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# Recent advances in single-cell sequencing technologies

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## Abstract

Single-cell omics sequencing was first achieved for the transcriptome in 2009, which was followed by fast development of technologies for profiling the genome, DNA methylome, 3D genome architecture, chromatin accessibility, histone modifications, etc., in an individual cell. In this review we mainly focus on the recent progress in four topics in the single-cell omics field: single-cell epigenome sequencing, single-cell genome sequencing for lineage tracing, spatially resolved single-cell transcriptomics and third-generation sequencing platform-based single-cell omics sequencing. We also discuss the potential applications and future directions of these single-cell omics sequencing technologies for different biomedical systems, especially for the human stem cell field.

**Keywords:** single-cell omics, genome sequencing, epigenome sequencing, lineage tracing, third-generation sequencing, human stem cell

## Introduction

A single cell zygote at the starting point of our life develops into 37 trillion cells in our body. The huge cellular heterogeneity and complexity represent a major bottleneck and pose great challenges for research on human development, stem cell biology and many other fields. In many organs of an adult individual, stem cells are continuously self-renewing and differentiating to maintain the lifelong physiological functions of the organs. Stem cells are generally rare in organs and are located at specific positions surrounded and controlled by well-organized niche cells. They are quite often heterogeneous, containing distinct subtypes or distinct biological states, such as quiescent and actively proliferating states, and there are also intermediate cell subpopulations during their multi-lineage differentiation processes. In recent years, single-cell omics sequencing technologies have been resolving many of these issues and revolutionizing the stem cell field. This starts from the first single cell RNA-seq (scRNA-seq) technique developed in 2009[1]. With rapid development of the technology, including tremendous improvement of throughput, accuracy, automation, and commercialization, scRNA-seq techniques have been widely applied to address critical biological and medical questions. In this review, we will discuss recent advances in single-cell omics sequencing technologies in four topics. First, epigenetic regulation stands at the center of the gene regulatory networks, study of which provides important insights into how transcription is regulated and how epigenetic memory is established and maintained in stem cells as well as their differentiated progenies. The epigenome of a cell is all of the epigenetic information stored and maintained in a cell. It is comprised of a variety of precisely regulated and tightly interconnected epigenetic features including chromatin states, 3D genome architecture, DNA methylation, histone modifications, as well as the specific binding of transcription factors or non-coding RNAs onto the chromatin.

Single-cell epigenome sequencing techniques for many of these features have been established and have been routinely used for stem cell biology studies. Second, another layer of information crucial for understanding stem cell biology is the trajectory or lineage 'history' of a cell during stem cell self-renewal and differentiation. This information is vital to rigorously confirm the multi-lineage differentiation potentials of stem cells *in vivo*, which is one of the defining features of stem cells. Since genetic manipulation is in general prohibited for humans, endogenous genetic variants in the genome of a cell in human bodies, including single-nucleotide variants (SNVs), copy number variations (CNVs), Indels, structure variations (SVs), variations of microsatellites or other repetitive elements, as well as mitochondrial DNA mutations, provide invaluable information for tracing the developmental trajectory of stem cells in intact human tissues *in vivo*. The rapidly improving single-cell genome sequencing technologies are satisfying such lineage-tracing requirements for human stem cell studies. Third, stem cells and their descendants, as well as the surrounding niche cells, are usually well spatially organized in the tissues. So the spatial organization and interaction information is important for understanding stem cell biology *in vivo*. This issue can be addressed by spatially resolved single-cell transcriptome sequencing techniques. Fourth, third-generation sequencing (TGS) technologies have progressed rapidly in recent years. Combining TGS and single-cell omics technologies provides novel information for alternative splicing and other crucial biological features of an individual cell.

## Single-cell epigenome sequencing

Single-cell epigenome sequencing technology is particularly challenging since the epigenetic information is scattered in the genome of a diploid cell, which has only two copies of the

genomic DNA. As a comparison, single-cell transcriptome analysis is somehow easier with many expressed genes in a diploid cell having over a dozen copies of the mRNAs. A highly sensitive enzyme or chemical reaction is essential for single-cell epigenome sequencing technology. To make the loss of DNA as minimal as possible, the experimental steps should be simple. Particularly if a barcode can be linked to a cell at an early step, hundreds to thousands of individual cells can be pooled together for subsequent operations, which will greatly increase the throughput of the method. Droplet- and microchip-based methods greatly facilitate automation and a combinatorial indexing strategy can increase the throughput in an exponential manner. It is noteworthy that Tn5 transposase shows excellent performance in sensitivity, simplicity, early barcoding and throughput, and thus has stood at the center of technological improvement of single-cell epigenome sequencing in recent years. Bioinformatics tools have also been developed to tackle the challenges of the sparse nature of single-cell epigenomic data. For example, chromVAR measures motifs or functional annotations together instead of individual open chromatin regions, while ArchR uses an iterative dimensionality reduction approach[2,3].

## Chromatin states

Chromatin states represent the active or repressive status of the regulatory genomic regions in a cell. Practically, the active chromatin states can be assessed by accessibility of an enzyme such as transposase, DNase I, micrococcal nuclease (MNase), or GpC methyltransferase, by which single-cell chromatin accessibility sequencing techniques have been established[4–9]. Among these strategies, the Tn5 transposon-based ATAC-seq (Assay for Targeting Accessible-Chromatin with high-throughout sequencing) method simultaneously inserts, fragments and adds adaptor tags to the active chromatin regions in a cell and thus is excellent for low-cost and high-throughput analysis[10]. Using plate-, droplet-, or combinatorial indexing-based methods, recent studies have shown that thousands to hundreds of thousands of individual cells can be analyzed in a single sample[11–15]. A major shortcoming of ATAC-seq is that it cannot directly detect the repressive chromatin states that can be reliably detected by the GpC methyltransferases-based methods. The latter can also simultaneously analyze both chromatin states and endogenous DNA methylation of a cell, and have relatively higher resolution, as a GpC dinucleotide (GCH) occurs approximately every 25 bp in the human genome. Both the GpC methyltransferases- and transposon-based methods have also been shown to be able to simultaneously analyze CNVs at megabase resolution[16,14].

## 3D genome architecture

While the chromatin state provides information on where the chromatin opens, 3D genome architecture analysis provides information on how a genome is spatially and structurally organized and compartmentalized, as well as how different genomic regions interact with each other in a cell. Since the first single-cell Hi-C technique was established, which detects ~1 000 contacts in an individual cell, the methodology has been continuously improved, and the latest techniques are able to detect >1 million contacts in an individual diploid cell[17–19]. The technological improvement includes omitting the biotin enrichment step, usage of highly efficient single-cell whole genome amplification methods such as multiplex end-tagging amplification (META) and multiple displacement amplification (MDA), as well as higher sequencing depth. This leads to a resolution of ~20 kb for analyzing the 3D structures in an individual cell, which is capable of distinguishing two parental genomes of a cell and different neuronal subtypes[20,19]. On the other hand, the throughput of the single-cell Hi-C technique has been increased by using combinatorial indexing and Tn5 transposase/plate-based strategies[21,22].

## Histone modifications and transcription factor bindings

Histone modifications contribute greatly to the organization of chromatin structures and regulation of gene transcription. Chromatin immunoprecipitation (ChIP) is a widely used method for detecting modifications of histones in nucleosomes of chromatin. Realizing that low sensitivity and specificity of antibody capture is the main obstacle in single-cell ChIP-seq analysis, cell-specific barcodes are added before aggregating the cells for immunoprecipitation[23–25]. Among these methods, Drop-ChIP and scChIP-seq add cell barcodes by MNase digestion and ligation with a droplet microfluidics workflow, while itChIP adds cell barcodes by Tn5 transposase tagmentation with a chromatin opening step. These methods are able to detect ~1 000 to ~10 000 unique reads per cell.

Enzyme-tethering represents a non-immunoprecipitation chromatin profiling approach that is becoming increasingly popular and has been adapted to single-cell analysis[26–31]. In these techniques, Tn5 transposase, MNase, or adenine methyltransferase is tethered to protein A that binds to the antibody, directly to the antibody, or directly to the target chromatin protein, which allows marking of the genomic regions with specific histone marks. ChIC, CUN&RUN, and scChIC use MNase, while scCUT&Tag, COBATCH, ACT-seq, and ChIL–seq use Tn5 transposase. A key cation-activation step, $Ca^{2+}$ for MNase and $Mg^{2+}$ for Tn5 transposase, allows activation of the enzyme activity in a short time window after washing off the nonspecifically-bound enzyme and drastically increases the signal-to-noise ratio. As the Tn5-based method simultaneously adds tagged sequences, it is more convenient for high-throughput single-cell epigenomics analysis. The current enzyme-tethering single-cell techniques are able to detect several thousand unique reads per individual cell.

## DNA methylation

DNA methylation comprises another critical epigenetic layer showing cell-type-specific patterns. Single-cell DNA methylome sequencing techniques have been established using various strategies including the reduced representation bisulfite sequencing (RRBS)- and the post-bisulfite adaptor tagging (PBAT)-based methods[32–34]. The mapping efficiency and throughput of PBAT-based methods can be increased by using 3′ tagging techniques such as adaptase and TdT tailing; yet since only one round of random amplification is used, the coverage is decreased[35,36]. A sci-MET method has applied a combinatorial indexing strategy for increasing the throughput, with the first and second rounds of barcodes being incorporated by Tn5 transposon and random priming, respectively[37]. Conventional RRBS enriches CpG-containing regions by selecting genomic regions between a pair of MspI (CCGG) sites, but also covers the whole genome with many randomly fragmented genomic fragments. Recently, a single-cell extended-representation bisulfite sequencing (scXRBS) method uses an alternative approach by ligating the adaptor to a single MspI site, and thus achieves a new balance between coverage and enrichment of functionally relevant genomic regions[38].

**Table 1.** Single-cell genome sequencing techniques.

| | Uniformity | Accuracy | Coverage | Operation | Reference |
|---|---|---|---|---|---|
| DOP-PCR | +++ | + | + | +++ | 46 |
| MDA | ++ | +++ | +++ | +++ | 47 |
| eMDA | +++ | +++ | +++ | ++ | 50 |
| SISSOR | +++ | ++++ | ++ | ++ | 49 |
| MALBAC | +++ | ++ | ++ | +++ | 52 |
| LIANTI | ++++ | +++ | ++++ | ++ | 48 |
| META-CS | +++ | ++++ | +++ | +++ | 51 |

## Joint analysis of chromatin states and transcriptome

Methods for joint analysis of multiple omics in the same individual cell have been achieved by physical separation of different omics molecules, parallel indexing, or parallel capturing[39]. In recent years, several methods have been reported for jointly detecting chromatin accessibility and transcriptome in an individual cell, which use various strategies for barcoding and separating two types of information[40–44]. sci-CAR uses well-specific barcodes to perform *in situ* reverse transcription and Tn5 transposition separately[40]. The scCAT-seq method physically separates the nucleus and cytoplasmic RNA by centrifugation[42]. Both Paired-seq and SHARE-seq use a combinatorial indexing strategy, in which two to three rounds of barcoding are performed, adding barcodes to the 5′ ends of both the Tn5 and RT primers by split-and-pool. To separate the chromatin accessibility and transcriptome libraries, Paired-seq uses a restriction enzyme strategy, while SHARE-seq uses a biotin affinity pull-down strategy[43,44]. SNARE-seq is a droplet-based method; it uses a barcoded oligo(dT)-bearing splint oligonucleotide for simultaneously performing a reverse transcription reaction for capturing the transcriptome and a ligation reaction for capturing the chromatin accessibility information[41].

## Single-cell lineage tracing

Single-cell lineage tracing techniques have recently been developed by using a combination of transposons or CRISPR/Cas9 genome editing and single-cell transcriptome sequencing[45]. However, these genetic manipulation-based methods are not suitable for *in vivo* study of humans. Genome sequence information is continuously changing during development of the human from zygote to adult and further ageing processes due to stochastic genetic mutations. So the genetic mutations are intrinsic and ideal 'markers' for lineage tracing of a cell in the human body. In fact, they have been widely used for lineage tracing of tumor cells at bulk levels for many years and are the basis for tumorigenesis studies. Recent studies have reported the use of these endogenous changes of genome and mitochondrial DNA (mtDNA) information for lineage tracing of human stem cells in development and ageing, using clones derived from single cells or single-cell genome sequencing technologies.

## Single-cell genome sequencing

Single cell whole genome amplification (scWGA) techniques such as degenerated oligonucleotide primer (DOP)-PCR[46] and MDA[47] have long been reported. More recently, several new methods, including MALBAC, eMDA, LIANTI, SISSOR, and META-CS, have been developed[48–52]. The general characters of these methods are shown in Table 1. MDA uses Phi29 for isothermal single-cell whole genome amplification[47]. As Phi29 is of high fidelity with about one nucleotide per $10^8$ error rate, MDA has relatively high accuracy for calling SNVs, and has been applied to single-cell genome lineage tracing[53]. One disadvantage of MDA is its exponential-like manner of amplification of genomic DNA, which results in amplification of initial extension errors and dampens coverage uniformity. LIANTI uses T4 RNA polymerase to linearly amplify the original template hundreds of times, which increases uniformity and accuracy[48]. Further, META-CS and SISSOR acquire information from both strands (Watson and Crick strands, or duplex) for reciprocal corrections, which gives even higher accuracy for calling SNVs[49,51]. The duplex information can also be recalled in MDA by using single-nucleotide polymorphism (SNP) information, though the number of informative SNVs is reduced[54]. Further investigations are needed to reach the extremely challenging requirement of single-cell genome sequencing-based lineage tracing.

Uniformity is measured by the coefficient of variation of the sequence-dependent bias along the genome. At a bin size of 1 000 kb, LIANTI shows the highest uniformity with a value of ~0.03, while eMDA, MALBAC (normalized), META-CS, and DOP-PCR show a range between 0.1 and 0.15, and MDA shows a value >0.21; the value of SISSOR has not been described and is expected to be similar to eMDA.

Accuracy is measured by the false positive rate (FPR). META-CS and SISSOR, which give strand-specific information, have the highest accuracy with the lowest FPR ($<2.4 \times 10^{-8}$). LIANTI measures the linear amplification product many times and has the second highest accuracy with a FPR of $5.4 \times 10^{-6}$. MDA and eMDA rank third with a FPR of $1.3 \times 10^{-4}$. MALBAC and DOP-PCR show FPRs of $3.8 \times 10^{-4}$ and $9.6 \times 10^{-4}$, respectively.

Coverage of LIANTI is the highest, covering 95% of the genome of a human diploid cell by sequencing 83 Gb data, while META-CS (64% by 18 Gb data), MDA (87% by 85 Gb data), and eMDA (72% by 30 Gb data) rank second, followed by MALBAC (Yikon, 73% by 94 Gb data), SISSOR (64% by 195 Gb data), and DOP-PCR (45% by 84 Gb data).

Experimental operations of DOP-PCR, MDA, MALBAC, and META-CS are easier than those of eMDA, SISSOR, and LIANTI.

## Genome sequence information in a cell for lineage tracing

Different types of genetic variants have different characters for the purpose of lineage tracing. Nucleotide substitution changes are estimated to occur at a frequency of ~1 per cell division in a human cell. As expected, the number of SNVs increases with accumulating cell divisions during development and ageing. A fetal hematopoietic stem cell has tens of somatic SNVs while a hematopoietic stem cell from a middle-aged adult has ~1 000 somatic SNVs[55,56]. Similarly, adult stem cells of colon, small intestine, and liver have a few thousands somatic SNVs, with an accumulation of ~36 mutations per year[57]. Thus SNVs represent a rich source of endogenous genetic polymorphisms for lineage tracing.

Microsatellites are expected to be an even richer source of endogenous polymorphism as the mutation rate of a microsatellite is as high as $10^{-3}$ to $10^{-5}$/locus/cell division. It is estimated that 50 microsatellite mutations occur per cell division in humans and the complete cell lineage tree can be reconstructed using this information[58]. Obstacles for full application of this information include error-prone sequencing, difficulty in capturing, and short read length.

The repetitive elements including long interspersed repeat elements (LINEs), short interspersed nuclear elements (SINEs), and long terminal repeats (LTRs) comprise a large portion of the human genome. A number of LINEs such as LINE1 and SINEs such as Alus are still active in humans, and transposition of LINE1 and Alus occurs at a rate of about 1 per 140 generation and 15 per generation, respectively[59]. Low-rate somatic mutations of LINE1 have been reported in individual neurons at rates ranging from <0.1 to >10 insertions per cell[60,61]. Also, insertions have been detected in normal gastrointestinal tissues and occur very early during the development of gastrointestinal tumors[62]. Though the frequency is low, the unique insertion sequences of new transposon elements can be definitely verified and detected, which facilitates using them as good markers for lineage tracing[63].

It is well known that CNVs occur in high frequency during human early development, although most of them lead to death of the embryo or are self-corrected by elimination of the aneuploid cells in chimeric embryos; how frequently the early CNVs result in human chimeras is not known. In recent years, somatic chimeric CNVs have been identified in many cell types including neurons, blood cells, and fibroblasts[64–69]. The advantage of CNVs as an endogenous marker for lineage tracing is that they can be detected by relatively low-depth single-cell genome sequencing. However, the disadvantages include low frequency and difficulty of verification.

mtDNA, which is a ∼16 kb long circular genome, is also a part of the cellular genome in addition to the nuclear genome. mtDNA mutation occurs 10–100 times more than genomic mutation, and there are hundreds of copies of mtDNAs in an individual cell. Also, mtDNA can be detected in other single-cell omics data, including scATAC-seq, scRNA-seq, and single-cell genome sequencing. These features make mtDNA a unique marker for simultaneous lineage tracing and cell-state detection[70].

Several recent studies have reported lineage tracing using single cells, clones derived from single cells, or microdissection[71–73,63,55,53,74,56]. Lee-Six *et al*. analyzed 140 colonies derived from single hematopoietic stem cells of a 59-year-old male individual and identified an average of 1 023 SNVs and 20 small insertion/deletions in each clone. They constructed a phylogenetic tree that revealed clonal relationships among these 140 colonies. The results showed a rapid population expansion of hematopoietic stem cells during early life and estimated that there are between 50 000 and 200 000 hematopoietic stem cells actively making white blood cells at any specific time[55]. In another study, Spencer Chapman *et al*. analyzed 511 colonies derived from haematopoietic stem cells of two human fetuses and identified 25.5 and 41.9 SNVs per clone in the 8 and 18 post-conception week fetuses, respectively[56]. These SNVs allowed for reconstructing phylogenetic trees for early human embryonic development, which revealed several interesting findings, including an unequal contribution of each of the two-cell stage blastomeres to the blood compartment, a higher mutation rate in the first three cell divisions, and hypoblast origin of the extra-embryonic mesoderm and primitive blood.

Cells like neurons are post-mitotic and not able to proliferate, limiting the use of clone amplification and bulk sequencing strategies. Evrony *et al*. analyzed 16 cerebral cortex neurons by MDA and whole genome sequencing, which allowed for identification of two new L1 retrotranspositions and one poly-A microsatellite mutation. They designed a custom droplet digital PCR (ddPCR) assay and analyzed these mutations in various brain regions, which revealed one clone limiting to the left middle frontal gyrus and another distributing over the entire left hemisphere[63]. In another study by the same group, 36 neurons were analyzed for SNVs and custom ddPCR was used for revealing the polyclonal architecture of the human cerebral cortex[53].

The field of lineage tracing at single-cell resolution is developing rapidly. The approach can be used to reconstruct the developmental history of stem cells, showing their precursors and progenies. It can also be used for estimating the number of stem cells that give rise to the differentiated cells, and whether or not they give equivalent contributions to these progeny cells. For example, Spencer Chapman *et al*. showed that two blastomeres in a two-cell stage embryo contribute unequally to the body[56]. Also, during tissue injury, it is important to know which types of cells contribute to repair the injured tissue and recover its physiological functions, as has been studied in mice by genetic lineage tracing. Single-cell multiple-omics sequencing for genome and epigenome or transcriptome may help in elucidating the situation in humans.

## Single-cell spatial transcriptome

Spatial localization is essential for determining cellular fate. Single cell spatially resolved transcriptome technologies have been developed and improved rapidly in recent years, and are particularly useful for human study for which genetic labeling techniques are not applicable[75]. The methods of spatially resolved transcriptomics are listed in Table 2. Among them, two types of technologies, single-molecule FISH (smFISH)-based ones and *in situ* sequencing-based ones, provide single-cell resolution. The first one, including seqFISH and MERFISH, uses combinatorial barcodes for smFISH[76,77]. seqFISH labels each RNA by a combinatorial set of colored probes, through multiple rounds of sequential hybridizations and clearance of the probes[77]. MERFISH uses a similar combinatorial labeling strategy with the use of readout probes instead of direct labeling probes[76]. The latest versions of both methods are able to image mRNAs for up to 10 000 genes in a single cell[78,79].

The second set of methods is *in situ* sequencing (ISS, FISSEQ, STARmap, ExSeq)[80,83]. All these methods use rolling cycle amplification for signal amplification before *in situ* sequencing analysis. ISS and STARmap are targeting methods. ISS uses padlock probes for targeting[80], and STARmap uses designed nucleic acids for directly targeting RNAs that bypass the reverse transcription step and increases detection efficiency[83]. FISSEQ and ExSeq are untargeted methods, and ExSeq is an improved technique of FISSEQ that adapts the chemistry of expansion microscopy to allow high spatial resolution mapping of RNAs[82,81].

Another set of spatial transcriptomics techniques use *in situ* capturing strategies[86,85]. These techniques are approaching single-cell resolution with the high density of on-slide capturing[87,88].

**Table 2.** Spatially resolved transcriptome techniques.

| | Full name | Strategy | Targeting | Single-cell resolution | References |
|---|---|---|---|---|---|
| seqFISH | Sequential fluorescence *in situ* hybridization (FISH) | Combinatorial barcodes for single-molecule FISH | Yes | Yes | 78,77 |
| MERFISH | Multiplexed error-robust (FISH) | Combinatorial barcodes for single-molecule FISH | Yes | Yes | 76,79 |
| ISS | *In situ* sequencing | *In situ* sequencing | Yes | Yes | 80 |
| FISSEQ | Fluorescence *in situ* sequencing | *In situ* sequencing | No | Yes | 81 |
| ExSeq | Expansion sequencing | *In situ* sequencing with expansion microscopy | No | Yes | 82 |
| STARmap | Spatially-resolved transcript amplicon readout mapping | *In situ* sequencing | Yes | Yes | 83 |
| TIVA | Transcriptome *in vivo* analysis | Photoactive tag | Yes | Yes | 84 |
| Spatial Transcriptomics | Spatial transcriptomics | Gene ChIPs with immobilized reverse-transcription oligo (dT) primers | No | No | 85 |
| Slide-seq | Slide-seq | Spatially resolved DNA-barcoded beads | No | Nearly | 86,87 |
| Stereo-seq | Spatial enhanced resolution omics-sequencing | Spatially resolved DNA nanoball | No | Yes/nearly | 88 |
| iTranscriptome | *In silico* spatial transcriptome | Combination of the low-input RNA sequencing with serial cryosection and laser capture microdissection | No | No | 89 |
| Tomo-Seq | RNA tomography sequencing | Combination of the low-input RNA sequencing with serial cryosection | No | No | 90 |

# Third-generation sequencing

Third-generation/real-time single molecule sequencing (TGS) methods have been developing especially fast in recent years. These include Nanopore sequencing (ONT) introduced by Oxford Nanopore Technologies, and single-molecule real-time (SMRT) sequencing by Pacific Biosciences (PacBio)[91]. Emerging as a new field of single-cell omics sequencing, TGS has several unique advantages and applications, some of which have been achieved (Fig. 1).

First, TGS-based scRNA-seq techniques are powerful for detecting alternative splicing or DNA rearrangements by directly sequencing the full-length intact cDNAs. Several studies including ours have recently developed TGS-based scRNA sequencing techniques including SCAN-seq, R2C2, ScISOr-Seq, ScNaUmi-seq, and RAGE-seq[93–96]. SCAN-seq is able to detect >8 000 genes in an individual mouse embryonic stem cell (mESC), exhibiting a similar sensitivity to the next-generation sequencing (NGS) platform-based scRNA-seq techniques such as SMART-seq2, and SUPer-seq. A large number of unannotated novel transcripts have been detected. SCAN-seq detected 6 487 unannotated transcripts corresponding to 3 834 genes in mESCs, and 27 250 unannotated transcripts corresponding to 9 338 genes in mouse preimplantation embryos[93]. ScISOr-Seq detected 18 173 known and 16 872 novel isoforms in mouse cerebellum[94]. In addition, RAGE-seq has shown the ability of the TGS-based approach for detecting fusion transcripts from somatic DNA rearrangements of T-cell-receptor (TCR) and B-cell-receptor (BCR) transcripts[95].

Second, while small variants such as SNVs and short indels can be accurately detected using NGS-based short reads, larger structural variations (SVs) are more challenging to detect and characterize. TGS-based methods have been developed rapidly to increase the reliability and resolution of SV detection[97]. Our group has recently developed a TGS-based single-cell genome sequencing technique SMOOTH-seq[98]. For individual cells, the technique gets long sequencing reads with an average length of 6 kb, and reaches 19% genome coverage by 0.4X sequencing depth. Except for insertions, deletions, duplications, and translocations, the technique also effectively detects extra-chromosomal circular DNAs (ecDNA), being able to cover the full-length ones of <10 kb in a single read.

Third, single molecular nanopore sequencing is able to directly detect epigenetic modifications such as 5mC and 6mA. Combining such power with the enzyme accessibility of N6-methyladenosine (m6A) methyltransferase or GpC methyltransferase, three groups have recently reported TGS-based enzyme accessibility techniques for detecting chromatin state along single-molecules over a long distance (SMAC-seq, Fiber-seq, and nanoNOMe)[99–101]. Although they have not been adapted to single-cell analysis, these methods display the value of TGS for investigating coordination between the states of neighboring regulatory elements over large genomic regions, which opens a new avenue for future study.
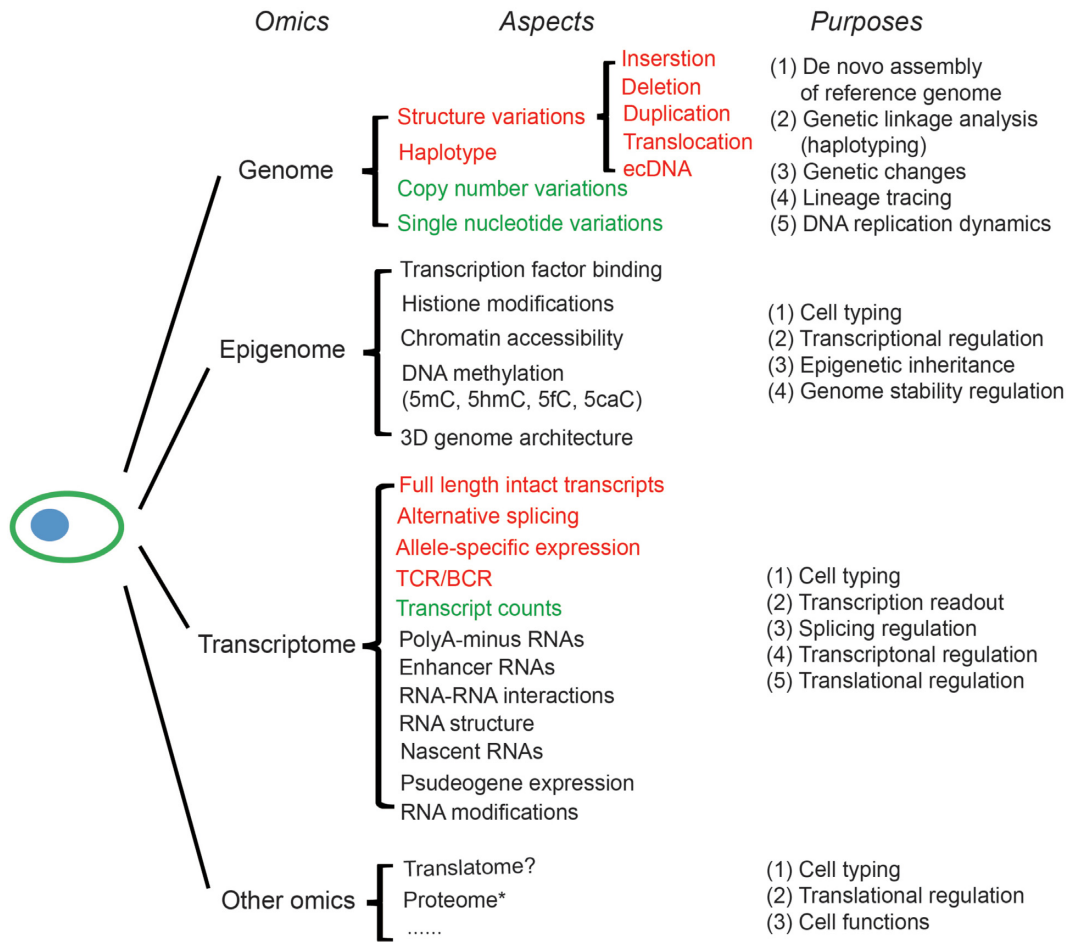
**Figure 1.** TGS-based single cell sequencing technologies. Red and green indicate the aspects that have been achieved, while the red indicates those where TGS-based methods have advantages over NGS-based methods. *The Nanopore-based technology may be used for directly sequencing the protein in the future[92]. Note the differences between NGS-based and TGS-based single-cell omics sequencing technologies (compare this figure to Fig. 1 of Ref. 39)

## Prospective

Single-cell omics sequencing technology has already made fruitful progresses in the stem cell biology field. However, the current techniques are still not ideal for human *in vivo* stem cell studies. We expect that the techniques will further be developed and improved within the next few years. The current single-cell omics sequencing technology has both strong (developed) and weak (developing) characteristics (Fig. 2). The technology has high universality as it is applicable in a wide range of biological research fields from plants to medicine. It gives high sensitivity and accuracy, but still requires improvement. The throughput, automation, and speed of the technology have increased greatly with the cost decreasing, but have not met clinical requirements. Particularly for stem cell studies, the temporal and spatial resolution of the technology is not satisfactory and is being improved. With its weaknesses being improved, single-cell omics sequencing technology will be routinely used to dissect the biology of stem cells, including (1) their self-renewal abilities or multiple lineage differentiation potentials under physiological conditions or under pathological conditions, (2) their premature differentiation or delayed leaving from self-renewal mode in diseased situations, (3) their microenvironments, (4) the consequences of their genetic perturbations, and (5) short-term responses to environmental changes or long-term maintenance of their fates.

Further, it will be ideal if genome, epigenome, and transcriptome can be simultaneously analyzed for an individual cell. The transcriptome will permit identifying and separating different types of cells. It will also act as a functional readout of the global transcriptional activity. Then how different layers of epigenomes regulate the organization of the genome and transcriptional activity of every gene can be delineated. Finally, the genome information can also be used to construct the lineage relationship. In addition, how genetic changes contribute to the abnormal behavior of a stem cell can be analyzed. If a genetic change perturbs the expression of a gene, it may change the physiological function and phenotype of a cell. In the future it is possible that through single-cell multi-omics sequencing we will identify genetic changes in the stem cells in our body and their potential connections to the phenotypic changes of a stem cell.

Of course, single-cell omics sequencing techniques are just a series of technologies and they cannot answer every question that arises in stem cell biology. Nevertheless, by integrating properly with other sets of powerful technologies such as gene editing tools and organoid 3D culture systems and biological concepts, they will definitely accelerate the transformation of our rich and deep knowledge of stem cells in animal models into more clinically relevant knowledge of stem cells in human.
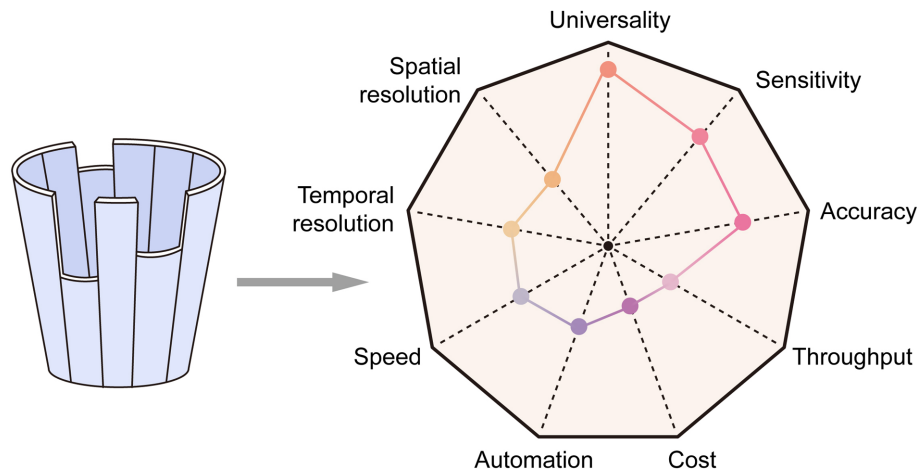
**Figure 2.** Characteristics of the current single-cell omics sequencing technology. Single-cell omics sequencing technology is shown shaped like a barrel with both long (developed) and short (developing) boards (left panel), and a radar chart shows the current technical states of nine major characteristics, with the more developed state of the character indicated by its being positioned more peripherally (right panel).

## Conflict of interest

None declared. In addition, as an Editorial Board Member of *Precision Clinical Medicine*, the corresponding author Fuchou Tang was blinded from reviewing and making decisions on this manuscript.

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