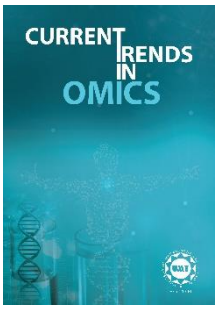

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Article: **Single-Cell Sequencing, its Application and Future Challenges**

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Single-Cell Sequencing, its Application and Future Challenges

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Abstract

Single-cell sequencing investigates the differences in the proteomic and genetic information available about individual cells by using next-generation sequencing technologies. The sequencing of the whole genome, epigenome, and transcriptome involves the heterogeneous process of diagnosis, progression, and treatment of disease(s). Previous studies showed that only a few selected proteins and RNAs can be measured. However, recent molecular studies suggest that advances in next-generation sequencing and whole genome amplification have allowed us to examine the differences among a variety of transcriptomic cells, gene expression, and phenotypic expressions. In our study, we summarized different technologies and their applications at single-cell level in diverse fields such as embryology, oncology, immunology, neurology, microbiology, tissue and organ development, antibody screening, and stem cell research.

Keywords: multiple displacement amplification, single cell proteomics, single cell RNA sequencing

Introduction

The fundamental unit of life is a cell. Various types of cells that perform similar functions are combined to make a tissue. The study of the bulk mass of tissue causes difficulty in the identification of a cell that undergoes mutation and leads towards serious diseases that ultimately cause destruction of body system and function [1]. The study of the specificity of single cell that make up the different systems by interactions is necessary to know about any dysfunctionality in the system. Previous studies on cancer progression and genome only provide limited genetic information for a cell population that mainly shows information of dominant cell subset. Due to this reason minor cell subset were ignored (2). It is very hard to distinguish properties of all cell subset in a population by using old methods so the biological

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information of single cells may be ignored. Because of fast development of sequencing, genome amplification, cell isolation techniques and single cell sequencing approach can overwhelm the limitations of old methods [3, 4].

Single cell sequencing give detailed information about systems of the body and diagnose many diseases by giving knowledge about genome, epigenome, and transcriptome. Any single mutation in an individual cell at these levels (genome, epigenome transcriptome, and proteome) can be observed by comparing with healthy cells. It also plays a great role in the diagnosis of diseases because cells from the same tissue show different genome during cell division. A developing cell may get any mutant gene that can't be recognized by tissue but can be recognized through analysis of single-cell that is responsible for developing and progression of a disease. So the isolation of single-cell is necessary for further analysis of disease diagnosis and treatment [5]. Single-cell genomics play an important role in the clinical side by sequencing every single gene from a whole genome in a single experiment. A gene that undergoes mutation can be detected through single cell genomics and its pathways can be detected. This technique helps in identifying the gene with a similar sequence.

Due to the isolation of a single cell, single cell sequencing has wide applications in clinical and fundamental research. Many different protocols for single cell sequencing (mRNA-protein and mRNA-DNA methylation) are applied to study gene regulation of specific cell type. Gaiti et al worked on DNA methylation and single cell transcriptome and got a lineage tree of human chronic lymphocytic leukemia after treatment and therapy. Using transcriptome data on lineage tree they found that drug treated cells showed irregularity in of genes which involved in toll-like receptor signaling and cell cycle [6]. Jia and her coworker use chromatin accessibility and single cell transcriptome to examine embryonic cardiac progenitor cells of mouse. They found marker genes which link epigenetic regulation with transcriptional regulation during development [7]. "Science" and "Nature Methods" have recorded SCS as one of the most recent techniques to study cell. In this paper, we summarize the methods and applications of SCS to provide better data in future studies.

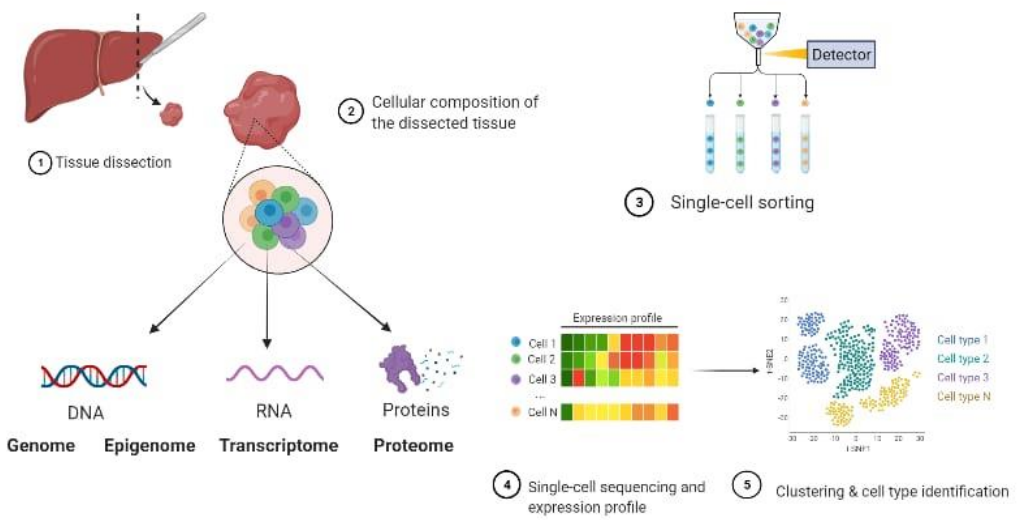
Single-Cell Isolation

Single-cell sequencing has emerged recently to explore the single cells multilayered status. Recent advances in single-cell sequencing technologies provide unprecedented access to DNA, RNA, and proteins at the single-cell level. Single-cell sequencing explicates the heterogeneity of cells and modalities at genomic, epigenomic, proteogenomic, and transcriptomic levels. To obtain the genomic

information of cells for the elucidation of cell types and omics layers from a clinical perspective, single-cell sequencing provides a wide platform. Recent advancements in conjugation with technologies provided the access to a broad range of single-cell profiling technologies such as single-cell genome sequencing [8] cell surface proteins [9, 10] chromatin accessibility [5, 11], and chromosomal conformation [12, 13] DNA (14-16), spatial and lineage evidence [17-19] and histone modifications [20].

Cell profile elucidation via single-cell sequencing technique requires cell isolation or cell dissociation. It is followed by amplification, sequencing, and data analysis. Among all these single-cell isolation and amplification are the core and crucial techniques. The methods implemented to carry out the cell dissociation directly affect the metabolomic profile, and stress of cells. Single-cell isolation techniques such as serial dilution, micromanipulation, fluoresce-activated cell separation, immunomagnetic separation, laser capture microdissection, and microfluidic techniques are implemented [21]. After isolation and amplification, the second most important step is sequencing which can also be performed using different platforms. The sequencing may be of the following types.

Figure 1. Step-Wise Single-Cell Sequencing Techniques: First The Qualified Single-Cell Isolated from Tissue Sample Followed by Extraction of Genetic Material and its Amplification and at The End Next-Generation Sequencing and Analysis is Performed



Single-Cell Genomic Sequencing

Single-cell genomics involve the isolation and extraction of DNA material from single-cell followed by amplification and sequencing analysis. The sequencing is performed using second-generation sequencing techniques. It precisely evaluates the genetic changes and detects mutations or abnormalities in DNA molecules [22, 23]. Moreover, it can be used to access new germ line as well as somatic mutations in normal and cancer cells. For the comprehensive and uniform amplification of the genomic DNA whole genome sequencing (WGC) methods such as multiple displacement amplification (MDA), degenerate oligonucleotide-primed PCR (DOP-PCR), and multiple annealing and looping based on amplification cycles are promising techniques [21, 24, 25]. However, whole genome sequencing is a challenging technique for human genome sequencing as there are only 2 copies of DNA available due to drawbacks such as allelic dropout and due to amplification bias, failure to attain uniform depth sequence. However, some of the advanced methods for the detection of single nucleoside variants have been developed keeping in view the allelic dropout and amplification defects. SCaller, LiRA, Monovar, and Conbase supported by C1 for automatic library construction can perform whole-genome sequencing as well as whole-exome sequencing efficiently [23, 26-28]. For the profiling of copy numbers in single cells, copy number variant has been recently employed for the chromium system. It has the advantage of a feasible library construction however it requires a large number of sequence reads which is a costly procedure [29].

Single-Cell Transcriptomic Sequencing

After the isolation of single-cell, transcriptomic sequencing is performed by the RNA extraction. At first extracted mRNA via reverse transcription converts to cDNA, after which the amplification of the reverse transcriptome sequence is done by PCR or alternative transcriptional techniques [30]. After the amplification, sequencing is performed to construct a library. Single-cell RNA sequencing transcript a mixture of cells or bulk. The analysis of the bulk RNA sequencing explicit the average transcript expression of specific cells population such as RNA-sequence of cancer tissue and transcript the expression of the cell population of tumor cell, immune cells andfibroblast; by this sequencing technique gene expression is reflected, moreover the cells at different differential stages can also be detected and distinguished.

For the highly sensitive appraisal and modeling of transcriptomic expression of heterogeneous cells population, single-cell RNA sequencing (scRNA-seq) techniques have found to be promising. Sc-RNA-seq is one of the most extensively

used and well-established single-cell sequencing approaches for precise, comprehensive, and conjugative profiling [31-33]. However, for the evaluation of the transcript of individual cells reverse transcription and cDNA amplification processes are required for the improvement of accuracy and highly efficient sequencing, several sequencing techniques incorporate recent innovations such as mRNA markers, and cell-specific DNA barcodes during reverse transcription and integration of single-cell optical phenotyping and expression sequencing [34, 35]. Whole transcriptome amplification (WTA) also known as smart-seq is the method that involves oligo priming and template switching for the amplification of whole cDNA [36]. The techniques of Smart-seq2, CEL-seq, and Quartz-seq can access the mRNAs profile of a single cell [37-39]. RamDa-seq technique detects noncoding and enhancer RNAs, along with non-poly (A) transcripts in a single cell. Several protocols for scRNA-seq detection and construction of library have been reported which are based on advanced technologies such as Drop-seq and DroNc-sec use microdroplet technology [40, 41] while microwell-seq approaches such as Nx1-seq and Seq-well are portable and easy to handle for a large number of cells [42-44]. Sci-RNA-seq3 is the most advanced low-cost high throughput combinatorial method that has been developed. A diverse range of techniques has been developed but still the limitations such as lower efficiency, lower cell capture, amplification, and the limited number of sequencing reads exist in different platforms used for sequencing [32].

Single-Cell Epigenomic Sequencing

Along with the genome epigenomic modifications and chromatin, components play a vital role in the regulation and expression of the genes such as methylation of DNA [14, 45]. Recent advancements in sequencing techniques made it possible to detect and evaluate DNA methylation at a single cell. The overall pattern of DNA methylation can be achieved using restrictive digestion and bisulfite transformation of CPG islands. This technique precisely affords the expression of genes indirectly [46, 47].

Single-cell epigenome sequencing detects the differentiation patterns of individual cells. By the elucidation of cells, epigenomic fingerprints such as DNA methylation and state of chromatin the cell lineage and differentiation patterns; states of individual cells can be observed by most widely used single-cell bisulfite sequencing [48], as well as single-cell, reduced bisulfite sequencing [49]. Chromatin material valuation several techniques can be used such as sc-ChIP-seq can detect histone modifications using microfluid based sequencing method Drop-ChIP [20]. Another method for the detection of chromatin component profile is by

cleavage under targets and tagmentation (CUT&Tag) [45]. CUT&Tag involves the identification of chromatin protein by antibody and then the binding of proteins (protein A and Tn5) to antibody tags the genomic regions at the target protein site. By using a small number of cells ATAC-seq explicitly open chromatin patterns [45, 50].

Single-Cell Proteomics

Proteins are of vital importance in biological systems, but the proteins that can be detected through single-cell profiling are limited because proteins cannot be amplified like other components such as DNA and mRNA. Several methods have been devised to detect proteome along with transcriptome of single-cell [51]. Different platforms such as proximity ligation assay for RNA (PLAYR), proximity extension assay and specific RNA target amplification (PEA/ STA), and cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq), RNA expression, and protein sequencing assay (REAP-seq) can carry out proteomic sequencing [9, 10, 52, 53].

PLAYR involves the labeling of the antibodies with isotopes and with the help of PLAYR probes antibodies bind to mRNA. After amplification mRNA levels are converted to labeled probes, the labeled mRNA and proteins can subsequently be quantified using mass cytometry. In PEA/STA technique, PEA tagged antibody binds are used for the proximity-dependent hybridization of DNA oligos, that convert proteins to DNA oligos, and cDNA is generated using RT primers. DNA oligos along with cDNA got amplified using PCR and sequencing is performed for quantification. While the recently developed CITE-seq and REAP-seq techniques can measure cell surface proteins as well as mRNA using oligonucleotide labeling to antibodies. While the single-cell RNA and immunodetection (RAID) can detect intracellular/phosphorylated proteins along with mRNA [54].

Applications of Single-Cell Sequencing

Single-cell sequencing technology has broad fields of application in clinics and medicines. Its application in clinic becomes beneficial when advances are made by reducing time and money of the sequencing technique. Auspicious expenses on DNA sequencing are going down quickly because of innovation and competition in industrial and technical fields. Sequencing can be used instead of microarrays to reduce the potential of huge sample multiplexing with the use of barcoding tactics. In this barcoding technique during amplification sequence of 4-6 base oligonucleotide is used in every library. In this way, many samples are collected together in a single sequencing reaction [55].

Single-cell sequencing techniques may be used in embryology, oncology, microbiology, and immunology. SCS strategies are also used in checking and predicting medicinal effects of disease progression, prognosis, drug resistance detection, and suggesting the accurate handling of individual medication. Single-cell sequencing has also been working in immune response, disease diagnosis, microorganism detection, and antibody screening [21, 56].

Figure 2. Versatile Clinical Applications of Single Cell Sequencing Techniques

Embryology

In mammals, during the early embryonic stage, only a few cells scientifically exhibit their properties. Significant research is conducted on embryonic development with respect to the clinical research at single-cell level. In one study step-wise scRNA-sequencing of mouse and a human embryo at different stages was seen (oocyte, a fertilized egg, 2-8 cell, morula, and blastocyte) [57]. In another research, scRNA-seq was used to elaborate the dynamics of transcriptome from first cell division to morula stage which controls the gene regulation, cell cycle, metabolism and translation [58]. Another group of researchers study the stem cells at single-cell level by using the microfluidic system with high data single-cell genome. They draw chromosome recombination maps at single-cell level by applying high-density genotyping. In this way, they observe the genomic diversity of single germ cells [59]. In recent years a study was done to measure cytosine

DNA modification in embryonic stem cells of the mouse by using single-cell bisulfite sequencing technique. Their study discusses that cell heterogeneity is clear in blastocyte level and these blastocyte cells pooled together to form nutrient ectoderm and primordial endoderm. This shows the massive global demethylation [49, 60]. Tang and his coworkers explore the transformational mechanism (in vitro) of embryonic cells into embryonic stem cells by the use of single-cell transcriptome sequencing technology. This technology was used to investigate transcriptional reprogramming as the cells transit from inner cell mass to pluripotent embryonic stem cells [61].

Oncology

Tumors arise from normal cells. When tumors form, cancer cells mutate and spread to form specialized lineage and subpopulations because of this tumor heterogeneity, diagnosis, and treatment of cancer patient. In many processes such as clonal evolution, metastasis, and invasion, progression of tumor cells play an important role by providing ways for evolution. Tumor cells survive selective pressure in the tumor micro-environment because of the diverse genome. Microenvironment includes geographic barriers, hypoxia, immune surveillance, chemotherapy, and immune surveillance. In short, it is really very difficult to study such a population of cancerous cells through standard sequencing methods [59]. This problem is resolved by using single-cell DNA and RNA sequencing techniques which give strong and novel ways to demarcate this clonal multiplicity and also note that there is a major involvement of these cells in cancer progression. Clinically these techniques help in profiling, monitoring, and detection of cancer cells that may be resilient to chemotherapy. Because of these techniques, the main themes of oncology have improved such as prediction, detection, and progression [55]. Bian and his coworkers also use these techniques in their work to investigate different characteristics found during metastasis of human colorectal cancer with the help of single-cell determination and multi-group level. Relationships and characteristics which was investigated at single-cell level are DNA methylation, gene expression variations, and genomic copy number changes [62, 63]. Chinese scientists present the immunological map of T-cells of lung cancer and the immune map of the microenvironment of liver cancer [64, 65]. They uncover tissue distribution, subgroup classification, drug target, and tumor heterogeneity of immunological cells of lung cancer and liver cancer. Casasent *et al.*, [66] publish in their work about the mechanism of transition between ductal cancer in situ and invasive carcinoma and polyclonal source of breast cancer by using single-cell topographic sequencing technique. They revealed pedigree between invasive tumor subpopulations and in situ tumor subpopulations in their study.

Immunology

Immunology is a very complex field having heterogeneous and difficult immune responses to antigens. Single-cell sequencing techniques are very useful to explore the unrevealed data about such immune responses. In the cell wall of gram-negative bacteria, lipopolysaccharides can bring crucial transcriptional changes. This is done by activation of toll-like receptors of dendritic cells [67]. Shalek and his coworker explained the heterogeneity of dendritic cells at single-cell level. They gave two reasons behind heterogeneity which are: random gene regulatory network and maturity of the dendritic cell. Gaublomme *et al.*, also explains this heterogeneity feature at single-cell level within the autoimmune Th17 compartment [68]. Holt et al discovered unique CD4 T-cells with the help of single-cell sequencing techniques in human beings [69]. Another group of scientists also discovered the heterogeneity of diverse oligo-clonal cells in peripheral blood CTLs and bone marrow before and after transplantation. es [70]. At the two-cell stage of mouse embryo when single-cell transcriptomics were conducted, *lv et al.*, discovered the several integrated responses to lighting such as injury effects, morphological changes and intracellular damage repair process [71].

Microbiology

Single-cell DNA and RNA sequencing techniques proved beneficial in resolving diverse microbial genomic populations and explore the cell to cell diversity of microbial genome. Bacterial cells and other micro-organisms have a very minute amount of DNA and RNA (femtograms) which made the amplification of the bacterial cells in comparison to the mammalian cells [72]. Blainey and his fellows sequence and arrange the 5 single cells with ammonia-oxidizing archaea by using MDA (microbial detection array) [73]. Woyke *et al.*, in their study gathered two marine flavobacteria genomes to 90% by using MDA and FACS to perform NGS [74]. In early research works, sequencing was limited to some microbial cells. After these studies, huge research was performed on 201 uncultivated bacterial and archeal cells from 9 different habitats. This study discovers 29 branches of trees of life that were uncharted which discover 3 official domains of life [75]. Five segmented filamentous bacterial cells were sequenced at single-cell in another study [76]. These studies resulted in metagenomic deep sequencing techniques which are necessary for single-cell sequencing. They revealed that microbial genome can't be cultured in the lab. Updated studies on analytical processes and technologies enabled microbiologist to investigate microbial genome at single-cell level.

Germ-Line Transmission

After fertilization of oocyte and sperm cells, the zygote is formed and their genetic material also transmits to the zygote which leads to genetic variation. Working on germline cells is complicated without single-cell sequencing techniques. Germline variation processes can be studied with the help of single-cell DNA sequencing techniques. Very first study explains the sequencing of sperm cells in which 5-15 gene conversion process, 25-36 de novo mutations, and 22.8 recombination phenomenon in each sperm cell were seen [77]. Most studies were made on sperm cells but in recent work, MALBAC was used to study fertilized oocyte [78]. In that study 8 different female samples were investigated which result in 43 crossovers in a single oocyte and the recombination rate noticed was 1.63 times larger than sperm cells. Aneuploidy in oocyte seen was also higher (17.6%) as compared to sperm cells which have 7% aneuploidy. Lu *et al.,* also use MALBAC to perform single-cell sequencing in sperm cells of Asian person which resulted in 26% recombination events and 4% aneuploidy per single cell of sperm [79].

Organogenesis

There were few dozen markers for traditional cell classification in most tissues. To identify a group of cells and to perform their transcriptional profiling, RNA single-cell sequencing is used. These cells have similar expression programs to signify every cell type. Single-cell genomic technique is valid to both mature and developing organs and tissues to accurately explain distinguished cell forms, identify linage specific regulatory factors, lineage hierarchies, and defined progenitors [80]. During kidney development in mice, RNA sequencing was used to investigate the gene expression configurations of a single-cell at E11.5, E12.5, and P4 [81]. In many other studies of the development of various tissues and organs, single-cell transcriptomics are publishing recently such as the inner ear. These studies reveal that single-cell RNA sequencing made it easy to distinguish transcriptomic variations in each cell type [82].

Antibiotic Screening

Antibodies are mostly screened from libraries of yeast and bacteriophage which have many disadvantages. One disadvantage is that heavy and light chains of antibodies are arbitrarily paired which hide their natural humoral immune response. A large sample size and a large number of an experiment are also required for this screening purpose. These screened antibodies have shown narrow range of neutralizing function. But single-cell sequencing made possible to identify antigen-specific memory B cells taken from peripheral blood lymphocyte. Single B cell sequencing made it possible to distinguish antigen-specific neutralizing antibodies. These antibodies portray a wide range of applications. Wang *et al.,* explore the Zika

virus-specific monoclonal antibodies which have high efficacy power [83, 84]. Using single-cell sequencing, neutralizing antibodies for West Nile virus was isolated from B-cells of human [85]. Dengue virus-specific antibodies are also isolated from antigen-specific memory B-cells [86].

Neurology

In each nervous system, there are distinct copy number variations in nerve cells. So everyone's neuron is different from others [87]. Because of the heterogeneity of neurons, it is very difficult to understand neuronal reconnections and brain circuits. Single-cell sequencing technology made it easy to study the various nerve cells, sketching it explained the neuronal map on single-cell level and has helped to identify many different types of neurons and their connections in the brain [88]. Lake *et al.*, study on the ^{s} second generation single-cell of the adult brain uses the novel single cell nuclear sequencing techniques [89]. Leu and his co-workers distinguished frontal cortical neuronal cells by using single-cell methylation sequencing techniques [15].

Conclusion and Future Perspective

Genomic, transcriptomic, and proteomic combined together with bioinformatics and biostatics. The coupling of these technologies provides great insight into the diseases. It opens the gateway for diagnoses and treatment of diseases at an earlier stage. Single-cell sequencing techniques are very expensive and difficult to run due to the sensitivity or small volume of the sample. With the advancement of technology, new methods are being looked into for single-cell sequencing. By using these new methods these difficulties can be overcome that we are facing nowadays. By merging pharmacogenomics, metabolomics, and epigenomics with single-cell genomic, transcriptomic, and proteomic can have widespread application [90].

Single-cell DNA, RNA, and protein sequencing techniques was carried out with bioinformatics advanced technology to get a clear image with great precision, accuracy, best quality, complete and clear gene expression. Alternative splicing, polyadenylation (adding phosphate group), and methylation makes the isolation and sequencing of a single gene easier, accurate, and acts as a marker for diagnosis of many diseases at earlier stages like cancer. The addition of these plays an important role in gene regulation so these can be used in the treatment of diseases [91, 92].

Nucleotide sequencing gives great insight into the cell of the organism. Sequencing at this stage plays an important role in curing and preventing diseases. The minor chance of developing the disease can be predicted with the help of nucleotide

sequencing [93]. Next-generation sequencing technology provides great insight into the organism. The development of single-cell next-generation sequencing of genomic, transcriptomic, and proteomic plays an important role in the diagnosis and prevention of suspected disorder occurrence [94].

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