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REVIEW ARTICLE

Recent Developments and Applications of Single-Cell RNA Sequencing Technology in Cell Classification

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ABSTRACT

Multicellular organisms have many cell types and are complex, and heterogeneity is common among cells. Single-Cell RNA Sequencing (scRNA-SEQ) is a new technique for studying the transcriptional activity of a single cell that is still in its early stages of development. It generates transcriptional profiles from thousands of parallel cells to reveal the differential expression of individual cell genomes. They reflect the heterogeneity between cells to identify different cell types and form cell maps of tissues or organs, which play an essential role in biology and clinical medicine. Based on the introduction and comparison of the scRNA-SEQ sequencing platform, this paper focuses on the application of scRNA-SEQ in the exploration of cell types in the nervous system and immune system and summarizes the research results of the combination of scRNA-SEQ and spatial transcriptome technology.

INTRODUCTION

Single-cell sequencing is a new technique for high-throughput molecular detection of genome and transcriptome based on a single-cell level. Previous high-throughput sequencing techniques aimed at sequencing a mixed population of cells. They ignored the results of gene expression differences between individual cells, so much of the low-abundance information was lost. TANG published a paper in 2009 describing the Single-Cell RNA Sequencing (scRNA-SEQ) method [1]. After that, the technology developed rapidly, and various technology platforms were developed. Simultaneous sequencing of many samples is realized, which provides an effective method for studying the molecular mechanism of cell classification and cell development of organisms. Its technical route is shown in figure 1. In this paper, the research on cell classification by using scRNA-SEQ technology alone at the single-cell level was described, and cell information was analyzed together with spatial transcriptome data, tissue gene maps were depicted at both the cellular and spatial levels, and the research results obtained by using scNA-SEQ technology were summarized.

SINGLE-CELL TRANSCRIPTOME SEQUENCING TECHNOLOGY

Single-cell capture

The key of scRNA-SEQ technology is how to accurately and completely capture individual cells with cellular activity from the population of somatic cells. Single-

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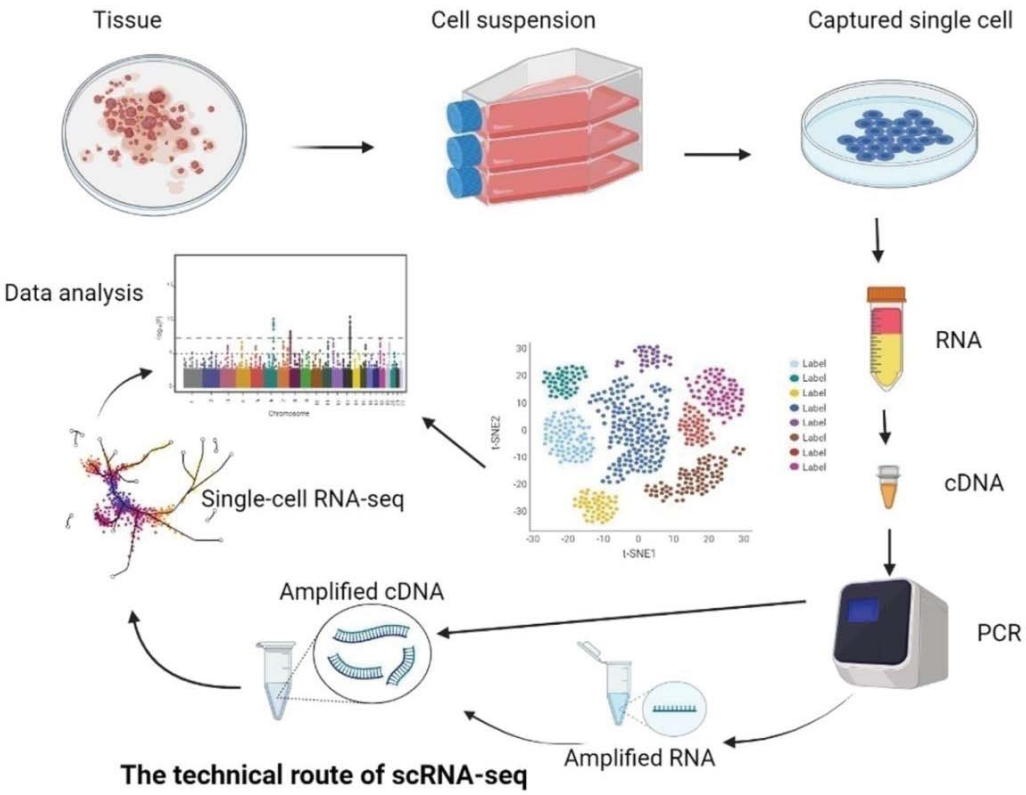

Figure 1 The technical route of scRNA-seq.

cell separation technology is becoming more mature, from manual micromanipulation to automated microfluidics systems are widely used in the market.

A market survey was conducted on single-cell capture technology and found that Fluorescence-Activated Cell Sorting (FACS) is the most widely used separation technology at present, and its samples cover most cell types [2]. Several studies, including [3]'s study on human ovarian cortex [3,4]'s study on zebrafish neurons [4,5] study on mouse hypothalamus [5], all utilized FACS to capture single cells in their respective experiments. Both FACS cell sorting techniques use flow cytometry instruments in advance for the specific antibody labeling cells from a single cell suspension sorting out; the advantage of this method is to set a preliminary enrichment type cells. However, the technical requirements for cell initial amount to a certain extent, so for small or rare cells [6] shall not apply. Under an inverted microscope, the microoperation method of directly sucking out a single cell with a pipette seems simple, but it is difficult to operate and has low flux. Only dozens or hundreds of cells can be detected in the same batch. Single-cell capture of solid tissue samples can be achieved by Laser Capture Microdissection (LCM) [2], in which laser pulses are used to melt the transparent thermoplastic film above the target cell. During the melting process, the film is fused with the target cell. After the film is removed, Target cells bind to the thin membrane, and non-target cells are left, thus isolating single cells [6].

Automated microfluidic systems are widely used in single-cell genome heterogeneity testing and tumor cell isolation. Compared with other separation methods, the microfluidic system requires less sample quantity, which is helpful to improve sample flux and mRNA detection sensitivity [6-9]. Fluidigm C1 microfluidic system enables rapid capture and preparation of more than 800 single-cell samples. The Droplets of the microfluidic chip are used to capture single cells, with a high capture efficiency of 65%. Drop-seq [10] and 10× Genomics [11] technology platforms use droplets to capture single cells.

scRNA-SEQ technology platform

In order to produce high-throughput sequencing libraries, scNA-SEQ first needs to obtain a single cell. Numerous scRNA-SEQ platform architectures have been developed (Table 1), roughly divided into two categories. One is the sequencing method based on the full-length sequence, such as smart-seq (Switching Mechanism at 5′ end of the RNA Transcript Sequencing) and SMARTseq2 (Switching Mechanism at 5′ end of the RNA Transcript Sequencing 2) [12]. The other is sequencing based on Unique Molecular Identifiers (UMIs), CEL-Seq2, Drop-Seq, and MARSseq (massively parallel RNA) Single-cell sequencing) are a few

Table 1: Summary of major scRNA-seq technologies.

Technology	Captured single cell			Amplified cDNA		Sequencing method
platform	Fluidigm C1	FACS	Droplets	PCR	IVT	Full-length UMIs
CEL-seq2	√				√	
Drop-seq			√	√		
MARS-seq		√			√	
SCRB-seq		√		√		
SMART-seq2		√		√		
SMART-seq	√			√		
10× Genomics			√	√		

of the technologies that fall into this category, as are 10 Genomics and Drop-Seq) [13-15].

- Full-length Sequence-based sequencing Method Smart-SEQ is a full-length scRNA-SEQ method [12,16-17]. The Fluidigm C1 microfluidic system was used to isolate and fragment single cells automatically, and using poly (T) primers, the first strand of cDNA was produced using reverse transcription, and the second strand was synthesized with oligo (dC) primers, and PCR was obtained nanogram DNA.

- Cell Expression by Linear Amplification and sequencing (CELseq) based on unique molecular identifiers was published in 2012. It is an amplification technique based on *Invitro* Transcription (IVT), with high reproducibility and sensitivity [18]. Cel-seq2 is a new system optimized based on CEL-SEQ, which has higher sensitivity, less manual operation time, and lower price than CEL-SEQ [13]. Drop-seq uses a microfluidic device to form nano-level droplets, in which individual cells and particles with obvious Barcode are encapsulated. Cells are cracked in the droplets, and associated particles capture the mRNA of specific cells. Single-Cell Transcriptomes Attached to Microparticles (STAMPs) are formed. STAMPs bar codes can be used to infer the cell origin of each transcript. Dropseq can perform horizontal analysis of thousands of single-cell mRNAs [10]. Mars-seq used FACS to sort single cells into 384-well plates, subsequently processed automatically. Which significantly improved cell yield and experimental repeatability by strictly controlling expansion bias and labeling errors [14]. 10× Genomics, a droplet-based nucleic acid barcode distribution system, is a rising star in the field of single-cell sequencing, featuring high sequencing depth, high cell flux, and UMIs based PCR sequencing bias elimination [15]. Two Scientists used 10× Genomics for single-cell sequencing. 10× Genomics uses a microfluidic system to wrap individual cells in gel beads with Barcode, Poly (dT) primers, and UMIs information [19,20]. Gel Bead in Emulsion Microsystem (GEM) is formed, which makes the cells have specific bar codes, and the mRNA is released after cell lysis, and finally, the cDNA with unique markers is generated for sequencing through reverse recording.

- Comparison of Various sequencing methods Researcher analyzed the scNA-SEQ sequencing data of mouse Embryonic Stem Cells (ESCs) [21]. The sensitivity, accuracy, and precision of six primary sequence assays for scRNA-SEQ were evaluated. UMIs cel-SEQ2, Mars-Seq, drop-SeQ, and SCRB-Seq were used to measure mRNA levels; smart-SEQ2 was found to be the most accurate (single-cell RNA barcoding and sequencing). Some of the most cost-effective techniques are Drop-seq, MarS-SEQ, SCRB-SEQ, and Smart-SEQ2. Because of the low cost of the library building, Drop-SEQ may be a better option for large-scale cell analysis with low coverage. However, when there are few cells, SCRB-SEQ and MARS-SEQ can be utilized instead of DROP-SEQ, which requires many cells. HANG compared drop-seQ, 10× Genomics, and interop high-throughput SCRNA-SEQ systems, and the results showed that 10× Genomics had higher sensitivity and precision and lower technical noise than the other two systems [22].

CELL CLASSIFICATIONS AT THE SINGLE-CELL LEVEL

scRNA-SEQ to studies of known cell types

The nervous system and immune circulation system are two major systems of the body, and most diseases are related to these two systems. Therefore, studying the structure and function of the nervous system and immune system is the key to understanding the human body's function and solving medical problems.

- Cell subsets of the nervous system the cell types of multicellular organisms are numerous and complex, especially the brain nervous system of mammals [23]. Mammalian neuron types are heterogeneous, but many cell types have not been discovered yet [24,25]. Therefore, it is a major biomedical initiative

to study the cell types of the nervous system by scNA-SEQ technology.

At present, the cell types of the nervous system mainly include neuronal cells, oligodendrocytes, astrocytes, vascular endothelial cells, microglia [26,27] and each cell type can be studied by scNA-SEQ technology. Neurons are the basic structure of the nervous system and the main component of the brain. Each neuron can realize information transmission and is an essential tool for the regular operation of the brain. Therefore, to comprehend how the nervous system works, it is essential to research neurotransmitters and their actions. Samples were obtained from 19 areas of the mouse spinal cord, sympathetic ganglion, dorsal root ganglion, and intestine, nervous system [26] and obtained WNT1-CRE samples by FACS technique. R26Tomato transgenic mice and VGat-Cre; Neural cresta-derived cells and inhibitory neurons from tdTomato cell line were sequenced on a 10× Genomics platform of about 500,000 cells. The primary cell types were identified by analyzing specific genes that perform specific functions of a particular cell type.

Moreover, the researchers found that neurons are among the most diverse nervous systems. Scientists used Illumina's smart ultra-low RNA kit for reverse transcription and cDNA amplification. Sequencing was performed on a total of 1679 cells in adult mice's central visual cortex, and more than 98% of the cells with 5 million sequenced transcripts were sequenced [28]. A total of 23 γ-aminobutyric acids (AMINObutyric acid) were identified. This study discovered that single-cell transcriptional characteristics relate to specific cellular traits such as electrophysiology and axonal projection.

Glial cells are widely found in the central nervous system and support and nourish neurons. Brain cell development also depends on glial cells. Oligodendrocytes, astrocytes, and microglia are all types of glia. The FACS and Fluidigm C1 platforms were used to collect and create a library of cells in mice's somatosensory cortex (S1) and hippocampal CA1 area [27]. After sequencing analysis based on UMIs, they developed a double-clustering method, BackSPIN, for identifying different cell types. Analysis of 3 005 cells revealed 9 groups of cells and 47 subgroups of cells that were different at the molecular level, including 6 oligodendrocyte species representing the development process from oligodendrocyte progenitor to mature oligodendrocyte, indicating that cells have specificity during maturation. The research could provide vital information for mapping the brain and studying the disease. Subsequently, the paper reported that the mouse nervous system observed 7 types of apQ4-expressing astrocytes, presenting region-specific distribution [26]. The researchers also found Olfactory-Specific (ACOB) astrocytes and telencephalic-specific (ACTE1 and ACTE2) astrocytes. Retinal nerve cells can convert light into nerve signals and transmit them to the brain *via* ganglion. The leading cause of adult blindness is hereditary retinal membrane disease. Therefore, it is imperative to understand the related genes of individual retinal cell types in the treatment of retinal diseases. MENON studied genetic variation related To Age-Related Macular Degeneration (AMD) using the 10× Genomics 3′ V3 platform [29]. The sequencing data of 3 248 retinal cells from 6 dead descendants were analyzed using the multi-resolution network. The major retinal cell types and gene expression characteristics were identified using known cell type-specific marker genes. It was found that AMD risk genes were significantly correlated with retinal microglia, microglia, and vascular cells.

- Circulating Immune Cell Subpopulations The immune system can be found all over the body, with many types of cells. The primary cell types known now are monocytes, dendritic cells. Hematopoietic Stem Cells (HSC) generate mature blood cells through intermediate progenitor cells, with proliferation, self-renewal, and differentiation [30]. NESTOROWA analysis [31] uses scRNA - seq technologies such as 1 656 hematopoietic stem cells and progenitor cells (hematopoietic stem/progenitor cell, HSPC) FACS cell sorting and captured in the first place, Illumina Nextera XT DNA kit was used for library construction. Illumina HiSeq 2500 and Illumina HiSeq 4000 systems were used to sequence the generated libraries. Finally, 12 HSPC cell groups were established, and the expression map of HSPC differentiation was obtained. VILLANI used FACS to separate the Dendritic Cell (DCs) and monocyte groups from human blood and used smart-SeQ2 technology for single-cell RNA sequencing. Unsupervised cluster analysis independent of known markers was used [32]. Six DCs and four monocyte subtypes were identified in 2 400 cells after Quality Control (QC), revealing a new DCs subpopulation with the same properties as plasmacytoid DCs (pDCs). However, they can effectively activate T cells, redefining pDCs. The discovery of a new DCs subgroup provides a new direction for studying DCs in diseases and provides practical information for constructing an immune cell map.

Another study used the 10× Genomics 3′ V2 platform to analyze single-cell sequencing on Peripheral Blood Mononuclear Cells (PBMC) of tuberculosis patients [33]. About 62 000 high-quality cells were retained after removing the doublet, total gene expression <500, and mitochondrial gene expression >7%. Unsupervised cluster analysis using Seurat R packages identified 29 cell subclasses. By comparing scNA-SEQ datasets from healthy control (HC), Latent Tuberculosis Infection (LTBI), and Tuberculosis (TB) patients, The density change of Natural Killer (NK) cell subclass (CD3 -- CD7+GZMB+) can distinguish healthy patients from latent patients and active tuberculosis patients, suggesting that NK cell subclass (CD3 -- CD7+GZMB+) can be used as biomarkers to identify tuberculosis. Even though vascular disorders are a leading

cause of death, our knowledge of the biological components of blood arteries is limited. 3436 brain cells and 1 504 lung colonies were sequenced using Smart-SEQ2. The BackSPIN algorithm was used to classify cells in the mouse brain and lung into various cell types and identify cell types related to vascular systems [34]. By establishing the molecular basis of blood vessel growth and illness in rat brain and lung, the researchers have laid a solid platform for their future work.

Exploration of new cell types and establishment of cell maps

Since the cell discovery by Robert Hook in 1665, cell types have been explored, but none of the existing cells are classified on the same basis and are not related. The scRNA-SEQ technology enables the simultaneous transcription of thousands of single cells to identify different cell types and create a complete cell atlas. Clarifying cell maps will bring new insights into cell development, histology, physiology, pathology, and extracellular regulation and improve the ability to judge cell changes and their effects.

The emergence of scNA-SEQ is a boon in biomedical history. As the technology continues to improve, more and more scientists are discovering rare and even new cell types that can facilitate the creation of complete cell maps and disrupt the understanding of specific systems or diseases. The following summarizes recent achievements in identifying new cell types by SCRNA-SEQ technology (Table 2). In 2018, Nature simultaneously reported two articles discovering the same new cell by scNA-SEQ technology. Two research teams sequenced mouse airway tissues [35], mouse tracheal epithelium, and human bronchial epithelium [36]. Lung ion cells, a novel type of lung cells, were the primary source of CFTR activity in cystic fibrosis. A scientist sequenced the airway tissues of mice using SMART seQ2 and 10× Genomics and analyzed 301 and 7 193 cells, respectively [35]. They reported mice and anti-FoxIL immune responses using FoxIL-GFP and confirmed that lung ion cells are new cell subsets. Furthermore, a mouse lung ion cell injury model was established to verify further the correlation between lung ion cells and the cystic fibrosis mutant gene CFTR, which provides a new way for the Medical community to think about the treatment of cystic fibrosis disease. The kidney is an organ closely related to human health. It acts as the body's filter, regulates its acid-base balance, and secretes hormones that regulate blood and blood pressure. PARK lab analyzed more than 40000 healthy mouse kidney cells by droplet scRNA-SEQ [37]. Three previously unreported cell subsets were identified, including a new cell type of the renal collecting duct and two cells that have not yet been defined, and a complete mouse renal cell map was mapped. They confirmed the existence of the new cell type of the renal collecting duct using dual immunofluorescence staining and in situ hybridization. Monocle R tool kit was used for cell trajectory analysis, and it was found that the new cells were a transitional cell type, which was located in the principal cell types responsible for the sodium-potassium balance. Between PCs and Intercalated Cells (ICs), which are responsible for acid-base balance, suggesting that there is a possibility of conversion between PCs and ICs and that new renal collecting cells may be one of the causes of metabolic acidosis.

Scna-seq can be applied to explore new cell types in model organisms and study new cell populations in human tissues. In 2019, Nature reported two studies on new human cell types. Parikh, et al. [38] analyzed ordinary people and ulcerative colitis disease using the 10× Genomics 3′ V2 platform. Cluster analysis of 11 175 colonic epithelial cells from IBD patients revealed the presence of a novel absorbent cell (BEST4/OTOP2 cells) that was sensitive to pH and expressed OTOP2 gene and satiated peptide (uroguanylin) in normal subjects. To further understand BEST4/OTOP2 cells, single-cell sequencing of BEST4/OTOP2 cells was performed using smart-SEQ2. The loss of BEST4/OTOP2 cells in inflammation and colon cancer was found by semi-supervised clustering, and goblet cells and intraepithelial immune cells were found in inflammatory samples. This will be a new therapeutic target for colitis. Later, Researcher studied about 10 000 cells from 9 normal human liver tissues, constructed a map of human liver cells by McEl-seq2 sequencing platform, and identified unknown endothelial cells, liver Kupfer cells, and liver cell types [39]. We identified a rare type of EPCAM+ cells capable of forming organoid cells. We confirmed that EPCAM+ cells are bidirectional precursor cells capable of differentiating into hepatocytes and bile duct cells by RaceID3d analysis, StemID2 and FateID pedigree construction, and organoid experiments. Recently, Han, et al. [40] used the self-developed Microwell-SeQ single-cell sequencing platform to determine cell types of all major organs in human embryos and adults. This

Table 2: Achievements of scRNA-seq in exploring new cell types.

Research team	New cell type	Action	Cell atlas
Montoro DT, et al. [35] Plasschaert LW, et al. [36]	Pulmonary monocyte	The primary source of CFTR activity of cystic fibrosis mutant gene	–
Park J, et al. [37]	Renal collecting duct transitional cell	It may be one of the causes of metabolic acidosis	Kidney cell atlas
Parikh K, et al. [38]	Crypt top absorptive cell	It expresses the proton channel OTOP2 and the satiety peptide uroguanylin, which is sensitive to pH and is dysregulated in inflammation and to pH and is dysregulated in inflammation and cancer	–
Aizarani N, et al. [39]	EPCAM+ cell	Progenitor cells with bidirectional dierentiation potential, which can dierentiate into hepatocytes and bile duct cells	Human hepatocyte atlas

technology platform has a low two-cell probability, broad cell applicability, and high cost-effectiveness compared with other platforms. Rare cell types can be identified even at low sequencing depths. HAN'S team [40] analyzed approximately 700,000 cells, sequenced approximately 3,000 transcripts per cell, identified 102 major cell types and 843 cell subtypes, including more than 30 new cell subtypes, and mapped the world's first human cell map. This is a significant milestone in single-cell research, and it has consequences for the development of human function and disease therapy.

scRNA-SEQ binding spatial transcriptome reveals cell niche

In multicellular organisms, the gene expression of a single cell is time-specific and spatial-specific. Scna-seq technology can analyze cell types and gene expression in the temporal dimension but cannot restore the original position of a single cell in the tissue and cannot obtain the spatial dimension information of cells [41]. The traditional in situ hybridization technique can obtain spatial specific information, but its flux is low and cannot meet the demand of high flux. Thus spatial transcriptome sequencing was born. Spatial Transcriptome (ST) mainly uses frozen tissue sections as samples without tissue dissociation, which reduces the probability of cell damage. Since the advent of ST, many technical methods have been reported. These include Slide-SEQ, LCM-SEQ, seqFISH (Single-molecule fluorescence in situ hybridization), Geo-seQ, TomO-SEQ, and 10× Genomics Visium [42-47]. In this technique, the tissue frozen section is placed on the fragment containing bar code primers, which can capture mRNA from the tissue, conduct library construction, and obtain the cDNA containing bar code so that each mRNA transcribed sequence corresponds to the original position of the tissue section.

Since the mRNA captured by ST technology is not necessarily the information of a single cell, while scNA-SEQ can effectively capture a single cell, but it cannot display the information of a single cell in space, so SCNA-SEQ can be complementary to ST to reveal the complete genetic information of the cell in time and space. Paper reports the used ST, 10× Genomics 3′ V2 and *In situ* Sequencing (ISS) for sequencing analysis of human embryonic hearts in different pregnancies, and detected 3115 spatial loci using ST [48]. 10× Genomics analyzed 3717 cells, a variety of cell types were identified by dimensionless clustering and differential expression analysis, and cell information was located in the original tissue location. ISS and pciSeq determined spatial cell maps of the embryonic heart. Thus, the human embryonic heart's comprehensive gene expression map was constructed. The Researcher identified resident cell types in Bone Marrow (BM) using 10× Genomics and identified several rare cell populations [49]. These cells include Schwann cells (Mog, Mag), smooth muscle cells (Tagin, Acta2), PDGFRA+ mesenchymal group, and endothelial cells (CDH5, PECAM). In order to further understand the spatial information of BM cells, the team integrated 7497 cell analysis data of 10× Genomics and LCM-SEQ data, verified the sequencing results with immunofluorescence staining, determined the spatial location of each BM cell type, and analyzed the source of bone marrow hematopoietic factors. Another lab performed Indrop-seq and ST sequencing on pancreatic cancer tissues, respectively, and found that groups expressing TM4SF1 and S100A4 in drop-SEQ data of 2 500 ~ 3300 UMIs represent cancer cells [50]. The drop-SEQ data and ST data of about 2 400 UMIs were integrated using MIA (Multimodal Intersection Analysis) method.

To reveal the relationship between the location of different cell types in the pancreatic tumor microenvironment and cell types in tumor subregions. All three research teams [49,50] used scRNA-SEQ and ST to study tissues and organs. Apart from identifying cell subsets, they also located different cell types in their original locations, providing detailed information at the cellular and spatial levels. The emergence of ST makes up for the shortcomings of scRNA-SEQ and provides an essential means for the construction of 3D models of organs and the treatment and prognosis of tumors, which is likely to become a popular tool in the field of life science in the future.

CONCLUSION

The cell is the fundamental unit of the organism, and due to the variability of cells and the distinctions between subclasses, each cell exhibits different physiological activities and functions. A variety of RNA-SEQ sequencing platforms have been developed over the last decade to enable the simultaneous expression profiling of thousands of cells, recognition of known cell lines, and exploration of new cell types, making it feasible to map human cells in order to understand the inner structure of biological organisms. For the first time in 2019, ECCITEseq (Expanded CRISPR-compatible Cellular Index of Transcriptomes and Epitope) was published in Nature Methods. Genome and epitope profiling in living cells by DNA sequencing (CELLULAR indexing of transcriptome and epitopes by sequencing) There are at least five different kinds of cell information detected by Excite-seq in a single cell. Based on 10× Genomics single-cell immunoassay, which has the potential to reconstruct clones of individual immune cells [51]. More multi-functional and diversified single-cell sequencing tools will be developed in the future. The rapid application of scRNAseq technology in biomedicine provides a necessary technical means for exploring the pathogenesis of diseases and realizing precise treatment.

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