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Single-cell profiling approaches to probing tumor heterogeneity

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Mini Review

Tumor heterogeneity is a major hindrance in cancer classification, diagnosis and treatment. Recent technological advances have begun to reveal the true extent of its heterogeneity. Single-cell analysis (SCA) is emerging as an important approach to detect variations in morphology, genetic or proteomic expression. In this review, we revisit the issue of inter- and intra-tumor heterogeneity, and list various modes of SCA techniques (cell-based, nucleic acid-based, protein-based, metabolite-based and lipid-based) presently used for cancer characterization. We further discuss the advantages of SCA over pooled cell analysis, as well as the limitations of conventional techniques. Emerging trends, such as high-throughput sequencing, are also mentioned as improved means for cancer profiling. Collectively, these applications have the potential for breakthroughs in cancer treatment.

Tumor Heterogeneity and Evolution

The tumor microenvironment is a complex heterogeneous system and consists of intricate interactions between the tumor cells and its neighboring non-cancerous stromal cells. The principal stromal cells in the tumor niche consist of endothelial cells, macrophages, immune cells, fibroblasts and stem cells. Each cell has unique behaviors due to variation in genetic and environmental factors, which has implications in pathogenic conditions.[1] In cancer, nonrecurring mutations and large genomic alterations generate vast heterogeneity, giving rise to tumors which comprised subpopulations of distinct cells. Other factors, including clonal evolution and positive selective pressure from therapeutics,[2] also play a role in inducing tumor heterogeneity.

Unlike prior literatures on cancer heterogeneity,[3,4] this review aims to evaluate and combine the issues of cancer heterogeneity with single-cell analysis (SCA),[5] summarizing the recent advances in single-cell cancer analysis which has allowed new understanding of cancer biology. These single-cell analytical techniques are classified into four different categories, including cell-based, nucleic acid-based, protein-based and metabolite-based methods and are explicitly discussed along with the advantages and limitations for each technology. Finally, the review highlighted the importance of SCA techniques over bulk tissue analyses, and summarized the application of next generation omics to single cells. The objective of this review is to familiarize the reader with the burgeoning field of SCA in oncology, thereby empowering them to select the best approach for their specific application.

Different characteristics of single cells

Cells work as single units or in organized tissues and organs. Despite the apparent synchrony in cellular systems, each cell

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Abbreviations: aCGH: array comparative genomic hybridization; CSCs: cancer stem cells; CTCs: circulating tumor cells; EMT: epithelial-mesenchymal transition; FACS: flow cytometry (FCM)/fluorescently activated cell sorters; FISH: fluorescence in-situ hybridization; FRET: fluorescence resonance energy transfer; NGS: next generation sequencing; q-PCR: quantitative polymerase chain reaction; SCA: single-cell analysis; SCBC: single-cell barcode chip; SINCE-PCR: single-cell PCR based approach

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can behave uniquely owing to their genetic and environmental factors, such as proximity to other cells and presence of extracellular signals. In pathological situations such as cancer, genetic mutation can give rise to vast heterogeneity of cells. Cell heterogeneity occurs even in newly divided cells,[6] so pooled sample analysis may mask certain unique characteristics that serve as determining traits for various cell types.

Cancer exhibits significant heterogeneity in terms of morphology, immunophenotype and genotype.[7] Although significant therapeutic advances have been made in the last decades, advanced metastatic cancers remain incurable as illustrated by targeted therapeutics in the treatment of BRAF mutant cutaneous melanoma[8] and ALK rearranged NSCLC.[9] Tumor heterogeneity and the emergence of resistant subclones are contributing factors to the lack of an efficient cancer therapeutic strategy.[10] Thus, current routine diagnostic methods for evaluating tumors, which employ bulk sequencing, such as biopsies, limit our ability to detect, predict and treat cancer.

Intertumoral and intratumoral variation

Cancer is often an "over-generalized" disease, and in fact displays vast intertumoral and intratumoral variation.[11] Tumors comprise subpopulations of distinct cells either within a primary tumor (intratumor heterogeneity), or between tumors of different tissues, including variations of the same tumor type within individuals (intertumor heterogeneity).[12]

Tumor heterogeneity presents a great range of clinical challenges. Different tissues and cell types have distinct mutational frequencies of oncogenes and tumor suppressors, and these can result in diverse signaling pathways depending on tissue context or microenvironment. For example, *APC* gene mutations were found to be more prevalent in colorectal cancer (CRC), probably due to the heightened role of WNT-signaling pathways in intestinal development.[13] This proteomic heterogeneity also contributes to variation in their response and resistance to a specific therapy.[14] At this point of time, the majority of existing molecular subtyping methods do not distinguish cancer cells from normal stroma tissue.

Both intertumoral and intratumoral variation have been well established in many forms of cancer, such as glioblastoma,[15] nonsmall-cell lung cancer (NSCLC),[16] renal cancer,[17] breast cancer,[18] prostate cancer[19] and ovarian cancer.[20] Clinically, intertumor variation is best handled by classifying tumors into subgroups typically based on morphology, mutations, copy number aberrations (CNAs) and gene expression profiles. Genomic profiles of multiple cancers showed that majority of cancer types clustered together under several subtypes, with exception of some distinct cancers, namely, lung squamous, head and neck, bladder cancers.[21] These cancer types only demonstrate similarities in genomic signatures in their TP53 genomic signatures. The integrative genomic and proteomic multiplatform analysis of single cancer cells have laid the foundation for a more comprehensive molecular classification of tumors. Hence, similar methods of classifying tumors are very informative for clinical decision making.

The common consensus for defined five breast cancer subtypes is the following: Luminal A, Luminal B, HER2 positive, basal-like and normal breast-like.[22] Treatment decisions are often based on clinical assays which sorts accordingly to a similar classification. However, this list is not conclusive. Several new subtypes are constantly being evaluated, such as the claudin-low subtype, which corresponds to cancer stem cells.[23] Although the five molecular subtypes were relatively well-defined,[24] it is increasingly obvious that tumors classified within the same subtype may still display varied clinical behavior.[25–27] The lack of better subtype definitions will be detrimental to the patient, as the efficacy of drug strategy can only be known after administration. However, this problem might be overcome in the future by the development of routine assays, which allow real-time monitoring of response, or by the unraveling of cancer heterogeneity with SCA approaches.

Nonrecurring mutations and large genomic alterations are linked to phenotype-based classification of cancer. Although SCA can be applied to resolve intertumor heterogeneity, such a resolution may not be required for classification based on morphology. However, intratumor heterogeneity is largely explained by applying SCA to study tumor progression by clonal evolution.[2] Introduction of therapeutics often induces a positive selective pressure which allow for the persistence of resistance variants.[2] According to the traditional model of clonal evolution, a selective sweep occurs when a series of clonal expansions occurs to dominate over the cancerous growth. Due to clonal evolution, conventional one-off genetic profiling often underestimates the diversity of the tumor. The resultant subclonal heterogeneity often generates variation in drug responses.[28]

SCA techniques may further serve to unravel phenotypes resulted from previous selective pressures and are not relevant to the metastatic disease status.[29] The distribution of mutations in subclones[30] is usually not revealed in pooled cell sample analysis. Although genetic heterogeneity may not eventually translate into phenotypic heterogeneity, specific mutations may still be impactful, depending on its location of occurrence. Inaccurate classification of a tumor also often results in partial clonal eradiation and subsequent relapse.[31] Cancer cell plasticity, which generates a range of epithelial-to-mesenchymal (EMT) phenotypes, is further often attributed to shifts in epigenetic regulation.[32] This phenomenon could be explored with Array Comparative Genomic Hybridization (aCGH).[33] Insights into the heterogeneity of cancer with single cell profiling will reveal distinct mutation patterns,[34] which could act as targets for future drug design and development, allowing for better personalized therapeutic development.

Using SCA to resolve tumor complexity

SCA is an up-and-coming approach for understanding cancer complexity. This approach is especially important since tumors comprise a highly diverse composition of cells[35] that

are either cancerous or noncancerous. With new techniques developed **(See Section "Modes of SCA for cancer cells")**, we are now able to characterize rare cancer subpopulations such as cancer stem cells (CSCs)[36] and circulating tumor cells (CTCs).[37] There are also *in-vivo* techniques to track single-cells motility within their intrinsic environment.[38]

SCA method is an ideal fit to resolve intratumor heterogeneity, since clonal evolution is of single-cell origin with multiple hypothesis-based models of evolution. The major clinical challenge is the fact that single tumor samples, obtained *via* needle biopsies, are likely to under represent the true extent of intratumor heterogeneity. Ideally, it would be valuable to resolve all the cells in a tumor *via* SCA techniques.

Our review focuses on the applications of SCA tools to resolve cancer heterogeneity, as distinct from recent reviews on independent topics on SCA[5] or cancer heterogeneity.[39] Here, we present various SCA techniques (cell-based, nucleic acid-based, protein-based, metabolite-based and lipid-based), and diskuss their strengths and limitations. Recent breakthroughs in cancer research *via* high-throughput sequencing[40] will also be highlighted to provide insights into their potential for cancer detection, classification, treatment and prognosis.

Modes of SCA for Cancer Cells

Cell-based analysis

Cellular events *in-vivo* are very dynamic in nature, and thus it is challenging to monitor them using end-point population-based analysis techniques. Flow cytometry (FCM) is a high-throughput and multi-dimensional technology which may overcome the drawbacks associated with end-point-based studies.[41] However, there is still a problem of spectral overlap and the technique suffers from a loss of spatio-temporal information due to the lack of imaging.

The issue of multiplexing profiling due to fluorescence-based spectral overlap is dramatically alleviated by mass cytometry.[42] This technique allows simultaneous measurement of parameters, and was recently shown to provide insights into the spatial information of breast cancer tissues and tumor heterogeneity *via* mass cytometry imaging.[43] Besides FCM and mass cytometry, other dynamic methods of live cell tracking, such as the use of dyes[44] or fluorescence proteins,[45] could be utilized to monitor these progressive events.

SCA techniques are also adopted for observing the effect of anticancer drugs at the individual cell level. Hydrodynamic stretching of single cells, also known as deformability cytometry (DC), was used in two opposing microfluidic channels to detect malignancy in pleural effusion[46] (Fig. 1a). This approach leads to unbiased label-free molecular characterization of single cells. Droplet-based cell encapsulation technology has emerged as a promising SCA tool for high-throughput drug screening by encapsulating single-cells in a droplet with different drugs under various concentrations.[47,48] Microscopy techniques such as single-cell image cytometry and ultrafast spectral imaging[49] can also be utilized to obtain morphological and spatio-temporal information of tumor cells in response to drugs. Such advances will allow probing of single particles or cells under high spatial, temporal and spectral resolution, allowing insights into enzyme kinetics, membrane protein studies *via* the precise manipulation and analysis of single cells.

Nucleic acid-based analysis

Nucleic-acid analysis of single cells is challenging, due to the detection or quantification of small amount of DNA (~7 pg), and total RNA (~20 pg). Although there are methods to amplify nucleic-acids, these methods usually start with low input materials and are hence replete with stochastic effects.[50] It is imperative to evaluate the principles of the amplification methods to understand how well they represent the original DNA or mRNA molecules and to avoid undesired biasness. This might lead to the better development of amplification technologies with greater accuracy and higher throughput. More recently, nucleic acid can be amplified by an exponential (PCR-based) or linear (isothermal-based) format. Introduction of amplification bias during PCR is a well-known concern. Therefore, sensitive methods, which can detect or sequence single molecules are highly desired.

Unlike genomics studies, transcriptomic tools help to acquire quantitative and qualitative information about genetic expression at a definite time point and under specific physiological conditions. The single-cell PCR based approach (SINCE-PCR)[51] enables the sorting of individual cells *via* FACS and also subsequent high-throughput analysis of small amount of mRNA (Fig. 1b). The main advantage of this technique is the parallel gene expression analysis of 96 genes from a single cell and the possibility to study expression from hundreds of cells of the same sample simultaneously. Fluidigm Corporation developed a fully automated microfluidic system (C_1 Single-cell Auto Prep System), which can capture single cells, followed by reverse transcription of mRNA and simultaneous detection of multiple genes using intercalating-dye based qPCR assay.[52] Also, the mRNA-Seq protocol (Smart-Seq) was developed to detect alternative transcript isoforms and single-nucleotide polymorphisms at a single-cell level with higher sensitivity and accuracy.[53]

Protein-based analysis

Proteomics analysis helps us gain understanding on the post-translational modifications that are crucial in cell signaling and cell-to-cell heterogeneity. One major bottleneck of the proteomics field is the minimum amount of protein required for detection by current technologies; usually many cells (a single-cell has 1×10^5 molecules of proteins)[54] are required for analysis.

Recent advancement in microfluidic technologies and mass spectrometric approaches has led to new single-cell proteomics studies that could be performed with greater

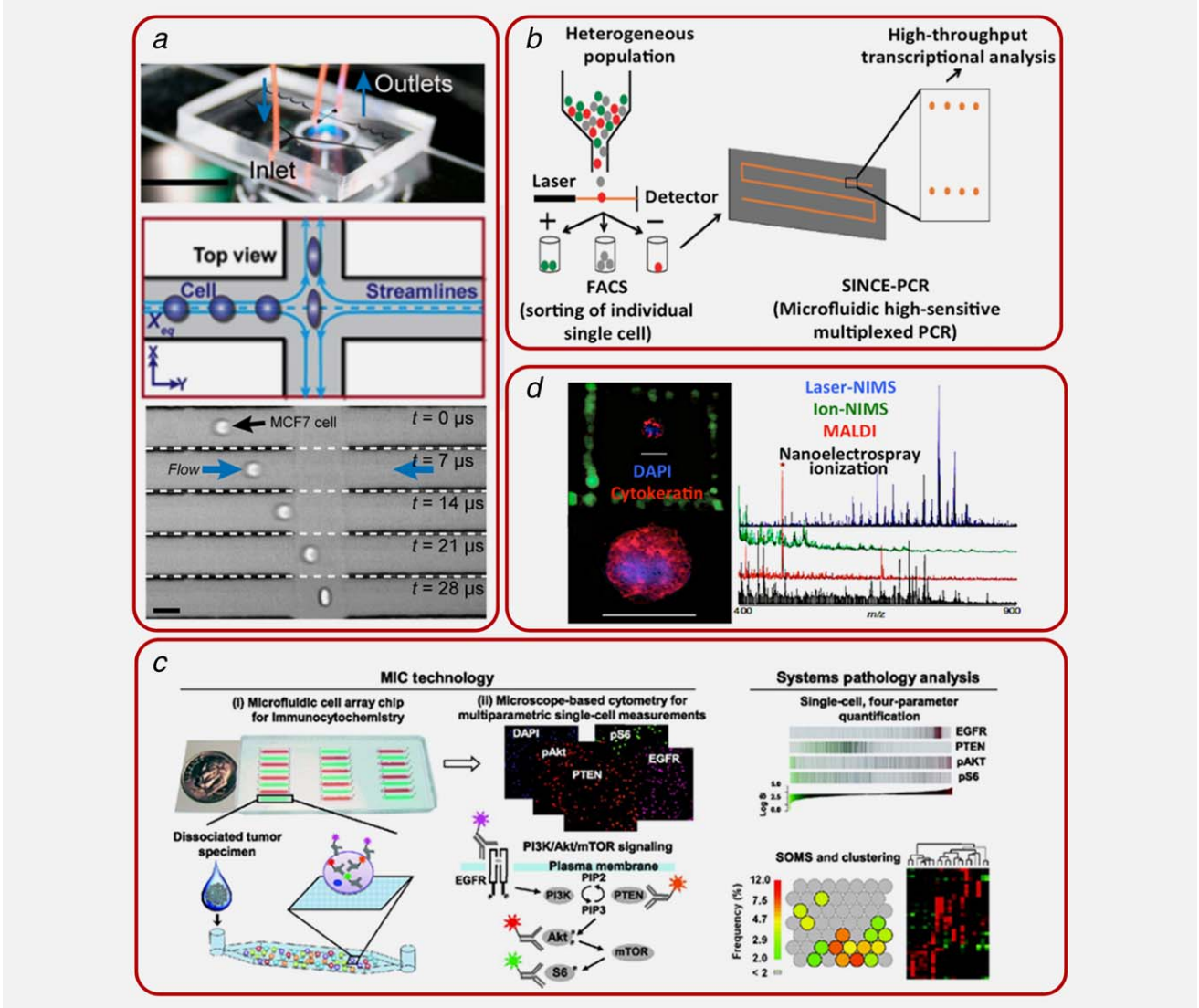

Figure 1. Different modes of SCA. **(*a*) Cell-based analysis -** Left: Schematic representation of a microfluidic device with inset showing channel design for single-cell mechanophenotyping (deformation junction is shown in red in the right FIG.). Right: Cells are injected into the device and stretched under continuous flow. The deformability and diameter of the cells are quantified to diagnose a cell as benign or malignant.[46] **(*b*) Nucleic acid-based analysis -** Working principle of SINCE-PCR. This method combines the single cell sorting capacity of FACS with the microfluidic PCR technology to perform high-throughput transcriptional analysis from limited amount of mRNA. (*c*) **Protein-based analysis –** Left: Diagrammatic representation of MIC technology that comprises two parts: (i) the microfluidic cell array chip and (ii) Fluorescence microscopy based image acquisition and cytometry. Four intracellular signaling proteins were immunologically labeled. Anti-EGFR: purple, anti-PTEN: orange, anti-pAKT: red and anti-pS6: green. Right: MIC platform provides complex data sets for multiparameter expression/phosphorylation of single cells (above). Representation of MIC parameters to qualitatively assess signal transduction mechanisms (below).[55] (*d*) **Metabolite-based analysis -** Laser- NIMS for direct mass analysis of a single cell at higher resolution and sensitivity. Left: Fluorescence microscopy image of DAPI (blue) and anti-cytokeratin (red) labeled cell. Right: Image of the cell at higher resolution (Scale bar 28 μm).[62] [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

sensitivity and specificity. Combining the advantages of the microfluidics platform and image cytometry analysis, microfluidic image cytometry (MIC; Fig 1c)[55] was developed to examine high-dimensional data from clinical samples at a single-cell resolution. This device can selectively lyse single adherent cells and capture cellular contents to determine kinase activities.[56] Further on, a novel photocleavable DNA barcode-antibody conjugate approach was introduced to identify protein biomarkers from single cancer cells. After binding of antibodies to the target protein, the DNA barcodes were photo-cleaved in solution and amplified by PCR to quantify various proteins from individual cells.[57] In a later study, the DNA-barcoded antibody sensing technology was further improved to analyze the heterogeneous expressions of ~90 proteins from fine-needle aspirate samples obtained from different patients.[58] More recently, a mass cytometry platform (CyTOF) was developed to highlight the phenotypic heterogeneity in acute lymphoblastic leukemia (ALL) at

single-cell resolution. In this work, the authors reported viSNE, a tool to map high-dimensional cytometry data onto two dimensions, and validated the functionality of this tool using 29 marker panel specific for ALL.[59]

Lipid and metabolite-based analysis

Single-cell lipidomics is still at its infancy stage of development. The time-of-flight secondary-ion mass spectrometry (TOF-SIMS) approach has been implemented to visualize lipids in individual breast cancer cells.[60] Prominent expression of stearoyl-CoA desaturase 1 in breast cancer SKBR-3 cell lines was reported, and static TOF-SIMS was concluded to be an effective method for determining the lipid molecular signature of the plasma membrane of individual breast cancer cells. Further applications in lipid detection of single cells are still being optimized.[61]

Single cancer cell metabolomics might be exploited to detect tumor cells that develop resistance against various chemotherapeutic agents. The nanostructure-initiator mass spectrometry (NIMS) was introduced for direct mass analysis of a single-cell at higher resolution and sensitivity (Fig. 1d). Using this device, desorbtion/ionization of endogenous phospholipids can be achieved from a single breast cancer cell with greater complexity as compared with that obtained from many cells by the conventional methods such as nanoESI, ion-NIMS and MALDI[62] or be used to monitor the response of individual tumor cells and xenografts to anticancer agents.[63]

Insights on Tumor Heterogeneity Obtained with SCA Techniques

Cancer cell profiling

The mutation distribution of cancer cells varies due to clonal evolution. Longitudinal SCA of cancer cells will serve to delineate the expansion, diversification and selective process. For example using nucleic-based approaches, mutation rates in breast cancer cells were modeled and it was found that clinically aggressive triple-negative breast cancer cells showed mutation rates at around 13 times greater than other subtypes of cancer cells.[64] aCGH could also analyze CNAs and massive parallel sequencing in CTCs enriched from CRC patients,[65] revealing distinct CRC-related CNAs in both the primary tumor and CTCs. Using Whole Genome Amplification (WGA) of the single CTCs, substantial inter and intra-patient heterogeneity in the expression of EGFR and genomic modifications in EGFR, KRAS and PIK3CA were observed.[65,66] Their data could explain the variation in the response of EGFR inhibitors in CRC patients. aCGH could also be applied on the studies of epigenetic status changes with cancer progression.[33]

It is well-known that cancer cells typically exhibit aberrant DNA methylation patterns that can drive malignant transformation.[67] Since single-cell epigenetic heterogeneity was recently highlighted in embryos,[68] we can expect similar studies to be performed for single cancer cells, potentially providing unique insights to cell-of-origin- specific epigenetic factors.

In another study using mRNA-Seq protocol (Smart-Seq), the expression profile of 84 epithelial-mesenchymal transition (EMT)-related genes was determined in rare prostate cancer cell subpopulations using single-cell microfluidics-based quantitative polymerase chain reaction (q-PCR).[69] Samples obtained from patients with castration-resistant cancer demonstrated an increased expression of a subgroup of EMT-associated genes (for example, PTPRN2, ALDH1, ESR2 and WNT5A) that could facilitate monitoring of disease. More recently, analysis of prostate CTCs with single cell RNA-seq also revealed heterogeneity in Wnt signaling pathways.[70] Another work also described analysis of the transcriptome from 430 single cells isolated from brain glioblastomas.[71] Their work highlighted that heterogeneity in tumor cells may reflect neural development. Similar SCA analyses on breast cancer cell lines reveal specific transcriptional programs, which were triggered as a stress response to drug treatment.[72]

In another study, 100 cells derived from a polygenomic tumor were examined with high throughput sequencing,[40] and three subpopulations, which demonstrate sequential clonal expansion were successfully identified. They analyzed the monogenomic tumor and its liver metastasis and observed that a particular clonal expansion was responsible for both cases. Analysis of the polygenomic secondary tumors clearly distinguished them from its monogenomic primary tumor, and they found subpopulations of nonmetastatic "pseudodiploid" cells in monogenomic primary tumors, suggesting presence of persistent intermediate cell types. Intertumor heterogeneity of tumors with similar morphology was also demonstrated with proteomic screening of single nuclei, which may potentially improve follow-up on treatment efficacy.[11] Traces of inter-[73] and intra-tumor heterogeneity[74] of KRAS, BRAF and PIK3CA expression were reported. These variations may be causative for the failure of existing EGFR antibody therapy in patients with CRC,[75] as the actual subtype may evade detection or be characterized wrongly as a different tumor subtype.

Besides distinguishing between cancer subtypes, SCA may be useful for investigating the non-cancer cells in tumors. The presence and concentration of tumor-infiltrating immune cells are gaining interest as they have been suggested to correlate with cancer relapse and treatment outcomes.[76] Single-cell techniques will enable closer scrutiny for the diversity and role of such immune cells.[77]

Variation in signaling mechanisms

Cancer cell exhibits differential responses to stimulation, such as acute myeloid leukemia blast cells (with or without certain Flt3 mutations) in response to granulocyte colony-stimulating factor (G-CSF) activation.[78] Insights on tumor cell responses demonstrate the ability of cancer cells to undergo dramatic remodeling with disease progression.

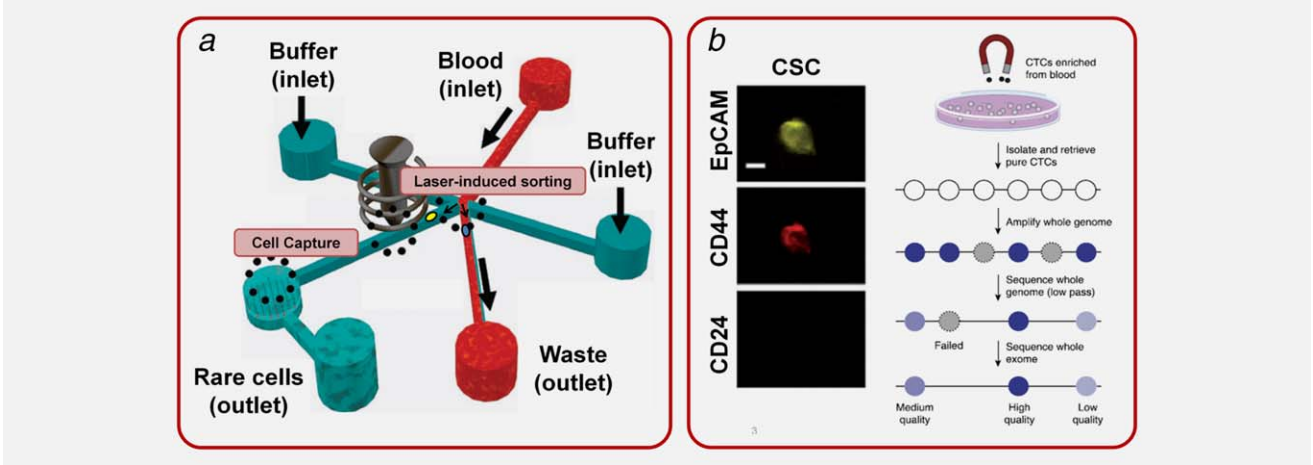

Figure 2. SCA for CTCs. (*a*) Schematic of working principle of the ensemble decision aliquot ranking device. The blood sample is broken down into nanoliter aliquots that were ranked according to the presence or absence of CTCs. The sorting of the aliquots with CTCs (indicated in yellow) is triggered with a laser-induced fluorescence and the cells are collected in the cell capture chamber. Fluorescent antibody labeled image of a captured breast cancer stem cell ($CD44^+/CD24^-$). Scale bar 20 μm. [Reprinted by permission from John Wiley and Sons][87] (*b*) Schematic representation of an integrative approach to separate, retrieve and sequence the whole exomes of the clinical CTCs. The Illumina MagSweeper was used to retrieve the EpCAM expressing CTCs from clinical samples. The purification of the CTCs is followed by whole genomic amplification and census-based sequencing of the whole exome. [Reprinted by permission from Macmillan Publishers Ltd: *Nature Biotechnology*, 2014][88] [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Intracellular signaling proteins from single-cells can be detected with single-cell barcode chips (SCBCs),[79] enabling characterization of downstream signaling pathways. In another work, heterogeneity in the Akt kinase activity in single hepatocellular carcinoma cells was detected in response to insulin stimulation, using MIC.[55] Recently, single melanoma cells were isolated using the C_1 System (from Fluidigm Corporation) and pluripotency factor POUF51 (OCT4) gene expression was identified to correlate with the tumorigenic potential of single cells.[52]

Other breakthroughs with SCA include the simultaneous observation of Src kinase and membrane type 1 matrix metalloproteinase (MT1-MMP) activities in response to epithelial growth factor (EGF) stimulation. Using fluorescence Resonance Energy Transfer (FRET), it was observed that although Src and MT1-MMP were part of the same signaling pathway, they were activated in different manners after EGF stimulation, due to the formation of dissimilar intermediates at different subcellular sites.[80] PI3Ki treatment response had also been monitored in cancer patients using various single-cell proteomic chip analysis, all of which revealed vast differences in protein-protein interactions.[79]

Identification of rare cancer cell populations

The diverse cancer cell sub-populations result in a substantial roadblock of detecting and distinguishing minority phenotypes, especially rare cell populations. High sensitivity techniques, such as pyrosequencing[81] and single-nucleus sequencing,[11] may enable the detection of unique and rare cancer-associated sequence variations, using as little as picoliter amounts of samples.

Perhaps the most intriguing application of SCA is on the characterization of rare cancer cell populations, such as CSCs and CTCs. CSCs are rare cancer cells with tumorigenic properties which are detected in tumors or at a low frequency among isolated CTCs.[82,83] The detection and sorting of rare CSCs subpopulation is of utmost importance in driving individualized cancer treatment. These cells are associated with tumor-initiating activity and may be resistant to certain anticancer therapeutics.[84] However, CTCs are cancer cells, which originate from tumors and invade the peripheral circulation to potentially form metastasis.[85] They may pave the way for early detection, diagnosis and monitoring of cancer treatment to various therapeutic agents. CTC isolation using biochemical marker (immunomagnetic, aptamer-mediated) or physical (size, deformability, electrical and magnetic)[86] properties were often utilized as a pre-enrichment step. In spite of the recent technological advancements in the isolation and characterization of CTCs, the clinical implementation of CTCs for routine diagnostic purpose of cancer has not been widely undergone due to several reasons, such as higher intra and inter-laboratory diskrepancies, different reagents and staining protocols used for evaluating these rare cells from peripheral blood.

Despite the challenges, transcriptional profiling of CTCs has since been made possible, revealing extreme differences in gene expression (Ensemble Decision Aliquot Ranking (eDAR); Table 1; Fig. 2a).[87] Using census-based sequencing technique, an integrative approach to separate, retrieve and sequence the whole exomes of the clinical CTCs derived from prostate cancer patients was introduced (Fig. 2b). A large proportion of the standard exome in CTCs was mapped (>99.995%) and 70% of the CTC mutations in the matched tissue were identified.[88] These profiles were also found to be significantly different from cell lines, which supports previous

Table 1. Summary of various modes of SCA

	Mode of analysis and principle	Cell type	Throughput	Application/published reports	Ref.
Cell-based	Ensemble Decision Aliquot Ranking (Bedard *et al*) device	Breast cancer (clinical samples)	1 ml in 20 min	Isolation of viable CTCs for SCA	(Schiro *et al*., 2012)
	Inherent dean vortex flow and inertial lift forces in a curvilinear microchannel	Breast and lung cancer (cell lines and clinical samples)	7.5 ml in 8 min	Ultra-fast, label-free isolation of clinical CTCs	(Warkiani *et al*., 2014)
Nucleic acid-based	Combination of single-cell adapter linker PCR (WGAM) with array comparative genomic hybridization (aCGH)	EpCAM positive cell lines, bone marrow of an esophageal cancer patient	–	Identification of copy number variations as small as 56 kb in single-cells	(Mohlendick *et al*., 2013)
	Amplifying DNA from single cells with WGA	CRC (clinical samples)	–	Investigate inter- and intra-patient heterogeneity	(Heitzer *et al*., 2013)
	Sequencing of the whole CTC exome using census based sequencing protocol	Prostate cancer (clinical samples)	–	Enables mapping for >99.995% of the standard exome in cells	(Lohr *et al*., 2014)
	Quantification of variation of genomic copy number change using flow-sorted nuclei and WGA	Breast cancer (cell lines)	~6% coverage of single-cell genome	Identification of subpopulations that signify sequential clonal expansion	(Navin *et al*., 2011)
	Single-cell PCR based approach (SINCE-PCR)	Colon cancer (cell lines)	Parallel analysis of 96 genes/cell	Transcriptional profiles of a subpopulation of cancer cells are similar to the distinct lineages of normal colon cells	(Dalerba *et al*., 2011)
	Single-cell microfluidics-based RT-PCR	Prostate cancer (clinical samples)	–	Increased expression of a subgroup of EMT-associated genes in the CTCs of castration-resistant cancer	(Chen *et al*., 2013)
Protein-based	SCBC	Melanoma (clinical samples)	1×10^4 cells/assay	High-throughput device to monitor cellular immunity and clinical outcome	(Ma *et al*., 2011)
	MIC	Human glioblastoma (cell lines and clinical samples)	1000–2800 cells/assay	Tumor progression could be predicted by cluster analysis of four intracellular signaling proteins	(Sun *et al*., 2010)

Table 1. Summary of various modes of SCA (Continued)

	Mode of analysis and principle	Cell type	Throughput	Application/published reports	Ref.
Metabolite-based	NIMS	Breast cancer (cell lines)	150 nm lateral resolution	Successful desorb/ionization of endogenous phospholipids from a single cancer cell	(Northen *et al.*, 2007)
	NIMS imaging to monitor the response of individual tumor cells and xenografts	Burkitt's lymphoma cells (CCL-86)	–	Drug induced metabolic changes detected by accumulation of FLT-MP within the cell	(O'Brien *et al.*, 2013)
Lipid-based	Time-of-flight secondary-ion mass spectrometry (TOF-SIMS)	Breast cancer (cell lines)	–	Lipid molecular signature of the plasma membrane of individual breast cancer cells	(Ide *et al.*, 2014)

work indicating that cell lines are not accurate representations of cancer *in-vivo*.[89]

Overall, CTCs were also found to be extremely varied in terms of biomarker expression, morphology and cell size.[90,91] They may also vary significantly from cells obtained from tumors of the same patient. In recent years, single-cell metabolomics[92] has also been suggested as a potential method to detect and enrich CTCs. Investigations are currently ongoing to better characterize the rare cancer cells, and their efforts may eventually reveal subpopulations with tumorigenic abilities or which play a substantial role in cancer recurrence.

Limitations

Data mined from SCA usually have to be carefully studied with rigorous computation, which is imperative to distinguish preexisting genetic alterations from amplification errors. Single cell studies also require multiple independent samples to detect reliable somatic changes. Improper computation may incur biasness and errors in data interpretation, especially in highly heterogeneous samples, such as CTCs.[86] In certain forms of cancer (*e.g.*, breast cancer), cells demonstrate extremely low prevalence of point mutations, as described in the full listing on the COSMIC database (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/). This has also been observed in many rare CTC samples (*e.g.*, PIK3CA (breast cancer, 3 out of 17 patients)[93] and p53 (breast cancer, 2 out of 6 single CTCs).[94] In fact, most literature describes cultured CTCs as cells with heterogeneity in mutation prevalence,[95] and usually demonstrates high diskordance between CTCs and the primary tumor and metastases.[96] Independent analysis from multiple single cells will be pivotal in distinguishing noise from low prevalence signals.

Since it is technically impossible to eradicate sampling noise[97] in SCA techniques due to the low amount of sample material, additional steps may be required to distinguish noise from low prevalence signals. These may be carried out with molecular fluorescence *in situ* hybridization (FISH)[98] or unique molecular identifiers.[99] Noise structure from single cell sequencing can also be determined to differentiate sampling noise from biological signals.[100] Independent analysis from multiple single cells may also serve to reveal repeated and specific mutational patterns, which will be pivotal in distinguishing technical noise from biological signals.

Current Application and Future Perspectives

SCA demonstrates heightened sensitivity over bulk sample analyses

SCA holds a major advantage over the analysis of rare cells in the absence of a pure sample cohort, since pooled samples may either mask important signals (Fig. 3) or generate false data due to the presence of contaminating components. These signals may be representative of various specialized cell types, or induced from stochastic changes,[101] which in turn may be amplified by downstream pathways to generate asymmetries that determine cell fate and play a crucial role in various developmental or pathological processes.[102]

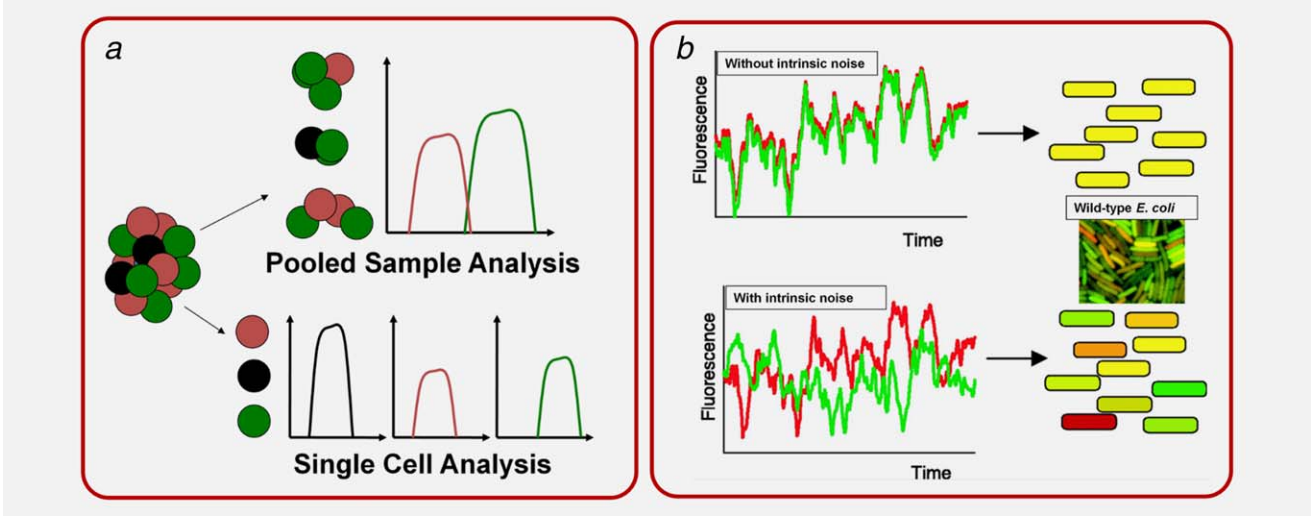

Figure 3. Schematic diagram to illustrate differences in signal detection with pooled and SCA. (*a*) Heterogeneous samples, such as cancer tumors, consist of vast cell subpopulations. When pooled samples of tumor biopsies are analyzed, rare subpopulations (in black) may not be present in the specimen tested or may contribute insufficient material for analysis, leading to false negatives. SCA with appropriate technology will enable individual and specific detection of signals without requiring further data interpretation to sort out various signals. Rare cells can be easily detected without requiring further enrichment procedures. (*b*) Stochastic nature of transcription in cells. In the absence of intrinsic noise, gene expression from all cells will be theoretically uniform (top). However, stochastic effects in gene expression generate huge degrees of cell–cell variation (bottom), creating differential gradients in gene expressions. These differences can be illustrated by the varied expression of *cfp*[103] and *rfp* (red) genes in wild-type *E. coli* (insert).[118] [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Current methods of evaluating cancer and their response to therapy remain restricted by analysis of bulk tissue and the feasibility of tumor biopsies in the clinical settings.[103] Single CTCs may be alternatively isolated from patients *via* a relatively non-invasive mode of liquid biopsy (blood withdrawal), and may serve to illuminate our understanding of mechanisms regulating metastasis. For example, recent work involving single CTC analysis suggests that multiple pathways in metastatic cascade.[104] However, these cells are rare and comprise CTC subpopulations, such as EpCAM±, Vimentin± and E-cadherin± populations.[83] These subpopulations are generated in part due to EMT.[105] Other specialized CTC sub populations include CSCs (*e.g.*, CD133 and CD166 cells for lung CSCs; Fig. 4a). These subpopulations may also vary after culture *in vitro*.[95,106] Sequencing and FISH[90] of single CTCs will highlight their genetic and proteomic variation in comparison to cell lines, and the studies can be further extended to investigate the differences among individual CTCs (Figs. 4b and 4c). Evolving techniques of SCA could overcome limitations imposed by low sample sizes, thus presenting an unprecedented opportunity to investigate and characterize CTCs, which could eventually translate into utility for clinical or biological aspects.

Application of next generation omics to single cells

There are probably two steps required to unravel the extent of cancer heterogeneity. First, is to involve the extensive use of next generation omics, such as single-molecule sequencing, which are able to reveal greater heterogeneity in cancer. Other extensive transcriptomic, methylomic and metabolomic data can be similarly obtained with various SCA techniques using multistage or meta-dimensional analyses.[107] The next step would involve computational integration of large scale databases, sometimes referred to as "big data."[110] There are currently existing databases to map cancer-associated phenotypes, such as the global International Cancer Genome Consortium,[111] but the use of SCA will fine-tune the search to reveal a greater extent of heterogeneity.

SCA techniques are constantly improving and evolving. One promising method with a potential to make an impact on single-cell biology is single molecule sequencing.

The first single-molecule sequencing was commercialized by Helicos Biosciences. In this approach, strands were tethered to a flow cell surface and sequencing was achieved by synthesis approach. Another single-molecule sequencing method, termed as single molecule real-time sequencing (SMRT) (Pacific Biosciences) can generate long reads (10–15Kb) and holds great potential as a long read platform, which can accurately uncover transcripts present. These platforms are known as third generation platforms (First generation: Sanger; Second generation: Massive parallel sequencing platforms). In the last 5 years, there has been huge interest in nanopore-based sequencing (Fourth generation).[112]

Both biological and solid state nanopores are being explored for nanopore-based sequencing. Oxford Nanopore released MinION,[111] a disposable DNA sequencing device with a footprint of a USB memory stick. Recent data indicate that the MinION is capable of average read length of 5.4kb with current upper limit at 10 Kb. The third and fourth-generation sequencing platforms have yet to stabilize with an

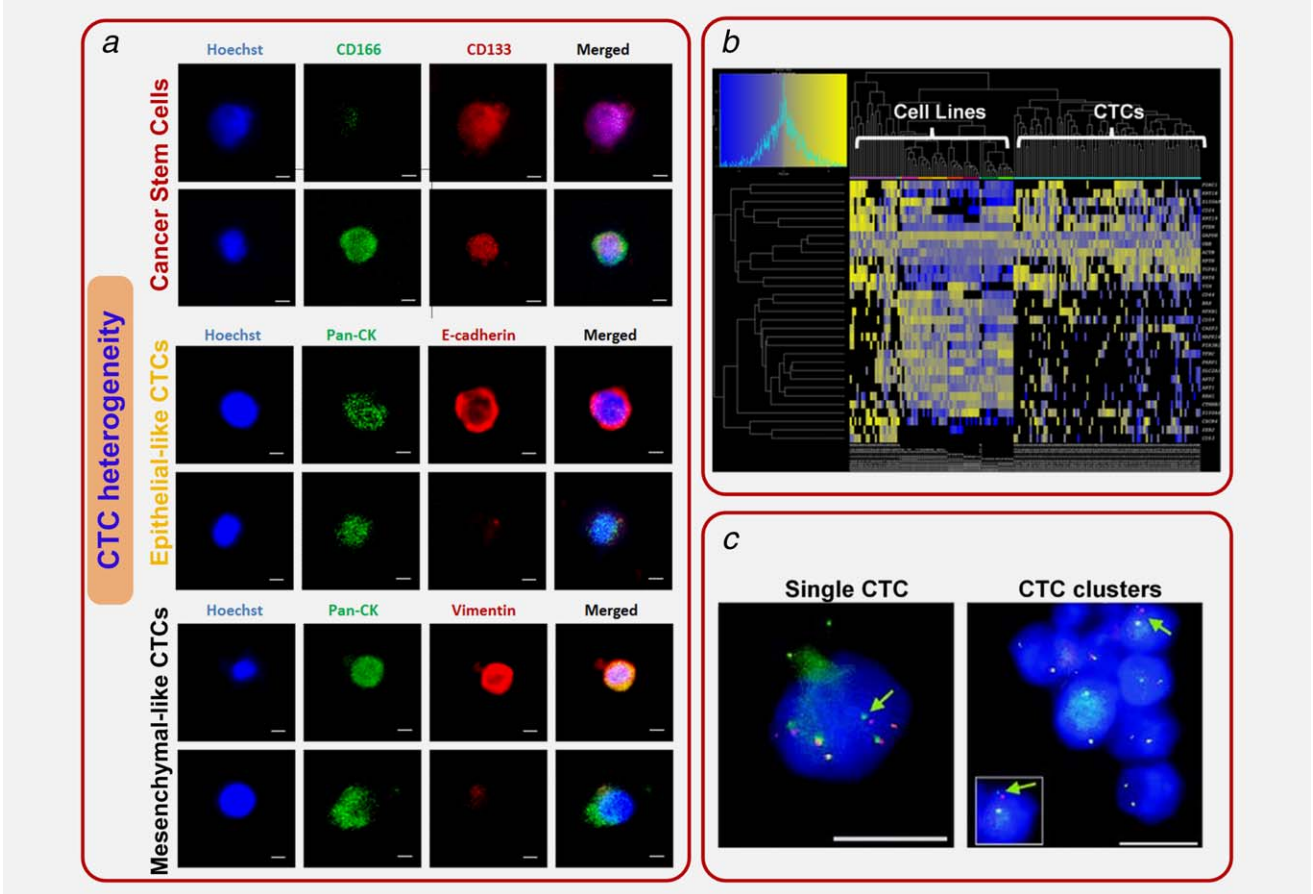

Figure 4. SCA revealing CTC heterogeneity. Single CTCs display vast heterogeneity in terms of morphology, biomarker expression and genetic composition. (*a*) Immunofluorescence staining with cancer stem cell biomarker (CD133), as well as EMT biomarkers such as E-cadherin and Vimentin.[105] (*b*) Heat map displaying subset data obtained from sequencing of single CTCs and the relevant comparison with cell lines.[91] (*c*) Fluorescence *in situ* hybridization[90] of enriched ALK gene rearrangements in single CTCs and CTC clusters.[119] [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

acceptable performance, except for SMRT. One key issue with single molecule sequencing is the high error rates, which require dramatic improvement in the system as a whole unit, including chemistry, instruments and nanopores or zero-mode waveguide. The application of single molecule sequencing on single cells is far from reality as these systems still start with high amounts of nucleic acids (μg). Another area, which needs attention is development of an interface to capture single cells and perform single molecule sequencing.

Concluding Remarks

Cancer is a heterogeneous disease, which demonstrates variation in terms of morphology, immuno-phenotype and genotype. Analysis of conventional one-off genetic profiling on bulk biopsy tissues are often employed as the current standard for evaluating tumors, limiting diagnostic and treatment efforts as mutations in subclones may be lost in the analysis or false data may be generated due to contaminating components. The profiling of bulk samples may also unravel phenotypes resulted from previous selective pressures and are not relevant to the current metastatic disease status. Inaccurate classification of a tumor often results in partial clonal eradiation and subsequent relapse.

Single cell profiling provides a means to reveal mutation patterns and allow for better therapeutic development. Efficacy of anti-cancer drugs can be investigated at the single cell level. The rise of combinational investigative platforms, such as MIC, could also generate high-dimensional data from clinical samples at a single-cell resolution. Single cancer cell metabolomics could also be exploited to examine tumor resistance development against chemotherapeutic agents. Array Comparative Genomic Hybridization (aCGH) has revealed distinct CRC-related CNAs in primary tumor and CTCs, as well as epigenetic status modifications with cancer progression. These efforts will pave the path for demonstrating the mechanisms involved in contributing to a varied response of various therapeutic strategies. Other applications include the characterization of rare cancer cell populations (*e.g.*, CSCs), which were also demonstrated to have specific drug resistant or tolerant properties. Besides distinguishing between cancer subtypes, SCA may be useful for investigating the non-cancer cells located within the tumor niche.

There are currently no SCA methods or devices that have received FDA approval and are currently employed for clinical decisions. However, notable advancement in the field for low amounts of CTC capture and analysis has been achieved through the development of FDA (Food and Drug Administration)–approved method, *via* the CellSearch system (Veridex).[112] This system consists of a semi-automated device for specifically capturing CTCs using ferrofluids loaded with anti-EpCAM antibodies and subsequently staining and identifying these cells with a cocktail of antibodies targeting the various epithelial cytokeratins. The CellSearch system has been utilized for CTC detection in clinical settings and it was observed that CTC evaluation could provide significant prognostic information (progression-free survival and overall survival) about patients with metastatic breast cancers.[113,114]

To further enhance single-cell analytics, novel methods and bioinformatics algorithms need to be established for improved throughput, lower cost, convenient handling and ease of use. Upcoming technologies, such as SCBC and SMART-seq, are evolving rapidly and may eventually reveal high resolution cellular diversity that was previously uncharacterized. Improving sequencing procedures will enable deeper scrutiny of RNA expression in individual cells, revealing heterogeneity in mRNA content and splice site usage beyond estimations previously obtained with pooled sample analysis.

Rapid improvements in single-cell quantification techniques have enabled detailed mapping of individual cell proteomic expressions and signaling network activity. These are aided by the emergence of super-resolution imaging techniques[115] and computational approaches,[116] which facilitates the use of such SCA techniques. Novel imaging approaches and programming methods enable detection of single signals and reduce protocol time frame by generating information from massive amounts of data. Enhanced methods for biomarker detection, such as isotopic labeling,[117] are also continuously improvised to better identify and catalog the new sub populations revealed. These advancements will no doubt enhance the applicability of SCA, making it more accessible, scalable and feasible for clinical use.

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