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Single-cell sequencing: the challenges and opportunities

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Recent technological advancements have made single-cell sequencing an increasingly powerful tool for understanding biology and cellular function, disease diagnosis, therapy response prediction, and treatment selection. Historically, sequencing technology only enabled an average analysis of a total cell population. In contrast, today tens of thousands of individual cells from a single tissue sample or patient can be analyzed, giving researchers an unprecedented opportunity to understand individual cell populations and their behavior in diseased tissue.

These opportunities are underpinned by the substantial developments in next generation sequencing (NGS) technologies in parallel with the decreasing cost to conduct these studies. This white paper describes the advances in single-cell sequencing methodologies, their workflows and applications, incorporation of GMP-compliance into single-cell workflows, and clarifies the important role of this technology within many research fields.

The emergence of single-cell sequencing

Named the 2013 "*Nature Methods*" technology of the year, single-cell sequencing has the power to characterize the genome and transcriptome of individual cells (1). Conventional sequencing approaches utilize many cells, and lack the sensitivity to analyze individual or a small number of cells. As a result, these sequencing modalities lose the heterogeneity information across the total population. In contrast, single-cell sequencing methodologies have the advantage of measuring individual cellular heterogeneity (2) and can distinguish a small sub-population of cells within a larger overall population.

The high cost and technical challenges of this technology initially restricted single-cell sequencing to just a few laboratories. Today, single-cell genome and transcriptome sequencing is more robust and broadly accessible throughout the research community. Figure 1 shows the overall single-cell workflow process.

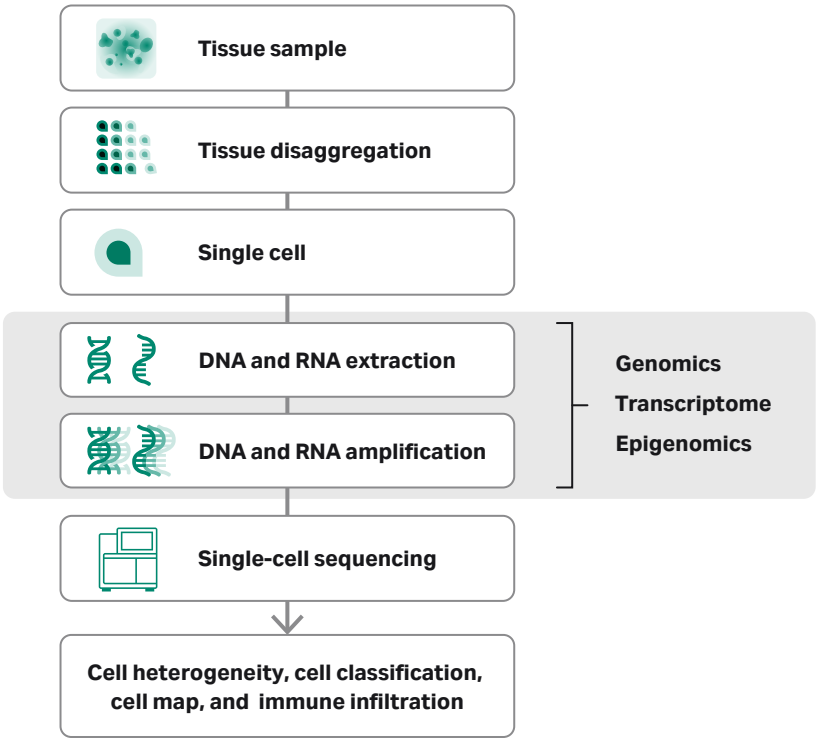

Fig 1. Overview of the single-cell sequencing workflow, including collection, isolation, amplification sequencing and data analysis.

One of the reasons for this increase in accessibility is the advent of new sequencing technologies and reagents that have considerably reduced the costs for single-cell sequencing. These improvements and savings have enabled the technology to be embraced by many fields, in particular oncology. For cancer biologists, the methodology has become intrinsically linked to fresh tissue biopsy and liquid biopsy processing to identify therapy resistant sub-populations, understand metastasis, and study tumor heterogeneity.

Development of single-cell sequencing methods

At present, there is no standardized single-cell sequencing method and this means that researchers need to choose from a range of options. In general, microfluidic-based approaches are the most popular, some of which are highlighted below.

Drop-seq

Drop-seq pioneered the microfluidic methodology for single-cell studies. This approach analyzes gene expression in individual cells by encapsulating and separating cells in tiny droplets. It demonstrated how large-scale single-cell analysis could be applied to complex tissues and whole cell populations in a cost effective manner (3).

High-throughput and low-deviation single-cell sequencing (SiC-seq)

Like Drop-seq, **SiC-seq** uses droplet microfluidics to separate, amplify, and barcode the genome of a single cell. This approach supports broader genomic studies for different cell populations (4). The microfluidic process pairs each genome with a unique single-cell oligonucleotide barcode, supporting the sequencing of up to 50 000 single cells.

Single-cell combinatorial marker (SCI-seq)

SCI-seq can simultaneously construct thousands of single-cell libraries and detect variations in somatic cell copy number (5). This approach increases the number of cells that can be detected individually and substantially lowers the cost of library construction.

Linear amplification via transposon insertion (LIANTI)

LIANTI can detect copy number variation (CNV) at kilobase resolution, identifying mutations in many tissue samples, especially biopsy samples (6). This technique has enabled the direct observation of DNA replication origins, which differ between individual cells under a range of conditions, for example, after gamma irradiation exposure.

Single-cell COOL-seq (scCOOL)

scCOOL-seq enables the simultaneous analysis of single-cell chromatin state, CNV, DNA methylation, and ploidy. These indicate different functions and patterns of chromatin state and gene expression in single cells (7). scCOOL-seq can analyze chromatin, nucleosome positioning, DNA methylation, CNV, and ploidy simultaneously in single cells.

Topographic single cell sequencing (TSCS)

TSCS reveals accurate spatial information for each cell, preserving their spatial context in tissue sections. This enables researchers to spatially map the specific characteristics of individual tumor cells in tissues, revealing clonal expansion, tumor cell invasion, and metastasis (8).

The challenge with these microfluidic approaches is to be able to identify the cell from which each mRNA transcript or genome originated. To meet this challenge, these microfluidic methods employ a molecular barcoding strategy to remember the cell-of-origin. This breakthrough has resulted in microfluidic approaches being widely embraced and adapted throughout the scientific community for many single-cell studies.

More recently, a **Microwell-seq** methodology has been developed as a high-throughput, low-cost scRNA-seq platform (9). Microwell-seq improves and reduces the detection costs compared to the coated oil droplets-based methods. Similarly, **SPLit-seq** technology, applies the same technology but with low-cost combined barcodes. These reduce the cost of single-cell transcriptome sequencing further still (10).

Each of these modalities has made single-cell sequencing studies markedly more accessible and cost efficient, opening the technology up to many researchers and fields.

The importance of single-cell sequencing in genomics

Single-cell DNA sequencing in oncology

Single-cell sequencing has made a remarkable impact in oncology, with many recent high-impact studies. Whole-genome and exome single-cell sequencing has been used to measure cells undergoing division in estrogen-receptor positive breast tumor tissue. This study revealed that aneuploid rearrangements, where the cell chromosome number deviates from a multiple of the haploid set, remained highly stable as the breast tumor clonally expanded (11). In contrast, point mutations evolved gradually, generating extensive clonal diversity at low frequencies. One limitation within this study is that many cancer cells are aneuploid, presenting an abnormal number of chromosomes (Fig 2). Consequently, this methodology might be restricted to cancers that do not exhibit aneuploidy.

In childhood lymphoblastic leukemia patients, single-cell capture and whole genome analysis revealed that there were different co-dominant clones in patients. Researchers describe important insights into both disease malignancy and treatment resistance (12). This provided an unprecedented view of the genetic events that resulted in the development of each patient's unique malignancy, and highlighted novel mechanisms for therapy resistance.

Single-cell DNA sequencing of colon cancer cells was applied to map the T-cell immunoreceptor, revealing tissue distribution, tumor heterogeneity, and expression variation in patients (13). This technology provided insights into the dynamic relationships of T cells in, and the application of immunotherapies for, these cancers.

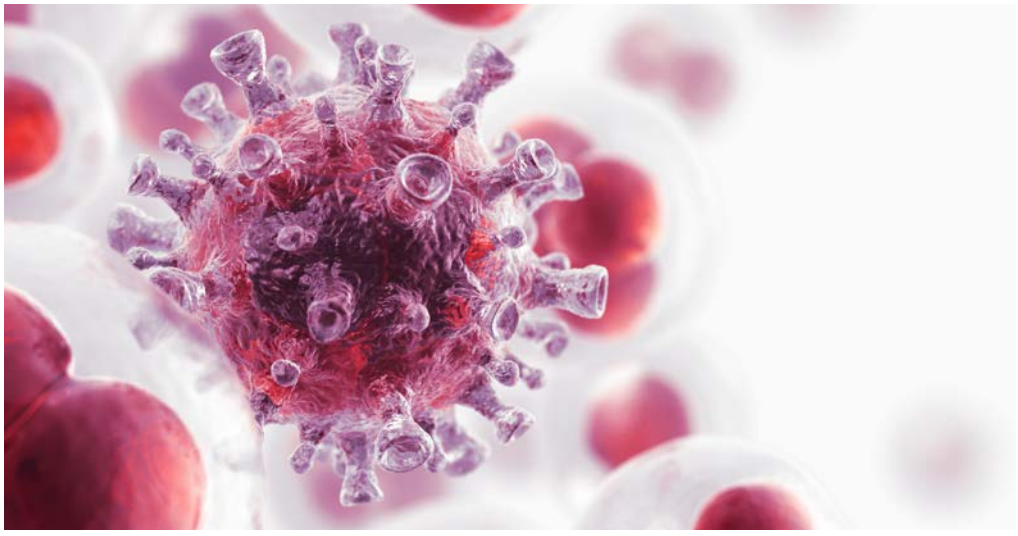

Fig 2. Many cancer cells are aneuploid, presenting an abnormal number of chromosomes.

In adult high-grade gliomas, researchers hypothesized that astrocytomas and oligodendrogliomas originated from different types of cells. Single-cell DNA sequencing revealed that transformed gliomas had a common origin (14). Critically, it was the respective tumor microenvironment that drove these differences. From a therapeutic standpoint, this single-cell sequencing study suggested that triggering cellular differentiation could arrest the growth of these tumors, highlighting a potentially novel avenue for brain tumor treatment.

Single-cell sequencing beyond oncology

This technology has applications throughout biology, for example, in neuronal development. Single-cell sequencing enabled the characterization of multiple sub-populations of cells in specific regions of the brain (Fig 3), revealing neuronal maturation profiles within these regions and associated them to gene expression (15). These findings shed new light on neural conditions, especially the autism spectrum disorders, and substantially contributed to understanding the regionalization within the brain. In doing so, the work provided a cellular foundation to dissect the molecular and cellular mechanisms of brain development and disease.

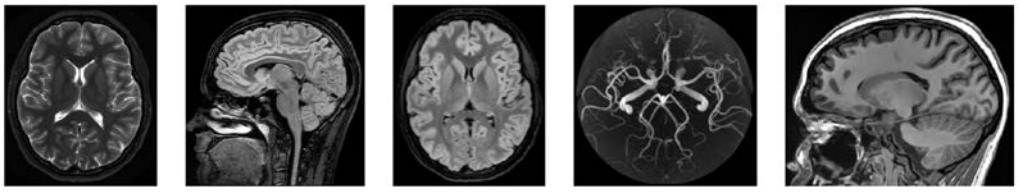

Fig 3. Single-cell sequencing enabled the characterization of multiple sub-populations of cells in specific regions of the brain.

Single-cell sequencing of the transcriptome

Single-cell RNA sequencing in oncology

Similar to genomics, single-cell RNA sequencing (**scRNA-seq**) has been used extensively in oncology to diagnose disease and understand treatment failure. For example, single cells were isolated from fresh intraductal papillary mucinous neoplasm tissues, which are the pre-cancerous lesions that develop into pancreatic cancer. The isolated single cells from these tissue samples revealed a more complex pattern of tumor evolution than previously thought and has implications in patient treatment and disease diagnosis (16).

A similar study examined fresh tissue samples from patients presenting with Barrett's esophagus, the precursor of esophageal adenocarcinoma. Using these tissue samples, this scRNA-seq study revealed that sub-populations of cells showed similar transcript profiles between the non-neoplastic submucosal gland cells and the Barrett's esophagus cells. These results revealed important biological traits for when planning patient treatment (17).

Single-cell RNA sequencing in developmental biology

Outside oncology, scRNA-seq has contributed substantially to the understanding of cell development. In 2018, scRNA-seq enabled researchers to generate a transcriptome map of the human prefrontal cortex during embryonic development (18). This single-cell map revealed the intrinsic development-dependent signals that regulate neuron generation and circuit formation as this highly specialized region of the brain develops.

Single-cell RNA sequencing in immunology

This modality is also having a substantial impact throughout the immunology field. scRNA-seq has identified natural killer cell sub-populations that originated from either the spleen or blood samples, and revealed unique features that differentiated them (19). Similarly, scRNA-seq identified a subtype of monocytes and dendritic cells (DC). Here, a new population of DC demonstrated the properties of plasmacytoid DC, a rare type of immune cell that secretes large quantities of type 1 interferon (20). A similar approach mapped the transcriptome of intestinal epithelial cells, elucidating how these cells maintain homeostasis and respond to pathogens (21).

These discussed examples all use scRNA-seq, where fresh, high-quality tissue was available. It is not always possible to obtain these types of sample. An alternative approach is single-nuclei sequencing (**snRNA-seq**). This method uses a nuclear dissociation protocol to isolate and sequence RNA within the nucleus. This modality is useful where fresh, high quality tissue is unavailable.

Utilizing snRNA-seq, frozen retinal tissue samples were examined and profiled. All major retinal cell types were observed and marker genes for each cell type could be identified (22). This study showed an improved power for prioritizing genes associated with human retinal diseases compared to both mouse single-cell RNA-seq and human bulk RNA-seq studies. It highlighted that single cell transcriptomes from human frozen tissues can provide important insights into disease that were missed by either human bulk RNA-seq or animal models.

Multiomic applications of single-cell sequencing

The power of these single-cell sequencing approaches become even more apparent when utilized in a multiomic manner (Fig 4).

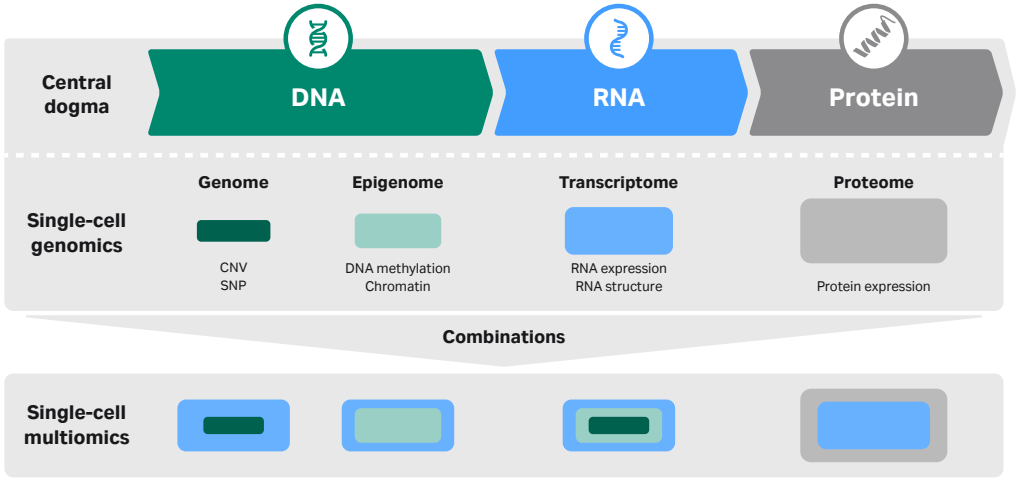

Fig 4. Strategies for multiomics profiling of single cells. Single-cell genomics methods profiling the genome, epigenome, transcriptome, and proteome, shown by different shapes with variable colors.

Multiomics provides even more information to the researcher, particularly in regard to the role of the tumor microenvironment in disease progression, mechanisms of drug resistance, or the process of cell differentiation. In 2016, the genotypic and phenotypic states of melanoma tumors were investigated using scRNA-seq to profile the tumor, immune, stromal, and endothelial cells. By incorporating scRNA-seq and proteomic data, these tumors were found to harbor malignant cells in two distinct transcriptional cell states, characterized by different levels of the AXL kinase protein. Single-cell analyses from these tissues suggested that it was the tumor microenvironment and cell-to-cell interactions that influenced this kinase. This multiomic approach has many implications for both targeted and immune therapies for melanoma tumors (23).

An alternate multiomic approach, **CITE-seq** (Cellular Indexing of Transcriptomes and Epitopes by Sequencing), incorporates scRNA-seq alongside obtaining quantitative and qualitative information on cell surface proteins. This dual approach enables the detection of genomic variants and protein expression simultaneously from the same cell. Utilizing CITE-seq, the relationship between mutational status and protein expression in peripheral blood mononuclear cells from six acute myeloid leukemia patients were analyzed. The study revealed pathogenic variants and mutational co-occurrence patterns in specific clones, raising substantial implications for the treatment of each patient and highlighting the impact of multiomic studies (24).

Adapting this modality for DNA sequencing also enables genomic variations such as single nucleotide variants (SNVs) and CNVs to be co-detected with specific proteins. This multiomic approach is rapidly expanding as more antibodies are validated and more targeted NGS panels become available to researchers.

The single-cell sequencing workflow

There are a diverse range of methodologies for conducting single-cell sequencing experiments, with critical steps conserved between them. There are key aspects that need to be considered throughout these processes:

- Minimal time between tissue collection and obtaining a single-cell suspension for downstream application(s).
- Protocol consistency to minimize variability throughout the entire workflow.
- High yield and viability of the disaggregated single cells.
- Representative and consistent transcriptome profiles of each cell relative to the original tissue sample.

In all cases, the starting material(s) needs to be of the highest possible quality. This can be highly variable, depending on the source. DNA and RNA from fresh tissue samples tends to be high quality whereas nucleic acids extracted from frozen tissue or formalin-fixed paraffin embedded tissues are typically much lower in quality.

Sample disaggregation and isolation

After collection, tissue samples need to be processed and single cells isolated as quickly as possible to make sure cell viability remains high and minimize any changes in the cellular transcriptome. Where possible, tissues should not be frozen because the thawing of frozen tissues can be highly damaging. However, when processing a fresh tissue sample, the disaggregation processes can still result in sample loss via ruptured or attached cells that are then unsuitable for library preparation. Indeed, this is frequently the primary source of unwanted technical variation and batch discrepancy in single-cell studies (25).

Tissue samples can also vary in extracellular matrix (ECM) composition, cell heterogeneity, and rigidity. Consequently, dissociation approaches require extensive optimization and care, as improperly disaggregated samples can reduce cell viability, decrease the efficiency of cell compartmentalization and block downstream instrumentation (Fig 5). The isolation process involves tissue dissection, mechanical dissociation, and enzymatic breakdown of the ECM. These steps are in parallel with mechanical agitation to obtain a viable, single cell suspension. There is a clear need for tissue dissociation systems for researchers to standardize the digestion and disaggregation of solid tissues samples to consistently obtain high quality, viable, single-cell solutions.

Systems for disaggregation

A small number of mechanical homogenizers have been re-purposed for single-cell sequencing. However, these systems rely on sheer force from beads, pistons, or sieves, and are prone to sample loss through cell rupture. The disaggregation process needs to be a mild procedure to make sure that cell yield and integrity remains high. Cell context must

Fig 5. Tissue dissociation approaches require extensive optimization and care to ensure cell viability is preserved.

be preserved, returning consistent and representative omic profiles relative to the original parent sample. In this regard, enzymatic approaches are well suited for these applications, and can be integrated with colloidal medium for cell separation by density centrifugation. This medium is nontoxic to biological materials and does not adhere to membranes. It is often unnecessary to remove the colloidal medium from the disaggregated cells as these could be transferred directly to cell culture systems or the individual cells separated for nucleic acid isolation (26).

A designated dissociation system for tissue sample digestion and disaggregation would be highly desirable to researchers. Such a platform could utilize single-use sample pouches, which limit exposure to the external environment, minimizing the risk of contamination. This approach would ensure that the platform could also be Good Manufacturing Practice (GMP)-compliant, tracking tissue sample dissociation and single-cell isolation for integration into GMP workflows.

Minimizing centrifugation within the workflow

The multiple centrifugation steps in the various single-cell sequencing methodologies can damage the cells and the nucleic acids being isolated. To minimize this damage, magnetic bead-based methods can integrate into the workflow. Alongside cell isolation, a magnetic bead-based approach mitigates the risk to the cells and nucleic acids from repeated mechanical force, and also enables the researcher to isolate DNA based on a size range. This can be tailored to suit the experimental requirements of the study and increase consistency in the workflow.

Standardizing single-cell sequencing sample acquisition

There is currently no standardized sample collection process, and this can introduce inconsistency throughout the protocol. In situations where samples need to be frozen, for example when they need to be shipped to a collaborator or service provider, this can become even more variable as the cryopreservation process itself can be uneven. Current processes for cryopreservation are generally manual and suffer from operator variability. Furthermore, the cryopreservation of whole tissues after collection can also lead to extensive cell death throughout the sample. There is also a risk for ischemic damage, resulting from a restriction in the blood supply to the sample immediately following collection. Each of these challenges can compromise tissue quality and substantially reduce the viability of the isolated single cells prior to downstream studies.

Another consideration is that existing cryopreservation systems are open, often manual, and not GMP-compliant. They tend to shed particles, and these can affect both single cell selection and sorting. A standardized automated approach, maximizing cell viability, that is amenable for scale-up in a GMP environment would be of substantial interest to many fields conducting single-cell sequencing studies. A clean room-compatible liquid nitrogen (LN2)-free cryopreservation system could eliminate the risks and substantial infrastructure costs associated with LN2-dependent technologies. There are platforms available that meet current GMP regulations, ensuring GMP-compliant cryopreservation can be incorporated into a single-cell sequencing pipeline.

These procedures require optimization and care as each might affect the expression signatures in the isolated cells. A defined, consistent tissue dissociation protocol would make sure that the most viable cells are isolated without preferentially depleting, substantially altering, or changing the expression characteristics of each cell.

Library preparation and NGS

Single-cell sequencing is underpinned by the substantial advances in NGS. NGS is well established with many optimized reagents and approaches for the detection of ultra-low concentration nucleic acids, critical when single cells will have, on average, ~20 picograms of DNA each.

Obtaining reliable, robust data in NGS is dependent on the quality of the nucleic acids used, defined by three key categories: quantity, integrity, and purity (Fig 6). Once isolated, the DNA or RNA require amplification and there are a range of reagents and kits on the market capable of producing microgram yields of DNA for NGS and genotyping. Importantly, some PCR-based kits have an amplification sensitivity down to one femtogram (fg) of genomic DNA (gDNA), an essential consideration for single-cell sequencing studies. Companies such as 10X Genomics provide a range of reagents for isolating, detecting, and sequencing these ultra-low concentrations of nucleic acids.

Alongside high sensitivity, minimizing sample contamination in library preparation is also a priority. Multiple strand displacement amplification (MDA), can amplify minute amounts of DNA to microgram levels for genomic analysis. This method utilizes random hexamer primers and the high fidelity Φ29 DNA polymerase to synthesize the cell's DNA. Compared with PCR-based methods, MDA generates much longer amplicons for NGS studies and substantially reduces the amplification bias commonly associated with PCR-based methods (27).

Utilizing an MDA mechanism, the GenomiPhi DNA Amplification system, can uniformly amplify gDNA and has a sensitivity of amplification down to 1 fg of gDNA. This kit includes an enzymatic cleanup protocol to ensure that any potential DNA contaminants are removed before each individual reaction. In this way, contaminating DNA, which is a common problem in single cell experiments, is effectively eliminated.

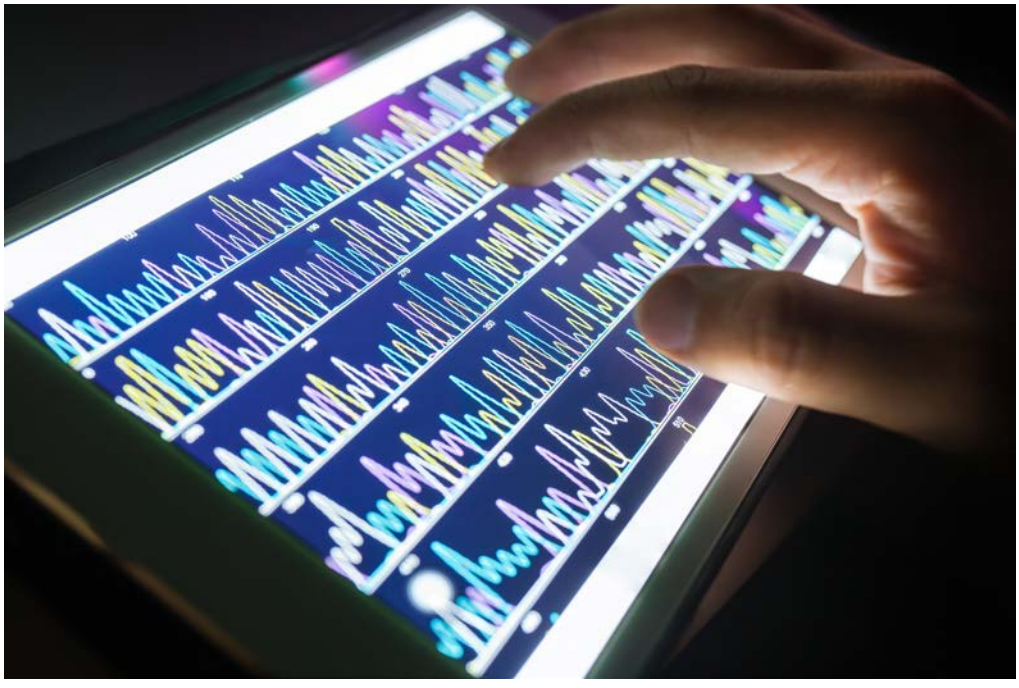

Fig 6. Obtaining reliable, robust NGS data is dependent on the quality of the nucleic acids used.

Current challenges with single-cell sequencing

Experimental design

A substantial challenge associated with single-cell sequencing is determining the number of cells needed to obtain data with the pre-requisite strength for downstream analysis. In this regard, the more cells per tissue sample the better. However, in every cell there are extremely low levels of nucleic acids that must be detected. The situation becomes even more complicated because, following detection, the researcher must be able to differentiate the sequencing data from every cell. The differentiation of single cells and their sequencing data emphasizes the importance of cell and sample barcoding throughout the single-cell workflow.

Additional challenges facing researchers in their experimental design include minimizing any stress-induced changes in the cells during tissue processing and single-cell isolation. The importance of minimizing any stress induced changes is underscored by the absolute need to obtain high-quality cells with representative epitopes on the surface, the recovery of all sub populations from the tissue being processed, the effective capture of all transcripts, and the unbiased amplification of gDNA.

Alongside these challenges, statistical models have been proposed that strengthen downstream analysis. These computational models require an estimation of the number and expected frequencies of cells and cell populations within the sample. These values are based on the actual number of cells available and the cost per experiment (28). Frequently, researchers sequence individual cells until they are satisfied that the specific population(s) of interest have been identified.

Closed systems for tissue processing and disaggregation

Tissue sample collection and preparation are the key steps that define the quality of the isolated single cells and therefore the downstream data. Currently there are no integrated platforms that enable these processes to be consistently conducted and tracked with GMP compliance. There remains huge variability in sample acquisition, quality, quantity, and viability, which can lead to sample loss, introduce errors, and remove consistency throughout the single-cell sequencing pipeline.

Cell heterogeneity

In developmental biology, specific regions and the cells that comprise tissues and organs are well defined. In oncology, clonal selection, microenvironmental pressures and genomic instability results in a poorly defined, heterogenic tumor mass (29). Single-cell sequencing studies of these tumor masses risk missing a cell population of interest or, will only analyze a particularly abundant cell population within the overall tumor. Taking into consideration both technical and sampling limitations, there is a possibility that low frequency or specific subclones will not be isolated. The source of tissue can be an additional challenge. Brain tissue can be difficult to disaggregate, process, and sequence due to its high lipid content. Moreover, the individual neurons in these samples are extremely fragile. As a result, when brain tissue samples are processed, the overall viability of each isolated cell is low and so requires care and attention in the design of the experiment, the sample collection process, and the preparation of the sample.

Data analysis

Bulk cell populations make sample isolation, NGS, and analysis of genomic and transcriptomic data relatively easy. In single-cell analysis, the small number of sequencing reads (compared to conventional NGS studies), the restricted data, and the cellular heterogeneity are considerable data analysis challenges. Computational biologists are starting to tackle these challenges, but several important concerns remain that need to be addressed throughout single-cell sequencing experiments (30, 31).

Transcriptome profiling by scRNA-seq is far noisier than bulk RNA-seq because the amplification of small amounts of starting material, in combination with sampling challenges, will affect the data. The recognition and detection of each gene in transcriptome studies is also critically important. Whether DNA- or RNA-seq, NGS experiments also involve the multiplexing of thousands of barcode sequences. These barcode sequences require demultiplexing when they are analyzed. Furthermore, errors are commonly introduced by barcode impurities alongside external background. The complexity of single-cell sequencing experiments has made resolving these challenges substantially more difficult as technological advances have empowered the investigation of many thousands of individual cells per study.

What does the future hold for single-cell sequencing?

Following the Human Genome Project, a human cell atlas program is now underway to map the 37 trillion cells in the human body. Single-cell sequencing technologies are an essential component for this huge project. The data collected will enable researchers to distinguish every cell among many types and provide far greater depth of understanding about cell-to-cell relationships. Essentially, this and specific single-cell sequencing studies will drive an increased understanding of the physiological processes and pathological mechanisms of disease at the single-cell level. These studies might result in the discovery of new diagnostic markers or therapeutic targets.

In regard to understanding disease and treatment, single-cell sequencing is revolutionizing oncology. As cancers develop, genomic instabilities increase the genetic and epigenetic diversity within tumors. Subclones within these masses display different molecular signatures, creating heterogeneity that drives metastasis and both intrinsic and acquired chemoresistance. Consequently, all cell populations, especially the rare ones, need to be characterized. The averaged analysis of whole tissue cannot describe this diversity and ignores rare but clinically important populations. Single-cell analysis of the genomes, transcriptomes, and proteomes provides researchers with a detailed molecular map of the cell types within a tumor and can reveal the mechanisms underlying cancer development, metastasis, and drug resistance.

While single-cell sequencing has many advantages, it certainly is not perfect. A plethora of approaches for obtaining single-cell sequencing data and single-cell whole genome sequencing exist, and each has distinct advantages and disadvantages (32, 33). This white paper has introduced single-cell sequencing methods, workflows, their applications in oncology, microorganism biology, neurology, and immunology, highlighting the great advantages of single-cell sequencing technologies in the study of highly heterogeneous cell populations. As single-cell sequencing technologies become more commonplace, with optimized workflows, and specific reagents and kits now available, the overall cost will further decline.

The introduction of disaggregation platforms for fresh or frozen tissue samples and the variety of methodologies available to the researcher highlight new opportunities for single-cell sequencing projects and is of particular importance for many fields, especially oncology. In parallel, the increasing computational biology power available today, alongside artificial intelligence, is empowering single-cell sequencing experiments to be conducted at a speed and scale previously deemed beyond all but a few laboratories and has an increasingly important role in disease diagnosis, understanding, and treatment.

Find out more about improving single-cell sequencing workflows or contact the Scientific Support team to discuss any other aspects of tissue processing and NGS.

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