REVIEW

Into the microscale: Low-input sequencing technologies and applications in medicine

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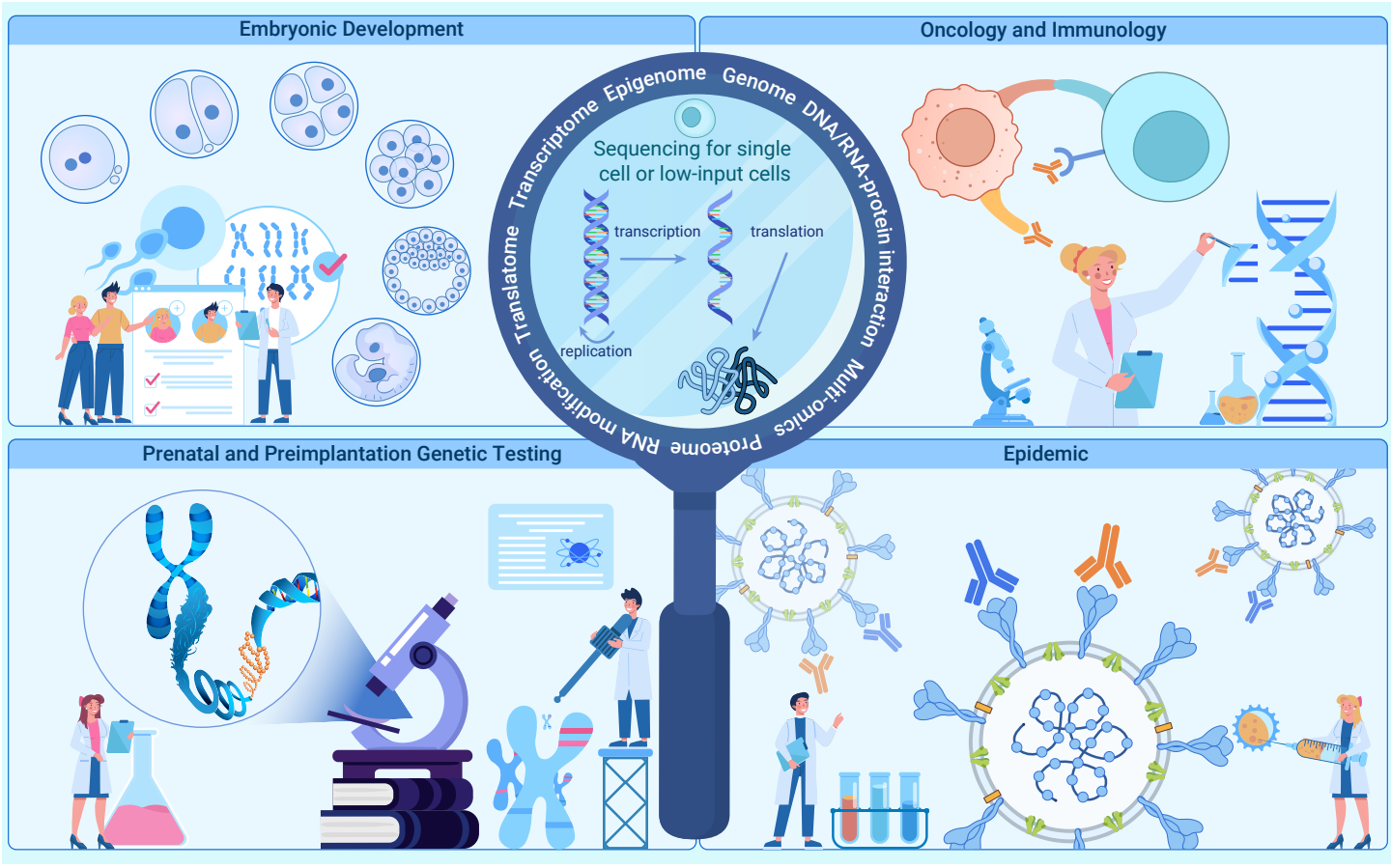
GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- Low-input and single-cell sequencing technologies are revolutionizing biological and medical research.
- Low-input and single-cell sequencing technologies investigate biological functions at multiple levels.
- Low-input and single-cell sequencing technologies facilitate precision medicine and targeted therapy.

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Sequencing technology has undergone major breakthroughs over time and has become indispensable in biological and medical research. Advances in protocols and analysis algorithms allow the analysis of small sample inputs, enabling the characterization of complex networks that govern biological processes in physiology and pathology, driving the development of precision medicine and targeted therapy. In this review, we comprehensively summarize low-input sequencing technologies that include methods for profiling the genome, epigenome, transcriptome, translatome, RNA modification, RNA-RNA interaction, RNA-protein interaction, and multi-omics. The key steps and innovations in different protocols are highlighted, and their advantages, limitations, and scope of application are described. With a focus on the impact of low-input sequencing technologies in biomedical fields, including embryonic development, prenatal and preimplantation genetic testing, oncology and immunology, and pandemic research, we discuss their potential to improve embryo implantation rates, prevent birth defects, develop prospective therapies, and predict prognosis. Lastly, we discuss current limitations and future prospects, providing new insights for medical research.

INTRODUCTION

Over the past few decades, our understanding of life and its nature has progressed by leaps and bounds. Sanger sequencing marked the first major milestone in sequencing history and became the dominant method at that time. Subsequent improvements and the introduction of automated machines further facilitated its widespread use. The emergence of parallel and high-throughput techniques ushered in the age of next-generation sequencing (NGS), making sequencing more affordable and efficient.[1] The third-generation sequencing (TGS), such as single-molecule sequencing in real time (SMRT) from Pacific Biosciences (PacBio) and nanopore sequencing from Oxford Nanopore Technologies, have reduced amplification bias and enabled the generation of extremely long reads.[2] Relevant researches sprung up at exponential speed and have contributed to diagnostics and targeted therapy, developmental biology, agriculture, forensic science, and so on.

These methods were originally suitable for a large number of cells and provide average information over the entire sample, which obscures the heterogeneity between individual cells. Technological innovations enable sequencing of rare samples such as clinical biopsies and embryos, reducing the amount of starting material required and laying the foundation for understanding developmental and disease mechanisms. Single-cell sequencing, an excellent example of low-input sequencing, investigates cell-to-cell variance, allowing for subpopulation identification and analysis of intercellular communication, greatly expanding the application of sequencing techniques. However, given the limitations of data sparsity and technical noise in single-cell sequencing, in some cases, using a small number of cells instead of individual cells as input can achieve better library complexity and data coverage.[3]

In this review, we will introduce state-of-the-art sequencing technologies specifically designed for low-input cells and single cells, covering different omics (Figure 1) and their promising applications in various fields (Table 1).

LOW-INPUT SEQUENCING TECHNOLOGIES

Whole genome amplification

NGS technologies offer new and rapid methods for characterizing and profiling the genome and epigenome, which is challenging since single cells contain only two copies of DNA molecules. In recent years, non-selective whole genome sequencing has become feasible for low amounts of starting material or even single cells.

Several proven whole genome amplification (WGA) methods have been established, which vary both in terms of protocols and quality of amplification. PCR-based protocols were some of the earliest single-cell WGA protocols available, mainly including Primer-Extension-Preamplification-PCR (PEP-PCR)[4,5] and Degenerative-Oligonucleotide-PCR (DOP-PCR).[6] These techniques differ in terms of primers and annealing temperature. PEP-PCR uses 15-base totally degenerate primers and reacts at relatively low annealing temperatures, while DOP-PCR uses partially degenerate primers that contain a six-random-nucleotide sequence in the middle and carry a defined sequence at the 5′ end, with an increasing annealing temperature.[7] A mixture of Taq and proofreading DNA polymerase was used to modify these two methods,[8,9] increasing the accuracy of genotyping.[10] However, these methods may have drawbacks such as non-specific amplification,[11] incomplete locus coverage,[12,13] and the generation of short products of less than 3 kb.[6,14]

Another WGA method, which is based on strand displacement synthesis rather than PCR,[15] was first proposed in 2001 by Lasken,[16] termed multiple-displacement amplification (MDA). Under isothermal conditions, MDA uses random hexanucleotide primers (6Ns) and Phi 29 DNA polymerase with a high processivity to form a branched structure, generating amplicons up to 100kb with an average length of 12kb.[17] MDA provides higher genomic coverage compared to PCR-based methods, but it still introduces a sequence-dependent bias due to its exponential amplification process.[7] On the other hand, Multiple Annealing and Looping-Based Amplification Cycles (MALBAC) is another isothermal method that adopts quasi-linear amplification in the first few cycles. This approach greatly reduces the bias associated with sequence-dependent amplification.[18]

In 2017, Chen and colleagues introduced Linear Amplification via Transposon Insertion (LIANTI),[19] achieving linear amplification of the entire genome with superior uniformity and fidelity compared to exponential amplification. This method randomly fragments genomic DNA by a specially designed transposon, which is then linearly amplified. A double-stranded DNA library is generated after reverse transcription and second-strand synthesis. Another linear amplification method for WGA, sci-L3, was developed in 2019. It utilizes a 3-level single-cell combinatorial indexing to improve throughput while maintaining the advantages of linear amplification.[20] Recently, a method called Multiplexed End-Tagging Amplification of Complementary Strands (META-CS) was reported. META-CS capitalizes on DNA complementarity and achieves the highest precision to date.[21]

DNA methylation

Epigenomic profiling encompasses a comprehensive analysis of multi-dimensional and precisely regulated epigenetic characteristics. However, achieving single-cell resolution remains a significant technical challenge for many techniques. In recent years, many methods for epigenomic profiling that require ultra-low input have emerged, greatly expanding our understand-

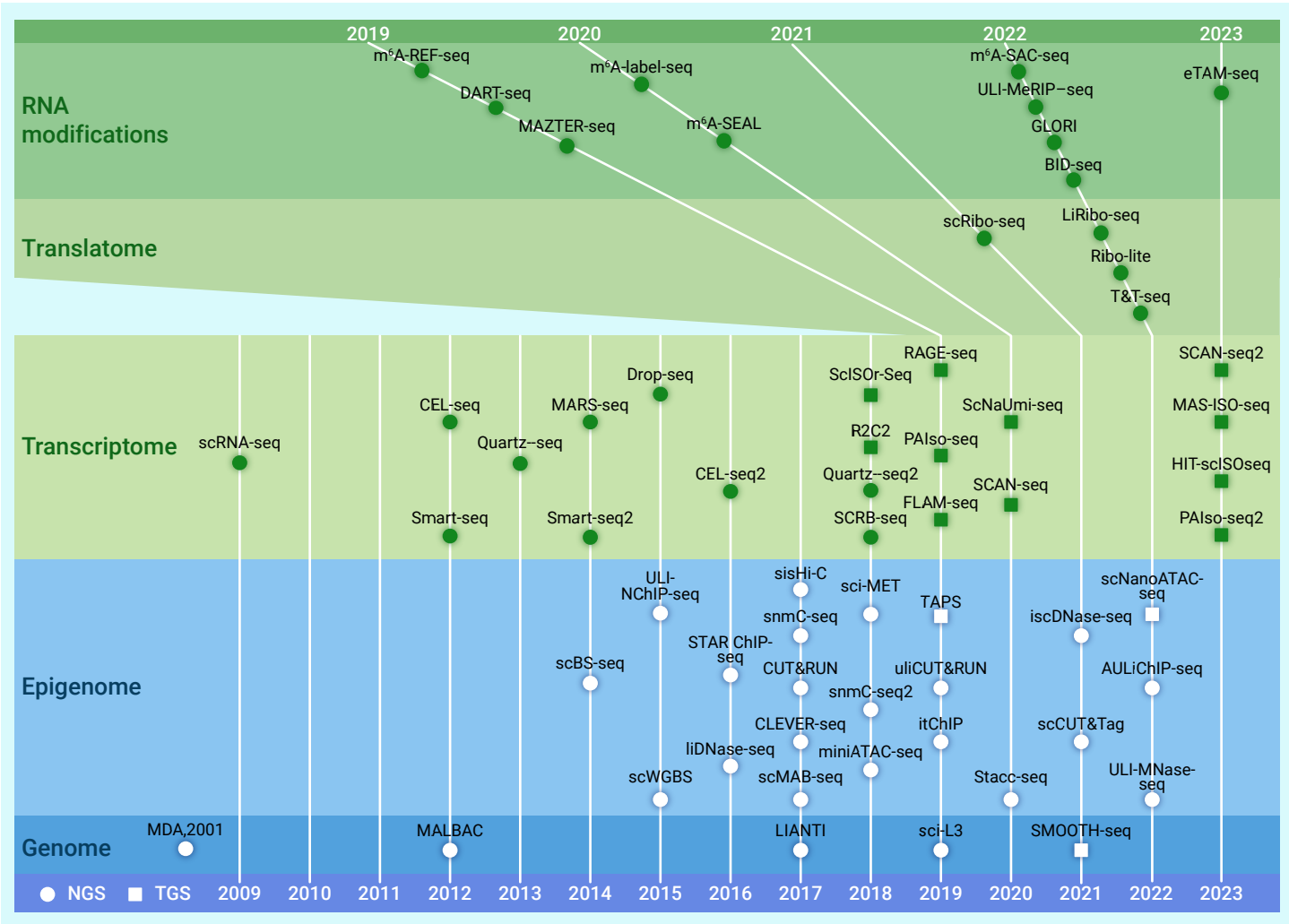

Figure 1. Representative sequencing technologies for low-input cells and single cells A chronological list of representative low-input and single-cell sequencing technologies used for profiling the genome, epigenome, transcriptome, translatome, and RNA modifications. Circles indicate next-generation sequencing technologies and squares indicate third-generation sequencing technologies (see the supplemental information for the definition of abbreviations).

ing of epigenetic regulation.

DNA methylation in the classical form of 5-methylcytosine (5mC) is one of the most important epigenetic regulators in mammals. Methods for sequencing single-cell DNA 5mC have been developed.

Bisulfite conversion-based sequencing is currently the most commonly used technique, in which 5mC and 5-hydroxymethylcytosines (5hmC) are both read as cytosines, making them indistinguishable.[22] Among them, the method of sequencing the whole genome is called whole-genome bisulfite sequencing (WGBS). The post-bisulfite adaptor tagging (PBAT)-based methods perform the bisulfite treatment prior to adaptor ligation to minimize the loss of DNA and reach single-cell resolution.[23] The complementary strands are synthesized by several rounds of random priming with oligos that contain adaptor sequences and random nucleotides. The strategies to incorporate the second strand vary from different protocols. scBS-seq utilizes another round of random-primed synthesis,[23] whereas scWGBS uses elongation blocked and tagged 3′ adaptor.[24]

The original PBAT-based method has a relatively low mapping efficiency partly due to the generation of chimeric reads,[25] which increases the sequencing costs and limits the application of the method to some extent.[26] Other 3′ tagging techniques have been adapted to increase alignment rate, but usually at the expense of coverage since only one round of random priming is performed to synthesize the first strand.[26,27] Another protocol, called snmC-seq, has been developed for sequencing single-nucleus methylcytosine.[28] snmC-seq2 improves mapping rates, coverage uniformity, and library complexity by using a modified random primer and inactivating dNTPs with shrimp alkaline phosphatase (SAP).[29] The sci-MET strategy applies a single-cell combinatorial indexing strategy to WGBS methylation analysis, breaking the confinement of individual reactions for each cell and achieving a mapping rate of nearly 70%.[30]

However, the harsh chemical conditions of bisulfite sequencing cause a significant amount of DNA fragmentation and degradation, which can lead to sequencing biases and reduced data quality.[31,32] Recently, a novel method based on milder reactions, called Tet-assisted pyridine borane sequencing (TAPS) has been established.[33] Using ten-eleven translocation (TET) enzyme, 5mC and 5hmC are oxidized to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). TAPS can retain long DNA fragments over 10kb in length. By combining TAPS with TGS, a more accurate long-read DNA methylation profile can be obtained.[34]

Unlike sequencing the whole genome, there are some methods that specifically target methylation-enriched regions. One such method is reduced representation bisulfite sequencing (RRBS). In RRBS, DNA is digested using a methylation-insensitive restriction enzyme (MspI) to enrich CpG-dense regions.[35] However, this method can only detect up to 10% of CpG sites, leaving a large number of regulatory elements cannot be effectively detected. Another method, extended-representation bisulfite sequencing (XRBS), pools barcoded DNA before bisulfite conversion. XRBS is designed to profile DNA methylation across a broader range, including promoters, enhancers and CTCF sites.[36]

Additional cytosine modifications, including 5hmC, 5fC,5caC, have also garnered attention in uncovering the mechanisms of methylation modification. Since 5hmC is resistant to bisulfite conversion, as is 5mC, it must be converted first to distinguish it from 5mC in sequencing. The first method

Table 1. Application of sequencing technologies in different fields

	Gametogenesis and embryonic development	Preimplantation genetic testing	Oncology and immunology	Epidemic
Genome	Whole genome sequencing of human gametes, revealing insights about meiosis[262,263]	Providing high accuracy and low bias amplification method for preimplantation genetic testing using limited material[18]	Mutational analysis in tumor study[264]	-
Transcriptome	Transcriptional atlas of human gametes and preimplantation embryos[198,208,201,265,266]	-	Profiling the features, markers and heterogeneity of various tumors, providing potential approaches for clinical treatment[237,238,267]	The immune landscape of COVID-19[268]
Translatome	Translatome atlas of human oocytes and early embryos, revealing key regulators of oocyte maturation and human ZGA[119,199]	-	-	-
RNA modification	Landscape and regulatory function of m^6A in mouse oocytes and embryos[121]	-	Regulatory mechanisms of RNA modifications in tumor development[269]	m^6A mediating the interaction between SARS-CoV-2 and host cells[270]
Epigenome	Mapping the epigenetic regulation of DNA methylation, chromatin accessibility, and histone modifications in gametes and preimplantation embryos[202-206,215,271]	-	Dynamic epigenetic regulatory mechanisms of tumor heterogeneity, microenvironment, and resistance to therapy[239,240]	Elucidation of epigenomic regulation of COVID-19 immunological characteristics[272,273]

developed for detecting 5hmC at single-nucleotide resolution is oxidative bisulfite sequencing (oxBS-Seq), in which 5hmC is transformed into 5fC and then to uracil in the bisulfite conversion reaction, leaving 5mC unchanged.[37] Another discriminating method involves blocking 5hmC through glycosylation, which protects it from further TET oxidation and bisulfite conversion. In this method, all 5mC is oxidized to 5caC by TET1 and then converted to 5caU through bisulfite treatment.[38]

In addition to traditional bisulfite conversion-based methods, an alternative approach called Aba-seq has been developed. Aba-seq utilizes 5hmC-dependent restriction endonucleases (AbaSI) and allows for the sensitive detection of 5hmC in low-occupied regions.[39] Another method combines hydroxymethylated DNA immunoprecipitation and multiple index sequencing, enabling different hMeDIP-seq data to be compatible.[40]

Since the abundance of 5fC is extremely low, most methods for detecting 5fC require a large amount of input. In 2017, two methods were adapted to address this issue: low-input Methylase-Assisted Bisulfite sequencing (liMAB-seq) and single-cell MAB-seq (scMAB-seq). These methods enable the profiling of 5fC and 5caC in a genome-wide manner using low input of cells, or even single cells. By first converting unmodified cytosine to 5mC by M.SssI methyltransferase, 5fC and 5caC can be read directly as thymine after bisulfite conversion.[41] Another method for single-cell resolution detection of 5fC is chemical-labeling-enabled C-to-T conversion sequencing (CLEVER-seq). This method uses malononitrile to selectively label 5fC, which then be converted from C to T during amplification.[42]

Chromatin accessibility

Chromatin accessibility, which refers to the degree of physical compaction of chromatin, provides an invaluable additional layer of gene regulation. Chromatin accessibility can be measured by treating chromatin with certain enzymes, including DNA endonucleases, micrococcal nuclease, GpC methyltransferase and transposase.

DNase-seq utilizes DNase I, one of the DNA endonucleases, to identify the genomic regions where active functional elements displace nucleosomes. This was one of the earliest methods used to profile genome-wide open chromatin regions.[43] Techniques were developed to improve the resolution of DNase-seq from millions of cells to the single-cell level using a plate-based strategy, which was a significant breakthrough.[44] However, interpreting the data from this scDNase-seq method relies on a previous DNaseI-Hypersensitive Sites (DHSs) map, raising doubts about its ability to discover *de novo* DHS. By modifying this method to lessen DNA loss, low-input DNase-seq (liDNase-seq) was established, enabling the identification of *de novo* DHS within the whole genome with an input of only 30 cells.[45]

Another single-cell DNase sequencing method, iscDNase-seq, has been designed using combinational indexing strategies.[43] This protocol is based on bulk analysis, which significantly increases cell throughput. The Assay for transposase-accessible chromatin with sequencing (ATAC-seq) employs the Tn5 transposase to simultaneously digest open chromatin regions and insert adaptors for library construction, making it feasible for low-input assays. Several methods based on different platforms have been established for single-cell ATAC-seq.[46-49] Tn5 identifies basically the same sites as DNase I, whereas in higher resolution analyses, ATAC-seq has distinct sequence biases, leading to a different footprint efficiency for some transcription factors (TFs).[50,51] A recent development in single-cell ATAC-seq is scNanoATAC-seq, which is based on the Nanopore sequencing platform and offers advantages in haplotype phasing and structural variation detection.[52]

Another method, Nucleosome Occupancy and Methylome sequencing (NOMe-seq), provides genome-wide nucleosome positioning information using the GpC methyltransferase M.CviPI, to methylate GpC dinucleotides that are not occupied by nucleosomes.[53] The uniqueness of this bisulfite conversion-based method lies in its ability to distinguish inaccessible chromatin regions and missing data,[54] which makes it well-suited for single-cell analysis.

DNA-protein interaction

DNA-binding proteins are a group of proteins containing certain domains that have a specific affinity for DNA. This group includes histones, polymerases, and transcription factors, which play a central role in chromosomal organization and transcriptional regulation.

Chromatin immunoprecipitation followed by sequencing (ChIP-seq) was one of the earliest high-throughput methods used to detect protein-binding sites.[55,56] In this method, DNA-binding proteins are first cross-linked to DNA using formaldehyde. Antibodies that specifically target certain proteins or nucleosomes are then used to enrich DNA fragments bound to these proteins or nucleosomes.[57] However, ChIP-seq has a limitation in its efficiency and requires a large amount of input.[58]

To address this limitation, several improvements to ChIP-seq have been developed to generate high quality data with a smaller input of cells. Linear DNA amplification (LinDA) is performed in a single tube to minimize the risk of sample loss.[59] Microfluidic oscillatory washing-based ChIP-seq (MOWChIP-seq) uses oscillatory washing to remove non-specific binding and increase ChIP efficiency.[60] Ultra-Low-Input micrococcal Nuclease based native ChIP sequencing (ULI-NChIP-seq) fragments the chromatin using MNase prior to immunoprecipitation, resulting in high-complexity libraries from a smaller input.[61] Another approach to improve resolution is by combining ChIP-seq

with DNA barcoding. By indexing and combining chromatin from different cells, genome-wide chromatin states can be profiled using as few as hundreds of cells or even single-cell samples.[62,63]

An alternative to ChIP-seq is to profile chromatin with tethered enzymes. This method differs from ChIP-seq in that it extracts DNA from live or unfixed cells without chromatin recovery by immunoprecipitation.[64,65] For example, Cleavage Under Targets and Release Using Nuclease (CUT&RUN) is based on the Chromatin Immuno-Cleavage (ChIC) strategy. By using TF-specific antibodies to tether pA-MN, a fusion of protein A and micrococcal nuclease, the CUT&RUN method can selectively cleave at the binding sites without the need for crosslinking. This approach produces high quality data with much lower background levels.[66] Another improvement is the use of a transposome, consisting of a pA-Tn5 fusion protein and sequencing adaptors, for factor-targeted tagmentation. This simplifies the processing steps and reduces DNA loss by generating fragments ready for sequencing. Other methods that utilize Tn5 include Chromatin Integration Labelling followed by sequencing (ChIL-seq),[67] Combinatorial Barcoding And Targeted Chromatin Release (CoBATCH),[68] and Antibody-guided Chromatin Tagmentation (ACT-seq).[69] A recently developed method called Small-scale Tn5-assisted chromatin cleavage with sequencing (Stacc-seq), which is based on pA/G-Tn5, has improved antibody binding efficiency by preincubating the antibody and Tn5-protein A/G and optimizing washing steps.[70] These methods address the limitations of ChIP-based methods, such as high signal-to-noise ratio, high background, and epitope masking, resulting in improved data quality for genome-wide profiling of specific chromatin components at a lower cost.

Transcriptome

The transcriptome refers to the complete set of all types of transcripts. Advancements in RNA-seq technology have enabled the sequencing of low-input materials and single cells without compromising the cellular heterogeneity. This has also expanded the application of RNA-seq to various cell species and samples. To date, single-cell RNA sequencing (scRNA-seq) has proven to be a robust and widely adopted strategy in biological research. It has achieved successful commercialization since it was first published by Tang and colleagues in 2009.[71]

Laboratorial workflow. The general workflow for scRNA-seq involves several steps: cell isolation, RNA capture, reverse transcription to cDNA, amplification, library construction, and sequencing. What sets scRNA-seq apart from bulk assays is the ability to capture RNA from each individual cell and amplify minute amounts of cDNA to a sufficient quantity for library construction. To ensure the reliability and comparability of gene expression data, unique molecules (UMIs) and spike-in RNA can be incorporated into the protocols.

Different strategies have been developed to refine protocols and meet specific requirements in researches. Ziegenhain et al. evaluated six prominent methods and concluded that Smart-seq2 has the highest sensitivity and uniformity.[72] It was also noted that, considering cost and efficiency, Drop-seq is a better option for large amounts of cells, while Smart-seq2, MARS-seq, and SCRB-seq are more suitable for low-input. To assess the reproducibility and reliability of different methods, a comparative evaluation of 13 popular scRNA-seq and single-nucleus RNA-seq (snRNA-seq) methods was conducted.[73] Each method was graded based on its ability to replicate tissue composition for a heterogeneous mixture of cells of different types and species. Quartz-seq2 performed the best, followed by Chromium and Smart-seq2, while CEL-seq2 had an advantage in detecting low expressed genes. Each of these methods has its own strengths, weaknesses, and usage scenarios that should be taken into account when choosing a suitable approach.

Computational workflow. The general process for scRNA-seq analysis consists of two parts: pre-processing and downstream analysis. The former includes quality control, data correction and normalization to minimize technical biases, while the latter focuses on mining gene expression features and functions that vary between cells and states.[74] For the upstream pipeline of scRNA data, commonly used tools for quality control are FastQC and Trimmomatic,[75] whereas Cutadapt[76] is used for adapter sequence removal. STAR[77] and HISAT2[78] are typical mapping tools used to align reads to the reference sequence. Additionally, holistic solutions such as Cell Ranger,[79] tailored for the 10X Genomics platform, provide end-to-end pipelines.

For downstream analysis, three widely used tools are Seurat,[80] scater,[81] and scanpy,[82] each offering integrated workflows that cover both data pre-processing and downstream tasks such as clustering, annotation, and the identification of differentially expressed genes (DEGs). DESeq2,[83] edgeR,[84] and limma[85] are commonly used R packages suitable for DEG analysis, which is the fundamental exploratory analysis within the bioinformatic pipeline. Other common analyses, such as cell trajectories and cell-to-cell interactions, have also spawned corresponding tools. Monocle is a powerful tool for pseudo-time analysis.[86] PAGE[87] is a scanpy-based tool that combines clustering and trajectory inference. For researchers interested in interpreting cell-to-cell communication, specialized tools such as CellphoneDB,[88] CSOmap,[89] and CellChat[90] allow profiling of the ligand-receptor interaction landscape.

TGS-based scRNA-seq. TGS-based methods have also been widely applied for single-cell transcriptome with the advancement of long read length, such as SCAN-seq,[91,92] R2C2,[93] ScISOr-Seq,[94] ScNaUmi-seq,[95] RAGE-seq,[96] and PAIso-seq.[97] In 2020, using the Nanopore platform, Fan et al. developed a method called SCAN-seq that can amplify and sequence the full-length RNA of single cells, providing higher sensitivity and reliability compared to NGS-based ones.[91]

In High-throughput single-cell isoform sequencing (HIT-scISOseq), multiple cDNAs are ligated in tandem and sequenced using the PacBio platform. This approach reduces the proportion of worthless TSO priming artifacts and improves capture efficiency and accuracy of transcripts.[98,99] Compared to HIT-scISOseq that connects cDNAs randomly, multiplexed arrays isoform sequencing (MAS-ISO-seq) first distributes cDNAs into 15 tubes, each with a specific adaptor, thus allowing oriented assembly and consistent length in subsequent concatenation, further improving sequencing yield and throughput.[100] The introduction of Spike-In RNA Variants (SIRVs) in full length sequencing improves the accuracy of RNA isoform quantification.

Full-length poly(A) and mRNA sequencing (FLAM-seq)[101] and Poly(A) inclusive RNA isoform sequencing (PAIso-seq)[97] are two PacBio-based methods that can sequence full-length mRNA and their poly(A) tails with the difference in adaptor ligation: the former uses G/I tailing and the latter uses templated extension. Besides, FLAM-seq requires about at least 500 ug RNA for input while PAIso-seq can be applied using single GV oocyte. These two TGS-based methods allow complete and reliable detection of long poly(A) tails among different transcripts and isoforms, paving the way for research on isoform-specific poly(A) tail regulatory function.[97] Both methods have reported the presence of non-A bases in poly(A) tails, which are involved in post-transcriptional regulation. Specifically, 3'end U residues promote mRNA degradation, while G residues stabilize mRNA.[102] An enhanced version, PAIso-seq2, can sequence mRNA with very short or even no poly(A) tails, or mRNA ends with non-A nucleotides. However, this increased capability comes at the expense of sensitivity (Table 2).[103] Thus, these two methods can complement each other, providing more holistic outcomes.

scRNA-seq and spatial transcriptomics (ST). Single-cell ST is a rapidly growing trend. It adds an additional dimension of spatial location, which provides valuable information such as cell-to-cell communication, subpopulation identification and subcellular localization of mRNAs.[104] Current ST techniques are either imaging-based or sequencing-based. Imaging-based techniques rely on microscopy to acquire positional information and can be further divided into *in situ* hybridization (ISH) (such as seq-FISH, MERFISH),[105,106] which is based on single molecule fluorescence *in situ* hybridization (smFISH), and *in situ* sequencing (ISS) (such as STARmap),[107] which applies sequencing by ligation (SBL) technology.[104] Sequencing-based methods preserve spatial information by constructing microarrays with spatially barcoded probes, as in techniques like Slide-seq,[108,109] HDST,[110] Seq-scope,[111] Stereo-seq,[112] and PIXEL-seq.[113] Alternatively, orthogonal microfluidic barcoding channels are employed in methods like DBiT-seq.[114] Most of the commonly-used ST techniques have achieved single-cell and even subcellular resolution. However, there are still pending optimization issues. The complexity of experimental operations and data structures present challenges for achieving higher detection resolution and developing analysis algorithms and, consequently, require further breakthroughs.

Table 2. Comparison of representative low-input RNA sequencing for full length-poly(A) tails

Method	Year	Input	Platform	Methods to capture and reserve poly(A) tail	Detection of non-A nucleotides within poly(A) tail	Detection of non-A nucleotides at 3′ end of poly(A) tail	Reference
FLAM-seq	2019	500 ng RNA	Pacbio	Ligate poly(A) tail with guanosines and inosines as a priming site for later adapter ligation	+	—	101
PAIso-seq	2022	single oocytes	Pacbio	Extend 3′ end using dU-containing DNA template	+	—	97
PAIso-seq2	2022	single oocytes	Pacbio	Directly ligate poly(A) tail with adaptor; Remove rRNA with Cas9	+	+	103,274

Translatome

Translatomics refers to the complete profile of components related to translation, such as translating mRNA, ribosomes, tRNAs, regulatory RNA, nascent polypeptide chains, and more. Among these components, translating mRNA is the primary focus.[115]

In 2021, single-cell ribosome sequencing (scRibo-seq) was developed, which achieved single-codon resolution.[116] In the process, ribosome-protected fragments (RPFs) are obtained after digestion of exposed RNA by micrococcal nuclease (MNase) without the use of marker and transgene. In 2022, Xiong et al. developed ligation-free, ultralow-input and enhanced Ribo-seq (Ribo-lite) by optimizing RNase I concentration and omitting markers during gel purification of RPFs to avoid contamination. They successfully applied Ribo-seq to as few as 50 cells and single oocytes, showing its potential for studying embryonic development.[117] Zhang et al. developed low-input ribosome profiling (LiRibo-seq), a protocol used to capture RPFs in active translation with the advantages of ligation-free, by reforming RiboLace.[118] LiRibo-seq is capable of detecting RPFs from as few as 5,000 mESCs.

To generate transcriptome and translatome profiles in the same cells, a method called transcriptome and translatome sequencing (T & T-seq) was developed. This method uses RiboLace beads to enrich actively translating ribosomes and has been used to detect translatome of single oocyte.[119] These novel tools expand the application of translatomics by allowing the study of precious and rare samples, such as embryos, and providing new insights into cell heterogeneity in translation.

RNA modification

N^6-methyladenosine (m^6A) modification. Chemical modifications of the RNA and the corresponding epitranscriptome play important roles in post-transcriptional control. Among the RNA modifications, m^6A is the most common type. It involves the methylation of nitrogen at position 6 in the adenosine base within mRNA. Recently, with the advancement of technologies such as m^6A sequencing, numerous studies have shown its crucial function in regulating gene expression.

In 2012, scientists conducted the first exploration of human and mouse RNA methylomes using MeRIP-seq. This method involves capturing target RNAs with anti-m^6A antibodies but is limited by the requirement of large amount of RNA.[120] Built on technical advancements, ultralow-input MeRIP-seq (ULI-MeRIP–seq) was developed, which can profile down to 50ng of total RNA.[121] The use of MaXtract High Density Gel Tubes and an optimized m^6A antibody improved immunoprecipitation (IP) efficiency, thereby reducing the amount of input RNA required.

There are many different strategies for optimizing enrichment via antibody, including PA-m^6A-seq,[122] miCLIP,[123] and m^6A-LAIC-seq.[124] However, antibody-dependent methods have limitations in terms of immunoprecipitation efficiency and resolution. Some researchers have developed antibody-free protocols,[125,126] which utilize MazF, an RNA endoribonuclease that specifically cleaves unmethylated ACA motif but not (m^6A)CA motif. Although MazF-based methods allow the quantification of methylation levels at single-base resolution and require minimal RNA input (ng or even pg), the ACA motif only accounts for less than a quarter of classical DRACH motifs (D = A/G/U, R = A/G, H = A/C/U) in m^6A modification. This necessitates more sensitive enzymes and strategies to cover global m^6A sites.

DART-seq introduces a fusion protein of cytidine deaminase and m^6A-binding domain to identify m^6A sites through the C-to-U deamination reaction adjacent to m^6A residues.[127] This protocol allows for as low as 10 ng of total RNA input. A tack of chemical reaction using FTO and dithiothreitol (DTT) are used to convert m^6A to active N6-hydroxymethyladenosine (hm^6A) and then to stable dm^6A, which can be labeled with biotin for RNA pull-down.[128] This FTO-assisted m^6A selective chemical labeling method (m^6A-SEAL-seq) allows more specific and sensitive profiling of the m^6A epitranscriptome with low-input samples.

Shu et al. reported m^6A-label-seq which achieved single-base resolution.[129] In this method, methyl is replaced with allyl on the S-Adenosyl Methionine (SAM), a cofactor enzyme involved in methylation, therefore metabolically obtaining N^6-allyladenosine (a^6A) instead of m^6A, which can be identified based on the iodination-induced misincorporation. With allylic-SAM, m^6A-selective allyl chemical labeling and sequencing (m^6A-SAC-seq) was introduced, but with a different enzyme-labeling protocol.[130] This method not only reduces the input to only 30 ng of poly(A) or rRNA-omitted RNA, but also provides stoichiometry information on the dynamic change of m^6A.

Recently, inspired by bisulfite sequencing for methylation detection in DNA, two novel methods, glyoxal and nitrite-mediated deamination of unmethylated adenosines (GLORI) and evolved TadA-assisted N^6-methyladenosine sequencing (eTAM-seq) were reported.[131,132] GLORI relies on glyoxal and nitrite-mediated deamination to convert all unmethylated adenosines to inosines, which are read as G. In contrast, m^6A is read as A, allowing for the absolute quantification of m^6A.[133] On the other hand, eTAM-seq relies on TadA, a laboratory-evolved adenosine deaminase, to keep RNA intact. This method enables the detection of m^6A at user-specified loci and requires as few as 10 cells for input material.[132]

Pseudouridine (Ψ) modification. Ψ is another abundant RNA modification, especially in rRNAs, tRNAs, snRNAs.[134] High-throughput methods have allowed profiling of mRNA pseudouridylation, revealing its impact on mRNA function.[135-137] N-cyclohexyl-N′-(2-morpholinoethyl) carbodiimide methyl-p-toluenesulfonate (CMC) can bind to Ψ and cause stop signature during reverse transcription.[138] Based on these characteristics, sequencing methods such as Pseudo-seq,[135] Ψ-seq,[137] PSI-seq,[136] and CeU-Seq were developed.[139] However, these methods lack base resolution and have a relatively low overlap ratio for identified Ψ sites.[140] An alternative approach for studying Ψ is based on bisulfite sequencing, which allows for base resolution detection of Ψ.[141] Khoddami et al. developed RBS-Seq that can detect Ψ, 5mC and N^1-methyladenosine (m^1A) simultaneously at single-base resolution. Recently reported bisulfite-induced deletion sequencing (BID-seq) is able to detect Ψ sites with 10-20 ng RNA as input material at base resolution, allowing detection of precious samples and low abundance RNA species.[142]

Modifications of tRNA. Extensive modifications, such as m^1A, m^5C, m^1G, and pseudouridine, have an impact on both the stability and function of tRNA, thereby affecting the translation process. Moreover, disturbances in tRNA modifications have been observed in various diseases, necessitating further research.[143] However, the unique structure of tRNA and the abundance of modifications hinder the reverse transcription process, posing difficulties in sequencing.

To address this, DM-tRNA-seq and ARM-seq were developed. These methods use the enzyme AlkB to remove methylated bases (m^1A, m^3C and m^1G) before library construction, facilitating the generation of longer cDNA. By combining these treated libraries with untreated ones, methylation landscapes can be profiled.[144,145] Another method called Nano-tRNAseq, based on nanopore technology, allows for the direct detection of tRNA modification dynamics at single-molecule resolution. Researchers ligated adaptors to both the 5′ and 3′ ends of tRNA to increase read length and optimized computa-

tional parameters, significantly increasing mapping rate and reducing length-dependent bias.[146] However, protocols for its application in single or low-input cells still require optimization.

RNA-RNA interaction

Growing findings of multiple types of RNA-RNA interactions have highlighted the pivotal role of RNA-RNA hybrids in gene regulation, expecting a comprehensive profiling of RNA-RNA interaction network and underlying mechanisms. These interactions are mostly mediated by RNA-binding proteins (RBPs).[147] Existing methods for profiling RNA-RNA interactions include, but are not limited to, RBP-based methods such as CLASH[148] and MARIO,[149] as well as chemical-based methods such as PARIS,[150] SPLASH,[151] and LIGR-seq.[152]

One innovative method called RNA *In situ* conformation sequencing (RIC-seq) utilizes RBPs for profiling.[153] The researchers optimized previous protocols by performing RNA proximity ligation *in situ* rather than in dilute solution, and using pCp-biotin for chimeric RNA enrichment. This enables unbiased mapping of RNA-RNA interactions at the single-nucleotide resolution, as well as the visualization of RNA structure and 3D interaction maps. However, RIC-seq still requires improvement for its application to low-input cells and even single cells, just as the authors planned.[154,155] Therefore, it is crucial to develop novel or refined sequencing methods for studying RNA-RNA interactions in rare samples and single cells.

RNA-protein interaction

RNA and protein interact with each other in various biological processes, such as RNA processing and modification, mRNA stabilization, mRNA localization and translation.[156] Basic sequencing methods include RNA immunoprecipitation with sequencing (RIP-seq),[157] ultraviolet light cross-linking and immunoprecipitation (CLIP)[158] and its variants high-throughput sequencing CLIP (HITS-CLIP or CLIP-seq),[159] photo-activatable ribonucleoside enhanced CLIP (PAR-CLIP),[160] individual-nucleotide resolution ultraviolet CLIP (iCLIP),[161] enhanced CLIP (eCLIP),[162] and infrared-CLIP (irCLIP).[163] However, these CLIP-based methods require large numbers of cells due to the limitations of antibody affinity and specificity. An alternative method called targets of RBPs identified by editing (TRIBE) allows pinpointing of RBP sites using as few as 150 cells. This is achieved by transfecting a fusion protein of RBP and a catalytic domain of the RNA editing enzyme.[164] Additionally, linear amplification of complementary DNA ends and sequencing (LACE-seq) is a method that can identify RNA-protein interaction at single-nucleotide resolution with a single oocyte, which pave the way for investigating RBP-mediated post-transcription.[165]

Proteome and post-translational modifications

Low-input sequencing technology relies heavily on amplification. While nucleic acids can be efficiently amplified by PCR, proteins, which are carriers that exercise functions in the cells, currently lack an effective amplification method. Previously, low-input protein analysis primarily relied on antibody-based methods. However, these methods were constrained by antibody specificity[166] and the limited number of proteins that could be analyzed simultaneously.[167] The mass spectrometry (MS)-based techniques offer higher sensitivity, lower sample requirement, and increased throughput compared to conventional antibody-based techniques.[168,169] The MS-based techniques can detect both proteins and post-translational modifications (PTMs), for the changes after PTM is significant in mass detection and the resolution can reach single amino acid,[170] it is considered as a more suitable approach for proteomics.

A variety of MS-based methods are now available to achieve low-input or even single-cell resolution.[171-173] One common approach is label-free MS, where proteins are extracted, digested into peptides, and separated primarily through capillary electrophoresis or liquid chromatography.[174] However, the complex process in this workflow can lead to protein loss.[175,176]

To optimize label-free MS workflow, the use of a nanodroplet-based approach has been introduced. This method reduces reaction volume and minimizes processing loss.[177,178] Another method developed for single-cell MS is label-based multiplexed analysis. One example is Single Cell ProtEomics by MS (SCoPE-MS),[179] which reduces protein loss through manual selection without using chemicals and utilizes tandem mass tags (TMT) labels for multiplexing. Its advanced version, SCoPE2 further optimizes the workflow for sample preparation and data processing, reducing costs and increasing throughput at the same time.[180] Other multiplexing labeling methods include isobaric tags for relative and absolute quantification (iTRAQ)[181,182] and easily abstractable sulfoxide-based isobaric-tag (EASI-tag).[183]

The rapid revolution in MS-based methods has greatly expanded our understanding of the dynamics and functions of the proteome and PTMs, effectively complementing the findings from RNA sequencing. Since the proteome and PTMs are essential regulators of both physiological and pathological processes, MS-based methods are unparalleled tools for further research.[174,184]

Multi-omics

The regulation of gene expression and cellular processes is an intricate network involving the coordination and interplay of multiple modules. Thus, a more comprehensive profile that combines genomic, transcriptomic, epigenomic, proteomic, and metabolomic landscapes can provide insights into the mechanisms governing cell fate determination and enable precise classification of cell states. However, integrating data from different omics is challenging due to variations among protocols and the scarcity and heterogeneity of certain materials, such as human embryos. Therefore, novel technologies are needed to sequence multiple layers of a cell simultaneously.

Single-cell multi-omics sequencing technology has greatly improved this situation. DR-Seq[185] and G&T-seq[186] were first developed to simultaneously sequence the genome and transcriptome of a single cell, linking differential expression to causative genetic variations.[187] Methods that profile the methylome and transcriptome link transcriptional and DNA methylation heterogeneity, revealing potential regulatory patterns.[188,189] Co-profiling of the transcriptome and chromatin accessibility enables the inference of target genes for candidate enhancers.[190] Single-cell triple omics sequencing (scTrio-seq) reveals the correlation among DNA copy number, gene expression, and promoter and gene body methylation.[191] Techniques such as snNMT-seq[192] and scNOMeRe-seq[193] crosslink different epigenetic layers, providing a more comprehensive view of their coordination and influence. Methyl-HiC profiles coordinate the DNA methylation landscape between spatially proximal DNA sequences and adjacent CpGs.[194] Based on previous joint ATAC-RNA workflows,[195,196] SHARE-seq[197] offers superior detection of epigenomic and transcriptomic landscape by simultaneously labeling mRNA and chromatin fragments in single cells using multiple hybridization blocking. Here we list some novel techniques for multi-omics profiling and summarizes their characteristics (Table 3, Figure 2).

APPLICATIONS OF LOW-INPUT SEQUENCING

Low-input sequencing in embryonic development

Embryonic development begins at fertilization, and undergoes a dramatic epigenetic reprogramming to form a totipotent embryo, followed by maternal mRNA decay and zygotic genome activation (ZGA). Subsequently, intercellular heterogeneity increases and cell fate differentiation occurs. The entire process is governed by a sophisticated and intricate regulatory network at multiple layers, which has been preliminarily profiled using low-input sequencing technologies (Figure 3).

The overall transcriptome dynamics across different stages, cell lineages, and species have been revealed by various studies.[198,207] Current research suggests that ZGA occurs at 4-cell to 8-cell stage in humans, while at 2-cell stage in mice.[198,208] Yan et al. profiled the transcriptome of human early embryos and observed significant gene expression changes between the 4-cell and 8-cell stage.[198] Combining Ribo-lite and Smart-seq2 (Ribo-RNA-lite), Zou et al. confirmed that translational regulation is critical for human ZGA and identified a group of pivotal transcription factors, *TPRXs*, which are highly translated around major ZGA and activate the expression of ZGA genes.[199]

Lineage specification in mice begins at the morula stage, during which the trophectoderm (TE) and inner cell mass (ICM) are formed after embryo compaction. At the blastocyst stage, the ICM further diverges into the epiblast (EPI) and primitive endoderm (PE).[201,209] However, numerous studies have demonstrated that key molecules involved in mouse lineage specifica-

Table 3. Summary of different multi-omics technologies

Methods	Year	Omics	Method features	DNA-RNA isolation method	Platform	Throughput (Cell numbers)	Gene number	Resolution	Reference
DR-seq	2015	Genome; Transcriptome	cDNA and gDNA part are split after first-strand cDNA synthesis, not involving physical separation		Tube-based	low	~10000	Single-cell	[185]
G&T-seq	2015	Genome; Transcriptome	MDA/ PicoPlex; Smart-seq2	Physical separation by magnetic beads	Plate-based	medium	>3500	Single-cell	[186]
SIDR-seq	2018	Genome; Transcriptome	MDA; Smart-seq2	Physical separation by magnetic beads	Microplate-based	low	2400~11000	Single-cell	[275]
TARGET-seq	2019	Genome; Transcriptome	cDNA and gDNA part are split after RT[d] and PCR, not involving physical separation		Plate-based	medium	2500~10000	Single-cell	[276]
scTrio-seq	2016	Genome; Transcriptome; DNA methylation	scRRBS; Smart-seq2	Physical separation by magnetic beads	Tube-based	medium	~5000	Single-cell	[191]
scM&T-seq	2016	Transcriptome; DNA methylation	G&T-seq; scBS-seq	Physical separation by magnetic beads	Plate-based	medium	4000-8000	Single-cell	[188]
scM T-seq	2016	Transcriptome; DNA methylation	Smart-seq2; scRRBS	Nucleus is transferred by a micro capillary pipette after lysis	Tube-based	low	>10000	Single-cell	[189]
scCOOL-seq	2017	Genome; DNA methylation; Chromatin accessibility	scBS-seq; NOMe-seq	-	Plate-based	medium	-	Single-cell	[277]
scNOMeRe-seq	2019	Genome; Transcriptome; DNA methylation; Chromatin accessibility	MATQ-seq; scBS-seq; NOMe-seq	Physical separation by magnetic beads	Tube-based	medium	>10000	Single-cell	[193]
scChaRM-seq	2021	Transcriptome; DNA methylation; Chromatin accessibility	Single-cell barcoded RNA sequencing; scCOOL-seq	Physical separation by magnetic beads	Tube-based	medium	~6000	Single-cell	[278]
scMethylHiC	2019	DNA methylation; Chromosome conformation	WGBS; *in situ* Hi-C	-	Plate-based	medium	-	Single-cell	[194]
sn-m3C-seq[a]	2019	DNA methylation; Chromosome conformation	snmC-seq2; *in situ* 3C	-	Plate-based	medium	-	Single-cell	[279]
sci-CAR	2018	Transcriptome; Chromatin accessibility	sci-RNA-seq; sci-ATAC-seq	RNA-seq and ATAC-seq part are split after second strand synthesis and purification	Split pool	high	>1000	Single-cell	[195]
Paired-seq	2019	Transcriptome; Chromatin accessibility	Nuclei tagmentation by ligation-based barcoding for DNA and RNA		Split pool	high	>200	Single-cell	[280]
SNARE-seq[b]	2019	Transcriptome; Chromatin accessibility	Co-capture of DNA and mRNA in one droplet, then enriched separately by different primers		Micro-droplet	medium	>200	Single-cell	[196]
SHARE-seq[c]	2020	Transcriptome; Chromatin accessibility	SPLiT-seq; Paired-seq	cDNA and DNA are split by magnetic beads after reverse crosslinking	Split pool	high	>300	Single-cell	[197]
CITE-seq	2017	Transcriptome; Cell-surface protein	Drop-seq / 10X Genomics Chromium system; DNA-barcoded antibodies	-	Droplet	high	>200	Single-cell	[281]
REAP-seq	2017	Transcriptome; Cell-surface protein	10X Genomics Chromium system; DNA-barcoded antibodies	Protein library is selected by agarose gel	Droplet	high	>250/500	Single-cell	[282]

[a] Single-nucleus methyl-3C sequencing; [b] Single-nucleus chromatin accessibility and mRNA expression sequencing; [c] High-throughput ATAC and RNA expression with sequencing; [d] Reverse transcription

tion are differentially expressed or even absent in humans,[209,210] revealing significant differences between species. There have been a few studies on human lineage specification by scRNA-seq, but they propose disparate models. Petropoulos et al. revealed that TE, EPI and PE lineages are established simultaneously and coincide with the formation of blastocyst at the E5 stage.[208] Stirparo et al. delineated a discrete development process where an

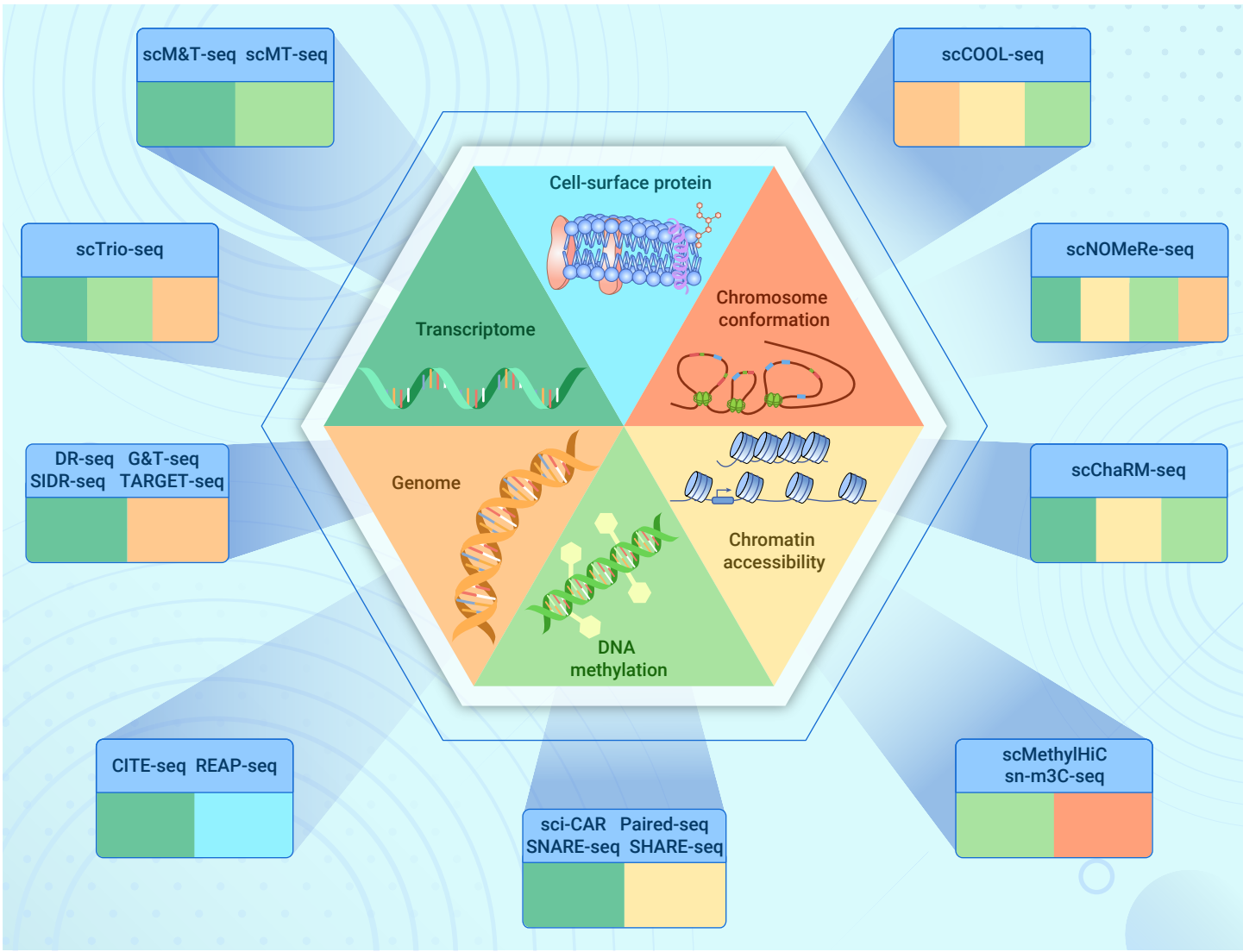

Figure 2. A brief schematic for representative multi-omics sequencing technologies Different colors correspond to different omics (as shown in inner hexagon), and the composition of color blocks in the figure show the omics that can be covered by each technique (see the supplemental information for the definition of abbreviations).

early ICM state was noted.[200] Meistermann and colleagues validated the two-step theory using integrated pseudotime analysis coupled with time-lapse annotation. However, when segregating from the TE, they identified an expression signature similar to the EPI rather than the ICM, and later on, the PE may differentiate from the EPI at the expanded blastocyst stage.[201,211] Given the variability of human *in vitro* development, as well as the diversity of sequencing protocols and bioinformatics strategies, the differences between various studies have made it challenging to determine the trajectory of human lineage specification.[200,201]

Epigenetic reprogramming is an important process in early embryonic development. Recent advancements in low-input sequencing technologies have allowed for significant progress in understanding the dynamic epigenetic regulatory networks. The dynamic patterns of DNA methylation during human embryonic development have been studied using RRBS and single-cell PBAT methylome sequencing.[202,212] In humans, the paternal and maternal genomes undergo rapid demethylation immediately after fertilization, reaching the lowest level in the blastocyst stage and then returning to a highly methylated state soon after implantation. This global demethylation is in balance with *de novo* remethylation, which is highly enriched in repeat elements.[202] Further studies of lineage specific methylated genes by scTrio-seq2 suggest that DNA methylation may play an essential role in cell fate determination.[213]

In terms of chromatin accessibility, the most significant chromatin remodeling occurs at 8-cell stage in human embryo, suggesting its involvement in the regulation of ZGA. A study on chromatin accessibility using DHS sequencing found that DHSs with OCT4 binding sites are enriched during ZGA in humans but not in mice, highlighting the essential role of OCT4 in human ZGA.[214] Another study found higher chromatin accessibility in the paternal genome compared to the maternal genome before 4-cell stage.[203]

The development of low-input ChIP protocols has enabled the mapping of genome-wide landscapes of histone modifications, such as H3K9me3,[205,206] H3K4me3, H3K27me3,[204] H3K27ac[215] in human early embryos. Unlike in mice, H3K27me3 in humans is largely erased at the 8-cell stage and is further reset on developmentally relevant genes at the morula and blastocyst stage. H3K4me3 is widely distributed at CpG-rich regions in pre-ZGA embryos, and stage-specific H3K9me3 shows divergent establishment patterns.

Databases are available that integrate multiple omics data of early embryos, making it easier to analyze the developmental dynamics of embryos. DevOmics[216] incorporates transcriptome and epigenome profiles of human and mouse early embryos from zygote to blastocyst stage. It also offers advanced functions like DEG analysis. Another similar database called dbEmbryo enables spatiotemporal analysis and compares different mammalian embryonic omics databases, which provides suggestions for researchers in selection of appropriate database for analysis.[217] With accumulating research, deciphering of embryonic development will facilitate our understanding of diseases such as embryonic developmental arrest and fetal malformations, as well as optimizing the assisted reproductive technologies (ART) for the benefit of more families.

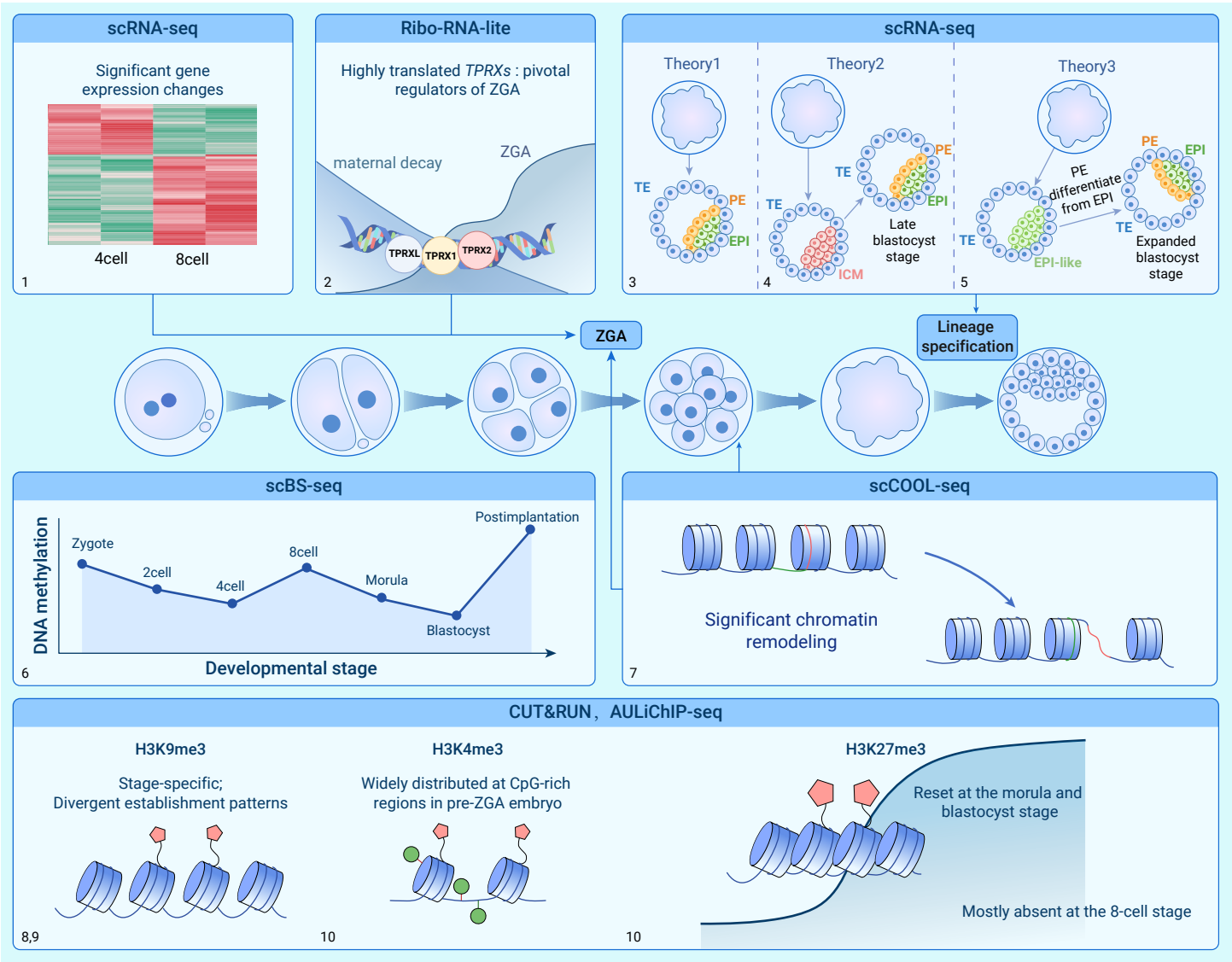

Figure 3. Representative advances in human early embryonic development using sequencing technology (1) Significant gene expression changes between 4-cell and 8-cell stage.[198] (2) Highly translated TFs, *TPRX*s, are found to be pivotal regulators of ZGA.[199] (3,4,5) Three mainstream theories of lineage specification. (3) TE, EPI, PE specification occurs concurrently and coincides with the formation of blastocyst.[208] (4) TE and ICM are established first, then EPI and PE are segregated at late blastocyst stage.[200] (5) TE and EPI-like transcriptome are identified first. PE differentiates from EPI at expanded blastocyst stage.[201] (6) DNA methylation reprogramming is in a dynamic balance between drastic global demethylation and *de novo* remethylation enriched in repeat elements.[202] (7) The most significant chromatin remodeling occurs at 8-cell stage in human embryo, involving in ZGA regulation.[203] (8,9,10) Dynamic patterns of histone modifications in human early embryos.[204-206]

Low-input sequencing in prenatal and preimplantation genetic testing

Genetic diagnosis for preimplantation embryos debuted in 1990 for preventing newborns from inheriting X-linked diseases by amplifying Y chromosome-specific repeat sequence, using a blastomere at the 6- to 8-cell stage.[218] Preimplantation Genetic Testing (PGT) is divided into PGT-A (for aneuploidies), PGT-M (for single gene/monogenic disorders), and PGT-SR (for chromosome structural rearrangements), each serving different indications.[219]

Currently, PGT has been widely applied in detecting both structural and numerical chromosomal aberrations, monogenic genetic disorders, human leukocyte antigen (HLA) typing, mitochondrial diseases and hereditary tumor syndromes (Figure 4).[220,221] Breakthroughs in sequencing technology and ART have expanded its clinical applications, including the detection of *de novo* mutations, benefiting many families. High-throughput sequencing has become an important technology for PGT due to its increased accuracy and cost-effectiveness. Compared to methods such as FISH, PCR, and microarray, sequencing-based methods can detect *de novo* single-nucleotide or short indel mutations.[222,223] Many large-scale PGT centers have adopted NGS technology, particularly for PGT-A, due to its relatively simple workflow, potential for semi-automation, and lower reagent costs.[224] Biopsies can be performed using polar bodies, blastomeres at the cleavage stage, or blastocyst TE cells. Among these, TE cells are widely preferred due to their minimal impact on embryonic development while offering valuable genetic information. However, it is important to note the potential occurrence of mosaicism, requiring comprehensive counseling and assessment in clinical situations.

For HLA typing cases, low-depth NGS combined with single nucleotide polymorphism (SNP) linkage analysis can simultaneously achieve chromosomal aneuploidy identification, target mutations detection and HLA typing. Compared to generic short tandem repeat (STR) linkage analysis, NGS-based SNP analysis provides a more accurate outcome for HLA matching.[225] As high-depth sequencing is expensive and unnecessary, low-depth NGS-based pipelines for PGT have been developed, such as the Mutated Allele Revealed by Sequencing with Aneuploidy and Linkage Analyses (MARSALA),[226] haploseek[227] and reduced representation genome sequencing with OnePGT.[228,229] MARSALA can acquire copy number variation (CNV), single nucleotide variant (SNV), and SNP-based linkage information simultaneously.[226] HaploPGT, a novel method that performs PGT-A, PGT-M, and PGT-SR in one assay, saves materials and time for detection.[230] HaploPGT can also identify triploidy, embryos carrying balanced translocation, and distinguish CNV origin.

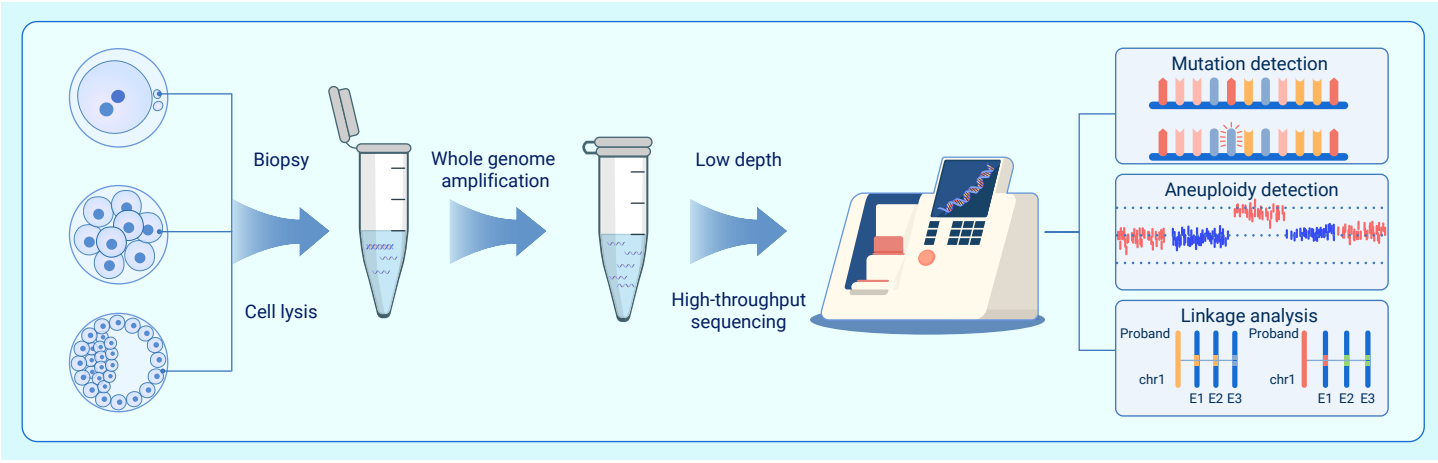

Figure 4. Common pipelines for PGT Cells biopsied from polar bodies, blastomeres, or trophectoderm are lysed for DNA amplification. Subsequently, data generated from low-depth, high-throughput sequencing is analyzed to meet various clinical needs, including mutation detection, aneuploidy detection, and linkage analysis.

Although not yet widely used, TGS has made an impact in the field of PGT. The ability to read large fragments and long reads greatly enhances the detection of complex structural variants that are often missed in short-read sequencing.[231] Researchers have found the Oxford Nanopore platform to be a portable and rapid method for embryo selection. It provides detailed information about structural rearrangements, such as the direct localization of translocation junction breakpoints at the single-base level and the identification of balanced translocation carriers from non-carrier embryos.[131,225,232-233] As TGS continues to evolve and become more accessible and affordable in the future, we expect that it will significantly contribute to the development of PGT technology in blocking the inheritance of genetic diseases and improving the birth rate of ART.

Low-input sequencing in oncology and immunology

Low-input sequencing is also making its mark in oncology. Previous studies have shown that tumor cells, due to their unstable genome, evolve heterogeneously during development in response to various stimuli.[234] Bulk RNA-seq veiled individual signals related to tumor characteristics, with the traditional focus mainly on the malignant cells themselves.[235] Additionally, non-tumor cells can confound bulk RNA-seq results. Therefore, research on intratumor heterogeneity (ITH) and tumor immune microenvironment (TIME), thanks to the advances in low-input sequencing technology, has provided additional insights into our knowledge of tumor and immunology, enabling precision and personalized treatment by identifying prospective therapeutic targets, predicting prognosis, and optimizing immunotherapy strategies (Figure 5).[236]

Intratumor heterogeneity. A wealth of sequencing data has revealed that coding mutations vary within tumors or between primary and metastatic or recurrence sites.[234] Bioinformatics tools based on sequencing have also been improved to decipher the temporal order of such genomic heterogeneity and construct tumor evolutionary trees.[234] One study identified a "cancer-primed" premalignant population based on transcriptomic disparities among normal-shaped thyrocytes.[237] Single-cell transcriptional and genotypic information obtained from acute myeloid leukemia (AML) patients identified distinct malignant cell types and compositions across different patients and tumor subclones.[238] Additionally, epigenetic remodeling plays an important role in tumor heterogeneity and predicts similar evolutionary pattern with genetic ITH, which indicates that epigenetic profiles can be applied to identify specific subclones for prognosis prediction and accurate diagnosis.[239,240]

Tumor immune microenvironment. The TIME, which comprises all sorts of immune cells and fibroblasts, is crucial in tumorigenesis, progression, invasion, metastasis, and drug response.[241] Advances in transcriptomic profiling of tumor tissue, coupled with bioinformatics algorithm, have empowered the identification of novel subpopulations and compositions of immune cell types with a higher resolution. This has shed light on the tumor immunity mechanism and provided guidance on different immunotherapy options.

A study employing paired single-cell analyses to construct an immune atlas of early lung adenocarcinoma revealed significant alterations in immune cell types even at stage I, including a reduced CD8+ T cells/Treg cells ratio, depletion of CD141+ DC, and an enrichment of PPARγhi macrophages. These results indicate that immune checkpoint inhibitor therapy could be tailored for patients at an early stage of the disease.[242] Sequencing data allows for the exploration of immune characteristics associated with immune escape, drug resistance, relapse, and other challenges in cancer diagnosis and treatment with higher resolution, thus providing guidance for the development and optimization of immunotherapy strategies. Table 4 lists some of the most recent studies that have profiled the tumor microenvironment from different perspectives using patient samples and highlights findings that contribute to enhancing the efficacy of immunotherapy.

Intercellular interactions. Communication among components of the tumor microenvironment has also been explored using scRNA-seq and analytical tools such as CellPhoneDB.[88,235] A study combining scRNA-seq data and TCGA-seq data revealed interactions within glioma cells and between macrophages and cancer stem-like cells. The authors further developed a computational prediction model for prognosis outcomes based on ligand-receptor interactions.[243] Interplays among immune cells have also been shown to be crucial in tumor immunity.[244]

Circulating tumor cells (CTCs) present promising sequencing materials for monitoring tumor progression and treatment. CTCs can be more easily obtained from blood samples compared to traditional biopsy methods, which expands the use of sequencing technologies in clinical applications.[245] As the associated techniques and algorithms evolve, it would dramatically change the way we study and combat tumors.

Low-input sequencing in epidemic

During the pandemic such as COVID-19, high-throughput sequencing technology has contributed greatly to the evaluation and treatment of urgent diseases, taking the advantage of its high-throughput and high efficiency. Scientists have leveraged scRNA-seq to analyze mononuclear cells from both peripheral blood and respiratory fluid (bronchoalveolar lavage fluid, sputum and pleural fluid). This approach revealed an association between immune subtypes and clinical features such as severity and stage of the disease. These distinctive immune features provide valuable insights into the understanding of disease pathogenesis, as well as the evaluation of patient conditions and treatment strategies.

Cao and colleagues used scVDJ-seq to detect antigen-enriched B cells for the rapid identification of neutralizing monoclonal antibodies (mAbs) against SARS-CoV-2.[246] While plasma with neutralizing antibodies from convalescent patients has been validated as effective in improving clinical conditions, it is often limited in availability for routine treatment. Moreover, traditional screening method is relatively laborious and time-consuming. Sequencing-based screening for potential antibodies demonstrates its potential in combating outbreaks of infectious diseases. As sequencing technology

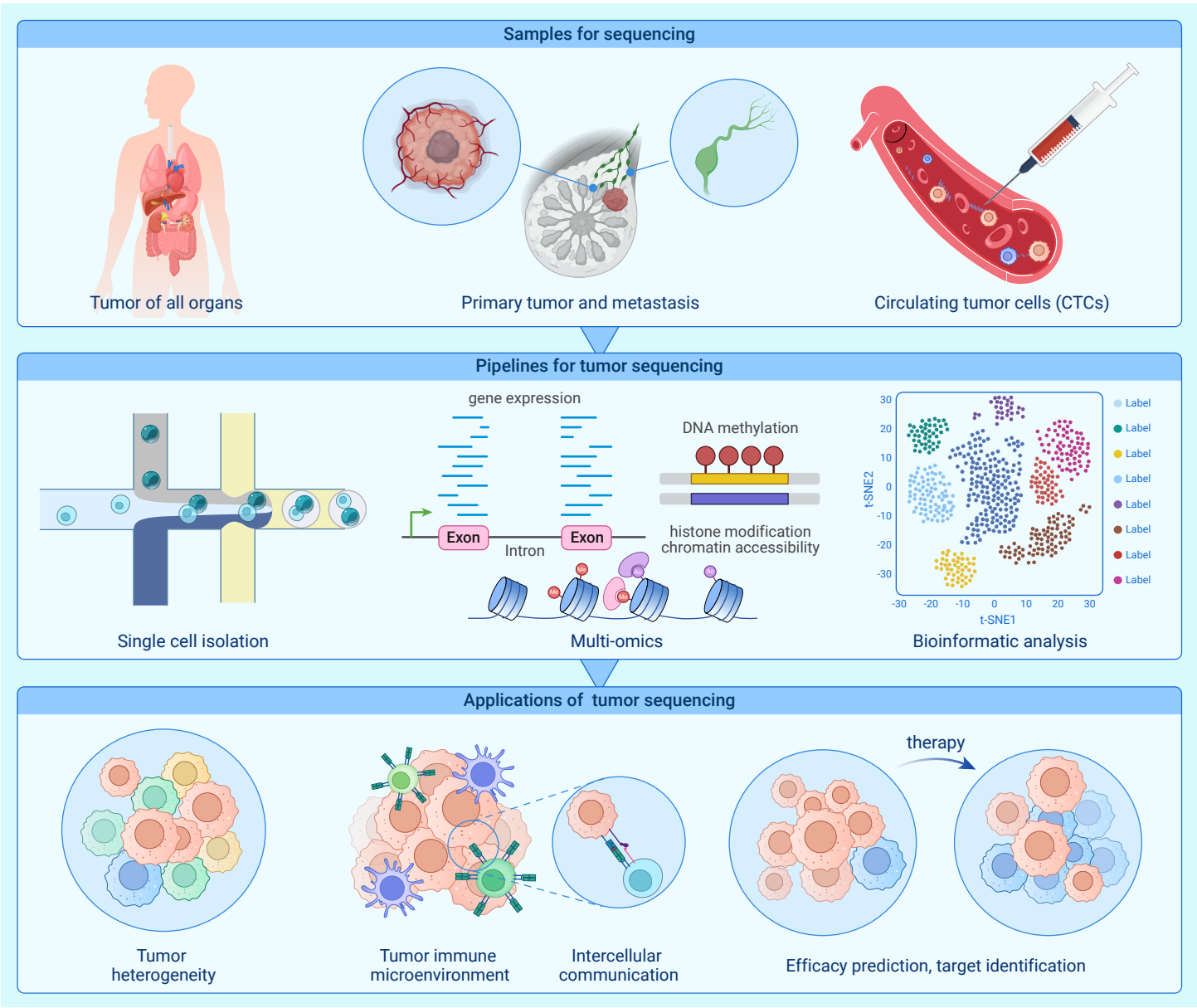

Figure 5. Low-input sequencing in tumor research Schematic of the application of low-input sequencing technologies in tumor research. Tumor research involves tumors of all organs throughout the body, such as lung, liver and colon. The samples mainly include the primary tumors and its surrounding tissues, metastatic lesion and circulating tumor cells (top). Multi-omics sequencing is privileged in characterizing the multi-level regulatory mechanisms of tumors and is widely used in tumor research (middle). Low-input sequencing profiling captures the tumor heterogeneity, reveals the tumor immune microenvironment, and provides potential therapeutic targets (bottom).

advances and becomes more efficient and convenient, it would be a powerful weapon in public health emergencies.

Low-input sequencing in other diseases

In addition to the areas mentioned above, sequencing technologies have also provided unprecedented insights into other areas and diseases (Figure 6). Scientists can now dissect individual signatures of all sorts of cells in convoluted brains and elaborate molecular heterogeneity across multiple levels.[247,248] Research into the pathogenic mechanisms of neurodegenerative diseases, such as Alzheimer's disease, has also been enhanced with the application of low-input sequencing.[249] Low-input and single-cell sequencing technologies have also facilitated in-depth research in other medical fields, including but not limited to cardiovascular diseases,[250] autoimmune diseases,[251] and infectious diseases.[252] This has provided a better understanding of the mechanisms of disease and opened up possibilities for new therapies.

CONCLUSIONS AND FUTURE PERSPECTIVES

Since its recent introduction, sequencing for low-input and single cells has undoubtedly become an essential tool for researchers in biology and medicine. The rapid advancements in laboratory protocols and bioinformatics tools have enhanced our understanding of gene regulation, disease causality, and clinical practice, including prognosis prediction and target identification.

However, there are still limitations and challenges in sequencing technology. Firstly, there is a need for improvement in resolution and accuracy. The stochastic and potentially biased capture of target sequences leads to confounding factors like drop-out events in scRNA-seq. Moreover, results from different computational strategies may vary, highlighting the importance of standard and reliable pipelines to extract heterogeneity from noise.[253] Meanwhile, as throughput explodes, a striking volume of data is being generated, requiring more efficient and high-speed algorithms and pipelines for integration and mining. Additionally, while the cost per cell has decreased significantly, it still tallies up to a substantial amount for studies that involve profiling large numbers of cells simultaneously or when TGS methods are required.[254] Besides, there is considerable scope for price reductions to make the standard clinical procedure more affordable.[255]

In addition to cost, there are other barriers to widespread clinical use of low-

Table 4. Recent studies on tumor immunity using single-cell sequencing technologies

Year	Tumor	Patient number	Mouse model	Method	Aim	Major conclusions	Reference
2019	Breast cancer	140	-	single-cell mass cytometry	Reveal the characteristics of tumor and TME[a] in breast cancer	1) High-grade tumors show enrichment of PD-L1 TAMs[b] and exhausted T cells; 2) Patient classification based immune landscape guides precise medical treatment	283
2019	Melanoma	25	-	scRNA-seq, TCR-seq	Reveal immune cell heterogeneity and the characteristics of dysfunctional $CD8^+$ T cells	1) Dysfunctional T cells are major clone of infiltrating immune cells but vary greatly among patients; 2) Dysfunctional T cells are highly proliferative and related to tumor reactivity	284
2019	Pancreatic ductal adenocarcinoma (PDAC)	24	-	scRNA-seq	Reveal the heterogeneity of PDAC and mechanism of tumor progression	Identified a ductal cell subtype with upregulation of proliferative and migratory genes associated with T-cell inactivation, potentially serving as a therapy response biomarker	285
2020	Bladder cancer	7	-	scRNA-seq, TCR-seq	Reveal mechanism of T cells-mediated tumor rejection to anti-PD-1 therapy	1) Cytotoxic $CD4^+$ T cell can predict response to anti-PD-L1 therapy; 2) Cytotoxic $CD4^+$ T cells can lyse tumor cells but are inhibited by regulatory $CD4^+$ T cells	286
2020	Colorectal cancer	18	+	scRNA-seq (10x scRNA-seq and Smart-seq2)	Depict landscape of myeloid cells in TAM and explore targeted immunotherapy	1) *C1QC* and *SPP1* TAMs are two major subtypes with distinct roles; 2) *C1QC* TAMs, related to antigen presentation, are initially depleted by anti-CSF1R therapy; 3) Anti-CD40 treatment activates cDCs and promotes BHLHE40 Th1-like cells expansion	287
2021	Clear cell renal carcinoma (ccRCC)	11	-	scRNA-seq, metaVIPER algorithm-based protein activity inference	Depict TME landscape in ccRCC	Tumor-specific $C1Q^+TREM2^+APOE^+$ macrophage subpopulation is enriched in relapsing patients and can serve as recurrence prediction biomarker and therapy target	288
2021	Glioma	31	+	scRNA-seq	Depict tumor-infiltrating T cell landscape in diffuse glioma	Identified potential target for immunotherapy: NK gene *KLRB1* and its encoding receptor CD161	289
2021	Hepatocellular carcinoma (HCC)	18	-	scRNA-seq	Depict immune landscape in early relapsed HCC	1) Relapsed HCC shows distinct immune subpopulations from primary tumor; 2) Identified a low cytotoxic $CD8^+$ T cell subtype with *KLRB1* overexpression, linked to poor prognosis	290
2021	Prostate cancer (with bone metastasis)	9	+	scRNA-seq	Reveal bone marrow microenvironment landscape in bone metastatic prostate cancer	1) Identified a tumor-specific immune state in the bone marrow: exhausted and dysfunctional T cells; 2) Identified overexpression of cytokine CCL20 in TIM[c] and TAM, associated with poor prognosis	291
2021	Small cell lung cancer (SCLC)	19	-	scRNA-seq	Reveal the heterogeneity of SCLC and landscape of TME; guide development of immunotherapy	1) Identified a pro-metastatic SCLC subpopulation with high *PLCG2* expression; 2) Exhausted $CD8^+$ T cells and profibrotic Mono/Mφ are linked to $PLCG2^+$ SCLC and metastasis	292
2022	Colorectal cancer (with liver metastasis)	17	-	scRNA-seq	Depict immune landscape and identify immune phenotypes that promote metastasis	1) Tumor malignancy and tissue niche impact immune cell subsets 2) $SPP1^+$ TAMs are a potential therapeutic target as they correlate with angiogenesis and metastasis	293
2022	Liver tumor	124	+	scRNA	Clarify TIME[d] subtypes and relationships to clinical outcomes	1) TAN[e] enrichment is associated with poor prognosis; 2) Depletion of TANs may serve as an immunotherapeutic strategy	294
2022	Non-small cell lung cancer (NSCLC)	10	+	scRNA-seq, TCR-seq, bulk RNA-seq	Depict Tas[f] cells landscape and develop personalized TCR-T cell therapy	1) T cells transduced with Tas TCRs showed tumor inhibitory effect; 2) Expression of Tas-specific *CXCL13* can predict the antitumor effect of ICB[g]	295

[a] tumor microenvironment; [b] tumor-associated macrophage; [c] tumor inflammatory monocyte; [d] tumor immune microenvironment; [e] tumor-associated neutrophil; [f] tumor antigen-specific T; [g] immune checkpoint blockade

input and single-cell sequencing technology. Laboratory findings of variants may have uncertain clinical significance in some cases. For instance, a variant of uncertain (or unknown) significance (VUS) in the PGT report can pose a dilemma for embryo selection. In a study profiling a drug resistance signature for therapy prediction, only a small number of cases provide enough evidence to justify a change in clinical practice.[256]

Despite its limitations, low-input and single-cell sequencing technology provides a promising blueprint for future scientific research and clinical applications. Recently, in combination with CRISPR-Cas9 technology, such as CRISP-seq,[257] Perturb-seq,[258] and CROP-seq,[259] it is now possible to dissect

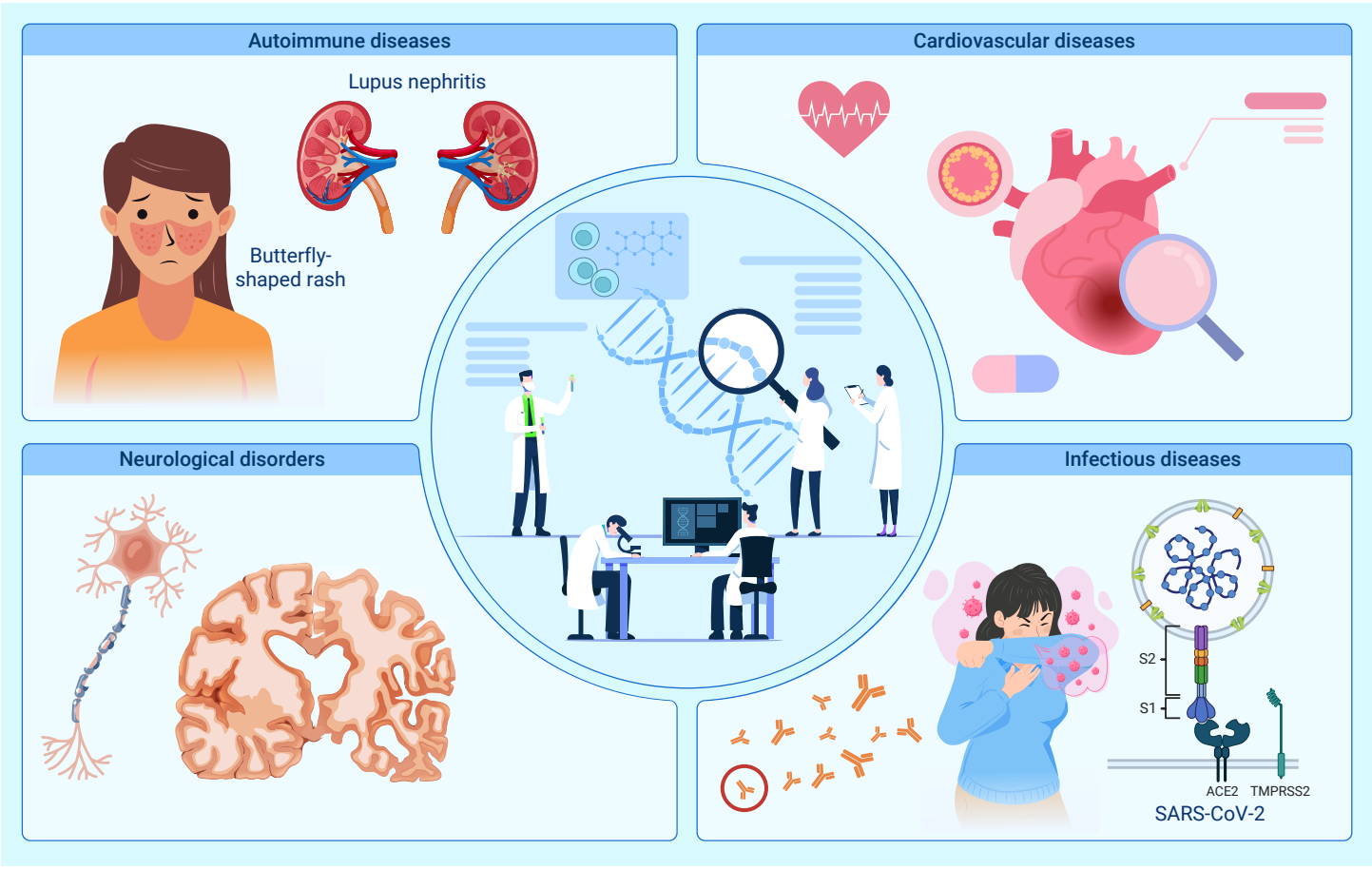

Figure 6. The application of low-input sequencing in other diseases and areas The figure enumerates several organs and diseases where low-input sequencing is also extensively implemented: autoimmune diseases (such as systemic lupus erythematosus, SLE), cardiovascular diseases, infectious diseases (such as SARS-CoV-2), and neurological disorders (such as Alzheimer's disease).

complex regulatory mechanisms and generate genotype-phenotype associations at a higher resolution.[260,261] With ongoing improvements in sequencing protocols and bioinformatics algorithms, armed with innovations in multi-omics, spatial omics, and CRISPR screening, this revolutionary technology is expanding our knowledge of genes, cells, and diseases on a comprehensive, multi-dimensional level. It holds great potential for clinical diagnostics and personalized medicine in the future.

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P.Y. and J.Q. designed and revised the manuscript. Y.L. and F.X prepared and wrote the manuscript. All authors approved the submitted version of this manuscript.

DECLARATION OF INTERESTS

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DATA AND CODE AVAILABILITY

Not applicable.

SUPPLEMENTAL INFORMATION

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