Trends in Biochemical Sciences

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Review

Single-Cell Proteomics

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The inability to make broad, minimally biased measurements of a cell's proteome stands as a major bottleneck for understanding how gene expression translates into cellular phenotype. Unlike sequencing for nucleic acids, there is no dominant method for making single-cell proteomic measurements. Instead, methods typically focus on either absolute quantification of a small number of proteins or highly multiplexed protein measurements. Advances in microfluidics and output encoding have led to major improvements in both aspects. Here, we review the most recent progress that has enabled hundreds of protein measurements and ultrahigh-sensitivity quantification. We also highlight emerging technologies such as single-cell mass spectrometry that may enable unbiased measurement of cellular proteomes.

Highlights

Single-cell proteomics holds the potential to create a mechanistic understanding of how the products of many genes interact to produce a cellular phenotype.

The recent growth in single-cell profiling has been focused primarily on nucleic acid measurement. There is now a pressing need for more comprehensive protein measurements of single cells, particularly in absolute quantification and multiplexed measurements.

Microfluidics is a core enabling technology for absolute quantification. Microfluidic devices can integrate traditional protein quantification technologies into single-cell chambers.

There has been rapid growth in multiplexing single-cell protein measurements by incorporating mass spectrometry or DNA sequencing. However, such measurements are still limited by the availability of antibodies.

Emerging technologies have the potential to eliminate the need for antibodies, providing an avenue for a minimally biased survey of single-cell expression at the protein level.

The Lagging Progress of Single-Cell Proteomics

Over the past several years, there has been rapid growth in techniques for measuring the properties of individual cells. Among the most surprising revelations that arose from these techniques is the high degree of variability that exists among cells previously thought to be identical. This variability can be crucial to understanding processes that are driven primarily by cell-intrinsic properties, such as oncogenesis and differentiation. Single-cell techniques have revealed processes in cell signaling, viral infection, cell fate decisions, drug resistance, and many other phenomena that simply cannot be addressed by studying at the level of individual genes or tissues [1–4].

The most rapid growth in single-cell analyte measurement has been single-cell RNA sequencing. A single experiment can measure thousands of transcripts from tens of thousands of individual cells [5,6]. However, to understand the mechanism of cellular processes, one must measure the effector molecules of that process, which are usually the proteins encoded by the mRNA rather than the mRNA itself. Simultaneous measurements of protein and mRNA levels from single cells have shown that their correlation ranges from modest to absent, severely limiting the capacity to estimate protein levels from mRNA measurements [7–9]. As such, single-cell proteomic techniques are essential to understanding how a cell functions.

If a biological system is entirely understood, then one should be able to predict the outcome of a perturbation. However, individual genes are rarely able to predict a cellular response. This is because cell-level biological processes are emergent properties arising from a set of dynamic interactions between several genes. Comprehensive measurements of a cell's proteome are a critical intermediate step between understanding individual genes and predicting a cell's response to perturbation. The most advanced techniques for studying such processes are dynamic models, which are often systems of differential equations. However, creating such models is difficult for two primary reasons. First, one must understand the phenomenon well enough to identify the simplest set of proteins that can model it. Second, one must quantify or estimate many different parameters that govern the dynamics. Single-cell proteomics has two areas of focus that can help overcome these obstacles. Research focused on absolute quantification of proteins in single cells provides precise parameters, and research focused on making many

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protein measurements simultaneously (**multiplexing**; see Glossary) provides a broad overview necessary to determine which sets of proteins are relevant for inclusion in the model.

Single-cell proteomics is a field in its infancy. The progress in measuring proteins from single cells stands in sharp contrast to progress in measuring nucleic acids. DNA sequencing acts as a unifying technology for the latter, enabling an extremely rapid pace of new measurement methods. One can measure mRNA, genomic sequences, genomic accessibility, epigenetic markers, and many more parameters using sequencing [5,6,10–12]. This is possible because many different measurements can be coupled to a single versatile output through DNA sequencing. It offers an extremely high capacity (hundreds of millions of measurements) with an unparalleled multiplexing capacity (with various measurements being barcoded with specific DNA sequences). With no such unifying technology, each single-cell proteomic method is unique to its specific purpose. Single-cell proteomics currently lacks a clear state-of-the-art method that is generally applicable to any research question. Despite this, there has recently been swift progress in absolute quantification of single-cell proteins and in highly multiplexed protein measurements. This review discusses successes and limitations of targeted proteomic methods, along with emerging technologies that offer the possibility to measure single-cell proteins in an untargeted manner.

Absolute Quantification of Proteins

Absolute quantification is an assay property that enables estimation of the exact amount of analyte in the sample. Doing so requires a standard of purified analyte that can be used to create a curve which relates the analyte to a measured signal (Figure 1A,B). All such assays have several figures of merit useful for comparison. The sensitivity of an assay is the minimum amount of analyte that can be reliably detected and is normally expressed as the **limit of detection (LOD)** (Figure 1C).

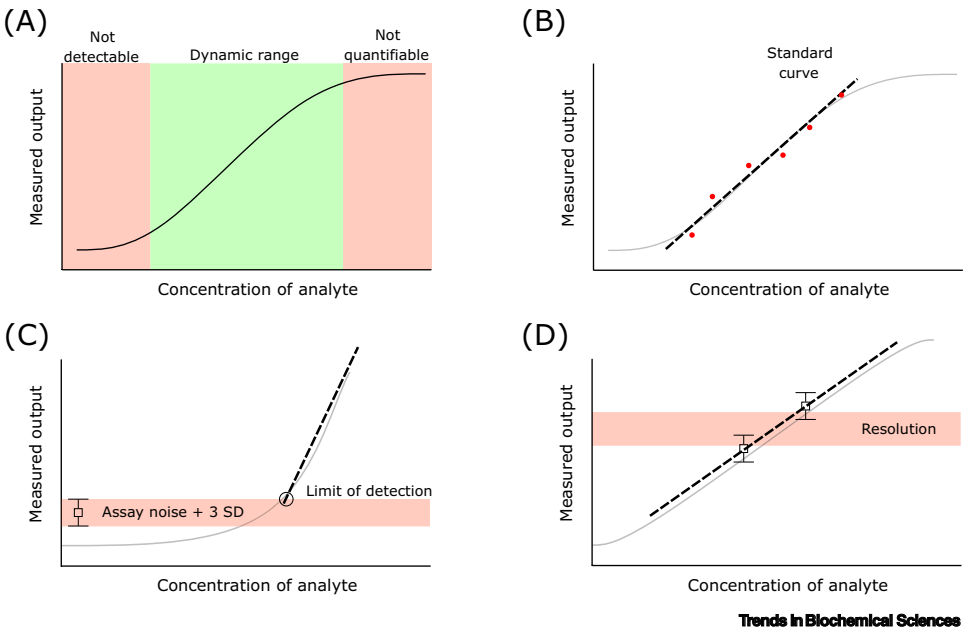

Figure 1. Properties of an Absolutely Quantitative Immunoassay. (A) Each assay has a dynamic range over which the measured output correlates with the concentration of analyte. (B) The correlation between analyte and output can be estimated using a standard curve with known analyte concentration. (C) The limit of detection (LOD) is the concentration of analyte that is three standard deviations (SDs) above the measured output in the absence of analyte. (D) The smallest difference between two values that can be reliably measured is the resolution. Similar to LOD, the resolution is determined by the SDs of measured values.

Glossary

CyTOF: CyTOF, short for cytometry time of flight, is the commercial name for mass cytometry. This technique applies metal isotope-labeled antibodies to single cells and analyzes them using mass spectrometry.
Dynamic range: an assay property which defines the range of analyte concentrations that can be quantified. Concentrations below the dynamic range are below the detection limit, and concentrations above the dynamic range result in signal saturation, wherein additional analyte does not increase the measured signal.
ELISA: enzyme-linked immunosorbent assay; one of the most popular versions of a sandwich assay wherein one antibody is used to capture a protein of interest on a surface and a second antibody is used to detect that protein and create a measurable signal. An ELISA uses an enzyme to produce the signal; however, other sandwich assays can use a variety of methods to produce an output.
Limit of detection (LOD): the smallest amount of analyte that generates an unambiguous signal during measurement. Typically, this is calculated as the signal that corresponds to three standard deviations above the measured signal in the absence of analyte.
Multiomic: refers to techniques that allow the simultaneous measurement of multiple different biomolecular classes, such as measuring both the transcriptome and proteome from the same sample.
Multiplexing: a multiplexed assay is capable of measuring several analytes simultaneously.
Proximity ligation assay (PLA): uses a pair of oligonucleotide-conjugated antibodies to translate the detection of a protein into the production of DNA. It has specificity similar to that of an ELSIA (due to requiring two antibodies) but does not require surface binding.
Resolution: the resolution of an assay is the smallest difference between two analyte concentrations that can be reliably detected.

This is typically calculated as the concentration of analyte corresponding to three times the standard deviation (SD) of the assay noise. **Dynamic range** indicates the total range of analyte concentrations that can be quantified (Figure 1A). Additionally, in some instances, **resolution**, which is the smallest difference that be identified between samples, can be a relevant property (Figure 1D). Finally, most assays are capable of some degree of multiplexing, which enables measurement of multiple analytes simultaneously. An ideal assay would perform well on all metrics; however, there is normally a trade-off between assay properties. A high dynamic range is often coupled with a low resolution, and high multiplexing causes a loss of sensitivity [13].

Protein quantification is a mature technology with many standard approaches available. The techniques most amenable to single-cell protein quantification are immunoassays, such as western blotting, **ELISA**, and the **proximity ligation assay (PLA)**. Each of these features an antibody affinity reagent to identify the protein of interest and a method to couple antibody binding to a measurable output. Western blotting separates proteins by size electrophoretically, followed by probing with an antibody specific to the target analyte. ELISA uses an antibody pair in which one antibody is immobilized on a surface to capture the analyte. After washing away unbound analyte, a second probe antibody binds to the same analyte and generates an output. PLA is conceptually similar to ELISA. It uses pairs of antibodies that have been functionalized with DNA oligomers. When two antibodies are in proximity (such as being bound to the same protein), the DNA can ligate, thus converting the detection of a protein into the generation of a specific DNA strand. The basic concept of each immunoassay is similar, with the primary difference being the way protein detection is coupled to the assay output. The challenge for single-cell proteomics lies in the fact that the common use of these techniques simply does not have the sensitivity necessary to quantify proteins from single cells.

Protein quantification methods are typically used at the microliter scale. If the protein of a single cell were diluted to this volume, it would be well below the detection limit of the assay. The most straightforward solution to the sensitivity limitation is miniaturization. As a result, microfluidics has become a core enabling technology for single-cell protein quantification. Microfluidic devices offer unparalleled liquid control by virtue of laminar flow at the micrometer scale and the automated devices that control liquid movement. Reactions can be run at the nanoliter scale in microfluidic chips and microwells or at the picoliter scale in droplets. Each single-cell protein quantification method features miniaturization, with the differences primarily concerning the method used to couple the quantification assay to the smaller scale. Microfluidic methods are featured throughout this review. In each case, the purpose of the device is to minimize reaction volume, control the chemical environment of the cells, or couple a protein measurement to another measurement in the same cell.

For every protein assay, the detection of the analyte is coupled to a measurable output. Care should be taken to differentiate between the sensitivity of the assay and the sensitivity of the output. Signal amplification schemes using advanced fluorescence optics, polymerase reactions, and nanotechnology have driven the output sensitivity to the single-molecule level [14–17]. However, this progress does not enable single-molecule quantification. Rather, it has shifted the bottleneck on sensitivity to other properties of the assay. The limit on sensitivity now comes from two places. The first is molecular shot noise, which is a fundamental noise source that arises from the statistical properties of counting individual molecules and limits accurate quantification to the low attomolar (aM) range [18]. The second limitation comes from the quality of the antibody [18,19]. Antibody properties generally limit sensitivity above the aM range. This is because in achieving the single-molecule detection required to count individual molecules, one must use antibodies with very low nonspecific binding and K_d values [19]. Care should be taken to avoid

interpreting improved ability to measure an output as a cause of improved sensitivity unless the same antibodies were used in both cases.

Miniaturization of Traditional Immunoassays

Single-cell western blotting (scWB) was achieved after several advancements in miniaturization of western blotting (Figure 2A). Microfluidic devices were used to create micrometer-scale polyacrylamide channels that enable quantification of very small amounts of protein [20]. This technology eventually yielded single-cell measurements by incorporating a microwell plate to isolate individual cells [20,21]. The first generation of scWB could individually assay thousands of cells. The LOD was comparable with that of flow cytometry and could be multiplexed to tens of proteins using stripping and reprobing, but at a relatively small dynamic range (1.3–2.2 logs). By combining

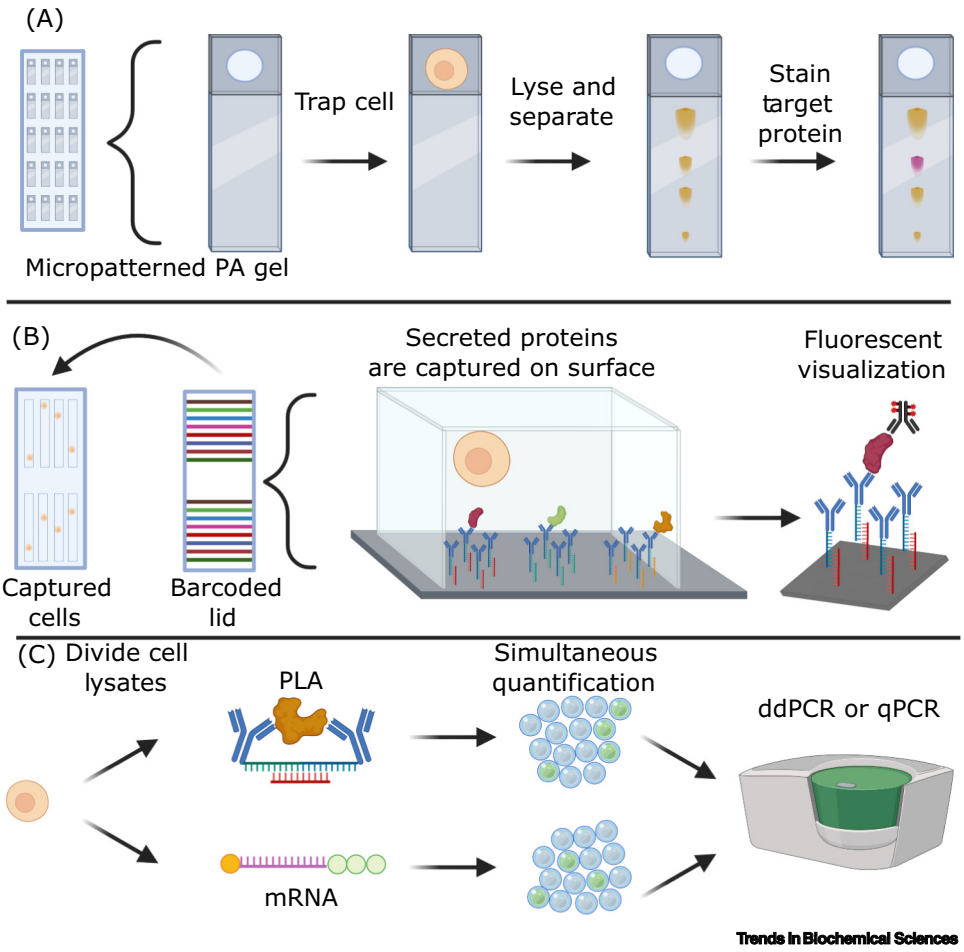

Figure 2. Single-Cell Absolute Quantification Methods. (A) Single-cell western blotting uses a series of microwells embedded in gel in order to trap individual cells. Upon lysis, the protein is electrophoretically separated directly in the gel. The separated proteins are photocaptured in the gel to enable probing and washing with detection antibodies. (B) The single-cell barcode chip measures single-cell proteins using an array of immobilized antibody strips. Cells are captured in chambers and enclosed with the antibody array. Upon lysis or secretion, proteins will bind to the array and are detected with a second fluorescent antibody, with the location of the fluorescence indicating the identity of the targeted protein. (C) The proximity ligation assay (PLA) detects protein by using pairs of DNA-functionalized antibodies. When both antibodies are bound to the same target, the proximity facilitates ligation. By encoding protein abundance into DNA, one can measure both a protein and mRNA in the same cell. Elements of the figure were generated with BioRender. Abbreviations: ddPCR, droplet digital PCR; PA, polyacrylamide.

scWB with microfluidic cell separation, rare circulating tumor cells were profiled for 12 proteins simultaneously [22]. Later devices incorporated live-cell manipulations prior to western blot analysis [23].

Sandwich assays, which include ELISAs, are considered the gold standard of quantitative immunoassays. An ELISA uses an enzyme output (colorimetric or luminescent); however, other sandwich assays feature a fluorescent output. Sandwich assays have excellent specificity provided by the requirement for two separate antibodies to bind to the analyte to generate an output. The additional specificity improves quantification over single-antibody methods, and the surface immobilization enables quantification from complex matrices. Standard ELISAs have detection limits as low as 1–1000 pg/ml [19]. Since typical mammalian cells have 500 pg of protein in total, miniaturization is essential to achieve robust quantification. Several laboratories have miniaturized sandwich assays into microfluidic devices [24–27]. In each case, the microfluidic component serves to minimize sample volume and thus maximize analyte concentration. The most straightforward approach was a direct application of an ELISA to the micrometer scale. This was sufficient to detect aM quantities of protein from single cells [24]. While the microfluidic components are largely similar, the devices differ in their detection method, throughput, and multiplexing capacity. This includes integrating a total internal reflection fluorescence (TIRF) output [26] and using microbeads to measure six proteins simultaneously [27].

The single-cell barcode chip (SCBC) developed by Heath *et al.* is one of the most advanced single-cell proteomic devices (Figure 2B). The design features micrometer-scale chambers that capture individual cells and a removable lid patterned with capture antibodies. Measurements are multiplexed through spatial encoding, whereby strips of capture antibodies are imprinted onto the microfluidic device, with the location of the strip encoding the identity of the antibody [28]. This device has a detection limit of hundreds to thousands of proteins, depending on the antibody, and a dynamic range of three to four orders of magnitude [25]. The basic design of the SCBC has been expanded to improve several technical aspects of the device, increasing the throughput of the SCBC to more than 1000 cells [29], expanding the multiplexing capacity up to 42 secreted proteins per cell [30], and simplifying the design to create a portable device [31].

The SCBC has been applied to understanding the epidermal growth factor (EGF) signaling pathway, measuring T-cell response by quantifying secreted proteins and profiling circulating tumor cells [25,27,28,32]. It was also modified to enable two-cell measurements, which was used to explore how intracellular signaling depends on the distance between two cells [33]. One study in particular highlights the power of coupling many different measurements to individual cells; Kravchenko-Balasha *et al.* showed that measurements of intracellular signaling, cellular communication, and cell distance could be integrated through a thermodynamic analysis to understand cell motility [34].

Encoding Protein Detection in a DNA Output

A PLA can offer specificity comparable with that of sandwich assays while providing the versatility to integrate into other single-cell methods. This versatility comes from translating the detection of a protein into the creation of a DNA molecule. The final output can be measured using qPCR, fluorescently, or through DNA sequencing [9,13,35]. These properties combine to push the detection limit down to the single-molecule level in ideal cases [35]. Using a DNA output also enables easy integration into existing single-cell sequencing methods. PLA and similar methods have been applied to measure various proteins and mRNA simultaneously from single cells [9,36–38] (Figure 2C).

Multiplexed Protein Measurements

Cellular phenotypes are an emergent property of many molecular components interacting in concert. Because of this, there is high value in being able to simultaneously measure several proteins in the same cell. One of the most active areas of research is driven by the goal of making tens to hundreds of protein measurements on single cells. These approaches must contend with the increasingly difficult task of encoding unique outputs for each analyte. For years, the most relevant limitation to multiplexing came from the spectral resolution of fluorophores. However, there are currently many technologies that have overcome this problem by clever use of various output methods.

Mass Cytometry: Encoding Proteins with Metal Isotopes

Perhaps the most broadly used technique for multiplexing single-cell protein measurements is mass cytometry. Conceptually, mass cytometry is similar to flow cytometry; however, cells are analyzed via mass spectrometry (MS) rather than optically [39] (Figure 3A). Antibodies used in this technique are labeled with metal isotopes. Metal ions do not suffer from spectral overlap, circumventing the difficulty in measuring specific fluorophores in highly multiplexed experiments. Currently, more than 40 isotopes can be measured simultaneously [40]. This method has been commercialized under the name **CyTOF** (Fluidigm, CA, USA) and has been broadly adopted and applied to many biological problems, including identifying brain cell subtypes, tracking immune cell differentiation, and monitoring signal transduction through cells [41–43]. The application to cell signaling is particularly interesting, as it illustrated a new obstacle that arises from highly multiplexed single-cell data. Such high-dimensionality data cannot typically be analyzed through simple inspection. Mass cytometry can be analyzed with biaxial plots commonly used in flow cytometry, but the higher dimensionality creates the possibility to cluster cells, analyze the clusters' expression levels, and perform more complex analysis such as spanning-tree progression analysis

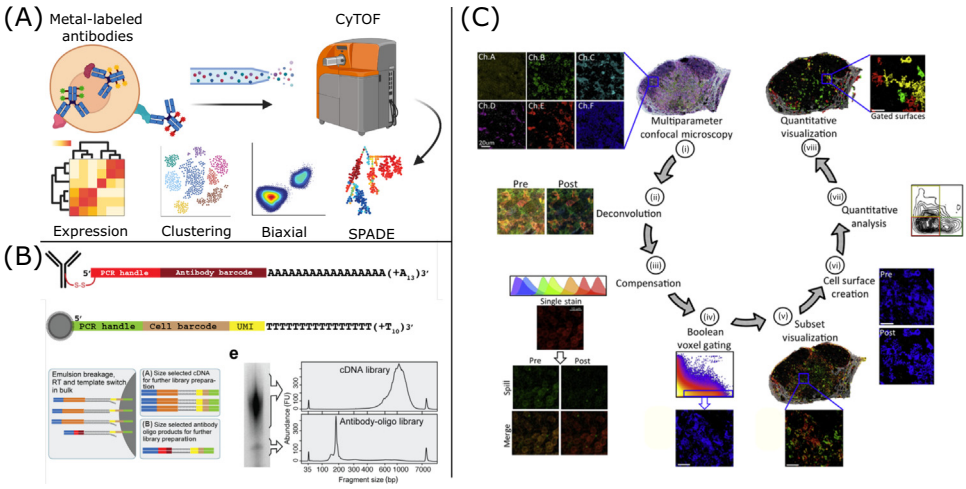

Figure 3. Highly Multiplexed Single-Cell Protein Measurements. (A) Cytometry time of flight (CyTOF) allows dozens of proteins to be measured in the same cell through mass spectrometry [39]. Data can be analyzed via traditional flow cytometry methods, such as biaxial plots, or more advanced methods designed to accommodate the additional dimensionality of CyTOF. (B) CITE-Seq (cellular indexing of transcriptomes and epitopes by sequencing) enables the measurement of even more protein targets than CyTOF by replacing the metal isotopes with DNA barcodes [47]. They are designed such that they integrate into DNA-sequencing protocols, providing the option to analyze mRNA and protein simultaneously. (C) Histocytometry uses an eight step (i–viii) analysis method to enable single-cell interrogation while simultaneously maintaining the spatial arrangement of each cell [51]. Elements of the figure were generated with BioRender. Abbreviations: FU, fluorescent unit; SPADE, spanning-tree progression analysis of density-normalized events.

of density-normalized events to identify cell state transition (Figure 3A) [39]. There is growing interest in using data-driven approaches to identify casual interactions between the proteins being measured in a highly multiplexed experiment [43–45].

Leveraging the Multiplexing Capacity of DNA

Just as a DNA output is a versatile option for absolute quantification, it is uniquely valuable as a multiplexing output. It draws on two major strengths. First, the multiplexing potential is functionally unlimited, as outputs can be coded by specific DNA sequences. Second, it can retain sensitivity even when highly multiplexed through well-established PCR techniques. Recently, two similar techniques were invented that leverage these advantages [46,47]. Both CITE-Seq (cellular indexing of transcriptomes and epitopes by sequencing) and REAP-seq (RNA expression and protein sequencing) use DNA-conjugated antibodies and sequencing to measure the abundance of their targets (Figure 3B). As an added benefit, the DNA oligos were designed to be integrated into existing single-cell DNA-sequencing techniques. These methods have produced some of the most comprehensive measurements of single cells to date, with over 1000 genes and 80 proteins measured from thousands of individual cells [46,47].

Multiplexing While Retaining Spatial Information

While most progress in multiplexing has been achieved using dissociated individual cells, much of the information needed to understand cellular function is encoded in the cell's spatial location. Indeed, studying tissues benefits greatly from enhanced multiplexing, as tissues generally consist of many cell types in various states that require many measurements. Multiplexed ion beam imaging (MIBI) and laser ablative CyTOF are two methods that expand on CyTOF to enable measurement of tissue proteins [48,49]. Both methods vaporize a small amount of tissue and transfer that material into a mass spectrometer to measure the abundance of the labeled protein. Laser ablative CyTOF can measure many proteins simultaneously, but at the cost of destroying the tissue and a modest spatial resolution of 1000 nm [50]. MIBI offers higher spatial resolution (200 nm) and preserves the tissue being imaged but can only measure seven proteins simultaneously [50].

The most widely applied technique for *in situ* single-cell multiplexing is histocytometry [51] (Figure 3C). This method features several cutting-edge fluorescence imaging techniques that are combined to enable visualization of individual cells in a tissue with up to eight proteins on each. Briefly, confocal images are analyzed to account for optical distortions and fluorophore spillover, identify voxels that contain cells, and create a volumetric surface composed of individual cells. In total, the technique enables tissue images with detailed information on individual cells, comparable with flow cytometry. Histocytometry has been used in many studies, primarily focused on understanding immune cell function [52–55].

Emerging Technologies

A mature single-cell proteomics field will be a great benefit to many disciplines, but to achieve its full potential, advances beyond simple detection capacity are required. There are clear challenges that will become more pronounced as measurement technology improves. New data analysis methods need to be created to specifically address questions that can benefit from single-cell proteomic measurements. There is a pressing need to create methods that can identify the most informative measurements and ideally data-driven approaches are needed for prediction of causal relationships. A high priority, after a network of proteins has been measured, is to link the properties of that network to other biological processes. This priority is apparent from the several technologies that enable **multiomic** measurements [38,46,47]. Another major limitation common to almost all single-cell proteomic techniques is the use of an affinity reagent. Moving past antibodies will be a challenging but necessary frontier for single-cell proteomics.

Moving Past Affinity Reagents

Both multiplexing and quantification research have one major bottleneck that limits their potential: antibodies. Antibody selection prevents unbiased analysis, in contrast to DNA sequencing. This problem is exacerbated by the fact that for many potentially interesting proteins, no suitable antibody exists. Furthermore, the process of creating new antibodies is outside the capacity of most research laboratories. Therefore, there is a growing body of research aimed at developing methods to circumvent the need for affinity reagents entirely.

The most well-developed technique for measuring protein abundances without affinity reagents is MS. This method can identify and quantify a high percentage of the proteome directly from lysates [56]. However, there are several impediments to the application of MS to measuring proteins from single cells. Traditional MS proteomic experiments have three phases after cell lysis (Figure 4A). First, the sample is prepared by isolating and fragmenting peptides, followed by cleanup steps to make the sample MS compatible. Second, peptides must be physically separated from each other, which is usually performed with a liquid chromatographic (LC) method. Finally, separated peptides are injected into a mass spectrometer, where they can be identified and quantified. All three of these phases require extensive modification to accommodate the miniscule amount of protein found in single cells. As with targeted methods, miniaturization has been key to achieving single-cell MS, specifically in regard to using small volumes for sample preparation and nanoscale separation techniques [57–60]. The technological improvements that made single-cell MS possible have recently been thoroughly reviewed [61]. However, this review highlights two particularly promising single-cell MS platforms: the nanodroplet processing in one pot for trace samples (nanoPOTS) platform and the single-cell ProtEomics by mass spectrometry (SCoPE-MS) platform (Figure 4B,C) [58,62].

The nanoPOTS platform integrates all sample preparation steps onto a nanowell-patterned slide [58]. The entire procedure generates MS-ready samples at a volume less than 200 nl (Figure 4B). In addition, the use of open droplets in nanowells minimizes surface area and thus prevents unnecessary protein loss to adsorption. Later iterations use nano-LC to further decrease the volume of sample that arrives in the mass spectrometer [63]. By coupling the platform to a single-cell sorter, nanoPOTS was able to identify hundreds of protein groups from individual cells [57]. The platform was used to measure the progression of proteome changes as hair cells developed into maturity [64].

A second platform that has proved capable of robust single-cell MS is SCoPE-MS. This platform incorporates tandem mass tags (TMTs) to improve throughput, peptide identification, and single-cell peptide quantification [62] (Figure 4C). In this study, the peptides from eight individual cells were uniquely barcoded with TMTs. This approach allowed the peptides of all eight cells to be quantified in a single MS run. This is an important advantage, as each MS run can take several minutes, which can severely limit the potential throughput of single-cell MS. In addition, a carrier sample can be included that is composed of the bulk proteome with a unique TMT. The carrier increased the total amount of protein being analyzed, which aided in peptide identification. The first version of SCoPE-MS was able to quantify over 1000 proteins in mouse embryonic stem cells (ESCs) [62]. The second iteration of this platform (SCoPE2) incorporates state-of-the-art data analysis methods, minimal ProteOmic sample preparation (mPOP), and high-throughput automation [65,66]. These improvements enabled the measurement of over 3000 proteins from over 1000 individual monocytes and macrophages.

Another technology that may be sensitive enough to measure unamplified cellular protein is single-molecule peptide sequencing [67]. This method combines cycles of Edman degradation

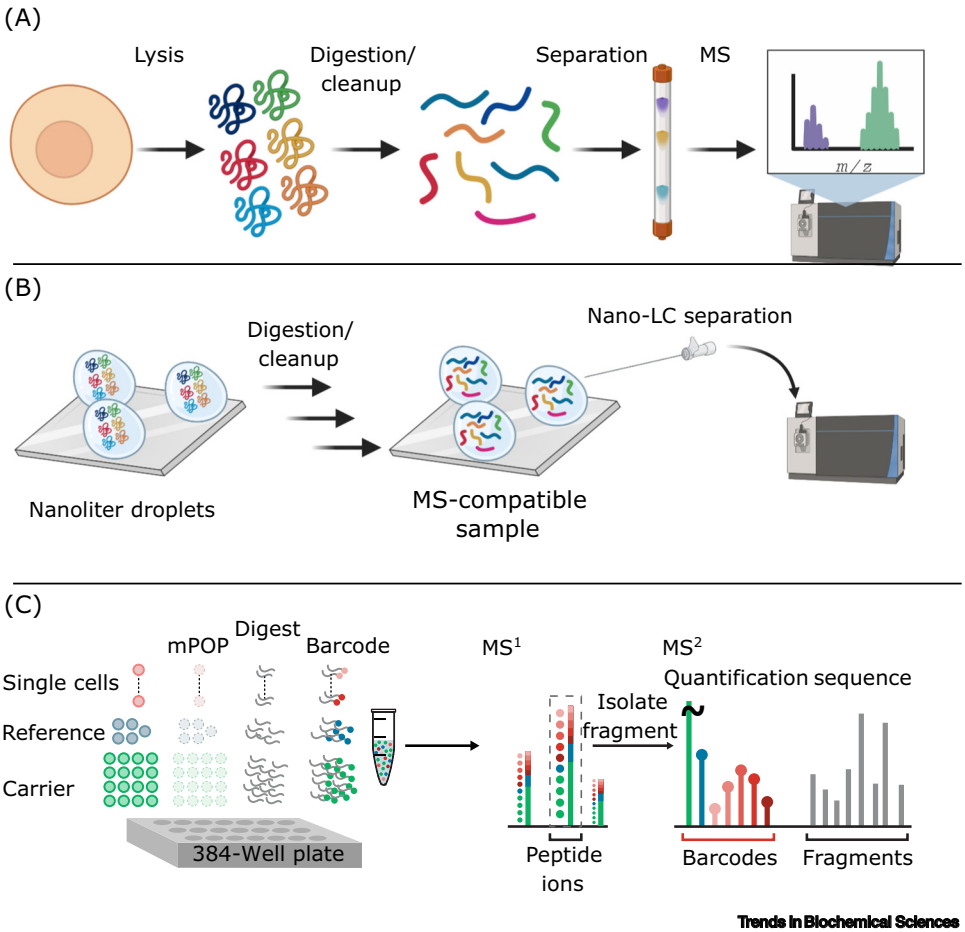

Figure 4. Single-Cell Mass Spectrometry (MS) Methods. (A) MS requires several steps between cell lysis and analysis that must be optimized in order to be compatible with single-cell samples. At a minimum, proteins must be digested into peptides, undergo cleanup to be MS compatible, and be separated from one another. (B) The nanoPOTS (nanodroplet processing in one pot for trace samples) platforms miniaturize each sample preparation step into 200-nl droplets atop a microwell plate. A nano-liquid chromatography (nano-LC) system then separates and injects each sample with minimal dilution. (C) The single-cell ProtEomics by mass spectrometry (SCoPE-MS) platform uses isobaric labeling and tandem mass spectrometry to both increase the sample throughput and improve detection by adding carrier and reference samples. These additions increase the total amount of peptide, making it easier to measure and quantify, while the isobaric labels enable quantification of the peptide in individual cells. Peptides ions selected in the first MS analysis (MS1) are fragmented and passed to the second MS analysis (MS2) for quantification and identification. Elements of the figure were generated with BioRender. Abbreviation: mPOP, minimal ProteOmic sample preparation.

applied to fluorescently labeled peptides, along with sophisticated TIRF imaging to determine the identity of individual peptides. While it has not been applied to single cells, it stands alone as a technology that can simultaneously identify a protein and offer the required sensitivity to apply to single cells.

Multimodal Measurements

The measurement aspect of single-cell proteomics is not an end unto itself. There is a growing realization that coupling multiple modes of high-content data to individual cells can be hugely valuable. This is most apparent in the popularity of multiomic approaches. Currently, methods exist to link together pairs of measurements from the genome, epigenome, and transcriptome [68]. Recently, our laboratory combined PLA with a single-cell sequencing output to create a

proximity sequencing method. It enabled measurement of mRNA, proteins, and potentially thousands of protein complexes from single cells [69]. Other studies have examined the relationship between the mRNA and protein of a particular gene in single cells [37]. However, there is still enormous potential for understanding gene expression through linking single-cell genomic, epigenomic, transcriptomic, and proteomic data. This positions DNA-encoded methods, such as REAP-seq, CITE-seq, and PLA, as potential leaders in the future of targeted single-cell proteomic research.

The cellular state that can be measured by a multiomic technique defines the phenotypic space that a cell can occupy. However, to understand the relationship between cellular state and phenotype, one must have methods that enable simultaneous measurement of both. Microfluidics is well positioned to address this need. Advanced devices can tightly control the cellular environment, perturb the cells, and monitor the result of the perturbation with optical microscopy. There is a significant potential in a technology that enables matching of a cell's 'phenotypic history' to a high-throughput proteomic measurement. However, this demands even more complex microfluidic devices in a field that already suffers from a high barrier to entry. One promising development is an effort to create a community-driven list of standard microfluidic features that may help lower these barriers [70].

Concluding Remarks

Single-cell proteomics has been a long-standing but elusive goal. A comprehensive understanding of cellular function requires measurement of the many proteins that act in concert to control the cell's behavior. The convergence of miniaturization, provided by microfluidics, and nonfluorescent outputs has spurred a rapid growth in the field. However, this growth has largely been focused on extending existing technologies. In order for the potential of single-cell proteomics to be fully realized, several questions need to be addressed (see Outstanding Questions). Current methods may have exhausted the utility of antibodies, creating a bottleneck that can only be relieved by moving past affinity reagents. Second, methods need to be created to unify multiplexed protein measurements with cellular behavior. These advances will need to come from outside the protein measurement field. While advanced microfluidic devices can couple proteomic and phenotype measurements, much more work is required to make such devices accessible and reliable. Once these measurements can be made, new data analysis techniques must be created to identify causal relationships between proteins and cell phenotypes.

Outstanding Questions

Microfluidics is a crucial technology for absolute protein quantification in single cells. However, there remains a high barrier to entry for microfluidics. What can the microfluidics community do to make devices more accessible to the cell biology community?

Single-cell proteomic experiments suffer from the curse of dimensionality. Proteomic data do not allow phenotypic insight from simple inspection. How can protein measurements be integrated into a predictive model of cellular behavior?

For proteomic methods, there is a trade-off between breadth (in the form of multiplexing) and depth (in the form of absolute quantification). Therefore, proteins that are critical for phenotypic outcomes need to be identified for more precise measurements. What methods can be used to identify which components of the cell are responsible for specific phenotypes?

The reliance on affinity reagents makes it difficult to make unbiased experiments. However, they also provide a convenient way to amplify signal and increase assay sensitivity. Is there a way to both identify and measure the minute amount of protein in single cells?

Declaration of Interests

There are no interests to declare.

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