriched in growth and immune signaling pathways with IL-16 playing a central role. Moreover, Treg cells in vitro exposition to tumor secretome increased the IL-16 processing into its bioactive form through caspase-3 activation, reinforcing Treg suppressive capacity [71].

Currently, MS imaging has been applied to study the protein heterogeneity as well as spatial pattern in multiple solid tumors, focusing on sub-histological classification as well as the discovery of new candidate biomarkers [72–75]. In breast cancer patients' samples, MS imaging revealed a correlation between high intra-tumor heterogeneity, high level of TILs, and better prognosis [76]. These findings suggest that unveiling the proteome heterogeneity is crucial for defining the extent of cellular heterogeneity within the TME. In recent years, MS imaging has been approved as a powerful tool to characterize immune cell population changes and to identify protein signatures in response to immunotherapy. Berghmans et al. [77] used MS imaging to measure anti-PD-L1 immunotherapy response in non-small cell lung cancer patients. Downstream analysis and IHC validation demonstrated that neutrophil defensins-1, -2, -3 are predictive biomarkers associated with a positive immunotherapy response. Indeed, in vitro experiments showed that these defensins activate immune cells against cancer cells. Importantly, MS imaging can be combined with LCM and subsequent bottom-up/top-down proteomics to facilitate the identification of putative proteins within the TME [78,79]. This combination revealed that the proteomes from TME cell subpopulations are associated with unique molecular signatures in breast cancer [78]. This proof-of-concept study demonstrates that the combination of proteomics approaches can reveal TME proteomics heterogeneity.

Top-down proteomics has not been widely applied to cancer immunological research but several studies exemplify the potential of this technique. Generally, top-down proteomics is combined with bottom-up proteomics or MS imaging. On one hand, top-down/bottom-up proteomics has been used to identify potential biomarkers in prostate cancer [80] and pediatric brain cancers [81–83] as well as to investigate the proteome landscape of breast cancer patient-derived mouse xenograft models [84]. Bottom-up proteomics has a higher coverage of the proteome, while top-down facilitates the identification of proteoforms with specific PTMs. These studies highlight the benefit of the integration of both approaches. On the other hand, combination of top-down proteomics and MS imaging can identify the spatial patterns of protein products from alternative Open Reading Frames within the TME. This integrative approach can detect potential biomarkers that were not

considered before. Interestingly, top-down proteomics also facilitates the identification of protein complexes [85], novel quaternary structures [86], and tumor mutant proteoforms [87].

In summary, MS-based proteomics has been widely applied in cancer immunology research. Studies have approved that novel insights into the current understanding of tumor-mediated immunosuppression have been gained by using these technologies. Systematic untargeted proteome characterization of different T cell subsets, other cell subtypes within the TME, and biofluids will facilitate the discovery of novel biomarkers and therapeutic targets to overcome tumor-mediated suppression of effector T cell activation.

Despite these great advances, several technical challenges must be addressed. MS-proteomics does not provide the full sequence of a protein but rather relies on the identification of unique peptides from a protein. Its sensitivity is limited by the number of acquired spectra to identify a specific peptide [88]. However, an average of 75% of collected spectra can remain unidentified [89]. This lack of sensitivity limits the dynamic range of mass spectrometers as well as the identification of low abundant proteins, especially in clinical samples such as serum, in which the dynamic range can overpass 10 orders of magnitude [90]. Once a peptide is correctly identified, another challenge is the identification of different isoforms of the protein, called proteoforms. These proteoforms are generated by posttranscriptional processing and PTMs, yielding multiple proteoforms from the same canonical amino acid sequence [91]. Despite the development of PTMs enrichment strategies, identification of modified peptides arises more complications due to their lower abundance, lower ionization and fragmentation efficiency, inaccurate mass determination, confusion with the assignment of residue substitutions, and uncertainty in the PTM site assignment [92,93]. Lastly, the high cost of MS instrumentation as well as the level of expertise required to perform MS-proteomics hinders its wider usage.

4. Antibody-based technologies to characterize immune responses in cancer

MS-based proteomics is widely used in discovery proteomics while antibody-based approaches are the most widely chosen for targeted proteomics, although the number of simultaneously detected proteins is limited. One of the main challenges in cancer immunology is to find novel biomarkers to guide the choice of therapeutic strategies to maximize patient benefit. Predictive biomarkers for immunotherapy require a more holistic approach with panels of biomarkers to identify the underlying biology and complexity of the tumor immune response [94]. Recently developed antibody-based detection techniques can detect from tens to hundreds of proteins simultaneously, being a powerful tool to identify these panels of biomarkers.

Multiplex immunoassays utilize antibodies as anchors that are immobilized on a solid surface or the surface of beads. In both, the protein of interest is bound to the specific antibody. This technology enables simultaneous detection and quantitation of tens of proteins. It is a powerful tool, especially for the detection of secreted proteins, such as cytokines and growth factors from a limited amount of biological and clinical materials. For example, this technique was applied to study the correlation between 59 serum-derived proteins and response to immunotherapy in gastrointestinal cancers. As a result, protein signatures characterized by higher levels of IC molecules, namely PD-L1, CD28, immunoglobulin and mucin domain 3 (TIM-3), LAG-3, and CTLA-4, correlated with better prognosis and higher response, being a promising panel of predictive biomarkers [95]. In addition to detecting proteins from serum or plasma samples, recently, this technique has been applied to characterize inflammation-involved proteins in CRC tumors and matched normal tissues, providing a panel of 32 biomarkers differentially expressed in CRC tumors [96].

Another antibody-based technology, **Proximity Extension Assay (PEA)** further extends the number of detected proteins from tens to hundreds and even thousands. The technology is based on target-specific

antibodies conjugated with unique complementary DNA. The antibody pairs targeting one protein bind to the target and a barcoded DNA duplex is formed, which is amplified by qPCR or next-generation sequencing (NGS), allowing quantification of up to 3072 proteins [97,98]. In a recent study, the oncology panel of PEA with 92 cancer-related proteins was utilized to identify potential circulating tumor biomarkers for meningioma. The pathway analysis revealed upregulation of immunomodulatory proteins such as CD69, C—C motif chemokine 24 (CCL24), IL-24, CCL9, and B-cell activating factor (BAFF) [99]. In another study, the PEA immune-oncology panel was applied to study the serum/plasma proteomic profiles of pancreatic neuroendocrine neoplasms patients. Many well-known immune regulators, such as CCL3, IL-7, IL-10, CCL20, were significantly elevated in patients compared to healthy controls, whereas FAS ligand (FASLG) was down-regulated [100]. The PEA technology has shown a promising potential to detect chemokine variability within metastatic melanoma patients subjected to anti-PD-1 therapy [101]. Likewise, it has also been used to assess the immune profile of chronic lymphocytic leukemia patients undergoing different treatments. [102]. PEA analysis of 29 CRC tumors using the immune-oncology panel resulted in only 9 tumors clustered together in unsupervised hierarchical clustering, which revealed the intra-tumor TME heterogeneity [103]. PEA technology possesses a validated specificity and sensitivity (sub-pg/ml) which allows multiplexed protein detection, consuming a minimal amount of sample. Further progress will have a powerful impact on the discovery of new diagnostic, predictive, prognostic, and monitoring biomarkers as well as on the understanding of the proteome of cancer patients [104].

Moreover, other antibody-based proteomics techniques, such as **Reverse Phase Protein Arrays (RPPA)** [105] and chip array cDNA-based **Nucleic Acid Programmable Protein Array (NAPPA)** [106] have been applied in cancer immunology research. RPPA has been used to correlate the tumor heterogeneity and immune response in melanoma patients [107], while NAPPA to analyze tumor autoantibodies in CRC patients [108]. However, antibody-based approaches are limited by the availability and the specificity of antibodies that implies cross-reactivity. Another disadvantage is the variability between batches, especially when the antibody is produced in a new population of antibody-producing animals [109]. Most importantly, these approaches only detect limited numbers of preselected proteins.

5. Emerging single-cell proteomics applied to characterize the immune TME

The interplay between cancer cells and their microenvironment plays an important role in many cancer-related biological processes, including progression, metastasis, drug resistance as well as immune response. These complex cellular interactions of the TME and cancer cells are driven by cell heterogeneity [110,111]. Therefore, to develop more effective immune therapies, it is fundamental to understand the interaction between immune and cancer cells. Single-cell protein measurements rather than a conventional bulk analysis can provide more precise information on this heterogeneity. This section reviews the different single-cell proteomics strategies applied or with potential application in cancer immunity and immune cell characterization. The following section includes a short description of antibody-based approaches, MS-based approaches, and multi-omics strategies applied to cancer immunity at the single-cell level.

5.1. Antibody-based approaches

For the past 30 years, FC has become the 'gold standard' in marker analysis at the single-cell level. Despite its popularity, this method is limited to a low number of markers for simultaneous analysis due to overlapping fluorescence spectra [112,113]. A recently developed modification of traditional FC, **full spectrum flow cytometry (FSFC)** overcomes the issue of overlapping fluorescence spectra of fluorophore-

conjugated antibodies, as the detection and measurement include an entire fluorescence spectrum. This enables the simultaneous detection of up to 64 proteins [114]. This technique has been applied to characterize specific cells populations within the TME. For instance, FSFC with over 30 markers found a tumor favorable environment formation caused by arginine-metabolizing myeloid cells co-localized with CD4⁺ T cells of unconventional phenotype in neuroblastoma mice models [115]. FSFC was applied to characterize the immune cells populations in syngeneic melanoma, breast, ovarian, and CRC cancer models with the focus on Tim-3 as a focal molecule [116]. Comparable higher cytolytic activity of Tim-3+PD-1+CD8⁺ TILs lead researchers to conduct the validation of combined treatment with Tim-3/PD-1 mAbs which indicated an enhanced anti-tumor effect [116].

By the combination of features of FC and MS, MC (CyTOF) has been developed to overcome the limitations of simultaneous analysis of up to 100 proteins at the single-cell level. In this method, cells are stained with metal isotope-tagged antibodies and separated in a mass cytometer, followed by TOF analysis of isotopes mass ratio in the analyzed samples. MC has been successfully applied in the study of the immune signature and immune response in cancer and exhibits potential in the discovery of novel cell populations in different types of cancer [117–125]. For example, MC and RNA-seq analysis of tumor and peripheral blood mononuclear cells (PBMC) of CRC patients revealed that exhausted T cells are induced and recruited by the TME at all stages of the tumor development, demonstrating the link between immunosuppressive TME and the lack of immunotherapy response [117]. This study demonstrated the superiority of MC analysis of TME over RNA-seq to characterize the single-cell proteome state. Interestingly, another CyTOF study identified a novel specific population of effector Tregs with protective function in CRC tumors [118]. In glioblastoma (GBM), MC provided data confirming the inter- and intra-tumor heterogeneity of glioma-associated macrophages (GAM). Moreover, the proportion of GAMs was decreased and exhausted T cells and Tregs were increased in recurrent tumors, contributing to the immunosuppressive environment [119]. In xenografts GBM models, MC was utilized as a comparative tool of immune landscape between tumor-silent and tumor-active models revealing distinct differences in the cells profiles [120]. Additionally, cell barcoding in MC enables sample multiplexing which is a very useful option when dealing with valuable clinical samples and low amounts of murine tissue samples. Recently, MC has been successfully applied in high-throughput clinical analysis, where multiple samples have been analyzed with more than 35+ isotope tags [121].

Further advances in antibody-based proteomics utilize the combination of already established antibodies properties and application with microchips or microfluidics to perform proteomic analysis in isolated single cells. **Single-cell barcode chips (SCBC)** separate single cells in microchambers and secreted or intracellular proteins are captured on an antibody array. Then, captured proteins undergo the staining and quantification with the corresponding biotinylated antibodies and fluorescent streptavidin [126]. Advances in this technology led to the development of a commercial platform that quantifies a panel of 40 key secreted proteins from a single, viable cell [127]. Among other applications, this platform was used to study the heterogeneity of CD8⁺ TILs in metastatic melanoma patients [128].

Multiplexed in situ targeting (MIST) technology uses microbeads hybridized with antibodies conjugated to single-stranded DNA. Once the secreted target proteins are captured, an ELISA assay with the usage of a second, complementary DNA-conjugated antibody is performed [129]. Both technologies, SCBC and MIST, have to compromise the multiplex capacity and detection sensitivity, i.e. increasing the number of different antibodies can increase the multiplexing capacity but, in parallel, decrease the amounts of particular antibodies used, decreasing the sensitivity [130]. **Antibody barcoding with cleavable DNA (ABCD)** is the next technology that improves multiplexing capacity by utilizing antibodies linked to a unique DNA barcode via a photocleavable linker. DNA barcodes are released after incubation by UV exposition and are

quantified by fluorescence hybridization [131]. Moreover, ABCD allows simultaneous analysis of hundreds of proteins from cancer cells and it was applied to characterize lung cancer cells from minimally invasive fine-needle aspirates [132].

TME heterogeneity does not only rely on the different cell types but also their spatial distribution and cell-cell interactions [133]. Whereas previous techniques analyze proteins in isolated single cells, the next antibody-based strategies are focused on comprehensive protein profiling in their natural spatial contexts. Multiplex immunofluorescence (mIF) is based on cycles of antibody staining, imaging, and antibody removal in tissue slides. This method allows the simultaneous identification of several immune markers in the same cell providing data about both the expression and location of target proteins (Fig. 2A). A combination of tissue microarrays with mIF has been optimized (e.g., for TME immune profiling) [134]. Gerdes et al. [33] applied mIF to analyze 61 proteins in CRC, revealing extensive tumor heterogeneity. Recently, mIF has been used to unveil the immune heterogeneity within the TME of melanoma and breast cancer ALNs [65,68].

Since the specific intracellular localization of the proteins is essential to performing their biological function(s), while localization abnormality may severely disrupt biological processes causing disease, characterization of protein expression as well as its localization in a high resolution is needed. Single-cell spatial proteomics aims at solving this problem in a comprehensive manner (reviewed in [135,136]). An mIF technique called **Multi-Epitope Ligand Cartography (MELC)** uses an automated microscopic robot that allows multiplexed protein characterization at subcellular level. In a pioneering work, MELC was applied to identify changes in key immune function-related proteins in CRC tissue at subcellular level [137]. In this study, 1930 clusters of proteins distinguished CRC from healthy tissue, and CRC tissue was enriched in T cells with altered T cell adhesion and NK cells with high nuclear factor-kappa B (NF- κ B) expression. Later, Bhattacharya et al. [138] used **Toponome Imaging System**, a similar mIF strategy, to compare CRC with a normal colon. 5708 clusters of proteins that are specific to colon cancer were identified, showing that CRC has a unique higher-order toponomy signature.

Since the application of mIF techniques carries a risk of damaging the epitopes' integrity, oligonucleotide conjugated antibodies alternatives have been explored [139–141]. **CO-Detection by indEXing (CODEX)** iteratively visualizes targets through in situ polymerization-based indexing procedure with oligonucleotide-conjugated barcodes and dNTPs analogs tethered to fluorophores (Fig. 2B) [142]. CODEX has been applied to study the immune TME of CRC with 56 markers, showing the importance of the spatial distribution and cell neighborhoods in CRC [143]. Despite the recent advances in multiplexed analysis, it was found that oligonucleotides negatively affect the specificity and the binding affinity of antibodies. To avoid this interference, other alternatives are used e.g., removable antibodies with fluorophores linked by an azido group [144].

In the context of cancer immunology, **imaging mass cytometry (IMC)** and **multiplexed ion beam imaging (MIBI)** are powerful tools to assess the complexity of the TME and networks of cell-cell interactions in their spatial context within the tissue. IMC is a technology that combines CyTOF (MC) and imaging to analyze proteins in situ (Fig. 2C). First, the tissue slide is stained with a panel of metal conjugated antibodies and then the stained tissue is converted to a stream of particles pixel-to-pixel by a laser. Next, the mass spectrometer determines and quantifies the metal isotopes linked to the antibodies in each particle and, finally, a computational algorithm combines the MS data of each pixel with its coordination information to generate a two-dimensional image [145]. IMC not only provides information on single-cell proteomics but also on the localization of the particular protein in the tissue and constructs the cellular interaction within the TME. This methodology gives additional data potentially relevant in the context of prognosis or treatment. IMC analysis with 35 biomarkers of patients' breast tumors samples, together with available survival data, yielded high-dimensional images providing

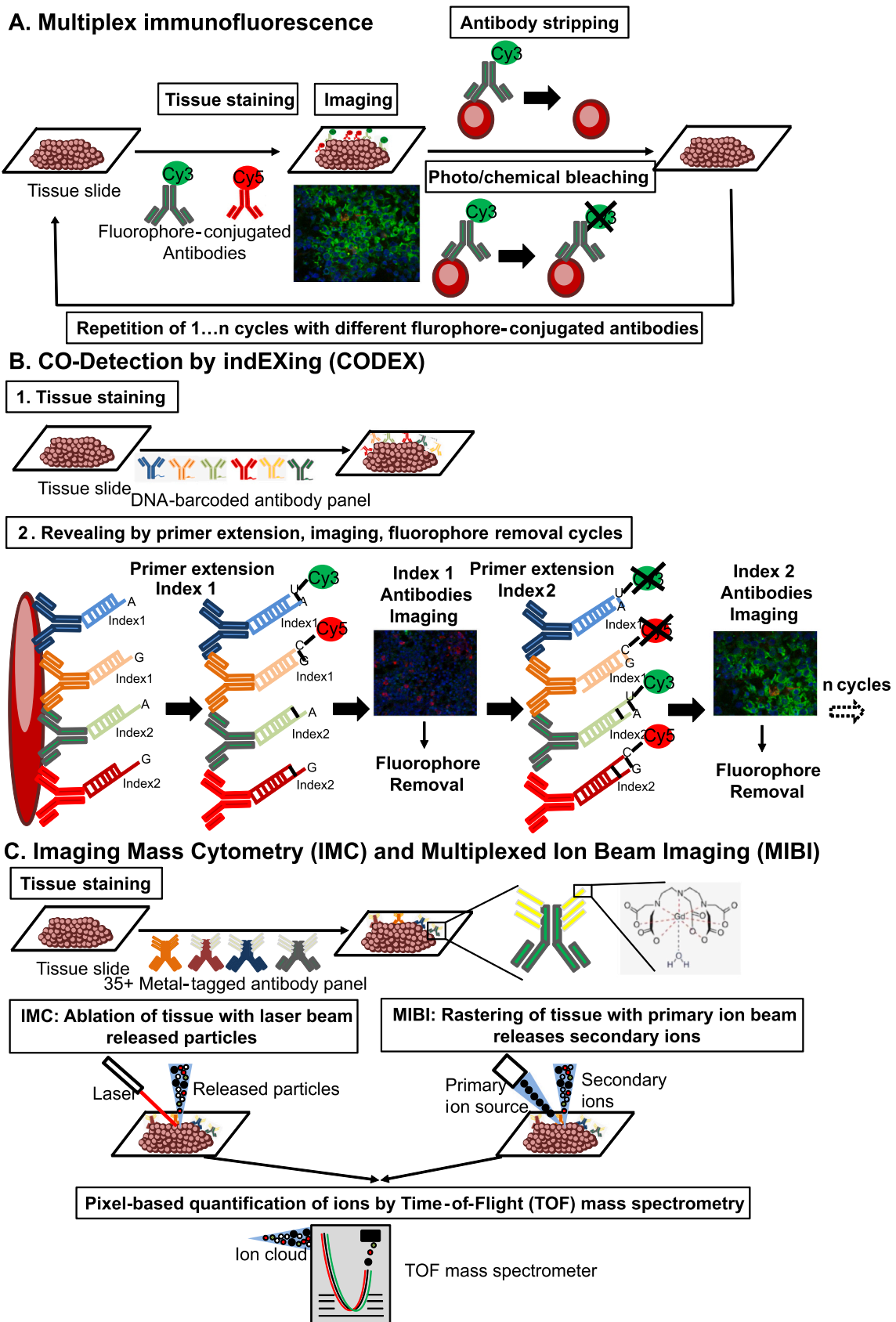


Fig. 2. Schematic representation of single-cell spatial proteomics approaches. (A) Multiplex immunofluorescence (mIF), (B) CO-Detection by indEXing (CODEX), (C) imaging mass cytometry (IMC) and multiplexed ion beam imaging (MIBI).

information on the complexity of organization of tumor and stromal cells, their location within the tissue, and distinct phenotypes of tumor cells. This study led to the proposal of novel breast cancer subgroups closely related to the particular patient's prognosis [146]. IMC was also used to explore the TME of different cancer types including Hodgkin lymphoma, and CRC, in which tertiary lymphoid structures in CRC were found to have abundant forkhead box P3 (FoxP3)+ Treg expression, demonstrating its potential for immune profiling in tumors [147].

The second technology, MIBI is a variation of IMC which operates an ion beam to release metal ion reporters, therefore increasing its multiplexing capacity to more than 100 targets at once [148]. An interesting application of MIBI is single-cell metabolic regulome profiling, which enables to study the composition of the metabolic regulome in combination with phenotypic identity with more than 110 antibodies against metabolite transporters, metabolic enzymes, or regulatory modifications. The study revealed the metabolic heterogeneity and spatial organization of CD8+ T cells in CRC, including subsets expressing the T cell exhaustion-associated molecules CD39 and PD-1, indicating their exclusion from the tumor-immune boundary [149]. Undeniably, IMC and MIBI are superior methods to fluorescence-based technologies because they detect simultaneously targeted proteins with a higher dynamic range avoiding staining/stripping cycles that can compromise epitope integrity [150]. However, their disadvantage is the availability of the number of antibodies conjugated with metal isotopes suitable for FFPE and fresh frozen tissue staining [151].

In summary, the bottleneck of single-cell measurements with antibodies is the limit of sensitivity, which stems from the molecular shot noise, limiting accurate quantification to the low attomolar (aM) range, as well as the quality of the antibody [152].

5.2. Single-cell MS-based approaches

Unbiased single-cell MS-based proteomics approaches are currently in development, being a promising alternative that can overcome the limitation of antibody-based approaches, potentially leading to an increased number of detected proteins [153]. However, single-cell MS analysis must overcome additional challenges apart from the above-mentioned for bulk MS proteomics. Proteins cannot be amplified as nucleic acids. Thus, one of the major challenges is the delivery of peptides to the mass spectrometer taking into account the low protein content of a single cell. Single-cell sample preparation requires miniaturization and automation to reduce protein losses and increases their concentration [154]. Single cells are separated by FACS or other alternative techniques and subsequently, protein extraction and digestion are performed in reduced volumes (1 μ l/cell or lower). Different strategies of sample preparation have been successfully developed such as nanodroplet processing in one pot for trace samples (nanoPOTS) [155], oil-air droplets [156], or minimal Proteomic sample Preparation (mPOP) based on freeze-heat cycles [157]. Moreover, peptide separation in the LC column and its corresponding ESI must be miniaturized with flow rates at low-nanoliter-per-minute or even picoliter-per-minute range. Therefore, the inner diameter of nanoLC columns is reduced from 75 μ m to 30 μ m which in consequence improves single-cell proteome coverage [158].

Importantly, single-cell MS analysis needs an increase in peptide sequence identification as well as in its multiplexing capacity to analyze the proteome from thousands of cells at an affordable cost [153]. A great advance has recently been achieved with an approach called **Single Cell Proteomics by mass spectrometry (SCoPE-MS)** [159]. SCoPE-MS prepares the sample by mPOP and adds an isobarically labeled carrier (e.g., the proteome of 100 cells) with tandem mass tags [160]. The usage of a proteomic carrier mitigates sample losses, facilitates peptide sequence identification, and increases the multiplexing capacity with a limit of 12 single-cell proteomes in one run due to the limited tandem mass tags available. With such technological development, SCoPE-MS found its application in heterogeneity studies. SCoPE-MS quantified

3042 proteins in 1490 single monocytes and macrophages, suggesting that heterogeneity of macrophages may emerge without the participation of polarizing cytokines [161]. Moreover, SCoPE-MS quantified 1500 proteins from 152 cells from three acute myeloid leukemia (AML) cell lines, revealing functionally distinct differences between the three cell clusters [162,163]. Additionally, the combination of nanoPOTS and SCoPE-MS quantified around 1000 proteins per cell of 3000 FACS-sorted cells from an AML culture model. It allowed resolving AML heterogeneity at a single-cell level along different hierarchical stages of differentiation [164].

Further improvements will be achieved through innovations in sample preparation and peptide separation, hardware advances of mass spectrometers as well as innovative acquisition and interpretation methods. These improvements will facilitate increased coverage of single-cell proteomes as well as the sensitivity and confidence of peptide sequence identification, revolutionizing cancer immunology [165].

5.3. Single-cell multi-omics strategies

For precision oncology, to deeply and comprehensively understand the complexity of the TME, in addition to proteomics, an integration of multi-omics data at the individual cell level with the molecular landscape of each cell is needed [166,167]. Proteogenomics approaches combine bulk MS-based proteomics with genomics and transcriptomics. This strategy has been applied to several cancer types, providing novel insights into somatic mutation consequences at the protein level as well as neoantigens discovery for immunotherapy [168–171]. However, the genomic and proteomic data integration at the single-cell level is currently in development. Recently, a pioneering study designed DAb-seq which allows analysis of 49 DNA targets and 23 protein markers by the combination of DNA barcodes conjugated to antibodies and multiplex PCR. Although this technology requires an increase in its multiplexing capacity, it demonstrated the heterogeneous interactions of somatic mutations and protein expression in AML single cells [172].

On the other hand, there are some techniques designed to link mRNA and antibody based protein analysis in single-cell approaches. **Proximity Ligation Assay for RNA (PLAYR)** is a method that uses FC/MC for simultaneous analysis of target proteins stained with antibodies and RNA. PLAYR probe pairs hybridize their targets and then the insert and backbone are hybridized and ligated to the probes. After rolling circle amplification, labeled oligonucleotides bind the insert regions for detection and quantification [173]. Recently, this method has been used to demonstrate intra-clonal heterogeneity in chronic lymphocytic leukemia cells [174]. **Cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq)** and its sister technology **RNA expression and protein sequencing (REAP-seq)** combine DNA-conjugated antibodies with scRNA-seq [175,176]. The difference is that CITE-seq uses biotinylated antibodies whereas REAP-seq uses antibodies covalently bonded to aminated DNA sequences. These methods integrate cellular surface protein and transcriptome measurements into single-cell readout. CITE-seq provides a more detailed characterization of cellular phenotypes compared to scRNA-seq alone and allows simultaneous protein expression and transcriptome profiling of thousands of single cells (Fig. 3). CITE-seq may also show quantitative differences in marker expression between subsets e.g., expression difference of CD8a between NK and T cells [176]. A CITE-seq panel of 157 antibodies was applied to immunophenotype breast cancer patients. 18 clusters of T cells and innate lymphoid cells (ILCs) were found with different proportions among clinical subtypes. Interestingly, IC molecules were also differentially expressed among breast cancer subtypes. These findings may lead to personalized immunotherapy strategies for each subtype [177]. Moreover, it was found that CITE-seq can be combined with single-cell sequencing assay for transposase-accessible chromatin (scATAC-seq) and used to study the RNA expression, surface proteins, and chromatin accessibility at the single-cell level. Granja et al. [178] applied such strategy to find distinct and shared molecular mechanisms of leukemia.

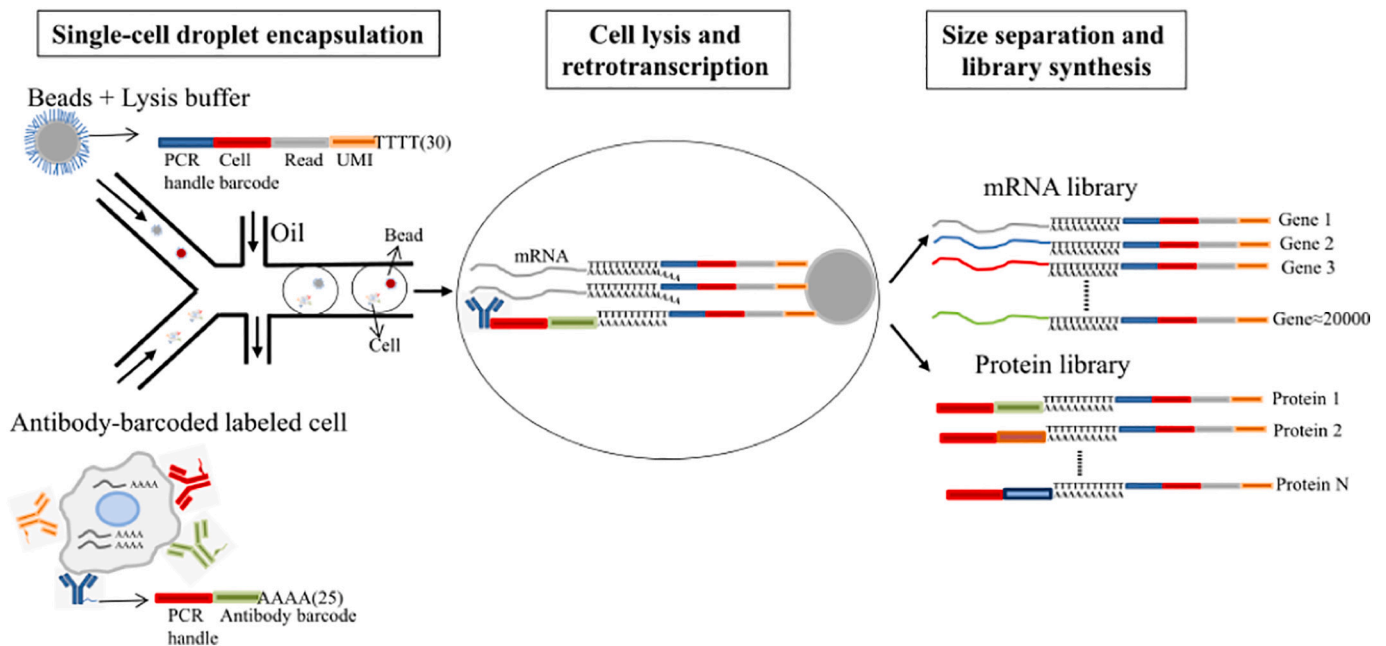


Fig. 3. Schematic representation of CITE-seq and REAP-seq. Antibody-barcoded labeled cells are mixed in a microfluidic system in which each droplet contains a cell, beads with the PCR adapters with the corresponding cell barcodes, and lysis buffer. After cell lysis within the droplet, mRNA and DNA barcodes from antibodies are hybridized with PCR adapters. Subsequent retrotranscription generates cDNAs and droplets are disrupted. Upon disruption, the respective cDNAs for mRNAs and proteins are separated by size. These synthesized libraries are sequenced, providing the single-cell expression profiles of mRNA and targeted proteins.

Among the challenges for both technologies (CITE-seq and REAP-seq), the efficiency of cell captures must be increased, the system requires total automation, and the multiplex detection must be extended to intracellular proteins which is currently limited to a reduced number of proteins [179,180]. Recently, **SURFACE-PROTEIN GLYCAN AND RNA-SEQ (SUGAR-seq)** has been designed to enable the detection and analysis of N-linked glycosylation, extracellular epitopes, and the transcriptome at the single-cell level. SUGAR-seq is an extension of CITE-seq in which glycans are captured with a biotinylated lectin and subsequently detected using an anti-biotin mAb conjugated to a DNA-barcode. Integrated SUGAR-seq and glycoproteome analysis identified TILs with unique N-glycan profiles as cellular T cell subsets with the altered epigenetic and functional state in CRC and melanoma mice models [181].

Zhang et al. [182] combined scRNA-seq and mIF to study the immune TME of CRC patients. They found that TILs showed an exhausted phenotype compared to T cells originating from normal tissue and peripheral blood. Moreover, they identified a population of Th1-like cells that were enriched in microsatellite instability (MSI) CRC, providing a possible explanation for MSI patients' good response to anti-PD-1 immunotherapy. Finally, de Vries et al. [183] combined MC with 36 markers, FC, scRNA-seq, and mIF to analyze T cells from CRC, matched associated lymph nodes, healthy mucosa, and peripheral blood. Different phenotypes of CD8⁺/γδ T cell and CD4⁺ memory T cells were observed in each examined tissue. Interestingly, an innate lymphoid cell (ILC) population was enriched in CRC tissues with high expression of cytotoxic molecules. Additionally, this ILC population correlated with the presence of tumor-resident cytotoxic, helper, and γδ T cells with similar activated profiles. This study not only sheds some light on the complexity of lymphocytes composition dependent on the sample type but also demonstrates that multi-omics data integration provides much more data and in-depth analysis, which otherwise would not be obtained.

6. Conclusions and future perspectives

Despite the great advances in cancer immunology and the

development of immunotherapy, the patients' response rate remains a clinical challenge. Understanding the complexity of TME and immunosuppression mechanisms may lead to design of more effective cancer immunotherapies. Proteomics is a powerful approach to accelerate the studies on immune responses in cancer. MS-based proteomics can uncover novel insights into molecular mechanisms and potential therapeutic targets, while the application of antibody-based proteomics approaches does not require specialized expertise as in MS and is widely applied as a tool to characterize selected proteins and discover new clinical biomarkers. However, both approaches possess limitations and technical challenges that complicate the characterization of the whole proteome of biological systems, especially to differentiate between proteoforms.

Emerging single-cell proteomics approaches will revolutionize our understanding of the complex cellular networks within the TME and interactions between cancer and immune cells. Several technologies have been recently developed with the potential for comprehensive proteomic characterization that facilitates the deep profiling of immune responses in cancer at the single-cell level. Novel technical solutions will provide higher sensitivity and higher resolution at the subcellular and molecular level [184–186]. Importantly, a new era in proteomics was born with single-molecule protein sequencing based on fluorescence-mediated *in situ* protein identification [187,188] as well as nanopores [189,190]. Further technical development of these next-generation proteomics approaches will ideally enable the whole proteome characterization and unveil the distribution of proteoforms at the single-cell level.

In summary, together with the technological advancements in single-cell analysis, progress in a holistic system of multi-omics approaches and data analysis is needed. To date, it was found that a combination of different 'omics' data with single-cell proteomics, may provide information on cancer origin, progression, and prognosis, which could remain undiscovered if were analyzed separately. It is well-recognized that a comprehensive approach to TME composition is crucial in personalized therapy and efficient treatment. In this review, we have discussed examples of immune heterogeneity studies of TME in cancer, focusing on both MS-based bulk/antibody-based and single-cell analysis

Table 1

Summary of the presented emerging single-cell proteomics techniques divided by approaches type and with their corresponding advantages and disadvantages.

| Type | Technique | Advantages | Disadvantages |
|---------------------------|---|--|---|
| Antibody-based approaches | Full spectrum flow cytometry (FSFC) | Up to 64 of markers analyzed simultaneously Possibility to use dyes with close peak emission, if the full spectra of fluorescence significantly differs | Increased need for a larger number of antibodies combined with dyes suitable for full spectrum detection Reproducibility (increased number of parameters analyzed simultaneously increases the number of factors influencing the outcome of the experiment) Requires specialized device |
| | Mass cytometry (MC or cytometry of time-of-flight; CyTOF) | Up to 100 of markers analyzed simultaneously in comparison to fluorescence methods Low background noise Multiplexing capacity crucial for the analysis of valuable samples | Infeasible to recover living cells after analysis Longer analysis time Requires specialized device |
| | Single-cell barcode chips (SCBC) | Dynamic range (up to 10,000 cells analyzed) Possibility to analyze proteins originating from cell-surface, cytoplasm or secreted Cells can be recovered for further analysis | Requires big number of barcoded antibodies Specificity of antibody binding might be hindered by oligonucleotides |
| | Multiplexed in situ targeting (MIST) | Potential to detect hundreds of proteins | Multiplexing vs. sensitivity – increase in the number of microbeads decreases the sensitivity |
| | Antibody barcoding with cleavable DNA (ABCD) | High multiplexing capacity due to infinite number of DNA barcodes can be discriminated | Low sample throughput May not detect low-expression proteins |
| | Multiplexed ImmunoFluorescence (mIF) | Unmodified primary antibodies can be used for this method Allows the detection for several markers at once Relatively short assay time No need for specialized equipment apart from fluorescence microscope | Incomplete removal of antibodies can interfere with the signal detection in subsequent staining cycle Risk of damaging the epitopes during the stripping step |
| | Multi-Epitope Ligand Cartography (MELC) | Allows the detection of up to 50 epitopes at once High resolution | Requires specialized device Long sampling time |
| | Toponome Imaging System | Allows the analysis of up to 100 proteins in a single cell | Requires specialized device |
| | CO-Detection by indEXing (CODEX) | Detection of up to 40 proteins Can provide information on relative abundance of the detected markers in spatial context | Specificity of antibody binding might be hindered by oligonucleotides Relatively long scanning time |
| | Imaging mass cytometry (IMC) | Up to 40 markers can be detected Provides spatial context | High cost of the analysis Availability of isotope-tagged antibodies Sample is destroyed during detection Time of the data acquisition and analysis Requires specialized device |
| | Multiplexed ion beam imaging (MIBI) | Higher detection capacity (up to 100 markers) | High cost of the analysis Availability of isotope-tagged antibodies Sample is destroyed during detection Time of the data acquisition |
| MS-based approaches | Single Cell Proteomics by mass spectrometry (SCoPE-MS) | High throughput and accurate quantification Decreased sample losses Increased identification of peptide sequences | Low accuracy (when comparing the abundance of proteins) Requires special device |
| Multi-omics approaches | Proximity Ligation Assay for RNA (PLAYR) | Possibility to analyze both mRNA and protein expression levels High throughput | Need to apply FC or MC to obtain the results Limited to the detection of 40 markers due to the availability of the suitable antibodies Multiple probes required to detect one transcript |
| | Cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq) | Can detect a protein even in the case when corresponding mRNA is of low abundance | Cell capturing efficiency needs to be increased Requires total automation Multiplex detection must be extended to intracellular proteins which is currently limited to a reduced number of proteins |
| | RNA expression and protein sequencing (REAP-seq) | Can detect a protein even in the case when corresponding mRNA is of low abundance | Cell capturing efficiency needs to be increased Requires total automation Multiplex detection must be extended to intracellular proteins which is currently limited to a reduced number of proteins |
| | Surface-protein Glycan And RNA-seq (SUGAR-seq) | Can analyze N-linked glycosylation, extracellular epitopes, and the transcriptome at the single-cell level | Similar limitations as for CITE-seq and REAP-seq Biased detection of glycans – detection depends on the type of lectin used in the assay |

(Table 1). Moreover, we reviewed emerging single-cell proteomic analysis methods with examples of the combination of multi-omics studies, which we believe become widely applied in cancer research in the future.

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Declaration of competing interest

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

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