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Single-cell protein analysis

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Heterogeneity of cellular systems has been widely recognized but only recently have tools become available that allow probing of genes and proteins in single cells to understand it. While the advancement in single cell genomic analysis has been greatly aided by the power of amplification techniques (e.g. PCR), analysis of proteins in single cells has proven to be more challenging. However, recent advances in multi-parameter flow cytometry, microscopy, microfluidics and other techniques have made it possible to measure wide variety of proteins in single cells. In this review, we highlight key recent developments in analysis of proteins in a single cell (excluding imaging-based methods), and discuss their significance in biological research.

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Introduction

Proteins are central to all cellular processes – including providing structure to cells, transporting molecules across cell membranes, controlling cell growth and adhesion, catalyzing biochemical processes by functioning as enzymes and regulating signal transduction. Characterizing the quantity and activity of proteins is therefore critical for understanding molecular mechanisms of cellular processes including those involved in disease progression, cell differentiation and fate, and for targeted discovery and development of novel therapeutics, vaccines and diagnostics. Measuring DNA and RNA can provide qualitative information on gene-products (proteins) but cannot provide information on protein concentration, location, post-translational modifications (PTMs) or interactions with other proteins and hence, we need tools and assays to directly measure proteins, their modifications and interactions. Numerous analytical methods have been developed to analyze proteins such as gel electrophoresis, immunoassays, chromatography and mass spectrometry. However, these methods require a large number of cells for analysis, resulting in a population-averaged measurement. Cells are heterogeneous in nature and hence, population-averaged data can mask the underlying molecular mechanisms; more desirable data in many instances could be data at the level of single cells [1–5]. A well-known example is response of bacteria to antibiotics, at certain doses many die but some survive. Similarly, one of the unanswered questions in cancer therapy has been why essentially identical cells respond differently to a drug. Single-cell level measurement of proteins (and other molecules) has provided valuable insight into mechanisms that dictate heterogeneity in cellular response to drugs and other internal and external stimuli. For example, it was reported that dynamic response of tumor suppressor protein p53 network derived from population studies was misleading [4]. Instead of damped oscillations seen in population-averaged data, individual cells show series of undamped p53 pulses with fixed amplitude and duration, independent of the amount of γ-irradiation. Similarly, real-time imaging of transcription factor RelA translocation revealed variability in the oscillatory dynamics of RelA translocation among single cells, and that RelA translocation dynamics determined the degree and timing of downstream gene expression [3]. Usefulness of single cell measurements is obvious for stem cell research as decisions in individual cells determine their fate. For haematopoietic stem cells, studying the varying levels of Sca-1 protein in individual cells revealed that Sca-1 protein abundance determines the timing and type of differentiation [6]. In a clinical context, single-cell level examination of T cell populations previously thought to be homogeneous were found to contain subpopulations with different cytokine profiles [25••], and these differences may serve to predict patient immune response to drug therapy.

Challenges in single-cell protein analysis

The biggest challenges to measuring proteins in single cells (assuming they have been successfully isolated from a tissue, microbial community or culture) are the vanishingly small amount of protein in a single cell and the enormous complexity of the proteome. Adapting traditional bulk protein analysis methods for single-cell level applications has met with varied degrees of success, with quantitative analysis being especially elusive. Proteomic measurements can be quite complex as there are various types of measurements to be made – protein abundance or expression levels, post-translational modifications, protein translocation, interactions with other proteins, DNA, etc., and protein activity (Figure 1). No single analytical method can measure all of these protein

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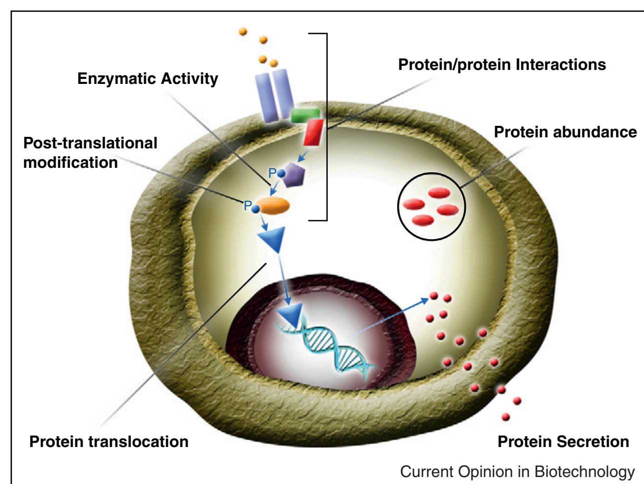
Figure 1



Protein analysis in single cells. Proteome of a cell is complex and requires measurements of many attributes including abundance or concentration of cytosolic, membrane-bound and secreted proteins, protein interactions with other proteins and molecules, inter-organelle and intra-organelle translocation of proteins, post-translational modifications, and enzymatic and other activities of a protein. Colored shapes represent different proteins. P – phosphorylation.

parameters in a single cell hence, a suite of biochemical and biophysical methods have been developed as discussed in the following sections.

Single-cell protein analysis by flow cytometry

The most established and user-friendly method for single cell protein analysis is flow cytometry. Its effectiveness derives from the fact that while the absolute amounts of proteins in a cell can be vanishingly small, the localized protein concentrations can be larger and measurable if the cells are kept intact. Since its invention in late sixties, flow cytometry has been transformed from a technique limited to measuring 1–2 fluorescent species in a cell to 10–15 species today, allowing profiling of entire pathways in single cells. This improvement has been enabled by advancements in both instrumentation and availability of highly specific antibodies. Roederer [7•,8] and Nolan [9••] groups pioneered the use of multi-parameter analysis using multi-color flow cytometers to measure 10–15 key proteins in signaling pathways simultaneously in single cells. The ability to perform correlated measurements of multiple proteins in single cells has turned cytometry into a powerful tool to semi-quantitatively analyze pathways underlying many diseases [10,11]. Tyramide signal amplification, which has long been used as a means to amplify nucleic acid detection in *in situ* hybridization protocols, has been added to traditional antibody staining techniques for analysis of low abundance proteins [12]. While multi-parameter cytometry allowed high content screening (for example, of multiple kinases) in cells, its throughput was still too limited to be useful for drug screening. This limitation was overcome by developing a barcoding method where differently treated cells are tagged with a combination of three dyes [13•]. Each dye, depending upon dilution, permits up to 7 intensity levels and hence, combination of the 3 dyes allows processing of up to 343 samples from one pool of cells.

While flow cytometry has been most commonly used for analysis of kinases and phosphatases, it is also useful for other types of protein measurements such as glycosylation levels and cytokine production. Venable *et al.* used a panel of 14 lectins to characterize glycans present on cell surface as potential markers of pluripotency in human embryonic stem cells [14]. A factor limiting the wider application of flow cytometry to glycosylated protein is that there are very few lectins available. Flow cytometry can also be used for analysis of secreted proteins such as cytokines. This requires treating cells with a vesicle formation inhibitor to trap synthesized cytokines in the Golgi, followed by fixation and permeabilization to stain the trapped cytokines with fluorescent antibodies for flow cytometric analysis [15].

Microfluidic flow cytometry

While commercial flow cytometers allow interrogation of cells one at a time, the sample preparation is still done manually and therefore, requires a large numbers of cells. This makes it hard to analyze samples that are limited in amount such as cells recovered from a biopsy sample, tissue specimens or small volumes of blood. To permit analysis of small number of cells (100–1000), microfluidic platforms have been developed that integrate sample handling with flow cytometry and sorting [16,17]. Srivastava *et al.* [18••] developed an integrated microfluidic device, retro-fitted to commercial microscopes, that can perform cell culture, stimulation and sample preparation in combination with conventional fluorescence imaging and microfluidic flow cytometry (Figure 2) to monitor immune response in macrophages. This microfluidic device not only drastically reduced the amount of sample and reagent required, it also provided a means to perform two orthogonal modes of measurements – imaging and cytometry, in one experiment. Similarly, McKenna *et al.* took advantage of microfluidics to implement a 384-channel flow cytometer for imaging cells. This approach overcomes the low throughput of CCD-based imaging flow cytometers by collecting one-dimensional, low-pixel images ($\sim$1/1000 information compared to a CCD image) in up to 384 fluidic channels simultaneously [19•].

Single-cell protein analysis by mass cytometry

The flow cytometry based analysis of single cells has permitted analysis of as many as 15 proteins simultaneously as described earlier. However, system-level interrogation of biological pathways requires the ability to do many more correlated measurements. A promising

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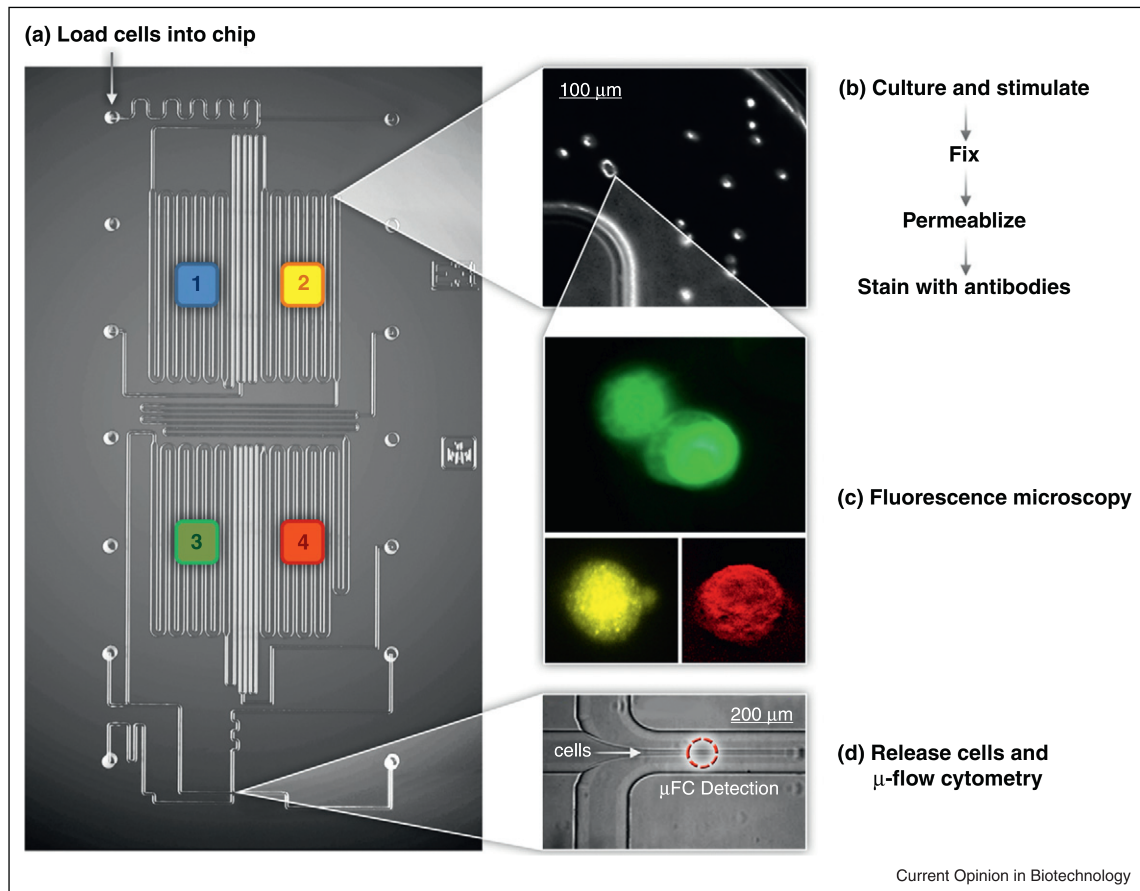
Figure 2



Microfluidic platform for single cell protein analysis. An integrated microfluidic platform that integrates cell culture and sample preparation with two orthogonal single-cell resolution techniques – flow cytometry and fluorescence microscopy [18].

development has been an approach called mass cytometry where the throughput of flow cytometry is combined with the ultra-high dimensionality and sensitivity of mass spectrometry [20•]. In mass cytometry, cells are stained with 20–30 antibodies conjugated to different metal isotope containing polymers [21]. The labeled cells are injected and nebulized and the metal tags are quantified using inductively coupled plasma time-of-flight mass spectrometry. This powerful technique was used to simultaneously profile 34 parameters in single bone marrow cells, including binding of 31 antibodies, viability, DNA content, and relative cell size [22••]. This technique should be capable of measuring even higher numbers of markers simultaneously considering that the precision of mass spectrometry detection overcomes the issue of spectral overlap confounding fluorescence measurements (Figure 3).

Affinity arrays for single-cell protein analysis

Surface immobilized antibody platforms can also be adapted to detect secreted proteins from single cells. For example, commercially available ELISpot method [23], which relies on antibody-coated PVDF membrane to bind target protein followed by detection with a second antibody, was modified to use fluorescence based detection of cytokines secreted from single cells [24]. However, it can take many hours, even days before cytokines are detectable and multiplexing is limited to 1–3 cytokines. A major stride towards multiplexing was made recently in the form of high-throughput single-cell barcoded chip [25••]. The chip contains 1040 3-nl volume microwells containing DNA-barcoded antibodies to more than 10 cytokines, with the quantitation standards at protein copy number resolution built into the antibody barcodes. 1–40 cells are isolated in each microwell, and their cytokine secretions are assessed by fluorescence immunosandwich assay. The chip was successfully used to profile cytokine secretion from tumor antigen-specific cytotoxic T cells and could be useful for profiling of other immune pathways. Another improvement to the affinity array was made by Choi *et al.*, where cytokines released from single cells were detected by antibodies with covalently attached fluorescent oligomers that can be amplified to increase detection sensitivity up to 200-fold [26].

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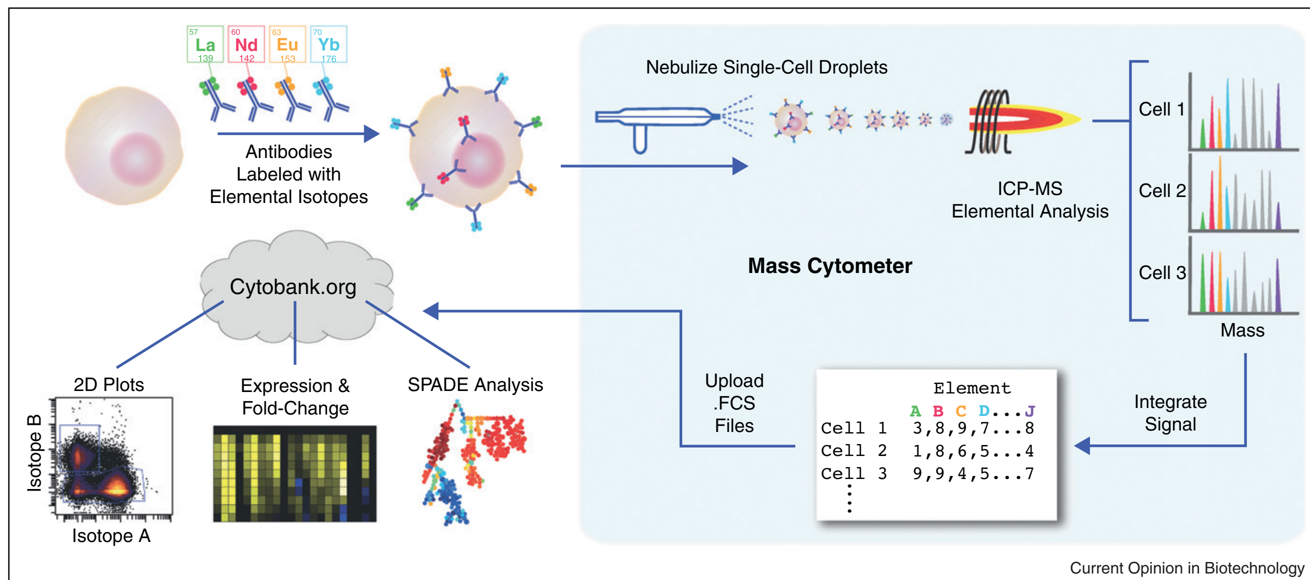
Figure 3



Mass cytometry measurement of immune cell response. Cells are stained with epitope-specific antibodies conjugated to transition element isotope reporters of different masses. Cells are nebulized into single-cell droplets, and an elemental mass spectrum is acquired for each. The integrated elemental reporter signals can then be interpreted using conventional flow cytometry data analysis methods and plotted as heat maps or tree plots. Reproduced with permission from Bendall *et al.* [22••].

Mass spectrometry based single-cell protein analysis

Single cell mass spectrometry (MS) has the potential to provide label-free quantitative analysis of the entire proteome of a single cell, including proteins, peptides and PTMs. The advantage of MS is that there is no need for molecular labels, and femtomolar sensitivity is routinely achieved for pure proteins. Mass spectrometry techniques used for single cell studies include electrospray MS, laser/desorption/ionization (LDI-MS), and secondary ion MS (SIMS). Matrix-assisted laser desorption (MALDI)-MS has been used to analyze neuropeptides in single neurons [27]. Using MS to study proteins in the single cells has its limitations. The biggest drawback is the lack of sensitivity (low signal/noise) to detect low amount of proteins typically found in single cells. Sample preparation methods that can fractionate proteins prior to mass spectrometry can help. In a recent article, integrating microfluidic cell lysis and capillary electrophoretic separation with electrospray mass spectrometry was used for high throughput detection of hemoglobin in individual erythrocytes [28]. A recent report combining microarray with MS analysis holds promise for increasing the throughput of single cell protein analysis [29•].

Separation based single-cell protein analysis

A cell typically has thousands of expressed proteins and high-resolution separation of the cell content into individual proteins by electrophoresis or chromatography can greatly aid measurements of those proteins. The advantage of separation methods, especially multidimensional separations, is that they allow unbiased measurement of the entire proteome in one experiment. This enables researchers to monitor changes in a cell's proteome at a global level as a function of an external stimulus. Conventional scale separations such as HPLC or slab gel electrophoresis are impractical for single cells because of their inability to process minute amounts of proteins. Capillary electrophoresis, implemented in capillaries that mimic the dimensions of cells (10–100 s of μm i.d.) or more recently in microfluidic chips with micron-sized channels, is more promising as it has the potential to separate and analyze proteome of a single cell, especially the large mammalian cells [30•]. To improve peak capacity, two-dimensional separation has been attempted to profile changes in cancer cells [31]. A microfluidic device that integrates capture, lysis, capillary electrophoresis of single cells with single-molecule fluorescence counting was used to quantify rare protein species (<1000 copies per cell) [32•]. Despite these advances, separation based methods are not routinely used in analysis of single cells because of two major factors – 1) the capillary-based or microfluidic chip-based separations do not have the peak capacity to resolve thousands of proteins in a cell and 2) most of the detection methods used do not have the ability to detect low-abundant proteins.

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Genetic and chemical probes for single-cell protein analysis

The accuracy and sensitivity of protein measurements rely largely upon the availability and functionality of the molecular probes that can selectively bind to proteins of interest. Antibodies enjoy widespread popularity owing to their high specificity, but they are not the universal remedy for all protein analysis applications. Antibodies do not enter live cells, so they cannot be used in live-cell studies. They are also cumbersome to generate against new antigens, and too expensive when many proteins are to be profiled simultaneously. Alternatives to antibodies include genetic probes – the GFP family of fluorescent proteins that can be genetically fused to the protein of interest, and translated into fluorescent fusion proteins that are detectable and quantifiable, even for large scale proteomic profiling [33]. The drawbacks of GFP and other genetic probes are (a) that they require genetic engineering of the cell, limiting their application to cultured cells, and (b) their large size can interfere with protein function. To tag proteins with smaller probes in a living cell, a new class of chemical molecules, referred to as bioorthogonal probes, has been developed. Bioorthogonal chemistry utilizes the cell's own machinery to covalently incorporate abiotic probes into specific cellular components including proteins and their post-translational modifications. Some of the highlights in bioorthogonal probe development include the biarsenical fluorescent dye FLAsH, which specifically reacts with target proteins genetically fused with the tetracysteine motif [34]; the carbonyl condensation of ketone-functionalized amino acids into proteins and detection by a hydrazide-functionalized fluorescent probe [35]; and the selective bioorthogonal chemical probes for metabolic labeling of glycans [36••].

Future perspective

The future of single cell protein analysis will depend on several factors. First, a larger repertoire of high affinity probes (other than monoclonal antibodies) are needed to detect low abundance proteins, post-translational modifications and protein interactions. Some promising candidates include aptamers, single-chain variable fragments, and biorthogonal reporters. Second, techniques that can effectively and reliably isolate single cells from culture, tissues, clinical and environmental samples will need to be further improved in their yield, ease-of-use and reproducibility. Third, most of the techniques described above use experiments at the population level followed by analysis at the single cell level. It will also be useful to have technologies that enable experimentation as well as subsequent analysis at the single cell level. For example, introducing one virus to one immune cell and recording the interaction in real time. While such experiments do not necessarily represent biological phenomena *in vivo*, they can simplify the interactions sufficiently to make basic observations that are otherwise hidden in a population experiment. Finally, in order for new single-cell technologies to become widely used, we also need to develop informatics and modeling tools that are customized for single cells and can integrate the protein measurements with other types of single cell measurements such as genomics, transcriptomics, and metabolomics.

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