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REVIEW

Emerging single-cell technologies for functional proteomics in oncology

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ABSTRACT

Introduction: Cellular heterogeneity has challenged current cancer therapeutics and hindered the discovery and development of cancer drugs. The heterogeneity in functional proteome is of particular interest because many cancer drugs are developed to target signaling proteins. The complex nature of tumor systems calls for more advanced multiplexed single-cell tools to address the heterogeneity issue.
Area covered: Over the past five years, there are a few single-cell functional proteomics tools introduced with unprecedented multiplexity and performance that are transforming the oncology field. Those tools are generally categorized as cytometry-based tools and microfluidics-based tools, and we discuss the representatives in both categories.
Expert commentary: The single-cell tools have provided an avenue to understand the multifaceted differences of cancer cells, the complex signaling networks, and the relationship of intercellular interaction and tumor architecture. We also provide an outlook of single-cell tools in five years and the challenges to address before a greater impact on oncology can be made.

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1. Introduction

Cellular heterogeneity occurs intrinsically and universally at multiple molecular-omics levels such as metabolomics, transcriptomics, and proteomics in otherwise identical cell populations [1–3]. The proteomic level of heterogeneity is of particular interest because proteins carry out the majority of cellular functions including signal transduction, cytokine/chemokine secretion, cell migration and invasion, and other physiological processes [4,5]. In oncology, cancer cells always bear aberrant signaling pathways, and therefore, signaling proteins are one of the major drug targets. Since functional proteins are on the frontier of cell response to environmental stimuli (e.g. signaling cues from neighboring cells), studying cellular heterogeneity in functional proteome would also be crucial for understanding the societal aspects of a cell population. With the advent of new multiplex single-cell tools, functional proteomics has recently constituted an emerging research area that is focused on monitoring and analyzing the spatial and temporal properties of molecular networks and fluxes involved in living cells [6–9]. With high multiplexity, the emerging single-cell analysis overcomes the old paradigm 'one gene-one protein' that does not reflect the real nature of a cell. Single-cell functional proteomic analysis not only reflects heterogeneity, but also possesses the power of early predicting cancer therapy outcomes and revealing regulatory mechanisms more detailed than ever [10]. Unconventional algorithms for data processing have been developed to uncover the hidden information in high-dimensional data, which is beyond the traditional bioinformatics in that many of those algorithms are borrowed from other disciplines such as thermodynamics and physics. With enough statistics, single-cell data have been exploited to rebuild the whole picture of a multicellular tumor system, providing a global view of tumorigenesis and disease progression in a great detail. All these are not achievable by conventional flow cytometry and bulk assays.

The advances of single-cell functional proteomics push technology development toward innovative solutions for technical and biological problems. There is a constant drive toward miniaturization, and in parallel, multiplexing, while increasing sensitivity and maintaining robustness. Along with technology development, the need for more extensive tools in data collection and analyses becomes more imperative [8]. The cost associated with any single-cell analysis must also be minimized in order for the technology to gain widespread use and encompass the broadest sectors of research and clinical audiences. Microfluidic tools are naturally suitable for manipulating cells and assaying biomolecules and thus are the future technologies to move forward the single-cell field. While flow cytometry still dominates the single-cell proteomic analysis field, microfluidics has recently been demonstrated to be comparable with flow cytometry in multiplexity and throughput, with additional capabilities that flow-based cytometry lacks.

We herein present a review on the state-of-the-art single-cell functional proteomic technologies and put the emphasis on microfluidics-based tools. These tools are important in that they gradually shift the paradigm of cell biology techniques and disease diagnosis, but they are still not quite familiar in the biomedical and oncology fields. Applications to oncology will be discussed, as well as the approaches for carrying out analyses on the large body of data generated from these

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technologies. Two-cell heterogeneity will also be discussed here as a study that bridges the gap between single-cell proteomics and tissue-level studies.

2. Challenges in single-cell proteomic assay

Major functional proteins, such as secreted proteins and intracellular phosphorylated proteins, can be associated with signaling networks stimulated by growth factors. The signaling mechanism could be, to some extent, revealed through accurate functional protein quantification. Although secreted proteins (e.g. growth factors and cytokines) have well-studied roles in immune response, few studies have been done on the function of secreted proteins and their interaction with intracellular signaling networks in cancer cells at the single-cell level. Prior investigation at the bulk level has inevitably missed important cell machinery information. Bulk-level technologies such as Western blot and protein mass spectrometry require lysis and mixing of >1000 cells together, and therefore, the heterogeneity information is lost and biomarkers of rare cells will not be identified. Another issue is that the large discrepancy of protein abundance (generally spanning several orders of magnitude) in single cells makes the accurate quantitation of multiple functional protein molecules challenging. Secreted signaling proteins and intracellular phosphoproteins could be present as low as tens of copies, which are already capable of triggering the response of signaling cascades. To address this issue, a single-cell functional proteomics assay first has to be sensitive enough to measure even very low quantities of proteins for isolated cells, two-cell systems, or for other small groups of *n* cells. The wide, linear range of assay quantification is equally important.

Another challenge lies in recapitulating the physiological conditions under which the protein is activated or produced. The *in vivo* microenvironment for cell populations is always complicated, and some *in vitro* artifacts from proteomic techniques, when not accounted for in data analysis, may threaten the validity of the research method. Live single cells separated in a tumor tissue would be expected to alter their regulatory network immediately to survive a new environment without cell adhesion and communication. These '*in vitro* artifacts' unfortunately exist more or less for all single-cell tools. Any conclusions from single-cell analysis must be validated in animal experiments and in low-multiplex techniques such as immunofluorescence staining. The emerging microfluidic technologies alleviate this problem by creating on-chip microenvironments to approach the *in vivo* conditions. Although most single-cell technologies are able to identify heterogeneous subpopulations, their dynamics during disease progression has never been obtainable, and the single-cell information has not been able to predict cancer metastasis and treatment efficacy. Thus, to address the differences of *in vitro* and *in vivo* microenvironments would be the key to advancing single-cell analysis in clinical diagnostics.

Measurement noise is very common in single-cell assays because of low quantity of proteins, and thus, those assays must possess much higher signaling-to-noise ratio than that of bulk assays. High noise background may significantly undermine the usefulness of the single-cell tools. For example, a distinctive subpopulation might be masked by the noise of detection measurement. However, high statistics can partially attenuate this problem. To obtain authentic biological information, the technologies must permit measurement of enough number of single cells in a high-throughput fashion so that the comparison is statistically significant evaluated by *P*value. This minimum throughput is mathematically dependent on both multiplexity and the depth of heterogeneity analysis. Normally, at least 1000 single cells should be measured to cover the major cell subsets and represent a whole tissue population. Measurement noise often stems from variable antibody quality and cross-reactivity. Almost all single-cell functional proteomic technologies are relying on antibodies for detection. Commercial antibodies provided by different vendors vary significantly in sensitivity and specificity. Before any single-cell assay, the antibodies must be fully validated using recombinant proteins or cell lysates by protein microarrays or Western blotting to ensure none of antibodies reacts with others and nontarget [11]. When the multiplexity is over 10, cross-reactivity occurs frequently and the assay cost increases dramatically (Table 1), so many groups cannot afford highly multiplexed single-cell analysis.

Finally, given that the single-cell proteomics techniques are still in their infancy, the new format of data generated from single-cell tools presents a challenge to the available analytical tools for protein quantitation and information extraction. There is still much to explore from the multiplexed single-cell data besides visualization of heterogeneity. It is also noteworthy that the kinetics associated with both the generation and degradation of the protein sample, or the proteomics assays described here, are typically carried out within 12 h. If these challenges are carefully considered, along with more general knowledge such as cell phenotypes, culture conditions, etc., it is possible to perform the single-cell functional proteomics assays for a wide range of applications.

An ideal single-cell functional proteomics tool would acquire the features of multiplexed readout, rapid manipulation of cells at single-cell level, and highly sensitive detection of the given proteins (e.g. cytoplasmic, membrane, and secreted) in small copy numbers. The representative tools to date basically include multiparameter flow cytometry, mass cytometry, enzyme-linked immunospot (ELISpot) technique, and microfluidics-based cytometry (Table 1). Microfluidics-based tools hold unique advantages over the other counterparts due to their powerful single-cell handling capability, flexible custom design, and multifunctional integration. These typical tools will be discussed in the following text, and the emphasis will be on microfluidics-based technologies.

3. Flow-based cytometry

Flow cytometry methods, such as fluorescence flow cytometry (FFC or fluorescence-activated cell sorting), have become the standard in single-cell protein detection for long. In FFC, cellular proteins are detected using fluorophore-labeled antibodies (Figure 1(a)) where each type of antibody corresponds to one type of fluorophore, limiting the multiplexing to be <17 due to overlap of fluorescence spectra [12,13]. With decades of

Table 1. Characteristics of single-cell functional proteomics tools.

Methods	Protein detection strategy	Multiplexity	Sensitivity (molecules/cell)	Comments	Refs
Fluorescence-activated cell sorting (FACS) and flow cytometry	Fluorophore-tagged antibody labeling	~17; routinely 1–3	~40	• High throughput with excellent statistics • Mature technique • Large sample size required	[12,13]
Mass cytometry (CyTOF)	Mass-tagged antibody labeling	~100; routinely <30	~400–500	• Throughput similar to FACS • High cost	[15–18]
Single-cell barcode chip	ELISA on spatially distributed antibody array	~42; routinely 5–12	~50	• Both secreted and intracellular proteins • Small sample size • Analysis of cell–cell signaling • Adjustable microenvironment	[4,26–30,32,33,35,42]
Microengraving	ELISA on coated glass slide	Routinely 1–3	~10	• Large sample size • Analysis of cell–cell signaling • Cost-effective • Recovery of analyzed cells for further analysis • Adjustable microenvironment	[21–24]
Single-cell Western blotting	Miniaturized gel electrophoresis and Western blotting	Routinely 3–12	10^3–10^4	• Many antibodies available • Fast (4 h) • Protein ladder available	[19,20]

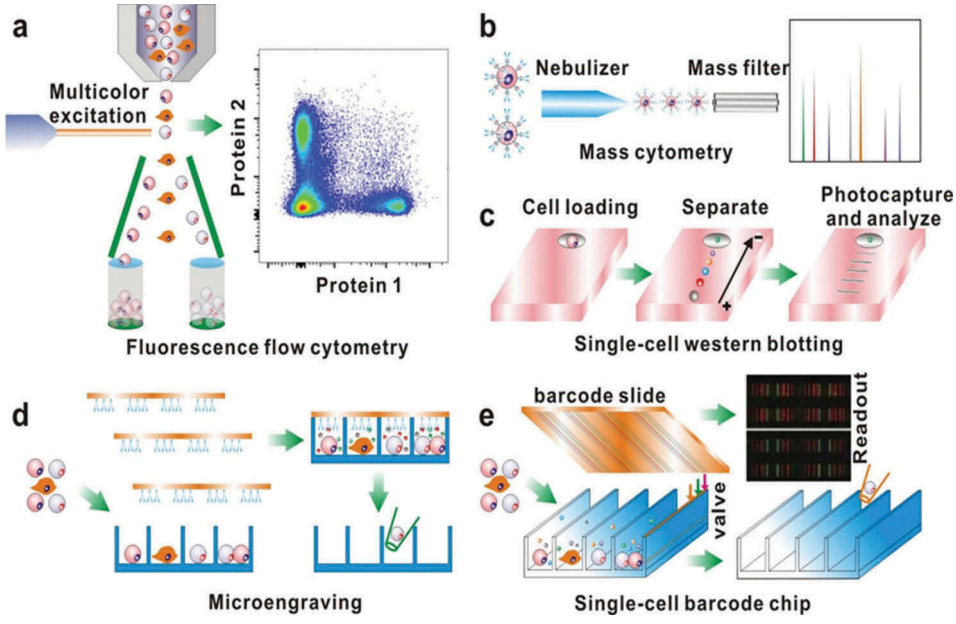

Figure 1. An overview of five technologies used for single-cell functional proteomics. (a) Multiparameter fluorescence flow cytometry. Cells are labeled with antibodies tagged with fluorophores. (b) Mass cytometry. Antibodies for labeling are encoded with transition metal-containing mass tags. Single cells are first atomized and then go through the mass filter to separate the transition metal atoms, which mass are subsequently analyzed. (c) Microchip-based single-cell western blotting. A miniaturized gel is used for electrophoresis and separation of proteins extracted from single cells. (d) Microengraving technology. Cells are isolated into nano-size chambers, and the secreted proteins are captured by antibodies on a cover slide. (e) Single-cell barcode chip. This assay also isolates single cells into respective chambers of the chip, but the antibody array on a glass slide is miniaturized in a barcode shape.

effort, FFC has been well developed and become the primary single-cell tool for studying biomarker distribution. However, protein measurement by FFC is usually not quantitative. FFC is not much used for assaying secreted proteins either, because secretion has to be blocked before immunofluorescence labeling, which may significantly interfere with intracellular signaling. The use of some new nanocrystal fluorescent probes, including multicolor nanoprobes, can potentially overcome the multiplexity limit of FFC, albeit likely toxic to cells [14]. The size and the requirement of extra resources have largely limited FFC as a point-of-detection platform in the applications outside of a laboratory environment. Portable

flow cytometry such as BD Accuri model personal flow cytometry has been introduced before, but they are still not standalone instruments applicable in a resource-limited environment.

Time-of-flight mass cytometry (CyTOF) revolutionizes the flow cytometry technique in that the detection multiplexity has been boosted to 100. This technique relies on metal isotopes, inductively coupled plasma mass spectrometry, and time-of-flight mass spectrometry (TOF-MS) for detection (Figure 1(b)), while cells are still flowing through channels like in flow cytometry [15,16]. This detection strategy, however, slightly limits the sensitivity. To achieve spectrally resolved protein assay, the antibodies of interest are first conjugated with isotopically rare-earth metal elements and then bind to the membrane protein or intracellular functional protein. After nebulization, these metallically tagged cells will be sent to an argon plasma for ionizing the multiatom metal tags, which are subsequently analyzed by a TOF-MS. The major advantage of CyTOF over conventional fluorescence-based flow cytometry is that the isotope spectra barely overlap with excellent separation, resulting in dramatic increase of multiplexity [17,18]. Similar to multiparameter FFC, CyTOF is a high-end instrument that is costly and therefore is not accessible for many institutes, and the operator needs extensive training.

4. Microfluidic methods

A few single-cell microfluidic prototypes have been demonstrated for analyzing single-cell functional proteome. Their sensitivity, multiplexity, and throughput are varied greatly. Single-cell Western blotting (scWestern) miniaturizes electrophoresis gel to obtain single-cell sensitivity and is aligned well to standard molecular biology practice. This technique uses a microscope slide supporting a layer of photoactive polyacrylamide gel (~30 mm thick) for Western blotting (Figure 1(c)) [19,20]. Single cells are loaded into the on-chip microwells, and the consecutive *in situ* lysis, gel electrophoresis, and photoinitiated blotting are executed to separate and immobilize proteins in bands. Such a streamlined scWestern method is capable of monitoring single-cell differentiation of rat neural stem cells and responses to mitogen stimulation. Compared to the sandwich ELISA-based protein assay, the scWestern only needs one antibody to target one protein, and there are plenty of antibody choices on the market for detecting intracellular proteins. Cross talk of detection is usually not a concern for this Western blotting-based technique, and the multiplexity has reached 11 protein targets per single cell as reported. Unfortunately, assay sensitivity remains disappointingly at about 10,000 or above copies per cells, while the quantity of signaling proteins often falls beyond that range.

The other prominent single-cell technology, namely microengraving technique, permits analysis of three secreted proteins in a high-throughput fashion (Figure 1(d)) [21]. Operation of the microengraved single-cell chip is accomplished by trapping cells on a specially designed chip containing hundreds of thousands of nanowells, all of which are collectively sealed by an antibody-coated glass slide. The cells in nanowells can be cultured on-chip for hours to allow for cytokine release and capture by the antibody array, and these cells are also accessible to further immunostaining. This technology can detect approximately three secreted proteins using sandwich ELISA, as well as permit the culture and recovery of specific cells [21]. Thus, this technique possesses notable similarities with ELISpot. Also of note is the ability to perform single-cell kinetic studies on the temporal secretion of cytokines [22]; this is carried out by replacing the glass cover of a microchip after designated periods of time. With those capabilities, Varadarajan efficiently identified antigen-specific responses of 10^4–10^5 single cells from blood and sorted T cells with specific functions in terms of cytokine production [23]. In addition, the microengraving platform can also be used to measure a few transcripts at the single-cell level by PCR [24]. The ability of creating cell-friendly microenvironment and directly detecting secreted proteins renders microengraving technique advantages over flow cytometry. However, the multiplexity of this technique is limited, mainly because it relies on fluorescence spectra to distinguish various binding events. Detection of intracellular proteins using this technique has not been achieved because the sealed nanowells could not permit *in situ* cell lysis. However, other researchers circumvent that issue by quickly isolating nanowells after adding lysis solution [25].

5. Single-cell barcode chip

The single-cell barcode chip (SCBC) is a similar but more multiplexed technology [26]. Advantages of this technique include a higher level of multiplexing, and the ability to assay for secreted proteins, cytoplasmic proteins, and membrane proteins, as well as still being able to fluorescently stain the cell. This method also enables the incorporation of cell interaction parameters into protein measurements while generating a controllable microenvironment similar to the *in vivo* one.

The SCBC is basically composed of a high-density, multiplexed antibody microarray and a polydimethylsiloxane (PDMS) replica. In general, capture antibodies are patterned in an array fashion, and such antibody arrays are aligned with single-cell microwells or microchambers, allowing for high-throughput immunoassays on the entrapped single cells (Figure 1(e) and Figure 2(a)). The platform may be extended to include analyses of pairs or larger groups of cells, thus providing information on cell–cell communication. A single microchip can contain as many as 10^4 microchambers for analysis with volumes ranging from 0.1 to 2 nL [4,27]. The method allows for sandwich-type ELISA of membrane proteins, as well as secreted proteins and intracellular proteins (Figure 2) [26]. The barcode antibody microarray is formed using perpendicular flow patterning of a DNA library followed by conversion to an antibody microarray using the aforementioned DEAL technique [28–30]. Each chamber encapsulates a complete repeat of the antibody microarray, so that all the microchambers of a chip are subjected to exactly the same sandwich ELISA procedures. A random number of cells can be entrapped within microchambers for detection of analytes of interest. To assay secreted proteins, cells are incubated in the SCBC for at least 6 h, and then the microarray slide is developed.

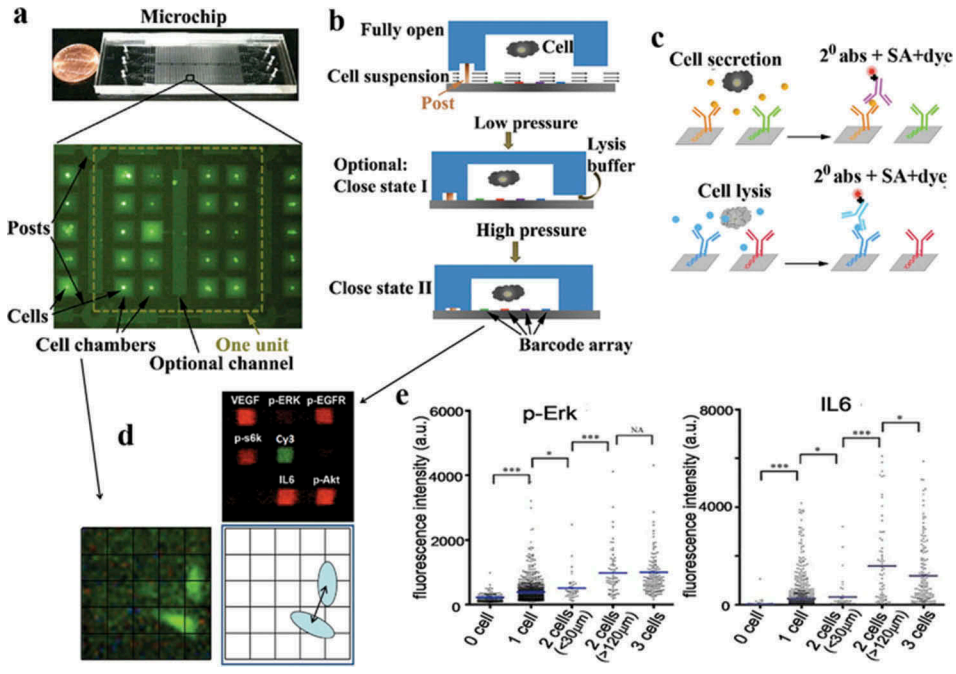

Figure 2. An overview of the SCBC platform. (a) A digital photo of the PDMS microchip with a fluorescence micrograph of a 20-microchamber unit (there are 435 of these per individual chip) with the central channel housing the cell lysate. (b) Schematic of the chip operation from the fully open to closed state. Cells are loaded in the open state, lysed in the close state I, and completely isolated in the close state II. (c) The stepwise illustration of protein capture and detection using a sandwich-type immunoassay, detecting secreted, cytoplasmic, or membrane proteins. (d) Fluorescence data collected from each microchamber, including the position of the cells and the fluorescence intensity from the respective assays. (e) Fluorescence intensity data for analyzed proteins, specifically secreted interleukin-6 (IL-6) and cytoplasmic phosphorylated signal-related kinase (p-ERK). Reproduced with permission from Wang et al. Quantitating cell–cell interaction functions with applications to glioblastoma multiforme cancer cells. Nano Letters, 12(12), 6101–6106 (2012) [4] . Copyright 2012 American Chemical Society.

To assay intracellular proteins, cell lysis buffer supplemented with phosphatase/protease inhibitors is introduced into the microchambers of SCBC after a sufficient incubation period, after which the microarray slide is scanned. In combination with microscopy imaging, single cells fluorescently stained in microchambers could be counted, and their corresponding microchamber addresses are recorded. Such information is needed in correlating cell number with the protein signal, since random number of cells fall into each microchamber. The empty microchambers are usually taken as the background or the base line against which protein signals will be measured.

The first version of SCBC had relatively low throughput – it was able to analyze about 100 cells per chip [26], with each specific protein being analyzed twice to generate statistically representative data. This generation of SCBC operated using a system of microfluidic pneumatic valves and a two-layer microfluidic assembly [31]. It featured a total of ~600 isolated microchambers for the entrapment of single or multiple cells on one microchip. The drawback to having two or more array repeats per microchamber (relatively larger than later versions, this microchamber had a volume greater than 2 nL) is that it usually gives rise to fluctuations in the fluorescence readout. One complete set of the array may not have the same fluorescence intensity as the repeat, because a single cell might be in closer proximity to one of these sets, and consequentially, more proteins are captured on that specific set. Shi and Wei carried out a thorough study on data analysis approaches that take these fluctuations into account [32]. They applied Monte Carlo simulations in order to extract the real biological signals while excluding measurement noises.

A more advanced variation of the SCBC has a sophisticated microchip design that obviates the need for pneumatic valves [4]. The valveless method allows 30-fold more throughput than the previous design. The access of cells, buffers, and antibody solutions, etc. to microchambers is controlled by altering the height of deformable PDMS posts (Figure 2(a,b)). When slight pressure is applied, the PDMS posts are compressed, and this change enables chemical communication between the microchannels and microchambers without allowing the cells to move between chambers. Upon application of sufficiently high pressure, the PDMS posts completely collapse, allowing the main body of the microchip to be in contact with the glass substrate, thereby sealing microchambers. Cells are incubated in the isolated microchambers, while the antibody array captures secreted proteins. With that convenience, the whole microchip-clamp assembly is portable and operable outside of a biology or chemistry laboratory without accessory facilities. The whole procedure of on-chip operation only needs turning adjustment screws and filling/taking away solutions by a pipettor, and thus an untrained operator would be able to handle it by following a protocol.

SCBC is currently the only technology possessing a high multiplexity comparable to CyTOF. Fan's group reports codetection of 42 immune effector proteins in single cells using a similar technology, which covers most cytokines and chemokines related with immune functions [33]. The SCBC permits routine quantification of >10 intracellular proteins without compromising sensitivity [26]. However, the common issue associated with multiplexed detection is antibody cross talk or low specificity. The ELISA antibody pairs used in those assays

have to be carefully validated using recombinant proteins or reagents with known antigens. To address this cross-reactivity issue, Juncker's group developed a snap chip for multiplexed sandwich immunoassays without mixing. A slide with multiple arrays of 10 more different capture antibodies is incubated with a sample, and then all detection antibodies transfer at once by snapping, each to the cognate spot [34]. This approach eliminates the chance of cross-reaction and may bring an opportunity to single-cell analysis if the array can be minimized.

6. Signaling networks generated by single-cell data analysis

The fluctuations of functional proteins across many cells imply the protein–protein connections and how signal is transduced. For example, in a cell population, the copy number ratios between protein 1 and protein 2 remain approximately the same for all the single cells. Then, with enough statistics, it is safe to conclude that protein 1 and protein 2 are in one signaling pathway. This is a simple approach to understand the network generated by single-cell functional protein assays. However, a signaling protein might be the joint point of multiple signaling pathways, and therefore, the ratios between protein copies may not be exactly integers. This is confirmed by the single-cell network analysis results (Figure 4). The thickness of connection lines and the color of edges reflect the strength of protein–protein correlations and the relative abundance of those proteins, respectively. The cutoff threshold is usually set at 0.5. Under various conditions, the correlation maps, or signaling networks, are significantly changed, indicating two individual cells are strongly influencing each other by communications.

As mentioned earlier, each functional protein may take part in multiple signaling pathways, and the signal transduction between any two proteins might be mediated by others. Therefore, the more meaningful derivation of signaling networks is through decomposition of the whole networks into different tiers to approach the real picture of signaling networks. Shin et al. employed an information theory, Le Chatelier's principle, to profile the signaling networks from single-cell data, and provided an approach to quantitatively predict the role of perturbations and characterization of a protein–protein interaction network [36]. The theoretical prediction is made by seeking that distribution of copy numbers that is of maximal entropy, meaning that the distribution is as uniform as possible subject to a given mean number of copies. This means that at the very global maximum of the entropy, the probabilities of the different proteins are not equal. Rather, as in any multicomponent system at thermal equilibrium, each protein will be present in proportion to its partition function where the partition function is the effective thermodynamic weight of a species at thermal equilibrium. The whole system, a single cell (or a small colony), is regarded as not being in an equilibrium state because it is under the action of constraints. When the constraints are present, the system is in that state of equilibrium that is possible under the constraints. Here, a single cell is a confined system, and the protein copies in this confined system are quantitated, so the modified Le Chatelier's principle combined with certain mathematic manipulations leads to newly constructed signaling networks with multiple tiers, with each tier representing the weight of the subnetwork in the whole system. This theory has been successfully applied to phosphoprotein networks in response to hypoxia as well as cancer cell interactions at various distances [32,35].

7. Applications in oncology

In the following content, we will discuss the use of SCBCs as the alternative to conventional proteomic tools and as useful devices in cancer immunotherapy and cancer biology studies. Shi described the first SCBC assay on single glioblastoma cells expressing epithelial growth factor receptor variant III (EGFRvIII) which consistently activates PI3K and MAPK pathways and promotes cell proliferation [26]. Eleven phosphoproteins related with PI3K pathway were quantitated for each single cell. It is found that the connectivity between phosphoproteins in EGFRvIII cells is much higher than that in wild-type EGFR cancer cells, implying that inhibiting of one signaling protein as drug target would not fully impede cell growth [26]. Because cancer cells might live in a hypoxia environment, Wei investigated the influence of hypoxia to cancer signaling by creating a low oxygen concentration culture condition on the SCBC while still being able to monitor signaling protein levels [32]. Signaling network associated with mammalian target of rapamycin (mTOR) was found responding to oxygen level quickly and switched the connectivity between proteins at near 1.5% oxygen content. This switch is similar to phase transition in physics, and thermodynamics-motivated principles are brought into understanding of cancer cell behavior under various conditions and predicting of drug responses. Bulk assay also proves that signaling proteins are connected in a particular way depending on the cell type [37]. Cancer cells may employ multiple signaling pathways and swiftly shift signaling connections to avoid inhibition and keep proliferating. This informs that the clinical therapies have to consider alternative survival pathways when using a targeted drug. However, due to cancer cell heterogeneity and plasticity, prediction of alternative signaling pathways, if achievable, would significantly improve clinical therapeutic effectiveness and personalize cancer treatment. The SCBC has been applied to test the possibility of predicting drug resistance mechanism by measuring alternations in the protein signaling coordinations (Figure 3) [10]. A patient-derived glioblastoma model of mTOR kinase inhibitor resistance was used in that study where coordination of phosphoproteins was measured. This approach may identify actionable alterations in signal coordination that underlie adaptive resistance, which can be suppressed through combination drug therapy.

Cancer immunotherapy has become one of the primary cancer therapies. It leverages the patients' own immune system to eliminate cancer cells and potentially keeps the body immune to cancer for longer time. Of particular interest in immunotherapy studies are leukocytes, or T cells from blood and tumor tissue, specifically the quantities of specific

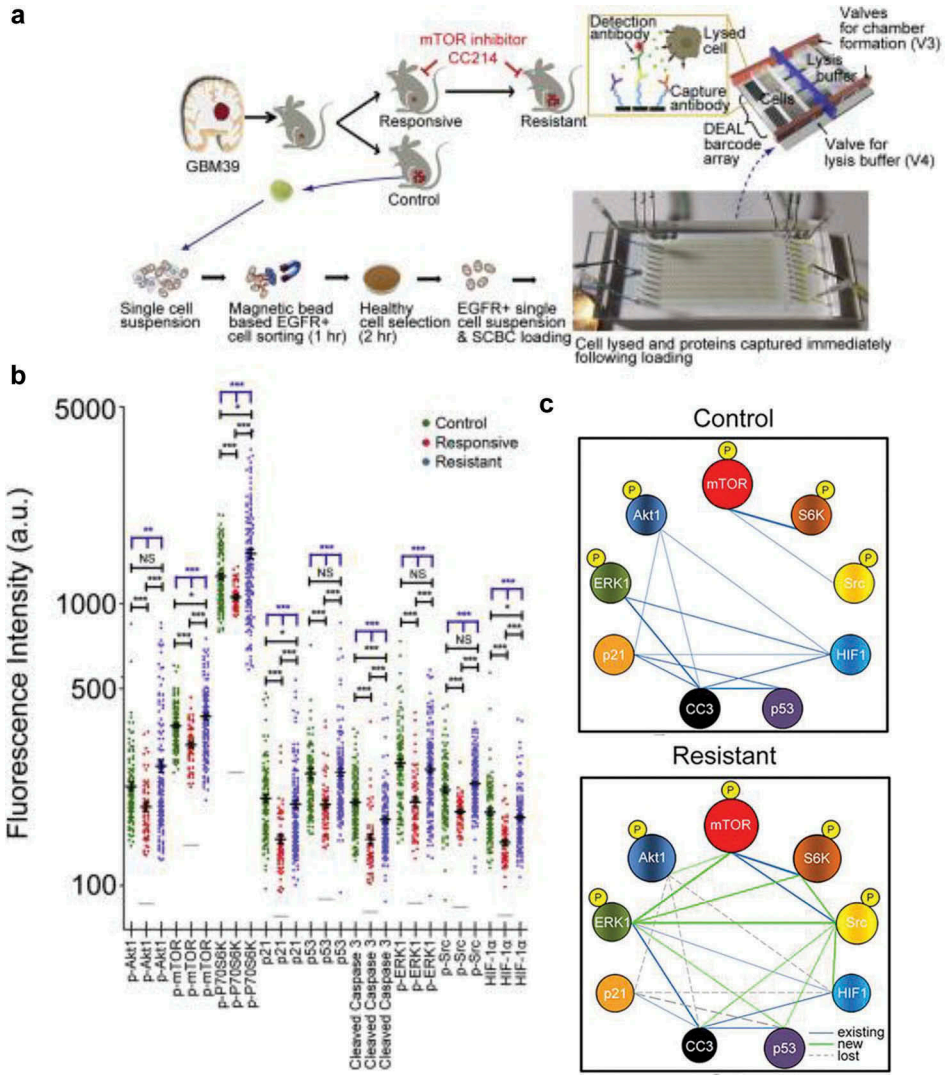

Figure 3. Single-cell intracellular protein analysis by single-cell barcode chip. (a) The procedure of cell separation and on-chip analysis with drug treatment. EGFR$^+$ cells were separated from the GBM39 models and loaded onto an SCBC. (b) Background-subtracted SCBC data represented as one-dimensional scatterplots with mean ± SEM for each protein shown as black horizontal bars. Gray bars indicate the background level of each protein assayed. Statistical uniqueness is evaluated by two-tailed MannWhitney test for pairwise comparison (*) and Kruskal-Wallis test for comparison among three groups (*). (c) Representative protein-protein correlation networks, extracted from SCBC data. Average protein levels are reflected in the sphere diameters, while correlation strengths are reflected in the thickness of the edges. For the resistant state, existing, new, and lost correlations, relative to control, are indicated. Reproduced from Wei et al. Single-cell phosphoproteomics resolves adaptive signaling dynamics and informs targeted combination therapy in glioblastoma. Cancer Cell, 29(4), 563–573 (2016), with permission from Elsevier [10] .

cell subsets, secretions, transcriptional states, and their proliferative profiles [38]. T cells could be engineered to be capable of recognizing cancer cells and subsequently release cytotoxic factors. T cells are naturally heterogeneous; that is, how they are able to recognize numerous pathogens and other invaders by surface T-cell receptors [39]. However, those functions, often indicated by secreted effector proteins that mediate the tasks of target killing, self-renewal, inflammation, and cell communications, are not always consistent with surface markers. Using single-cell functional proteomic tools, recent studies show that only a small subset of T cells of a particular type can secrete large amounts of cytokines and chemokines, and therefore, such a subset serves as the major player in the immune response [27,40]. In addition, the secretion profiles of different types of T cells defined by surface markers have significant overlaps. It is also found that a portion of hematopoietic stem cells and progenitor cells are the active responders in immune defense against pathogen invasion [41].

The single-cell tools for studying cancer cells are also trending toward integrating multiple omics together to provide a more comprehensive picture. For example, simultaneous quantification of both signaling proteins and metabolites can potentially reveal the physiological connections between the two important classes of oncology biomarkers, the protein-mediated signaling networks that are signified in tumor maintenance and growth, and the metabolite molecules that produce energy for cell growth or drive the metabolic signal transduction [42]. Xue applied an SCBC-type chemical method to measure a few phosphoproteins and metabolites including cyclic adenosine monophosphate, cyclic guanosine monophosphate, and glutathione, which are closely related to

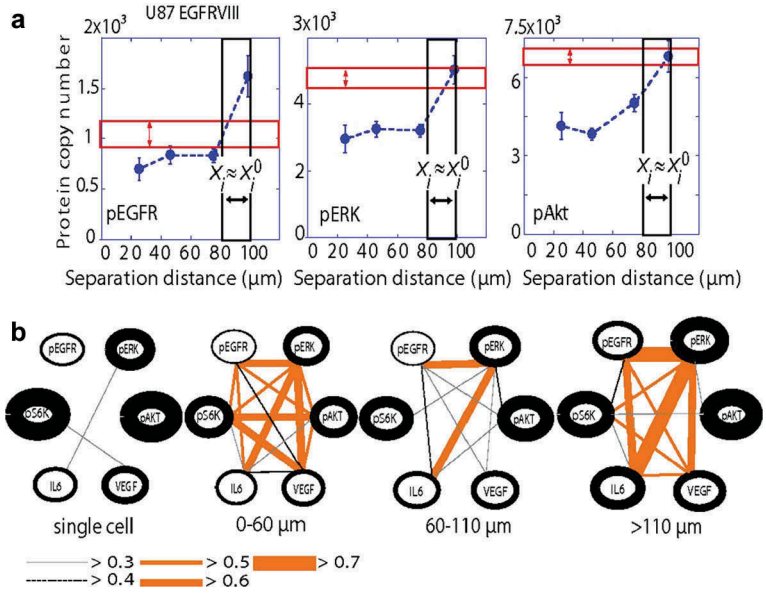

Figure 4. Comparisons of noninteracting U87EGFRvIII single cells with interacting cell pairs. (a) Mean values of protein levels, as measured for U87EGFRvIII single cells, are compared against measurement of those same proteins for cell pairs, at cell separation distance ranges close to the steady state (80–100 μm for U87EGFRvIII cells) or deviating significantly (20–30 μm for U87EGFRvIII cells). (b) Protein–protein coordination maps were generated using U87EGFRvIII two-cell and single-cell data. The thickness of the lines encircling the protein names reflects the relative abundance of those proteins. The thickness and color of the edges reflect the extent of the protein–protein coordination ($P < 0.05$) as provided in the key below the networks. Reproduced from Ref [35]. with permission.

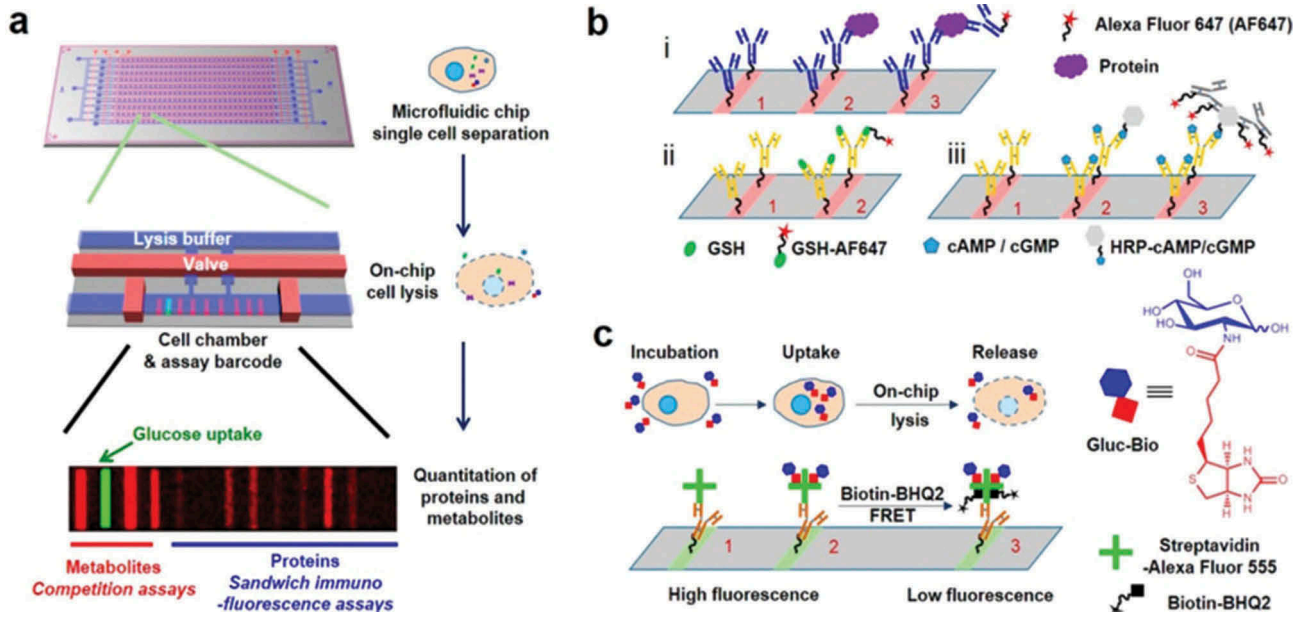

Figure 5. Co-detection of functional proteins and metabolites. (a) Illustration of the SCBC layout and the individual miniaturized cell chambers, and a typical fluorescence image of one set of barcode. (b) Scheme of the immunofluorescence assay for (i) proteins, (ii) GSH, and (iii) cAMP or cGMP. (c) Scheme of the Gluc-Bio probe detection. Reproduced with permission from Xue et al. Chemical Methods for the Simultaneous Quantitation of Metabolites and Proteins from Single Cells. J Am Chem Soc, 137(12), 4066–4069 (2015). Copyright 2015 American Chemical Society [42].

metabolic activities and intracellular signaling (Figure 5). Drug intervention on aberrant signaling leads to change of metabolism in the same single cells. Another notable multi-omics technique is proximity ligation assay for RNA (PLAYR) which is based on flow and mass cytometry to measure simultaneously both proteins and mRNAs. PLAYR permits multiplexity as high as 40 and is compatible with standard antibody staining. Shi group pushes forward the omics integration by a technique co-detecting metabolic activity, intracellular functional proteins, and genetic mutations from the same single cells [25]. This technique is also based on a high-density antibody array coupled with a microengraving PDMS replica. Circulating tumor cells isolated from blood samples were assessed by measuring their glucose update and phosphoprotein oncogenic signaling activities, while their genomes were analyzed by capillary sequencing.

8. Beyond single cells: two-cell assay

Cancer is a heterogeneous system with multiple coexisting subclones. It has been postulated for three decades that individual subpopulations of cancer cells behave rather like societies and substantially interact with one another. However, despite our growing knowledge about cellular heterogeneity in cancer, the understanding of that dynamic process that operates among the heterogeneous subpopulations is particularly difficult in patients due to the lack of tools. The concept of cell competition, one format of cell interactions, is widely accepted by scientists, because the complex interactions and selective sweeps are in concordance with the 'survival of the fittest' aspect of Darwinian evolution [43–45]. But competition alone will lead to tumor collapse. Recently, increasing attention is being directed toward the cooperative behavior of subclones that can influence disease progression [46]. The collaboration of clonal populations in model systems has been observed before, such as basal $Wnt1^{low}$ subclones and luminal $Wnt1^{hi}$ subclones [47], $CD29^{hi}CD24^{hi}$ tumor initiating cells and more differentiated $CD29^{hi}CD24^{low}$ mesenchymal populations [48], IL-11 overexpressing cells and FIGF overexpressing cells [49], etc.

Although cell–cell signaling and its importance in tumor growth and metastasis are well acknowledged, little is known about heterogeneity of cell–cell interactions, which is expected to be heterogeneous since individual cancer cells are different. Traditionally, these interactions are studied in bulk through the analysis of the transcriptome or proteome of different cell types cultured together [50,51], as compared to cultures of pure cell types. Microchip-based technologies allow for cell interaction assay at the single-cell level. Wang quantified a few signaling proteins to implicate the consequence of cell–cell interactions [4]. It was discovered that oncogenic signaling is highly correlated with cell–cell distances, and there exists an optimal distance for each cell type. This is the first try to investigate heterogeneous cell interactions. Further study on protein–protein coordination confirms that at certain cell–cell distances, the coordination reaches the minimal level, which may be the characteristics of particular cancer cells. When cells were randomly loaded into each of 10,000 microchambers, statistically, there could be over 1000 two-cell chambers. Cells were incubated in the isolated microchambers for hours to sufficiently communicate with their neighbors, and subsequently, secreted proteins and intracellular proteins were assayed by sandwich ELISA.

Models have been developed to understand the behavior of a whole multicellular system by reconstructing the whole system through pairwise interactions. A physical model was built to predict three-cell interaction based on two-cell interactions [4]. In the model, each cell is treated as a particle that is able to exert some 'force' or influence other particles as a function of distance. Protein levels are taken as a measure of such influence or strength of interaction, and such are correlated with intercellular separations. Data from two-cell experiments are used to give rise to protein-dependent interaction functions. So far, this model has been applied to glioblastoma multiforme cancer cells and is limited to protein copy numbers from 6-h incubation periods. Kravchenko and Wang further applied surprisal analysis to understanding cancer cell interactions and how the preferred cell–cell distance is formed [35]. They identified a steady-state separation distance at 80–100 μm for EGFRvIII cells where the free energy reaches the minimum, whereas wild-type EGFR-expressing cells have a steady-state at much shorter distances. This signaling-dependent architecture was confirmed by bulk assay using radial distribution function.

The high-throughput cell interaction assay is still at the beginning stage as the above-mentioned work is the only one that studied heterogeneous cell interactions. Similar technologies and research are highly demanded to develop a detailed landscape to identify fundamental principles underlying cellular organization in tumor and all tissues, because single-cell analysis alone is not capable to achieve that goal. The next step of cell interaction assay and algorithm development should be directed toward analysis on more complicated xenografted tumors in mice and further on human tumor samples, where stroma–tumor interactions should be also counted in. In addition, how to reconcile the *in vitro* two-cell interactions with tissue-level tumor growth needs much investigation experimentally and theoretically. Only when those issues are addressed, we can start to understand many cellular level dynamics in tumor growth and progression, and possibly predict those biological events.

9. Expert commentary and five-year view

The advances in single-cell proteomics technologies have been rapid within recent 5 years, with a few fresh perspectives offer on cellular heterogeneity and cell–cell interactions. These technologies are essential for advancing cell signaling studies and have important potential in clinical and diagnostic applications. The clinical and biomedical drivers of this technology are propelling this field at an ever-faster rate, aiming at inexpensive and easy to use methods that will be available to a broad audience. Though only a few microfluidic proteomics methods including the SCBC have matched the statistics and throughput of cytometry tools, the robustness and user interface still need improvements. The single-cell barcode technology affords the capability to enhance the multiplexity to 42, higher than the most state-of-the-art multiparameter flow cytometry. It is likely, in the future, the multiplexity of a single-cell proteomic technology can advance to over 100, as the newly introduced CyTOF already demonstrates such a capacity. Beyond 100, all single-cell technologies including microchips and CyTOF will be limited by the availability of antibodies and their cross talks, which is a common problem in the multiplexed assays [34]. Thus, the development of high-performance, inexpensive, and robust antibodies is equally imperative. Compared with genomic analyses, the cost of protein assays per amino acid is generally 100 times higher.

The future single-cell functional proteomics technologies are expected to be more diversified. The reason is that many tools are developed specifically to address certain technology bottlenecks and are driven by clinical needs, which are changed time-by-time. For instance, the rapid growth of immunotherapy field pushes forward the two-cell functional proteomic assays

[52]. The increasing use of targeted drugs in cancer therapies, coupled with confounding modest performance of those drugs in the clinic, requires the point-of-use single-cell tools to analyze phosphoprotein signaling networks that are targeted by those therapies. Furthermore, the major proteomics tool, mass spectrometry, has been becoming more accurate and quantitative for clinical diagnostics and may continuously place a key role in single-cell analysis in the future.

One underlying challenge that single-cell tools have to address is the discrepancy between single-cell information and tissue-level knowledge. A multicellular system comprised of many single cells is a homeostatic system where cells dynamically interact and change their functions, instead of being the add up of all individual cells. The intrinsic weakness of most high-throughput single-cell technologies is that cells are isolated from tumor issue first before analysis, and thus the tissue-level information cannot be captured. High-throughput two-cell functional proteomics has alleviated that issue to certain degree. Alternatively, installing a single-cell sensor may also keep the integrity of a tumor tissue [53]. In the near future, it is expected that new tools may address that discrepancy in a better way, while maintaining similar multiplexity and throughput. These tools are already available for mRNA analysis without separating cells from a tissue [54]. Multiplexed tools for real-time signaling protein detection in live cells would be another direction that a few groups are working on. If existent, such a tool may inform drug resistance of tumor cells immediately without lengthy procedure and may revolutionize cancer therapeutics by matching the right drugs to individual patients.

Key issues

- Cellular heterogeneity occurs intrinsically and universally at multiple molecular-omics levels such as metabolomics, transcriptomics, and proteomics in otherwise identical cell populations.
- Cancer cell heterogeneity is the underlying problem that impedes effective clinical therapies.
- Currently there is no clinical single-cell tool available at the bedside or operable in a field setting. Microfluidic technologies can potentially address this gap.
- Emerging single-cell proteomic tools mainly include CyTOF, microengraving technology, single-cell western blotting, and single-cell barcode chip. They all have advantages and disadvantages. CyTOF is currently commercialized and possesses the highest multiplexity and throughput.
- Single-cell barcode chip and the variants permit high-throughput two-cell assays and bridge the gap between single cell analysis and whole tissue architecture.

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