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Review

ADVANCEMENTS IN SEQUENCING TECHNOLOGIES: FROM GENOMIC REVOLUTION TO SINGLE-CELL INSIGHTS IN PRECISION MEDICINE

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Abstract

Single-cell Sequencing (SCS) technologies, methods for analyzing genetic material at the single-cell level, offer extensive insights into cellular heterogeneity. This has broadened oncology research by enabling the exploration of functional and genetic diversity within tissues of different cell types. Furthermore, SCS facilitates the study of complex biological processes like metastasis tracking and tumor microenvironment analysis. However, the implementation of SCS methods is furrowed by a lack of clinical accessibility and high application costs. This review examines the development of SCS technologies, analyzing trends in throughput, accessibility, and cost of various commercial platforms, by focusing on the domain of cancer research and precision medicine. Despite the significant advancements offered by third-generation sequencing platforms, which provide high accuracy, versatility, and throughput for sequencing single-cell genetic information, these methods face challenges such as high error rates, insufficient funding, and complex data analysis. Furthermore, we've determined that the advancements of the previous decade have enabled personalized medicine and in-depth analysis of cellular heterogeneity, revolutionizing fields like medicine, biotechnology, and biological research. We anticipate our assay indicating extensive advancements in healthcare through

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the adoption of precision medicine concerning individual genomes and helping to demonstrate the promise of advancement in general understandings of complex biological systems. Furthermore, our research indicates that efforts to overcome technical, analytical, and cost-related challenges are essential in future clinical application, distribution, and growth of SCS methods.

Keywords

Genetics, Single-cell sequencing, Next-generation sequencing, Cancer genomics, Precision medicine

1. Introduction

Cells are the basic functional and structural units of life, in which genetic mechanisms and complex cellular environments interplay to form complex structures such as tissues and organs, eventually leading to intricate organ systems and, therefore, organisms, showcasing the dynamic nature of life. Interestingly, each cell itself is governed by DNA, which contains the genetic blueprint of an organism. The human genome, for example, comprises approximately 3 billion base pairs, equating to around 3 gigabytes of data per cell [1]. This genomic information encodes instructions critical for development, function, and maintenance of the organism. The flow of genetic information from DNA to RNA, and subsequently to proteins, is a fundamental process, often influenced by various epigenetic factors such as DNA methylation and histone modification [2]. Hence sequencing genetic information encoded in DNA and transcribed in RNA, emerged as an immediate need to understand the functioning of a single cell as well as interactions in its external and internal environment.

Traditionally, scientists have used methods like Sanger sequencing and Polymerase Chain Reaction (PCR) to decipher this genetic code. While these techniques have led to significant advancements, they fall short in capturing the full complexity and individuality of the human genome. For instance, traditional bulk sequencing methods provide an average readout from a mixture of cells, potentially masking critical differences between individual cells [3]. Precision medicine, which aims to tailor medical treatments to the unique genetic makeup of each individual, requires more detailed and personalized genomic data. This is essential for understanding how genetic variations influence disease susceptibility and treatment response, and SCS has enabled pragmatic insights in this regard [4].

In recent years, starting around 2010, the advent of single-cell sequencing (SCS) has revolutionized the field of genomics. Unlike bulk sequencing methods, SCS allows for the analysis of individual cells, offering insights into cellular heterogeneity and genetic diversity [5]. This technology has significantly impacted fields such as cancer research, neurology, and developmental biology by providing a more nuanced and personalized understanding of genetic changes and their implications. For example, SCS has been instrumental in identifying rare cell populations and understanding tumor heterogeneity, which are critical for developing targeted therapies [6].

Despite its transformative potential, SCS technology faces several challenges, including technical issues, high costs, and complex data analysis. Understanding and addressing these challenges is crucial for the broader adoption of SCG in clinical practice with its full potential. Advances in technology and protocols for cell isolation, library preparation, and data analysis are required to continually improve the accuracy and efficiency of SCS. Moreover, efforts to reduce costs and enhance accessibility are underway, aiming to democratize access to these transformative technologies across diverse research and clinical settings [7].

This review traces the evolution of single-cell genomics from its origins in traditional bulk sequencing methods to the current state of high-throughput, single-cell techniques. By exploring the historical progression and recent technological innovations in SCS, alongside an analysis of throughput, accessibility, and cost-effectiveness, we aim to provide insights into its impact on precision medicine and the broader biomedical research and clinical studies.

2. Historical Overview

The emergence of Sequencing technologies in the past few decades has fundamentally transformed medicine, biotechnology, and biological research. Sequencing technologies have progressed in various domains and have significantly furthered scientific progress. This section will explore history from the beginning of Sequencing technologies through their improvements to where they are today.

2.1. Genomic Revolution : Emergence of first generation sequencing

The Genomic revolution, a metaphorical kickstart to the development of Sequencing technologies, began in 1953 with the creation of a double helix structure of DNA [8]. Throughout the 1970s, sequencing methods continued to increase with

the introduction of Sanger sequencing(The Chain-Termination Method), allowing researchers to sequence small DNA splinters [9]. PCR was introduced in 1983, allowing smaller samples to generate large quantities of DNA sequences. By 2003, scientists had successfully developed a reference of a human genome illustrating complex genetic variation [10]. In addition to pure scientific discovery, Genomic science has made way for exceptional improvements in personalized medicine and agricultural trait breeding. With sequencing technologies, care treatments may be tailored to the individual's genetic makeup, expediting diagnosis and recovery treatment [11]. Furthermore, sequencing has begun to appear more commonly in agriculture through its use in Genetically Modified Foods (GMOs), increasing food accessibility [12]. Through the prolific discoveries of the Genomic Revolution, it becomes apparent that the modern foundation of genomics has been well underway for more than half a century.

2.2. Next-Generation Sequencing

Closely following the development of the human genome reference, NGS sequencing methods began rapidly diffusing in numerous scientific fields. NGS(High-Throughput Sequencing) is a technology that allows for the swift processing of large quantities of DNA and RNA. As a more affordable, profound scalable approach, NGS has rendered itself significantly more efficient than its predeceasing bulk sequencing methods.

Table 1 below acknowledges and summarizes NGS technologies and provides a thorough context of their abilities. The technologies featured were the most prominently developed platforms during the peak of NGS's popularity. They quite successfully demonstrate the degree to which genomics had evolved by the mid-2000s.

Sequencing technologies that succeeded Sanger sequencing,(i.e. first generation of technology which was also used in the Human Genome Project) are classified according to their read length. Compared to the relatively short read length of NGS of 'second generation' technologies, 'third generation' or NNGS technologies are much longer. Today, Illumina and Ion Torrent represent the majority of NGS short-read technologies. Third-generation sequencing (TGS) is achieved through single-molecule real-time (SMRT) technology by PacificBiosciences and the nanopores technology provided by Oxford NanoporeTechnology.

2.2.1. Advantages of NGS Compared to Bulk Sequencing Methods

Speed and High Throughput: NGS methods consistently surpass Bulk Sequencing methods in effectiveness. One of the most notable abilities of NGS sequencing is its capacity to generate millions of sequences instantaneously. Where genomic sequencing methods might take months or years to sequence a human genome, NGS can do it in a handful of days. Furthermore, modern NGS platforms produce terabases of sequencing data, surpassing bulk methods through the enablement of simultaneous analysis of multiple samples [17].

Affordability: With the emergence of NGS technologies came a notable decrease in the cost of sequencing per base, making NGS more accessible in varying research domains.

Platform	Technology	Key Features	Applications
Illumina (Solexa Sequencing)(2nd gen. NGS)[13]	Sequencing-by-synthesis	High accuracy, short reads (150-300 bp)	Whole genome sequencing, RNA sequencing, epigenetic studies
Ion Torrent (Thermo Fisher)(2nd gen. NGS)[14]	Semiconductor sequencing	Measures changes in pH as nucleotides are incorporated	Targeted sequencing, exome sequencing, amplicon sequencing
Pacific Biosciences (PacBio)(3rd gen. NNGS)[15]	Single-molecule real-time (SMRT) sequencing	Long reads (up to 30 kb), higher error rate	De novo genome assembly, structural variation analysis
Oxford Nanopore Technologies(3rd gen. NNGS)[16]	Nanopore sequencing	Very long reads (up to 2 Mb), portable devices	Real-time sequencing, fieldwork applications, metagenomics

Table 1. *Emerging next gen sequencing technologies.*

What's more, NGS reduces implementation costs through its ability to evaluate multiple samples simultaneously [18].

Versatility: An added benefit of NGS is its application in various fields as it is applicable in domains ranging from transcriptomics to epigenomics. Moreover, NGS technologies

provide great insight into rare cell populations and cellular heterogeneity as they can derive the genomic content of individual cells [19].

2.2.1. Limitations and Challenges of NGS Sequencing Methods

Error Rates: Unfortunately, due to technical limitations regarding sample preparation, bioinformatics analysis, and sequencing platforms, NGS is sometimes susceptible to PCR amplification errors, sequencing bias, etc. As a result, NGS has a high error rate in downstream analysis, thereby comprising the reliability of the data it generates. Although individuals apply quality control strategies and error-correction algorithms to operation processes to improve accuracy, NGS data is still sometimes liable to base-calling errors with combined complexity due to multiplexing and automation [20].

Read Lengths: Compared to traditional Sanger sequencing methods, NGS needs to improve its yields of shorter DNA fragments, resulting in shorter sequence reads. As a result, when reading complex genomic regions, NGS struggles to analyze structural variations and certain insertions, deletions, and rearrangements, making NGS a challenging technology to use when assessing genomes with high heterogeneity levels. Compensating for these discrepancies, researchers now optimize library preparation and use long-read sequencing technology protocol to apply NGS to complex genomes [21].

Data analysis: NGS's tendency to succumb to data analysis challenges results from the sheer size of data accumulated during the implementation of NGS technologies and the specialization of the tools used to evaluate this data. When using NGS methods without a bioinformatics background, this can be quite challenging as the tools used to assess data provide a suitable barrier to entry. With the development of standard benchmarking protocols and analysis pipelines, researchers have made good progress in increasing the accessibility of NGS operation-specific knowledge [22].

The emergence of Sequencing technologies in the past few decades has fundamentally transformed medicine, biotechnology, and biological research. Sequencing technologies have progressed in various domains and have significantly furthered scientific progress. This section will explore history from the beginning of Sequencing technologies through their improvements to where they are today.

2.3. Single-Cell Sequencing Technologies

Appearing for the first time in the late 2000s, single cell sequencing (SCS) began with the development of microfluidic devices enabling the isolation of single cells, a critical function in SCS technologies. This advancement was followed by the invention of Molecular Barcoding, allowing researchers to label RNA and DNA from different cells, thereby verifying SCS lineage tracing. Molecular Barcoding then instigated the development of High-throughput sequencing, an early combination of SCS and NGS technologies, to drastically increase sequencing throughput [23]. Table 2 elaborates on the three most explored SCS technologies being Single-cell RNA Sequencing, Single-cell DNA Sequencing, Single-Cell Epigenomic Sequencing and details their traits. For epigenomic profiling, single-cell assay for transposase-accessible chromatin (ATAC) sequencing (scATAC-seq) has become the most widely used assay to measure chromatin accessibility of single cells to derive transcription binding sites and regulatory elements in an individual cell [26,7].

Sequencing Name	General Description
Single-cell RNA Sequencing (scRNA-seq)[24]	Measures transcriptome in individual cells and generates an overview of gene patterns and cellular functions.
Single-cell DNA Sequencing (scDNA-seq)[6]	Analyzes cellular genomes to detect genetic variations.
Single-Cell Epigenomic Sequencing [25]	Investigates epigenetic modifications.

Table 2. *Types of single cell sequencing technologies.*

2.2.1. Modern Application of SCS Technologies

Cancer Research: SCS traces clonal evolution to reveal tumor heterogeneity, allowing it to evaluate cancer progression and developed resistance. With the information necessary to understand the complexity of advanced tumors and their development and progression, SGS effectively contributes to research toward more effective therapies[27].

Neurology: As SCS can map neural cell development and characterize neuron cell types, the technology offers a deep perspective on brain development and nervous system complexity. SCS sequencing methods have significant potential to uncover the cellular bases of neurological disorders such as Alzheimer's [28].

Developmental Biology: With its ability to sequence

individual cells and generate lineage relationship data, SCS facilitated the generation of maps detailing the formation of organs and tissues. By analyzing Cells in different stages of their development, SCS has also allowed for substantial progress in research on cellular differentiation [29].

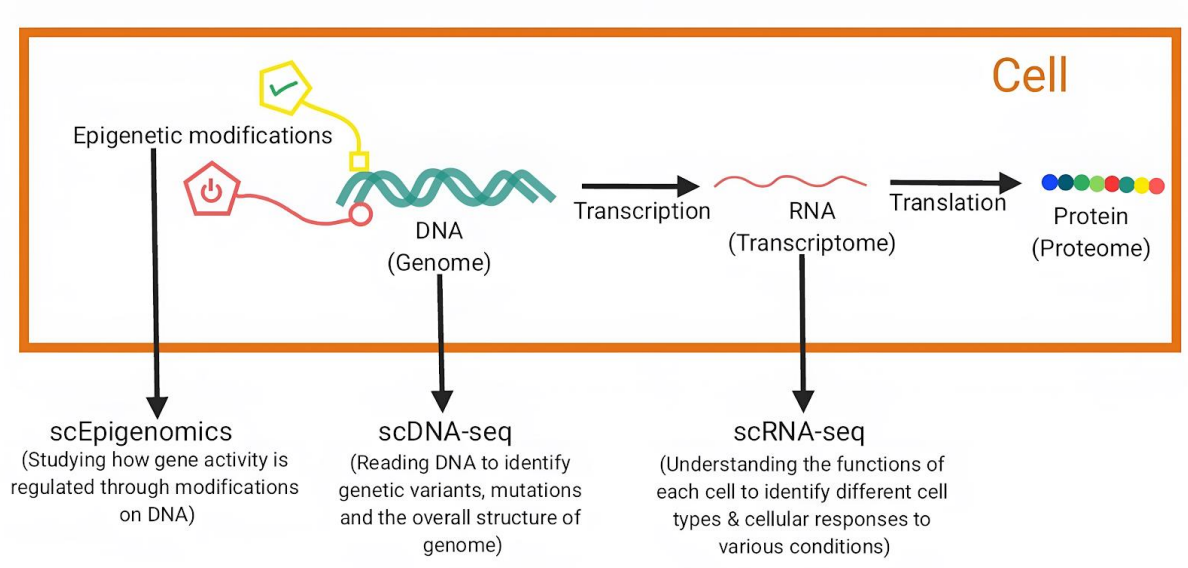

Figure 1 *Based on the manner in which genetic information flows and regulates in a cell, three approaches are adopted for single-cell sequencing,i.e. ScDNA-seq, ScRNA-seq and scEpigenomics. In Sequencing DNA (by scDNA-seq) the aim is to understand the structure of the genome and identify Genetic variants and mutations, while sequencing RNA(by scRNA-seq)helps to understand the function of cells and identify cell type. Profiling Epigenetic modifications(by scEpigenomics)helps to determine which region of DNA can be turned off or on for transcription in response to environmental conditions*.

2.2.1. Challenges in Dissemination of SCS Technologies

Technical Challenges: Although techniques such as LCM (Laser Capture Microdissection) and FACS (Fluorescence-Activated Cell Sorting) avoid cell isolation errors, these error prevention tactics inevitably have significant implementation issues concerning cost and throughput. It is possible that SCS technologies also have variability introduced as a result of data processing batch effects. Furthermore, SCS technologies are also at risk of Low Input Material, where cells don't provide the necessary data to evaluate without biases [30].

Interpretive Challenges: SCS is iconic for single cell sequencing results only sometimes transferring between multiple laboratories. Inconsistencies that highlight sometimes large variability in conditions. The validation of SCS might also be up in the air as the accuracy of cellular heterogeneity is sometimes questionable. Finally, biological insights from SCS data require considerable prior knowledge in biological fields, meaning interpretations can vary starkly.

3. Single Cell Genomics Framework

3.1. Cellular Heterogeneity in Biological Systems

All life forms, unicellular or multicellular, plant or animal, exhibit complexity in functionally organizing themselves, contributing to the overall performance of the whole organism. Humans, just like any other higher life form, consist of a variety of distinct tissues and cell types.Hence, the diversity within an ecosystem of unicellular elements (e.g. a tissue, a colony of volvox, or a tumor) can not be

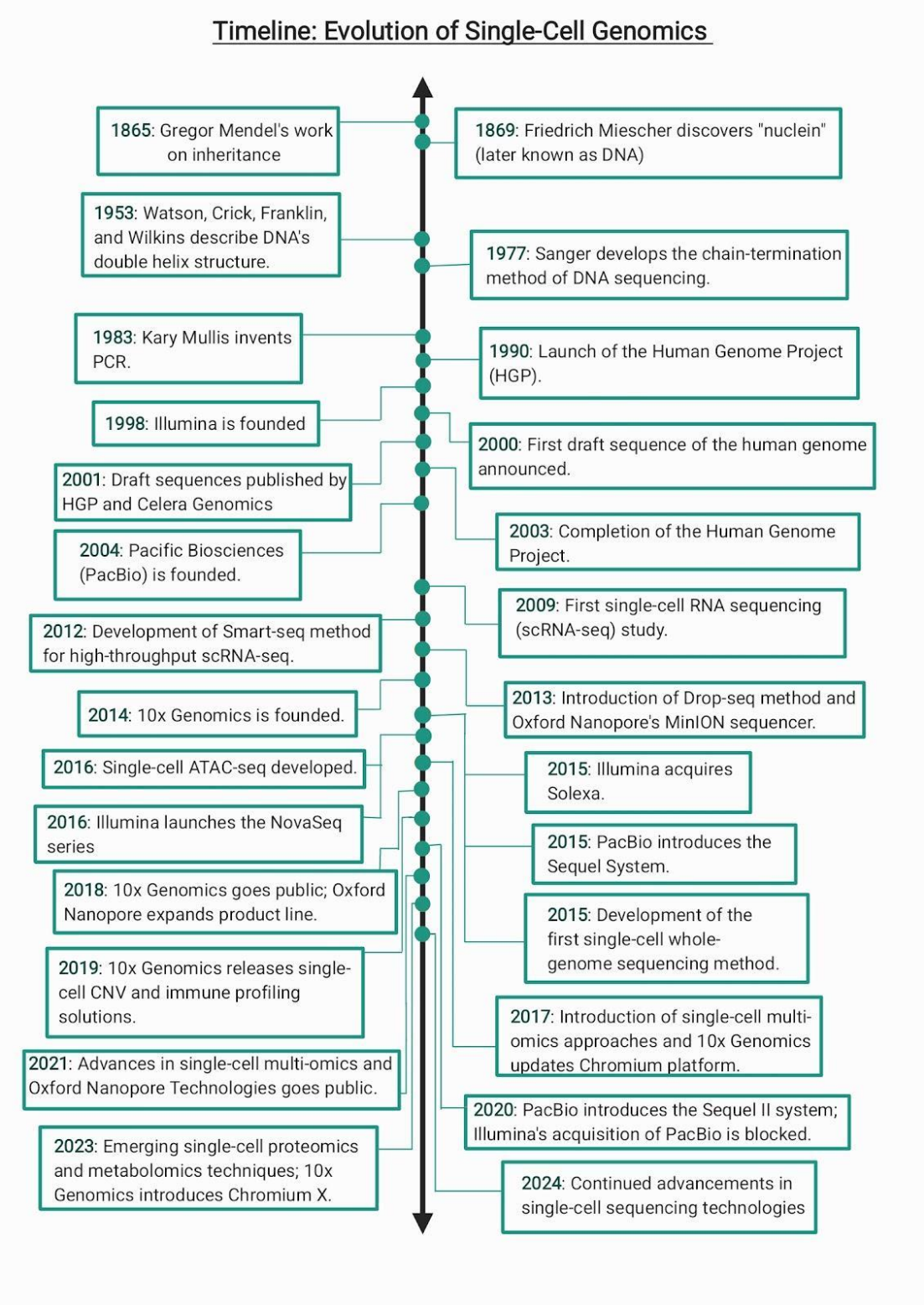

Figure 2 *Timeline Highlighting major breakthroughs in the Development of Single-cell Sequencing Technologies.*

accurately measured by studying mixed groups of cell types, and the Genomes within the cells of an individual multicellular organism are not always the same [31].

By analyzing bulk samples, containing a mixture of heterogeneous tissue, only average measurements of everything present in the sample can be performed, thereby often preventing a correct interpretation of results (especially because some of the cell types build the majority of the tissue whereas others

are present only temporarily in small amounts) [32]. Hence, using bulk sequencing methods, averages signals over thousands or millions of cells, potentially masking the heterogeneity that defines complex biological systems.

To tackle this challenge, single-cell sequencing (SCS), based on next-generation sequencing, has progressed in recent years. SCS aims to investigate cellular heterogeneity, by studying a single DNA molecule from individually isolated cells, thereby providing individual details to each cell type instead of providing broad population-based estimates [31].

Isolation methods	Description	Key Features	Common usage
Manual Cell Selection [34]	Manual manipulation to isolate single cells from suspension using techniques like serial dilution, micropipetting, microwell dilution, and optical dilution	Requires manual effort; suitable for small-scale applications	Isolation of single cells from suspension
Random Seeding/Dilution [34]	Isolating cells by randomly seeding or diluting cell suspensions	Simple and cost-effective	Basic cell isolation
Fluorescence-Activated Cell Sorting (FACS) [34] [33] [31]	Uses fluorescent markers and flow cytometry to sort and isolate single cells	Highly precise; allows isolation of live cells and cell nuclei; advantageous for frozen library samples	Most commonly used for live cell isolation from tissues or cultures
Microfluidic/Microplate Methodology [34]	Automated methods using droplets or micromechanical valves in microfluidic devices	Enables high-throughput and precise cell manipulation; can be automated	High-throughput single-cell isolation
Micromanipulation [34] [31]	Uses mechanical tools to collect live cells from tissues that have been dissociated or from in vitro cultures	Allows collection of live cells	Live cell collection from tissues or cultures
Laser Capture Microdissection (LCM) [34] [31]	Captures cells from tissue sections using a laser; typically results in cell death due to tissue fixation	Suitable for fixed tissue samples; often results in cell death	Cell isolation from fixed tissue samples

Table 3. *Cell isolation methods.*

SCS has revolutionized the ability to interrogate the transcriptional, genomics, and epigenomic characteristics of thousands of cell types in depth.

3.2. Isolation to Data Analysis

The first step in SCG is Isolation of individual cells from primary samples. Several single-cell isolation methods have been developed including manual cell selection, random seeding/dilution, laser microdissection, (FACS), and microfluidic/microplate methodology [33]. Confirming the isolation of single cells is crucial, as this step ensures the accuracy of subsequent analyses. Mistakes like analyzing empty chambers or those with multiple cells can lead to misleading results. This confirmation is typically achieved by obtaining microscopy data for each chamber or well, verifying the presence of

only one cell [31].

Following isolation, the next step involves lysing the single cells to extract genomic DNA (gDNA). The lysis method chosen must strike a balance between being harsh enough to effectively lyse cells and gentle enough to maintain the integrity of the gDNA. Depending on the cell type, lysis can be performed using physical techniques such as sonication and freeze/thaw cycles, chemical methods involving strong bases followed by neutralization, or enzymatic lysis using agents like lysozyme or proteinase K [33].

Next-generation sequencing technologies require micrograms of DNA, so amplification is a de facto prerequisite to sequence cells that typically contain femtograms of DNA [33]. WGA aims to amplify the DNA accurately and uniformly, preserving the original genetic information as much as possible. The goal of WGA is to increase the amount of DNA available for analysis while trying to minimize errors and distortions that can occur during the amplification process. These potential errors include: (a) Amplification Bias: Uneven amplification of different regions of the genome, which can lead to some parts being overrepresented and others underrepresented in the final amplified product. (b) Genome Loss: Loss of parts of the genome during the amplification process, which can result in incomplete genome coverage. (c) Mutations: Introduction of errors or changes in the DNA sequence not present in the original genome. (d) Chimeras: Formation of artificial DNA sequences that combine segments from different regions of the genome, which can create misleading results.

MDA (Multiple Displacement Amplification) is proven to be the best method for WGA (when compared with pure PCR amplification), but it presents limitations of uneven genome coverage, genomic rearrangements due to chimeric sequences, and non-specific amplification [35]. SCS technologies are effective in examining the genetic and epigenetic features of individual cells, uncovering cellular diversity in various biological settings such as cancer, development, and immunology. ScDNA-seq enables the examination of the genomic DNA of each single cell. This method consists of separating individual cells, increasing the amount of their genomic DNA, and sequencing it to detect mutations, copy number variations, and other genomic characteristics. This is especially beneficial for comprehending genetic variation in tumors and monitoring clonal evolution [7]. ScRNA-seq examines the transcriptome of single cells, offering information on gene expression patterns. This method consists of separating individual cells, formation of cDNA from RNA, and analyzing the cDNA. scRNA-seq is commonly employed to discover cell types, conditions, and roles, especially in intricate tissues and during growth stages [36] [37].

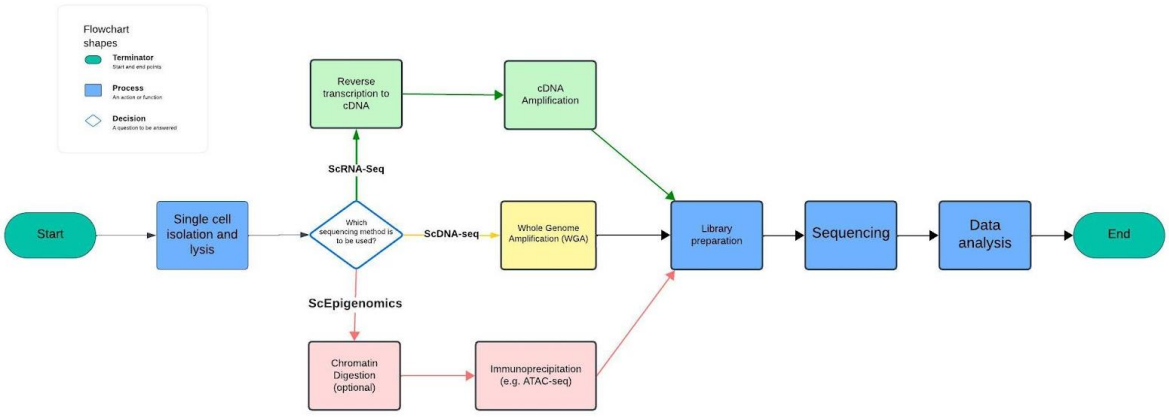

Figure 3 *Workflow comparison of scRNA-seq, scDNA-seq and scEpigenomics.*

Various techniques are used to analyze the epigenetic makeup of individual cells in the field of single-cell epigenomics. Methods such as scATAC-seq and single-cell methylome sequencing offer information on chromatin accessibility and DNA methylation patterns. These techniques aid in the comprehension of how epigenetic alterations control gene expression and play a role in cellular identity and function [37] [7].

Both second-generation sequencing and third-generation sequencing platforms are used in single-cell sequencing, each with its strengths and limitations. Single-cell sequencing is favored for its accuracy and high throughput, making it ideal for large-scale single-cell RNA sequencing projects. Third-generation sequencing platforms, such as those from Pacific Biosciences (PacBio) and Oxford Nanopore Technologies (ONT), with long-read capability, are better suited for applications requiring detailed structural information and full-length transcript analysis. The choice between these technologies depends on the specific requirements of the research project, such as the need for read length, accuracy, throughput, and cost considerations [38].

Collectively, these tools and platforms empower researchers to analyze the intricate nature of biological systems at a cellular level, leading to breakthroughs in areas like cancer

biology and immunology. After sequencing, the data is processed and analyzed to extract meaningful information. This includes identifying gene expression patterns (After sequencing, reads are aligned to a reference genome, and expression levels are quantified. Techniques such as expression quantitative trait loci or eQTL mapping help in understanding how genetic variants influence gene expression across different conditions and tissues), genetic variants (Genotyping from sequencing data involves identifying single nucleotide polymorphisms (SNPs) and other genetic variants. Tools like the Genome Analysis Toolkit (GATK) are used for variant calling, and the results can be imputed to improve accuracy. This process helps in detecting variations that may contribute to diseases or different phenotypes) [39], and epigenetic modifications (e.g. for understanding developmental processes and disease mechanisms, such as in cancer, where specific epigenetic changes can differentiate between normal and cancerous cells) [40] [41]. This step helps to understand how genes are turned on or off, what genetic changes are present, and how these changes affect the cells.

3.2. Commercialization Techniques

Since the development of the first single-cell RNA-sequencing(in 2009) and single-cell DNA sequencing (in 2011) methods,the field of single cell genomics has progressed rapidly. Till 2016, three generations of sequencing technologies of various types have been developed. A major shift observed in the last decade is the transfer of technologies from few sophisticated laboratories into hands of cancer research groups around the world. This democratization of SCS methods has been facilitated both by open sharing of protocols as well as by commercialization of technologies by various companies [7, 36, 42].

Some popular second-generation sequencing platforms were developed and commercialized by Roche Life Sciences, Thermo Fisher Scientific, Illumina, BD biosciences, and 10X Genomics. Overall, Illumina emerges to be the champion player in SCS utilizing NGS, 10x Genomics, and BD Rapsody are also widely used [7, 42].

4. Commercialization

The emergence of NGS technologies has significantly hastened genomic research, facilitating the rapid sequencing of DNA and RNA in large volumes. Illumina, Ion Torrent, Oxford Nanopore Technologies (ONT), and PacBio are prominent commercial platforms with unique mechanisms and applications.

4.1. First Generation

The first genomes sequenced by Sanger were about 5374 bp in since, and by 1980 a genome with a size of about 4851 bp were sequenced. But it took years of improvement for Applied Biosystems to be the first company to automate Sanger sequencing. This eventually added to the groundbreaking decoding of the first Human Genome. However, the technology was extremely expensive, thus remaining confined to sophisticated laboratories. First generation sequencing technologies were the most common sequencing technologies used by biologists until Roche's 454 technology, based on sequencing by synthesis approach,involving pyrosequencing were introduced and commercialized in 2005 as higher throughput and lower cost that its predecessors [43]. This event brought about a new era of High-throughput sequencing technologies or Next Generation sequencing technologies, opening new ways for genome exploration and analysis.

4.2. Second Generation

Following, sequencing by synthesis method of Roche's 454, and by improving it, The Solexa company introduced and improvised sequencing platform. However, Illumina acquired solexa and started to commercialize the Genome Analyser. Although it produced short reads but it was overshadowed by its ability to produce paired end, to sequence both ends of DNA cluster [43].

As of now, Illumina has a series of sequencing platforms with varying read lengths and throughput , but notably Illumina sequencers can generate gigabases (Gb) of data in a single run, with the HiSeq X Ten system reaching up to 1.8 Tb per run. Moreover, The cost for equipment utilizing Illumina technology typically ranges from US$20,000 [44]. Yet, Cost per gigabase is comparatively low, making it ideal for extensive genomic projects. Illumina's popularity is also due to its easy-to-use interface , ability to handle large amounts of data efficiently and widespread availability in research institutions.

However, Illumina technology presents a drawback in high requirements of sample loading control as overloading can result in overlapping of clusters and poor reading quality. The error rates are impressively low about 1% and mainly due to substitutions [43]. Overall, due its high throughput systems and cost-effectiveness, Illumina has dominated the commercial landscape of NGS Technologies in recent decades.

After Illumina, Life Technologies followed the footsteps of 454 pyrosequencing technologies by involving the detection of Hydrogen Ion released during sequencing, differentiating itself from pre-existing second generation technologies that relied on detecting fluorescent labeled nucleotides. Hence, the Ion Torrent semiconductor-based sequencing technologies appeared in the market in 2010.

IIon Torrent technology provides fast and cost-effective sequencing and is capable of producing read lengths of 200 bp,400bp and 600bp. For instance, the Ion proton sequencer can generate up to 10 gigabytes per run. Although it has lower

throughput compared to Illumina, Ion Torrent's shorter run times of about 2 to 8 hours and cost-effectiveness in smaller projects make it valuable in clinical and diagnostic contexts. However, its susceptibility to homopolymer errors(~1%) with insertions and deletions and shorter read lengths restrict its utility in certain research domains [44].

4.3. Third Generation

Although second generation sequencing technologies revolutionized the analysis of DNA, they required PCR amplification, which is a time and cost consuming procedure. To counter this challenge The third generation of sequencing technologies emerged that offered lower cost, easy sample preparation and higher resolution without any need of PCR amplification. Companies like Pacific Biosciences and Oxford Nanopore adopted Single Molecule Real-Time sequencing approach (SMRT) and Companies like 10X Genomics adopted synthetic approaches that rely on existing short read technologies. As of now, SMRT based platforms are in wide use.

PacBio's SMRT sequencing offers several advantages, firstly It gives long reads, averaging at 10 Kbp and impressively reaching a higher limit of 60kbp crucial for understanding intricate genomic regions and sequencing entire genes. Secondly, it takes about 4-6 hours for a run. With the Sequel II system capable of producing up to 100 gigabases (Gb) per run, PacBio stands out. While its cost per gigabase is higher compared to Illumina, its exceptional capability to generate long reads is indispensable for tasks such as de novo genome assembly and studying structural variations. However, PacBio's accessibility is moderate, as it involves higher operational costs and technical complexity.Moreover its error rate of about 13% is dominated by deletions and insertions and randomly distributed along the long read [44].

The Oxford nanopore sequencing (ONT) utilizes nanopore technology, which reads DNA by monitoring electrical changes as nucleotides move through a nanopore. In 2014 ONT released a device named MinION promising to generate longer reads and a better resolution. This mobile device measuring four inches in length and connected by USB type 3.0 port to a laptop computer ONT stands out for its ability to generate exceptionally long reads, surpassing 150 kilobases (kb). MinION can produce terabytes of data, with the latter tailored for high-throughput studies. The portability, low cost and real-time data collection (i.e. data is displayed on screen and generated without waiting for run to complete), and minimal sample preparation requirements of ONT make it well-suited for field and on-site applications. However, its higher error rates of about 12%(~3% mismatches,~4% insertions and~5% deletions) and lower throughput compared to Illumina pose ongoing challenges [44, 45]. ONT has then released PromethION which competes with PacBioRSII Sequencer in terms of read length and with Illumina's HiSeq in terms of cost [43]. Considering, the fact that ONT technologies are relatively new, yet they have competed with the well-established predecessors, and the veritality ONT, this technology has the potential to emerge as leader of sequencing technologies, dethroned the current Illumina based sequencing approaches if it adopts and integrates well with the emerging spatial and multi-omic approaches [46].

4.4. Comparative Analysis

Sequencing technologies have evolved to cater to diverse research and clinical needs. Illumina's high throughput and cost efficiency make it ideal for large-scale projects, while Ion Torrent excels in rapid and cost-effective clinical diagnostics. ONT's portability and real-time sequencing are unmatched for field applications, whereas PacBio's long-read capabilities are crucial for complex genomic analyses. The second generation sequencing platforms are both the pioneers and current leaders to produce high throughput with low error rates.However, Second generation sequencing tools like Illumina and Ion Torrent, with their brief reads, face challenges in piecing together intricate genomes and detecting structural variations due to limited read lengths. On the other hand, Third generation sequencing platforms such as PacBio's SMRT and ONT generate significantly longer reads, up to multiple megabases. PacBio captures single DNA molecules, while ONT directly sequences single-stranded DNA using nanopore technology. Despite their higher error rates and lower throughput compared to NGS platforms, PacBio and ONT offer advantages in capturing long sequences. To overcome limitations, hybrid sequencing approaches have emerged, combining both technologies' strengths to improve accuracy and mappability [47].

Hybrid sequencing methods, leveraging both PacBio and

Platform	Throughput (Gb per run)	Cost	Accessibility	Merits	Demerits
Illumina	Up to 1.8 Tb (HiSeq X Ten)	Equipment: ~$20,000; Low cost per gigabase	Widely available, user-friendly, efficient data handling	High throughput, cost-effective for large projects, easy-to-use, low error rate (~1%)	Requires careful sample loading to avoid cluster overlapping, errors mainly due to substitutions

Ion Torrent	Up to 10 Gb (Ion Proton)	Cost-effective for smaller projects	Valuable in clinical and diagnostic contexts	Fast sequencing, shorter run times (2-8 hours), cost-effective for specific applications	Lower throughput than Illumina, homopolymer errors (~1%), shorter read lengths
PacBio	Up to 100 Gb (Sequel II)	Higher operational costs; Higher cost per gigabase	Limited to specialized labs in well defined research institutions due to higher technical complexity	Long reads (average 10 Kbp, up to 60 Kbp), crucial for de novo genome assembly and structural variation studies	Higher error rate (~13%), dominated by deletions and insertions
Oxford Nanopore (ONT)	Terabases of data (MinION)	Low cost; Competitive with Illumina's HiSeq in some models	Well-suited for field and on-site applications	Exceptionally long reads (surpassing 150 kb), portable, minimal sample preparation, real-time data collection	Higher error rates (~12%, with mismatches, insertions, deletions), lower throughput compared to Illumina

Table 4. *Comparison of various NGS platforms in the commercial landscape.*

ONT data have proven especially useful for transcriptome research, enhancing sensitivity and accuracy. By integrating the strengths of long-read sequencing with the efficiency of short-read technologies, hybrid-Seq approaches offer comprehensive solutions for transcriptome studies (Table 4).

5. Precision Medicine

Single cell is the ultimate unit of life activity, in which genetic mechanisms and the cellular environment interplay with each other and shape the formation and function of such complex structures as tissues and organs. Dissecting the composition and characterizing the interaction, dynamics, and function at the single-cell resolution is crucial for fully understanding the biology of almost all life phenomena, under both normal and diseased conditions [4]. Precision medicine goes in-depth and identifies aspects of the cell to make treatment more personalized.

The term precision medicine has grown popular in recent years, it is labeled as the term personalized medicine as it focuses on personalized treatment strategies targeting the needs of individual patients based on numerous factors such as genetics, biomarkers, proteins, environment, and psychosocial characteristics [48]. Single-cell RNA sequencing and single-cell genomics have created an entirely new paradigm to study human tissues at a very small scale and investigate each cell at a time. It has been used in various fields to improve our understanding and contribute to multiple discoveries such as, in the areas of embryonic medicine, immunology and immunotherapy, tumor ecosystems, and oncogenic processes.

Single-cell sequencing is widely known to predict alterations in transcriptomic, genomic, epigenomic, and proteomic levels in healthy versus malignant cells. Numerous reports have been released presenting evidence, for instance, single-cell profiling of tumor heterogeneity and the microenvironment in advanced non-small cell lung cancer [49].

5.1. scRNA-seq on Tumor Microenvironments

Infected cells can manifest resistance to various therapeutic drugs through cellular heterogeneity and plasticity. This infection is viewed as a ‘tumor ecosystem’, a community in which tumor cells coexist with other cells containing tumors or host cells in their microenvironment, and can also evolve and adapt according to the changing conditions [4]. Single-cell RNA sequencing (scRNA-seq) helps us understand tumor microenvironments at a larger scale by focusing on every individual tumor cell, highlighting their biomarkers and genome. This plays a significant role as it provides a lot of information regarding the tumor microenvironment. On testing, it was discovered that tumors with differing anatomical origins portray distinct T cell properties. In scRNA-seq, studies of T cells from patients with hepatocellular carcinoma, non-small cell lung cancer, and colorectal cancer that have numerous autologous control tissues available, similar T cell compositions are observed in peripheral blood of all three cancer types [50].

Recently, scRNA-seq made it feasible to unveil the complex heterogeneity in the PDAC microenvironment with unprecedented resolution [51, 52, 53]. The scRNA-seq can be utilized to determine biomarkers based on genome-wide expression data, indicating the developmental trajectories of specific cells by pseudotime analysis. It is also used to identify gene-coregulated networks. For instance, scRNA-seq

revealed distinct immune subsets with different states in the breast tumor microenvironment, which promoted the understanding that immune cell subsets play different roles in promoting and opposing tumor progression.

5.2. Metastasis research and single-cell sequencing

Metastasis is the spread of cancer cells from the place where they originate to other parts of the body. In metastasis, cancer cells break the first primary tumor and usually travel from the lymph system (sometimes, they travel through the blood) and form new tumors in other sections or organs of the body. Metastasis research gives us a better understanding of metastasis and the reasons behind its occurrence. Numerous factors affect the growth, development, and occurrence of metastasis cancer, including genetic aspects and in vivo microenvironment. Single-cell sequencing (SCS) can be used to examine the relationship between tumor metastasis and tumor heterogeneity, tumor drug resistance, and tumor microenvironment, to propose a new cure to treat tumor metastasis [54]. SCS can play a substantial role in the prediction and monitoring of tumor metastasis. Currently, knowledge on tumor metastasis is limited and many questions remain unanswered, for instance, which cell subtypes or clones in primary tumors can spread, how many times tumors can metastasize to different organs at what speed, and can tumor metastasis be tracked [55]. With increasing and unwavering progress in SCC, it has become more feasible to answer these questions.

SCS identifies when the tumor will metastasize by identifying biomarkers present in our body, for example, an SCS study of 190 distinct metastasis breast cancer tissues. SCS of tumors at different times of its growth can help predict mutations during tumor progression. This was seen in a method by Davis et al to monitor global transcriptome changes of metastatic cells in the process of breast cancer metastasis [56].

6. Clinical Barriers & Challenges

SCS is a sequencing method that allows for the analysis of genetic and transcriptomic data at a singular cell level, allowing the technology to offer invaluable perspective in cellular heterogeneity research. Holding great promise for clinical application through potential insight into rare cell populations and disease mechanisms, SCS's role in medicine is pivotal in advancing the scientific community's research into personal medicine tailored to patients' genomes [57].

Stemming from the potential of SCS technology in developing early disease detection strategies, evaluating responses to treatment, and monitoring disease progression, there is a growing interest in integrating such methods into clinical practice. As sequencing methods evolve, they will most likely be responsible for large progression in personalized healthcare [58]. Although there are many revolutionary potential applications of SCS, the path toward clinical implementation is fraught with multiple barriers to entry. Through data analysis struggles, technical complexity in the sequencing process, and regulatory hurdles, research is still considerably impeached by its inability to harness the full potential of SCS technologies [59].

Technical Complexity: Although crucial to ensure the integrity of cells undergoing SCS sequencing, isolating these cells from other organic matter can be extremely time-consuming and technically challenging. As a result of technical errors and variations, SCS results may appear inconsistent, thereby not accurately registering the genetic information of those cells.

Data Analysis and Interpretation: Due to a lack of standardization in research protocols, there is a range of variability between different studies, hampering the validity of many claims. Furthermore, as SCS generates vast amounts of data only interpretable using specialized tools, processing information for studies becomes unbelievably complicated [60].

Cost and Resource Intensity: SCS technologies require significantly specialized facilities and expertise, something not available in all clinical settings. SCS also has a high cost of single-cell sequencing equipment, computational resources, and reagents. As a result, implementing SCS in research requires extensive funding and space, making it sometimes inaccessible [57].

Regulatory and Ethical Considerations: Although helpful in treatment and diagnosis, SCS's access to personal genetic information sparks debate over patient confidentiality and ethical concerns about patient consent and data security. Moreover, obtaining permission to use SCS is notoriously difficult and time-consuming, and then maintaining regulation standards creates a whole new layer of difficulty in an already aggravating situation [61].

High Throughput and Sensitivity: A significant challenge of SCS technologies is their lack of enhanced sensitivity towards subtle genetic variation. Low-abundance molecules not being detected in cells poses an issue as it prevents the technology from locating rare transcripts. Furthermore, SCS is required to process large quantities of data simultaneously while maintaining a high sensitivity to prevent compromising the data quality [62].

Technical Variability and Reproducibility: Unfortunately, batch effects are a somewhat regular occurrence as variations in sampling are inevitable. However, it does compromise the reliability of the analysis. It has become apparent that developing rigorous protocols to standardize clinical experiences with SCS is necessary as it prevents technical variations during library preparation and sequencing [63].

Data Processing and Analysis: Today, there is still a great need for bioinformatic tools that successfully support and

interpret SCS technologies. There is great demand for algorithms capable of trajectory inference, cell type identification, and differential expression analysis, as these domains are currently lacking. There is also a great need to develop computational infrastructure and algorithms to manage the massive data sets generated by SCS sequencing methods [64].

Integration of Multi-Omics Data: For holistic insight and seamless integration from SCS programs, it is essential to ensure compatibility between different omics data sets. Moreover, developing integrated multi-omics approaches to interpret proteomic data is necessary if research is to develop a deeper understanding of cellular states and functions [65].

Cost Reduction: Moving towards the facilitation of SCS sequencing in both clinical and research settings, developing cost-effective methods for cell isolation, library preparation, and sequencing might facilitate its broader use. For widespread adoption, there must be a price reduction in the cost of SCS, including reagents, consumables, and computational resources. What is needed most is innovations that don't compromise data quality but lower expenses.

Within the past decade, the field of single-cell genomics (SCG) has rapidly evolved with the introduction of the first single-cell DNA sequencing (scDNA-seq) and RNA sequencing (scRNA-seq) methods [36]. Initial methods for analyzing individual cells were limited in capacity, time-consuming, and costly, limiting their use in sophisticated research projects, but they laid the groundwork for future advancements. The advent of high-throughput sequencing technologies, particularly next-generation sequencing (NGS), has revolutionized this field by facilitating the simultaneous analysis of numerous individual cells and enabling the identification of distinct cell groups and differences at unprecedented levels [66].

Innovative solutions from Illumina, 10x Genomics, Pacific Biosciences, and Oxford Nanopore Technologies have democratized single-cell genomics (SCG) by developing accessible genomics platforms and openly sharing protocols. Although early SCS technologies were extremely costly, advances such as Illumina's sequencing by synthesis (SBS) method and portable tools like Oxford Nanopore's MinION have significantly reduced costs, making high-throughput sequencing more accessible to a wider range of laboratories.

SCG has now shifted from few sophisticated labs to leading scientific research, promoting innovation and exploration in studying biological systems at the single-cell level, such as advancements, innovation and collaboration in cancer research.

Single cell genomics has played a significant role in providing a deep understanding of tumor microenvironment, tumor metastasis, and therapeutic resistance. With the examination of individual tumor cells, identification of unique subgroups within tumors has elucidated their specific genetic and transcriptomic characteristics and uncovered mechanisms behind drug resistance. Techniques like scRNA-seq have identified various immune cell types and their communication with cancer cells, shedding light on immune evasion strategies and potential therapeutic targets. Furthermore, SCS has significantly advanced our understanding of the genetic and epigenetic changes driving cancer metastasis by identifying critical biomarkers and mutations associated with cancer spread, thus influencing the development of targeted therapies. Addressing therapeutic resistance remains a major challenge in cancer treatment; however, SCS has illuminated the cellular mechanisms behind resistance by uncovering resistance pathways and informing strategies to overcome them through the analysis of genetic and transcriptomic alterations in cancer cells before and after treatment.

The potential for advancing precision medicine and cancer treatment is significant with the future integration of SCS into clinical settings. Anticipated enhancements in efficiency, availability, and expenses are set to boost the utilization of SCS in medical settings, facilitating the creation of customized treatment strategies based on the genetic and transcriptomic characteristics of patients' tumors. Advancements in SCS technologies will enhance our knowledge of cellular heterogeneity and disease mechanisms by improving sensitivity and accuracy. Combining genomic, transcriptomic, and epigenomic data through multi-omics integration will offer extensive knowledge on cellular functions and disease advancement, ultimately aiding in the creation of improved treatments.

7. Conclusion

With the introduction of Single Cell Sequencing in the 1970s, Sequencing technologies have undergone remarkable evolution leading to the development of three generations of sequencing technologies by 2016 [42, 67]. Along with this, the incorporation of SCS methods, and advancements in sequencing technologies have revolutionized medicine, biotechnology, and biological research by enabling personalized medicine and in-depth analysis of cellular heterogeneity. Commercial platforms like Illumina, Ion Torrent, and Oxford Nanopore Technologies offer high accuracy, throughput, and versatility, while SCS methods allow for the analysis of individual cells, providing detailed insights into cellular diversity and interactions. This democratization of sequencing technologies, facilitated by the open sharing of protocols and commercialization efforts by various companies, has led to their widespread adoption by research groups worldwide [7]. However, despite their immense potential, sequencing technologies face challenges such as high error rates, data analysis complexities, and cost considerations. Efforts to address these challenges, including improving library preparation, enhancing data analysis techniques, and reducing costs, are crucial for the broader distribution and application of sequencing technologies in

various fields, including their clinical applications. This study focused on the evolution of major commercial sequencing platforms in the last decade from throughput, cost and accessibility analysis, but there is a need to delve deeper and consider technologies that have emerged in the 2020s, e.g. collaboration of 10x Genomics with ONT and PacBio aiming the ubiquitous adoption of Single-cell and Spatial Full-length Isoform Transcript Sequencing across research laboratories, [46] which will enable the researcher to look beyond what has been limited by current Illumina based sequencing methods and will be helpful in further advancing the third generation sequencing approaches. For now, albeit the efforts to overcome technical, analytical, and cost-related challenges have greatly improved sequencing technologies, it is essential to fully harness the potential of sequencing technologies for wider implementation in clinical setups. But as sequencing technologies continue to advance in the 2020s, they hold promise for further enhancing our understanding of complex biological systems and transforming healthcare through precision medicine tailored to individual genomes.

Conflicts of Interest

The authors declare no competing financial interests or conflicts of interest.

Author Contributions

K.G.B., K.D., S.V. conceptualization. I.K., T.G., E.R., K.G.B., K.D., S.V. writing original draft, methodology, writing review & editing.

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