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CRITICAL REVIEW

Beyond single cells: microfluidics empowering multiomics analysis

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Abstract

Single-cell multiomics technologies empower simultaneous measurement of multiple types of molecules within individual cells, providing a more profound comprehension compared with the analysis of discrete molecular layers from different cells. Microfluidic technology, on the other hand, has emerged as a pivotal facilitator for high-throughput single-cell analysis, offering precise control and manipulation of individual cells. The primary focus of this review encompasses an appraisal of cutting-edge microfluidic platforms employed in the realm of single-cell multiomics analysis. Furthermore, it discusses technological advancements in various single-cell omics such as genomics, transcriptomics, epigenomics, and proteomics, with their perspective applications. Finally, it provides future prospects of these integrated single-cell multiomics methodologies, shedding light on the possibilities for future biological research.

Keywords Microfluidics · Single-cell · Multiomics · scRNA-seq

Introduction

As the essential structure of living organisms, cells have been primarily studied in bulk population in the past decades. However, given the inherent cell heterogeneity, even the cells with the same cellular history can exhibit differences in physical characteristics, chemical compositions, as well as biological activities, which can be masked by traditional bulk analysis [1, 2]. Moreover, the hidden variations among cells are prominent especially for understanding the mechanism underlying diseases, such as the initiation and progression of cancers. For this reason, single-cell technologies have emerged to uncover the heterogeneity underlying different cells. Specifically, single-cell sequencing technologies have been increasingly developed for the observation of multilayered information ranging from transcriptomic, genomic to epigenomic, and proteomic heterogeneity of individual cells [3–5].

While characterization of single modality within individual cells is valuable, it may not capture the whole picture of cellular modalities. To overcome this limitation, it is important to integrate multiple modalities and consider the interactions between different cells within a system [6–8]. Single-cell multiomics analysis has revolutionized our ability to investigate the complex biological system at an unprecedented level of resolution. By integrating data from different modalities, such as transcriptomics, genomics, epigenomics, and proteomics, researchers can obtain a comprehensive understanding of molecular profiles within individual cells and unravel previously inaccessible molecular intricacies potentially [9, 10].

Microfluidic technology, which allows for precise manipulation, control, and analysis of small volumes of fluids at the microscale level, has emerged as a highly efficient and versatile tool for single-cell analysis [11]. With the merits of minimalization, integration, automation, low reagent consumption, and high throughput, microfluidics has boosted the analysis of single-cell multiomics [12, 13]. For instance, using a specially designed microfluidic chip, single cells can be isolated individually in micro-scaled compartments,

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followed by the subsequent cell lysis and chemical reactions. Independent chambers eliminate the possible contamination from other cells, thereby improving the accuracy of single-cell analysis. Moreover, the picoliter-to-microliter reaction volume can significantly enhance the reaction efficiency and shorten the analytical time. Furthermore, microfluidics offers precise control over the surrounding microenvironment of single cells, enabling the study of cell behavior under controlled conditions with high throughput. Overall, microfluidic technology has revolutionized single-cell analysis by offering improved control, throughput, and integration.

In this review, we outline various state-of-the-art microfluidic platforms employed in single-cell analysis, encompassing droplets, valves, microwells, and digital microfluidics (DMF) (Fig. 1). Moreover, we expound on different layers of omics, with a primary focus on transcriptome, genome, proteome, etc. Notably, we undertake a comparative analysis of these approaches, examining their respective merits and demerits. Finally, we discuss the challenges and provide a visionary outlook for the future of microfluidic-based single-cell multiomics analysis.

Typical microfluidic platforms and commercial microfluidic devices for single-cell analysis

The implementation of microfluidics in single-cell multiomics analysis presents a groundbreaking opportunity to intricately manipulate target cells and acquire multiomics data from individual cells with high sensitivity and resolution. Microfluidic devices can process a large number of individual cells in parallel, improving the throughput of single-cell multiomics analysis. Moreover, microfluidic platforms can be designed to integrate different omics assays, which allows researchers to obtain a comprehensive view of a cell's molecular profile in a high-throughput manner. Microfluidic devices can be adapted for dynamic and temporal studies, enabling researchers to track changes in a cell's multiomics profile over time or under various conditions. Typically, different types of microfluidic-based platforms display varied throughput including low (less than one hundred), median (hundreds to thousands), and high (more than thousands). The required number of cells depends on the complexity of samples. Usually, the suggested number increases with the heterogeneity of samples [14]. High-throughput methods are suitable for atlas construction where large numbers of heterogeneous cells are required. On the contrary, low-throughput strategies find their applications especially in the cases where in-depth and high coverage analysis is necessary [15]. Overall, these cutting-edge approaches possess the potential to revolutionize our understanding of cell biology and diseases. While various types of microfluidic-based devices have been extensively covered in previous reviews [11–13], this overview will provide a concise introduction to these platforms.

Droplet-based

Droplet-based microfluidic technologies have gained immense popularity due to the merits of high throughput and high speed [16–18]. Numerous droplets, typically in

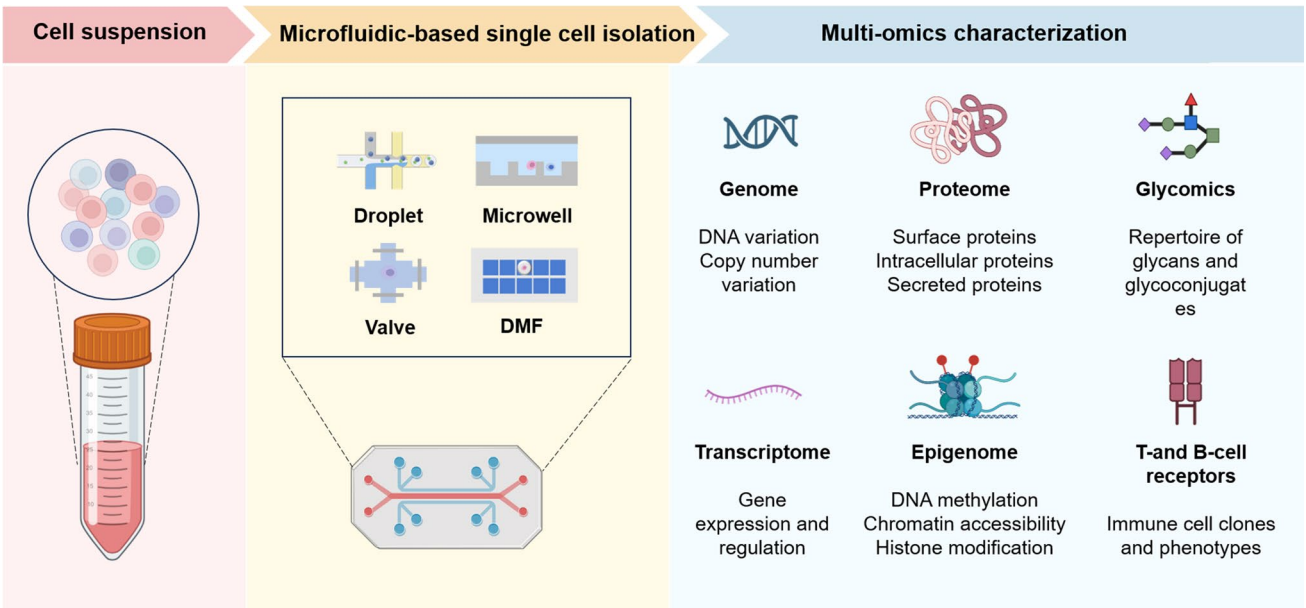


Fig. 1 Schematic diagram of single-cell multiomics characterization empowered by microfluidics

the range of picoliters to microliters, can be generated as independent compartments by carrier oil, which enables the processing of thousands to millions of cells. More importantly, these droplets can be precisely controlled and subjected to subsequent manipulation such as merging, heating sorting, and storage, facilitating on-chip single-cell analysis such as fluorescence-based profiling of cells. Drop-seq [17] and InDrops [18] were back-to-back pioneering work for droplet-based scRNA-seq (Fig. 2A). These techniques have found great suitability in various applications, including single-cell drug discovery and high-throughput screening. However, to eliminate the potential for cell doublet formation, large numbers of samples are required to be diluted, leading to limited cell availability. Thus, this strategy is less suitable for trace samples.

Valve-based

Valve-based microfluidics is a technique that utilizes valves to regulate the movement of fluids within microchannels. These valves are commonly made of soft materials such as polydimethylsiloxane (PDMS) and can be controlled using various methods such as pneumatic pressure or electrostatic forces. By controlling the movement of individual cells through a series of processing steps, valve-based microfluidics enables the isolation, lysis, and library preparation of single cells with exceptional precision [19–22]. For instance, Paired-seq was developed for precise pairing of single cell and single barcode bead, enabling highly efficient scRNA-seq (Fig. 2B) [19]. With their exceptional ability to precisely control the fluid flow and execute complex fluidic operations, valve-based microfluidic platforms are well-suited for integrated single-cell analysis that necessitate precise manipulation of diverse analytes, especially the identification of rare cell populations. But valve-based platforms face limitations related to its complexity and cost. And the throughput (usually hundreds to thousands of cells) is lower compared with droplet-based methods.

Microwell-based

Microwell-based devices are a class of prevailing microfluidic platforms that feature an array of interconnected micro-wells or micro-chambers. These platforms facilitate the distribution and manipulation of cells, particles, or other small targets, allowing for parallel analysis [23–27]. Compared with other microfluidic technologies, microwell-based platforms are easy to handle because cells can be seeded based on gravity without the need for additional instruments. Typically, Well-Paired-Seq leveraged a dual-layer microwell structure to ensure highly efficient pairing of single cell and single barcode bead based on size-exclusion principle (Fig. 2C) [26]. By adjusting the size and design of the microwells, researchers can control the number and distribution of cells, enabling high-throughput single-cell sequencing experiments. This strategy is particularly well-suited for studying cellular interactions and lineage tracing, as it allows for the longitudinal analysis of single cells over time and space due to the static location of each cell and the transparent characteristics for direct monitoring. Nonetheless, the size and shape of the microwells can affect the capture efficiency of different cell types, posing challenges when studying heterogeneous populations.

DMF-based

DMF is an advanced technology that enables the manipulation of small volumes of fluids on an array of electrodes based on electrowetting-on-dielectric (EWOD). Different from other microfluidic strategies, DMF offers automatic control over the movement of droplets, allowing for a wide range of chemical and biological processes, such as mixing, dispensing, diluting, and reacting [28–31]. Using DMF, single cells can be lysed, cellular analytes extracted, and libraries prepared in a highly controlled and efficient manner, facilitating in-depth single-cell analysis (Fig. 2D) [30]. With the advantages of automation, high precision, and addressability, DMF is well-suited for customized workflows of various single-cell sequencing applications. Since it relies on precise control of limited numbers of electrodes, DMF-based strategies usually face the challenges of the relatively small numbers of cells that can be processed simultaneously compared with other techniques.

Commercial microfluidic devices

Commercial microfluidic platforms are become increasingly popular in recent years due to their capability to execute a wide range of researches, spanning various fields such as cancer biology, medical diagnosis, and drug discovery (Table 1).

A typical example of commercialized droplet-based platform is the Chromium™ system released by 10 × Genomics company, which pioneered the market of single-cell sequencing using droplet-based strategies [32]. In this way, approximately tens of thousands of cells can be encapsulated into the drops per run, followed by the subsequent automated workflows. A similar droplet-based Tapestri system

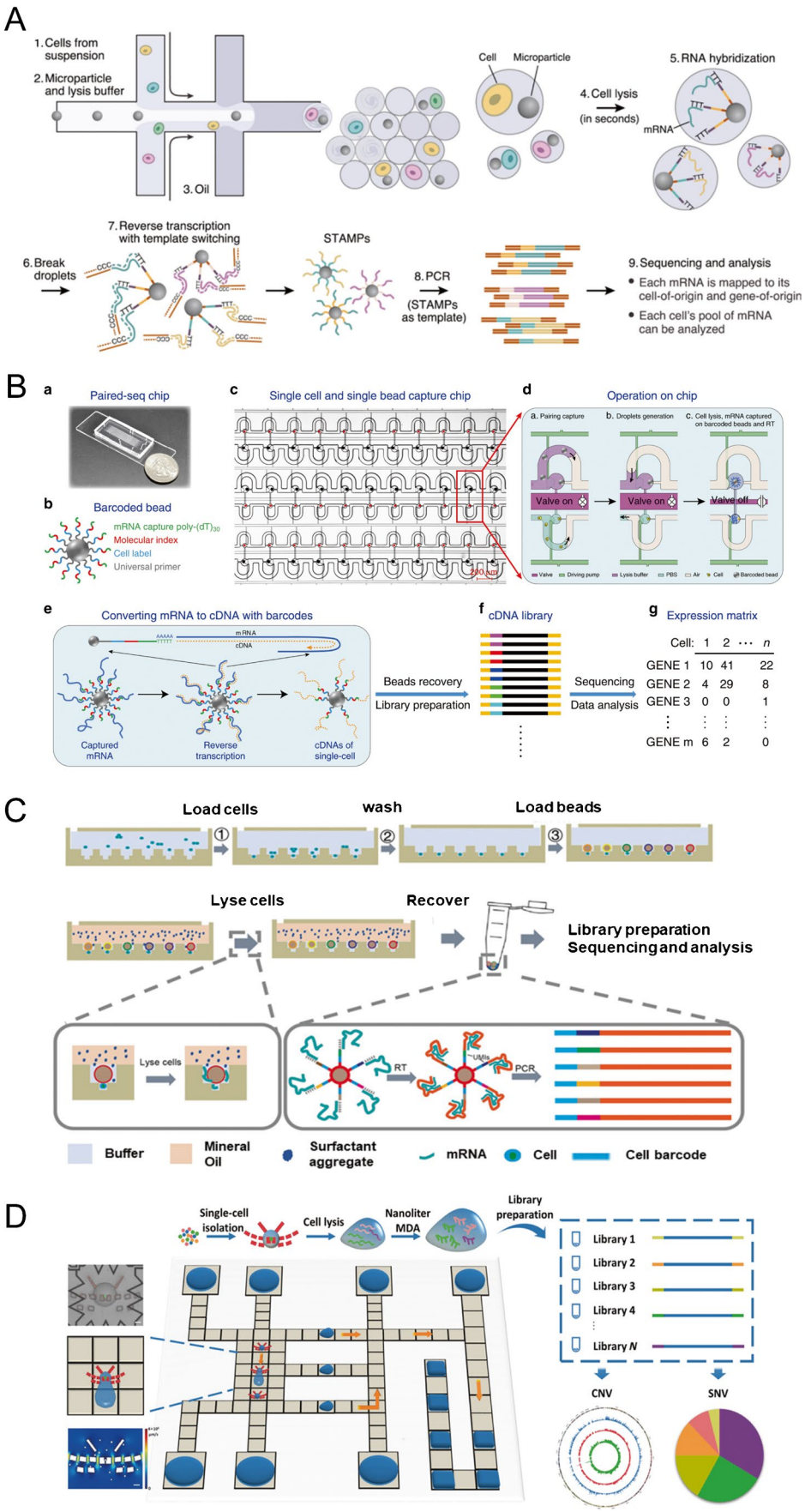


Fig. 2 Typical microfluidic platforms for single-cell analysis. (**A**) Droplet-based platforms. Reproduced with permission from ref. [17] Copyright 2015, Elsevier. (**B**) Valve-based circuits. Reproduced with permission from ref. [19] Copyright 2020, Springer Nature. (**C**) Microwell-based chips. Reproduced with permission from ref. [26] Copyright 2022, Wiley–VCH GmbH. (**D**) DMF-based strategies. Reproduced with permission from ref. [30]. Copyright 2020, The American Association for the Advancement of Science

Table 1 Comparison of different commercial microfluidic systems

Name	Throughput (per run)	Parameters	Technology
Chromium™	> 10,000 cells	Transcriptome Genome Proteome Epigenome TCR/BCR	Droplets
Tapestri	~ 5000 cells	Genome Proteome	Droplets
C1 Autoprep	96/384 cells	Transcriptome Proteome	Valve
Rhapsody™	20,000 cells	Transcriptome Proteome	Microwell
Singleron Matrix	> 10,000 cells	Transcriptome Proteome TCR	Microwell
Well-Paired-Seq	> 10,000 cells	Transcriptome	Microwell

was released by Mission Bio to allow both characterization of genomics and surface proteins [33].

The C1 Autoprep System, which harnesses integrated fluidic circuits with multiple valves as well as channels, was released by Fluidigm to perform single-cell capture and a series of reactions of up to 96 single cells [21]. Recently, the system has been updated that up to 394 single-cell samples can be analyzed in parallel, which offered increased throughput and reliability.

As the representative of commercial microwell-based devices, the Rhapsody™ Single-Cell Analysis System released by BD Company allows as high as 20,000 cells to be isolated in microwells within a chip. Likewise, Singleron Matrix was established by Singleron Bio to perform the pipeline of scRNA-seq from cell isolation to library construction. More recently, a double-layered microwell-based system was released by Dynamic Biosystems, where improved barcoding efficiency of single cells was achieved (82%).

Characterization of different layers of omics

Linking transcriptome with genome

The transcriptome consisting of mRNA transcripts can reflect gene expressions that cause cellular heterogeneity. Single-cell transcriptional analysis provides comprehensive pictures of the diverse transcriptional signatures of single cells under internal and external influences, thus enabling identification of cell subsets and cell states [5]. In most cases, single-cell transcriptional analysis is performed by single-cell isolation, lysis, reverse transcription, and amplification of cDNA. Subsequently, the amplified cDNA can be analyzed using PCR for targeted analysis or sequencing for whole transcriptome profiling. For single-cell RNA sequencing (scRNA-seq), barcoding strategy has been implemented for paralleled analysis. Typically, barcode beads were incorporated to label individual cells. The bead barcodes were composed of four parts: oligo dT to capture polyadenylated mRNA, cell barcode for identification of each cell, unique molecular identifier (UMI) to benchmark the absolute gene expression, and a PCR primer for the subsequent amplification. In this way, thousands of single cells and single barcode beads were co-trapped. After single cell was lysed, and the transcriptomic information was transferred to the paired barcode bead during reverse transcription. Afterwards, barcode beads were pooled and the transcripts were amplified and sequenced [17, 23].

Single-cell genome analysis allows for the characterization of genetic mutations and variations such as copy number variations (CNVs) and single-nucleotide variants (SNVs), thereby revealing the genetic heterogeneity during cell proliferation as well as differentiation. Such genetic heterogeneity has been reported as a leading cause of cancer and development disorders [34]. Due to the limited amounts of the genetic materials in single cells, the genomic DNA (gDNA) should be amplified by whole-genome amplification (WGA) using strategies such as PCR [35], multiple displacement amplification (MDA) [36], multiple annealing and looping-based amplification cycles (MALBAC) [37], and linear amplification via transposon insertion (LIANTI) [38]. However, it still remains challenging to achieve WGA with high uniformity and fidelity. In this case, microfluidic-based platforms with small reaction volumes can minimize contamination and reduce amplification bias, thereby serving as ideal tools for single-cell genomic analysis.

Joint analysis of transcriptome and genome allows the profiling of RNA and DNA from the same single cell, thereby linking the transcriptional variation with the genetic differentiation unambiguously, and enabling the identification of disease-causative genetic variations among the diverse gene expressions. DNA-RNA sequencing (DR-seq) [39] and genome and transcriptome sequencing (G&T-seq) [40, 41] were developed successively for co-measurement of DNA and RNA from the same cell using different separation strategies. Both methods required multiple tube-based handling processes and were constrained by low coverage and sample losses.

To solve these limitations, Fan's group proposed the first microfluidic platform enabling joint profiling of genes and transcripts at the single-cell level [42]. Specifically, they designed a valve-based microfluidic device to trap single cells, enabling selective cytoplasmic lysis from the nucleus. Following the separation of lysed RNA and reverse

transcription, the nucleus was lysed to release gDNA. The platform seamlessly integrated single-cell capture and multistep reactions with precise control. The small chambers facilitated rapid mixing, leading to the enhanced efficiency of nucleic acid amplification, which was imperative for the limited amounts of DNA and RNA from a single cell.

Similarly, Zaag et al. proposed a pressure-driven microfluidic platform for differential on-chip extraction of both DNA and RNA from single cells [43]. Using this method, they characterized the functional activity of the Wnt pathway from both cytosol and nuclear fraction. Leveraging DMF technology, Yang's group devised a DMF-based platform for joint measurement of DNA and RNA (DMF-DR-seq) (Fig. 3A) (Table 2) [44]. It facilitated streamlined analysis of genome and transcriptome from the same single cells with higher genome coverage and less amplification bias at lower costs in comparison with other strategies. Given the limited throughput, DMF-DR-seq was well-suited for trace samples like circulating tumor cells (CTCs) or stem cells where deep information is needed.

Current droplet-based single-cell sequencing platforms usually profile all the cells in each sample but cannot differentiate cell subsets, which is unsuitable for analysis of rare cell subpopulations. To this end, Abate's group introduced a novel droplet-based microfluidic device with fluorescence-activated droplet merger for RNA and DNA sequencing of selected cells (Fig. 3B) [45]. The droplet containing the targeted cell could be triggered to merge with the droplet with a barcode bead, thereby achieving pairing of targeted cells and barcode beads. With such technology, rare subpopulations of cells were identified while large numbers of uninformative cells were discarded, thereby saving the sequencing cost.

Linking transcriptome with proteome

Proteins are principal biological executors of important cellular behavior such as DNA replication and metabolic reactions. Proteome profiling allows large-scaled characterization of protein signatures within cells, promotes the understanding of biological mechanisms, and facilitates the discovery of disease-related biomarkers or therapeutic targets [46, 47]. Compared with transcripts, proteins have less stochastic noises, higher stability, and higher expression levels than cognate mRNA [48]. Antibodies conjugated with fluorophores or metal isotope tags have been widely used to measure protein expressions by flow cytometry or mass cytometry [49]. However, the number of markers available for the identification of cell subpopulations was limited due to the spectral overlap of fluorophores. Furthermore, while

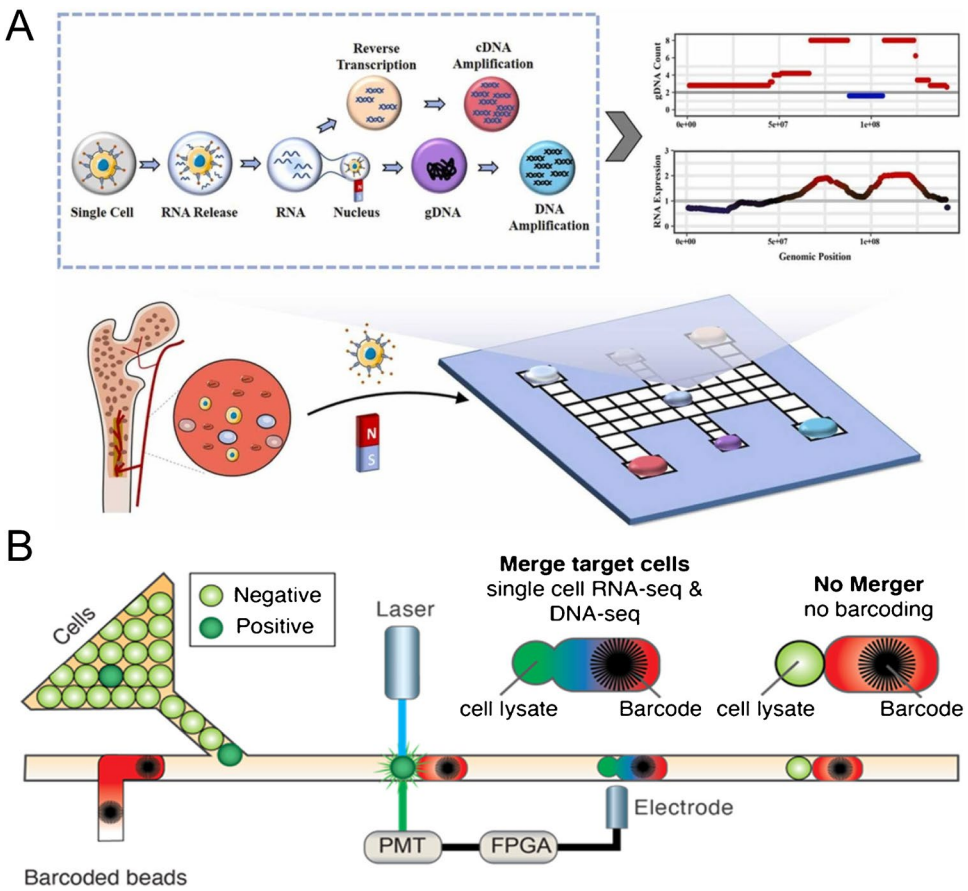


Fig. 3 Microfluidic-based strategies for co-analysis of transcriptome and genome. (**A**) Schematic of DMF-DR-seq using an integrated DMF platform. Reproduced with permission from ref. [44]. Copyright 2022, Elsevier. (**B**) Schematic of RNA and DNA sequencing of selected cells using droplet technology. Reproduced with permission from ref. [45] Copyright 2020, American Chemical Society

Table 2 Comparison of different microfluidic-based multiomics analysis

Name	Analytes	Technology	Strengths	Weakness	Ref
DMF-DR-seq	Genome and transcriptome of clinic myeloma samples	DMF	High accuracy and sensitivity	Limited throughput	[44]
CITE-seq	Transcriptome and surface proteins of cell lines	Droplets	High throughput	Limited cell availability	[51]
REAP-seq	Transcriptome and surface proteins of PBMCs	Droplets, valve	High throughput and scalability	Limited cell availability	[53]
multi-Paired-seq	Transcriptome and surface proteins of cancer cell lines	Valve	High accuracy and availability	Limited throughput	[54]
Prox-seq	Extracellular protein complexes and transcriptome of human peripheral blood mononuclear cells	Droplets	High throughput and ability to analyze protein interaction	Limited cell availability	[55]
Protein expressions with targeted transcriptomes	Targeted transcriptomes and protein expressions in T cells	Valve	Decreased cost with minimal sequencing depth	Require prior knowledge of targets and median throughput	[56]
INs-seq	Transcriptome and intracellular proteins of tumor models	Droplet	High throughput and ability to analyze intracellular proteins	Limited cell availability	[57]
TRAPS-seq	Transcriptome and surface proteins in PBMCs	Droplet	High throughput and time-resolved characterization of protein secretion	Limited cell availability	[58]
inCITE-seq	Intracellular transcriptome and proteins in mouse models	Droplet	High throughput and access to solid tissues	Limited cell availability	[60]
Apt-seq	Transcriptome and surface proteins of cancer cell lines	Droplet	High throughput and replace antibodies with versatile aptamers	Limited cell availability and available aptamers	[63]
Dab-seq	Immunophenotype and genotype of AML cells	Droplet	High throughput	A prior knowledge of the targeted loci and epitopes	[53]
sc-GEM	Transcriptome and methylation in human fibroblasts	Droplet	High throughput and less sample degradation	Limited cell availability	[70]
SNARE-seq	Single-nucleus chromatin accessibility in neonatal and adult mouse cerebral cortices	Droplet	High throughput	Limited cell availability	[72]
T-ATAC-seq	Chromatin accessibility and T cell receptors of T cell leukemia	Valve	Unbiased pairing of chromatin accessibility and	Limited throughput	[74]
ISSAAC-seq	Transcriptome and chromatin accessibility from adult mouse cerebral cortex	Droplet	High throughput and sensitivity	Limited cell availability	[75]
scTrio-seq	Genomic copy-number variations, DNA methylome dynamics, and transcriptome of single mammalian cells	Droplet	High throughput	Limited cell availability	[77]
DOGMA-seq	Accessible chromatin, transcriptome and surface proteins in PBMCs	Droplet	High throughput and able to analyze none polyA-tailed mRNA	Limited cell availability	[78]
TEA-seq	Transcriptome, surface epitopes, and chromatin accessibility of permeabilized PBMCs	Droplet	High throughput	Limited cell availability	[79]
NEAT-seq	Nuclear protein epitope abundance, chromatin accessibility, and transcriptome in CD4 memory T cells	Droplet	High throughput	Limited cell availability	[80]
SUGAR-seq	Glycoproteome, epitopes, and transcriptome of TILs	Droplet	High throughput and ability to characterize glycans	Limited cell availability	[81]
LacNAc-seq	Glycans, transcriptomics, and TCR signatures of human immune cells	microwell	Ability to map LacNAc of human immune cells	Dependence on glycosyltransferases	[82]
Perturb-Seq	Transcriptome and genomic perturbation in immune cells	Droplet	High throughput, provides functional information about genomic perturbation	Limited cell availability	[84]

Table 2 (continued)

Name	Analytes	Technology	Strengths	Weakness	Ref
CRISP-Seq	Transcriptome and genomic perturbation in myeloid cells	Droplet	High throughput, provides functional information about genomic perturbation	Limited cell availability	[85]
CROP-seq	gRNA expression associated with corresponding transcriptome responses	Droplet	High throughput, ability to dissect gRNA expression	Limited cell availability	[86]
ECCITE-seq	Transcriptome, epitopes, clonotypes, and CRISPR perturbations in PBMCs	Droplet	High throughput, ability to integrate multiple modalities	Limited cell availability	[87]

DNA and RNA can be amplified by various amplification strategies using universal probes, it is challenging to amplify proteins directly, which adds additional circumstances to single-cell proteomic analysis [50]. To this end, antibodies conjugated with DNA barcodes have been implemented for unlimited simultaneous analysis of markers as well as converting protein signals into DNA readout [51].

Recent reports have revealed unexpected discrepancy between mRNA and corresponding proteins, emphasizing the importance of constructing the dynamic relationship between transcriptome and proteome [52]. Considering the close relationship between transcriptome and proteome, integrative analysis of both omics offers a holistic view of gene expressions and protein synthesis, thus bridging a gap between genotype and phenotype. Generally, current strategies can be classified into antibody- and aptamer-based approaches.

Antibody-based strategies

Antibodies conjugated with DNA barcodes have been extensively used for simultaneous measurement of RNA and proteins. For instance, CITE-seq (cellular indexing of transcriptomes and epitopes by sequencing) was developed for multiplexed quantitation of proteins and transcripts from the same single cells [51]. In this case, cells were incubated with labelled DNA-barcoded antibodies, which was similar to the flow cytometry staining process but with DNA-antibody conjugates instead of fluorophores. The antibody-DNA complexes are also comprised of three parts including oligo-dA, antibody barcodes, and PCR handle. After routine droplet-based scRNA-seq workflow, mRNA-derived labels (~ 500 bp) can be size fractioned from the antibody-derived tags (~ 150 bp). The strategy was applied for enhanced characterization of immune cell phenotypes. Similarly, the RNA expression and protein sequencing assay (REAP-seq) was also reported [53]. Different from CITE-seq using streptavidin–biotin to link antibodies and oligos, REAP-seq employed the conjugation of DNA and antibodies using unidirectional chemistry with a covalent bond. Such conjugation strategy was more stable and minimized the steric hinderance, and was crucial for enhancing the scalability of protein analysis. REAP-seq has been commercialized by Fluidigm as C1 REAP-seq for simultaneous characterization of scRNA-seq and surface proteins. Taking advantage of DNA-antibody conjugates, Yang's group introduced a strategy termed as multi-Paired-seq for resolving the expression dynamics of both the transcriptome and proteins of single cells based on previously developed Paired-seq (Fig. 4A) [54]. Single cells and barcode beads could be precisely manipulated and paired by the control of valve/pump structures, and background noises such as free mRNA and conjugates could be removed efficiently.

Considering the significance of protein interaction and challenges where the scale of paired proteins increased quadratically, Tay's group recently presented proximity sequencing (Prox-seq) combining PLA with scRNA-seq to simultaneously measure extracellular protein complexes and mRNA (Fig. 4B) [55]. Prox-seq used a pair of DNA-antibody conjugate probes with index information of corresponding proteins to identify protein conjugates. Initiated by a universal connector, paired probes were ligated to generated complete PLA products, including barcode information of protein complexes, UMI, and a poly-A tail. Integrated with scRNA-seq, Prox-seq allowed for joint characterization of protein complexes and transcriptomes in a similar way to CITE-seq and REAP-seq. Using Prox-seq, the authors observed Toll-like receptor (TLR)–associated protein interactions.

Instead of combining phenotypes with whole transcriptome, Prlic et al. characterized protein expressions with targeted transcriptomes in microwells based on a BD Rhapsody platform (Fig. 4C) [56]. Hundreds of immune-related genes were selected for the establishment of immunophenotypes and transcripts in T cells. In comparison with routine methods, such strategy only required minimal sequencing depth (approximately 10% of other methods). Therefore, the overall cost was reduced without compromising high sensitivity. But upfront decisions on targets of interest were needed.

Aside from surface proteins, intracellular staining and sequencing (INs-seq) was proposed for high-throughput scRNA-seq with characterization of intracellular proteins (Fig. 4D) [57]. Different from other strategies, cells were permeabilized, intracellularly stained by fluorophore-labelled antibodies, and sorted to droplet-based microfluidic systems, followed by conventional scRNA-seq protocols, allowing for coupled analysis of transcriptome and intracellular protein activity.

More recently, a groundbreaking strategy termed "time-resolved assessment of protein secretion by sequencing" (TRAPS-seq) was developed to characterize secreted proteins using scRNA-seq [58]. Released proteins were captured onto the cell surfaces using bispecific antibodies, followed by the formation of immunosandwich structure with barcode antibodies. TRAPS-seq was leveraged to dissect the interplay between cytokines as well as cellular gene expressions.

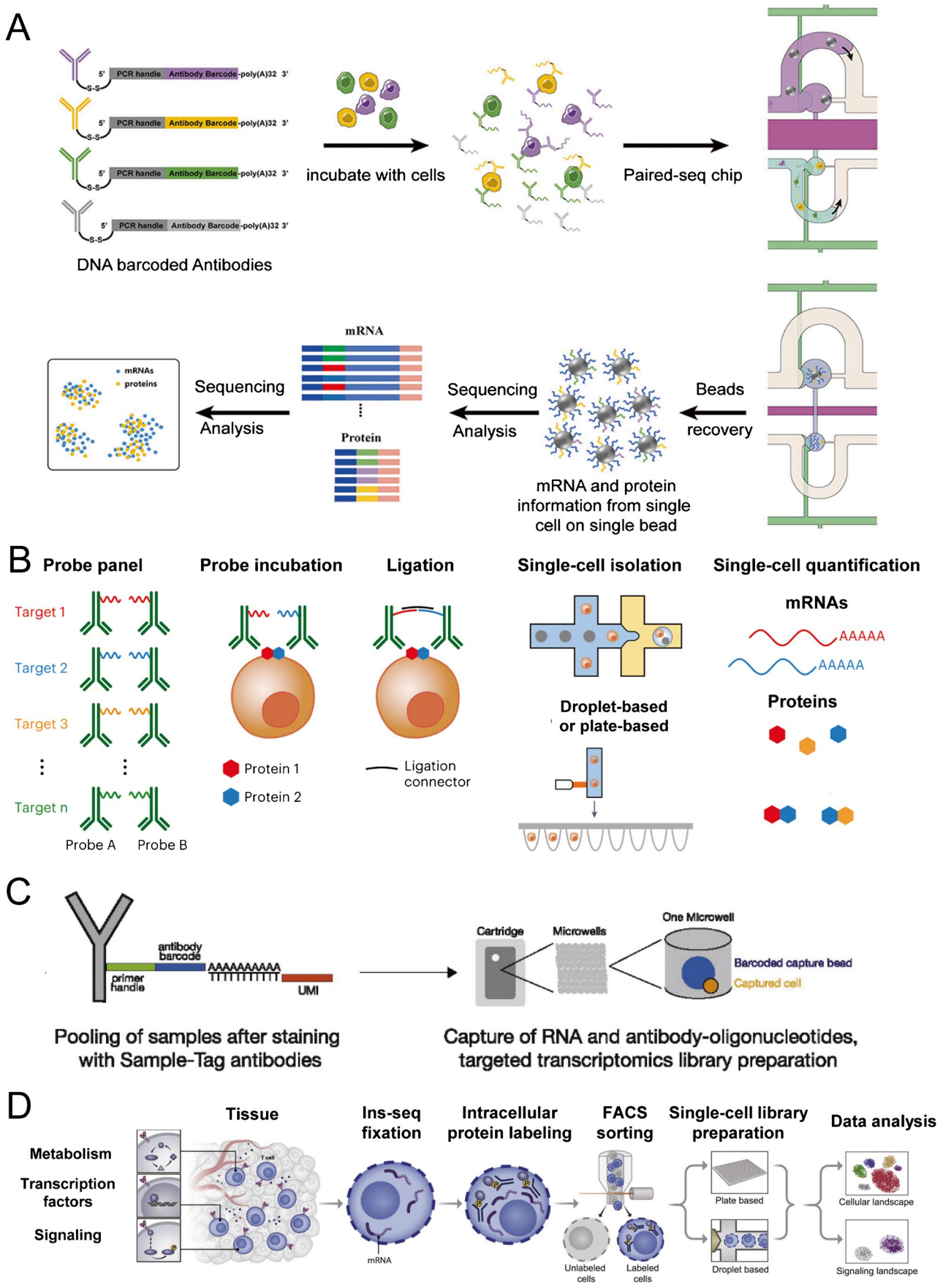
Single-nucleus (sn)RNA-seq is paramount for characterization of solid tissues where cellular membrane integrity is damaged during dissociation. Joint measurement of nuclear transcriptome and proteins provides deeper insight into how nuclear proteins and transcriptomes shape gene expressions and pathways [59]. As an expansion of CITE-seq, inCITE-seq was proposed for intracellular indexing of transcriptomes and epitomes with droplet-based microfluidic platforms [60]. Cells were fixed and permeated, followed by intracellular binding of DNA-antibody conjugates and nucleus-hashing antibodies for multiplex sampling. inCITE-seq allowed the deciphering of cells from solid tissues especially those that are hard to dissociate, and shed light on gene regulation during dynamic responses.

Aptamer-based strategies

In most cases, antibodies are ideal tools for proteomic profiling. However, antibodies are limited by batch effects and high costs. Moreover, the steric hindrance caused by the relatively large size could impact binding affinity. Alternatively, aptamers are single-stranded nucleic acids with unique tertiary structures that could specifically recognize targets [61]. Aptamers are evolved by Systematic Evolution of Ligands by EXponential enrichment (SELEX) [62]. Compared with antibodies, aptamers hold unique merits such as ease of modification, controllability, facile chemical synthesis, versatile structural design, and flexibility. Abate et al. reported Apt-seq for simultaneous profiling of surface proteins and transcriptomes of single cells with aptamers [63]. Certain types of aptamers corresponding to epitopes were incubated with cells, followed by general droplet-based scRNA-seq protocols. In this way, independent information of cell types can be inferred from aptamer binding. And additional information could be obtained to solidate the differentiation of cell states. Taking 3T3 and Ramos cells as an example, the authors confirmed that aptamers clustered in transcriptional fingerprints, indicating that aptamers could be leveraged to differentiate cell types and states in line with transcriptional data.

Linking genome with proteome

While combined analysis of transcriptome and proteome has been widely studied, co-measurement of single-cell genotype–phenotype was rarely reported. Although scRNA-seq provides a valuable tool for the study of genotype–phenotype linkage, where the sequences of mRNA and their abundance reflect the genotype and phenotype, respectively, the genotyping from mRNA only reflects the expressed part of the genome, indicating that non-transcribed information and other mutations are lost. In the circumstances, Abate's group proposed Dab-seq for combined profiling of immunophenotype and genotype of single cells from patients with acute myeloid leukemia (AML) (Fig. 5) [64]. Dab-Seq was based on Abseq that enabled sequencing of single-cell surface proteins using barcoded antibody-DNA conjugates for immunostaining of single cells, but with additional cell lysis for genome amplification. Using a commercial Mission Bio Tapestri instrument, droplets were paired with those containing barcode beads and subjected to PCR using targeted primers for amplification of specific genomic regions. As a result, DAb-seq identified 49 target DNA and 23 hematopoietic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Single-cell isolation
Single-cell quantification
Target 1
Target 2
Target 3
Target n
Probe A
Probe B
Protein 1
Protein 2
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Intracellular protein labeling
FACS sorting
Single-cell library preparation
Data analysis
Metabolism
Transcription factors
Signaling
T cell
mRNA
Unlabeled cells
Labeled cells
Plate based
Droplet based
Cellular landscape
Signaling landscape

◂**Fig. 4** Microfluidic-based strategies for joint characterization of transcriptome and proteome. (**A**) Schematic of multi-Paired-seq using microwell-based microfluidics. Reproduced with permission from ref. [54] Copyright 2022, American Chemical Society. (**B**) Schematic of Prox-seq for simultaneous measurement of extracellular protein complexes and mRNA using commercial droplet-based systems. Adapted from ref. [55]. (**C**) Schematic of targeted transcriptomes and protein expressions in commercial microwell devices. Reproduced with permission from ref. [56] Copyright 2020, Elsevier. (**D**) Schematic of INs-seq for integrated analysis of transcriptome and intracellular proteins. Reproduced with permission from ref. [57], Copyright 2020, Elsevier

markers in AML, revealing the variability of the genotype and phenotype of blast cells over different clinical scenarios. Since Dab-seq required a prior knowledge of the targeted loci and epitopes, it was inaccessible to profiling disease-related mutations beyond the targeted loci or phenotypic markers.

Linking epigenome with transcriptome

Epigenomic analysis focuses on revealing how functional genomic elements informs the phenotype via regulatory variations, including DNA methylation, post-translational histone modifications, and chromatin accessibility, which is essential for investigating diverse cellular behavior and uncovering disease development [65]. For instance, DNA methylation, the most generally studied epigenetic modification, is related with repression of gene expression and regulation of diverse cellular behavior. Notably, 5-methylcytosine (5mC) at guanine base (CpG dinucleotide) serves as an epigenetic mark, and the strategy of analyzing 5mC usually relies on using bisulfite to convert cytosine to uracil, while the leftover 5mC residues remain unchanged [66]. Besides, histone modifications indicate the likelihood of gene expression near a DNA locus and reflect cellular differentiation trajectories. Chromatin immunoprecipitation (ChIP), which leveraged specific antibodies to pull down the associated chromatin, comes as one of the conventional approaches for detection of histone modifications [67]. For instance, droplet-based single-cell chromatin immune-precipitation sequencing (Drop-ChIp) was considered as a gold standard for analyzing posttranslational histone modifications at the single-cell level [68]. Moreover, chromatin accessibility can reflect the dynamic contact of macromolecules and packed DNA, providing insights of transcription factors and cis-elements, revealing the regulatory variation between single cells. Chromatin accessibility can be quantified via enzymatic methylation or DNA cleavage. For example, valve-, droplet-, and microwell-based single-cell assays for transpose accessible chromatin using sequencing (scATAC-seq) were developed [69]. Basically, scATAC-seq leveraged transposases (Tn5) to fragment and insert adaptors to DNA in open chromatin, where the regulatory proteins were attached and transcription was initiated. Afterwards, the regions were amplified by PCR and enriched for library construction, enabling the discovery of trans-factor related cell-to-cell variation.

Linking epigenetic and transcriptomic analysis allows for simultaneous interrogation of the gene expression and epigenome at multiple loci, offering a powerful tool to deepen the understanding of linkage of cell fates during dynamic processes. Burkholder et al. developed an automated, integrated, and high-throughput microfluidic platform (sc-GEM) for implementation of gene expression and DNA methylation based on a commercial Fluidigm C1 AutoPrep IFC [70]. sc-GEM combined the single-cell restriction analysis of methylation (SCRAM) measurement proposed by the same group [71] with RT-qPCR as well as sequencing, utilizing methylation-sensitive restriction endonuclease (MSRE) instead of bisulfite conversion to digest the CpG methylation at specific locations, which ensured less sample loss and DNA degradation. Taking reprogrammed human fibroblasts as examples, sc-GEM allowed identification of specific epigenetic signatures in tumor subpopulations.

While sc-GEM targeted specific gene expressions, Zhang et al. proposed single-nucleus chromatin accessibility and mRNA expression sequencing (SNARE-seq) based on the Drop-seq platform for joint profiling of chromatin accessibility and whole-transcriptome with high throughput [72]. The accessible genomic sites in nuclei were pre-tagged by

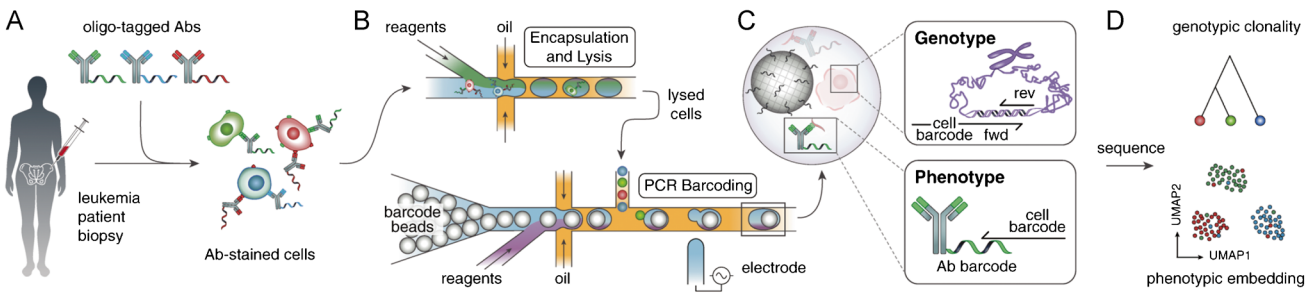


Fig. 5 Schematic of Dab-Seq for combined analysis of genotype and phenotype using droplet-based microfluidics. Reproduced with permission from ref. [64] Copyright 2023, Springer Nature

Tn5 transposase, and emulsified with barcode beads. And the fragmented chromatin captured by designed sequences as well as poly (A) tailed mRNA were captured, enabling the two layers of omics information to share with cellular barcodes. SNARE-seq enabled reconstruction of the transcriptome and epigenome landscape from thousands of mouse cerebral cortices cells, as well as the identification of linkage-associated epigenomic sites. Subsequently, this technique was updated to SNARE-seq2, which dramatically increased the throughput by the incorporation of cellular combinatorial indexing [73].

In addition, Transcript-indexed ATAC-seq (T-ATAC-seq) was designed for combined analysis of chromatin accessibility and T cell receptors (TCRs) using a commercial C1 system [74]. Tn5 was incorporated for transposition of accessible chromatin and primers targeting the encoding region of TCR chains were introduced for TCR-seq. T-ATAC-seq was applied for identification of tumor-related epigenomic signatures in T cell leukemia, holding potential for uncovering specific regulatory elements underlying different cell states. However, due to the limited throughput of C1 systems (96 cells per chip), T-ATAC-seq was better suited for the identification of TCRs of interest, and increased throughput is anticipated.

To streamline the workflow, in situ sequencing hetero RNA–DNA-hybrid after assay for transposase-accessible chromatin-sequencing (ISSAAC-seq) was devised for highly sensitive investigation of both chromatin and transcriptome within the same cell [75]. ISSAAC-seq was established based on Sequencing HEteRo RNA-DNA-hYbrid (SHERRY) [76], a simplified and versatile strategy of RNA-seq library construction. Unlike traditional scRNA-seq protocols where RNA is usually converted into dsDNA via reverse transcription, SHERRY employed Tn5 to cleave RNA/DNA hybrids with adapters, mimicking the process utilized for dsDNA, thus dramatically simplifying the scRNA-seq workflow and curtailed manual involvement to a mere 30 min. Incorporating SHERRY with scATAC-seq, ISSAAC-seq facilitated the interrogation of chromatin accessibility and gene expression with high sensitivity and flexibility. It is noteworthy that ISSAAC-seq was compatible with either FACS or droplet-based microfluidic systems regarding the number of samples.

Linking diverse omics

While most multimodal single-cell analytical methodologies were confined to the integration of transcriptomic data with an auxiliary omic dataset, multiplex single-cell measurements have illuminated a path towards comprehensive snapshots of intricate cellular compositions across diverse layers of characterization. For example, Peng et al. reported single-cell triple omics sequencing (scTrio-seq), which combined single-cell reduced representation bisulfite sequencing (scRRBS) [66] and RNA-seq for joint analysis of genomic copy-number variations, DNA methylome dynamics, and transcriptome of single mammalian cells by concessive cell lysis, separate library construction, and data analysis [77]. Such concept was extended for high-throughput single-cell profiling integrating protein measurement, transcriptome analysis, and mapping of chromatin accessibility using 10×Genomics systems. Greenleaf et al. combined CITE-seq and scATAC-seq to construct molecular features of patients with mixed-phenotype acute leukemias (MPALs), which displayed multiple hematopoietic lineages. Using integrative analysis of single-cell multiomics, the authors constructed the framework of cancer development and identified both common and distinct molecular signatures across different patients with individual phenotypes [16]. Likewise, ATAC with select antigen profiling by sequencing (ASAP-seq) was presented for simultaneous analysis of accessible chromatin, transcriptome, as well as surface proteins [78]. Instead of simply combining CITE-seq with scATAC-seq, ASAP-seq optimized the enzymatic steps to minimize sample loss and ensure high-quality analysis of multiple modalities. To tackle the challenge that most scRNA-seq was constrained by profiling of poly A-tailed mRNA, ASAP-seq introduced bridging identifier sequences (UBI) and UMI sequences for poly A-tailed mRNA and other none polyA-tailed mRNA, respectively. And ASAP-seq was applied for profiling of epigenomic, transcriptomic, and proteomic analysis of bone marrow mononuclear cells. Furthermore, ASAP-seq was transferred to 10×Chromium single-cell multiome platform as DOGMA-seq, which allowed profiling of gene regulation from chromatin accessibility to mRNA expression and proteome over the central dogma.

Meanwhile, TEA-seq was also developed as a trimodal platform for measurement of transcriptomic, surface epitopes, and chromatin accessibility of permeabilized cells using a 10×Chromium system [79]. Considering that the release of ambient RNA during cell lysis could impair cell barcoding, the authors selected hypotonic lysis to weaken the perturbation of non-cell barcodes, thus improving the signal-to-noise ratio of sequencing data. Afterwards, a technique called NEAT-seq was developed, enabling the study of quantitative impact of epigenetic regulators on chromatin and gene expression states in primary human samples with 10×Genomics Multiome kits (Fig. 6A) [80]. This technique has been successfully applied to study CD4 memory T cells and identify regulatory activities gated by transcription, translation, and chromatin binding.

Aside from routinely characterized omics including transcriptome, genome, and proteome, other molecular signatures have also been profiled for comprehensive understanding of cellular stages. For instance, SUrface-protein Glycan And RNA-seq (SUGAR-seq) incorporated posttranslational

modification with transcriptional and phenotypic characteristics (Fig. 6B) [81]. Specifically, lectins of *Phaseolus vulgaris agglutinin* (L-Pha) that could selectively bind with N-linked glycosylation (N-glycans) on the T cell surface were utilized. With the integration of CITE-seq and TCR-seq based on 10×Genomics platforms, SUGAR-seq drew a comprehensive snapshot of glycoproteome, epitopes, and transcriptome of tumor-infiltrating T cells (TILs) and could be readily adaptable with commercial microfluidic strategies, thus allowing deeper multi-faceted single-cell analysis. However, the selectivity of SUGAR-seq was less desirable since L-Pha could label multiple glycan structures. And the multivalent binding sites could induce the crosslinking to change cellular status. Alternatively, Li's group proposed a chemoenzymatic-based approach to characterize N-acetyllactosamine (LacNAc), a natural disaccharide unit on cell surfaces (Fig. 6C) [82]. LacNAc-seq leveraged the fucosylation reaction, where guanosine diphosphate β-L-fucose (GDP-Fuc) as the donor could be transferred to LacNAc as the receptor with the help of fucosyltransferase (FT). By introducing barcode DNA to GDP-Fuc analogs, the level of cellular LacNAc could be translated into DNA sequences that could be further compatible with commercial microfluidic-based scRNA-seq such as 10×Genomics, paving a new way for the understanding of the relationship between glycans, transcriptomics, and TCR signatures.

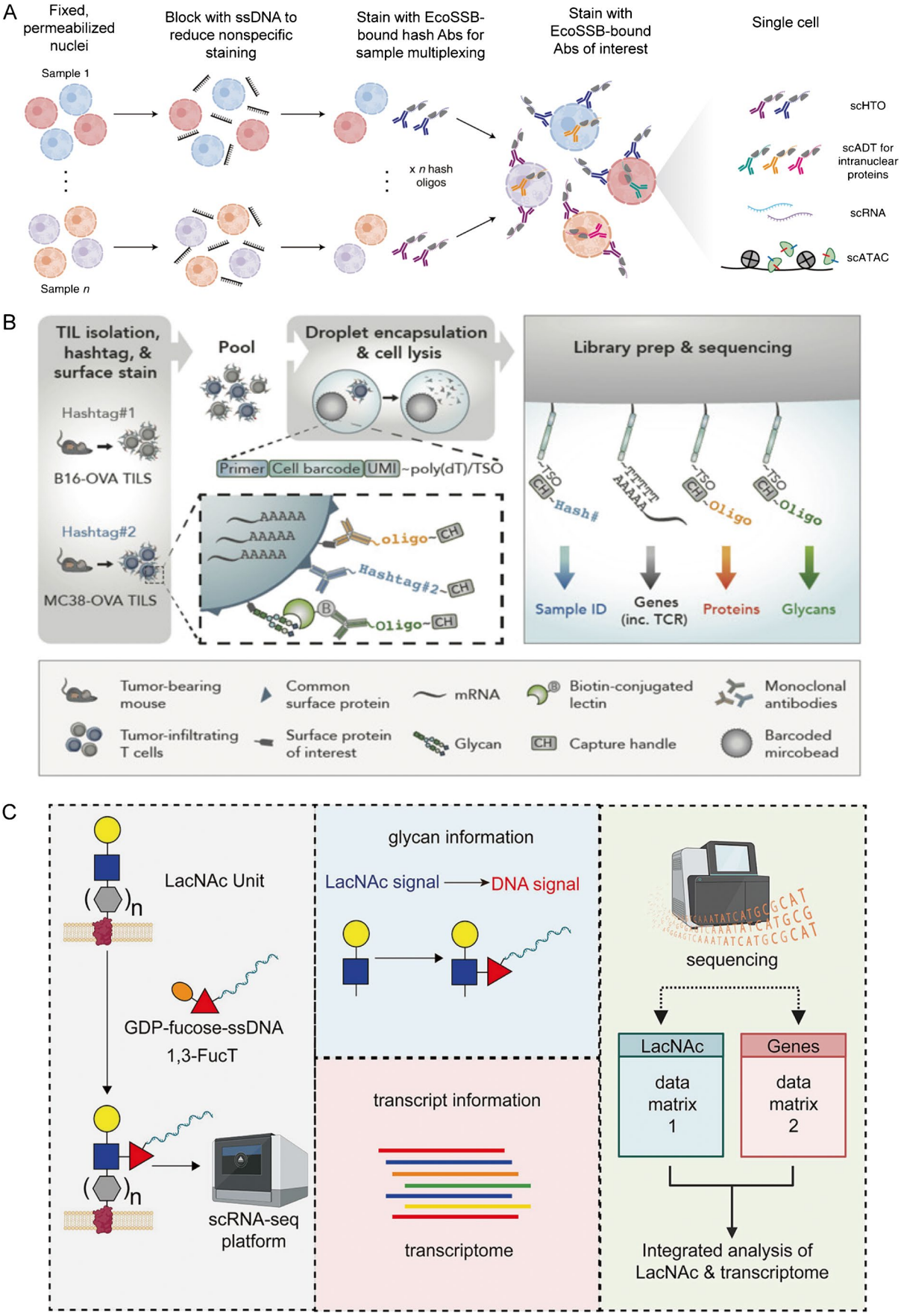
Although scRNA-seq facilitates the identification of cells by nature, it lacks the ability to resolve cells with genetic perturbations, thereby impeding the dissection of genetic function as well as causality. To this end, clustered regularly interspaced short palindromic repeats (CRISPR/Cas)–based technologies have been incorporated for genetic screens. The CRISPR/Cas system consists of a single-guide RNA (gRNA) and a Cas protein, which can trigger the cleavage of the target site upon the recognition of gRNA and the corresponding sequences. The controllability, accuracy, and versality have propelled its broad applications in identification of genes underlying diverse biological mechanisms [83]. For instance, Perturb-Seq [84] and CRISP-Seq [85] integrated droplet-based microfluidic systems for parallel profiling of both transcriptome and genomic perturbation within individual cell, achieving accurate elucidation of molecular circuits and identification of independent gene signatures during genetic perturbations.

Meanwhile, CROP-seq combined pooled CRISPR screening with droplet sequencing, allowing direct reading of gRNA expression associated with corresponding transcriptome responses, and simplifying tedious CRISPR screening processes [86]. To incorporate CRISPR-based genetic screening with omic analysis beyond transcriptome, Smibert's expanded CITE-seq, which allowed joint profiling of transcriptome and surface proteins by DNA oligo-labeled antibodies, for CRISPR-compatible cellular barcoding (ECCITE-seq), achieving massive parallel analysis of multiple modalities from transcriptome and epitopes to clonotypes and CRISPR perturbations [87]. Rather than using 3′tag barcoding strategies, ECCITE-seq leveraged 10×Genomics 5P/V(D)J systems with custom reverse transcription primers based on template switch oligos (TSO) during reverse transcription. Moreover, the invariant scaffold at the 3′ end of gRNAs was taken as the annealing site to append the barcode beads. In this way, gRNAs can be directly captured, and transcripts especially non-polyadenylated ones as well as epitopes were interrogated to the same barcode bead, enabling multimodal assessments within each single cell. As a result, ECCITE-seq allowed clonotype determination with immunophenotyping of PBMCs from patients with cutaneous T cell lymphoma (CTCL).

Conclusion and future developments

Microfluidic-based single-cell multimodal analysis has revolutionized the ability to provide comprehensive molecular profiles of individual cells from the perspective of transcriptome, genome, proteome, and epigenome, etc. Characterization of multi-modalities offers unprecedented opportunities for the exploration of cellular regulatory circuitry and heterogeneity in a more comprehensive way. For instance, joint measurement of transcriptome and genome revealed the correlation of transcriptional dynamics and genomic alterations for disease-associated genes. Besides, integrative analysis of epigenome and transcriptome established the link between regulatory networks and gene expression. On the other hand, microfluidics with the merits of minimalization, integration, high throughput, and automation has emerged as a powerful tool for large-scaled single-cell analysis. Large numbers of commercial microfluidic systems represented by 10×Genomics Chromium and Fluidigm also dramatically propelled the wide adoption of single-cell multiomics analysis. It is expected that by developing more robust and user-friendly platforms, researchers can process larger numbers of samples, resulting in increased throughput. Moreover, automation will reduce the need for highly specialized skills and enhance accessibility. For enhanced multiplexing capabilities, sophisticated microfluidic design is anticipated to enable the simultaneous processing of multiple samples or assays. Furthermore, the development of standardized microfluidic platforms as well as open-source designs can promote widespread adoption and makes it easier for laboratories to create and implement their own microfluidic systems.

Single-cell multiomics analysis is a transformative approach with profound current and potential future applications. In the present, it offers insights into cell typing, lineage tracing, and the unraveling of tumor heterogeneity, aiding in precision medicine [9]. Additionally, the profiling of immune cell responses and the


A
Fixed, permeabilized nuclei
Block with ssDNA to reduce nonspecific staining
Stain with EcoSSB-bound hash Abs for sample multiplexing
Stain with EcoSSB-bound Abs of interest
Single cell
Sample 1
Sample n
x n hash oligos
scHTO
scADT for intranuclear proteins
scRNA
scATAC
B
TIL isolation, hashtag, & surface stain
Hashtag#1
B16-OVA TILS
Hashtag#2
MC38-OVA TILS
Pool
Droplet encapsulation & cell lysis
Primer
Cell barcode
UMI
~poly(dT)/TSO
AAAAA
oligo-CH
Hashtag#2-CH
Library prep & sequencing
Sample ID
Genes (inc. TCR)
Proteins
Glycans
Tumor-bearing mouse
Tumor-infiltrating T cells
Common surface protein
Surface protein of interest
mRNA
Glycan
Biotin-conjugated lectin
Capture handle
Monoclonal antibodies
Barcoded mircobead
C
LacNAc Unit
GDP-fucose-ssDNA
1,3-FucT
scRNA-seq platform
glycan information
LacNAc signal
DNA signal
transcript information
transcriptome
sequencing
LacNAc
data matrix 1
Genes
data matrix 2
Integrated analysis of LacNAc & transcriptome

◂**Fig. 6** Microfluidic-based strategies for multi-modular characterization. (**A**) Schematic of NEAT-seq for integrated analysis of intranuclear proteins, epigenome, and transcriptome. Adapted from ref. [80]. (**B**) Schematic of SUGAR-seq for integrated analysis of glycoproteome, epitopes, and transcriptome. Reproduced with permission from ref. [81] Copyright 2021, The American Association for the Advancement of Science. (**C**) Schematic of LacNAc-seq for simultaneous characterization of glycans, transcriptomics, and TCR signatures. Reproduced with permission from ref. [82] Copyright 2023, American Chemical Society

amalgamation of scRNA-seq with functional genomics and epigenomics will expand our understanding of cellular regulation. Ultimately, the clinical realm is poised to benefit from this technology's evolution, fostering personalized medicine, early disease detection, and the monitoring of treatment efficacy.

Despite the enormous advances in multiplex single-cell analysis based on microfluidics, there remains several challenges regarding technical improvements and further applications. First, improved sensitivity and accuracy of multimodalities were expected. For instance, conventional scRNA-seq usually required more than 10 mRNA molecules for characterization of gene expression [88]. It is anticipated that such threshold could be lowered for detection of other low-copy-number mRNAs. Efforts focusing on optimizing enzymes [70] and advancing microfluidic platforms [26, 89] have been reported for enhanced sensitivity. In addition, the accuracy of different layers of omics analysis could be subjected to sample pretreatment. For example, bisulfite treatment usually required by methylome analysis could result in DNA damage, which impaired intrinsic genomic information. Moreover, fixed cells were prone to biased measurement and lost information. Thus, it is crucial to integrate mild sample pretreatment that is compatible with multi-layered characterization. Recently, a series of newly developed DMF-based strategies have paved a way for streamlined multiomics analysis with high sensitivity and less sample damage [89].

Second, although huge strides have been made in multiomics measurements, a large proportion of reported strategies focus on dimension transcriptome, genome, proteome, and epigenome, excluding metabolism, which is crucial for elucidating life process of organisms and revealing tissue heterogeneity. The lack of microfluidic-based single-cell metabolomic is possibly due to huge diversity and remarkably high dynamics of cell metabolites. Moreover, most single-cell metabolomic methods depend on mass spectrometry, which is hard to integrate with other omics characterization, and are not well suited for measurement of short-lived metabolic molecules. Therefore, it is anticipated that extended or refined strategies can be proposed to combine single-cell metabolism with other omics, which will be paramount to investigate how metabolic molecules affect other omics profiling at the single-cell level.

Third, innovative computational tools capable of interrogating multiple modalities and lowering information loss are solely needed. As bioinformatic approaches advance by leaps and bounds, it becomes accessible for researchers to restore the characterization of original molecules in single cells and establish biological regulatory networks. However, it remains challenging to distinguish the biological variance between technical noises. It is also crucial to eliminate the interference between individual modalities. More importantly, most current multimodal computational strategies were based on independent modality. A common approach entails the transformation of multimodal data into a unified feature space based on a priori knowledge, followed by the utilization of single-omics data integration methodologies [90]. Although conceptually straightforward, it leads to information loss. As such, the data quality and information content of each modality should be guaranteed, and the information variance among different modalities presented a challenge for integration of multi-omics datasets. An alternative avenue involves the alignment of cells from different omics layers through nonlinear manifold-based techniques, which has been applied to relatively modest datasets featuring a limited diversity of cell types [91]. Therefore, strategies like "weighted-nearest neighbor" [92] and "graph-linked unified embedding (GLUE)" [93] that could integrate multimodal data were highly desired. The introduction of AI technology is also expected to boost the bioinformatic analysis of multiomics.

Overall, the field of single-cell multiomics analysis is experiencing rapid growth and holds immense potential to transform our comprehension of biological systems. As the technology continues to evolve and become more accessible, it is expected to assume a progressively significant role in uncovering novel insights into the intricacies of life.

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Declarations

Competing interests The authors declare no competing interests.

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